Plant Signaling Molecules

Role and Regulation Under Stressful Environments

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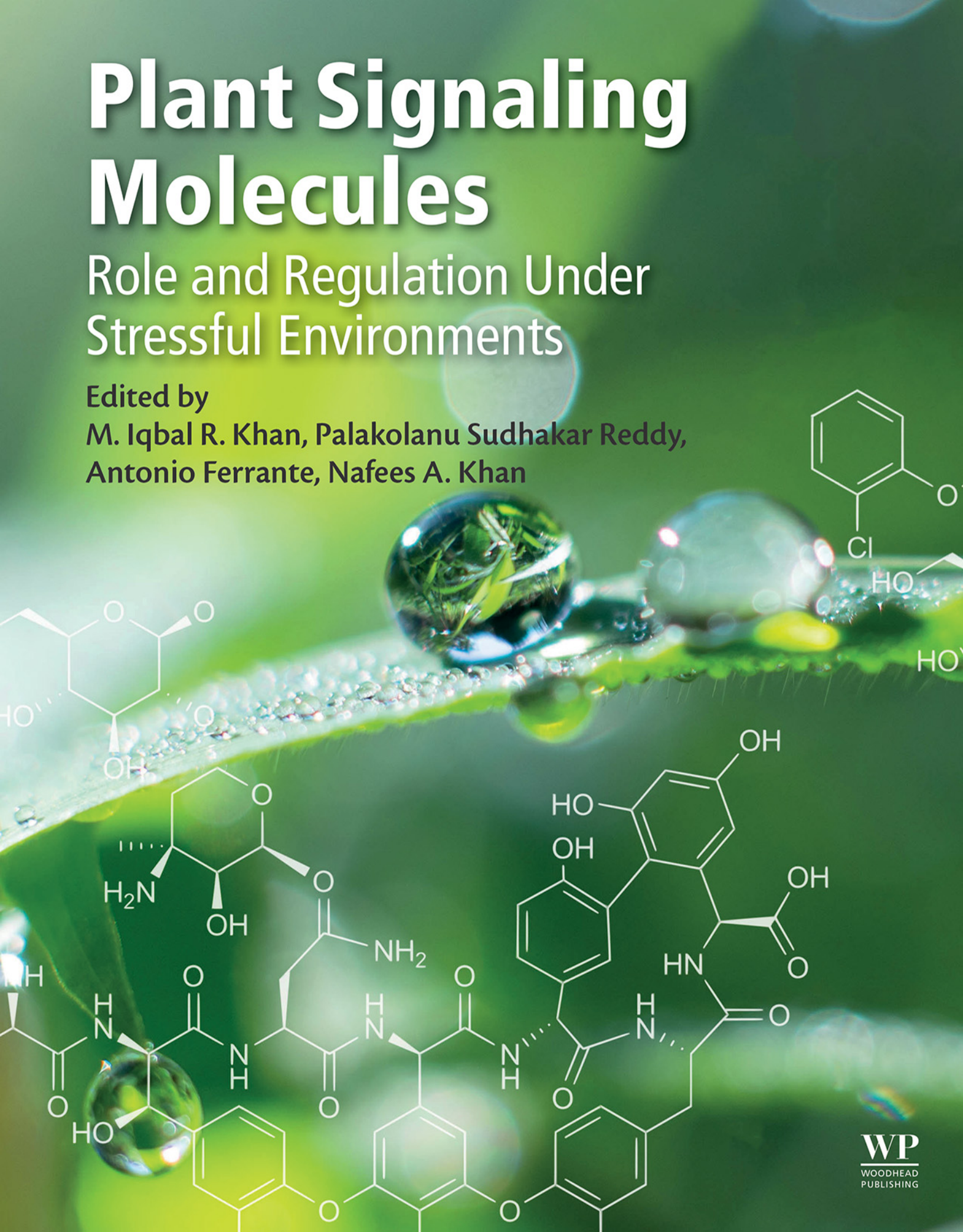

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PLANT SIGNALING MOLECULES

Role and Regulation Under Stressful Environments

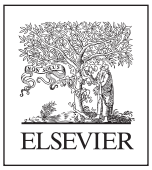
Edited by

M. Iqbal R. Khan
Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India

Palakolanu Sudhakar Reddy
Cell, Molecular Biology and Genetic Engineering Group, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Telangana, India

Antonio Ferrante
Department of Agricultural and Environmental Sciences, University of Milano, Milano, Italy

Nafees A. Khan
Department of Botany, Aligarh Muslim University, Aligarh, India



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Contents

List of Contributors

Aamir Raina Department of Botany, Mutation Breeding Laboratory, Aligarh Muslim University, Aligarh, Uttar Pradesh, India; Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Aditya Banerjee Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

Aisha Kamal Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India

Alessandra Francini Institute of Life Sciences, Sant'Anna School of Advanced Studies, Pisa, Italy

Alice Trivellini Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa, Italy

Altaf Ahmad Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Amarjeet Singh National Institute of Plant Genome Research, New Delhi, India

Ananya Singh Department of Biosciences, Integral University, Lucknow, Uttar Pradesh, India

Andrea Giro DAFNAE—Department of Agronomy Animals Food Natural Resources and Environment, University of Padua, AGRIPOLIS—University Avenue, Legnaro, Italy

Antonio Ferrante Department of Agricultural and Environmental Sciences, Università degli Studi di Milano, Milano, Italy

Appa Rao Karumanchi Department of Genetics, Osmania University, Hyderabad, Telangana, India; Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, India

Arajmand Frukh Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India

Aryadeep Roychoudhury Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

Ashwini Talakayala Plant Molecular Biology Laboratory, Agri Biotech Foundation, Hyderabad, India

Asim Masood Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Attila Ördög Department of Plant Biology, University of Szeged, Szeged, Hungary

Badar Jahan Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Banashree Saikia Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India; Academy of Scientific and Innovative Research (AcSIR), CSIR-NEIST, Jorhat, Assam, India

Bilal Ahmad Plant Physiology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

BilalAhmad Mir Department of Botany, School of Life Sciences, Satellite campus Kargil, University of Kashmir, Srinagar, Jammu and Kashmir, India

Challa Surekha Department of Biochemistry and Bioinformatics, Institute of Science, Gandhi Institute of Technology and Management (GITAM Deemed to be University), Visakhapatnam, Andhra Pradesh, India

Chandra Obul Reddy Puli Plant Molecular Biology Laboratory, Department of Botany, Yogi Vemana University, Kadapa, Andhra Pradesh, India

Chandra Sekhar Akila Department of Biotechnology, Yogi Vemana University, Kadapa, Andhra Pradesh, India

Channakeshavaiah Chikkaputtaiah Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India

Chiara Pucciariello Institute of Life Sciences, Sant'Anna School of Advanced Studies, Pisa, Italy

Daniele Massa CREA, Research Centre for Vegetable and Ornamental Crops, Council for Agricultural Research and Economics, Pescia (PT), Italy

Debasis Chakrabarty Council of Scientific and Industrial Research – National Botanical Research Institute (CSIR-NBRI), Lucknow, India; Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

Deepesh Bhatt Department of Biotechnology, Shree Ramkrishna Institute of Computer Education and Applied Sciences, Veer Narmad South Gujarat University, Surat, Gujarat, India

Devineni Lakshmi Punita Department of Genetics, Osmania University, Hyderabad, Telangana, India

Dipali Srivastava Council of Scientific and Industrial Research – National Botanical Research Institute (CSIR-NBRI), Lucknow, India

Durdana Shah Plant Tissue Culture Laboratory, Centre of Research for Development, University of Kashmir, Srinagar, Jammu and Kashmir, India

Eray Simsek Department of Plant Protection, Harran University, Şanlıurfa, Turkey

Fareed Ahmad Khan Plant Ecology and Environment Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Farhan Ahmad Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India

Fariha Raghib Plant Ecology and Environment Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Fauzia Naushin Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Gandra Jawahar Department of Genetics, Osmania University, Hyderabad, Telangana, India

Giacomo Cocetta Department of Agricultural and Environmental Sciences, Università degli Studi di Milano, Milano, Italy

Gian Attilio Sacchi Department of Agricultural and Environmental Sciences—Production, Landscape, Agroenergy, University of Milan, Milan, Italy

Giti Verma Council of Scientific and Industrial Research − National Botanical Research Institute (CSIR-NBRI), Lucknow, India

Giulia Franzoni Department of Agricultural and Environmental Sciences, Universià degli Studi di Milano, Milano, Italy

Guddimalli Rajasheker Department of Genetics, Osmania University, Hyderabad, Telangana, India

H.P.D. Boruah Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India

Hassan Jaleel Plant Physiology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Iffat Zareen Ahmad Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India

Indra Dutt Bhatt G. B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, Uttarakhand, India

Indrani Baruah Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India; Academy of Scientific and Innovative Research (AcSIR), CSIR-NEIST, Jorhat, Assam, India

Johni Debbarma Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India; Academy of Scientific and Innovative Research (AcSIR), CSIR-NEIST, Jorhat, Assam, India

Kanika Khanna Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Kapil Sharma Department of Plant Sciences, Repository of Tomato Genomics Resources, University of Hyderabad, Hyderabad, Telangana, India

Kiran K. Sharma Cell, Molecular Biology & Genetic Engineering Group, Research Program—Genetic Gains, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

Krishna Kumar Guduru Plant Molecular Biology Laboratory, Department of Botany, Yogi Vemana University, Kadapa, Andhra Pradesh, India

Kummari Divya Cell, Molecular Biology & Genetic Engineering Group, Research Program—Genetic Gains, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

Luciano Freschi Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, Brazil

M. Iqbal R. Khan Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India

Madhu Tiwari Council of Scientific and Industrial Research − National Botanical Research Institute (CSIR-NBRI), Lucknow, India

Mallikarjuna Garladinne Plant Molecular Biology Laboratory, Agri Biotech Foundation, Hyderabad, India

Manoj Nath Indian Council of Agricultural Research, New Delhi, Uttar Pradesh, India

Mayank Sharma Martin Luther University of Halle-Wittenberg, Halle, Germany

Megha D. Bhatt GSFC AgroTech Ltd. Gujarat State Fertilizers & Chemicals Ltd., Vadodara, India

Meher Fatma Plant Molecular Biology, School of life Sciences, Jawaharlal Nehru University, New Delhi, India

Mohammad Abass Ahanger Department of Botany, Govt PG College Rajouri, Rajouri, Jammu and Kashmir, India

Mohammad Israil Ansari Department of Botany, University of Lucknow, Lucknow, India

Mohd Irfan Naikoo Plant Ecology and Environment Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Mudasir Irfan Dar Plant Ecology and Environment Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Murat Dikilitas Department of Plant Protection, Harran University, Şanlıurfa, Turkey

Nafees A. Khan Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Nageswara Rao Reddy Neelapu Department of Biochemistry and Bioinformatics, Institute of Science, Gandhi Institute of Technology and Management (GITAM Deemed to be University), Visakhapatnam, Andhra Pradesh, India

Naravula Jalaja Department of Biotechnology, Vignan University, Vadlamudi, Guntur, India

Naser A. Anjum Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Nasreena Sajjad Department of Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, India

Natarajan Velmurugan Branch Laboratory Itanagar, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India

Nazish Nazir Plant Tissue Culture Laboratory, Centre of Research for Development, University of Kashmir, Srinagar, Jammu and Kashmir, India

Neha Handa Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Nese Sreenivasulu Grain Quality and Nutrition Center, Plant Breeding Division, International Rice Research Institute, Metro Manila, Philippines

Noemi Negrini Department of Agricultural and Environmental Sciences—Production, Landscape, Agroenergy, University of Milan, Milan, Italy

Noushina Iqbal Department of Botany, Hamdard University, New Delhi, India

Palak Bakshi Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Palakolanu Sudhakar Reddy Cell, Molecular Biology & Genetic Engineering Group, Research Program—Genetic Gains, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

Palavalasa Hima Kumari Department of Genetics, Osmania University, Hyderabad, Telangana, India

Parankusam Santisree International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

Parminder Kaur Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Parvaiz Ahmad Department of Botany, Govt PG College Rajouri, Rajouri, Jammu and Kashmir, India; Department of Botany and Microbiology, Faculty of Science, King Saud University, Riyadh, Saudi Arabia

Parveda Maheshwari Department of Genetics, Osmania University, Hyderabad, Telangana, India

Péter Poór Department of Plant Biology, University of Szeged, Szeged, Hungary

Polavarapu Bilhan Kavi Kishor Department of Genetics, Osmania University, Hyderabad, Telangana, India

Polavarapu Rathnagiri Genomix CARL Pvt. Ltd., Kadapa, Andhra Pradesh, India

Pooja Bhatnagar- Mathur Cell, Molecular Biology & Genetic Engineering Group, Research Program—Genetic Gains, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

Pooja Sharma Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Poonam Saini Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Poonam Tiwari Council of Scientific and Industrial Research − National Botanical Research Institute (CSIR-NBRI), Lucknow, India

Pradyumna Kumar Singh Council of Scientific and Industrial Research − National Botanical Research Institute (CSIR-NBRI), Lucknow, India; Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

Priya Arora Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Priyanka Sharma Department of Biotechnology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, Maharashtra, India

Ramesha A. Reddy Seri-Biotech Research Laboratory, Central Silk Board, Bangalore, Karnataka, India

Renata Callegari Ferrari Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, Brazil

Renu Bhardwaj Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Riwandahun Marwein Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India; Academy of Scientific and Innovative Research (AcSIR), CSIR-NEIST, Jorhat, Assam, India

Roberta Bulgari Department of Agricultural and Environmental Sciences, Universià degli Studi di Milano, Milano, Italy

Rohaya Ali Department of Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, India

Ruhi Afreen Department of Genetics, Osmania University, Hyderabad, Telangana, India

Samiullah Khan Department of Botany, Mutation Breeding Laboratory, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Sara Melito Department of Agriculture, University of Sassari, Sassari, Italy

Saurabh Badoni Plant Breeding Division, International Rice Research Institute, Metro Manila, Philippines

Sema Karakas Department of Soil Science and Plant Nutrition, Harran University, Şanlıurfa, Turkey

Shabir H. Wani Mountain Research Centre for Field Crops, Sher-e-Kashmir University of Agricultural Sciences and Technology, Srinagar, Jammu and Kashmir, India; Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, United States

Shahid Umar Department of Botany, Hamdard University, New Delhi, India

Shelja Sareen Department of Biotechnology, BBK DAV College for Women, Amritsar, Punjab, India

Shyamal Kumar Nandi G. B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, Uttarakhand, India

Silvia Morgutti Department of Agricultural and Environmental Sciences—Production, Landscape, Agroenergy, University of Milan, Milan, Italy

Snober Shah Cytogenitics and Molecular Biology Laboratory, Centre of Research for Development, University of Kashmir, Srinagar, Jammu and Kashmir, India

Somanaboina Anil Kumar Department of Genetics, Osmania University, Hyderabad, Telangana, India

Srivani S. Adimulam International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

Sumaya Hassan Department of Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, India

Sunitha Mellacheruvu Plant Molecular Biology Laboratory, Agri Biotech Foundation, Hyderabad, India

Sushma Sagar National Institute of Plant Genome Research, New Delhi, India

Syed Uzma Jalil Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow, Uttar Pradesh, India

Tariq Omar Siddiqi Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India

Thammineni Chakradhar Thakora Crop Sciences Pvt. Ltd., Anantapur, Andhra Pradesh, India

Thummala Chandrasekhar Department of Environmental Sciences, Yogi Vemana University, Kadapa, Andhra Pradesh, India

Titash Dutta Department of Biochemistry and Bioinformatics, Institute of Science, Gandhi Institute of Technology and Management (GITAM Deemed to be University), Visakhapatnam, Andhra Pradesh, India

Vandana Gautam Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India

Veena Pandey G. B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, Uttarakhand, India

Vinod Verma Department of Botany, DAV University, Jalandhar, Punjab, India

Yogita N. Sarki Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India; Academy of Scientific and Innovative Research (AcSIR), CSIR-NEIST, Jorhat, Assam, India

Zalán Czékus Department of Plant Biology, University of Szeged, Szeged, Hungary; Doctoral School in Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

Zebus Sehar Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Zhong-Guang Li School of Life Sciences, Yunnan Normal University, Kunming, P.R. China; Engineering Research Center of Sustainable Development and Utilization of Biomass Energy, Ministry of Education, Kunming, P.R. China; Key Laboratory of Biomass Energy and Environmental Biotechnology, Yunnan Province, Yunnan Normal University, Kunming, P.R. China

CHAPTER

1

Physiological Responses and Mechanisms of Signaling Molecules in Plants Stress Tolerance

Daniele Massa[1] *and Sara Melito*[2]

[1]CREA, Research Centre for Vegetable and Ornamental Crops, Council for Agricultural Research and Economics, Pescia (PT), Italy [2]Department of Agriculture, University of Sassari, Sassari, Italy

OUTLINE

1.1 INTRODUCTION

The signaling pathways involved in the response to salinity are very complex and highly heterogeneous due to the many biological, botanical, and environmental variables that actively interact with one another. Salinity produces several changes in plant physiology that range from osmotic effect to ion toxicity and nutritional disorders (Läuchli and Grattan, 2007; Munns, 2002; Khan et al., 2014a,b) thus causing a number of growth and development alterations from seed germination to plant maturity. It has been observed that saline soils (with at least 4 dS/m electrical conductivity, roughly equivalent to 40 mM NaCl) significantly affect the growth rate of most crops, thus causing significant decreases in produce yield (Shrivastava and Kumar, 2015). The plant response to salinity depends on several factors such as genotypes, plant age, stress duration, salt concentration, and the plant organ involved. Under salinity, plants show typical morphological traits: reduction of leaves numbers, reduction of plant size and of roots length and biomass production (Shannon and Grieve, 1999). These traits are the consequence of the negative effects of high salt concentration on membrane permeability, ions imbalance, stomatal conductance, and lower photosynthesis efficiency (Ivanova et al., 2014). Tolerant species generally show high capacity in sensing saline environments and prompt reaction to change their physiological status. Stomatal closure indeed is one of the first mechanisms to counteract the detrimental effects of osmotic stress (Munns and Tester, 2008; Robinson et al., 1997).

Based on their salt-tolerance capacity, plant species are classified into glycophytes and halophytes. The

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plant tolerance to Na^+ largely varies among plant species; Flowers (2004) reported a low tolerance to Na^+ in tomato, citrus, and avocado species, while a high tolerance was found for barley and cotton. In general, most of the crops require water with a very low Na^+ concentration (micromolar) and are referred to as glycophytes. Growth alteration, lack of fruiting and seed germination, occur for several crops under 100 mM NaCl (Flowers, 2004; Park et al., 2016; Zhu, 2001). For other plants (i.e., halophytes), such as *Atriplex*, *Salicornia*, *Rhizophora*, and *Suaeda*, growth and development depend on high NaCl concentration (200 mM) (Bohnert and Cushman, 2000; Flowers et al., 2010). The NaCl cellular concentration is the result of the dynamic fluxes in and out of the plant cell. Halophytes show high capacity of controlling NaCl concentration, because of specific ion-gated channels while glycophytes have a more limited ability to regulate the NaCl fluxes (Glenn et al., 1999). To control the Na^+ level inside the cell, a plasma-membrane antiport Na^+/H^+ is required (Horie and Schroeder, 2004). Some halophytic species use this antiport system to compartmentalize Na^+ and Cl^- ions in vacuoles within the cell, maintaining a low NaCl level in the cytosol (Zhang et al., 2010a). Both glycophytes and halophytes are not able to tolerate high salinity in their cytoplasm; consequently they regulate the salt concentration in the cytosol compartmentalizing ions in vacuoles or translocating them in different plant tissues (Turkan and Demiral, 2009).

1.2 GROWTH AND DEVELOPMENT

1.2.1 Plant Response to Salinity: Signaling Pathway at Tissue and Organ Level

Each phenological stage responds differently to the salinity stress (Munns, 2002; Sairam and Tyagi, 2004). For instance, germination and seedling growth are deeply sensitive to ion imbalance, while the following stage of vegetative growth, in general, shows less sensitivity to salt concentration. Finally, the salinity stress is more damaging during the initial flowering stage as compared with seeds that are already set. Overall, in susceptible plants the shoot growth showed a greater size reduction compared with the root size (Läuchli and Grattan, 2007).

At the germination level, the first signal-molecule received as stress signal is NaCl. High salinity, due to the accumulation of Na^+ and Cl^-, affects the germination because it induces a variation of the osmotic potential that decreases the water uptake, thus leading to a reduction in seed water absorption. In this case, salinity enforces the "dormancy" seed status inhibiting water absorption and the metabolic processes associated with the first stage of germination (Hannachi and Van Labeke, 2018). In addition to the osmotic effect, also the toxic effect of ion accumulation showed to negatively affect plant germination (Bajji et al., 2002). High Na^+ and Cl^- levels inhibit the expanding and division cell process, causing reductions in the germination percentage and further lengthening the time of germination in several plant species such as tomato and eggplant (Cuartero and Fernandez-Munoz, 1999; Hannachi and Van Labeke, 2018). Halophyte and glycophite plant seeds present different germination response. In both groups, salinity induces a reduction of germination: in halophytes the altered osmotic potential is the main cause, while for glycophytes the ion toxicity has an additive role (Bajji et al., 2002). As an adaptive mechanism, seeds of salt-tolerant species have a low osmotic potential inside the seed coat, which ensures adequate water absorption. Two principal strategies are used to create a water potential difference between the environment and the seeds: the use of organic solutes, to keep the osmotic potential, and the NaCl compartmentalization (Zhang et al., 2010b).

Osmotic adjustment, ion flux regulation, and Na^+ and Cl^- compartmentalization are salt tolerance strategies with a high-energy cost that compete with the seed germination and plant growth and development (Zhang et al., 2010b). The metabolic cost for the salt tolerance absorbs carbon reserves and energy otherwise required for the germination and seedling development, inducing a reduction of successful germination and an increase of the germination time. As well as the ion concentration and the quantitative variation of endogenous plant hormone regulates the plant response to salt stress.

One of the principal factors involved in salt stress response is abscisic acid (ABA). ABA is a plant hormone that plays a key role in several plant growth and development aspects under abiotic stress (Devinar et al., 2013; Fahad et al., 2015a; Huang et al., 2012). ABA is considered as the major internal plant signal molecule allowing the plant survival in adverse environmental conditions (Keskin et al., 2010).

Under salinity, seed germination typically decreases because of the higher level of ABA, which generates a sort of "induced dormancy" (Kang et al., 2015). The effect of salinity in seed germination has been deeply studied in *Arabidopsis thaliana*. The germination is regulated by ABA and gibberellins (GA) phytohormones, which have an opposite activity. ABA blocks the seed germination, while GA promote this process (Finkelstein et al., 2008; Piskurewicz et al., 2008; Yamaguchi, 2008). While the ABA biosynthesis is upregulated, at the same time, salt stress induces an

upregulation of genes involved in GA inactivation and suppression of GA signaling, with the result of a germination reduction (Achard and Genschik, 2009; Colebrook et al., 2014; Kim et al., 2008; Magome et al., 2008; Yuan et al., 2011).

In other plant stages, ABA and GA interactions play crucial roles. The ABA biosynthesis increases in roots as a consequence of osmotic stress and water deficit generated by salinity conditions (Cabot et al., 2009; Cramer and Quarrie, 2002; Gupta and Huang, 2014; Kang et al., 2015). The high level of ABA has been found to induce the activation of the salt tolerance mechanisms in plants that mitigate the negative effects of salinity on plant growth and development, photosynthesis, and assimilated translocation (Yoshida et al., 2004; Zhu et al., 2014). Despite ABA produced in roots and transported to leaves through the xylem as response to salinity stress, which has an important local and systemic effect, Seung et al. (2012) showed that also the new ABA produced in diverse plant tissues can regulate the physiological response in plants.

GA influence as signaling molecule, in plant processes other than germination (leaf expansion, stem elongation, flowering, and fruit development), is also relevant (Yamaguchi, 2008). The regulation of these processes is associated to the role of GA on the photosynthetic enzymes, which is also associated to leaf area index, light interception, and efficiency in nutrient absorption and translocation (Khan et al., 2007; Yamaguchi, 2008). The reduction of plant growth, under salinity stress, is therefore the most important physiological response due to altered hormonal balance in plants. Nonetheless, high level of GA has been associated to an increased activity of the source potential and to an efficient redistribution of photosynthates in the sink sites (Khan et al., 2007) while reduced level of GA highlighted a close correlation between salt induced growth reduction and alteration of GA pathway (Achard and Genschik, 2009).

In *A. thaliana*, a reduction of GA content has been observed as consequence of salt stress. The role of GA in vegetable salt stress tolerance has been elucidated through DELLA mutants, a quadruple mutant defective of the GAI, RGA, RGL1, and RGL2 genes associated to the GA pathway; DELLA mutants were less tolerant to salt stress as compared with the wild type (Achard and Genschik, 2009). In addition, a minor reduction of flowering delay was shown in DELLA mutants, which supports the association between flowering and salt stress. These data were confirmed by the "spy" mutant, in *A. thaliana*, which showed a higher GA level and an increased salt tolerance compared with the wild type (Olszewski et al., 2002, 2010; Qin et al., 2011).

Despite several studies have been conducted on plant response to salinity in different plant development stages, little exploration has been done on the impact of salinity effect on the young seedlings during the early phases of plant growth. In general, most of the studies showed that annual crops are more salt-tolerant at the germination and less resistant at the following stages such as emergence and early vegetative development (Läuchli and Grattan, 2007). Furthermore, the classification and evaluation of salt tolerance is not the same during all the stages. While for germination and seedling, the tolerance is evaluated as percentage of germinated plant, for the other stages the evaluation is based on growth rating.

Beside plant development stages (seed, seedling, and mature plant), salinity response also shows organ-specific trends. Shoot and root are both affected by salinity, but the recovery mechanism is probably different. A reduction of leaves growth under salinity is mainly caused by the osmotic effect nearby the roots. One of the secondary effects of the salt exposure is the osmotic imbalance that leads to water deficit, with a consequent temporary and partial plant loss of turgor. Cells recover the original volume using osmotically active metabolites to properly adjust the osmotic cell conditions (Munns and Tester, 2008). The type of osmolytes is species-specific and beside the tolerance to salt stress, these metabolites are used as active molecules for resistance to other unfavorable environmental conditions, such as freezing, heat, and drought stress. Sugars (glucose, sucrose, fructose, trehalose, and raffinose), polyols (sorbitol, mannitol, glycerol, arabinitol, and pinitol), ions (predominantly K^+), specific proteins, and charged metabolites (betaine, proline, glutamate, aspartate, glycine, choline, putrescine, oxalate, and malate) (Cheong and Yun, 2007; Sairam and Tyagi, 2004) are the most common osmolytes used to balance the osmotic stress, and to protect proteins and membranes.

Some of the above-mentioned molecules such as sugars and proteins have shown multiple roles: metabolic resources, structural components, osmolytes, and signaling molecules under salt stress. The role of sugars as signaling molecule in salt stress conditions has been better explained in *A. thaliana* (Hanson and Smeekens, 2009). In this species, hexokinase 1 (HXK1) is a sugar (glucose) sensor that integrates nutritional and hormonal plant status with plant growth and gene expression under abiotic stress including salt stress (Cho et al., 2006; Moore et al., 2003). HXK1 sugar sensor can be found in cytosol and organelles (chloroplasts and mitochondria), (Rolland et al., 2002; Wiese et al., 1999). Furthermore, HXK1 can be transferred to the nucleus (Aki et al., 2007). When the glucose level increases, the

HXK1 nuclear complex suppresses the expression of nuclear photosynthetic genes (Cho et al., 2006).

Among proteins and amino acids, the role of proline in plant abiotic stress response has been more deeply explored. Proline accumulation has been observed in several plant species as response to abiotic stress, especially under salt and drought conditions (Mattioli et al., 2009; Szabados and Savoure, 2010; Trovato et al., 2008; Verbruggen and Hermans, 2008). Beside its role as compatible osmolytes, proline accumulation acts as cell redox balancer (Hare and Cress, 1997) and as stress-related growth regulating signal (Khedr et al., 2003; Maggio et al., 2002). In *A. thaliana*, transcriptomic analysis revealed that the exogenous application of proline activated defense or detoxification genes, and in the meantime repressed genes involved in photosynthesis (Deuschle et al., 2004; Hellmann et al., 2000; Nanjo et al., 2003). The association between proline accumulation and photosynthesis reduction suggests that high concentration of proline in plants may also affect the function of chloroplasts.

K^+ and Ca^{2+} are the most important nutrient ions involved in the physiological response of salt-stressed plants. Under salinity stress, indeed K^+ plays a strategic role in boosting plant growth and development due to its activity in maintaining Na^+/K^+ homeostasis. The decrease in extracellular K^+ concentration causes the root epidermal plasma membrane hyperpolarization, thus increasing the accumulation of Ca^{2+} in the cytosol (Demidchik et al., 2002). The presence of Ca^{2+} in the cytosol has also been related to improved plant nutrition under salinity (Wilkins et al., 2016). Furthermore there are evidences for possible connections between nitric-nitrogen nutrition and Ca^+ accumulation in the cytosol and vice versa (Wilkins et al., 2016). The above relationships between Ca^{2+} and plant nutrition underline the importance of the crosstalk between nutrient ions and stress signaling under salinity.

In conditions of moderate salt stress, several other symptoms of growth and development alteration appear as late plant responses. An inhibition of lateral shoot development, as well as early flowering or a reduced number of florets, is observed over weeks and/or months (Munns and Tester, 2008). In the meantime, older leaves die with a reduction of production of new leaves. These effects are mainly due to the osmotic stress caused by salt outside the roots. Beside the osmotic impact, NaCl affects plant growth through the toxicity of Na^+ accumulation in cytosol (Sumer et al., 2004). The mechanism of leaves and shoot development reduction is still to be well understood. As shown by Fricke and Peters (2002), carbohydrates supply and water status only marginally affect the leaves growth. In addition, also the nutrient deficiency and ion toxicity seems to play only a minor role in the shoot development (Hu et al., 2007, 2005; Munns and Tester, 2008). The most probable cause is associated to long distance signals from roots such as hormones that are involved in the plant development alteration and that induce a cascade signal in other plant organs. Shoot and root elongation are both influenced by salinity. The impact of NaCl exposure on root growth is less important than on leaves and its recovery is faster (a few hours, for a moderate stress, and a few days for a severe stress conditions) (Guo et al., 2015; Munns and Tester, 2008). In contrast to leaves, the root recovery occurs even if the turgor is not completely recovered, suggesting different response mechanisms.

1.2.2 Temporal Signature and Related Cell Response Mechanisms

Under salinity conditions, plant growth is reduced due to salt accumulation in shoots or due to processes independent of the shoot salt accumulation. These two phenomena are closely related to the plant time-response: the immediate response to salinity (from a few minutes to a few days) and longtime response (from several days to few weeks). In the early response, the plant reacts in a few minutes and a rapid and transient change in growth can be observed (Sirault et al., 2009). These effects can be considered independent of the salt shoot accumulation because of the quick plant response mechanism. The overall effect is the reduction of new leaves and a general reduction of shoot size caused by the "osmotic phase" (Munns and Tester, 2008). Roots are tissue directly involved in the salt stress perception and its signal transduction. From the roots, hormonal signals are generated inducing a general growth reduction during the osmotic phase (Ismail et al., 2014). The second and slower phase causes growth inhibition because of the salt accumulation during a longer period, mainly in older leaves. Salt accumulation in leaves induces cellular damage and necrotic lesion and later cell death. In this case, the cell death and consecutive abscission is an adaptive mechanism of the plant to remove the salt accumulated in older leaves. This phase is called the ionic phase (Bera et al., 2014).

In highly susceptible plants, the rate of dead leaves (for salt accumulation) is higher than the rate of new leaf production. This is a consequence of the photosynthetic activity, which is not sufficient to supply the carbohydrate required for the growth and plant development. The photosynthates produced by the plant are not sufficient to reach the reproductive stage and produce viable seeds (Läuchli and Grattan, 2007). This two-stage response concept implies that the

osmotic effect of salt detected by roots causes an initial growth reduction, in both resistant and susceptible plant genotypes. However, the differences between tolerant and non-tolerant plant species is mainly associated to the second phase and consequently, to the plant ability to prevent the toxic salt accumulation in transpiring leaves (Munns et al., 2006).

Early and late phases involve specific signal molecules that deeply affect growth and development in plants. Temporal pattern of signal molecules induces adaptation or cell death. High salt level causes a fast increase of Na^+ in the cytoplasm through the nonselective cation channel (NSCCs) and later, through the K^+ transporter called HKT1 (Essah et al., 2003; Ismail et al., 2014) (Fig. 1.1). Two principal types of NSCCs are involved: hyperpolarization-activated (HA) NSCCs and depolarization-activated (DA) NSCCs. HA-NSCCs are activated later than DA-NSCCs and have low selectivity for monovalent cations and are reported to be predominant in salinity susceptible plants; by contrast, DA-NSCCs are mainly detected in tolerant species. The NSCCs have three principal functions: to regulate the Na^+ flux into the cytosol, to activate the Ca^{2+} signal, and modulate K^+ flux in the cell. The quick DA-NSCCs determines a fast Na^+ influx with the consequent activation of Ca^{2+} signal pathway and the rapid inhibition of K^+ loss thus maintaining the Na^+/K^+ homeostasis in the plant (Shabala et al., 2006). In less tolerant plants, the slower response will be less efficient to regulate the water potential with a consequent loss of water. The inhibition of Na^+ influx is not sufficient to tolerate the stress condition; ions at this point need to be removed from cytosol to prevent accumulation in plant organelles.

The osmotic stress activates the synthesis of ABA, which upregulates the gene expression of the Na^+/H^+ antiport for the accumulation of Na^+ in vacuoles (Shi and Zhu, 2002; Yokoi et al., 2002) (Fig. 1.1). In addition, ABA induces increasing concentration of Ca^{2+} in the cytosol that is associated to the stomatal closure under exposure to salinity. Turkan and Demiral (2009) reported that one of the early plant responses to salinity associated to osmotic stress consists of the increase of Ca^{2+} in the cytosol (after 1 min). The normal cytoplasmic Ca^{2+} is between 100 and 200 nM, while in plant cell organelles is 1−2 mM (Ismail et al., 2014). Ca^{2+} channels are distributed on the surface of cytoplasmic membrane and the membranes of different organelles (vacuoles, chloroplasts, and mitochondria). Variation of Ca^{2+} levels regulated downstream signals for the stress response activation, which includes plant development hormones (Park et al., 2016). The fast increase of Ca^{2+} activates the antiport ATPase responsible of the Na^+ vacuole accumulation (Fig. 1.1). Ca^{2+} is reported to be involved both in the early response and long-term plant response tolerance to salinity (Tuteja, 2007).

The role of Ca^{2+} as signal molecules for salinity tolerance has been better elucidated in association with the salt overly sensitive (SOS) pathway. Tolerance to salt stress and the maintaining of a favorable ion ratio in plant cytoplasm was associated to the SOS1−SOS3 genes pathway in *A. thaliana* (Zhu, 2002). The study of the SOS pathway function highlighted the role of Ca^{2+} in the cell ion equilibrium. SOS3 is a Ca^{2+} binding protein strategic for the salt tolerance; its role as Ca^{2+} sensor is, in fact, crucial to transduce the salt stress signal in plant. SOS3−SOS2 complex stimulates the Na^+/H^+ antiport activity of SOS1 with the purpose to reestablish the cellular ion homeostasis. In addition to the SOS1−SOS3, also two other proteins involved in SOS pathway have been characterized: SOS4 and SOS5 (Mahajan et al., 2008). These two proteins are involved in cell wall structure and integrity under salinity conditions.

In cross-talking with the Ca^{2+} signal and with the hormonal response, H^+ also acts as an efficient salt stress signal molecules in plants (Gao et al., 2014; Ismail et al., 2014). Increasing level of H^+ has been reported as an early plant salt response occurring immediately after NaCl stress recognition (Ismail et al., 2014) (Fig. 1.1). Protons regulate the plant metabolism acting on the proteins (enzymes) conformation and activity. In *A. thaliana*, the cytosol alkalization, mediated by ABA and methyl jasmonate (MeJa) increasing level, causes stomatal closure (Suhita et al., 2004). The increase in H^+ influx, often associated with the Ca^{2+}, induces the activation of Ca^{2+} channels (Ismail et al., 2014) and the Ca^{2+}-mediated signal pathway. In addition, variation of pH regulates the ratio between inactive and active jasmonic acid (JA), which act as stress signal molecules (Fonseca et al., 2009).

A more rapid and persistent alkalization of the apoplast has been correlated to an increased salt tolerance in *Vitis vinifera* (Ismail et al., 2014). Furthermore, H^+ is associated with the Ca^{2+} fluctuations. Under salinity stress Ca^{2+} and H^+ influx can occur simultaneously: the increase of apoplast pH, in fact, is a strong signal for the rapid Ca^{2+} influx (Felix et al., 1999). Briefly, the alkalization of cytosol provokes a temporary accumulation of Na^+ and Ca^{2+}, which then represent the early signal molecules of salinity conditions.

After a few minutes of exposure to salinity conditions, salt stress has a general effect on the plant metabolism that involves specific signal molecules. Under salinity, the reduced photosynthesis rate and alternated respiration process generate a general metabolism imbalance in the oxidative processes, which causes an increasing level of reactive oxygen species (ROS) (Fig. 1.1; Khan and Khan, 2017). The

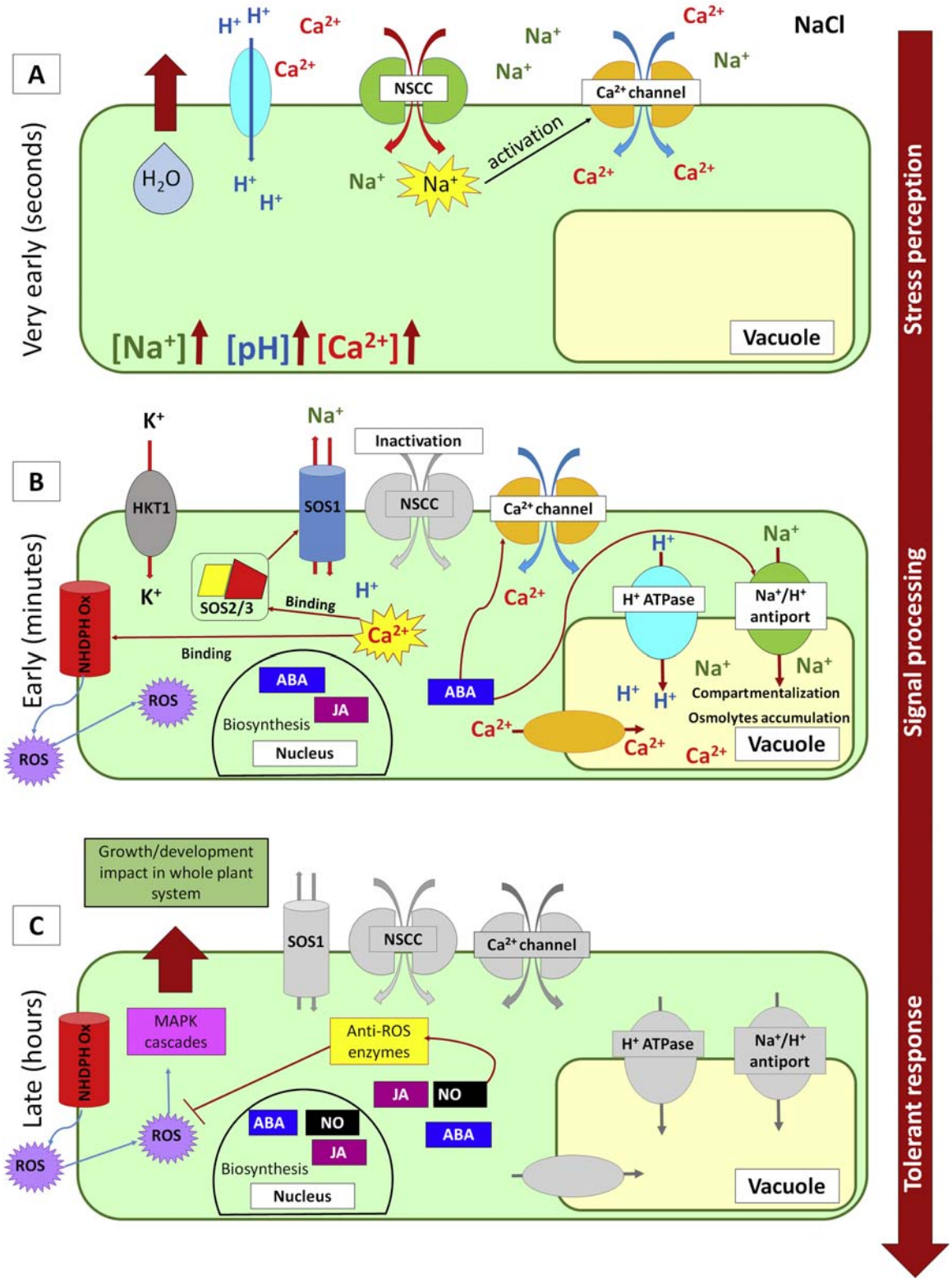

FIGURE 1.1 Plant response signals under salinity stress. (A) During the very early response, tolerant cells are mainly involved in the salt stress perception. High NaCl levels induced the activation of Ca^{2+}, H^+, and Na^+ (NSCC) channel. In a few seconds, cytosol alkalization occurs as well as an increasing level of cytosolic Ca^{2+} and Na^+ levels. Osmotic potential changes inducing a loss of cell water content. (B) During the early response (minutes), the high Ca^{2+} level activated the SOS system. Ca^{2+} binds to SOS2/SOS3 complex, which activates the SOS1 membrane protein. SOS1 is a membrane pump that excretes Na^+ outside the cell, working on the rebalancing of the Na^+ level in the cytosol. In the meantime, the Ca^{2+} binds to the NHDPH oxidase inducing the ROS production. The increased level of ABA, associated to the cell stress condition, induced the compartmentalization of Na^+, Ca^{2+}, and H^+ into the vacuole through antiport systems. Meanwhile the biosynthesis of ABA, JA, and NO is activated in the nucleus. (C) In the late responses (hours), the influx of Na^+, Ca^{2+}, and H^+ is blocked and NO activates the production of anti-ROS enzymes for the reduction of ROS level in the cytosol. The initial high level of ROS induced the MAPK cascade with general growth and development consequences in the whole plant system. In a tolerant cell, the osmotic potential at this stage is normalizing and the vacuole compartmentalization is not active anymore.

high level of apoplastic ROS is caused by the apoplastic depletion of proton and the ROS influx into the cytoplasm. ROS are recognized by the membrane-localized histidine kinase, which activates the mitogen activated protein kinase (MAPK) signaling cascade (Fig. 1.1). The MAPK cascade is a highly conserved signaling pathway in eukaryotes (Ichimura et al., 2002). MAPK cascades, which include a minimum set of three subsequential proteins (MPK, MAPKK, and MKK or MEK), activate downstream targets by phosphorylation system. MAPKs are associated to several plant growth and development aspects (gametogenesis, embryogenesis, morphogenesis, senescence, abscission, flowering, and seed development), (Xu and Zhang, 2015). The *A. thaliana* genome analysis revealed 20 different MPKs; 3 of which (MPK3, MPK4, and MPK6) are involved in both stress and developmental responses (Colcombet and Hirt, 2008; Lampard et al., 2009; Liu et al., 2010). Studies conducted on MPK6 loss of function mutants, highlighted alteration of embryo and root development (Bush and Krysan, 2007; Müller et al., 2010; Wang et al., 2010). Primary roots originate from the embryo and generate lateral roots during vegetative growth. A well-developed root system architecture has an important role in plant−environment interaction, especially in case of drought and salinity stress (Casimiro et al., 2003; Dubrovsky and Forde, 2012). Müller et al. (2010) demonstrated a strong relationship between MPK6 activity and cell division. MPK6, in fact, interact with γ-tubulin and the microtubule plus end protein EB1 associated to the cell division during stress conditions (Kohoutova et al., 2015). Furthermore, Li et al. (2017a,b) have demonstrated that MPK6 is involved in the MAPKs cascade that modulates the ABA response regulating the cell division and elongation in root.

Taj et al. (2010) showed that three MAPKs (MPK4, MPK6, MAPKK1) are triggered under salinity; the overexpression of MAPKK2 in *A. thaliana* caused a constitutive MAPK4 and MPK6 activity that increased plant salt tolerance. In addition, MPK4 was found to regulate salicylic acid (SA) and ROS production in *A. thaliana* (Gao et al., 2008; Takac et al., 2016). Besides SA and ROS production, MPK4 and MPK6 activate the ACC synthases enzymes responsible for the ethylene biosynthesis, which is involved in several biological processes (from senescence to fruit maturation) (Liu and Zhang, 2004). Considering the cascade of signals generated by the ROS, their production needs to be strongly regulated, otherwise their overproduction could result in a cell death. In addition, a close connection has been demonstrated between ROS and Ca^{2+} in response to stress, which generated alteration in plant growth (Hu et al., 2007; Jiang et al., 2003; Shoresh et al., 2011). ROS, such as hydrogen peroxide (H_2O_2), activated the Ca^{2+} channel increasing its concentration in the cytosol (Pei et al., 2000). The increased level of Ca^{2+} controls the plant cell expansion. On the other hand, cytosolic Ca^{2+} regulated the NADPH oxidase, which generates ROS (Fig. 1.1). The ROS, generated by NADPH oxidase, in the plasma membrane, activates the ABA signals cascade. In this intricate signal network, nitric oxide (NO) has an important role for plant salt tolerance. The action of NO is more closely related as signal molecules that cooperate with JA and ethylene. The cross-talking of NO with hormones associated to plant stress response induced alteration of plant growth and development (mainly during seed germination and roots formation). In *Lupines liteus*, for instance, NO increased the antioxidant activity of "anti-ROS" enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), better known as antioxidant enzymes (Kopyra and Gwozdz, 2003). Because of high SOD and CAT activities (NO induced) a decreased ROS level (mainly O^{2-} in the mitochondria) is reported, which makes it easier to report cells to a redox homeostasis (Lamattina et al., 2003).

In addition to the ABA and ethylene, ROS also influences JA biosynthesis in roots, which is known to act as an important hormone regulating plant growth and development (Xiong and Zhu, 2003). JA is produced as an early signal, occurring between minutes and hours after the NaCl stress recognition (Ismail et al., 2014). As a hormone, JA is involved in several plant biological processes, determining alteration of seed germinations, primary root growth, flowering, and plant senescence (Fahad et al., 2015b; Riemann et al., 2013; Robson et al., 2010; Tani et al., 2008). The JA biosynthesis is localized in roots' and leaves' peroxisomes and chloroplasts (Cheong and Choi, 2003). The relationship between JA and salinity tolerance has been demonstrated in several species such as *Solanum lycopersicum* (Pedranzani et al., 2003) and *Iris hexagona* (Wang et al., 2001) in which high level of JA enhanced the salinity tolerance. Salt-tolerant cultivars of *Oryza sativa* showed higher JA levels than the susceptible cultivars (Kang et al., 2005); the increase of JA was detected either in roots or in leaf tissues (Tani et al., 2008). It has been shown that salt-tolerant plants at the beginning activate the JA pathway (through a transient pick of ROS), and later JA production is shut off because of the activation of the ABA signaling. ABA and JA signaling are in fact antagonist, mainly for mutual competition for common signaling factors (Anderson et al., 2004). Overaccumulation of JA caused, for instance, the delay of Ca^{2+} signaling activation thereby leading to membrane damage and cell death (Ismail et al., 2014).

Both salt-tolerant and sensitive plants use the same signal molecules. Timing appears to have a crucial role

in plant cell adaptation to NaCl or death. The ability to survive and adapt to salinity is associated to the cell timing of the transient status of salt stress signal pathway. Tolerant plants can efficiently regulate the crosstalk between different signal pathways controlling the temporal intensification of the signal. If the stress signals persist longer, or start later, other plant pathways not associated to the salt tolerance response are activated. A delay in the switching on (and in the turning off) of Ca^{2+} signal will let increase the ROS production (over the plant cell acceptability) activating the JA pathway, which culminates with plant stress symptoms and eventually cell death.

1.3 LEAF GASEOUS EXCHANGES

1.3.1 Stomatal Conductance and Water Relations

The reduction of the stomatal activity is one of the most rapid reactions in plants exposed to saline environments. Reduced gaseous exchange rates are associated to limited leaf transpiration (Mittler and Blumwald, 2015), which in turn limits water uptake and fluxes within and between plant tissues, and limited CO_2 and O_2 exchanges. Such phenomena are known to negatively affect carbon intake and/or sugar respiration (Koyro et al., 2011; Mittler and Blumwald, 2015; Munns and Tester, 2008; Park et al., 2016). Limited stomatal activity causes disorders in primary plant metabolism leading to photoinhibition, oxidative stress, and eventually reduced growth and development in the medium-long period, until cell and plant death (Mittler and Blumwald, 2015; Park et al., 2016).

Salt stress drastically reduces the tolerance to salinity causing significant reduction in both crop quality and yield (Munns and Tester, 2008). Stomatal activity regulation is one of the most important plant defense/modulation mechanisms to face salinity stress. In fact, stomatal closure occurs both in halophyte and glycophyte plants when exposed to saline conditions (Tester and Davenport, 2003). Indeed, plants that are sensitive to salinity commonly show poor capacity to reduce stomatal conductance (Robinson et al., 1997). Salinity induces plant stress at different levels including osmotic stress, ion toxicity, and nutrient imbalance. Therefore, many different response mechanisms were found to be associated to different botanical families and growth environments (Munns and Tester, 2008). The osmotic stress rapidly influences stomatal conductance that is reduced as a response to the presence of stress-signaling molecules. Yet, it has been demonstrated that lack of molecules signaling saline stress, or the absence of connections between these molecules and adequate ecophysiological plant response, are typical of plants sensitive to salinity (Apel and Hirt, 2004; Basu and Rabara, 2017; Hetherington, 2001; Huang et al., 2012; Munns and Tester, 2008; Zhu, 2002).

Stomatal conductance in salt-stressed plants is primarily reduced, to keep optimal tissue water relations and to maintain cellular turgor, by ABA as the main signaling molecule (Dar et al., 2017; Mittler and Blumwald, 2015; Park et al., 2016). In addition, the reduced photosynthetic activity induces feedback mechanisms on stomatal closure in which ROS play a crucial role as second messengers (Apel and Hirt, 2004; Koyro et al., 2011; Shi et al., 2015). ROS, therefore, largely contribute to the stomatal reactions to salinity exposure, showing many different functions that are directly or indirectly related to abiotic stresses, including salinity (Mittler and Blumwald, 2015; Tester and Davenport, 2003). Among ROS, H_2O_2 has been found to decrease stomatal conductance both in earlier (McAinsh et al., 1996) and in recent studies conducted on different plant species (An et al., 2008; Li et al., 2017b; Rodrigues et al., 2017; Shi et al., 2015). The effects of H_2O_2 on stomata behavior have mainly been related to the role of this molecule in the activation of Ca^{2+} channels to mediate ABA signaling in guard cells (Basu and Rabara, 2017; Kim et al., 2010; Mittler and Blumwald, 2015; Pei et al., 2000). Nonetheless, recent studies underline the importance of the combined action of ROS and the reactive nitrogen species, NO, as connection point between Ca^{2+} and ABA signaling cascade in the control of stomatal closure (Li et al., 2017b; Saxena et al., 2016). Possible interactions of the different signaling molecules that play strategic role in the stomata control are summarized in Fig. 1.2.

ABA plays a major role as signaling molecule in salt-stressed plants. Hence, several reports have been produced in the last few decades leading to increased knowledge on this hormone and its role in controlling stomatal behavior in higher plants (Apel and Hirt, 2004; Basu and Rabara, 2017; Danquah et al., 2014; Hetherington, 2001; Huang et al., 2012; Munns and Tester, 2008; Saxena et al., 2016; Taiz et al., 2015; Zhu, 2002). ABA promptly promotes stomatal closure by acting on ion fluxes in guard cells of plants exposed to salinity (Mittler and Blumwald, 2015; Parida and Das, 2005). Its action is explicated through different mechanisms that basically aim to favor the release of K^+ from guard cells thus leading to K^+ concentration levels not sufficient to maintain cellular turgor and stomata opening. In sweet pepper exposed to saline environment and different levels of atmospheric CO_2, the accumulation of ABA was correlated with stomatal conductance (Piñero et al., 2014). In transgenic cotton, the overexpression of a gene encoding for a bZIP transcription factor, which can improve the ABA signaling

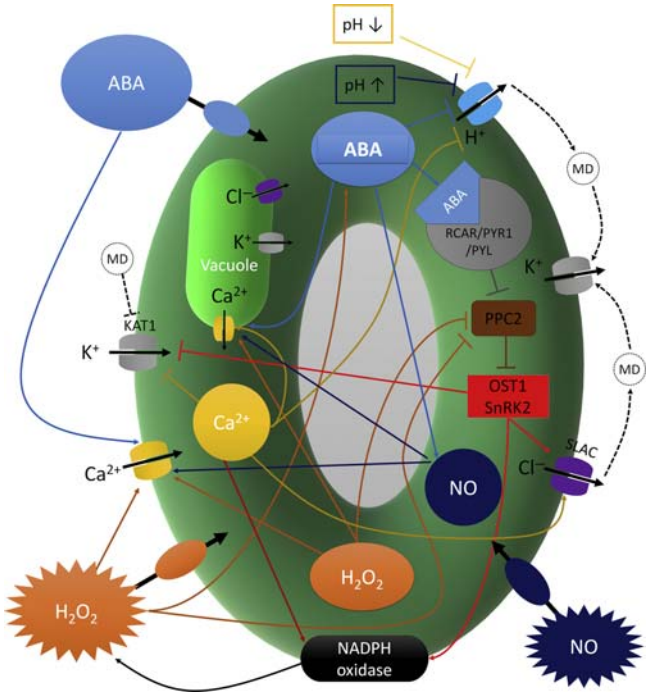

FIGURE 1.2 Stomatal response signals under salinity stress. The main biochemical mechanisms behind the induction of stomata closure are schematically reported in the figure without taking into consideration the real cell guard shape. The possible interactions among the different primary and secondary signaling molecules, which lead to membrane depolarization (MD) and turgor loss, are summarized from various authors (see the text for details). *ABA*, abscisic acid; *H_2O_2*, hydrogen peroxide; *KAT1*, inward potassium channels; *MD*, membrane depolarization; *NO*, nitric oxide; *OST1*, open stomata 1; *SnRK2* protein kinase; *NADPH*, reduced form of nicotinamide adenine dinucleotide phosphate; *RCAR/PYR1/PYL*, ABA-receptor/pyrabactin resistant protein/PYR-like proteins; *SLAC*, slow anion channels.

pathway, resulted in higher resistance to different stresses, including salinity, showing increased chlorophyll, proline, and soluble sugar content (Wang et al., 2017). The rapid increase in ABA at local (leaf) level, after a few minutes of NaCl exposure, has been detected in barley (Fricke et al., 2004). However, longer expositions (hours), to the saline environment still induced a reduction in the stomatal conductance, while ABA concentration at leaf level decreased significantly (Fricke et al., 2006, 2004). Therefore, the stomatal response to salinity, in the medium-long period, has mainly been related to signaling mechanisms responding to ABA produced at the root level (Chaves et al., 2009; Davies et al., 2005). However, many works have highlighted the importance of ABA produced in the various organs during the physiological response of plants exposed to stress conditions (Park et al., 2016). Exogenous ABA has commonly been found to activate genes involved in the response to osmotic stress (Huang et al., 2012) despite that there are genes active against osmotic stress that are not ABA-dependent (López-Pérez et al., 2009; Zhu, 2002). Table 1.1 reports a non-exhaustive compendium of recent works that describes the role of ABA and other signaling molecules involved in the control of stomatal movements under salinity.

ABA-induced stomatal closure can be associated to an ABA-activated protein kinase that controls anion channels in the plasma membrane (Ashraf and Harris, 2013; Danquah et al., 2014; Mittler and Blumwald, 2015; Fig. 1.2). The ABA receptors RCAR/PYR1/PYL (regulatory components of ABA-receptor/pyrabactin resistant protein/PYR-like proteins) binds ABA and forms a complex that inhibits the 2C protein phosphatases (PP2C) responsible for dephosphorylation, and then, inactivation of the protein kinase open stomata 1 (OST1) in absence of stress. OST1 belongs to a large category of protein kinases (SnRK2) that respond to ABA-dependent and ABA-independent signals under osmotic stress (Yoshida et al., 2014). After phosphorylation, the activated OST1 induces stomatal closure mainly through (1) depolarization of the intracellular plasma, operated by the activation of anion channels, which causes the release of K^+; and (2) inhibition of guard cell−inward KAT1 K^+ channels, which causes higher osmotic potential (Sato et al., 2009). OST1, via a plasma membrane-bound NADPH oxidase (Sirichandra et al., 2009), also promotes the production of H_2O_2, which catalyzes the inactivation of PP2C showing a feedback loop on OST1 activity (Mittler and Blumwald, 2015).

In summary (Fig. 1.2), ABA influences stomatal activity following different mechanisms based on the induction of stomatal closure and the inhibition of stomatal opening (Danquah et al., 2014). In this process, many different signaling molecules, which represent powerful second messengers, support ABA actions. ROS play a relevant role in these signaling mechanisms (Gudesblat et al., 2007). The accumulation of ROS in the apoplast can indeed be linked to stomatal closure (An et al., 2008). This was demonstrated through ABI1 and ABI2, which are encoding enzymes involved in stomatal closing. Using the *abi1* and *abi2* mutants, which show limitation in the phosphatase activities, ABA was found to be unable to generate ROS only in *abi1* mutants showing different ROS upstream and downstream action by ABI1 and ABI2, respectively (Murata et al., 2001). Other ABA-dependent mechanisms act through MAPKs, which are involved in stomatal development (Wang et al., 2008). MAPKs were found to be sensitive to the presence of H_2O_2 thus enhancing the transduction of H_2O_2 signaling at the cellular level (Danquah et al., 2014; Pitzschke and Hirt, 2009).

TABLE 1.1 Overview of Recent (2010–17) Works Assessing Relationships Between Primary or Secondary Signaling Molecules and Stomatal Activity in Salt-Stressed Plants

Species	Stomata measurements	Other performance parameters	Signaling molecule/s	Methodology	References
Dianthus superbus	Stoma and chloroplast development	Growth; photosynthesis; antioxidant enzyme activity	H_2O_2; $O^{\bullet}{}_2^-$	Pot experiment; increasing NaCl concentrations	Ma et al. (2017)
Triticum aestivum	Stomata movements and development	Growth; leaf water loss; proline and soluble sugar accumulation	ABA; H_2O_2	Liquid solution culture; 200 mM NaCl + exogenous ABA	Yang et al. (2016)
Medicago truncatula	Stomatal conductance	Growth; antioxidant enzyme activity; proline accumulation	H_2O_2; NO	Pot experiment (perlite/sand); growth chamber; 200 mM NaCl	Filippou et al. (2016)
Arabidopsis thaliana	Stomatal movements	Cotyledons characteristics	ABA	MS agar medium; 0, 125, 150 and 175 mM NaCl + exogenous ABA	Singh et al. (2015)
Arabidopsis thaliana	Leaf osmotic potential	Biometric parameters; leaf water loss; enzyme activity	H_2O_2	Hydroponic culture; growth chamber; 80 mM NaCl + exogenous tunicamycin	Ozgur et al. (2014)
Ocimum basilicum	Stomatal conductance and density	Growth; volatile compounds	ABA	Greenhouse/growth chamber; 0, 100, 200 mM NaCl; two cultivars	Barbieri et al. (2012)
Pisum sativum	Stomatal conductance	Growth; photosynthesis; antioxidant enzyme activity; mineral content	H_2O_2	Hydroponic system; growth chamber; 150 mM NaCl	Martí et al. (2011)
Solanum lycopersicum	Stomatal conductance	Growth; yield; proline accumulation	ABA	Greenhouse experiment; soilless rockwool; 0, 20, 40 mM NaCl	Orsini et al. (2010)

The role of ABA in membrane depolarization is relevant for stomatal closure (Fig. 1.2). The phosphorylation of the slow anion channels (SLAC), which release anions outside the guard cells, is a fundamental mechanisms for the depolarization process (Brandt et al., 2012; Lee et al., 2009). In addition, ABA acts through the inhibition of the cell plasma membrane H^+-ATPase (Danquah et al., 2014; Hayashi et al., 2011) and the activation of Ca^{2+} channels in which H_2O_2 takes part (Pei et al., 2000; Wang et al., 2016a). Membrane depolarization is also driven by the alkalization of the cytosol (Fig. 1.2) since pH increases have been observed to precede ROS signaling in stomatal closure (Sobahan et al., 2015). Cytosolic acidification has instead been associated to ROS removal in guard cells. Conversely, in presence of osmotic stress, stromal acidification in chloroplast has been observed at increasing ROS concentration (Wang et al., 2016a) as reversed conditions of optimal photosynthetic activity when stromal pH is typically higher (Taiz et al., 2015). Elevated concentrations of Ca^{2+} in the cytosol promotes the activation of SLAC1 thus causing the inhibition of inward KAT1 K^+ channels due to membrane depolarization (Osakabe et al., 2014; Wang et al., 2016b). Yet, Ca^{2+} would stimulate the activity of NADPH oxidase (Fig. 1.2) responsible for increased H_2O_2 (Sirichandra et al., 2009) acting in a feedback loop to boost Ca^{2+} influx and to improve stomatal closure efficiency in general (Mittler and Blumwald, 2015; Munemasa et al., 2013; Saxena et al., 2016). Together with ROS, Ca^{2+} therefore appears to be a strategic second messenger in the stomatal response of plants exposed to saline stress (Basu and Rabara, 2017). Recently, it has been highlighted that the interaction between H_2O_2 and NO would improve the signaling cascade between Ca^{2+} and ABA in the control of stomatal movements (Li et al., 2017b; Saxena et al., 2016). Yet, the interaction between Ca^{2+}, H_2O_2, and NO in the control of stomata closure has been observed in *A. thaliana* during pharmacological experiments (Niu and Liao, 2016). Wang et al. (2016a,b) have proposed a model in which chloroplast Ca^{2+} firstly, and then ROS, are involved in the achievement of the early state for stomatal closure.

NO takes part in many mechanisms of response to stress conditions thereby resulting in a strategic signaling molecule in the ecophysiology of plants growing under saline environments (Niu and Liao, 2016). Endogenous NO is synthetized by NO synthase but also by nitrate reductase via reduction of nitrite (Neill et al., 2008). The increase in H_2O_2 concentration, induced by ABA, brings about NO generation via

nitrate reductase and nitrogen oxide synthase activity (Neill et al., 2008). The positive effect of NO in stomatal closure has largely been demonstrated through exogenous NO application in many different plant species (Desikan et al., 2002; Mata and Lamattina, 2001; Neill et al., 2002). However, the production of endogenous NO has been observed when ABA-induced stomatal closure occurs, while the presence of treatment against NO partially limited the closure mechanism (Neill et al., 2008). In *A. thaliana*, it has been demonstrated that NO and H_2O_2 are both involved in the stimulation of stomatal closure (He et al., 2013). Yet, transgenic plants of *A. thaliana* were modified to overexpress NO synthase activity, which resulted in an increased accumulation of NO and eventually displayed improved resistance to salt stress (Foresi et al., 2015). Indeed, a strong interaction between H_2O_2 and NO, as second messengers in signaling stressful conditions, has been well documented for higher plants growing under saline conditions (Niu and Liao, 2016).

Other than stomatal movements, ABA produced under salinity stress acts on plant water balance also by increasing guard cell permeability to water, and by the activation of water channels. ABA has, in fact, been identified to be a signal hormone for aquaporins influencing their activity through different mechanisms from gene expression to the modification of gene products (Dar et al., 2017). In ABA-deficient barley mutant Az34, the presence of exogenous ABA induced the proliferation of aquaporins, and improved hydraulic conductivity of root cells (Sharipova et al., 2016). Corpas et al. (2017) have supposed that ABA-activated aquaporins could have a relevant role in the translocation of H_2O_2 produced by peroxisomes as second messengers of abiotic stress in plants. Yet, aquaporins have also been reported to facilitate the intake of H_2O_2 into guard cells during ABA-induced stomatal closure (Rodrigues et al., 2017). Undoubtedly, the abundance of aquaporins can be associated to possible plant strategies of resistance under salinity (López-Pérez et al., 2009).

1.3.2 Photosynthesis

The main consequence of stomatal closure is the reduced photosynthesis (Mittler and Blumwald, 2015; Munns and Tester, 2008; Neill et al., 2008; Park et al., 2016) since the photosynthetic machinery is generally inhibited by salinity (Parida and Das, 2005). In higher plants, the photosynthetic activity is one of the most important performance, and then stress, indicators for its implications in the production of primary and secondary metabolites, and signaling molecules as well (Ashraf and Harris, 2013). Net photosynthesis shows heterogeneous responses when plants undergo saline stress depending on time of exposure, intensity, and plant tolerance. Increase in the assimilation of CO_2 per unit area has been observed in salt-stressed wheat (James et al., 2002). This apparently controversial plant response is due to the higher concentration of chloroplast caused by the positive association between salinity and leaf thickness (Munns and Tester, 2008). On the other hand, when photosynthesis is expressed per unit chlorophyll reductions are commonly observed in salt-stressed plants (Munns and Tester, 2008). The lower carbon intake which, in turn, limits plant growth, is therefore a phenomenon that mostly occurs at the whole-plant level and mainly relates to the lower leaf area showed by salt-stressed plants (Parida and Das, 2005). However, the reduction in plant growth causes the accumulation of carbohydrates in plant tissue thereby stimulating feedback signals to control photosynthetic activity (Munns and Tester, 2008).

When photosynthesis is inhibited the production of ROS increases, due to photoxidative stress, as well as the enzyme activity involved in their removal (Apel and Hirt, 2004; Chaves et al., 2009; Foyer and Noctor, 2005; Munns and Tester, 2008; Suo et al., 2017). Photoxidative stress is one of the main metabolic processes producing ROS in plants due to many reactions involved in the photosynthesis and photorespiration process (Foyer and Noctor, 2003), which in presence of reduced stomatal conductance cause the increase of the O_2/CO_2 ratio (Koyro et al., 2011). The production of ROS at low level is normal when plants are exposed to optimal growing conditions but it increases dramatically in presence of salinity stress thus causing their abnormal accumulation in the production sites then translocated within plant tissues. The limited photosynthesis activity under salinity is therefore one of the most relevant mechanisms triggering stress signaling related to ROS, especially superoxide and H_2O_2, which take part in many plant growth and development processes as second messengers (Apel and Hirt, 2004; Foyer and Noctor, 2005; Koyro et al., 2011; Khan et al., 2013).

ROS in salt-stressed plants are largely produced at the chloroplast and mitochondrial levels other than in the peroxisomes (Apel and Hirt, 2004; Miller et al., 2010). In chloroplasts, oxygen reduction by PSI brings about the production of superoxide, then converted in H_2O_2 through CuZn-superoxide dismutase (Asada, 1999; Asada et al., 2000) as an intermediate product of the water−water cycle that, in normal conditions, would eventually end with the production of H_2O (Miller et al., 2010). Furthermore, when the intracellular concentration of CO_2 declines, due to stomatal

closure, the photorespiratory oxygenation of ribulose 1,5-bisphosphate becomes strategic to preserve the integrity of PSII. Nevertheless, this process leads to the production of 2-phosphoglycolate, which is then converted to glycolate, whose oxidation process causes the accumulation of H_2O_2 in plant cells (Apel and Hirt, 2004). Under salinity stress, singlet oxygen is also accumulated in chloroplasts due to PSII activity (Miller et al., 2010). In mitochondria, one of the most relevant mechanisms that generates ROS has been related to the overreduction of the electron transport chain. However, the contribution of these organelles to the total ROS balance is lower than chloroplasts and peroxisomes (Miller et al., 2010). In peroxisomes, H_2O_2 and superoxide are both abundantly produced as a result of many metabolic processes (Miller et al., 2010). The oxidation of glycolate to glyoxylic acid would cause the production of H_2O_2 in peroxisomes at increasing rates of photorespiration (Mittler et al., 2004). In addition, the dismutation of superoxide, fatty acid b-oxidation, and the flavin oxidase pathway are all processes causing the formation of H_2O_2 in these organelles. In salinity-like stress (i.e., drought stress) experiments, polyamine catabolism has also been found to regulate ROS concentration in peroxisomes (Miller et al., 2010).

When plants are able to counteract the detrimental presence of ROS in cells, a steady state between ROS formation and removal is achieved while H_2O_2 is kept at a level optimal for cell signaling (Mittler et al., 2004; Munns and Tester, 2008). The production of ROS due to photoxidative stress triggers the activation of many genes involved in antioxidant activities by various enzymes such as superoxide dismutase, glycolate oxidase, catalase, and ascorbate peroxidase, which play a strategic role for the removal of ROS (Apel and Hirt, 2004). The progressive rise of the above enzyme activity has been observed in plants under salinity stress induced in the root zone (Hussain et al., 2015). However, enzymatic ROS scavenging activities are not the only mechanisms in counteracting salinity stress (Miller et al., 2010, 2007) while the complex pathway surrounding ROS as signaling molecules is still not well understood (Mittler et al., 2004).

The above discussed aspects underline the toxicity of ROS, while also highlighting their beneficial effect as powerful molecules improving the transduction of stress-related signals (Miller et al., 2010). Nonetheless, many experiments have been set to prove the role of ROS and NO in the stomatal response as plant strategy to face salinity stress (Desikan et al., 2005; Niu and Liao, 2016). The higher ability of pumpkin-grafted cucumber to counteract the effects of osmotic stress was associated to the increase of H_2O_2 in plant tissue that caused earlier stomatal closure relative to self-grafted plants (Niu et al., 2017). Similar findings were obtained using tomato grown under standard and increased concentration of atmospheric CO_2 (Yi et al., 2015), and pea as test plants (Desikan et al., 2004; Yi et al., 2015). In *Vicia faba*, it was observed that NO and ROS act together to mediate the ABA-induced inhibition of stomatal opening (Yan et al., 2007). While the role of ROS and NO in stomatal control was rarely investigated under salinity stress (Filippou et al., 2016), many authors (Table 1.1) report that ABA plays a relevant role in both stomatal closure and opening to control salinity effects with the main aim of reducing water loss (Dar et al., 2017; Kim et al., 2010).

The signaling molecules that regulate gaseous exchange activity of plants exposed to salinity are mostly revealed and the basic action mechanisms are often well understood. Since many processes follow multigenic mechanisms of activation and action, one of the main difficulties lies in the individuation of key genes and proteins. However, the ongoing challenge is to turn the knowledge of those mechanisms and the related physiological responses into tools for boosting cropping systems in areas affected by salinity.

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CHAPTER

2

Stress Responsive Signaling Molecules and Genes Under Stressful Environments in Plants

Murat Dikilitas[1], *Eray Simsek*[1] *and Sema Karakas*[2]

[1]Department of Plant Protection, Harran University, Şanlıurfa, Turkey [2]Department of Soil Science and Plant Nutrition, Harran University, Şanlıurfa, Turkey

OUTLINE

2.1 INTRODUCTION

The changing climatic and environmental conditions and human-oriented pollution increase the possible occurrences of combined stresses of pathogens and abiotic stresses. Evidence suggests that abiotic stresses will affect the distribution and pathogenicity of quite a range of pathogens with increased virulent strain or race development (Garrett et al., 2006; Dikilitas and Karakas, 2012; Ramegowda and Senthil-Kumar, 2015). Although the possible interactions between biotic and abiotic agents in plants were analyzed in the past through the examination of stress parameters via the biochemical and molecular methods, the molecular and biochemical basis of combined interactions between abiotic–abiotic, and abiotic–biotic interactions remain elusive in terms of signaling molecules such as the production of nitric oxide (NO), reactive oxygen species (ROS), and accumulation of plant growth regulators such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA), and abscisic acid (ABA) (Ramegowda et al., 2013; Khan et al., 2015; Khan and Khan 2017; Lai et al., 2018; Lalithya et al., 2017; Per et al., 2018; Zheng et al., 2018). When individual stress agents are examined, their low-level effect could increase the transmission of signaling molecules, which

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induce the defense responses. When the stress is prolonged and becomes severe, these molecules could act as prooxidants and become signals of diseases.

In nature, plants are exposed to the combination of abiotic and biotic stresses either simultaneously or sequentially. Although this type of stress has been recognized for many years, their molecular and biochemical pathways should be evaluated by producing new pathways. The interactions between stresses could be negative, positive, or additive. When plant faces both abiotic and biotic stress factors simultaneously or sequentially, their behaviors in terms of molecular, physiological, and biochemical pathways have potential to modify themselves to the changing conditions. The changing pattern of host plants to the stress could be slow or fast depending on the severity and duration of the stress factors as well as the resistance of crop plants. In most cases, the response of crop plants is slow and therefore, crop plants die quickly due to sudden depletion of biochemical metabolites that have already been prepared for only one type of stress in advance. The response of plants to the simultaneous or sequential actions of several combined stress factors is different from those of plants exposed to a single stress factor. Ramegowda and Senthil-Kumar (2015) stated that concurrent occurrence of drought and pathogen during plants' development provoked complex pathways controlled by various signaling events. Plants tolerating two or more independently occurring stresses need not necessarily tolerate when these stresses occur simultaneously or sequentially (Nostar et al., 2013; Dikilitas et al., 2017). The reaction of plants, in general, depends on the sensitivity of species or cultivars, the intensity of stress factors, duration of exposure, and the mode of action of stress agents on plant metabolism.

In abiotic and biotic stress combinations, the instant simultaneous effect is not very common. One of the stress agents, in general, develops gradually. For example, pathogens can infect plants that are already stressed with abiotic stress agents or abiotic stress gradually develops on already pathogen-infected plants. Under these circumstances, the outcome of these interactions varies depending on the severity of each stress (Xu et al., 2008; Dikilitas et al., 2017). Plants exposed to mild abiotic stress or low pathogenicity of pathogens activate their basal defense systems. On the other hand, severe abiotic stress causes membrane leakage, which fills up the apoplast with cellular nutrients and enhances the sporulation and development of attacking pathogens and as a result of that, it facilitates successful pathogen infection. The same case is also valid for the attacking of virulent pathogens. They could destroy the defense barrier of the host via secretion of cell wall degrading enzymes or toxins, which could stop the many functions of cell metabolites and predispose the plants to abiotic stressors (Dikilitas et al., 2018). For example, a significant positive correlation between spot blotch (caused by *Cochliobolus sativus*) disease severity in wheat genotypes and average nighttime temperature was evident (Sharma et al., 2007). Again, high-temperature stress in combination with *Puccinia striiformis* f. sp. *tritici* (causal agent of stripe rust) increased the disease incidence in spring wheat (*Triticum aestivum*) (Milus et al., 2009). They stated that temperature-adapted "new" isolates (12−28°C) were more aggressive than "old" isolates adapted to cool temperatures (10−18°C) for all cultivars treated. At low-temperature, "new" isolates sporulated earlier, grew faster, and produced more spores per lesion per day compared with those of "old" isolates. Similar patterns were also observed at high-temperature regime. The authors suggested that wheat rust fungi could cause severe disease in unfavorable conditions via adaptation to extreme temperatures. Similar issues were observed with those of salinity, high temperature, and drought stresses. For example, simultaneous exposure of common bean (*Phaseolus vulgaris*) to drought stress and a fungal pathogen, *Macrophomina phaseolina* (causal agent of charcoal rot and seedling blight), resulted in high transpiration rate and leaf temperature when compared with plants subjected to only drought stress (Mayek-Pérez et al., 2002). Again, exposure of tomato plants (*Lycopersicon esculentum* Mill.) to salinity stress either before or after inoculation with *Pseudomonas syringae* pv. tomato or *Phytophthora capsici* induced root and crown rot with severe symptoms when compared with nonstressed control or pathogen-inoculated plants (Pye et al., 2013; Bostock et al., 2014). Salt stress significantly reduced fresh weights and increased root and crown necrosis under pathogen infection. Similar findings were also made by Dikilitas (2003) who stated that even very low concentrations of NaCl (50 mmol/L) along with the inoculation of wilt pathogen *Verticillium albo-atrum* on tomato plants resulted in more severe symptoms than those of NaCl (50 mmol/L) salinity or *V. albo-atrum* infection alone (Fig. 2.1). The pathogen was able to survive in a large range of NaCl concentrations (Fig. 2.2). Again, under salinity stress, the performance of cell wall degrading enzymes of *Rhizoctonia solani* and *Sclerotium rolfsii* and their pathogenicity varied profoundly (El-Abyad et al., 1997). The activities of cell wall degrading enzymes of *R. solani* decreased and those of *S. rolfsii* increased with the increase of salt concentrations when the cell wall material was used as a sole carbon source. Recently, Shankar et al. (2016) reported that the increased Na^+ uptake and accumulation by stressed plants resulted in increased root permeability in chickpea. The increased severity of plants

FIGURE 2.1 Tomato plants (A) control; (B) inoculated with *Verticillium albo-atrum* (isolate V2), growing under hydroponic conditions showing symptoms such as wilting and flaccidity on the lower leaves; (C) treated with NaCl showing wilting and chlorosis; and (D) inoculated with *V. albo-atrum* (isolate V2) and treated with 50 mmol/L NaCl (Dikilitas, 2003).

under NaCl stress was also attributed to the stimulatory effect of Na^+ ions on mycelial growth of fungi as in the case of *Botrytis cinerea* (Boumaaza et al., 2015; HanumanthaRao et al., 2016). Similarly, Huot et al. (2017) reported that high-temperature stress promoted translocation of bacterial effector proteins into plant cells and caused a loss of ICS1-mediated SA biosynthesis. Therefore, the combined stress of temperature and pathogen prevented the SA-based defense mechanism.

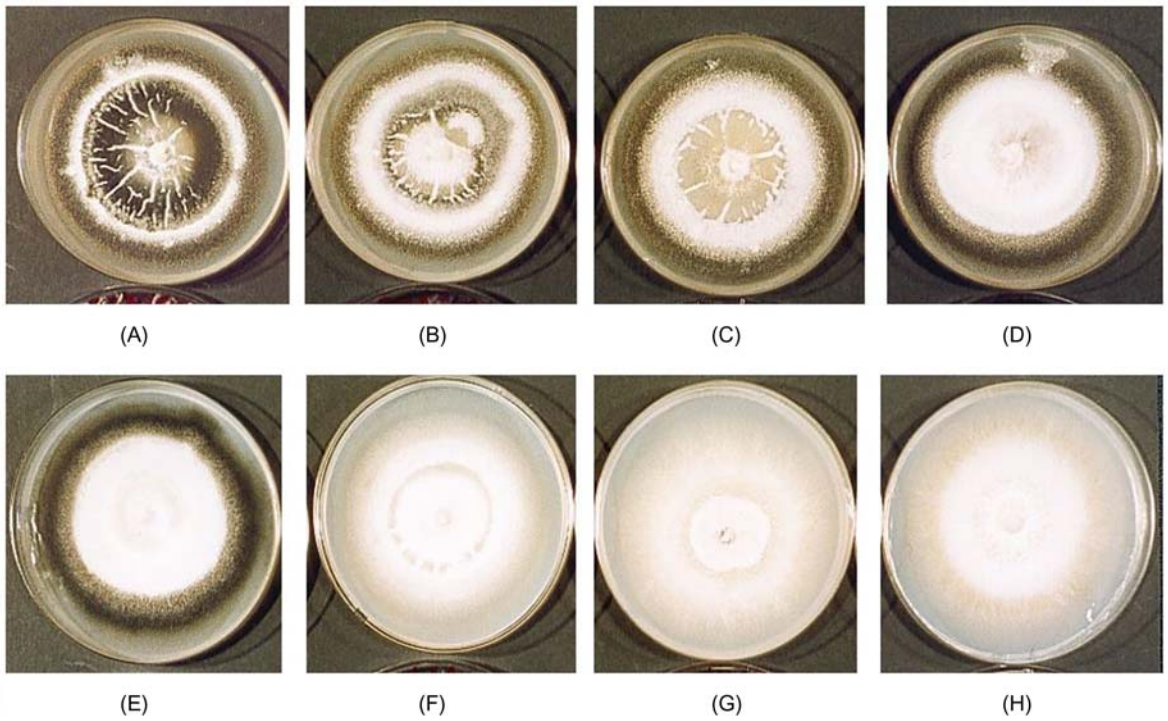

FIGURE 2.2 Development of mycelium of *V. albo-atrum* (isolate V1) on Dox medium containing different concentrations of NaCl. (A) Control, 0 mmol/L NaCl; (B) 25 mmol/L; (C) 50 mmol/L; (D) 100 mmol/L; (E) 150 mmol/L; (F) 250 mmol/L; (G) 300 mmol/L; and (H) 350 mmol/L NaCl (Dikilitas, 2003).

To prevent the crop plants from secondary stress agents, scientists suggested some ideas related to plant nutrition such as application of $CaSO_4$ or K_2SO_4 or plant growth substances, etc., which could have adverse effects on growing fungi in leaf surfaces and positive effects on salt tolerance of crop plants (Stockwell et al., 2012; Jabnoun-Khiareddine et al., 2016). However, under abiotic stress conditions such as salinity, drought, temperature, and heavy metal pollution stresses, plants tend to accumulate appreciable amounts of chloride, sulfate, and other toxic ions in tissues that limit the growth and yield potential as well as defense responses. By supplying beneficial substances to stressful plants could be a limited solution. The microorganisms could adapt themselves to the conditions of harsh environments. For example, Abbas and Mandeel (1995) isolated *Fusarium oxysporum*, *F. sambucinum*, *F. clamidosporum*, *F. solani*, *F. moniliforme*, etc., from the soil characterized with high salinity. Similar issues were made by Maggi et al. (2013) who stated that macrofungal community of some Italian alpine habitats, in which *Inocybe microfastigiata*, *Laccaria Montana*, and *Lactarius salicis-herbaceae* fungi were found adapted to very cold temperatures. Previously, Hasan (2002) reported that fungal species such as *Aspergillus flavus*, *A. niger*, *F. oxysporum*, *Penicillium* spp., *Rhizopus* spp. inhabiting in rhizosphere and rhizoplane of faba bean, sesame, soybean, etc. produced more gibberellin and indole-acetic acid (IAA) at 0.5% and 1% NaCl after 5 days. Therefore, application of fertilizers or chemicals that help crop plants grow better in harsh conditions would have no efficient use due to the adaptation of pathogens. Since virulence was positively correlated with the activities of cell wall degrading enzymes exerted by the pathogens, it is important to note the level of pathogenic enzymes before or after the stress, especially after long-term stress, which could lead to increase of pathogenic enzymes.

Interactions between abiotic stress and virus diseases were evident as well. Simultaneous exposure of *Arabidopsis* plants to drought and turnip mosaic virus (TuMV) led to a higher reduction in plant weight and leaf numbers compared with those of individual stresses (Prasch and Sonnewald, 2013). Similarly, Moury et al. (1998) and Király et al. (2008) stated that the combined effects of high temperature and tomato spotted wilt virus or tobacco mosaic virus (TMV) suppressed the resistance of pepper (*Capsicum annuum* and tobacco (*Nicotiana tabacum*), respectively. Similar findings were also made by Dossa et al. (2017) who stated that plant height and dry shoot biomass of rice plants were remarkably reduced by drought stress treatments. On the other hand, the authors concluded that effect of drought stress response could vary according to rice

genotype. It is possible that drought stress could interfere with accumulation of ABA over the plant defense response. For example, Mohr and Cahill (2003) stated that both additions of 100 μM ABA to plants and drought stress resulted in increased susceptibility of *Arabidopsis thaliana* to an avirulent isolate of *P. syringae* pv. *tomato*. In contrast, an ABA-deficient mutant of *Arabidopsis* exhibited reduced susceptibility to virulent isolates of *Peronospora parasitica* or *P. syringae* pv. *tomato*. Similar issues were also made by Sherwood et al. (2015) in which the drought-induced proline accumulation and ROS metabolism invoked susceptibility toward *Diplodia pinea* in Austrian pine.

There is a synergistic relationship between frost and pathogen occurrences as well. Ferrante and Scortichini (2014) reported that autumn and winter frosts resulted in a sudden outbreak of bacterial canker caused by *P. syringae* pv. *actinidiae* in *Actinidia chinensis* and *A. deliciosa* trees. During thawing stages, bacterial colonization was observed at a 2-cm distance upwards and downwards from the site of inoculation within 3 min and the leaves were extensively colonized with the pathogen. It appears that the defense mechanisms of plants are weakened, and further damage caused by sequential stress agents result in quality and quantity loss at large. At this point, it is very difficult to assess the level of damage in advance due to changing mechanisms of plant cell response to multiple attacks.

There can also be an antagonistic effect among stressors that the effect of one stress factor is reduced while the effect of other stress factor stays the same as if acting alone. It should be kept in mind that two different stressors would not nullify each other, therefore, plants have to deal with at least one of the stress factors all the time. This antagonistic scenario gets further complicated by a combined stress occurrence. For example, salinity stress increased resistance of barley (*Hordeum vulgare*) plants to *Blumeria graminis* (causal agent of powdery mildew) in a concentration-dependent manner (Wiese et al., 2004). They stated that salinity stress exerted both osmotic and ion toxicity on the pathogen growth, which eventually reduced the pathogenicity. Similarly, Kissoudis et al. (2016) stated susceptibility of susceptible or partial resistant lines of tomato increased at mild salt stress (50 mmol/L NaCl) toward *Oidium neolycopersici* (a causal agent of powdery mildew in tomato). However, NaCl stress over 150 mmol/L reduced the disease symptoms in resistant cultivars. The authors stated that Na^+ and Cl^- accumulation in the leaves was linearly related to the decreased pathogen symptoms under combined stress. They concluded that increased susceptibility under combined stress was associated with the induction of ET and JA pathway genes. Similarly, drought stress reduced fungal pathogen, *B. cinerea* (causal agent of gray mold) infection by 50% in tomato plants and suppressed the spread of another fungal pathogen, *O. neolycopersici* (causal agent of powdery mildew) due to a concomitant increase in endogenous ABA levels (Achuo et al., 2006). Ramegowda et al. (2013) stated that drought acclimation in advance in *Nicotiana benthamiana* plants imparted tolerance to necrotrophic fungus, *Sclerotinia sclerotiorum*, and to the hemibiotrophic bacterial pathogen, *P. syringae* pv. *tabaci*. Acclimated plants with drought stress led to less disease-induced cell death and reduced disease symptoms compared with non-acclimated plants. They stated that the generation of ROS during drought acclimation resulted in increased disease resistance and primed a defense response that subsequently led to tolerance against pathogens. Ramegowda et al. (2013) also suggested that ABA could change pattern for the attacking pathogens via closing stomata or reduction photosynthesis. Therefore, early exposure of plants to drought stress results in the onset of increased ABA and ROS levels and induction of PR-protein coding genes and contributed to disease resistance under combined stress (Ramegowda and Senthil-Kumar, 2015).

As seen so far, the role of ROS and ABA has been controversial in plant defense systems. The susceptibility of plants could be affected by abiotic stress agents in both ways. Ramegowda and Senthil-Kumar (2015) stated that pathogen infection on already drought-stressed plants can either lead to plant resistance to pathogen infection through drought-induced activation of basal defense or can result in susceptibility due to weakened basal defense. Drought-induced pathogen resistance was attributed to the enhanced expression of antimicrobial and PR-proteins activated by drought. On the other hand, the susceptibility of crop plants was attributed to high levels of ABA under drought stress, which can interfere with pathogen-induced plant defense signaling and thereby reducing the expression of defense-related genes (Li and Luan, 2014; Ali et al., 2017). For example, Ali et al. (2017) reported that pretreatment of *Brassica juncea* with SA and JA stimulators downregulated each other's signature genes suggesting an antagonistic relationship between SA and JA. They also reported that after ABA treatment, SA signatures were downregulated while JA signature genes were upregulated. ABA-SA interaction is two-sided. SA signaling via pathogen inoculation negatively affects ABA responses. However, ABA contributes to the formation of callose, which prevents pathogen penetration. On the other hand, ABA signaling mainly inhibits signals that activate biotic stress signaling. Therefore, combined stress enhances pathogen spread from the initial site of infection. Combined stress, if one of them is not detoxified, is able to affect the nucleus and its contents. Just like ROS, the

concentration of ABA plays significant roles in resistance to disease. It can either prevent the pathogen infection through stomatal closure or make plants more susceptible to invading pathogens by suppressing JA, SA, or ET-mediated signaling. Here, it is important to note that the level of ROS or ABA should not exceed a certain level that would be characterized as toxic. For example, Gupta et al. (2016) stated that the sporulation of *P. syringae* pv. *tomato* under drought stress was reduced due to activation of basal defense response through ABA-mediated gene regulation in *A. thaliana* plants. This also reduced ROS generation and cell death and suppressed the effect of pathogen infection (Fujita et al., 2006). Sinha et al. (2016) stated that drought-stressed chickpea plants challenged with *P. syringae* pv. *phaseolicola* (leaf pathogen) and *Ralstonia solanacearum* (xylem inhabiting wilt pathogen) exhibited reduced bacterial colonization in chickpea plants as compared with pathogen infection alone. In a similar manner, proline, a stress-related amino acid, could be accumulated upon drought-, salinity- or heavy metal−exposed plants (Per et al., 2017). In this response, one of the stress agents, especially biotic ones, should have low or mild effects. On the other hand, nonvirulent or less virulent biotic stress agents act as elicitors and enable protection for plants to further abiotic stress agents. However, if the stress agents such as drought, salinity, heavy metal pollution, etc. have a continuous effect, then the antagonistic effect of the biotic stress agents would have no beneficial effects after some stage.

The antagonistic interactions were also found between virus and abiotic stress factors as well. *N. benthamiana* plants infected with brome mosaic virus (BMV), cucumber mosaic virus (CMV), and TMV showed delayed the appearance of leaf wilting and stem dehydration under the combination of drought and virus stress when compared with the only drought-stressed plants (Xu et al., 2008). BMV- and CMV-inoculated plants exhibited increased accumulation of osmoprotectants such as glucose, fructose, and sucrose (Xu et al., 2008; Tauzin and Giardina, 2014). In addition, virus-infected plants showed lower transpiration rate due to the partial stomatal closure.

Antagonistic effects could be observed among abiotic stress factors as well. For example, mild stress factors could trigger physiological and biochemical defense mechanisms that enable plants to continue growth under stressful conditions. For example, Bandurska et al. (2013) reported that UV-B radiation enhanced the resistance of *A. thaliana* to water stress. They concluded that H_2O_2, NO, ABA, JA, ET, and SA participated in the activation of defense mechanisms. However, if the duration of stress is prolonged or one of them is severe, the stress tolerance cannot be achieved, leading to cell death and tissue necrosis. Similar results were also made by Majsec et al. (2016) who stated that toxic effects of cadmium alone were more prominent on tobacco than that of the combined stress of cadmium and copper. However, mixtures of high concentrations of both cadmium and copper had the most adverse effects. This was directly related to the increase of ROS, malondialdehyde (MDA), and protein carbonyl (PC) contents.

Sometimes antagonistic responses between stresses result in synergistic effects in crop plants. For example, during heat stress, plants open their stomata to cool down their leaves by transpiration. However, heat stress with a combination of drought would not allow plants to open their stomata and their leaf temperature would be higher resulting in more damaging effects on plants (Rizhsky et al., 2002; Mittler, 2006). It is known that the stomatal opening and closing organization is regulated by signaling molecules. However, the sequence of stress would determine the function of stomata. Lu et al. (2017) stated that the combination of *P. capsici* infection and heat caused more severe damages to pepper seedlings than either of the individual stresses. Under heat stress, plant leaves make more transpiration by opening their stomata to cool down the extra heat, however, this predisposes crop plants and makes them more susceptible to invading pathogens (Prasch and Sonnewald, 2015). If plants close their stomata to prevent pathogen infection and spread within leaf tissues, this increases the canopy temperature and leads to heat injury (Atkinson and Urwin, 2012). It is possible that overaccumulation of ROS may cause dysfunction of the cell membrane and results in susceptibility in susceptible and moderately resistant cultivars. When signaling molecules and gene expressions were tested, *C. annuum* heat shock protein (*CaHSP*) genes were upregulated in resistant (CM334) and susceptible (EC) varieties of pepper plants under stress alone. However, under heat stress, *P. capsici* infection, the gene expression increased in the resistant cultivar *CM334* while decreasing in the susceptible cultivar EC. The defense-related genes were more upregulated under the combined stress than under the individual stresses. On the other hand, the Ca^{2+}-signaling pathway genes were enhanced in susceptible cultivar "EC," they were inhibited in resistant cultivar "CM334." *HSP* genes were expressed during heat stress alone to alleviate the conditions of plants under heat stress, but these genes were stable under *P. capsici* inoculation alone in both the susceptible "EC" and resistant "CM334" cultivars (Bokszczanin et al., 2013; Lu et al., 2017). Similarly, pathogenesis-related (PR) proteins play significant roles to prevent further pathogen attack and improve the conditions of plants under pathogenic stresses (Sudisha et al., 2012). Under

P. capsici inoculation alone, *CaPO1* (peroxidase), *CaPR1* (pathogenesis-related protein), CaSAR82A (systemic acquired resistance) genes were upregulated in resistant *CM334* pepper cultivar, and the response was increased when *P. capsici* was combined with heat stress treatment. However, no significant expression in pathogenesis-related genes was noticed in the susceptible cultivar EC. Since a higher antioxidant capacity along with lower ROS accumulation is associated with plant tolerance/resistance to abiotic or biotic or to both stresses (Suzuki et al., 2014), cultivars that could not express signaling molecules and stress-related genes would not be able to survive under the combined stresses in the long run. Similarly, calcium plays important roles in mediating abiotic and biotic stress signals. Lu et al. (2017) stated that Ca^{2+}-signaling-related genes under heat stress were suppressed in susceptible pepper cultivar EC but enhanced in resistant cultivar *CM334*. However, this did not follow a clear pattern between abiotic and biotic stresses. Therefore, the molecular responses could not be predicted in advance by examining the responses of individual stresses.

In this chapter, interactions between abiotic and biotic stressors were evaluated and their combined stressful effects were outlined in crop plants in terms of signaling molecules, gene expression patterns and enzymatic and hormonal responses in the light of new molecular and biochemical findings and approaches. The mechanisms of interactions were briefly outlined in Table 2.1 before detailed evaluation.

2.2 SIGNALING MOLECULES UNDER STRESS CONDITIONS

When crop plants get exposed to stress factors, they respond by enhancing production of ROS, which eventually leads to protein-, lipid-, and carbohydrate-oxidation and DNA damage (Ahmad et al., 2010). Although ROS have destructive effects on cell metabolism, they also act as signaling molecules in the cellular process. Low concentrations of signaling molecules participate in the activation of defense-related mechanisms involving enzymatic and nonenzymatic antioxidant systems, flavonoid biosynthesis, accumulation of low−molecular weight compounds, vitamin C, anthocyanins, etc. (Liang et al., 2018). Therefore, the signal transduction pathways, in general, lead to activation of biosynthesis of proteins, osmoprotectants, and detoxification enzyme systems (Prasch and Sonnewald, 2015). However, under continuous stress or severe short-term stress, the efficiency of the antioxidant system decreases, and the capacity of the defense system becomes exhausted leading to membrane damage and even cell death (Bandurska et al., 2013). Plants have developed effective stress detection mechanisms and efficient signal transduction pathways to respond to various pathological and environmental stresses quickly (Petrov et al., 2015; Hoque et al., 2016). The signaling molecules include various plant hormones such as SA, JA and ET, ABA, auxin, gibberellin (GA), cytokinin (CK) and brassinosteroid (BL), ROS, lipid, mRNA accumulation, vitamins, ion homeostasis, proteins, sugar, NO, Ca^{2+}signaling, etc. They play crucial roles in regulating developmental processes and signaling networks in a coordinated way in a wide range of biotic and abiotic stresses (Robert-Seilaniantz et al., 2007; Baxter et al., 2014; Prasch and Sonnewald, 2015). Signal transduction is the metabolism in which extracellular physiological stimuli are transmitted via signaling cascade into intracellular signals that involve multiple genes/proteins to regulate the expression patterns of the key genes. When these are orchestrated properly, a quick response will be triggered to protect plants from further damages. Signal transduction starts from the receptor activation and simply involve secondary messengers, transcription factors, stress-responsive genes, phosphoprotein cascades (Gong et al., 2013). Therefore, all signaling molecules take part in the regulation of the defense and growth mechanisms. Many scientists have reported that the plasma membrane (PM) is responsible for perceiving and transmitting external stress signals (Gong et al., 2013). Sensors located in membranes perceive the signals. For example, phytohormones magnify the initial signals and trigger a new signaling event either following the same pathway or using other signaling pathways with different components (Huang et al., 2012; Prasch and Sonnewald, 2015). For example, leaf stomata or leaf surfaces are closed under drought stress and cell osmotic potential decreases. As a result, photosynthesis is reduced. Plants, therefore, must constantly adjust stomatal conductance to allow enough CO_2 uptake and avoid unnecessary water loss during water stress. In another way, plants must always sense the water stress. ABA, a phytohormone, plays important roles in stress signaling such as transcriptional changes, stomatal closure, etc. (Sreenivasulu et al., 2012; Dar et al., 2017). The accumulation of ABA in roots is one of the fastest responses to water or drought stress. It is loaded to xylem vessels and transported to the leaf cells via the transpiration stream. This hormone plays a crucial role in regulating the expression of many stress-related genes. Crop plants under stress synthesize proteins, enzymes, and nonenzymatic metabolites such as ascorbic acid (vitamin C), glutathione, proline, etc. to remediate the harmful effects of the stresses. All these responses are triggered by ABA-dependent and ABA-independent

TABLE 2.1 Interactions Between Abiotic–Abiotic; Abiotic–Biotic Stress Agents

Stress interactions	Biotic stress		Abiotic stress	Host plant(s)	Physiological, biochemical, and molecular response	References
	Causal agent	Disease name				
Biotic & Abiotic stress combinations (synergistic or additive)	*Cochliobolus sativus*	A causal agent of Spot blotch disease	Temperature	Wheat	Increased disease symptoms	Sharma et al. (2007)
	Puccinia striiformis f.sp. *tritici*	A causal agent of Stripe rust disease	Temperature	Wheat	Increased disease symptoms	Milus et al. (2009)
	Macrophomina phaseolina	Charcoal rot and seedling blight	Drought	*Phaseolus vulgaris*	Higher transpiration rate	Mayek-Pérez et al. (2002)
	Phytophthora parasitica	Root and crown rot disease	Salinity	Tomato	Severe root rot symptoms	Swiecki and MacDonald (1991)
	Verticillium albo-atrum	Wilt disease	Salinity	Vegetables	Increased wilting and root rots	Dikilitas (2003)
	Turnip mosaic virus	Mosaic disease	Drought	*Arabidopsis thaliana*	Higher reduction in plant weight	Prasch and Sonnewald (2013)
	Rhizoctonia solani	Root rot disease	Salinity	Sugarbeet	Synthesis of cell wall degrading enzymes decreased (salinity caused an antagonistic effect on the fungus)	El-Abyad et al. (1997)
	Blumeria graminis	Powdery mildew	Salinity	Barley	Salinity caused antagonistic activity	Wiese et al. (2004)
	Botrytis cinerea, *Oidium neolycopersici*	Gray mold, Powdery mildew	Drought	Tomato	Infection and the growth of fungal pathogens were suppressed by drought	Achuo et al. (2006)
Biotic and abiotic stress combinations (antagonistic)	*Oidium neolycopersici*	A causal agent of powdery mildew in tomato	Salinity	Tomato	Accumulation of Na^+ and Cl^- ions in the leaves was linearly related to the decreased pathogen symptoms under combined stress	Kissoudis et al. (2016)
	Sclerotium rolfsii	Root rot disease	Salinity	Sugarbeet	Synthesis of cell wall degrading enzymes increased (Salinity caused a synergistic effect on the fungus)	El-Abyad et al. (1997)
	Botrytis cinerea	Gray mold disease	Salinity	Tomato	Increased Na^+ ion uptake stimulated the growth of fungus (salinity caused a synergistic effect on the fungus)	Boumaaza et al. (2015)
	Tomato spotted wilt virus, *Tobacco mosaic virus*	Mosaic disease, Spotted wilt disease	Temperature	Pepper and tobacco	Suppressed the resistance of plant, synergistic effect	Király et al. (2008)
	Pseudomonas syringae pv. *actinidiae*	Kiwifruit canker disease	Frost	*Actinidia chinensis*, *A. deliciosa*	Bacterial colonization increased during thawing, synergistic effect on the pathogen	Ferrante and Scortichini (2014)
		Mosaic disease	Drought	Rice, beet		Xu et al. (2008)

(*Continued*)

TABLE 2.1 (Continued)

Stress interactions	Biotic stress: Causal agent	Biotic stress: Disease name	Abiotic stress	Host plant(s)	Physiological, biochemical, and molecular response	References
	Brome mosaic virus Cucumber mosaic virus				Drought caused an antagonistic effect on pathogens with delayed disease symptoms	
	Blumeria graminis	Powdery mildew	Ozone	Wheat	Ozone cause some adverse effects including necrosis and chlorosis in plants, however, it can trigger different defense mechanisms against powdery mildew	Pazarlar et al. (2017)
Abiotic and abiotic stress combinations (synergistic)	–	–	UV-B radiation and water stress	*Arabidopsis thaliana*	UV-B radiation caused a synergistic effect for *Arabidopsis* sp. with enhanced resistance to water stress	Bandurska et al. (2013)
Abiotic stress causing pathogen adaptation	*Fusarium oxysporum*	Wilt disease	Salinity	–	Saline-adapted *Fusarium oxysporum*	Abbas and Mandeel (1995)
	Laccaria montana	–	Cold	–	Cold-adapted *Laccaria montana*	Maggi et al. (2013)
	Penicillium spp. *Aspergillus niger* *Fusarium oxysporum*	Plant–fungal disease	Salinity	*Vicia faba*, *Corchorus olitorius*, *Sesamum indicum*	Saline-adapted fungus species produced high gibberellic indol acetic acid (IAA) at moderate or high NaCl levels	Hasan (2002)
Abiotic stress agents	–	–	Heat	Wheat	Heat stress significantly reduced seed germination and seedling growth, plant water-use efficiency	Akter and Islam (2017)
	–	–	Salinity	Wheat	Salinity stress caused oxidative stress resulting in enhancement of lipid peroxidation	Ahanger and Agarwal (2017)
	–	–	Cold	Wheat	Cold stress caused flower abortion, pollen and ovule infertility, and affects seed filling, leading to low seed set and ultimately low grain yield	Thakur et al. (2010)
	–	–	Heavy metal	Wheat	Heavy metal stress caused inhibition of root growth, enzyme inactivation, and plant death	Karataglis et al. (1991)

transduction pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). ABA stimulates the activity of NADPH oxidase and H_2O_2 generation to enhance the production of NO that triggers the defense mechanism via synthesis of ET, which leads to stomatal closure (He et al., 2011).

It is important that signaling molecules should be at low concentrations. For example, under low UV-B conditions, the ROS level is controlled through a specific signaling pathway involving UVR8, COP1, and HY5/HYP transcription factors triggering activation of a gene involved in antioxidant defense system (Bandurska et al., 2013; Rejeb et al., 2014). However, under high UV-B conditions, this may not be achieved due to insufficient antioxidant scavenging capacities after signaling mechanism and gene expression pathway, because overproduction of ROS causes damage to organelles and DNA and disrupts the membrane structure and downregulates the defense-related genes (Brosché and Strid, 2003; Jenkins, 2009). Generation of ROS differs from one stress to another. The sense and expression of ROS are different in abiotic and biotic stress conditions. ROS induces tolerance or resistance by activating stress-related mitogen-activated protein kinases (MAPKs), transcription factors, antioxidant enzymes, and stress-related and pathogen-related proteins (Rejeb et al., 2014). ROS were initially considered as a byproduct of cell metabolism, however, they are

now regarded as triggering molecules of signal transduction. This means that cells use ROS as biological stimuli and signals to activate and regulate various genetic stress response processes (Gong et al., 2013). ROS contain both free radical ($^{\bullet}O^{-}_2$, $RO^{\bullet}$, $HO_2^{\bullet}$, $OH^{\bullet}$) and nonradical forms (H_2O_2, $^{\bullet}O_2$). They are highly reactive toxic and harmful byproducts of normal cellular metabolism that damage proteins, lipids, carbohydrates, DNA and cause cell death eventually. ROS are produced at various parts of plants such as chloroplasts, mitochondria, plasma membranes, peroximoses, apoplasts, endoplasmic reticulum and at cell walls in both unstressed and stressed cells (Sharma et al., 2012). Although H_2O_2 was recognized as a toxicant, recently, it has been regarded as a signaling molecule and a regulator of the expression of some genes encoding antioxidants and defense proteins and signaling proteins such as kinase and phosphatase (Shu-Hsien et al., 2005).

Under stress, production of ROS is balanced with the production of enzymatic and nonenzymatic antioxidants. However, this equilibrium is perturbed by abiotic and biotic stressors such as salinity, drought, heavy metals, UV light, pathogens, etc. Once the balance is disturbed, various signals are preceded via different pathways to level the imbalance and to protect cells from extra ROS. Whether ROS would act as signaling molecules or could lead to oxidative damage to the tissues or organs, this totally depends on the fine equilibrium between ROS production and their scavenging capacity (Sharma et al., 2012). It has been estimated that 1% of O_2 consumed by plants is diverted to produce ROS (Asada and Takahashi, 1987). From them, oxygen radicals and H_2O_2 are produced. H_2O_2 is moderately reactive and relatively long-lived molecule. It can readily cross biological membranes and cause cellular damage far from the site of its formation (Torres et al., 2002). At low concentration, H_2O_2 can regulate biological processes and trigger tolerance against various environmental and biotic stresses. At high concentration, it can oxidize the cysteine or methionine residues and inactivate enzymes through oxidation of their thiol groups (Halliwell and Gutteridge, 2015).

Reactive nitrogen species (RNS) also play a significant role in signaling. NO, NO_2, as well as nonradical nitrous acid (HNO_2), act as signaling molecules to trigger different essential pathways involved in plant–stress interactions (Hossain et al., 2018). These molecules are present at very low concentrations at every developmental stage of plants. During stress development, NO quickly diffuses across the plant membranes due to its lipophilic properties (Vaishnav et al., 2018). The NO is mainly produced from agricultural soils. Soil nitrogen contents, pH level, temperature, and moisture content affect the production of NO. Increase in concentrations of NO plays a crucial role in neutralizing ROS by directly interacting with them or inducing an antioxidant enzyme activity. Therefore, application of NO thorough sodium nitroprusside (SNP) enhances plants resistance against stressors by activating the antioxidant system and plasma membrane transporter, which leads to decrease in ROS accumulation (Fu et al., 2015). As a signaling molecule, NO is able to change the metabolic activities of plants, bacteria, and fungi. Therefore, it would be appropriate to employ NO-producing plants or microorganisms in polluted soils for sustainable agriculture practices. However, the signaling functions of NO should be evaluated in detail under considered stress conditions, because combined stress may not allow NO to function as a signaling molecule by decreasing the concentration of it.

Methylglyoxal (MG) has been noticed as an emerging signaling molecule recently in plant stress responses and tolerance (Hoque et al., 2016). It is produced as a byproduct of a number of metabolic reactions. Under natural growth conditions, MG remains low in plants. However, under stress conditions, it can be accumulated in much higher levels. When it reaches high levels, it functions as a toxic molecule and it inhibits growth and development including germination, root growth, photosynthesis, etc. At low levels, like other oxidants, it acts as a signaling molecule (Li, 2016). It regulates many physiological and biochemical events such as cell proliferation and survival, cell homeostasis, and physiological and biochemical functions. It can also modulate stress responses by adjusting stomatal opening and closure, the production of ROS, cytosolic calcium ion concentrations and the expression of many stress-signal transduction and functions for promising adaptation of plants growing under stress conditions. To explore the role of MG in detail, it is important to find out its relationships with other signaling molecules, that is, whether it has synergistic or antagonistic pathways with them. Since this signaling molecule is new, it would be appropriate to find its role under the combined stress conditions to sustain crop plants.

Calcium is one of the important secondary messengers. Calcium ions regulate ion homeostasis thereby enhancing stress tolerance (Huda et al., 2013). It acts as a crucial signaling molecule in both abiotic and biotic stress tolerance. Calcium is bound to different organic molecules including proteins and lipids. In plant cell, calcium ions are placed in endoplasmic reticulum, vacuoles, plastids, and mitochondria and internal concentrations of calcium levels are maintained by various channels, pumps, and transporters (Dixit and Jayabaskaran, 2014). It regulates gene expression and

defense mechanism. For example, Goswami et al. (2015) reported that exogenous Ca^{2+} application showed negative correlation with lipid peroxidation and positive correlation with total antioxidant capacity of the cell system under the elevated temperature stress. They also reported that efflux of Ca^{2+} triggered the activities of kinase and peroxidase enzymes in tolerant *T. aestivum* plants as compared with those of susceptible cultivars.

MAPK cascades are highly conserved signaling modules that transduce extracellular stimuli into intracellular responses (Bitrián et al., 2012). Plant MAPK cascades play important roles in signaling defense mechanism against pathogen attack. Activation of MAPKs is one of the earliest signaling events after sensing pathogen effectors. MAPK is also involved in signaling of multiple defense responses including plant hormones, ROS generation, stomatal closure, gene expression, phytoalexin synthesis, cell wall strengthening. However, pathogens are able to produce effector proteins to suppress plant MAPK activation and downstream defense responses to promote pathogenesis (Bitrián et al., 2012).

Another signaling molecule is lipid and its derivatives. They are one of the major constituents of biological membranes that can sense the stress at first stage. Lipids provide the structural basis for cell membranes and provide energy for cell metabolism. Lyophospholipid, fatty acid, phosphatidic acid, inositol phosphate, and N-acylethanolamine have been proposed to function as signaling lipids (Okazaki and Saito, 2014). Studies have demonstrated that each lipid has class-specific signaling cascades, which activate defense reactions. Lipids can also function as stress mitigators to reduce the intensity of stressors. The increased amount of synthesis of lipids has been noticed under stress conditions (Okazaki and Saito, 2014). The accumulation of oligogalactolipids has recently been found to mitigate freezing and nutrition-depletion stresses. Signaling lipid molecules are usually present in small quantities in tissues and very quickly synthesized from preexisting membrane lipids (Gill and Tuteja, 2010; Markham et al., 2013).

Stress treatment also affected sugar metabolism and sugar signaling (Baena-González and Sheen, 2008). In general, apoplastic accumulation of hexoses is shifted during stress. This leads to increase in sugar concentration to initiate the defense response. In most plant stress relations, a high level of sugars in plant tissues enhances plant resistance. Sugars constitute the primary substrate and provide energy and structural material for defense responses in plants. On the other hand, they also act as signal molecules interacting with the hormonal signaling network, which regulates the prime immune system (Morkunas and Ratajczak, 2014). Sugars enhance oxidative burst at early stages of infection, increase lignification of cell walls, stimulate the synthesis of flavonoids, and induce PR protein. Like other signaling molecules, sugars at low concentrations play important roles for defense mechanisms. They regulate cellular activity at multiple levels, from transcription and translation to protein stability and activity (Rolland et al., 2006). Hexokinase is the best-investigated glucose sensor, while this protein also serves in the enzymatic processes, and catalyzes the first step of glycolysis (Smeekens et al., 2010). Apart from glucose, sucrose also functions as a signaling molecule (Wind et al., 2010). It enhances the expression of anthocyanin biosynthesis genes. Control of sugar and energy metabolism in cells is a highly important plant defense mechanism. It should be remembered that in combined stresses, higher accumulation of sugar prior to pathogen attack has a significant role for increasing the virulence and spread of pathogens since sugars are good sources of carbon for attacking pathogens. Santino et al. (2013) showed that phytohormones played a key role in plant defense as well as in plant development. They function as signaling molecules, that is, ET, JA, ABA, SA, etc.

2.3 SIGNALING MOLECULES AND PLANT RESPONSES UNDER COMBINED STRESS CONDITIONS

Plants have to respond and cope with various abiotic or biotic stress all the time. To survive in stressed conditions, plants must recognize the stress and rapidly exert multiple defense reactions. Their responses depend on the speed and ability to sense the stress. So far, plant molecular and biochemical responses have been evaluated considering single stress factors. For example, drought, soil flooding, high or low temperatures, pathogens, insects, etc., have now been in interaction with each other more than ever due to changes in climate and environmental conditions. Recently, biochemical or molecular responses of crop plants have been elucidated more commonly in combined interactions (Bonnet et al., 2017). These interactions could be between abiotic and biotic stressors or between abiotic and abiotic or between biotic and biotic stressors. However, interactions between abiotic and abiotic or abiotic and biotic stressors have now been noticed more due to changes in global temperature and environmental pollution (Guerret et al., 2016). These interactions have been modulated by evaluating a complicated network of signaling pathways examining Ca^{2+} signaling (Seybold et al., 2014; Nguyen et al., 2016), ROS and RNS (Wang et al., 2013; Baxter et al., 2014), and phytohormones (De Vleesschauwer et al., 2014; Kazan, 2015). Plants are often simultaneously or sequentially

exposed to multiple biotic and abiotic stresses that result in substantial yield loss. Simultaneous occurrence of an abiotic stress with a biotic stress either aggravates or inhibits the effect of the latter, which leads to either enhanced or reduced susceptibility to pathogens. Thus, abiotic stress can change plant behavior in terms of tolerance or susceptibility toward pathogens by different mechanisms. Signaling molecules have been evaluated under single abiotic or biotic stress factors. However, the pathway of these molecules is different at combined stress conditions from those of single stress conditions, Fig. 2.3. The outcome of interactions between biotic and abiotic stress signaling is not easy to predict by evaluating the signaling pathways in single stress responses. More experimental outputs are needed not only in model plants like *A. thaliana* but also in nonmodel crop plants including grains and vegetables and even trees.

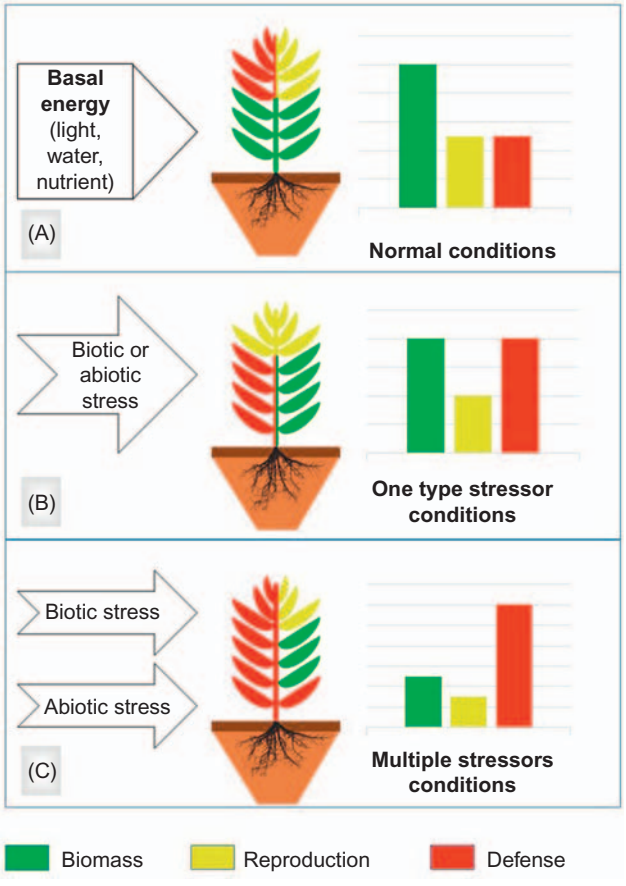

FIGURE 2.3 Energy and metabolic levels of plants under single or multiple stress conditions. The interactions between abiotic and biotic stress agents are not straightforward. Under one type of stress condition, a great amount of energy is diverted into defense. Under the combined stress, most of the energy is diverted into defense, and a very low level of energy is spent for reproduction. Flowering or fruiting stage would be accelerated and fruits would be very small and less compared with those of normal metabolic conditions. In severe cases, early maturity or death of crop plants could be inevitable.

Concentrations of these molecules could change or cross-talk with other signaling molecules. Thereby, new pathways should be produced to understand the complex interactions if we aim to remediate the conditions of crop plants under combined stress conditions (Fig. 2.4). Because, if a stress-related protein or enzyme is to be produced under stress conditions, plants would not produce this metabolite from different pathways, instead it will produce this metabolite using a common or shared pathway to use their energy resources efficiently under the combined stresses. This pathway should be quick and cost-effective as compared with those of pathways under single stress conditions. Therefore, plant adaptation strategy to a combination of two or three stresses consists of both "shared" and "unique" response. Many researchers refer to the "shared" response as the molecular, biochemical, and physiological responses using common pathways by two or three different stresses. They refer to "unique" responses as the specific individual responses in combined stress conditions (Atkinson et al., 2013; Prasch and Sonnewald, 2013). Signaling molecules under combined stress include production and detoxification of ROS, calcium-, phytohormone-, and MAPK-signaling pathway (Atkinson and Urwin, 2012; Suzuki et al., 2012, 2014; Rejeb et al., 2014). Most signaling molecules for abiotic and biotic stresses share common pathway and molecules including ROS (Møller et al., 2007; Wong and Shimamoto, 2009), calcium ions (Galon et al., 2010), transcription factors (Walley and Dehesh, 2010), hormones (Fonseca et al., 2009; Ton et al., 2009), (MAPK) cascades (Pitzschke et al., 2009), etc.

The shared responses play important roles in the adaptation of plants to harsh conditions. Some unique adaptation strategies tailored for stress combinations have been identified in the recent reports (Choi et al., 2013; Prasch and Sonnewald, 2013). For example, a combination of heat stress and virus infection resulted in upregulation of cytosolic invertases instead of cell wall–bound invertases (Prasch and Sonnewald, 2013).

The combination of abiotic and biotic stresses shares the unique or shared pattern (Prasad et al., 2011; Bostock et al., 2014; Rejeb et al., 2014; Pandey et al., 2015; Dikilitas et al., 2017). However, it is not easy to predict the results of stress combination by elucidating each stress alone. Even if they share unique or shared response, it is crucial to test the combination of these stresses in vitro or in vivo conditions. The combination of stresses, in general, is sensed as a new state of stress (Mittler, 2006). Here, the response of plants to stress combination is mainly characterized by the dominant stress factor. This could be described as a kind of a

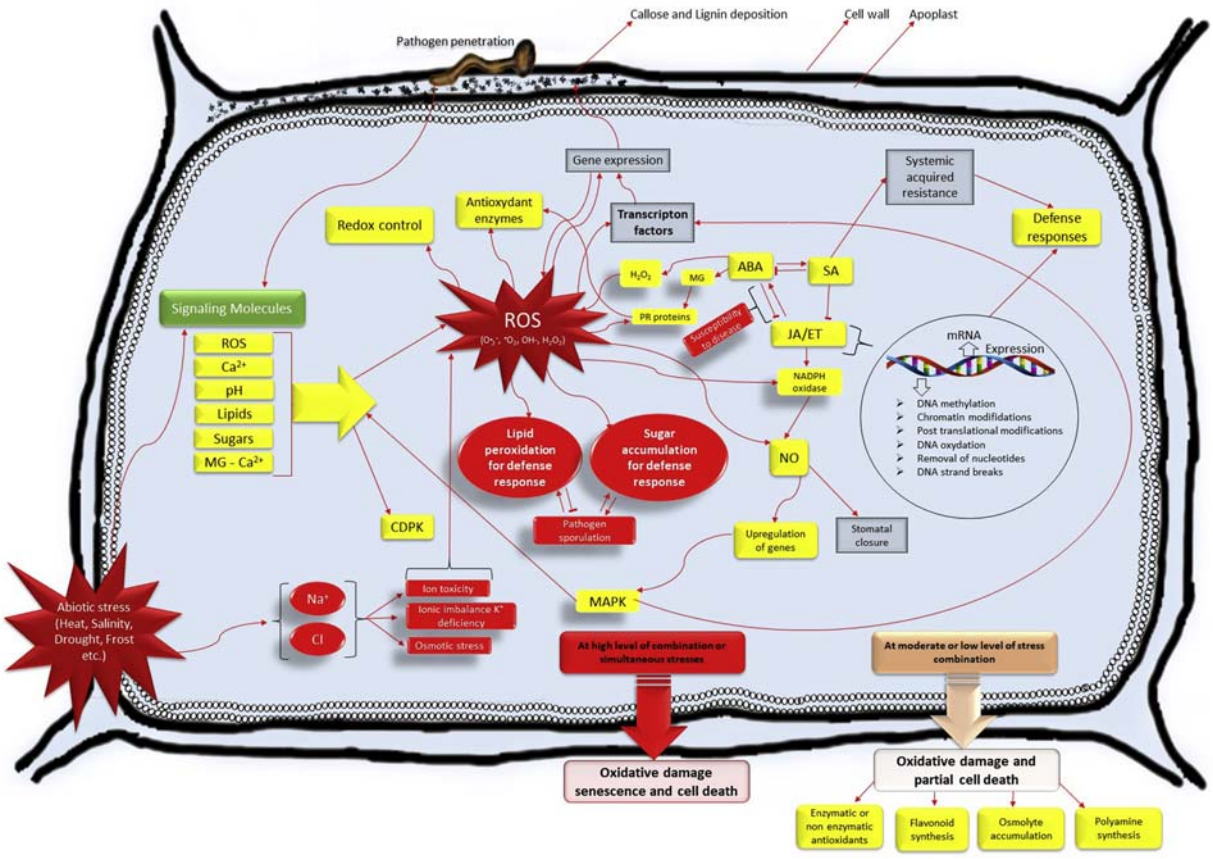

FIGURE 2.4 Signaling pathways of abiotic and biotic stress at the cellular level. Arrows indicate induction and bars indicate inhibition. The combination of stresses could be additive, synergistic, or antagonistic in terms of symptoms or disease progress on crop plants. Abiotic and biotic stress factors affect the homeostasis of chemical signals at the apoplastic space. Abiotic stress disrupts the structure and properties of physical barriers of the cell wall that protects the cell from pathogenic attacks, therefore, abiotic stress predisposes the plants to pathogenic attack. ABA signaling negatively affects hormonal signaling exerted by pathogens. ABA results in closure of stomata, this prevents water loss and pathogen entry, however, overproduction of ABA results in enhancement of pathogen spread and sporulation, because ABA and SA, JA, ET negatively affect each other, and therefore, overproduction of ABA resulted from abiotic stress could suppress the SA-based defense mechanism of plants against pathogenic attack. Therefore, under the combined stress, crop plants might stay defenseless due to cross-talk of signaling molecules. Also, high level of abiotic stress such as toxic ions or high temperatures or drought promote the translocation of effector proteins of pathogens into the host cell (plant). Here, one of the important mechanisms of the combined stress is the order of stresses; the stresses could be sequential or concurrent (simultaneous). Which type of signaling molecules would be prevalent and play a decisive role simply depends on the severity of the stress that would synthesize the signaling molecules. Since detoxification mechanisms would be slow and under capacity, the excess accumulation of ROS could, therefore, damage proteins and lipids and DNA in severe cases. Studies related to genomics, proteomics, transcriptomics, and metabolomics are needed to further elucidate the combined stress interactions.

more severe stress of an individual stress. Although it is not easy to predict the result of stress combination in terms of physiological, molecular, biochemical, or disease progression in advance, we could state whether the different stresses lead to the same or different kinds of changes in plants. The identification of signaling molecules involved in shared and unique response under combined stresses would be an important step to develop resistant crop plants.

Overlap responses between cross-talk of signaling pathways can be a source of potential stress tolerance traits that can be engineered into crops to confer multiple stress resistance into plants (Kissoudis et al., 2016). For example, different calcium-dependent protein kinase (CDPK) genes in *T. aestivum* showed that 12 CDPKs were expressed in response to *B. graminis* pv. *tritici* (causal agent of powdery mildew in wheat) attack, eight of them also responded to abiotic stresses (Li et al., 2008). Similarly, genes involved in ROS scavenging pathway like *ascorbate peroxidase* (*APX*) gene have been shown to take part against various abiotic and biotic stresses (Choi and Hwang, 2012). The transcriptomic and proteomic analysis of plants under combined stresses can produce useful information regarding common and unique genes regulated under combined stresses.

Under combined stress, hormonal signaling interacts with each other. How such interactions affect plant responses under multiple stresses is not well known although such interactions frequently occur in nature. For example, Vos et al. (2013) stated that drought stress enhanced insect resistance due to synergistic interactions between JA and ABA signaling. On the other hand, flooding or waterlogging decreased resistance to chewing herbivores due to its negative cross-talk with JA. It is clear that ABA synthesis and signaling is required to activate defense responses and resistance against abiotic and biotic stresses. However, very low or high concentration of ABA predisposes crop plants to pathogenic attack. For example, Kissoudis et al. (2016) stated that powdery mildew resistance in tomato plants was affected by salt stress in a genotype- and stress intensity−dependent manner. Mild salt stress (50 mmol/L NaCl) increased the susceptibility of tomato while 150 mmol/L NaCl reduced disease symptoms. They suggested that accumulation of Na^+ and Cl^- ions in the leaves was linearly associated with the decreased pathogen symptoms under severe stress. They concluded that complete resistance was enabled in tomato genotypes carrying *Ol-2* and *Ol-4* resistance genes. Increased susceptibility and senescence under combined stress was associated with the induction of ET and JA pathway genes. The majority of these studies conclude that there is a negative impact of abiotic stress (mostly drought and salinity stress) on pathogen resistance (Suzuki et al., 2014). ABA is mainly involved in responses to abiotic stresses whereas SA, JA, ET have important roles in biotic stress signaling (Sebastiani et al., 2017). ABA appears to be a central modulator of the regulatory cross-talk, directly impacting biosynthesis of SA, the major regulatory hormone for defense responses against biotrophic pathogens (De Torres Zabala et al., 2009). Therefore, antagonistic relations were evident between ABA and other signaling molecules. However, under abiotic stress conditions, the role of ABA is significant. For example, enhanced callose deposition to prevent membrane leakage of plants was reported to be regulated by ABA pathway (Cao et al., 2011). Fujita et al. (2006) stated that the ABA-deficient tomato mutant cultivars (deficient in functional enzyme activity at the final step in ABA biosynthesis) had increased resistance to pathogens and application of exogenous ABA restored the susceptibility (Audenaert et al., 2002; Thaler and Bostock, 2004). High ABA concentrations inhibited the SA-dependent defense responses in tomato. ABA and ET are well known antagonistic hormones (Beaudoin et al., 2000; Ghassemian et al., 2000). Additionally, exogenous application of ABA resulted in the downregulation of JA- or ET-responsive defense gene expression in wild-type plants, whereas higher expression levels of these defense genes were observed in ABA-deficient mutants without any treatments (Anderson et al., 2004). From the above findings, exogenous application of JA and ET cannot restore the defense gene expression of plants suppressed by exogenous ABA application, because the ABA-regulated abiotic stress response is a dominant stress at the severe case when compared with the biotic stress process (Anderson et al., 2004). Verma et al. (2013) reported that ABA was produced during water stress. Various genes induced during drought and cold stress also get induced by application of ABA exogenously, indicating ABA-dependent signal transduction pathways. On the other hand, several genes may not be induced by application of ABA exogenously indicating that the existence of ABA-independent signal transduction is evident as in the case of *Arabidopsis* (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Whenever a plant encounters any kind of stress, this is sensed by the potential sensors, which generate secondary messengers (e.g., ABA, ROS, Ca^{2+}, inositol phosphates, etc.). The secondary messengers can alter the levels of intracellular Ca^{2+}. Any changes in cytoplasmic Ca^{2+} is sensed by Ca^{2+} sensors, that is, calcium-binding proteins to regulate stress-related genes. The expression of stress-regulated genes leads to the production of various kinds of regulatory molecules like the phytohormones ABA, ET, and SA and transcription factors, etc. These signaling networks are interconnected at many levels for transferring information to generate firm plant response. A successful signal transduction pathway demands proper coordination of all signaling molecules (Suzuki and Katano, 2018). Fujita et al. (2006) reported that although a few studies dealt with revealing the pathway under combined stress interactions, most of the signaling stress pathways need to be elucidated. However, recent studies have revealed several molecules, including transcription factors and kinases, involved in cross-talk (antagonistic or synergistic actions) between signaling pathways. For example, heavy metal ($CuSO_4$) stress with incompatible necrotrophic pathogen infection revealed a significant overlap between responses to biotic and abiotic stresses in terms of gene expression (Fujita et al., 2006). This relationship may simply suggest that plants have developed strategies to avoid simultaneously producing proteins that are involved in abiotic stress and disease resistance responses (Anderson et al., 2004). Suzuki et al. (2014) stated that despite a certain degree of overlap, each combined stress requires a unique mechanism. In addition, the concurrent occurrence of different biotic and abiotic stresses was shown to result in a high degree of complexity in plant responses. Therefore, the mechanisms should be clarified to tackle

the combined stress. Abiotic stress alters pathogen-related signaling networks, which could lead to the deactivation of defense responses and the higher susceptibility of plants. Many authors reported that when combined stress triggered the defense mechanism, signaling molecules activated the defense-related genes (Li et al., 2008). For example, increase of cytosolic calcium (Ca^{2+}) levels in response to pathogen attack, osmotic stress, water stress, cold, or wounding triggers simultaneous pathways and activates calcium-interacting proteins such as Ca^{2+}-dependent protein kinases (CDPKs) and calmodulin, which are involved in transcription factors. In a combined stress, ET, JA, SA regulate the defense mechanism regarding biotic stress while AB regulates the abiotic stress responses. In general, the biotic defense signaling networks regulated by phytohormones are mainly dependent on the nature of the pathogen and its mode of action. SA plays an important role against biotrophic and hemibiotrophic pathogens through the establishment of systemic acquired resistance (SAR). By contrast, JA and ET are usually associated with defense against necrotrophic pathogens and insects. JA and ET function synergistically to activate the expression of defense genes as reported by various workers (Thomma et al., 2001; Glazebrook, 2005). For example, Audenaert et al. (2002) stated that tomato mutants (ABA-deficient) showed a reduction in susceptibility to *B. cinerea* and virulent isolates of *P. syringae* pv tomato DC3000 (Thaler and Bostock, 2004; De Torres-Zabala et al., 2007). Similarly, ABA-deficient *Arabidopsis* showed reduced susceptibility to the oomycete *Hyaloperonospora parasitica* (Mohr and Cahill, 2003). In general, it could be stated that ABA is mainly involved in the negative regulation of plant defenses against various biotrophic and necrotrophic pathogens. However, the positive side of ABA was reported in pathogen-involved cases (Mauch-Mani and Mauch, 2005). For example, ABA-activated stomatal closure played an important role in preventing bacterial infection (Melotto et al., 2006). However, there are conflicting results in combined stresses even though these results may come from the same authors. ABA-deficient mutants do not always prevent the disease occurrence. Ton and Mauch-Mani (2004) showed that ABA-deficient mutants showed susceptibility to *P. syringae* pv. *tomato* while ABA-applied plants showed resistance against *Alternaria brassicicola* and *Plectosphaerella cucumerina*. Biochemical and molecular analysis showed that the effects of combined stress were not linear when the effects of single stress value were summed. The increase of ABA under the effects of abiotic stress induces stomatal closure, therefore, virus, bacteria, or fungi like powdery mildew and *B. cinerea* may have faced a barrier before infection. However, if the infection gets started, the progress of the disease gains speed due to cross-talk between ABA and SA pathways. ABA and SA have an antagonistic role in plant defense against stressors (Bowler and Fluhr, 2000; Robert-Seilaniantz et al., 2007).

Massa et al. (2013) stated that gene expressions of *Solanum tuberosum* in triple combined stress (abiotic stress + hormone + biotic stress) differed from those of individual stress. For example, 40% of the genes were upregulated under abiotic stress while 19% and 10% of the genes were upregulated under biotic and hormonal stress conditions, respectively. Only 6% of the genes were upregulated under triple combined stress conditions. It could, therefore, be concluded that defense mechanisms of plants are reduced due to decreasing number of defense-related genes under multiple stress conditions. Similarly, Prasch and Sonnewald (2013) stated that double or triple or multiple stress responses (heat, drought, virus) by transcriptome and metabolome analysis revealed that gene expressions were downregulated. The increased cytoplasmic response was also evident; however, this was not observed under single stress conditions. The authors suggested that abiotic stress factors significantly altered virus-specific signaling networks, which led to the deactivation of defense responses and a higher susceptibility of plants. For example, TMV was able to overcome the M gene-mediated resistance at temperatures above 28°C in tobacco (*N. tabacum*) (Király et al., 2008). Massa et al. (2013) stated that overlap between abiotic, biotic, and hormone stresses showed that hormones (ABA, IAA, GA_3) and abiotic stresses (heat, salt) shared more differentially expressed genes than each showed with the biotic stress (*Phytophthora infestans*) agent.

ROS signals after pathogen inoculation may change stomatal conductance and ion uptake transport. Abiotic stress such as salt and drought affect ABA signaling and systemic acquired resistance, therefore, pathogen spread is quickly enhanced and colonizes the whole plant. For example, SA signaling, induced after infection with biotrophic fungi, can attenuate ABA signaling, which takes important roles in adapting plants to abiotic stresses (Kim et al., 2011).

2.4 DNA DAMAGE OF PLANTS UNDER CONCURRENT OF ABIOTIC AND BIOTIC STRESS COMBINATIONS

The plant defense mechanism has also been examined in terms of genetic responses in combined stress conditions. Many authors reported that genes and gene products have been expressed under combined stress. Abiotic and biotic stresses affect genome stability and even cause multiple DNA damages. The DNA

damage could be direct or indirect depending on the source, duration, and level of stress as well as the resistance traits of organisms. During stress, plants are able to recover from the stress, however, this depends on the duration, severity, and source of stress. However, combined stress may not allow plants to recover (Dikilitas et al., 2018). If the concentrations of antioxidant molecules exceed a certain level that the host cannot tolerate, these molecules damage the cell compounds including DNA (Wan-Ibrahim et al., 2010). For example, Čabarkapa et al. (2017) reported that dry olive leaf extract (DOLE) had radical scavenging activity in peripheral blood lymphocytes (PBL), however, the concentrations over 1 mg/mL led to a severe increase of DNA damage exhibiting prooxidant rather than antioxidant effect.

Although most of these molecules act as signaling molecules at low concentrations, however, overexpression of them is highly toxic and cause protein oxidation, carbohydrate degradation, lipid peroxidation, pigment breakdown, and DNA strand breaks. Although there is no report that the combined stress has made strand breakages in DNA molecules, DNA methylation, a prestep of DNA damage, is evident. This damaging effect could lead to DNA damage and the damage, if not repaired, has an ongoing effect.

The concentration of signaling molecules and DNA damage has not been correlated in plant crop science studies to the best of our knowledge. It should be one of the important steps in identifying the pathway in terms of signaling molecules and DNA damage parameters to generate resistant plants. Plants have to face enormous challenges to maintain their genome integrity due to global warming and environmental pollution, and their immobile character makes this case worse. For example, UV light, ionizing radiation, high soil salinity, drought, heavy metal, chemical mutagens, free radicals, alkalyting agents, pesticides, and even sometimes biological agents and their toxins have the potential to result in DNA methylation and DNA damage (Dikilitas et al., 2015; Georgieva et al., 2017; Silveira et al., 2017). DNA damage generated by abiotic and biotic stress affects plant growth, development, and crop productivity. Therefore, to maintain the stability of genome, plants have developed an extensive array of mechanisms for detection and repair of DNA damage. DNA damage could vary such as DNA base oxidation, single and double strand breaks, DNA intercross-links, DNA methylation, etc. (Roy, 2014). Lesions in the DNA structure as a result of stress agents may lead to biochemical and physical changes in the DNA structures. These lesions have tended to accumulate and have potential to break DNA strands at the end. Under combined stress conditions, the damaging effects of both abiotic and biotic stresses could be more prominent and be able to break the single and double strands of DNA. Individual stress factors, even at low doses, may produce additive or synergistic interactions between them and their negative effects could be visible on DNA. For example, the interactions of ultraviolet lights (UV-B) with different concentrations of cadmium or lead on *Bacillus cereus* resulted in DNA damage, cytotoxicity, depletion of glutathione, and formation of lipid peroxidation. Although UV-B rays alone enhanced glutathione production, the combined stress exhibited high genotoxic activity due to inhibition of the DNA repair mechanism (El-Sonbaty and El-Hadedy, 2015).

2.5 GENOMIC AND BIOCHEMICAL APPROACHES FOR PLANTS UNDER COMBINED STRESSES

If a stress is beyond tolerance level, defense mechanisms are activated at molecular, physiological, and biochemical level. When stress is managed and controlled, the cell signaling returns to normal level. The signals are able to induce expression of specific genes that lead to the assembly of defense reaction (Jaspers and Kangasjärvi, 2010; Ji et al., 2011). Advances in plant genomics research have opened up new perspectives and opportunities to improve crop plants in terms of quantity and quality. For example, many of the key enzymes (peroxidase, catalase, ascorbate peroxidase, and superoxide dismutase) involved in ABA synthesis have been used in transgenic plants to improve abiotic stress tolerance (Sun et al., 2017). Transgenic plants overexpressing the genes involved in ABA synthesis showed increased tolerance to drought and salinity stress (Ji et al., 2011). Also, the genes involved in the synthesis of osmoprotectants such as proline, glycine betaine, sugars showed increased tolerance to abiotic stresses. These transgenic modifications of biosynthetic and metabolic pathways indicated that higher stress tolerance and the accumulation of compatible solutes may also protect plants against damage by scavenging of ROS and maintain protein structures and functions (Umezawa et al., 2006; Hu et al., 2016). Low molecular weight compounds, polyamines, were regulated with arginine decarboxylase (*ADC*). Therefore, transgenic plants overexpressing *ADC* gene showed an increase in biomass and better performance under salt stress conditions. Genetic engineering in plant signaling pathways is also a valuable option to improve tolerance of crop plants to various stresses (Radwan, 2015).

Although most of the pathways have concentrated on the quick signaling pathway to reduce the effect of stress or stresses, one of the most appropriate ways is

to eliminate or reduce the impact of stress factors from the very beginning. For example, phytoremediation is one of the most secure and cost-effective ways to reduce the salinity or heavy metals from the vicinity of root area of crop plants. The cultivation of crop plants with halophytes (plants that remove salt or heavy metals from the soil) could reduce the toxic ions that could exert toxicity on crop plants (Karakas et al., 2016). Thus, crop plants would have to deal with at least one stress factor. Also, halophytes would provide plants with minerals, hormones, and other beneficial metabolites during the stress period.

Another approach for the plants under combined stress is the use of organic metabolites. For example, Wani et al. (2017) stated that the effect of foliar spray of 24-epibrassinolide (EBL) on *Cicer arietinum* L. increased nitrogen metabolism, antioxidant system, photosynthetic characteristics, and chlorophyll fluorescence under cadmium (Cd; 50 μM) and/or NaCl (100 mM) stress. In addition to that, EBL application enhanced the activities of enzymes such as nitrogenase, glutamate synthase, glutamine synthase, and glutamate dehydrogenase under both stress and stress-free conditions (Gupta et al., 2017; Wani et al., 2017). It is possible that the remediation effect was due to increase in signaling molecules. Similar findings were made by Derevyanchuk et al. (2017) who stated that the phospholipid signaling was the early steps of brassinosteroids signal transduction. On the other hand, Yusuf et al. (2017) stated that cumulative effort of proline metabolism and enhanced antioxidant system along with increased soluble sugar content, via EBL supply, successfully reduced the negative effects of the combined stress of aluminum and salt. There are other activators that result in similar findings. It is important that these bioactivators should be environmentally friendly and should lead to quick signaling response under the combined stress conditions and should be carefully evaluated for possible antagonistic effects toward other signaling pathways under the stress conditions. Therefore, suitable markers such as SA, NO, ET, and ABA measurement should be employed to test the biochemical signaling pathways.

To sustain the effect of bioactivators naturally, the stress-tolerant plant growth-promoting rhizobacteria (PGPR) and arabuscular mycorrhizal fungi (AMF) have been recently used under stressed conditions (Sinclair et al., 2014; Mishra et al., 2018). The secondary metabolites produced by PGPR are capable of improving crop plants. The secondary metabolites, although they have little or no effect on the primary metabolism, are important for survival and protection of the cell, particularly in stressed conditions. Accumulation of osmoprotectants such as soluble sugars and amino acids help in maintaining the equilibrium across the plasma membranes and reduces cell osmotic potential and increases the turgor pressure in the cell. Recent studies have proved that volatile organic compound (VOC) secreted by *B. subtilis GB03* could trigger phytohormone signaling including auxin, cytokinins, SA, and gibberellins in *A. thaliana* (Liu and Zhang, 2015). Similarly, Orhan (2016) stated that IAA produced by halotolerant and halophilic PGPRs increased the root and shoot length and total fresh weight of the wheat plants under saline conditions. Like other bioactivators, stress-tolerant PGPRs may stimulate plants to selectively take up Ca^{2+} to maintain a high Ca^{+}/Na^{+} ratio (Sharma et al., 2016). They stated PGPR inoculated peanut seedlings accumulated less ROS and exhibited high transcript levels of antioxidant genes. The authors thus concluded that selective ion uptake and redox homeostasis was an important protective mechanism of PGPRs.

Another promising approach to improve the conditions of stress tolerance of plants under both abiotic and biotic stress conditions is to prime the plants with chemical compounds to enable better stress tolerance. This could be achieved with chemicals having high signal transduction traits. Since conventional priming is time-consuming and others are not acceptable (genetically modified organisms, GMO) in many places of the world, an alternative way is priming or hardening the plants under single or multiple combined stress conditions. Savvides et al. (2016) showed that sodium nitroprusside (SNP), hydrogen peroxide (H_2O_2), melatonin, and sodium hydrosulfide enhanced tolerance of plants when exposed to multiple stresses. They stated that chemical priming agents shared similar components in their modes of action.

Recent studies have also shown that polyamines (PAs) took a great part in a large number of abiotic and biotic stresses (Alcázar et al., 2010). PAs are low molecular weight polycationic compounds found in most living organisms. Diamine putrescine (Put), triamine spermidine (Spd), tetramines spermine (Spm), and thermospermine (tSpm) can be in free and conjugated forms. Recently, the synthesis and accumulation of PAs were found to be connected to other signaling molecules and took great part in the signaling network. Depletion of PAs in cells is generally detrimental. Therefore, it is necessary to study the interconnections of PA with other signaling molecules especially under combined stress conditions.

Because the exogenous application of PAs might play significant roles to protect plants from further damage even if different stressors use antagonistic pathways (SA or JA pathways), polyamine coding generally engineered plants or PA synthesizing plants or exogenously PA applied plants would protect themselves from stress only when needed without

significant modifications in biochemical pathways responsible for protein and enzyme synthesis.

2.6 CONCLUSIONS AND FUTURE PROSPECTS

Scientists try to generate more resistant or tolerant plant species to get higher crop production in adverse conditions, however, so far only one particular stress agent such as drought, salinity, pathogen, heat, weed, etc. was considered to make plants resistant or tolerant. Now, the problems are more complex and interwoven due to changes in climatic and environmental conditions. Therefore, crop plants have to be generated or bred for multiple stress factors. However, when multiple or combined stresses are involved, we should consider the interactions between abiotic and biotic stress agents. For this reason/matter, stress-responsive signaling molecules including genes, hormones, enzymes, and antioxidant and oxidant molecules should be evaluated in response to multiple stress factors.

Understanding the combined stress involves a fine description of the underlying mechanisms at the cellular and molecular levels. The major challenge is to identify and differentiate the pathways at single or combined stress conditions. Many studies have revealed that the molecular and metabolic responses of plants to combined or multiple stresses is unique when compared with those of single stresses and cannot be predicted from the responses of each individual stress factor. Therefore, any combined stress interactions even if studied with the physiological and biochemical parameters should be identified at the molecular level including signaling pathways to draw a reliable pathway. This is because the majority of pathways produced so far have been composed of the combination of two or more different stress factors, which need to be evaluated in detail to generate resistant crop plants against multiple stress factors occurring simultaneously or sequentially in nature.

Transcriptome analyses and proteomic studies have given rise to rapid progress in the field of plant signal transduction and gene regulation studies. GeneChip and cDNA microarray studies with genome sequencing are useful in identifying novel signaling molecules. Proteomics approach is another useful study in signal transduction studies. To reveal the cross-talk between signaling molecules, more studies, especially in DNA microarray technology, transcriptomic studies, and new generation sequencing techniques, would be very crucial. Nejat and Mantri (2017) stated that when a plant faces one stress, it may tolerate another stress; this phenomenon is called cross-tolerance. Two different stress factors have antagonistic, additive, or synergistic effects, and each stress factor should be verified, and their simultaneous or sequential effects should be considered at first. This is because the sequence of stress plays important roles in the determination of characteristics of stress. Here, each signaling molecule has been discussed and their possible interactions with other signaling molecules have been tried to be evaluated in combined stress issues. A significant gap has been noticed in terms of mechanisms of signaling molecules in combined stress issues. Therefore, mechanisms of signaling molecules should be evaluated individually and possible interactions should be considered. Gene expression studies and signaling molecules would open new pathways and clarify the complex defense mechanisms. Exposure to combined stress can either be more detrimental than a single stress due to synergistic or additive effects or have an attenuating effect due to antagonistic effects.

So far, only a few studies have been conducted on the whole genome responses under multiple stress conditions; this mainly has been performed on *Arabidopsis* spp. We could simply state that the survival of plants under combined stress conditions depends on the ability to perceive stress signals and transmit the signal into cellular components to biochemical changes to start molecular, biochemical, and physiological defense mechanisms.

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CHAPTER

3

Engineering Signaling Molecules to Improve Abiotic Stress Tolerance in Crop Plants

Krishna Kumar Guduru[1], *Chandra Sekhar Akila*[2] *and Chandra Obul Reddy Puli*[1]

[1]Plant Molecular Biology Laboratory, Department of Botany, Yogi Vemana University, Kadapa, Andhra Pradesh, India
[2]Department of Biotechnology, Yogi Vemana University, Kadapa, Andhra Pradesh, India

OUTLINE

3.1 INTRODUCTION

Plants as sessile organisms come across several biotic and abiotic stresses during their course of growth and development. Global climatic changes impose abiotic stresses such as drought, salt, and extreme temperatures, which majorly affect geographical distribution of plants and agricultural productivity and cause a dearth of food (Fedoroff et al., 2010). It has been estimated that globally 70% of plants' potential yield is reduced by abiotic stresses (Acquaah, 2007). Rapid changes in global climate are predicted to increase the intensity of abiotic stresses in the near future; at the same time global population is expected to reach 9 billion by 2030 (Husaini and Tuteja, 2013). Plant scientists anticipate a need to improve plants to enhance productivity under adverse climatic conditions to meet the global population demands. Conventional breeding methods proved unsuccessful for complex traits, that is, abiotic stress. Advancements in the field of plant improvement technologies such as transgenic approaches permit to introduce gene(s) from a wide range of organisms into plants and to develop transgenic plants to cope with complex abiotic stresses in a faster way. Identification of candidate genes is the foremost step in the development of transgenic plants. It is of utmost importance to understand how the plants sense abiotic stress signals and transduce at the molecular level for successful

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development of stress tolerant plants (Xiong et al., 2002; Zhu, 2016). Plants respond to this hostile environment, assimilate several molecular and cellular responses, and develop defense mechanisms to overcome the effect of abiotic stresses. Abiotic stress signal perception and transduction at the cellular level are important steps to ignite molecular adaptive responses of plants to extreme environments. The key genes involved in abiotic stress signal sensing, perception, and transduction comprise receptor molecules/osmosensors, phospholipid-cleaving enzymes, reactive oxygen species (ROS), mitogen-activated protein kinases (MAPKs), and Ca^{2+} sensors. Several signaling molecules have been successfully used for the development of transgenic plants for abiotic stress tolerance in plants. In this chapter we discuss mainly the abiotic stress responsive signaling molecules used for the engineering of plants for abiotic stress tolerance.

3.2 STRESS SIGNAL SENSORS

Plant cells apparently display precise gene expression, metabolic, and phenotypic response to different environmental stresses (Zhu, 2016). However, despite a lot of research to date only a few putative stress sensors have been identified. Most of the genes encoding stress sensors display functional redundancy for a variety of environmental stresses. Environmental stresses persuade incursion of transient Ca^{2+} into the cytoplasm $(Ca^{2+})_{cyt}$ of the cell (Sanders et al., 1999; Knight, 2000; Xiong et al., 2002). The Ca^{2+} channels are mainly involved in the transport of $(Ca^{2+})_{cyt}$. Osmosensing Ca^{2+} channels might serve as osmosensors in bacteria, yeast, and animal cells (Kung, 2005; Arnadottir et al., 2010). Recently, a hyperosmotic responsive putative plasma membrane bound Ca^{2+} sensor gene *OSCA1* (reduced hyperosmolality-induced calcium increase 1) was identified in *Arabidopsis* roots through a calcium imaging–based unbiased forward genetic screen (Yuan et al., 2014a,b). *Arabidopsis* wild plants accumulated more $(Ca^{2+})_{cyt}$, displayed drought and salt stress phenotypic symptoms when grown in the media supplemented with sorbitol and mannitol compared with mutant plants with loss-of-function of *osca1* gene (Yuan et al., 2014a,b). This data suggests that OSCA1 might function as an osmotic sensor in response to environmental stress to induce $(Ca^{2+})_{cyt}$. Nonetheless, the mechanism of how OSCA1 senses the osmotic stress signals is not known yet; probably it might decrease the turgor pressure and affect the plasma membrane–cell wall interactions (Zhu, 2016) and osmosensors. Several eukaryotic organisms apart from plants have numerous mechanosensitive channels, a subtype of osmosensors including MscS-like, Piezo, K2P, DEG/ENaC, and TRP (Arnadottir et al., 2010; Hedrich, 2012). Among them, MscS-like family proteins and Piezo mechanosensitive channel proteins were well characterized (Hedrich, 2012). One of the *Arabidopsis* mechanosensitive ion channel proteins, MSL8, an MscS-like family protein is critical for protection of pollen from hypoosmotic shock during repeated desiccation and rehydration cycles of pollen germination and pollen tube growth (Hamilton et al., 2015). The MSL8 are confined to plasma membrane of tricellular, mature pollen, and pollen tubes, express only during osmotic stress, regulate the movement of ions, and protect the cell membrane from bursting (Hamilton and Haswell, 2017).

Further, plants also encompass a family of glutamate receptor-like (GLR) channels and a large family of nonspecific cation channels cyclic nucleotide-gated channels (CNGCs) to sense environmental signal and modulate cytosolic calcium signals (Finka et al., 2012; Steinhorst and Kudla, 2013; Swarbreck et al., 2013). The CNGCs family consists of 20 members in *Arabidopsis*, which mainly interact with a self-activated α-subunit of heterotrimeric G proteins to activate the Ca^{2+}channels through GTPase activity (Ma et al., 2015). Recently, Ma et al. (2015) identified a QTL harboring gene, chilling-tolerance divergence 1 (COLD1), which confers cold tolerance to rice. COLD1 belongs to the CNGC family transmembrane protein; it is localized in the plasma membrane and endoplasmic reticulum (ER), acts as a cold-sensing calcium channel protein or regulates calcium channel proteins to sense low temperature stress signal, and might interact with RGA1 of the α-subunit of G-protein (Ma et al., 2015). Further, overexpression studies of COLD1 gene in Zhonghua10 of japonica cultivar clearly conferred enhanced cold tolerance nature of the gene; in contrast antisense expression of COLD1 in transgenic plants displayed decreased cold tolerance (Ma et al., 2015).

Another important group of proteins that sense the changes in the fluidity of plasma membrane during environmental stress are membrane bound receptor-like kinases (RLKs) (Sangwan et al., 2002). RLKs are serine/threonine protein kinase gene family proteins consisting of large numbers of genes including 610 in *Arabidopsis* and 1132 members of rice (Shiu et al., 2004). The RLK protein comprises an extracellular ligand-binding domain (ECLB), a transmembrane domain (TM), and a protein kinase catalytic domain (PKC) (Walker et al., 1995; Greeff et al., 2012). The ECLB domain combines the stress signal and transmits through a TM domain; a further signal is transmitted to the intracellular region by the PKC domain, which activates or represses the gene expression based on phosphorylation and dephosphorylation of protein conformation changes (Ye et al., 2016). Several RLK

genes have been identified and functionally characterized in several other plant species in response to environmental stimuli (Ye et al., 2016). A drought and ABA-induced LRR-RLK gene of rice, FON1, when overexpressed in transgenic rice, conferred drought tolerance and sensitivity to ABA compared with FON1-RNAi suppressed transgenic rice plants (Feng et al., 2014).

3.3 SALT STRESS SENSORS

Salt concentration (Na^+) in the soil significantly influences the growth and development of plants; it also leads to hyperosmotic stress and oxidative stress (Zhu, 2002). Information is extremely limited on how the soil salinity (Na^+) is sensed by the multicellular organisms (Zhu, 2016). Unicellular organisms such as yeast sense the Na^+ ions through calmodulin and calcineurin. However, plants do not have calcineurin; as a substitute they use calcium-binding protein kinase proteins SOS (salt overly sensitive) (Zhu, 2002). In the root cells, the soil salt concentration educes cytosolic calcium levels, and the calcium sensor protein SOS3 senses the signal and activates a serine/threonine protein kinase protein (SOS2), which phosphorylates and positively regulates the SOS1, a plasma membrane bound sodium/proton antiporter (Zhu, 2002). The SOS1 localizes in both the root epidermis as well as in xylem cells; the one that is localized in the root epidermal cells excretes the Na^+ into the soil media and protects the cells from sodium toxicity, and the one that is localized in the xylem parenchymatic cells excludes the Na^+ from xylem cells to the apoplastic space of leaf mesophyll cells for long-distance transport (Shi et al., 2002; Zhu, 2016). The SOS3 along with SOS3 homolog protein SCaBBP8/calcineurin B-like 10 (CBL10) augments SOS2 kinase activity and protect the shoots and roots from salt-induced damage (Du et al., 2008). In *Arabidopsis* several (sucrose nonfermenting 1)-related protein kinase (SnRKs) families whose catalytic domain was similar to SOS2 protein kinases were identified. A total of 37 member SnRKs belonging to 3 subfamilies interacts with SOS3-like calcium-binding proteins (ScaBPs) (Guo et al., 2001). The ScaBP further interacts with PKS/CIPK and inhibits H^+-ATPase on the plasma membrane (Fuglsang et al., 2007). Recently, genetic analysis in *Arabidopsis* revealed that a cellulose synthase like D6 (AtCSLD6)/SOS6 provides osmotic tolerance through regulation of ROS levels (Zhu et al., 2010).

Transgenic overexpression of SOS1 cDNA in the salt sensitive *Arabidopsis sos1* mutant cells enhances the salt tolerance (Shi et al., 2000). Transgenic tomato plants overexpressing the SISOS1 revealed that SOS1 is not only important for salt tolerance, but also it is vital for the distribution of Na^+ between plant organs (Olías et al., 2009). Constitutive overexpression of SOS2 in *Arabidopsis* (AtSOS2), tomato (SISOS2), apple (MdSOS2), and poplar (PtSOS2) increased the salt tolerance in transgenic plants by enabling optimal ion homeostasis and enhanced antioxidative capacity (Yang et al., 2015). Transgenic overexpression of SOS pathway genes SOS1, SOS2, SOS3 along with *AtNHX1* with different combinations moderately improved the salt tolerance similar to the overexpression of the any of the genes alone (Yang et al., 2009). However, in contrast transgenic coexpression of *Arabidopsis thaliana SOS1 + SOS2 + SOS3 + CBL10* genes under a stress inducible promoter *rd29a* in tall fescue plants improved the excretion of Na^+ and uptake of K^+ in transgenic plant roots and improved the salt tolerance in transgenic tall fescue (Ma et al., 2014). Similarly, stacked overexpression of *AtNHX1* and *SOS1* in transgenic *Arabidopsis* plants significantly improved the salt tolerance in transgenic *Arabidopsis* plants (Pehlivan et al., 2016). This data clearly revealed that genetic manipulation of SOS pathway improves the salt tolerance in plants (Table 3.1).

3.4 OSMOTIC STRESS SENSORS

As discussed earlier the calcium channel OSCA1 senses the osmotic stress signal and elicits cytosolic calcium levels (Yuan et al., 2014a,b). The calcium levels activate the CPKs and ScaBP/CBL-PKS/CIPKs (Zhu, 2016). In *Arabidopsis* several SnRK2 family protein kinases were activated by osmotic stress (Boudsocq et al., 2004). However, it is not clear how the osmotic stress activates the SnRK2s. The genetic studies in *Arabidopsis* where all 10 members of SnRK2 family members were deactivated revealed that the hyperosmotic stress retards the growth, gene expression, accumulation of compatible osmolyte proline, secondary messenger inositol 1,4,5-trisphosphate (IP3), and abscisic acid (ABA) content in snrk2 mutant plants, however in the absence of osmotic stress they grow like wild-type plants (Fujii et al., 2011). This study clearly disclosed that SnRK2 are important players; they are upstream components of ABA accumulation and are critical for osmotic adjustment during osmotic stress signaling (Fujii et al., 2011). A recent study in the moss *Psyscomitrella patens* revealed that the ABA and abiotic stress-responsive Raf-like kinase (ARK) play a major role in activation of SnRK2s under both osmotic as well ABA stress (Saruhashi et al., 2015).

Tobacco NtC7 might be involved in sensing of osmotic stress signals. Transient expression of

TABLE 3.1 Transgenic Plants Overexpressing Signaling Molecules for Abiotic Stress Tolerance

Functional category	Genes	Protein function	Origin	Transformation receptor	Promoter	Abiotic stress regulation	References
Protein kinase	SOS1	Salt overly sensitive 1	*Arabidopsis*	*Arabidopsis*	–	Salt tolerance	Qiu et al. (2004)
Protein kinase	SOS2	Salt overly sensitive 2	*Arabidopsis*	*Arabidopsis*	–	Salt tolerance	Ohta et al. (2003)
Protein kinase	SOS2	Salt overly sensitive 2	*Arabidopsis*	*Arabidopsis*	–	Salt tolerance	Liu et al. (2011)
Protein kinase	SOS3	Salt overly sensitive 3	*Arabidopsis*	*Arabidopsis*	–	Salt tolerance	Guo et al. (2001)
						Ion homeostasis	
Protein kinase	SOS3	Salt overly sensitive 3	*Arabidopsis*	*Arabidopsis*	CaMV35S	Salt tolerance	Ishitani et al. (2000)
Protein kinase	SOS2-1 and SOS3-1	Salt overly sensitive 2-1 and 3-1	*Arabidopsis*	*Arabidopsis*	T7 promoter	Salt tolerance	Kamei et al. (2005)
Protein kinase	SOS6/ AtCSLD5	Salt overly sensitive/ Cellulose synthase like protein	*Arabidopsis*	*Arabidopsis*	Rd29A	ROS signaling	Zhu et al. (2010)
						Osmotic tolerance	
						Salt tolerance Drought tolerance	
Protein kinase	SCABP8/ CBL10	SOS3-like calcium binding protein 8/ Calcineurin B-like-10	*Arabidopsis*	*Arabidopsis*	CaMV35S	Salt tolerance	Quan et al. (2007)
Protein kinase	LKS1	Low K + sensitive 1	*Arabidopsis*	*Arabidopsis*	Super	Ion homeostasis	Xu et al. (2006)
Protein kinase	CBL-CIPK 3/9/23/26	Calcineurin B-like-CBL interacting protein kinases	*Arabidopsis*	*Arabidopsis*	CBL2	Mg^{2+} tolerance	Tang et al. (2015)
Protein kinase	SnRK2	Sucrose nonfermenting 1-related protein kinase 2	*Arabidopsis*	*Arabidopsis*	CaMV35S	Hyper osmotic tolerance	Boudsocq et al. (2004)
						Saline tolerance	
Protein kinase	SnRK2	Sucrose nonfermenting 1-related protein kinase 2	*Arabidopsis*	*Arabidopsis*	–	Osmotic tolerance	Fujii et al. (2011)
Protein kinase	SnRK2.2/ 2.3/2.6	Sucrose nonfermenting 1-related protein kinase 2.2/2.3/2.6	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Fujii et al. (2009)
Protein kinase	SnRK2.2/ 2.3/2.6	Sucrose nonfermenting 1-related protein kinase 2.2/2.3/2.6	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Wang et al. (2013)
Protein kinase	SnRK2.6	Sucrose nonfermenting 1-related protein kinase2.6	*Arabidopsis*	*Arabidopsis*	Native promoter	ABA signaling	Wang et al. (2015)

(*Continued*)

TABLE 3.1 (Continued)

Functional category	Genes	Protein function	Origin	Transformation receptor	Promoter	Abiotic stress regulation	References
Protein kinase	SnRK2	Sucrose nonfermenting 1-related protein kinase 2	*Arabidopsis*	*Arabidopsis*	CaMV35S	Dehydration tolerance	Yoshida et al. (2006)
Protein kinase	ARK	ABA and abiotic stress responsive Raf-like kinase	*Physcomitrella patens*	*Physcomitrella patens*	CaMV35S	Hyper osmotic tolerance	Saruhashi et al. (2015)
Protein kinase	ROP11	Plant specific rho-like small GTPase	*Arabidopsis*	*Arabidopsis*	CaMV35S	ABA signaling	Li et al. (2010)
Protein kinase	SLAC1/ OST1	Open stomata 1	*Arabidopsis*	*Arabidopsis*	–	Drought stress	Geiger et al. (2009)
Protein kinase	OST1	Open stomata 1	*Arabidopsis*	*Arabidopsis*	–	ROS signaling	Sirichandra et al. (2009)
						ABA signaling	
Protein kinase	OST1	Open stomata 1	*Arabidopsis*	*Arabidopsis*	CaMV35S	ABA signaling	Grondin et al. (2015)
Protein kinase	OST1	Open stomata 1	*Arabidopsis*	*Arabidopsis*	CBF3	Freezing tolerance	Ding et al. (2015)
Protein kinase	OST1	Open stomata 1	*Arabidopsis*	*Arabidopsis*	POST1	ABA signaling	Mustilli et al. (2002)
						ROS signaling	
Protein kinase	SLAC1	S-type anion currents 1	*Arabidopsis*	*Arabidopsis*	pUBQ10	ABA signaling	Brandt et al. (2015)
Protein kinase	SLAC1	S-type anion currents 1	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Geiger et al. (2010)
Protein kinase	SLAC1	S-type anion currents 1	*Arabidopsis*	*Arabidopsis*	pUBQ10	ABA signaling	Brandt et al. (2015)
Protein kinase	SLAC1	S-type anion currents 1	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Geiger et al. (2010)
Protein kinase	CIPK26	CBL-interacting PK26	*Arabidopsis*	*N. benthamiana*	EF1α	ROS signaling	Drerup et al. (2013)
						Ca^{2+} signaling	
Protein kinase	Tyr nitration	Tyrosin nitration	*Arabidopsis*	*Arabidopsis*	CaMV35S	ABA signaling	Castillo et al. (2015)
Protein kinase	CBK3	CaM binding protein kinase-3	*Arabidopsis*	*Arabidopsis*	CaMV35S	Heat tolerance	Liu et al. (2011)
Protein kinase	ABI1 and PP2CA	Type 2C protein phosphatase	*Arabidopsis*	*Arabidopsis*	CaMV35S	ABA signaling	Rodrigues et al. (2013)
Protein kinase	SrlK	Leucine-rich repeat RLK gene	*Medicagotrancatula*	*Medicagotrancatula*	SrlK promoter	Salt tolerance	de Lorenzo et al. (2009)
His kinase	AtHKT1	Histidine kinase 1	*Arabidopsis*	*Arabidopsis*	HKT1	Salt tolerance	Maser et al. (2002)
His kinase	AtHKT1	Histidine kinase 1	*Arabidopsis*	*Arabidopsis*	HKT1	Salt tolerance	Rus et al. (2004)

(*Continued*)

TABLE 3.1 (Continued)

Functional category	Genes	Protein function	Origin	Transformation receptor	Promoter	Abiotic stress regulation	References
His kinase	AtHKT1	Histidine kinase 1	*Arabidopsis*	*Arabidopsis*	–	Salt tolerance	Rus et al. (2001)
His kinase	AtHKT1	Histidine kinase 1	*Arabidopsis*	*Arabidopsis*	CaMV35S	Vegetative osmotic stress signaling	Wohlbach et al. (2008)
ABA receptor	PYL8	Pyrabactin resistance 1-like protein 8	*Arabidopsis*	*Arabidopsis*	IAA19	Promote lateral root growth	Zhao et al. (2009)
ABA receptor	PYL6/ RCAR9	Pyrabactin resistance 1-like protein 6	*Arabidopsis*	*N. benthamiana*	Ubiquitin 10	ABA and jasmonic acid signaling	Aleman et al. (2016)
ABA receptor	PYR/PYL	Pyrabactin resistance	*Arabidopsis*	*N. benthamiana*	Rd29A	ABA signaling	Park et al. (2009)
ABA receptor	PYR1 PYL1/2/ 4/5/8	Pyrabactin resistance 1/ PYR1-like 1/2/4/5/8	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Gonzalez-Guzman et al. (2012)
ABA receptors	PYR/ PYL/ RCAR	Regulating components of ABA receptors	*Arabidopsis*	*N. benthamiana*	CaMV35S	ABA signaling	Rodriguez et al. (2014)
Transcription factor	AREB1	ABA responsive element binding protein 1	*Arabidopsis*	*Arabidopsis*	CaMV35S	ABA signaling	Furihata et al. (2006)
Transcription factor	CBF and COR	C-repeat binding factors and cold responsive genes	*Arabidopsis*	*Arabidopsis*	CBF3	Cold tolerance	Norén et al. (2016)
Transcription factor	AtMYC2 and AtMYB2	MYB-related transcription factors	*Arabidopsis*	*Arabidopsis*	CaMV35S	ABA signaling Drought tolerance	Abe et al. (2003)
Transcription factor	CBF2/ DREB1	C-repeat binding factor2/dehydration responsive element-binding factor 1	*Arabidopsis*	*Arabidopsis*	CBF2	Freezing tolerance	Novillo et al. (2004)
Transcription factor	MBF1C	Multi-protein binding factor 1 c	*Arabidopsis*	*Arabidopsis*	CaMV35S	Heat tolerance	Suzuki et al. (2011)
Transcription factor	WRKY39	–	*Arabidopsis*	*Arabidopsis*	CaMV35S	Heat tolerance	Li et al. (2010)
Transcription factor	HD-START	Homeodomain-START-TF	*Arabidopsis*	*Arabidopsis*	CaMV35S	Drought tolerance	Yu et al. (2008)
MAPK	MPK9&12	MAPK-protein kinase9&12	*Arabidopsis*	*Arabidopsis*	Actin 2	ROS signaling ABA signaling	Jammes et al. (2009)
MAPK	MKK2	MAPK kinase kinase 2	*Arabidopsis*	*Arabidopsis*	ADH	Cold tolerance Salt tolerance	Teige et al. (2004)

(*Continued*)

TABLE 3.1 (Continued)

Functional category	Genes	Protein function	Origin	Transformation receptor	Promoter	Abiotic stress regulation	References
MAPK	CRLK1	Calcium/Calmodulin-regulated receptor like kinase-1	*Arabidopsis*	*Arabidopsis*	–	Cold tolerance	Yang et al. (2010)
MAPK	TPC1	Two pose channel 1	*Arabidopsis*	*Arabidopsis*	CaMV35S	ROS signaling	Choi et al. (2014)
						Salt tolerance	
MAPK	MKK2	MAPK-kinase kinase 2	*Arabidopsis*	*Arabidopsis*	CaMV35S	Cold tolerance	Teige et al. (2004)
						Salt tolerance	
MAPK	OsMEK1 and OsMAP 1	–	*Arabidopsis*	*Arabidopsis*	–	Low temperature tolerance	Wen et al. (2002)
HSFs	HSFA1S	A1 heat shock factors	*Arabidopsis*	*Arabidopsis*	–	Heat tolerance	Liu et al. (2011)
HSFs	AtCaM3	Arabidopsis Calmodulin 3	*Arabidopsis*	*Arabidopsis*	CaMV35S	Heat shock signal transduction	Zhang et al. (2009)
PMRK (Plasma membrane receptor like)	GHR1	Guard cell hydrogen peroxide resistant 1	*Arabidopsis*	*Arabidopsis*	SUPER	ROS signaling	Hua et al. (2012)
						ABA signaling	
ERK (Extra cellular signal regulated kinase)	HAMK	Heat shock activated MAPK	Alfalfa	Alfalfa	–	Cold tolerance	Sangwan et al. (2002)
						Heat tolerance	
CDPK	CPK5	Calcium dependent PK 5	*Arabidopsis*	*Arabidopsis*	CaMV35S	Defense mechanism	Dubiella et al. (2013)
Peroxidases	FBP1	French bean peroxidase type 1	*Phasiolus vulgaris*	*Arabidopsis*	CaMV35S	Defense mechanism	Bindschedler et al. (2006)
CBFTPs	ICE1	Inducer of CBF expression 1	*Arabidopsis*	*Arabidopsis*	CBF3	Freezing tolerance	Chinnusamy et al. (2003)
ST gene	ST6-66	Salt-tilerace 6-66 gene	*Thellungiellahalophila*	*Arabidopsis*	CaMV35S	Salt tolerance	Du et al. (2008)
Stress inducible gene	VuNCEDI	9-cis-epoxy carotenoid dioxygenase	*Vignaunguiculata*	*Vignaunguiculata*	CaMV35S	Drought tolerance	Iuchi et al. (2000)
USP	SpUSP	Universal stress protein	*Solanum pennellii*	*Solanum pennellii*	CaMV35S	Drought tolerance	Loukehaich et al. (2012)
Protein phosphatase	PP_2C	Type 2C protein phosphatase	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Ma et al. (2009)
G-proteins	GTG1 and GTG2	GPCR-type G protein	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Pandey et al. (2009)
ABA-binding START protein	PP2C	Type2c-protein phosphatases	*Arabidopsis*	*Arabidopsis*	–	ABA signaling	Park et al. (2009)

(*Continued*)

TABLE 3.1 (Continued)

Functional category	Genes	Protein function	Origin	Transformation receptor	Promoter	Abiotic stress regulation	References
Osmosensors	OSCA1	Hyper osmolality induced (Ca^{2+})i increase 1	*Arabidopsis*	*Arabidopsis*	Rd29A	Osmotic tolerance Drought tolerance	Yuan et al. (2014a,b)
MscS (Mechano sensitive channel of small conductance)	MSL8	Mechanical stresses	*E. coli*	*Arabidopsis*	LAT52 (pollen specific promoter)	Hypo-osmotic tolerance	Hamilton et al. (2015)
Stress sensor	COLD1	Activates Ca2 + ion channel	Rice	Rice	CaMV35S	Chilling tolerance	Ma et al. (2015)
ROS signaling	Chy1	Peroxisomal β-hydroxyisobutanyl Co-A hydrolase	*Arabidopsis*	*Arabidopsis*	CBF3	ROS signaling Cold tolerance	Dong et al. (2009)

receptor-like protein gene NtC7 in onion epidermal cells confirmed that it is a transmembrane protein and its overexpression in tobacco provides osmotic stress tolerance to seeds induced by mannitol, but not by NaCl (Tamura et al., 2003). Several genetic and biochemical studies on the salt stress tolerance revealed that MAP kinases are important signal transducers and they were regulated by osmotic and salt stress both at transcriptional and protein levels. In plants such as *Arabidopsis* the protein AtHK1 (yeast osmosensor SLN1 homolog) senses the osmotic signal and transmits the signal to an MAPK downstream cascade and activates MEKK1, AtMPK3, AtMPK4, and AtMPK6 (Mizoguchi et al., 1996; Ichimura et al., 2000; Droillard et al., 2002; Wohlbach et al., 2008). Transgenic overexpression of AtHK1 in *Lycium barbarum* exhibited tolerance to salt and water stress by modulating the antioxidative enzymes and osmolytes (Chen et al., 2010). In the MAPK signaling pathway the activated MEKK1 further elicits the activity of MKK4 and MKK5 (Asai et al., 2002). During salt and cold stress the activated MKK2 as an activator subsequently activates MPK4 and MPK6. The transgenic overexpression of the MKK2 gene in *Arabidopsis* plants upregulates several stress responsive genes and improves cold and salt tolerance (Teige et al., 2004). Under salt stress conditions the MAP6 kinase activates are regulated by phosphatidic acid (PA) and activated MAP6 associates with MYB41 TF, improves the salt tolerance in *Arabidopsis* plants (Yu et al., 2010; Hoang et al., 2012).

The osmotic stress-induced MKK and MPK members might activate the upstream activators such as rd29A and rd29B gene promoters under salt stress conditions (Hua et al., 2012). Transgenic overexpression of maize ZmSIMK1 in *Arabidopsis* plants triggers several known stress responsive genes such as rd29A and P5CS1 under salt stress conditions and improves the salt tolerance of transgenic plants (Gu et al., 2010). In a recent study Kim et al (2012) identified an osmotic regulator gene in *Arabidopsis*, MKKK20, a new MAP kinase kinase gene, by activating MPK6. Transgenic *Arabidopsis* plants overexpressing MKKK20 genes displayed osmotic tolerance nature compared with mutant mkkk20 plants (Kim et al., 2012). Earlier findings suggest that *Arabidopsis* MKK4 improves the osmotic tolerance at cells and seedling stage by activation of MPK3 (Kim et al., 2011). Osmotic stress also activates the MPK17 in *Arabidopsis* and its homolog ZmMPK17 in maize (Pan et al., 2012). As part of the hypoosmotic stress-signaling pathway *Arabidopsis* MPK20 interacts with proline dehydrogenase (ProDH) under hypoosmotic stress conditions and provides osmotic tolerance to plant cells (Moustafa et al., 2008). Transgenic tobacco plants overexpressing maize MAP kinase kinase ZmMKK4 improved the osmotic stress tolerance and its transcript levels were modulated by salinity stress (Kong et al., 2011). Several salt-induced osmotic stress MAP kinases were identified in rice, that is, OsMPK44 (Jeong et al., 2006), OsMSRMK3 (Agrawal et al., 2003), OsMSRMK2 (Agrawal et al., 2002), OsEDR1 (Kim et al., 2003), OsMAPK5 (Xiong and Yang, 2003), and OsMAPK4 (Fu et al., 2002). Ectopic overexpression of rice MAP kinase genes, OsMPK5 and OsMPK44, in rice plants improved tolerance to salt stress (Jeong et al., 2006; Xiong and Yang, 2003). In contrast stress responsive overexpression of

rice OsMAPK33 in transgenic rice plants increases sensitivity to salt stress by altering the expression of several ion transport genes such as efflux pumps and the K^+/H^+ antiporter. These data clearly suggest that OsMAPK33 could have a negative role in salt stress tolerance (Lee et al., 2011).

Transgenic overexpression of cotton MAP kinase GhMPK2 in cotton plants improved the osmotic adjustment of the transgenic plants and enhanced tolerance to salt stress (Zhang et al., 2011). Osmotic stress responsive MAP kinase of cucumber root CsNMPAK, upon overexpression in tobacco plants, improved the germination rates of seeds under salt stress conditions compared with wild type plants (Xu et al., 2010a,b). Transgenic lines overexpressing *Populus trichocarpa* MAP kinase PtMAPKK4 displayed tolerance to salt stress by improving oxidative stress tolerance compared with wild type plants (Yang et al., 2017) (Table 3.1).

3.5 ABA SIGNALING PATHWAY

The plant hormone ABA plays a crucial role in response to several stresses, regulates the movements of stomata, regulates the gene expression, and modulates various metabolic pathways (Cutler et al., 2010). The concentration of ABA varies in response to abiotic stresses, particularly under drought and salinity (Khan and Hakeem, 2014). The phytohormone ABA synchronizes the complex regulatory network of drought and salt stresses, hence it is vital for the plants to cope with extreme environmental conditions (Zhu, 2002). Two plasma membrane proteins such as ATP-binding cassette (ABC) transporters AtABCG40/AtPDR12/ and AtABCG25 were identified in *Arabidopsis* through genetic and biochemical analysis studies (Kang et al., 2010). Among them AtABCG25 export ABA from ABA biosynthesis cells to apoplastic areas, further AtABCG40 import ABA from apoplastic cells to guard cells to close the stomata (Kuromori and Shinozaki, 2010). Further, to elucidate the genetic network of ABA signaling, comprehensive genetic screening tests and protein-interaction studies have been carried out in several model plant species and have identified pyrabactin (4-bromo-N-[pyridin-2-ylmethyl] naphthalene-1-sulfonamide) resistance (PYR)/regulatory component of ABA receptor (RCAR) proteins, also called PYL family proteins, or soluble START domain proteins, as receptor for ABA chemical signal (Park et al., 2009; Ma et al., 2009). The hormone ABA interacts with PYL family receptors through affinity binding; in the presence of co-receptors such as type 2C protein phosphatases (PP2Cs), ABI1, ABI2, HAB1, and PP2CA. (Ma et al., 2009).

The SnRK2 kinases such as SnRK2.2, 2.3, and SnRK2.6 bind to PP2Cs in the absence of ABA, and inactivate the catalytic domain of PP2Cs by dephosphorylating the activation loop (Soon et al., 2012). The structural studies revealed that PYL/RCAR protein forms a central hydrophobic ligand-binding pocket that plays a vital role as an ABA binding site. ABA enters into this pocket and stimulates the gate and latch loops to close and lock the pocket, and forms the binding surface to PP2Cs (Melcher et al., 2009). In the pocket the tryptophan residue of PP2C tightly binds to the ABA and stabilizes the ABA-PYLs-PP2C complex, and the ABA-PYL complex inhibits the active site of the PP2C and liberates the SnRK2s (Park et al., 2009). The liberated SnRK2s autophosphorylates and activates, and the activated SnRK2s further modulate several downstream effectors (Furihata et al., 2006; Fujii et al., 2009). The SnRK2 family comprises three subfamilies, SnRK1, SnRK2, and SnRK3 (Hrabak et al., 2003). The members of SnRK2 were activated by both osmotic stress and ABA; however subfamily II members, except SnRK2.2, SnRK2.3, and SnRK2.6, were weakly activated by ABA (Boudsocq et al., 2004). The ABA activated SnRK2 kinase proteins phosphorylate bZIP transcription factors such as ABA responsive element binding factors (ABFs) and AB15 (Furihata et al., 2006); the plasma membrane anion channels SLAC1 (slow anion channel-associated 1) and KAT1 (K^+ channel in *Arabidopsis thaliana* 1) that are critical for ABA regulation of stomatal movement, and RBOHF (respiratory burst oxidase homolog F) that functions in ROS generation in response to ABA (Sirichandra et al., 2009); miRNA regulation, chromatin regulation, and RNA splicing (Wang et al., 2013).

Calcium is an important molecule within the ABA dependent signaling pathway and plant guard cell regulation. Genetic analysis studies revealed that *Arabidopsis* mutant plants with four redundant, nonfunction, calcium-dependent protein kinases, CPK5, CPK6, CPK11, and CPK23, are unable to close the stomata in response to ABA (Brandt et al., 2015). The ABA activated CPK simultaneously phosphorylates two key phosphorylation sites in S-type anion channel (SLAC1) and closes the guard cell (Geiger et al., 2010; Brandt et al., 2015). The ABA activated calcium signal activates the CBL-interacting protein kinase (CIPK26)/ calcineurin B-like (CBL), which interacts with the N-terminal of RBOH protein and phosphorylates it and enhances the production of ROS (the RBOH Ca^{2+} sensor proteins together with their interacting kinases (CIPKs) (Drerup et al., 2013). Furthermore, recent genetic analysis studies in mutant plants revealed that

ABA also induced the production of nitric oxide (NO) and phosphatidic acid (Hou et al., 2016). Further, NO deactivates the SnRK2 and PYLs by modifying the S-nitrosylation of a cystenine residue (Wang et al., 2015; Castillo et al., 2015).

The protein PYRs upon binding to ABA modulates several gene expressions and plays a vital role in plant growth and development. Knockout studies and protein interaction experiments revealed that in the presence of ABA the protein PYL6 strongly interacts with a key basic helix−loop−helix transcription factor MYC2 of jasmonic acid (JA) signaling pathway and modulates the expression of JA pathway genes ZA6 and ZA8 (Aleman et al., 2016). However, it was shown that under stress conditions higher concentrations of ABA limit the growth of primary and lateral roots. It was shown that a higher concentration of ABA limits the growth and development of primary and lateral roots. However, the protein PYL8 at low concentrations of ABA directly interacts with the transcription factors MYB77; and MYB 77 triggers the induction of several auxin-responsive genes as resulted lateral roots growth enters into the recovery phase (Zhao et al., 2014).

Several studies have shown the potential applications of engineering ABA signaling molecules through transgenic overexpression/suppression of signaling pathway genes. Transgenic constitutive overexpression of ABA receptor proteins (clade A PP2C interacting proteins) PYL5 and HAB1 in *Arabidopsis*, displayed disparity in phenotypes in responses to ABA. The PYL5 OE lines exhibit hypersensitivity to ABA, whereas HAB1 OE lines showed reduced sensitivity to ABA. Simultaneous overexpression of PYL5 and HAB1 (PYL5-OE HAB1-OE) transgenic lines exhibited a phenotype similar to transgenic PYL5-OE lines, which revealed that PYL5 has alienated HAB1 function in the transgenic plants. Further, this study demonstrates PYL5−OE lines displayed enhanced drought tolerance in transgenic plants (Santiago et al., 2009). Constitutive overexpression of another ABA receptor protein PYL2 in *Arabidopsis* activated the ABA signaling compared with mutant plants (Mosquna et al., 2011). Genetic analysis studies in *Arabidopsis* mutant plants identified that in the absence of ABA, the receptor molecule PYL4A^{1947} interacts with PP2CA. Transgenic overexpression of PYL4A^{1947} under a constitutive promoter enhances the sensitivity of transgenic *Arabidopsis* plants to ABA. The transgenic plants also displayed increased tolerance to drought stress compared with nontransformed or transformed 35S:PYL4 plants (Pizzio et al., 2013). Similarly, overexpression of a rice cytosolic ABA receptor OsPYL/RCAR5 in rice plants under a maize ubiquitin promoter trigger the expression of several stress responsive genes such as LEA, dehydrin, and Hsp under normal growth conditions and enhanced the drought and salt tolerance of rice at the vegetative growth stage. However, transgenic plants yielded reduced seed and plant height under field conditions. This study demonstrates that fine regulation of expression OsPYL needed to overcome the detrimental effects in transgenic plants (Kim et al., 2012; Kim et al., 2014). Transgenic overexpression of two rice ABA receptor genes *OsPYL3* and *OsPYL9* in rice plants demonstrated that in the presence of ABA, *OsPYLs* expressed lines showed positive seed germination and improved drought and salt tolerance compared with wild type plants (Tian et al., 2015). Likewise, constitutive overexpression of rice OsPYL3 in *Arabidopsis* plants displayed an ABA hypersensitive phenotype and improved tolerance to cold and drought stress (Lenka et al., 2018). Transgenic overexpression of poplar ABA receptors PtPYRL1 and PtPYRL5 in *Arabidopsis* plants improved hypersensitivity to ABA and drought stress tolerance (Yu et al., 2016). In a recent work, two different types of transgenic plants were developed in *Arabidopsis* by transgenic overexpression of two tomato monomeric subgroup ABA receptors, AtPY4-6 and AtPYL7-10, and a tomato dimeric receptor from the subgroup AtPYL1. The *Arabidopsis* transgenic lines carrying tomato monomeric-type receptors displayed improved drought tolerance, while transgenic *Arabidopsis* lines carrying a tomato dimeric receptor did not show drought tolerance (Gonzalez-Guzman et al., 2012).

The transcription factors bZIP play vital roles in the ABA signaling pathway. Transgenic overexpression of one of the positive regulators of the ABA and a member of rice bZIP, OsbZIP72, in rice transgenic plants demonstrated hypersensitivity to ABA and modulated the expression of ABA responsive genes such as LEAs and improved drought tolerance in transgenic rice plants compared with wild-type plants (Lu et al., 2009). Rice third subfamily member bZIP transcription factor OsbZIP46 was activated by drought, heat, hydrogen peroxide, and ABA, but not by salt and cold. Transgenic overexpression of OsbZIP46 displayed hypersensitivity to ABA, but no tolerance to drought stress. Mutational modification with a deletion of domain D created a constitutive active form of OsbZIP46 (OsbZIP46CA1). Transgenic overexpression of the active form in rice seedlings improved tolerance to drought and osmotic stress by modulating several downstream stress responsive genes (Tang et al., 2012). These studies proved that

engineering ABA signaling pathway genes was an ideal strategy to improve abiotic stress tolerance in plants (Table 3.1).

3.6 CALCIUM SENSORS AND SIGNALING

Various extracellular factors such as biotic and abiotic stress and intracellular responses stimulate calcium (Ca^{2+}) as a secondary messenger (Snedden and Fromm, 1998, 2001; DeFalco et al., 2010). A number of Ca^{2+} sensors including calmodulin (CaM), calmodulin-like (CML), calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs)/calcium dependent protein kinases (CDPKs) (Yang and Poovaiah, 2003; Bouche et al., 2005) have been reported in plants. The CaMs are small acidic proteins, unique Ca^{2+} sensor proteins, which consist of four EF-hand domains located at both N-terminal and C-terminal to bind Ca^{2+} in plants and animals (Perochon et al., 2011). Osmotic stress-induced CaM-mediated signaling pathway is well documented in several plant species (Bouche et al., 2005) such as AtCML8, an ortholog of GmCaM4 (Park et al., 2010), and AtCML9 (Magnan et al., 2008). Transgenic *Arabidopsis* plants overexpressing soybean CaM gene GmCaM4 improved drought and salt tolerance by modulating the transcriptional activity of an MYB2 transcription factor, which in turn activated the expression of salt and drought responsive genes (Abe et al., 2003; Yoo et al., 2005). A novel rice calmodulin-like gene OsMSR2 (multistress-responsive gene 2) overexpressed in transgenic *Arabidopsis* plant exhibited drought and salt tolerance by modulating the known stress responsive genes in an ABA-mediated pathway (Xu et al., 2011). Several CaMBPs involved in the calcium signaling pathway were induced by drought, salt, or osmotic stresses (Zeng et al., 2015). Overexpression of wheat CaMBP TaCCaMK in *Arabidopsis* plants reduces the ABA sensitivity and acts as a negative regulator of ABA-mediated signaling pathway (Yang et al., 2011). The transgenic expression of one of the Ca^{2+}/CBP proteins *BjGly-I* of *Brassica juncea* in tobacco plants conferred tolerance to methylglyoxal and high salt stress (Veena et al., 1999). In *Arabidopsis* drought, low temperature and high salt stress-induced protein AtCaMBP25 reduced the osmotic tolerance nature of transgenic plants by acting as negative regulator, silencing of AtCaMBP25 through antisense approach increased the osmotic tolerance (Perruc et al., 2004). Transgenic overexpression of AtCaM3 in *Arabidopsis* plants increased the temperature tolerance (Zhang et al., 2009). High temperature responsive signaling pathway genes were AtCBK3, AtPP7, AtHSF, and AtHSP elevated in the transgenic *Arabidopsis* plants overexpressing rice $OsCaM_{1\text{-}1}$.

Calcineurin B-like protein-interacting protein kinases (CIPKs) are important components of Ca^{2+}-mediated CBL-CIPK network in response to various stresses (Xiang et al., 2007; Zhao et al., 2009). Several CIPK family genes have been identified and a few of their functional roles have been characterized in plant species such as *Arabidopsis* (Kolukisaoglu), rice (Kolukisaoglu et al., 2004), canola (Zhang et al., 2014a, b), maize (Chen et al., 2011), and wheat (Sun et al., 2015). Numerous CIPK genes involved in the SOS signaling pathway mediated salt tolerance have been well characterized (Qiu et al., 2004; Tang et al., 2011). In *Arabidopsis*, root salt tolerance and Na^+ efflux are controlled by the Na^+/H^+ antiporter; the interaction between AtCBL4 and CIPK24 (SOS3-SOS2) complex mainly regulates NHX activity (Liu et al., 2011), whereas in the shoots AtCBL10-AtCIPK24 complex protects from salt stress (Quan et al., 2007). Transgenic overexpression of maize CIPK21 enhanced salt tolerance of *Arabidopsis* by activating the downstream stress responsive transactivator dehydration-responsive element-binding (DREB) proteins, decreased accumulation of Na +, and enhanced root length under salt stress conditions (Chen et al., 2014). Ectopic constitutive expression of halophyte *Hordeum brevisubulatum* kinase HbCIPK2 improved the osmotic stress tolerance in *Arabidopsis* mutant sos2-1 plants, as well as in wild-type *Arabidopsis* plants during seed germination under salt stress (Li et al., 2010). Heterologous expression of *Populus euphratica* CBL1 (PeCBL1) improves the ion homeostasis by interacting with CIPK24, CIPK25, and CIPK26 and enhanced salt tolerance (Zhang et al., 2013). Transgenic tobacco plants overexpressing a wheat TaCIPK2 displayed enhanced tolerance to drought by improving antioxidative enzymes and regulating stomata movements (Wang et al., 2016). A CBL-interacting protein kinase of wheat TaCIPK29 when overexpressed in transgenic tobacco plants expresses throughout the cells and interacts with genes such as TaCBL2, TaCBL3, NtCBL2, NtCBL3, NtCAT1; regulates ROS homeostasis and cations; and improves salt stress tolerance of transgenic plants (Deng et al., 2013).

The CDPKs are known to be involved in abiotic stress-induced Ca^{2+} signaling pathway. The ion channel proteins and transporters are the substrates for CDPKs. Several CDPKs have been observed to induce drought stress (Xu et al., 2010a,b). Genetic analysis revealed that CPK3 and CPK6 regulate ion channels of guard cells. CPK3 localizes in the plasma membrane and vacuole, and regulates abiotic stress signaling independent of MAPK (Mehlmer et al., 2010). However, CPK6 is a functionally redundant positive regulator for abiotic stress, and enhanced constitutive expression of CPK6 in *Arabidopsis* plants and improved

the drought and salt tolerance of transgenic plants by modulating expression of several known stress responsive genes (Xu et al., 2010a,b). The *Arabidopsis* transgenic plants overexpressing CPK10 displayed drought tolerance, in the transgenic plants the CPK10 protein might interact with HSP1 and plays a critical role in regulating ABA- and Ca^{2+}-mediated stomatal movements (Zou et al., 2010). The CDPK proteins of *Arabidopsis* phosphorylate the subfamily of bZIP transcription factors, ABA-responsive element binding factors (ABFs). The CPK4 and CPK11 proteins, phosphorylates ABF1 and ABF4 improve the tolerance to salt and drought stress by regulating various physiological process (Zhu et al., 2007). Similarly, CPK32 interacts and phosphorylates ABF4, the transgenic overexpression of ABF4 regulated expression of several ABA-responsive genes (Choi et al., 2014). In contrast, the closely related CPK21 and CPK23 act as negative regulators in abiotic stress-induced signaling pathway. The mutant lines with loss of function of CPK21 and CPK23 displayed enhanced drought and salt tolerance (Ma and Wu, 2007; Franz et al., 2010). The overexpression of CPK23 in transgenic lines exhibited drought and salt stress sensitivity by increasing stomatal apertures and altered ion homeostasis (Ma and Wu, 2007). Several CDPK genes, OsCDPK7 (Saijo et al., 2000), OsCDPK 13 (Abbasi et al., 2004), and OsCPK21 (Asano et al., 2011), were transformed into rice and the transgenic rice plants displayed enhanced tolerance to drought, salt, and cold, suggesting that all of these CDPKs are involved in ABA and abiotic stress signaling pathways (Table 3.1).

3.7 ROS SIGNALING

Aerobic organisms in response to different environmental cues produce ROS, such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH), and singlet oxygen (O) from excitation or incomplete reduction of molecular oxygen, which are harmful to organisms. Furthermore, ROS play a crucial role in signaling pathways to regulate plant growth and development under various biotic and abiotic stresses (Apel and Hirt, 2004; Miller et al., 2010; Chakradhar et al., 2017; Khan and Khan, 2017). Genetic analysis studies revealed that ROS molecules such as $O_2^{\bullet-}$ and H_2O_2, which are considered as primary ROS in plants, act as secondary messenger molecules in the signaling pathway to regulate various biological functions in plants (Foyer and Noctor, 2005). Due to high reactive nature ROS they react with various biological molecules such as membrane lipids, carbohydrates, proteins, and DNA. ROS molecules such as H_2O_2 rapidly diffuse through biological membranes through aquaporins. Moreover, plants also develop efficient enzymatic and nonenzymatic antioxidative systems to protect from various stresses. ROS scavenging systems including ascorbate peroxidases (APX), catalase (CAT), dehydroascorbate reductase (DHAR), glutathione peroxidases (GPX), glutathione S-transferase (GST), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), peroxiredoxin (PRX), and superoxide dismutase (SOD) are localized in different parts of the plant to detoxify ROS. Avoiding of excess ROS production is an important process to maintain ROS homeostasis under stress conditions. Maxwell et al. (1999) reported that alternative oxidases (AOX) prevent excess ROS production in mitochondria. Other mechanisms like leaf movements, leaf curling, and rearranging photosynthetic apparatus maintain limited ROS production through balancing absorbed energy levels with availing of CO_2 (Mittler, 2002).

Genetic analysis studies in the past few decades identified ROS molecules as important signaling molecules in plants (Pitzschke and Hirt, 2006). The overexpression of OsHK3 in transgenic rice regulated NADPH oxidase expression and H_2O_2 production in ABA signaling (Wen et al., 2002). The plant NADPH oxidases also referred to as respiratory burst oxidase homologs (RBOHs) are mostly enzymatic sources to produce ROS as a signaling molecule during abiotic stress. Transgenic overexpression of NADPH oxidase of tomato SlRBOH1 induced the production of H2O2, ABA accumulation, stomatal closure/opening and oxidative stress tolerance (Xia et al., 2014; Zhou et al., 2014). Transgenic overexpression of pepper receptor-like protein kinase 1 (CaRLK1) improved the expression of RBOH genes and production of O_2; the resulting transgenic plants displayed tolerance to pathogen infection (Yi et al., 2010).

These ROS molecules regulate the expression of several genes as a signaling molecule. The ROS levels vary the gene expression by oxidating the signaling pathway components and activating the redox sensitive transcription factors (Laloi et al., 2004). The overexpression of various families of transcription factors including WRKY, Zat, RAV, GRAS, and Myb resulted in increased ROS signaling in genetically modified plants (Desikan et al., 2001; Vranova et al., 2002; Epple et al., 2003; Pnueli et al., 2003; Rizhsky et al., 2003; Rizhsky et al., 2004). In *Arabidopsis*, the expression of AtPep1 increased H_2O_2 synthesis and ROS signaling defense mechanism (Orozco-Cardenas et al., 2001; Huffaker et al., 2006). Rentel et al. (2004) reported that the overexpression of OXI1 (oxidative signal-inducible 1) gene in *Arabidopsis* enhanced the H_2O_2 signaling. Similarly, transgenic alfalfa expressing H_2O_2 induced OMTK1 activates the MAPK pathway downstream genes MMK3 revealed that OMTK1 can be activated not only by H_2O_2; but also by ethylene and other elicitors. (Nakagami et al., 2004).

In rice, h-type thioredoxin (Trx) OsTRXh1, which is involved in the regulation of apoplastic redox state, has been identified and transgenic plants with overexpression of OsTRXh1 induced less concentration of H_2O_2 regulated accumulation of ROS in apoplast, which influenced the expression of several developmental and stress responsive genes leading to the salt sensitive phenotype (Zhang and Guo., 2012). A rice NADPH thioredoxin reductase (NTRC) which utilizes the NADPH to reduce the chloroplast 2-Cys PRX BAS1 decreases the amount of H_2O_2 and protects the chloroplast against oxidative damage (Perez-Ruiz et al., 2006). The Ca^{2+}/CaM-dependent protein kinase (CCaMK) of rice OsDMI3 is required for the ABA dependent regulation of ROS by enhancing the expression of NADPH oxidase genes (Shi et al., 2012). A C2H2-type ZFP protein ZFP182 is a component of ABA-induced antioxidant defense system. ABA-activated MPKs induce the expression of ZFP proteins such as ZFP36, which activates the expression of NADPH oxidase, MAPK genes, and ABA-induced production of ABA (Zhang et al., 2014a,b). Transgenic rice plants with overexpression of ZFP36 recorded greater activities of antioxidative enzymes and tolerance to water stress (Zhang et al., 2014a,b).

Several MAPK cascades are crucial players in the ROS signaling pathway (Kovtun et al., 2000; Pitzschke and Hirt, 2006; Pitzschke et al., 2009). Transgenic overexpression of cotton MAPK kinase protein GhMKK1 in tobacco exhibited greater levels of antioxidative enzymes and improved tolerance to salt and drought stresses (Lu et al., 2013). Similarly, transgenic overexpression of GhMKK5 in tobacco plants activated several ROS and apoptosis related genes (Zhang et al., 2012). The CDPK proteins are known to be involved in abiotic stress-induced ROS regulation (You and Chan, 2015). Rice transgenic plants overexpressing OsCPK12-OX expressed several ROS scavenging enzyme genes (OsAPx2 and OsAPx8) and recorded lower concentration of H_2O_2 in the leaves compared with wild-type plants. Transgenic plants also displayed low levels of NADPH oxidase gene compared with wild-type plants (Asano et al., 2012). Transgenic overexpression of an apple CIPK gene MdSOS2L1 induced the expression of several ROS scavenging proteins and enhanced tolerance to salt stress (Hu et al., 2015).

3.8 CONCLUSIONS AND FUTURE PERSPECTIVES

Plants have refined and compactly regulated abiotic stress signal sensing and transduction mechanisms at the cellular or at the whole plant levels to protect them from various environmental cues. Plants exhibit genotypic variation in their signal sensing and transduction. Classical and advanced technologies were applied to develop stress tolerant plants by targeting signal transduction pathways. The classical breeding programs were not successful for abiotic stress tolerance as it is a multigenic trait. Thus, a genetic engineering approach offers a new way towards the development of stress tolerant transgenic plants for multiple abiotic stresses. The most critical step towards the development of an abiotic stress tolerant plant is the identification of candidate stress sensors. So far, forward and reverse genetic analysis in combination with chemical screening methods identified a limited number of sensors involved in the intricate abiotic stress signaling pathway due to gene redundancy problems. Advancement in the technologies such as genome editing and chemical genetic screening approaches would enable to overcome these problems. To date research on the abiotic stress signaling pathway have been carried out in plants grown in the culture media; however in nature, plants growing in a complex environment simultaneously face multiple abiotic and biotic stresses and there would be cross-talk between the abiotic and biotic signaling pathways. Hence, more research focus should be on field grown plants to decipher the signaling molecules involved in the multiple stresses.

The transgenic plants developed using the abiotic stress signaling molecules have significantly improved the stress tolerance under laboratory conditions and greenhouse experiments; however, these transgenic plants need to be evaluated in the field before being integrated in the molecular breeding programs. Furthermore, transgenic plants depend on the foreign genetic source, hence before release these transgenic plants need to undergo risk assessment tests. To overcome these regulatory issues, recently, genome editing technologies such as zinc finger nucleases (ZFNs), a transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/CRISPR associated 9 (CRISPR/Cas9) have been identified as viable tools and have started being used in several crop species not only for genetic improvement of desired traits, but also for functional characterization of genes.

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CHAPTER

4

Genetic Engineering/Genome Editing Approaches to Modulate Signaling Processes in Abiotic Stress Tolerance

Riwandahun Marwein[1,2], *Johni Debbarma*[1,2], *Yogita N. Sarki*[1,2], *Indrani Baruah*[1,2], *Banashree Saikia*[1,2], *H.P.D. Boruah*[1], *Natarajan Velmurugan*[3] *and Channakeshavaiah Chikkaputtaiah*[1]

[1]Biotechnology Group, Biological Sciences and Technology Division, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India [2]Academy of Scientific and Innovative Research (AcSIR), CSIR-NEIST, Jorhat, Assam, India [3]Branch Laboratory Itanagar, CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat, Assam, India

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4.1 INTRODUCTION

Global climatic changes and human anthropogenic activities have exhibited detrimental effects of abiotic stresses on crop productivity (Savvides et al., 2016). Rapidly unexpected climate change will challenge world food security by 70% of global food production to feed up to an additional 9 billion people by 2050 (Commission on Genetic Resources for Food and Agriculture), and the World Bank extrapolated the decrease of 20% crop yield by 2050 due to climate change (Gill et al., 2014). Climate variation in temperature, precipitation, and ozone concentration is adversely affecting plant growth and development quantitatively and qualitatively (Tripathi et al., 2016; Ramirez-Cabral et al., 2017). Plants being sessile are naturally exposed to both biotic and abiotic stresses, but exceeding a threshold level of these stress deteriorated plant growth and productivity (Meena et al., 2017). Abiotic stresses imposed on plants induce changes in the physiological metabolism affecting growth and yield (Ben Rejeb et al., 2014; Khan et al., 2016; Pasala et al., 2016; Hasanuzzaman et al., 2018). Although plants exhibit an adaptive defense response against abiotic stress, concurrent exposure to these stresses limits their potential, hindering their growth and development (Mohanta et al., 2017). About 50% of world agricultural yield is affected by abiotic stresses, which include drought, flooding, extreme temperature (heating or freezing), nutrient deficiency, ion toxicity (salinity, heavy metals), excess light, UV radiation, and soil hardness, impacting agricultural productivity (Khan et al., 2012, 2015, 2016; Anjum et al., 2015; Chikkaputtaiah et al., 2017). The combined effects of these stresses have a significantly detrimental effect on plant growth and development compared with the impact they have individually (Prasad et al., 2011; Pandey et al., 2017). Further, interaction between abiotic (mainly drought and salinity) and biotic stresses had a negative impact on the pathogen resistance of the plant (Suzuki et al., 2014; Kissoudis et al., 2016). Environmental stress also leads to the reduction in arable land due to soil degradation, erosion, and salinization, which requires the development of stress tolerant plants adaptable to unfavorable land to feed the growing world population (Koyro et al., 2012). Concerning the future food scenario and global population, rapid development of climate resilient plants is imperative and could be achieved through breeding or by engineering plant tolerance to abiotic stress (Rodziewicz et al., 2013).

Although use of conventional breeding for abiotic stress tolerant plants is achievable, its slow process is limited by the incompatible transfer of traits between species, involvement of thousands of genes, requirement of several back crosses to obtain a desired trait (Ahmad and Mukhtar, 2017), and a suitable gene for breeding (Abdallah et al., 2014; Parmar et al., 2017). Genetic engineering surmounts conventional breeding by its ability to modify a target gene of interest within the same or distantly related species (Singh and Singh, 2014). With the advent of structural and functional genomics approaches, understanding metabolic regulatory pathways of stress responsive genes and identifying valuable genetic traits through the use of modern molecular markers, genome wide association studies, and next generation sequencing approaches, facilitates the incorporation of these traits through genetic engineering in the making of abiotic stress tolerant plant cultivars (Mohanta et al., 2017).

This book chapter describes the plant response to abiotic stress in developing tolerance through stress signaling molecules such as late embryogenesis abundant (LEA) proteins, HSPs, methylglyoxyl, HyPRPs, and protein kinases. Various genetic engineering approaches to modulate abiotic stress signaling process in crop plants were also discussed along with recent case studies. The topic highlights a special emphasis on ubiquitination pathway genes, epigenetic regulation, small RNAs, and helicases in modulating signaling processes in abiotic stress tolerance. The chapter also explains the scope of modern genome editing system (CRISPR-Cas9 and CRISPR-Cpf1) in developing multiple abiotic stress tolerance in crop plants.

4.2 PLANT RESPONSE TO ABIOTIC STRESS IN DEVELOPING TOLERANCE

Plants complete their life cycle in one place and have to encounter different parameters of stress, that is, biotic or abiotic. Thus, plants have acquired the capability to respond to sudden change in the environment by activating signals positively or negatively. In response to abiotic stress, plants undergo a series of morphological, biochemical, physiological, and molecular changes thus reprogramming their genetic makeup, which determines their survival. Understanding the adaptive mechanism underlying the response of plants to multiple abiotic stresses causing disturbance in cellular homeostasis is crucial to developing abiotic stress tolerant plants. The response of plants to abiotic stress is highly complex because multiple stress response is governed by complex signaling pathways that either activate or inhibit downstream processes (Suzuki et al., 2014). However, living in an era where omics and bioinformatics technologies are greatly advancing, understanding the molecular mechanisms, interactions, and interconnected signaling

pathways of stress responsive genes in both model and crop plants gives us an insight into the biological functions, gene expression, and finally key genes regulating the tolerance (Ramesh, 2017), which might alleviate stress tolerance and possibly memory. Upon stress recognition, downstream signal transduction led to the activation of various stress responsive genes. These stress responsive genes have been divided into two classes: (1) genes encoding protective proteins like heat-shock proteins (HSPs) or chaperones, LEA proteins, osmoprotectants, antifreeze protein, detoxification enzymes and free radical scavengers, aquaporins, transporters (sugar and proline), enzymes involved in fatty acid metabolism, inhibitors of proteinase, ferritin and proteins that transfer lipid, ROS and RNS produced during abiotic stress; and (2) genes involved in the downstream signaling processes such as transcription factors, mitogen activated protein kinase (MAPK), calcium dependent protein kinase (CDPK), SOS kinase and phospholipase, and DEAD-box RNA helicases (Ciarmiello et al., 2011; Fang et al., 2016; Baruah et al., 2017). Another important plant product is methylglyoxylase, which is toxic in stress conditions (Hoque et al., 2016). In contrast, plant hormones such as abscisic acid (ABA) play a pivotal role in defense against abiotic stress by triggering the activation of short term responsive genes regulating osmotic balances, and maintaining root and shoot architecture, root hydraulic conductivity, and stomatal closure by regulation of ion channel thus reducing water loss in plants and the upregulation of other downstream stress inducible genes (Lata and Prasad, 2011; Verma et al., 2016) through an ABA dependent or ABA independent pathway (Shao et al., 2015). Fig. 4.1 describes the signaling mechanism modulating the plant response to abiotic stress.

4.2.1 Late Embryogenesis Abundant Proteins

LEA proteins are a group of hydrophilic proteins that were first identified in cotton (*Gossypium hirsutum*) seeds during the late stage of seed development in occurrence of desiccation. Their function in protecting orthodox seeds, pollen, and anhydrobiotic plants from dehydration suggests their protective role in plant tolerance to water scarcity. Their expression is induced by plant stress hormones, ABA during seed maturation, and by various abiotic stresses (Dalal et al., 2009). LEA proteins have been described as intrinsically unstructured proteins characterized by high proportion of charged amino acid residues such as alanine, serine, or threonine enabling them to change conformation to variable cellular environments such as water deficit thus facilitating their interactions with other macromolecules and stabilizing the negative impact caused by abiotic stress (Saucedo et al., 2017). Their functional protective role during desiccation is dedicated to their ability to prevent protein aggregation; to act as a molecular shield and membrane stabilizer, particularly those of dehydrins in cold stress; in ion sequestration; and to prevent inactivation of enzymes such as lactate and malate dehydrogenase 2 (Reddy et al., 2012; Battaglia and Covarrubias, 2013; Amara et al., 2014; Liang et al., 2016; Saucedo et al., 2017). In the model plant *Arabidopsis* LEA proteins are widely distributed subcellularly in the cytosol, nucleus, plastids, mitochondria, endoplasmic reticulum, and pexophagosomes, signifying their importance in protective mechanisms against desiccation or cold stress (Amara et al., 2014; Candat et al., 2014).

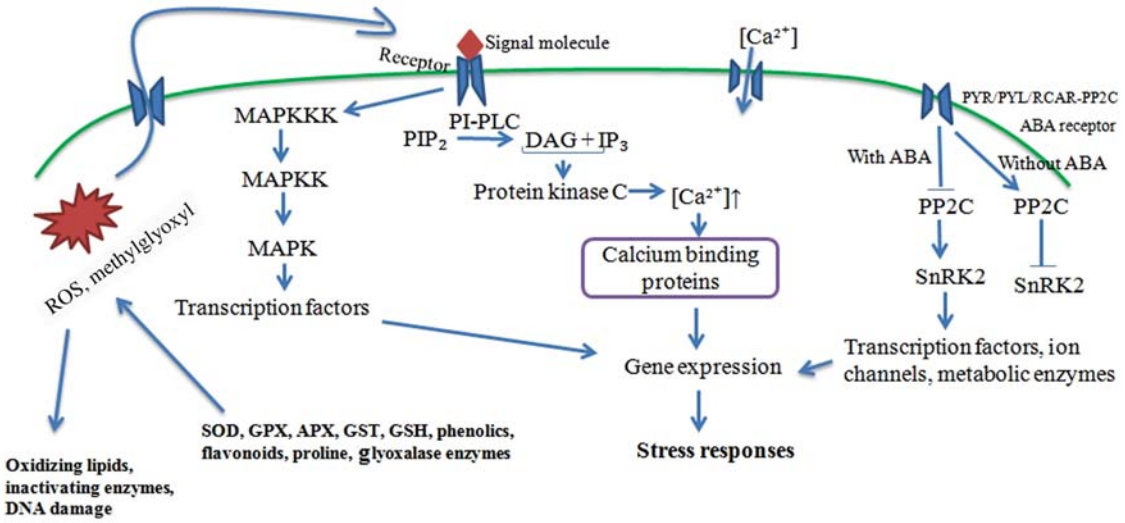

FIGURE 4.1 Overview of signaling mechanism modulating the plant response to abiotic stress.

4.2.2 Production of Methyglyoxyl Under Abiotic Stress

Methylglyoxyl ($CH_3C(O)CHO$) is a byproduct form from glycolytic intermediates via enzymatic and nonenzymatic elimination of phosphate from glyceraldehydes-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) and lipid metabolism, occurring in all living systems, however, in plants it is a 20th century discovery formed spontaneously by nonenzymatic mechanisms under physiological conditions from glycolysis and from photosynthetic intermediate (Hoque et al., 2016). Methyglyoxyl (MG) at low concentration acts as a signaling molecule wherein it regulates functional events such as cell proliferation and survival, maintaining redox reaction and cellular homeostasis (Hossain et al., 2012; Kaur et al., 2014; Hoque et al., 2016). Higher accumulation is toxic to the cell by directly disrupting cellular metabolism causing oxidation of macromolecules leading to formation of reactive oxygen species (ROS), inactivating antioxidant enzymes, photoreduction of O_2 to superoxide (O_2^-) in photosystem I increasing oxidative stress, membrane disruption, ion leakage, and DNA strand cleavage leading to death of plant cells. In rice *OsDJ-1C* converts MG into D-lactate in a single step, acting as a shorter route to MG detoxification (Ghosh et al., 2016). In *Arabidopsis* DJ-1/PfpI superfamily has been reported to exhibit glyoxylase activity against MG (Kwon et al., 2013). With understanding the metabolic pathway of MG and glyoxalase system, several studies have shown improved crop tolerance to abiotic stress by modifying one or two of these pathways (Singla-Pareek et al., 2003). Since both antioxidants and glyoxalase system are involved in the detoxification of ROS, coordination of both pathways can mitigate the accumulation of ROS in the plant cell (Nahar et al., 2015; Hasanuzzaman et al., 2017). In *Arabidopsis*, the gene encoding for glyoxalase II enzymes, *GLX2-1*, which is found to be nonfunctional during normal growth, is highly expressed in response to abiotic stress such as hypoxia, drought, and salt stress. The role of MG was also studied in induction of glycolytic enzymes triose phosphate isomerize (TPI) in a concentration dependent manner in rice resulting in decreased DHAP by shifting reactions toward GAP thus decreasing MG via feedback mechanism, which results in survival mechanism under abiotic stress (Sharma et al., 2012). More information on methylglyoxal, a novel signaling molecule in plant responses to abiotic stress, is covered in Chapter 10, Bioactive Molecules as Regulatory Signals in Plant Responses to Abiotic Stresses.

4.2.3 Hybrid Proline Rich Proteins (HyPRPs) and Abiotic Stress Responses

HyPRPs represent a group of putative cell wall proteins characterized by their unusual domain structure, which was deduced only from the primary amino acid sequence (Dvořáková et al., 2012). HyPRPs constitute a family of cell wall proteins that is phylogenetically related to nonspecific lipid transfer proteins. Members of the HyPRP family are involved in basic cellular processes and their expression and activity are modulated by environmental factors. Extensins (hydroxyproline-rich glycoproteins), proline-rich proteins (PRPs), and glycine-rich proteins (GRPs) are structural proteins of the primary plant cell wall. According to recent research, expression of a *HyPRP1* is suppressed by various abiotic stresses, including drought, high salinity, cold, heat, oxidative stress, and phytohormone ABA in *Solanum pennellii* (Li et al., 2016a). Investigation of transgenic functional analysis and transcriptional level *SlHyPRP1* and *SpHyPRP1* that were isolated from cultivated tomato *Solanum lycopersicum* and wild tomato *S. pennellii*, respectively, demonstrated that *HyPRP1* possibly plays a negative role in stress tolerance (Li et al., 2016a). Loss-of-function and gain-of-function studies revealed that overexpression plants showed a significant increase in cold tolerance than the wild type plants, which is conferred by the accumulation of OsPRP3 protein during cold treatment (Gothandam et al., 2010). In a recent study, *GhHyPRP4*, gene encoding putative HyPRP, was isolated from cotton cDNA library under cold stress. *GhHyPRP4* expression was significantly upregulated in leaves of cotton seedlings. *GUS* (b-glucuronidase) gene driven by *GhHyPRP4* promoter was specifically expressed in leaves and cotyledons of the transgenic *Arabidopsis thaliana*, which was remarkably induced by cold stress (Huang et al., 2011).

4.2.4 Role of Protein Kinases in Response to Abiotic Stress

Plants' adaptive tolerance to multiple abiotic stress relies on the coordinated signaling pathway perceived via multiple cell surface receptors and translating it into cellular response by activation of protein kinases such as mitogen activated protein (MAP) kinases, SNF1, CDK, etc. in response to various external and internal stimuli (Agarwal et al., 2010). MAP kinases' multifunctional role in plants involves the growth and development, immune defense system, hormone signaling, and response to biotic and abiotic stresses (Moustafa et al., 2014). These evolutionary conserved signal transduction modules are functionally interlinked through phosphorylation events and include the MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and a MAPK activating each other and other proteins in a sequential manner (Moustafa, 2014). The coordinated signaling is brought about by the activity of MAPK phosphatase that functions as an

on/off switch signal regulating gene expression of target proteins such as cytoskeletal proteins and transcription factors in response to environmental stresses (Moustafa et al., 2014). Many studies have revealed the functional role of plant MAPK kinase in adaptation to abiotic stresses such as drought, salinity, temperature fluctuations, etc. Because abiotic stress interferes with translational processes, Xu and Chua (2012) reported the role of MPK6 in promoting mRNA decapping by phosphorylating a decapping protein DCP1 thus getting associated with DCP5 conveying rapid response to dehydration stress in *Arabidopsis*. In rice, overexpression of *DSM1* gene encoding a putative MAPKKK of the Raf family protein kinase increases tolerance to dehydration stress at seedling stage by regulating the scavenging activity of ROS (Ning et al., 2010). Cotton MAPK gene (*GhMPK2*) conferred drought and salt stress tolerance in transgenic tobacco plant by upregulating various stress responsive genes such as *DIN1*, *Osmotin*, and *NtLEA5* and proline accumulation (Zhang et al., 2011). Functional role of *Arabidopsis* transcription factors *AtMYB41* in salt tolerance was found to be activated and interact with MPK6 (Hoang et al., 2012). In vitro assay of *Arabidopsis* MKKK20 was shown to regulate MPK6 activity under salt and cold stress and that MKKK20 overexpressing plant increased tolerance to salt stress (Kim et al., 2012). Rice *OsMKK6* and *OsMKK6DD* were found to phosphorylate *OsMPK3* enhancing chilling tolerance, confirming the signaling pathway for moderately induced low temperature (Xie et al., 2012).

CDPKs play a major role as calcium sensors to a changing intracellular calcium ion concentration, as well as transducers, translating signals into downstream phosphorylation events (Singh et al., 2018). Increased cytosolic Ca^{2+} concentration occurred as a result of exposure to various biotic and abiotic stresses initiating signaling cascades, a feature of an acclimatization response in plants (Tang and Luan, 2017). Realizing their role in abiotic stress response, studies have started exploring functions of CDPKs genes exposed to multiple stresses. The protective role of rice *OsCPK4* gene towards salt and drought stress was found to be effective by preventing membrane lipid peroxidation and reduced electrolyte leakage, thus protecting from stress induced oxidative damage (Campo et al., 2014). Similar function was reported by Bundo and Coca (2017) for *OsCPK10* gene isoform during drought stress and was also found to positively regulate blast disease resistance by a reduced production of hydrogen peroxide interfering with fungal growth. Reducing water loss and maintaining osmotic potential is crucial under drought conditions; this has been reported for rice CPK gene *OsCPK9*, which when overexpressed improved drought stress tolerance by enhancing stomatal closure and maintaining osmotic balance in an ABA dependent manner, in addition to increased spikelet fertility (Wei et al., 2014). Transgenic *Arabidopsis* plants expressing maize CPK genes such as *ZmCPK4* enhanced drought tolerance through ABA mediated regulation of stomatal closure (Jiang et al., 2013); *ZmCPK12* conferred tolerance to drought and salt stress (Wang and Song, 2013) and *ZmCK3* towards drought and heat stress (Wang and Song, 2014). In response to salinity stress overexpression of *OsCPK12* gene increases tolerance to salt stress by regulating expression of ROS genes such as *OsAPX2* and *OsAPX8*, however showing ABA sensitivity and susceptibility to blast fungal disease (Asano et al., 2012). Transportation of toxic metal arsenite in *Arabidopsis* was shown to be negatively regulated by CPK31 protein interacting with the nodulin 26-like intrinsic protein (NIP 1;1), an aquaporin involved in As (III) uptake (Ji et al., 2017). Gene encoding CPK of barley *HVCPK2a* was found to be upregulated by drought stress and undergo autophosphorylation; however its overexpression in *Arabidopsis* reduced tolerance to drought, reduced nitrogen balance index, increased chlorophyll content, and decreased relative water content (Ciesla et al., 2016).

4.2.5 Cross-Talk Signaling of Jasmonate and Ethylene Biosynthesis in Abiotic Stress Tolerance

The developmental processes in plants are governed by plant growth regulators such as ethylene (ET), jasmonic acid (JA), auxin, gibberellin, ABA, cytokinin, and salicylic acid (SA) (Khan and Khan, 2014; Khan et al., 2015, 2016; Huang et al., 2017). Under adverse environmental conditions, a plant's metabolic reactions and hormonal signaling pathways are disrupted. To avoid these, plants have set up a modified mechanism by which they can trigger a cascade to develop resilience. Ethylene is an important phytohormone that is involved in maintenance of seed germination, fruit ripening, abscission, and senescence (Abeles et al., 1992; Iqbal et al., 2017). The MAPK cascade pathways have integrated in major signaling systems with ethylene in various plants (Cristina et al., 2010). Jasmonate (JA) acts as a crucial signal to modulate multiple stress development in plants (Per et al., 2018). Recent studies have demonstrated the involvement of JA in leaf senescence and cold stress tolerance. Exogenous JA exhibits the expression of senescence related genes and leaf programmed cell death (PCD) in *Arabidopsis*. In response to cold stress, exogenous application of JA enhances *Arabidopsis* cold stress tolerance. Thus, JA also shows interaction with other hormone signaling pathways like ethylene to enhance tolerance upon cold stress (Hu et al., 2017). Phytohormone shows transport

activity from their sites of synthesis to mediate physiological and molecular responses of plants under stressful conditions. JA has been exercised for its biological role in response to abiotic stresses in growth and development (Clarke et al., 2009; Brossa et al., 2011; Zhao et al., 2013). Despite all environmental cues, JA and ET inducible *AP2/ERF* genes have been reported to confer stress tolerance when overexpressed in diverse species of plants (Chen et al., 2012). It has also been suggested that modification of upstream components may affect plant development. Moreover, it probably can say that downstream elements have a key positive regulatory role for genetic improvement of stress mediated crops. Thus, application of genetic engineering of JA and ET signaling pathways can offer a new platform to improve efficiency of abiotic stress tolerance in crops.

4.3 GENETIC ENGINEERING APPROACHES TO MODULATE ABIOTIC STRESS SIGNALING PROCESS IN CROP PLANTS

4.3.1 Transgenic Approaches

We are in a situation where a tremendous increase of crop productivity must be achieved with no increase in arable land and in the face of multiple environmental stresses. To overcome this problem, in the past few decades, a great deal of efforts have been devoted to breeding and biotechnology of stress tolerant crops with higher yields and improved qualities against multiple environmental stresses. However, it remains as one of the greatest challenges faced by modern agriculture (Takeda and Matsuoka, 2008; Newton et al., 2011; Liu et al., 2014a). Apart from the conventional breeding approaches that have marginal success due to the complexity of abiotic stress tolerance traits, modern transgenic technology has been in the limelight, which deals with introducing novel exogenous genes or altering the expression levels of endogenous genes to improve stress tolerance. The molecular mechanisms by which plants discern and transduce stress signals to cellular machinery to initiate adaptive responses can be deciphered proving it to be an essential prerequisite for identification of the key genes and pathways to genetically engineer stress-tolerant crop plants (Heidarvand and Maali Amiri, 2010; Ray et al., 2010; Sanchez et al., 2011). Significant progress has been made to elucidate the molecular mechanisms of abiotic stress responses in plants by means of high throughput sequencing and functional genomics tools. Pea DNA Helicase 45 (*PDH45*) has been discovered in combating multiple abiotic stresses in chili (Shivakumara et al., 2017). Molecular genetics and functional genomics of abiotic stress responsive genes (dehydrins and LEA) in crop plants have been recently reported (Singh et al., 2015; Chikkaputtaiah et al., 2017). A scheme of genetic engineering approaches to develop abiotic stress tolerance in crop plants is given in Fig. 4.2. More information on transgenic approaches to modulate signaling processes in abiotic stress tolerance is covered in Chapter 6, Drought Tolerance in Plants: Role of Signaling Molecules and its Molecular Mechanisms and Regulation.

4.3.1.1 Genetic Engineering for Biosynthesis of Osmoprotectants and LEA

Osmoprotectants are small molecules having low molecular weight, and are electrically neutral, highly soluble, and nontoxic at molar concentrations (Ahn et al., 2011). The accumulation of osmoprotectants is a common plant response under abiotic stresses. They basically stabilize proteins and membranes and contribute to the cell osmotic pressure (Zandalinas et al., 2018). In particular, proline accumulates in many plant species in response to environmental stress. *Ailanthus altissima* (Miller), when exposed to salinity and drought conditions showed the activity of proline biosynthesis enzyme, 1-pyrroline-5-carboxylate synthetase (Filippou et al., 2014). Maize plants truly suffered with abiotic stress and accumulated more proline to defend salt, dehydration, and heat stresses (Huang et al., 2018).

Thousands of *LEA* genes have been isolated and cloned to study functional role in abiotic stress responses. A novel gene *KvLEA* has been isolated and expressed from *Kosteletzya virginica*, a plant of economic importance that showed tolerance to multiple abiotic stresses (Tang et al., 2016). Thirteen LEA genes from *Pinus tabuliformis* introduced in *Escherichia coli* cells showed enhanced resistance under salt and heat stress (Gao and Lan, 2016). A dehydrin LEA gene *CsLEA11* was identified and characterized from *Cucumis sativus* that is induced by heat and cold stress. Overexpression of this gene in *E. coli* protects cell viability and confers tolerance to heat by preventing inactivation of lactate dehydrogenase enzymes and cold stress (Zhou et al., 2017). Molecular genetic characterization of LEA proteins have shown enhanced drought tolerance in upland cotton *Gossypium tomentosum* (Magwanga et al., 2018).

4.3.1.2 Aquaporin Genes Associated With Abiotic Stress Tolerance

Aquaporins are intrinsic membrane channel proteins, members of the major intrinsic protein (MIP) family, ubiquitously present in all kingdoms except archaea and intracellular bacteria. They facilitate the

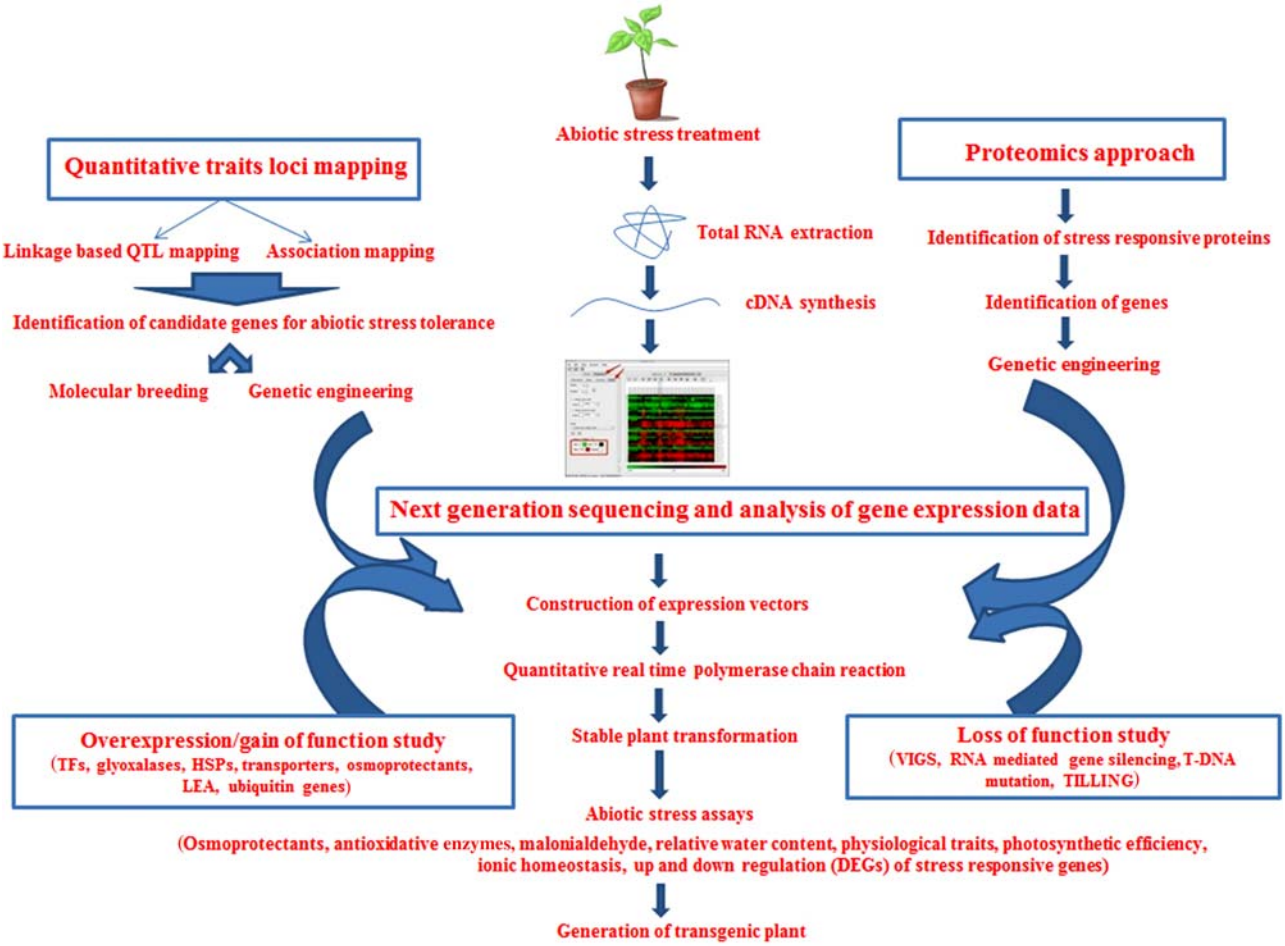

FIGURE 4.2 Scheme of genetic engineering approaches to develop abiotic stress tolerance in crop plants.

bidirectional flow of water across cellular membrane and other solutes such as glycerol, urea, CO_2, NH_3, metalloids, and ROS that are important for the growth and development of plants. Abiotic stresses such as drought, water logging, and salinity regulate the expression and activity of aquaporins maintaining water content to short and long term exposures, regulating the hydraulic conductivity in root, stem, and leaf and abiotic stress signaling (Srivastava et al., 2014; Moshelion et al., 2015). A considerable amount of work has been done on aquaporin subfamilies PIP (plasma membrane intrinsic proteins) and TIP (tonoplast intrinsic protein) because of their abundance in plants and their role in plant water transport. Their expression pattern is differentially regulated depending on the isoform, tissue, species, or level of stress (Šurbanovski and Grant, 2014). Overexpression functional studies of aquaporins genes have been carried out in both model and crop plants when imposed with different stresses. Among these, PIP genes from tomato *SlPIP2;1*, *SlPIP2;7*, and *SlPIP2;5* enhance drought tolerance by improving plant water content and osmotic balance (Li et al., 2016c); wheat aquaporins genes *TaAQP8* (Hu et al., 2012) and *TaNIP* (Gao et al., 2010) enhance salt tolerance in transgenic *Arabidopsis* plant. *Arabidopsis AtTIP5;1* increased tolerance to boron toxicity with a better phenotypic development (Pang et al., 2010). Improved drought tolerance in *Solanum tuberosum* L. was observed in a constitutively overexpressed *StPIP1* gene by improving water use efficiency, increasing nonstructural carbohydrates thus minimizing carbon starvation, and increasing biomass yield (Wang et al., 2017b). Under high salt and osmotic stress, transgenic *Arabidopsis* plant expressing barley exhibits stress tolerance conditions by minimizing oxidative damage, higher chlorophyll and water retention, and increases expression of osmoprotectant genes (Alavilli et al., 2016). The functions of aquaporins in CO_2 conductance were studied in the T-DNA insertion line in *Arabidopsis AtPIP1;2* observing a reduced net photosynthesis under drought stress (Boudichevskaia et al., 2015).

However some aquaporin genes display contrasting effects when subjected to abiotic stress, such as the

wheat *TaTIP2;2* that acts as a negative regulator to both drought and salt stress downregulating various stress responsive genes and decreasing proline content in an ABA independent manner (Xu et al., 2013a). In a study conducted by Li et al. (2015), *Arabidopsis* plant expressing *GoPIP1* transcript from *Galega orientalis* increased sensitivity to drought stress as a result of increased transpiration, fluctuated osmolyte accumulation, and changes in ABA signaling, however, no changes were observed in response to salt stress conditions. Negative impact on drought tolerance was also observed when *Arabidopsis PIP1b* was expressed in tobacco plant causing faster wilting under drought stress with no response under salt stress (Aharon et al., 2003). Further study into the mechanism of aquaporin genes in regulating abiotic stress tolerance is needed to decipher their functional role in different plant species.

4.3.1.3 Genetic Engineering of Molecular Chaperones, HSPs, and Plant Transcription Factors for Abiotic Stress Tolerance

Molecular chaperones are involved in protein folding, assembly, translocation, and degradation in a broad array of normal cellular processes. It has been reported that a wide range of proteins have chaperone activity. However, many molecular chaperones are stress proteins and many of them were originally identified as heat-shock proteins (HSPs) (Wang et al., 2004). HSP gene expression is strongly correlated with heat stress transcription factors (HSFs) that are cytoplasmically located (inactive) regulatory proteins and are activated by stress conditions. They undergo oligomerization and recompartmentalization to the nucleus, under in vivo conditions (Baniwal et al., 2004). HSPs are classified into five major families based on their molecular weight (15–42 kDa): Hsp70 (DnaK), chaperonins (GroEL and Hsp60), Hsp90, Hsp100 (Clp), and small Hsp (sHsp) family (Wang et al., 2004). Under heat stress condition, five *Hsp90* genes of *Glycine max* were overexpressed in *A. thaliana*. This resulted in higher biomass production, pod setting, and reduction in lipid peroxidation and loss of chlorophyll (Xu et al., 2013b). In transgenic rice, overexpression of *OsHSP18.6* produced crops with increased tolerance to heat, drought, salt, and cold temperatures. The exogenous expression of the *Hsp70* gene significantly improved salinity tolerance in transgenic rice at the whole plant level (Hoang et al., 2015). Overexpression of *PgHsc70* and *PgHsp90* improves the salt and heat stress tolerance in *E. coli* cells (Reddy et al., 2010, 2011). Overexpression of *EaHSP70* in transgenic sugarcane plants showed enhanced tolerance to water deficiency and high salt concentrations, cell membrane thermostability, enhanced RWC and chlorophyll content, and enhanced photosynthetic efficiency (Augustine et al., 2015). It was reported that a strongly heat-induced *HsfA2* gene from maize, *ZmHsf04*, was isolated and characterized. The gene has shown its function in the heat/salt-stress response and is localized to the nucleus. Transcription activation data demonstrate that an AHA2 domain protein is necessary for the transcriptional activity of *ZmHsf04*. The function of ZmHsf04 was characterized in detail in transgenic plants of *A. thaliana* where it was observed that overexpression of *ZmHsf04* in *Arabidopsis* enhanced thermotolerance and positively regulated short-term acquired thermotolerance (Jiang et al., 2017).

4.3.1.4 Epigenetic Regulation of Abiotic Stress Tolerance

Plant gene regulation in response to abiotic stress is also epigenetically controlled without involving changes in the underlying sequence information, in a heritable and reversible manner. This epigenetic changes are brought about by modification in chromatin structure mediated by DNA methylation, histone posttranslational modification, and small noncoding RNAs (miRNA and siRNA) inducing RNA dependent DNA methylation, modulating the transcriptional and posttranscriptional gene expression (Kumar, 2016). These modifications brought about the differential expression of stress responsive genes by altering the binding efficiency of transcription factors (Zhu et al., 2014), and such changes working together or individually enable plants to adapt to unfavorable stresses (Yaish, 2017). Modification of histones occurred in the N terminal region called the histone tail via methylation, acetylation, phosphorylation, and ubiquitination of lysine and arginine residues altering gene activity that is wrapped around the core histone, and such modification can lead to activation as with acetylation or silencing of abiotic stress related genes (Kim et al., 2015b). Alteration in histone modification has been associated with abiotic stress such as drought, temperature, and salinity maintaining plant physiological status under adverse conditions. Histones, which form the structural unit of chromatin, and any changes, be they through enzymatic modification or incorporation of histone variants, impact chromatin stability and accessibility of transcriptional factors regulating gene expression (Asensi-Fabado et al., 2017). This concept has been explored with several findings, such as that the transcriptional activation of *OsDREBb* rice gene in response to cold stress is marked by hyperacetylation of histones H3K9, H3K14ac, and H3K27ac resulting in enhanced tolerance to salinity and cold stress (Scott et al., 2014). DNA methylation has also been shown to differentially regulate miRNA gene expression under temperature stress conditions. Through genetic

engineering approaches the role of DNA methylation and histone modification in the transcriptional activation or repression of transcription factors MYB, bZIP, and AP2/DREB in soybean tolerance to salinity stress has been experimentally proved (Ci et al., 2015). Studies on the functions of histone modification on various stress responsive genes such as the transcription factors are widely explored on the model plant *Arabidopsis* by the loss−of-function mutation analysis (Song et al., 2012). Histone deacetylase gene *HDA6* is required to survive under cold stress (To et al., 2011), and is involved in salt stress inducing response (Chen and Wu, 2010). HD2 proteins function in increased tolerance to salt stress (Luo et al., 2012). Histone modification is also correlated with the transcriptional regulation of cell cycle genes during various abiotic stresses. Under abiotic stress in maize seedlings, modifications of histones at specific lysine residue on the cell cycle gene promoter region regulate gene expression by inhibiting cell proliferation, inhibiting plant growth and development. Increased histone acetylation at H3K9ac and H4k5ac was observed with heat stress. Different stress regulates different phase of the cell cycle and reduced expression of cell cycle kinase CDKs during abiotic stress. And because increased acetylation functions in chromatin activation, such studies imply the role of modification in abiotic stress tolerance (Zhu et al., 2014). The altered modification might reset to their normal conditions upon relief from stress or persist for future adaptations to recurrent stress, referred to as stress memory (Chinnusamy and Zhu, 2009). This has been observed for several abiotic stress factors such as in the case of drought. Further understanding the epigenetic regulation mechanism for different abiotic stresses through various genetic engineering approaches will facilitate the development of stress tolerant plants by controlling expression of various stress related genes epigenetically modified.

4.3.1.5 Functional Role of Ubiquitination Encoding Proteins in Abiotic Stress Tolerance

Ubiquitination is an intrinsic posttranslational protein modification playing an important role in plant development and environmental stress. Plants adapting to environmental stresses such as drought, salinity, water deficit, and fluctuating temperature undergo changes in their physiological metabolism by modulating the amount and activity of proteins involved in cellular defense (Lyzenga and Stone, 2011). Ubiquitination mediates degradation of a target protein, fine tuning their abundance and stability, regulating gene expression and signaling (Ding et al., 2015). Regulated protein response to abiotic stress involves the regulation of stomatal opening, ROS, hormonal signaling, protein stabilization, and maintaining cell membrane integrity in addition to the degradation of proteins accumulated during abiotic stress (Dametto et al., 2015). The process is mediated by a conserved protein ubiquitin that covalently binds to a target protein on lysine residue and is subjected to 26S proteosomal degradation. The degradation is accomplished by sequential action of three enzymes: the ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligase (E3) (Sharma et al., 2016). The role of ubiquitin proteasome complex has been widely studied with respect to abiotic stress response, mainly on the E3 complex that plays a major role in the system (Stone, 2014). The highlights of a few of the recently identified ubiquitin encoding genes in response to different abiotic stresses through genetic engineering approaches are given in Table 4.1.

4.3.1.6 Genetic Engineering of Helicases in Plant Abiotic Stress Tolerance

Helicases are motor enzymes catalyzing the unwinding of duplex DNA (DNA helicases), RNA secondary structures, or RNPs (RNA helicases) in an ATP dependent manner (Macovei et al., 2012; Tuteja et al., 2012). DNA helicases function in DNA replication, repair, recombination, and transcription whereas RNA helicases are involved in transcription, ribosomes biogenesis, and translation initiation. Most of them are members of the DEAD-box protein subfamily (Sanan-Mishra et al., 2005). Because abiotic stresses interfere with the transcriptional and translation machinery, it is clear that molecules such as helicases involved in maintaining the integrity of nucleic acids are affected, and many studies have reported the positive role of these helicases to counteract with abiotic stresses (Sahoo et al., 2012; Raikwar et al., 2015). DEAD-box RNA helicases are known to be enriched for multiple abiotic stresses like drought, salinity, cold, and oxidative stress in both prokaryotes and eukaryotes as well (Baruah et al., 2017). Overexpression of a DEAD-box pea DNA helicase PDH45 in tobacco exhibited salinity tolerance probably by stabilizing protein synthesis and association with DNA multi subunit complexes regulating gene expression (Sanan-Mishra et al., 2005). OsABP, a DEAD-box RNA helicase ATP binding protein was found to be responsive to multiple abiotic stresses by interacting with proteins involved in RNA metabolism, signal transduction, and stress response (Macovei et al., 2012). Transgenic chili plant overexpressing pea DNA helicase *PDH45* was found to alleviate multiple abiotic stresses such as drought, salinity, senescence, and oxidative stress by improving plant physiological parameters, production of antioxidative enzymes, and enhancing expression of

TABLE 4.1 List of Ubiquitin Encoding Genes in Response to Different Abiotic Stresses Through Genetic Engineering Approaches

Plant	Ubiquitin complex	Encoding genes	Stress	Functional Expression studies	References
Arabidopsis	U box E3 ligase	*PUB46* and *PUB48*	Drought	Negatively regulate plant response to drought stress by degrading target proteins	Adler et al. (2017)
Arabidopsis	RING finger E3 ligase	*SDIR1*	Salt	SDIRIP1 is a substrate for SDIR1, which negatively regulates ABA and salt stress response	Zhang et al. (2015)
Apple	RING E3 ligase	*MdMIEL1*	Salt and oxidative stress	Negatively regulates salt stress and oxidative stress tolerance by increasing ROS concentration	An et al. (2017)
Arabidopsis	F-Box E3 ligase	*AtDIF1*	Salt and drought	Induced by salinity, drought, ABA but repressed by cold treatment. Confers salt tolerance by maintaining Na^+/K^+ homeostasis and promote seedlings growth. However, negatively regulates drought stress tolerance with retarding seed germination and survival rate	Gao et al. (2017)
Grimmia pilifera	RING E3 ligase	*GpDSR7*	Drought	Induced by water deficit, enhances tolerance to drought stress pre and post recovery by reducing water loss	Li et al. (2016b)
Watermelon	RING E3 ligase	*GdRZF1*	Drought	Reduced expression enhances tolerance to drought stress showing reduced leaf wilting, higher relative water content, lower ion leakage and MDA content, higher proline content in the antisense lines	Chung et al. (2017)
Wheat	E3 ligase	*TaSAP5*	Drought	Promotes degradation of DRIP proteins leading to the accumulation of DREB2A protein enhancing survival rates of wheat seedlings under drought stress	Zhang et al. (2017)
Rice	RING E3 ligase	*OsHTAS*	Heat	Enhances rice heat tolerance through an ABA dependent and DST (drought and salt tolerance) mediated pathway via an induced H_2O_2stomatal closure in the leaf blade	Liu et al. (2016a)
Brassica napus	RINGv E3 ligase	*BnTR1* (thermal resistance)	Heat	Modest expression confers thermal tolerance likely by regulating calcium channels altering heat-shock factors and heat-shock proteins expression	Liu et al. (2014b)
Ryegrass	Ubiquitin like protein family	*LpHUB1* (homology to Ub1)	Drought	Overexpression improves drought tolerance with higher relative water content, leaf water potential, and chlorophyll content under drought stress	Patel et al. (2015)
Maize	RING finger E3 ligase	*ZmAIRP4* (*Zea mays* abscisic acid insensitive RING protein)	Drought	Overexpressing transgenic *Arabidopsis* enhance drought tolerance by reducing water loss rate and altering other stress responsive genes	Yang et al. (2018)
Pepper	RING type E3 ligase	*CaREL1*	Drought	Overexpressing transgenic *Arabidopsis* exhibits low sensitivity to abscisic acid, increased water loss rate, and impaired stomatal closure, confirming as a negative regulator to drought stress	Lim et al. (2017)
Cotton	RING finger E3 ligase	*GhSARP1* (salt-associated ring finger protein)	Salt	Overexpressing transgenic *Arabidopsis* reduces tolerance to salt stress with reduced germination rate	Liu et al. (2016b)

stress responsive genes (Shivakumara et al., 2017). Many plant DEAD-box RNA helicases have been identified and their active participation in the stress signaling pathway has been reported. Overexpression of Pea p68 transgenic tobacco plants accumulates less Na^+ and more K^+ as compared with the WT plants. Higher K^+ content that controls endonuclease and caspase-like protease activity results in delayed leaf senescence in the transgenic lines under stress conditions (Tuteja et al., 2014). Similarly, *SIDEAD30*, *SlDEAD31*, *AtRH36*, and *AtRH9* confer resistance against salinity, drought, cold, and ABA stress conditions, and also regulate the expression of other genes involved in the stress signaling pathway (Zhu et al.,

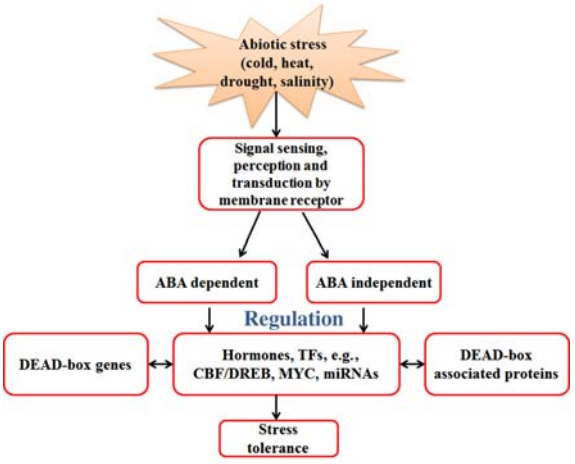

FIGURE 4.3 Schematic presentation of abiotic stress response of DEAD-box genes by interacting with transcription factors (TFs), miRNAs, and DEAD-box associated proteins.

2015). Transgenic groundnut plants expressing DEAD-box RNA helicase gene *PgeIF4A* were recently developed through genetic engineering that exhibit tolerance to stress conditions like oxidative, salt, and drought stress (Santosh Rama Bhadra Rao et al., 2017). The role of DExD/H-box RNA helicase member of SF_2 superfamily in rRNA biogenesis has been attributed during cold stress, where missense mutation of *AtRH10* impaired rRNA processing with physiological defects under high temperature; similarly *AtRH7* mutants exhibit severe growth retardation in plants exposed to cold stress (Liu and Imai, 2018). The negative role of DEAD-box RNA helicases has been identified for *Arabidopsis* encoding genes stress response suppressor 1 (*STRS1*) and *STRS2*. They are found to be downregulated by multiple abiotic stresses whose mutation increased tolerance by inducing expression of multiple stress responsive genes (Kant et al., 2007). A schematic representation of abiotic stress response of DEAD-box genes by interacting with transcription factors (TFs), miRNAs, and DEAD-box associated proteins are given in Fig. 4.3. The role of the DEAD-box RNA helicases in regulating responses to multiple abiotic stresses in plants has been recently reported in detail (Baruah et al., 2017).

4.3.1.7 Genetic Engineering of Small RNAs for Abiotic Stress Responses

MicroRNAs are endogenous small interfering noncoding RNAs of 20−24 nt long that negatively regulate the posttranscriptional expression of specific genes in response to abiotic stresses. Regulation is brought about by mRNA degradation, translational repression, and epigenetic modification. Upregulation of miRNA downregulates stress responsive genes and their inhibition causes expression of functional genes. Identification of these RNAs through various computational approaches could help in engineering plant genes improving plant abiotic stress tolerance (Shriram et al., 2016). miRNA mediated transcriptional regulation was based on the presence of abiotic stress response elements on the promoter region of miRNA (Barciszewska-Pacak et al., 2015). miRNAs are encoded by MIR genes and transcribed by RNA polymerases II forming double stranded stem loop primary miRNA. The duplex is then processed by a micropressor DICER like protein (DCL 1), HYL1, SE protein, and stabilized by HEN1 protein forming mature miRNA-miRNÂ. This is then transported in the cytoplasm, where the miRNA associate with the argonaute protein in the RISC complex while its cognate miRNÂ is degraded. Guided by the argonaute protein miRNA complementary base pair with the target mRNA leading to its degradation and transcriptional inhibition (Gielen et al., 2012). miRNA has been widely accepted to regulate stress responsive genes under abiotic stress and any changes can affect expression conferring adaptation advantages, thus identifying these targets would be beneficial in understanding the regulatory role of miRNA in plant abiotic stress response (Chen et al., 2017). Expression analysis revealed the downregulation of TCP (teosinite branched/cycloidea/proliferating cell factors) and a homolog OsNAC domain in transgenic plant. Overexpression of cold responsive OsmiR156K in rice decreased tolerance to cold stress and suggested the downregulation of cold stress responsive genes *01g22249*, *OsP5CS*, and *SPL* (Cui et al., 2015). *OsSPL2* and *OsSPL14* genes were targeted by miR529a in overexpression rice improving plant tolerance to oxidative stress (Yue et al., 2017). Transgenic chickpea overexpressed miR408 increases drought tolerance and it is involved in the expression of transcription factor *DREB* genes (Hajyzadeh et al., 2015). Role of soybean *miRNA172c* for drought and salt stress and *MIR394a* for drought was investigated in transgenic overexpression lines of the *Arabidopsis* (Ni et al., 2012; Li et al., 2016d). The overexpression plant improved the plant's physiological metabolism, promoted early flowering under drought stress, and regulated ROS accumulation suggesting the positive role in drought and salt stress tolerance; however it showed hypersensitivity to ABA. The overexpression of two conserved microRNAs *miR319a* and *miR319b* in rice improved leaf morphology and enhanced tolerance to cold induced stress (Yang et al., 2013). Suppressing the expression of a gene *ESK1* with siRNA together with overexpression of *CBF* gene in *Arabidopsis* enhanced drought tolerance. However, suppression of this gene reduced respiration rate during drought stress and reduced seed production in normal watering conditions (Xu et al., 2014).

4.4 GENOME EDITING APPROACHES TO MODULATE ABIOTIC STRESS SIGNALING PROCESSES IN CROP PLANTS

Genome or gene editing technology is a new technique of precisely manipulating genome structure in a simple and efficient manner. It involves modifying the known sequence in a genome, targeting only a few nucleotide bases, facilitating functional studies of the gene related to the phenotypic traits. This system encompasses use of site-specific nuclease (SSN) and site-specific recombinase (SSR) to generate the knockout of an undesirable trait or knock-in gene activation in the genome of an organism. In plants this technology has been widely used to study and improve crop nutritional quality and yield, disease management, and improve crop tolerance to abiotic stresses. It is also regarded as a non-GM technology since it does not involve manipulating a large sequence and/or introduction of foreign gene species (Abdallah et al., 2016). Site-specific nuclease generates a double-strand break (DSB) in the targeted gene sequences and uses the cell endogenous DNA repair mechanism, nonhomologous end joining (NHEJ), and homology directed repair (HDR) to repair breaks, creating a new gene function. This includes the zinc finger nuclease (ZFN), transcription activator like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) system (Kim et al., 2015a). NHEJ is an error prone repair system resulting in gene knockout by creating an indel mutation causing frameshift mutations in the coding region of a gene or disrupting the *cis*-regulatory elements of promoters and enhancers, whereas homology dependent repair uses a template with sequence homology flanking the region of interest resulting in insertion of the desired sequence mediated by homologous recombination repair (Kamburova et al., 2017b).

4.4.1 Zinc Finger Nucleases

Zinc finger nucleases are engineered restriction nucleases consisting of a sequence specific zinc finger DNA binding protein domain fused to a nuclease domain FokI (dimer) for generating double-strand break cleavage at specific loci (Petolino, 2015). ZFN specifically creates the DNA double-strand breaks on the chromosome, and cause site-specific mutagenesis and base substitution, which can alter the expression of a target gene, preferably gene knockdown (Li et al., 2013). In *Arabidopsis*, ZFNs have been generated that recognize target DNA to introduce indel mutations (de Pater et al., 2009; Hou et al., 2014). Site-specific mutagenesis and base substitution play a very important role in the plant genetic improvement. Thus, ZFNs are one of the potential genome editing tools to be explored for abiotic stress tolerance in diversified plants.

4.4.2 Transcription Activator Like Effector Nucleases

TALENs are the proteins derived from the plant pathogenic bacterium *Xanthomonas campestris*, which binds to specific DNA sequences (Curtin et al., 2012). TALEN allows site-directed mutagenesis in the target gene of interest (Hou et al., 2014). TALENs are based on fusion of a Fok1 nuclease domain to the DNA binding TALE repeats (Mahfouz et al., 2011). TALENs have been successfully introduced in plants such as *Arabidopsis*, tobacco, rice, and *Brachypodium* (Curtin et al., 2012; Shan et al., 2013). Recently, TALEN was shown to induce different heritable mutation in rice (Zhang et al., 2016). Improved cold storage and processing traits in potato have been engineered through TALENs (Clasen et al., 2016). Multiple assembly methods based on golden gate cloning have revolutionized the rapid adoption of TALEN technology for genome editing in plants for several applications including developing abiotic stress tolerant crop plants.

4.4.3 CRISPR-Cas9 Genome Editing

The clustered regularly interspaced short palindromic repeat/CRISPR associated protein (Cas9) is a novel genome editing tool that precisely creates a double strand cleavage of target DNA (Khatodia et al., 2016). Discovered from the adaptive defense mechanism of *E. coli* and archaea against invading foreign DNA, this type II system is commonly used to study the function of a gene by transcriptional activation or repression, altering the metabolic pathway, which can be used to improve crop quality and for drug development because of its simplicity and ability for multiplex genome editing, especially for plant abiotic stress complex trait (Arora and Narula, 2017; Liu et al., 2017). Active Cas9/sgRNA complex cleaves targeted DNA upon binding and the resultant blunt end DSB is then repaired either by NHEJ using DNA ligase IV introducing frameshift or indel mutation leading to gene knockout or by HDR repair pathway causing the insertion of oligotemplate resulting in gene replacement or knock-in of foreign DNA (Song et al., 2016). The basic strategy of the CRISPR-Cas9/strand-based genome editing system is represented in Fig. 4.4.

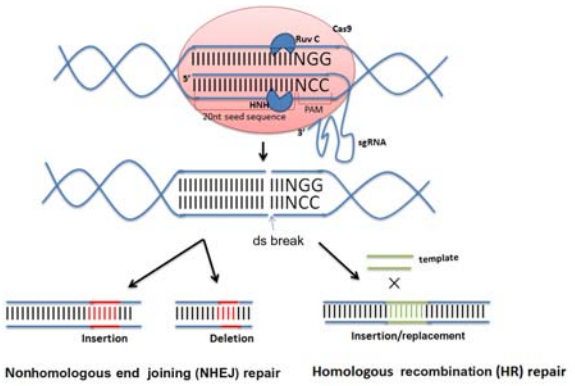

FIGURE 4.4 The basic strategy of the CRISPR-Cas9/sgRNA based genome editing system.

4.4.3.1 *Application of CRISPR-Cas9 in Plant Abiotic Stress Tolerance*

The role of annexin genes in conferring abiotic stress tolerance such as drought and salinity through overexpression studies has been investigated (Ijaz et al., 2017), however the role in cold tolerance is exploited using CRISPR-Cas9 knockout gene mutation and decreased tolerance to cold stress has been observed (Shen et al., 2017). The CRISPR-Cas9 technology using HRD mediated gene replacement has been used to generate novel ARGOS8 variant in maize, which in its native form has low expression level. Modified ARGOS8 variant increased expression transcript, and was found to increase grain yield by five bushels per acre compared with wild type under drought conditions and no yield loss under well-watered conditions (Shi et al., 2017). MAPKs are important signaling molecules for drought stress. Tomato mutant line SlMAPK3 generated using CRISPR-Cas9 exhibits severe leaf wilting, increased hydrogen peroxide content, reduced antioxidant enzymes, and caused membrane damage under drought conditions, thus confirm the positive role of SlMAPK3 in drought tolerance by maintaining cell membrane structure (Wang et al., 2017a). CRISPR-Cas9 has been also used to target noncoding regulator element, the promoter region of rice *OsRAV2* for its functional role with salt treatment (Duan et al., 2015).

4.4.4 CRISPR-Cpf1

While the ease and efficiency of using CRISPR-Cas9 complements other SSNs such as ZFN and TALEN, its limitations in generating off target effects and its uncertain intellectual property landscape reduce its scientific adoption (Begemann et al., 2017). Alternative to CRISPR-Cas9, a recently discovered approach for genome editing is the CRISPR-Cpf1 a type V CRISPR-Cas system, which is an RNA guided endonuclease (Xu et al., 2017). This system discovered from Prevotella and Francisella has emerged as a new, efficient tool for target gene modification including DNA free editing in plants, and shows higher efficiency and potential to the CRISPR-Cas9 system, exhibiting fewer off target effects in plants (Zaidi et al., 2017). The CRISPR-Cpf1 uses a single crRNA with approximately 44 nucleotides guiding cleavage by Cpf1 nuclease, in contrast to 100 nucleotide in Cas9, which requires tracrRNA to pair with crRNA for its functional role. Thus a shorter gRNA sequence is required for designing CRISPR-Cpf1 simplifying cloning process. The functional role of Cpf1 nuclease is governed by only RuvC without HNH domain. Cpf1 recognizes a thymine rich 5′-TTTN-3′ PAM site distal to its target DNA at the 5′end, and generates cohesive sticky ends compared with the blunt end of CRISPR-Cas9, improving efficiency for NHEJ gene insertion (Malzahn et al., 2017; Verwaal et al., 2017). The advantage of multiplexing genome editing using Cpf1 has been demonstrated (Zetsche et al., 2017), where using a single crRNA can target up to four genes simultaneously in mammalian cells, possibly because of the ability of Cpf1 to process its own crRNA, thus minimizing the limits of generating a large expression construct by CRISPR-Cas9. However, further studies on its advantages and disadvantages need to be explored as well as the generation of mutated lines. In rice targeted gene editing using Cpf1 generated precise gene insertion and indels with up to 8% greater efficiency compared with other nucleases (Begemann et al., 2017), and generated a biallelic mutation of 100% efficiency using Cpf1 from *Lachnospiraceae bacterium* (Tang et al., 2017).

Using these approaches genome editing has been widely used for the functional characterization of plant genes and genetic improvement of agricultural crops. A detailed mechanism employed by different genome editing approaches (ZFNs, TALENs, and CRISPR-Cas9), and their relative comparisons and applications in improving plant tolerance to abiotic stress are briefly explained in Table 4.2. A scheme of the development of multiple abiotic stress tolerance in a crop model using target specific multiplex multigene CRISPR-Cas9 genome editing approach is given in Fig. 4.5.

4.5 CONCLUSIONS AND FUTURE PROSPECTS

The impact of rapid global climate change on crop production has emerged as a major research priority during the past decade. The major abiotic stress factors such as drought, salinity, and extreme

TABLE 4.2 Comparison of Mechanism, Application, Advantages, and Disadvantages of Different Genome Editing Approaches (ZFNs, TALENs, and CRISPR-Cas9)

Section	Zinc finger nuclease	TALEN	CRISPR-Cas9
Origin	Zinc finger motifs	*Xanthomonas campestris*	*Streptococcus pyogenes*
Mechanism	Cleavage occur by binding of 4–6 tethered zinc finger protein domains (Cys2-His2 fingers) on both strands of target DNA fused with a dimerized nuclease FokI	Cleavage occurs by binding of pairs of TAL effectors fused to restriction endonuclease FokI for one target site	Cleavage occurs by binding of a chimeric sgRNA to the target sequence mediated by Cas9 nuclease
Catalytic domain	Required dimerize FokI	Required dimerize FokI	Only a single Cas9 nuclease required
Sequence recognition	18–24 bps	30–40 bps	20–22 bps
Advantage	Can target any sequence	• Can target any sequence • Higher specificity than CRISPR • Reduced off target	• Easily engineered, cost effective, versatile • Multiplex genome editing • Can target methylated DNA • Can identify off target using design tools CGAT, CRISPR-P, CHOPCHOP, and CRISPR • Can minimize off target using CRISPR nickase, truncated sgRNA
Limitations	• Engineering is difficult because of the context dependent effects • Low affinity and specificity • High off target and toxicity • Determining off target is difficult due to context dependence of protein–DNA interaction	• Difficult for engineering nuclease and TALEN, time consuming and expensive • Cannot target methylated DNA, which plays important role in abiotic stress tolerance • Determining off target is difficult due to context dependence of protein–DNA interaction • Binding site starts with T base	• Higher off target effects than TALEN • Required PAM sequence for recognition • Suitable for targets containing high GC content
References	Kamburova et al. (2017a), Zhao and Wolt (2017)	Malzahn et al. (2017), Zhao and Wolt (2017)	Malzahn et al. (2017), Zhao and Wolt (2017)

temperatures have negatively impacted the agricultural productivity worldwide. Most plants have developed genetic, biochemical, and physiological mechanisms to cope with different abiotic stresses, which include activation and repression of key signaling pathways; negative and positive regulation of specific stress signaling genes such as DEAD-box RNA helicases, hybrid proline rich proteins, dehydrins, methylglyoxalases, ethylene response factors, and transcription factor genes; and hyper- and hypoaccumulation of compatible salts, and stress proteins such as aquaporins, molecular chaperones, HSPs, kinase proteins, and helicases. With a greater understanding of genetic engineering and functional genomics approaches such as transgenics, overexpression of elite genes, next generation sequencing, loss-of-function and gain-of-function mutations, and small RNAs several abiotic stress tolerant crop plants have been developed in the past decade to date. Major genetic engineering has also been extensively used in recent years for development of abiotic stress tolerance in crop plants through epigenetic regulation, ubiquitination, and transcription factor regulation. Very recently the CRISPR-Cas9 and CRISPR-Cpf1 genome editing systems have become the significant discovery of modern day agricultural biotechnology. Widely regarded as a non-GM approach, CRISPR technology has emerged as a breakthrough genome editing system that could be a potential game changer for expanding our research on developing sustainable multiple abiotic stress tolerance in crop plants for the future. Considering the huge loss of crop productivity majorly due to abiotic stress factors, there is an urgent need to direct our research focus to develop sustainable multistress crop tolerance in combination with high yields using genome editing technology to counter the climate change related adverse effects on the productivity of crops.

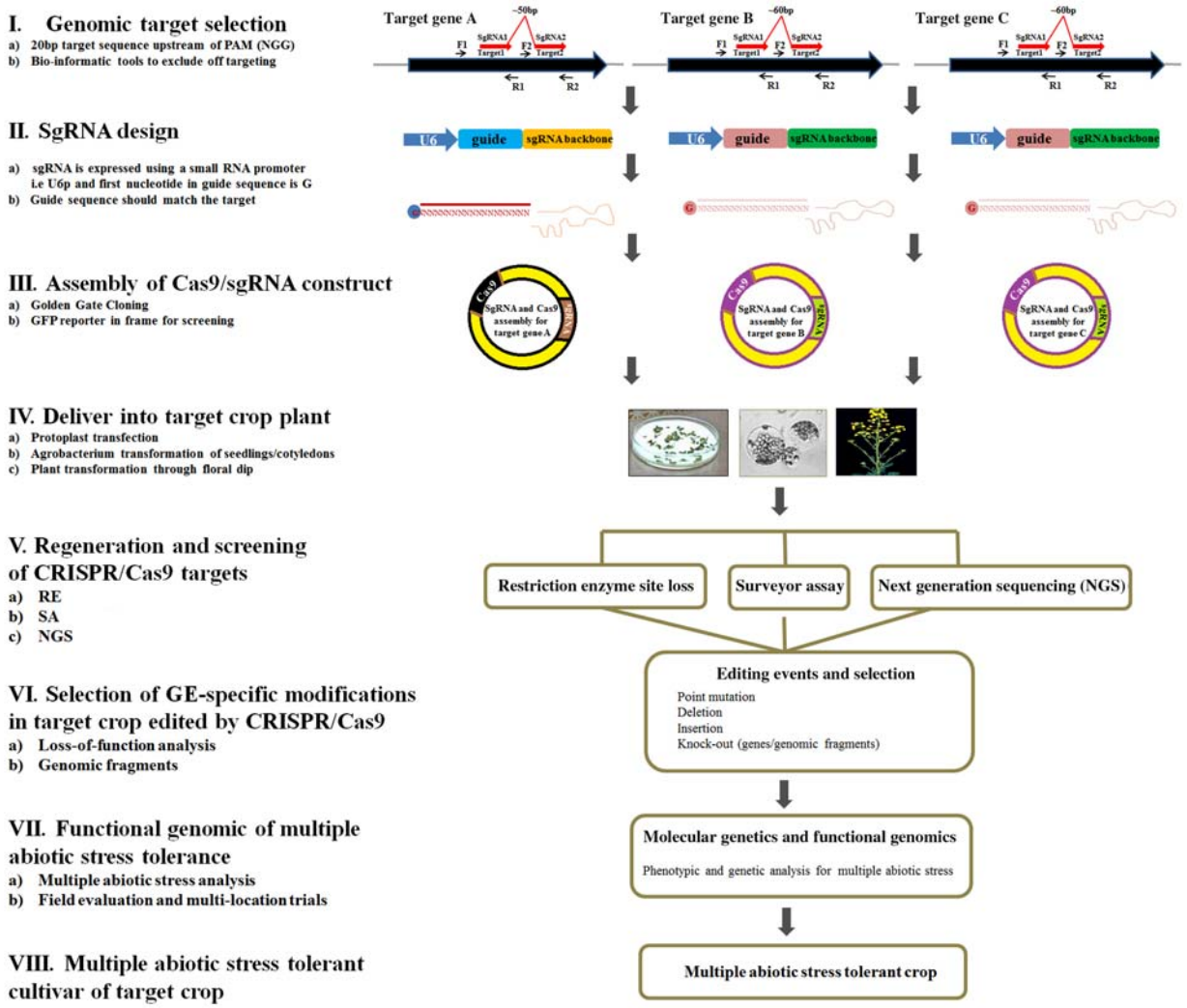

FIGURE 4.5 Scheme of development of multiple abiotic stress tolerance in a crop model using target specific multiplex multigene CRISPR-Cas9 genome editing approach.

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CHAPTER

5

Measurement of Signaling Molecules Calcium Ion, Reactive Sulfur Species, Reactive Carbonyl Species, Reactive Nitrogen Species, and Reactive Oxygen Species in Plants

Zhong-Guang Li[1,2,3]

[1]School of Life Sciences, Yunnan Normal University, Kunming, P.R. China [2]Engineering Research Center of Sustainable Development and Utilization of Biomass Energy, Ministry of Education, Kunming, P.R. China [3]Key Laboratory of Biomass Energy and Environmental Biotechnology, Yunnan Province, Yunnan Normal University, Kunming, P.R. China

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5.1 INTRODUCTION

Plants commonly perceive, respond, and transform environmental signals by many second messengers, and finally adapt to changing environments (Tardieu and Tuberosa, 2010; Zhu, 2016; Jeandroz and Lamotte et al., 2017). These signaling molecules include calcium messenger systems (mainly calcium ion: Ca^{2+}), reactive sulfur species (RSS, mainly hydrogen sulfide: H_2S), reactive carboxyl species (RCS, mainly methylglyoxal: MG), reactive nitrogen species (RNS, mainly nitric oxide: NO), and reactive oxygen species (ROS, mainly superoxide radical: $O_2\bullet^-$; hydrogen peroxide: H_2O_2; and hydroxyl radical: •OH) (Gill and Tuteja et al., 2010; Li, 2016; Campbell, 2018; Sami et al., 2018). Plants have developed multiple biosynthetic pathways (such as H_2S, MG, NO, and H_2O_2) or stored pools (such as Ca^{2+}) of second messengers and their scavenging mechanisms (Gill and Tuteja et al., 2010; Li, 2016; Campbell, 2018; Sami et al., 2018). These signaling molecules can be rapidly produced when needed, while expediently and efficiently eliminated when not needed by plants (Neill et al., 2002; Dodd et al., 2010; Li et al., 2016; da-Silva and Modolo, 2018). In addition, intracellular and extracellular cues can rapidly trigger an increase in second messengers simultaneously or successively in cytoplasm or other organelles (Neill et al., 2002; Dodd et al., 2010; Li et al., 2016). Therefore, signaling molecules (Ca^{2+}, H_2S, MG, NO, and H_2O_2) play a crucial role in plant metabolism, growth, development, and response to environmental stress including abiotic and biotic stresses. Nowadays, messenger transportation and signal transduction are research hotspots in the field of plant biology, especially in plant stress biology, that are receiving extensive attention. Additionally, qualitative and quantitative analysis of signaling molecules, especially imaging in living cells, is an important fundamental for investigating signaling molecules in plants. In this chapter, based on the current protocols (Mustafiz et al., 2010; Rodriguez and Taleisnik, 2012; Xie and Shen, 2012; Li and Gong, 2014; Li, 2015; Angelini et al., 2018; Antoniou et al., 2018; Campbell, 2018; Park and Roubelakis-Angelakis, 2018) and research papers (Schopfer et al., 2001; Gay and Gebicki, 2000, 2003; Fraisse et al., 2002; Yu et al., 2003; Ashtamker et al., 2007; Maeda et al., 2007; Liu et al., 2011; Wild et al., 2012; Shaheen et al., 2014; Qian et al., 2015), in vivo (including living cell imaging) and in vitro methods, namely histochemical staining method, fluorometry, and spectrophotometry, for determination of Ca^{2+}, H_2S, MG, NO, $O_2\bullet^-$, H_2O_2, and •OH, were investigated. Its aim is to arouse the research and development of signaling molecules in the field of plant biology, especially plant stress biology.

5.2 SECTION 1: CA^{2+} QUANTIFICATION

5.2.1 Method 1: MTB Method

1. Principle

Free calcium ion (Ca^{2+}) can rapidly react with methylthymol blue (MTB) in alkaline conditions and produces a blue dye-Ca^{2+} complex. The complex has a maximal absorbance at 610 nm (Fig. 5.1; Qian et al., 2015). The light absorption is proportional to the content of Ca^{2+} in plant samples. Therefore, free Ca^{2+} content can be measured by spectrophotometry at 610 nm.

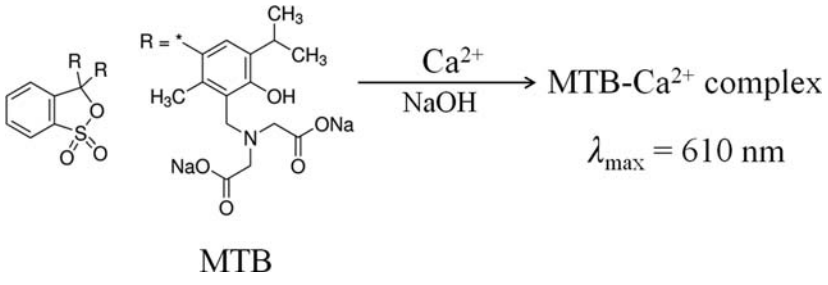


FIGURE 5.1 Ca^{2+} reacts with MTB under alkaline conditions.

2. **Materials**
   - **2.1.** 100 mL of MTB solution: 100 mL of 0.1 mol/L HCl (mix 0.84 mL concentrated HCl with 99.16 mL of deionized water) containing 0.25 mM MTB (21.1 mg, MW = 844.74) and 0.6% polyvinyl pyrrolidone (PVP, 0.6 g).
   - **2.2.** 100 mL of alkaline solution: 100 mL of solution containing 0.2 M Na_2SO_3 (2.53 g, MW = 126.04), 0.1% (w/v) glycine (0.1 g), and 0.23 M NaOH (0.92 g, MW = 40).
   - **2.3.** Spectrophotometer or microplate reader.
3. **Methods**
   - **3.1.** Plant tissue roots or leaves (0.1 g FW) are homogenized in 1 mL deionized water with mortar and pestle.
   - **3.2.** Centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant was collected and used for Ca^{2+} assay.
   - **3.3.** Add 50 μL of supernatant and 1 mL of MTB solution to 2 mL alkaline solution.
   - **3.4.** Read absorbance at 610 nm.
   - **3.5.** Calculate the amount of Ca^{2+} from a standard curve prepared using dilutions of a 2.5 mM Ca^{2+} stock solution and expressed as μmol/g FW.
4. **Precaution**

   Alkaline solution should be freshly prepared.

5.2.2 Method 2: Fura-2 AM Fluorescence Method

1. **Principle**

   Fluorescent probes Fura-2 AM and Fura-3 AM are not able to bind Ca^{2+}. When they enter freely into the cytosol, Fura-2 AM and Fura-3 AM are converted by esterase into Fura-2 and Fura-3, respectively, in the cytosol. Both can rapidly bind to Ca^{2+} and produce a green fluorescent substance (Fig. 5.2; Campbell, 2018). The intensity of fluorescence is proportional to the content of Ca^{2+} in plant samples. The fluorescence can be visualized by fluorescence microscope with excitation at 506 nm and emission at 525 nm.
2. **Materials**
   - **2.1.** 100 mL of 100 mM Tris-HCl (pH 8.0): Add 50 mL of 200 mM Tris (2.42 g/100 mL) to 26.8 mL of 200 mM HCl (1.68 mL concentrated HCl/100 mL). Mix and make up to 100 mL with deionized water.
   - **2.2.** 5 μM of Fluo-2 AM: Dissolve 1 mg Fluo-2 AM (MW = 1001.85) in 1 mL of dimethylsulfoxide (DMSO) to obtain 1 mM Fluo-2 AM. Dilute to final concentration with 100 mM Tris-HCl (pH 8.0).
   - **2.3.** Fluorescence microscope.
3. **Methods**
   - **3.1.** Plant tissues (tissues or cells) are incubated in 100 mM Tris-HCl (pH 8.0) buffer containing 15 μM Fluo-2AM in darkness at 25°C for 30 min.
   - **3.2.** The fluorescent image was captured using a fluorescence microscope with 506/525 nm (excitation/emission) filter set.
   - **3.3.** Take image of tissues or cells using fluorescent microscope.
4. **Precautions**
   - **4.1.** Fluo-2 AM should be freshly prepared or stored at −20°C after subpackage of stock solution of 1 mM.
   - **4.2.** Inverted fluorescence microscope is used for tissues, tissue slices, and cells, while laser confocal fluorescence microscopy for tissues and organs such as roots and leaves.

5.3 SECTION 2: H_2S QUANTIFICATION

5.3.1 Method 1: DTNB Method

1. **Principle**

   Under neutral conditions, H_2S can react with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's Reagent) to produce a yellow product 5-thio-2-nitrobenzoic acid (TNB). The TNB exhibits a maximal light absorption at 412 nm and the molar extinction coefficient is 1.4 × 10^4 M^{-1} cm^{-1}

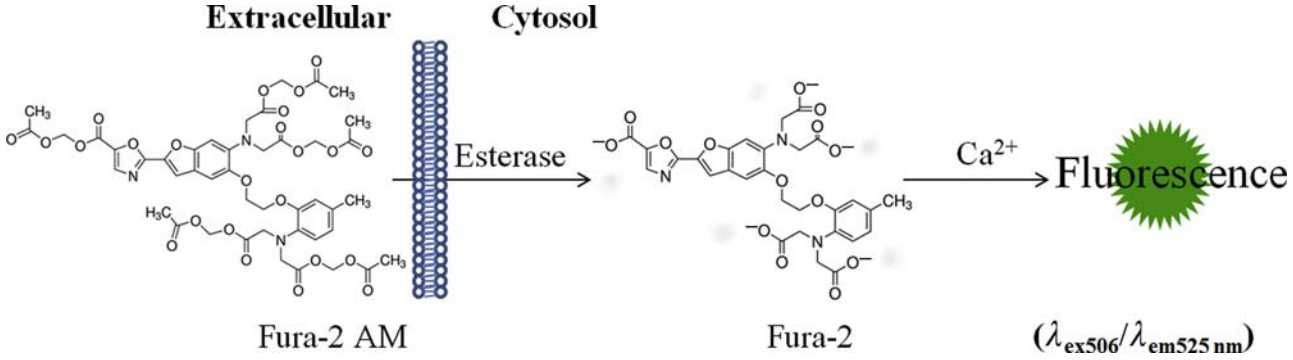


FIGURE 5.2 Fura-2 AM, which can freely enter into cytosol, is deacetylated by intracellular esterase to generate Fura-2, which can react with NO to generate fluorescence.

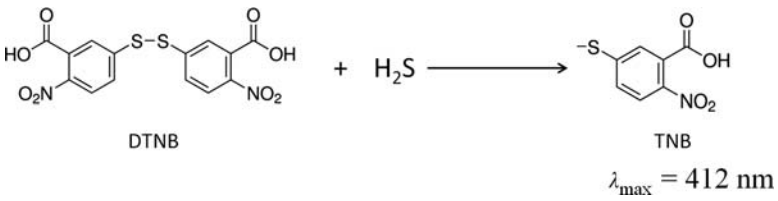


FIGURE 5.3 H_2S reacts with DTNB under neutral conditions.

(Fig. 5.3; Li, 2015; Antoniou et al., 2018). The absorbance is in direct proportion to the content of H_2S in plant samples. So, H_2S content in plant samples can be determined by spectrophotometry.

2. **Materials**
   - **2.1.** 100 mL of extraction buffer: 100 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA, MW = 292.2). Mix 6.15 mL of 1 M K_2HPO_4 (17.418 g/100 mL) with 3.85 mL of 1 M KH_2PO_4 (13.609 g/100 mL), add deionized water to reach a final volume of 100 mL. Then dissolve 0.292 g EDTA in K-phosphate buffer.
   - **2.2.** 20 mM Ellman's reagent: Namely 20 mM DTNB: Dissolve 39.6 mg DTNB (MW = 396.35) in 5 mL of extraction buffer (see note).
   - **2.3.** Spectrophotometer or microplate reader.
3. **Methods**
   - **3.1.** Plant roots or leaves (100 mg) are homogenized in liquid nitrogen with mortar and pestle and then add 1 mL of extraction buffer.
   - **3.2.** Centrifuge at 10,000 × g for 15 min at 4°C.
   - **3.3.** Add 1880 μL of extraction buffer into 100 μL of supernatant.
   - **3.4.** Add 20 μL of 20 mM DTNB (add 1980 μL of extraction buffer to 20 μL of 20 mM DTNB as the blank).
   - **3.5.** Incubate at room temperature for 2 min.
   - **3.6.** Transfer 300 μL of the reaction mixture to 96-well plate and read absorbance at 412 nm in the plate reader spectrometer, or read absorbance using spectrophotometer.
   - **3.7.** A standard curve is performed with known concentrations of a sodium hydrosulfide hydrate (NaHS, H_2S donor) ranging from 0 to 100 μM.
   - **3.8.** The H_2S content is calculated using standard curve or the molar extinction coefficient of $1.4 \times 10^4\ M^{-1}\ cm^{-1}$ for TNB and expressed in μmol/g FW.
4. **Precaution**

   DTNB should be stored in the dark between 0°C and 5°C in where it can be stable for 6 months.

5.3.2 Method 2: MB Method

1. **Principle**

   H_2S can react with *N,N*-dimethyl-*p*-phenylenediaminedihydrochloride (DMPD) under acidic conditions and generates a blue product methylene blue (MB). The MB has a maximal light absorption at 670 nm and the extinction coefficient of $1.5 \times 10^7\ M^{-1}\ cm^{-1}$(Fig. 5.4; Li, 2015). A positive correlation between the light absorption (A_{670}) and the H_2S content in plant samples can be noted.

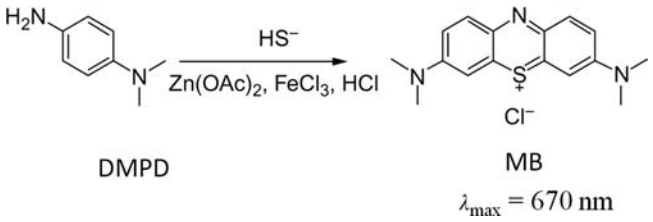


FIGURE 5.4 H_2S reacts with DMPD under acidic conditions.

2. **Materials**
   - **2.1.** 100 mM sodium phosphate buffer (pH 7.0): Mix 61 mL of 200 mM Na_2HPO_4 (53.65 g $Na_2HPO_4 \cdot 7H_2O$ or 71.64 g $Na_2HPO_4 \cdot 12H_2O$/1000 mL) with 39 mL of 200 mM NaH_2PO_4 (27.6 g $NaH_2PO_4 \cdot H_2O$ or 31.2 g $NaH_2PO_4 \cdot 2H_2O$/1000 mL). Mix and make up to 200 mL with deionized water.
   - **2.2.** 100 mL of extraction buffer: 100 mL of 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA (0.292 g, MW = 292.2) and 20 mM $Zn(OAc)_2 \cdot 2H_2O$ (0.439 g, MW = 219.51).
   - **2.3.** 100 mL of 30 mM $FeCl_3$: Dissolve 0.487 g $FeCl_3$ (MW = 162.2) in 1.2 M HCl (10.08 mL concentrated HCl/100 mL).
   - **2.4.** 100 mL of 20 mM DMPD: Dissolve 0.418 g DMPD (MW = 209.12) in 7.2 M HCl (60.48 mL concentrated HCl/100 mL).
3. **Methods**
   - **3.1.** Plant roots or other tissues (2 g) are ground into fine powder with a mortar and pestle under liquid nitrogen, and then are homogenized in 2 mL of extraction buffer.
   - **3.2.** Centrifuge at 10,000 × g for 15 min at 4°C.
   - **3.3.** Add to 1 mL of 30 mM $FeCl_3$ and 1 mL of 20 mM DMPD to 1 mL of the supernatant.
   - **3.4.** Incubate at room temperature for 15 min and read the absorbance at 670 nm.
   - **3.5.** Graph the standard curve as absorbance (A_{670}) versus known NaHS or Na_2S concentrations from 0 to 10 μM.

3.6. Calculate the H_2S content from the graph or the extinction coefficient of $1.5 \times 10^7\ M^{-1}\ cm^{-1}$ and expressed as nmol/g FW.

4. Precaution

$FeCl_3$ and DMPD can be dissolved in 3.5 and 100 mM H_2SO_4 to obtain 5 mM DMPD and 50 mM $FeCl_3$, respectively.

5.3.3 Method 3: WSP-1 Fluorescence Method

1. Principle

WSP-1 (Washington StateProbe-1), 3′-methoxy-3-oxo-3H-spiro(isobenzofuran-1, 9′-xanthen)-6′-yl 2-(pyridin-2-yldisulfanyl)benzoate, is a fluorescent probe for detecting H_2S within living cells or plant extraction with high-sensitivity and selectivity. WSP-1 can freely enter into the cells across membrane and reacts with H_2S to generate benzodithiolone and a fluorophore with excitation and emission maxima of 465 and 515 nm, respectively (Fig. 5.5; Liu et al., 2011). The intensity of fluorescence is positively pro rata to the content of H_2S in plant samples. The fluorescence is detected by fluorescence microscope or fluorospectrophotometer with excitation at 465 and an emission at 515 nm via in vivo or in vitro methods.

2. Materials

2.1. 100 mL of 20 mM Hepes-NaOH (pH 7.5): Dissolve 0.477 g Hepes (MW = 238.31) in approximately 80 mL of deionized water. Adjust pH to 7.5 with 1 M NaOH, and then make up to 100 mL with deionized water. Store at 4°C.

2.2. 15 μM WSP-1: Dissolve 1 mg WSP-1 (MW = 561.65) in 1 mL of DMSO to obtain stock solution of 1.78 mM, and then dilute to final concentration with 20 mM Hepes-NaOH (pH 7.5).

2.3. Fluorescence microscope or fluorospectrophotometer.

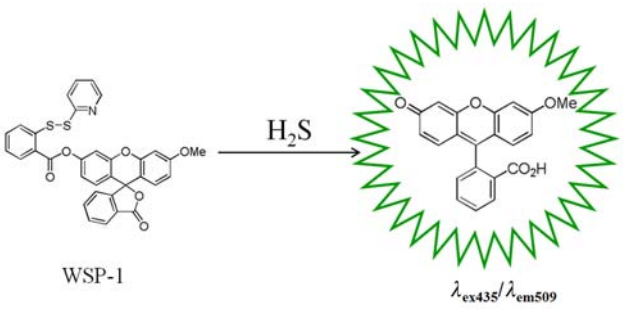


FIGURE 5.5 H_2S reacts with WSP-1 and generates green fluorescence.

3. Methods

3.1. For in vitro method, H_2S extracted from plant samples as DTNB method.

3.2. Plant extraction or tissues is incubated in 20 mM Hepes-NaOH (pH 7.5) containing 15 μM WSP-1 for 40 min (in vitro method needs a short time, approximately 10 min).

3.3. The plant tissues were washed with distilled water three times (this step ignored in in vitro method).

3.4. Detect immediately by a fluorescence microscope or fluorospectrophotometer with a 465/515 nm (excitation/emission) filter set.

3.5. Photograph or calculate H_2S content using standard curve with known concentrations of NaHS or Na_2S from 0 to 50 μM.

4. Precautions

WSP-1 should be freshly prepared or stored at −20°C after subpackage of stock solution of 1.78 mM.

5.4 SECTION 3: METHYLGLYOXAL QUANTIFICATION

5.4.1 Method 1: DAB Method

1. Principle

Methylglyoxal (MG) is produced during normal cellular metabolism, such as glycolysis and photosynthesis. This aldehyde has long been considered as a cytotoxin, which inhibits the growth of cells in all types of organisms, but now is found to be a signaling molecule. MG can react with 1,2-diaminobenzene (DAB) and produces a product hydrazone. The hydrazone has a maximal optical density (OD) at 336 nm (Fig. 5.6; Mustafiz et al., 2010). The light absorption (OD_{336}) is positively proportional to the content of MG in plant samples.

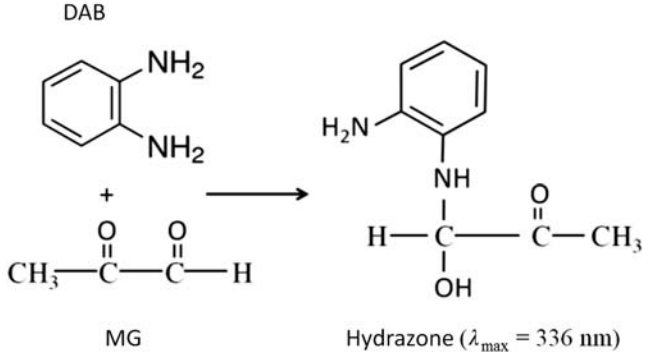


FIGURE 5.6 MG reacts with DAB to produce hydrazone.

2. **Materials**
   - **2.1.** 100 mL of 0.5 M perchloric acid (PA): Mix 4.17 mL of PA ($M = 12$) in 100 mL of deionized water.
   - **2.2.** 100 mL of 5 M PA: Mix 41.7 mL of PA ($M = 12$) in 100 mL of deionized water.
   - **2.3.** Charcoal.
   - **2.4.** Saturated solution of K_2CO_3: Dissolve K_2CO_3 in water in excess amount so that some amount of the solute remains undissolved in the solution.
   - **2.5.** 100 mL of 7.2 mM, 2-diaminobenzene (DAB): Dissolve 77.9 mg DAB (MW = 108.14) in 100 mL of deionized water.
   - **2.6.** Methylglyoxal (40%, $\rho = 1.178$, 6.54 M).
   - **2.7.** Spectrophotometer or microplate reader.
3. **Methods**
   - **3.1.** Fresh plant tissue (250 mg) is homogenized in liquid nitrogen with mortar and pestle.
   - **3.2.** Add 2.5 mL of 0.5 M PA and mix well.
   - **3.3.** Centrifuge at 11,000 × g for 10 min at 4°C.
   - **3.4.** The supernatant is decolorized using charcoal (10 mg/mL) and kept at room temperature for 15 min. The mixture is centrifuged at 11,000 × g for 10 min. The clear supernatant is collected and used for MG assay.
   - **3.5.** The solution is neutralized using saturated K_2CO_3 which should be added gradually (initially 20−30 μL should be added, and then one should add 2 μL at a time) and the pH of the supernatant should be checked using a pH paper time to time. With each addition of K_2CO_3, the solution should be mixed properly and the bubbles of CO_2 gas should be allowed to come out.
   - **3.6.** The neutralized extract is kept at room temperature for 15 min and centrifuge at 11,000 × g for 10 min.
   - **3.7.** Add 250 μL of 7.2 mM DAB and 100 μL of 5 M PA to 650 μL of neutralized supernatant.
   - **3.8.** Incubate at room temperature for 30 min and take the absorbance at 336 nm with spectrophotometer or microplate reader.
   - **3.9.** A standard curve of different concentrations of MG (10, 25, 50, and 100 μM) is made using stock solution of MG.
   - **3.10.** MG content is calculated using the standard curve and expressed as μmol/g FW.
4. **Precautions**
   - **4.1.** MG solution should be freshly prepared and stored in the dark due to its sensitivity to light.
   - **4.2.** MG should be handled carefully due to its high toxicity.

5.4.2 Method 2: DNP Method

1. **Principle**

   MG, similar to other aldehydes, can react with 2,4-dinitrophenylhydrazine (2,4-DNP) in acidic conditions and produces a pink product MG-bis-2,4-DNP-hydrazone. The hydrazone has a maximal absorbance at 432 nm and the molecular absorption coefficient of $3.36 \times 10^4\ M^{-1}\ cm^{-1}$ (Fig. 5.7; Wild et al., 2012). The absorbance is proportional to the content of MG in plant samples. Therefore, MG can be quantified by spectrophotometry.
2. **Materials**
   - **2.1.** 0.2 mM 2,4-dinitrophenylhydrazine (DNP): Prepare first 100 mL of stock solution of 10 mM 2,4-DNP (0.198 g, MW = 198) in ethanol, and then dilute freshly to 0.2 mM (1:50) using a mixture of HCl and ethanol (12:100, v/v).
   - **2.2.** 1 mM MG working solution: Mix 15.3 μL of stock solution of MG ($M = 6.54$ M) with 100 mL deionized water. Freshly prepare from the stock solution every day.
   - **2.3.** Spectrophotometer or microplate reader.
   - **2.4.** Shaker.
3. **Methods**
   - **3.1.** MG extracted from plant samples as per DTNB method.
   - **3.2.** Take 50 μL of neutralized supernatant (volume can be adjusted according to the amount of MG in plant samples) to 950 μL of 0.2 mM 2,4-DNP.
   - **3.3.** Incubate at 42°C for 45 min in a shaker at 600 rpm.
   - **3.4.** Recovery at room temperature for 5 min.
   - **3.5.** Read absorbance at 432 nm.
   - **3.6.** Calculate MG content according to the molecular absorption coefficient of $3.36 \times 10^4\ M^{-1}\ cm^{-1}$ for MG-bis-2,4-DNP-hydrazone and expressed as μmol/g FW.

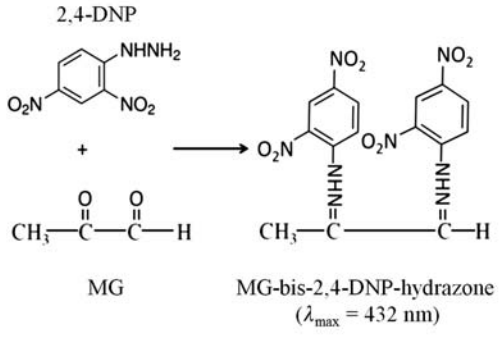


FIGURE 5.7 MG reacts with 2,4-DNP to produce MG-bis-2,4-DNP-hydrazone.

4. **Precautions**
   - **4.1.** MG solution should be freshly prepared and stored in the dark due to its sensitivity to light.
   - **4.2.** MG should be handled carefully due to its high toxicity.

5.4.3 Method 3: NAC Method

1. **Principle**

   Under neutral conditions, MG can react with *N*-acetyl-L-cysteine (NAC) to produce *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl) cysteine (NASC). The NASC exhibits a maximal absorbance at 288 nm and the molecular absorption coefficient is 248 M^{-1} cm^{-1}(Fig. 5.8; Wild et al., 2012). The light absorption (A_{288}) is positively related to the content of MG in plant samples. Herein, MG can be detected by spectrophotometer.
2. **Materials**
   - **2.1.** 500 mM NAC: Freshly dissolve 81.6 mg NAC (MW = 163.2) in 1 mL of deionized water.
   - **2.2.** 100 mM sodium phosphate buffer (pH 7.0): Mix 61 mL of 200 mM Na_2HPO_4 (53.65 g $Na_2HPO_4 \cdot 7H_2O$ or 71.64 g $Na_2HPO_4 \cdot 12H_2O$/1000 mL) with 39 mL of 200 mM NaH_2PO_4 (27.6 g $NaH_2PO_4 \cdot H_2O$ or 31.2 g $NaH_2PO_4 \cdot 2H_2O$/1000 mL). Mix and make up to 200 mL with deionized water.
   - **2.3.** Spectrophotometer.
3. **Methods**
   - **3.1.** MG extracted from plant samples according to DTNB method.
   - **3.2.** Pipette 50 μL of neutralized supernatant (volume can be adjusted according to MG content in plant samples) into 930 μL of 100 mM sodium phosphate buffer (pH 7.0).
   - **3.3.** Set to zero with a spectrophotometer.
   - **3.4.** Add 20 μL of 500 mM NAC (final concentration up to 10 mM).
   - **3.5.** Reaction at room temperature for 10 min.
   - **3.6.** Read absorbance at 288 nm.
   - **3.7.** Calculate MG content as the molecular absorption coefficient of 248 M^{-1} cm^{-1} for NASC and expressed as μmol/g FW.
4. **Precaution**

   NAC should be freshly prepared.

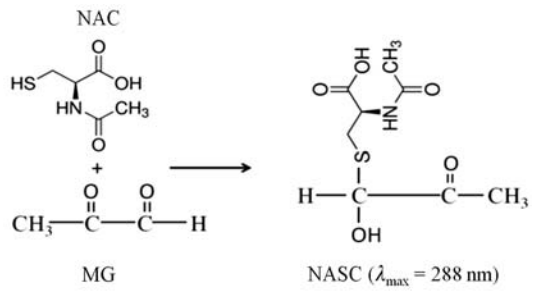


FIGURE 5.8 MG reacts with NAC to generate NASC with maximal absorbance at 288 nm.

5.4.4 Method 4: DAF-2 and DAR-1 Fluorescence Methods

1. **Principle**

   Fluorescent probes 4,5-diaminofluorescein (DAF-2) and 4,5-diamino-rhodamine (DAR-1) can react with MG and produce fluorescence substances DAF-2MG and DAR-1MG, respectively (Fig. 5.9; Shaheen et al., 2014). The intensity of fluorescence is proportional to the amount of MG in plant materials. Therefore, the imaging of DAF-2MG and DAR-1MG can be visualized by fluorescence microscope with excitation at 435 nm and emission at 509 nm for DAF-2MG, as well as excitation 545 nm and emission at 566 nm for DAR-1MG. The amount of MG in plant samples can be detected by in vivo or in vitro fluorometry.
2. **Materials**
   - **2.1.** 200 mL of 100 mM sodium phosphate buffer (pH 7.4): Mix 81 mL of 200 mM Na_2HPO_4 (53.65 g $Na_2HPO_4 \cdot 7H_2O$ or 71.64 g $Na_2HPO_4 \cdot 12H_2O$/1000 mL) with 19 mL of

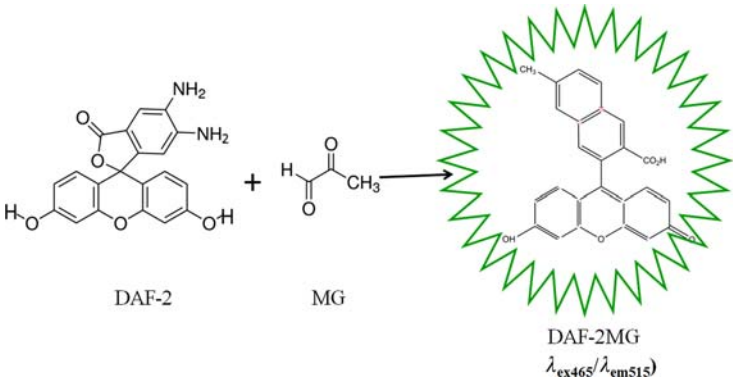


FIGURE 5.9 MG reacts with DAF-2 to generate a green fluorescence.

200 mM NaH_2PO_4 (27.6 g $NaH_2PO_4 \cdot H_2O$ or 31.2 g $NaH_2PO_4 \cdot 2H_2O$/1000 mL). Mix and make up to 200 mL with deionized water.

2.2. 10 μM DAF-2: Dissolve 1 mg DAF-2 (a pack, MW = 446.4) or DAR-1 (a pack, MW = 472.6) in 1 mL of DMSO to obtain 2.24 mM DAF-2 or 2.12 mM DAR-1, and then dilute to final concentration with 100 mM sodium phosphate buffer (pH 7.4).

2.3. Fluorescence microscope or fluorospectrophotometer.

3. Methods

3.1. For the in vitro assay, MG extracted from plant samples as DTNB method.

3.2. Add 5 μL of 2000 μM DAF-2 or DAR-1 (final concentration is 10 μM) to 995 μL of 100 mM sodium phosphate buffer (pH 7.4) and incubate at 37°C for 20 min (depending the amount of MG).

3.3. Develop fluorescence in a fluorospectrophotometer as excitation at 435 nm and emission at 509 nm for DAF-2-MG, and 545 and 566 nm for DAR-1-MG.

3.4. 3.4 Calculate the content of MG in plant samples using standard curve with known concentrations of MG from 0 to 50 μM.

4. Precautions

4.1. This method can detect MG in vivo using 100 mM sodium phosphate buffer (pH 7.4) containing 10 μM DAF-2 or DAR-1.

4.2. DAF-2 and DAR-1 should be freshly prepared and stored in the dark due to their sensitivity to light.

5.5 SECTION 4: NO QUANTIFICATION

5.5.1 Method 1: Griess Reagent Method

1. Principle

Signaling molecule nitric oxide (NO) can be rapidly converted into NO_2^- in solution in the presence of oxygen (O_2). Under acidic conditions, NO_2^- can react with sulfanilamide (SA) and *N*-naphthylethylenediamine (NNED) to form a chromophoricazo product. The pink product exhibits a maximal absorbance at 530 nm (Fig. 5.10; Antoniou et al., 2018). A direct correlation between the absorbance (A_{530}) and the concentration of NO in plant samples can be found.

2. Materials

2.1. Extraction buffer: Namely 50 mM acetate buffer (pH 3.6) containing 4% (w/v) zinc acetate: Mix 46.3 mL of 200 mM acetic acid (11.55 mL/1000 mL) with 3.7 mL of 200 mM $C_2H_2O_2Na$ (16.4 g $C_2H_2O_2Na$ or 27.2 g $C_2H_2O_2Na \cdot 3H_2O$/1000 mL), and then fill it up to 200 mL with deionized water. Dissolve 4 g of zinc acetate in 100 mL buffer.

2.2. Griess reagent: Reagent consisting of 0.5% (w/v) SA and 0.05% (w/v) naphthyl ethylenediamine dihydrochloride (NNED). For SA solution, dissolve 1 g of SA in 100 mL of 5% phosphoric acid (add 5.88 mL of phosphoric acid to 94.12 mL deionized water). For NED solution, add 0.1 g to 100 mL deionized water. Before measurement, mix NED solution with SA solution in equal volume.

3. Methods

3.1. Plant tissue (100 mg) is homogenized in liquid nitrogen with mortar and pestle, and then add 1 mL extraction buffer.

3.2. Mix well and centrifuge at 15,000 × g for 15 min at 4°C.

3.3. Extract again with 0.5 mL extraction buffer from pellet and centrifuge.

3.4. Mix supernatant for NO assay.

3.5. Add 1 mL of supernatant to 1 mL of Griess reagent.

3.6. Mix and incubate at room temperature for 30 min.

3.7. Transfer 300 μL of the reaction mixture to 96-well plate and read absorbance at 530 nm or read in a spectrophotometer.

3.8. A standard curve is performed with known concentrations of $NaNO_2$ ranging from 0 to 10 μM.

3.9. NO content is expressed as nmol/g FW.

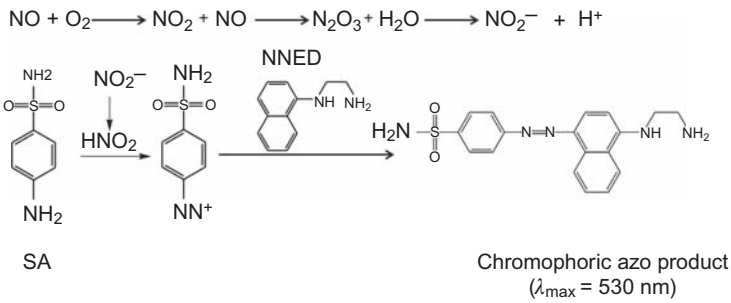


FIGURE 5.10 NO reacts with Griess reagent to generate a chromophoric azo product.

4. **Precautions**
   - **4.1.** All extraction buffers should be kept cool in a container with crushed ice.
   - **4.2.** Store the two solutions (NNED and SA) at 4°C with NNED covered in aluminum foil due to its sensitivity to light.
   - **4.3.** If your supernatant contains chlorophylls (green tissues), they can be remove from the supernatant by adding ~0.1 g active carbon.
   - **4.4.** Griess reagent from the fridge should be equilibrated at room temperature before the reaction takes place.
   - **4.5.** Sulfanilamide and NNED solutions also can be separately and orderly added to supernatant without mixing.

5.5.2 Method 2: DAF-FMDA Fluorescence Method

1. **Principle**

   Fluorescent probes 3-amino,4-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA) can freely enter into the cytosol across the biomembrane where it is deacetylated by intracellular esterase to produce DAF-FM, which is trapped. The DAF-FM can react with NO to produce a green fluorescent substance (Fig. 5.11; Xie and Shen, 2012), which has a maximal emission at 525 nm after excitation at 490 nm. The intensity of fluorescence is positively proportional to the amount of NO in plant samples. Therefore, NO in plant can be detected by in vivo or in vitro methods.
2. **Materials**
   - **2.1.** 100 mL of 20 mM of Hepes-NaOH (pH 7.5): Dissolve 0.477 g Hepes (MW = 238.31) in approximately 80 mL of deionized water. Adjust pH to 7.5 with 1 M NaOH, and then make up to 100 mL with deionized water. Store at 4°C.
   - **2.2.** 15 μM of DAF-FM DA: Dissolve 1 mg DAF-FM DA (MW = 496.42) in 1 mL of DMSO to obtain the stock solution of 2 mM, and then dilute to final concentration with 20 mM of Hepes-NaOH (pH 7.5).
   - **2.3.** Fluorescence microscope.
3. **Methods**
   - **3.1.** Plant tissues are incubated in 20 mM Hepes-NaOH (pH 7.5) containing 15 μMDAF-FM DA at 25°C for 15 min.
   - **3.2.** Wash plant tissues with distilled water three times, and determine the fluorescence using a fluorescence microscope with excitation 490 nm and emission 525 nm.
   - **3.3.** Photo is taken by using fluorescent microscope.
4. **Precautions**
   - **4.1.** DAF-FM DA should be freshly prepared and stored in the dark.
   - **4.2.** This method can detect NO in vitro using DAF FM instead of DAF-FM DA after NO extracted from plant samples following Method 1.

5.6 SECTION 5: H_2O_2 QUANTIFICATION

5.6.1 Method 1: KI Method

1. **Principle**

   Iodine ion (I^-) in KI solution can be reduced to iodine (I_2) in the present of H_2O_2, which in turn forms the yellow product triiodide (I_3^-). Triiodide exhibits a maximal light absorption at 390 nm, the absorbance is positively related to the content of H_2O_2 in plant samples (Fig. 5.12; Li and Gong, 2014; Antoniou et al., 2018).

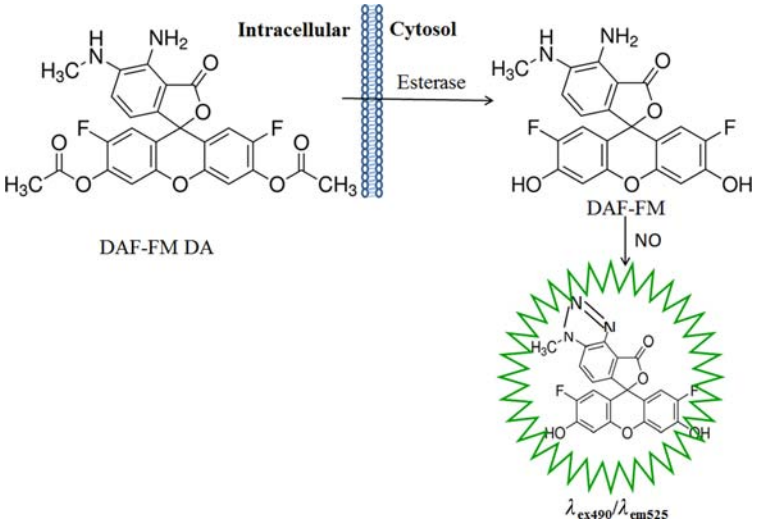


FIGURE 5.11 DAF-FM DA, which can freely permeate into cytosol, is deacetylated by intracellular esterase to produce DAF-FM, which can react with NO to generate fluorescence.

$$2I^- + H_2O_2 + 2H^+ \longrightarrow I_2 + 2H_2O + I^- \longrightarrow I_3^-$$
$$(\lambda_{max} = 390\ nm)$$

FIGURE 5.12 H_2O_2 reacts with KI to generate triiodide with maximal absorbance at 390 nm.

2. **Materials**
   - **2.1.** Extraction buffer: Namely 0.1% (w/v) trichloroacetic acid (TCA). Add 227 mL deionized water to 500 g TCA (a pack) and forms 100% (w/v) TCA, and then dilute to final concentration with deionized water. TCA should be stored at 4°C.
   - **2.2.** 10 mM potassium phosphate buffer (pH 7.0): Mix 6.15 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 3.85 mL of 100 mM KH_2PO_4 (13.609 g/100 mL), and add deionized water to reach a final volume of 100 mL.
   - **2.3.** 1 M potassium iodide: Dissolve 16.6 g KI (MW = 166) in 100 mL deionized water. Store at 4°C.
   - **2.4.** Spectrophotometer or microplate reader.
3. **Methods**
   - **3.1.** Plant tissue (100 mg) is homogenized in 1 mL of 0.1% (w/v) TCA with mortar and pestle.
   - **3.2.** Centrifuge at 15,000 × g for 15 min at 4°C.
   - **3.3.** Add 0.5 mL of the supernatant to 0.5 mL of 10 mM phosphate buffer (pH 7.0) (use 0.5 mL of 0.1% (w/v) TCA instead of the supernatant as the blank).
   - **3.4.** Add 1 mL of KI to initiate the reaction as quick as possible.
   - **3.5.** Mix gently and incubate in the dark for 2–15 min (depending on plant tissue).
   - **3.6.** Transfer 300 μL of the reaction mixture to 96-well plate and read the absorbance at 390 nm or read in a spectrophotometer.
   - **3.7.** A standard curve is performed using the known concentrations of H_2O_2 ranging from 0 to 250 μM.
   - **3.8.** H_2O_2 content is calculated following standard curve and expressed as μmol H_2O_2/g FW.
4. **Precaution**

   Because KI is sensitive to light and liberates free iodine, it should be freshly prepared and covered with aluminum foil. Store at 4°C.

5.6.2 Method 2: $Ti(SO_4)_2$ Method

1. **Principle**

   H_2O_2 can react with Ti^{4+} in $Ti(SO_4)_2$ or $TiCl_4$ solution under acidic conditions to form a yellow titanium–hydrogen peroxide complex (Ti-H_2O_2). The complex exhibits a maximal absorbance at 410 nm and has a molar extinction coefficient of $2.8 \times 10^5\ M^{-1}\ cm^{-1}$ (Fig. 5.13; Yu et al., 2003; Li and Gong, 2014). The yellow product (the amount of Ti-H_2O_2 complex) is positively proportional to the content of H_2O_2 in plant samples.

$$H_2O_2 + Ti(SO_4)_2 \longrightarrow \text{Ti-}H_2O_2\ \text{complex}$$
$$(\lambda_{max} = 410\ nm)$$

FIGURE 5.13 H_2O_2 reacts with $Ti(SO_4)_2$ to generate a yellow Ti-H_2O_2 complex.

2. **Materials**
   - **2.1.** Extraction buffer: Namely 50 mMK-phosphate buffer (pH 6.5): Mix 31.5 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 68.5 mL of 100 mM KH_2PO_4 (13.609 g/100 mL), and add deionized water to reach a final volume of 200 mL.
   - **2.2.** 0.1% (w/v) $Ti(SO_4)_2$: Dissolve 0.1 g $Ti(SO_4)_2$ in 100 mL of 20% (v/v) H_2SO_4.
   - **2.3.** Centrifuger.
   - **2.4.** Spectrophotometer.
3. **Methods**
   - **3.1.** H_2O_2 is extracted by homogenizing 0.5 g plant tissues with 3 mL of 50 mM K-phosphate buffer (pH 6.5) at 4°C. The homogenate is centrifuged at 10,000 × g for 15 min, and supernatant is collected and used for H_2O_2 assay.
   - **3.2.** A 3-mL sample of supernatant is mixed with 1 mL of 0.1% $TiCl_4$, and reacted at room temperature for 5 min.
   - **3.3.** The mixture was then centrifuged at 10,000 × g for 10 min at room temperature and supernatant was collected.
   - **3.4.** The optical density of the supernatant was measured by spectrophotometer at 410 nm.
   - **3.5.** H_2O_2 content is calculated as the extinction coefficient of $2.8 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as μmol/g FW.
4. **Precautions**
   - **4.1.** To avoid the degradation of H_2O_2 by catalase, the process of H_2O_2 extraction must be carried out at 4°C, or 1 mM hydroxylamine (final concentration) is added to 50 mMK-phosphate buffer (pH 6.5) to inhibit catalase.
   - **4.2.** $Ti(SO_4)_2$ can be replaced with $TiCl_4$ (correspondingly dissolved in 20% HCl) in this method.
   - **4.3.** If plant sample contains pigment, it should be removed using active carbon from supernatant.

5.6.3 Method 3: XO Method

1. Principle

Under acidic conditions, Fe^{2+} can be oxidized by H_2O_2 to Fe^{3+}, which reacts with xylenol orange (XO) to generate a purple complex (Fe^{3+}-XO). The complex has maximal absorbance at 560 nm and molar extinction coefficient of $2.24 \times 10^5\ M^{-1}\ cm^{-1}$ (Fig. 5.14; Gay and Gebicki, 2000, 2003; Li and Gong, 2014). The purple product (the amount of Fe^{3+}-XO) is positively correlated to the content of H_2O_2 in plant samples and the sensitivity can be improved when proper concentrations of sorbitol are added to the reaction system.

2. Materials

2.1. Extraction solution: Namely 200 mM perchloric acid (PA): Add 1.7 mL of $HClO_4$ ($M = 12$) to 98.3 mL of deionized water.

2.2. Working reagent: 100 mL of working reagent containing 500 μM ammonium ferrous sulfate (19.6 mg, MW = 392), 50 mM H_2SO_4 (0.28 mL, $M = 18$ M), 200 μM xylenol orange (tetrasodium salt, 14.3 mg, MW = 716.6), and 200 mM sorbitol (3.64 g, MW = 182).

2.3. Centrifuger.

2.4. Spectrophotometer or microplate reader.

3. Methods

3.1. Plant samples roots or leaves (0.2 g) are extracted in 200 mM PA with mortar and pestle.

3.2. Centrifuge at 4°C, 10,000 × g for 15 min, collect supernatant and use for H_2O_2 assay.

3.3. To 1.5 mL of supernatant, add 1.5 mL of working reagent. Develop at 30°C for 30 min.

3.4. The optical density (OD_{560}) of the purple solution was measured by spectrophotometer at 560 nm.

3.5. H_2O_2 content is calculated as the extinction coefficient of $2.24 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as μmol/g FW.

4. Precautions

4.1. The specificity for H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture with catalase (CAT) after being neutralized with 1 M NaOH.

4.2. If plant sample contains pigment, it should be removed using active carbon from supernatant.

5.6.4 Method 4: ABTS Method

1. Principle

H_2O_2 can react with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) in the present of the peroxidase such as horseradish peroxidase (HRP) to generate a soluble end product (radical monocation ABTS•$^+$) that is green in color. The ABTS•$^+$ can be detected spectrophotometrically at 405 or 734 nm (Fig. 5.15; Angelini et al., 2018). The light absorption (A_{405} or A_{734}) is positively proportional to the content of H_2O_2 in plant samples.

2. Materials

2.1. 100 mM K-phosphate buffer (pH 7.0): Mix 61 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 39 mL of 100 mM KH_2PO_4 (13.609 g/100 mL).

2.2. Extraction buffer: 100 mL of 100 mM K-phosphate buffer (pH 7.0) containing 10% polyvinyl pyrrolidone (PVP, 10 g) and 1 mM hydroxylamine (0.695 g, MW = 69.5).

2.3. 10 mM H_2O_2: Add 10 μL of 30% H_2O_2 solution to 9990 μL of 100 mM K-phosphate buffer (pH 7.0) to obtain 10 mMH_2O_2. Measure the absorbance at 240 nm to correct its concentration following the extinction coefficient of $43\ M^{-1}\ cm^{-1}$.

2.4. Horseradish peroxidase (HRP) solution: Dissolve 1 mg HRP (~150 U/mg) in 1 mL of 100 mM K-phosphate buffer (pH 7.0).

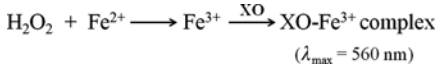


FIGURE 5.14 Fe^{2+} is reduced by H_2O_2 to Fe^{3+} that can react with XO to produce a purple XO-Fe^{3+} complex.

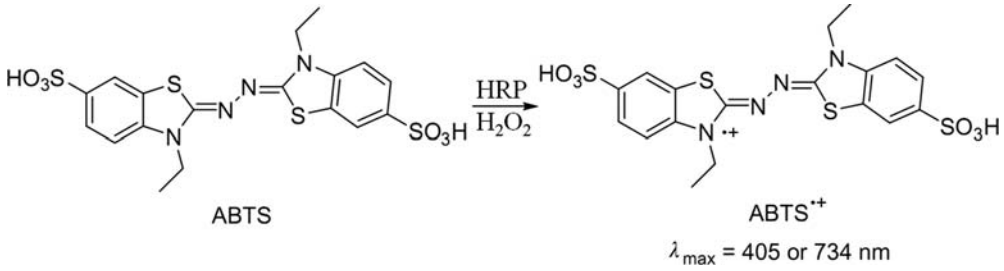


FIGURE 5.15 ABTS reacts with H_2O_2 in the present of peroxidase to ABTS•$^+$.

2.5. 50 mM ABTS: Dissolve 0.24 g ABTS (MW = 548.68) in 10 mL of deionized water.
2.6. Spectrophotometer or microplate reader.

3. Methods

3.1. Set up standard curve
1. Dilute the 10 mM H_2O_2 solution to 0, 50, 100, 150, and 200 1 μM with 100 mM K-phosphate buffer (pH 7.0).
2. Mix 200 μL of the different concentrations of H_2O_2 with 10 μL of HRP, and then add 10 μL of 50 mM ABTS.
3. Incubate for 3 min at room temperature, and then measure the absorbance at 405 nm.
4. Draw the standard curve with absorbance values on the *Y*-axis and H_2O_2 amount (nmol) on *X*-axis or establish regression equation.

3.2. Extraction and measurement of H_2O_2
1. Plant tissues (500 mg) are homogenized in 1 mL of extraction buffer with a prechilled mortar and pestle at 4°C.
2. Centrifuge at 10,000 × g, 4°C for 10 min, collect supernatant and use for H_2O_2 assay.
3. Add 10 μL of HRP and 10 μL of 50 mM ABTS to 200 μL supernatant.
4. Incubate at room temperature for 3 min.
5. Read absorbance at 405 nm.
6. Calculate H_2O_2 content using the standard curve and express as μmol/g FW.

4. Precautions

4.1. The molar concentration of 30% (w/w) H_2O_2 with a density of 1.11 g/mL is 9.8 M.

Due to the instability of H_2O_2, it should be freshly prepared and immediately corrected using the molar extinction coefficient of 43 M^{-1} cm^{-1}.

4.2. This method can in vivo detect H_2O_2 in plant materials by incubating in 100 mM K-phosphate buffer (pH 7.0) containing HRP (45 μg/mL) and 2.5 mM ABTS for the indicated time, and then taking a photo.

5.6.5 Method 5: DCHBS-AAP Method

1. Principle

The reaction of H_2O_2 and 3,5-dichloro-2-hydroxybenzene sulfonic acid (DCHBS) generates a radical DCHBS• in the presence of peroxidase such as horseradish peroxidase (HRP). The DCHBS• further reacts with 4-aminoantipyrine (AAP) to form a nonradical byproduct that is pink in color. The pink product exhibits a maximal absorbance at 515 nm and the molar extinction coefficient is 2.6 × 10^4 M^{-1} cm^{-1} (Fig. 5.16; Fraisse et al., 2002; Li and Gong, 2014). The light absorption is proportional to the content of H_2O_2 in plant samples. Therefore, the H_2O_2 production can be measured as the formation of a pink adduct (nonradical byproduct) via in vivo and in vitro methods.

5.6.5.1 In Vivo Method

2. Materials

2.1. 100 mM K-phosphate buffer (pH 7.0): Mix 61 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 39 mL of 100 mM KH_2PO_4 (13.609 g/100 mL).
2.2. 5 mM AAP: Weigh 10 mg AAP (MW = 203) and transfer to 5 mL of 100 mM K-phosphate buffer (pH 7.0). Mix and make up to 10 mL with 100 mM K-phosphate buffer (pH 7.0).
2.3. Reaction mixture: The solution containing 100 μM AAP, 1 mM DCHBS, 60 μg/mL HRP: Weigh 13 mg DCHBS (MW = 265) and 3 mg HRP, respectively, and then add 1 mL 5 mM AAP solution. Mix and make up to 50 mL with 100 mM K-phosphate buffer (pH 7.0).
2.4. Vacuum pump.
2.5. Spectrophotometer.

3. Methods

3.1. Submerge the plant material completely in the reaction mixture by vacuum infiltration for 1−3 times (1 min each time).

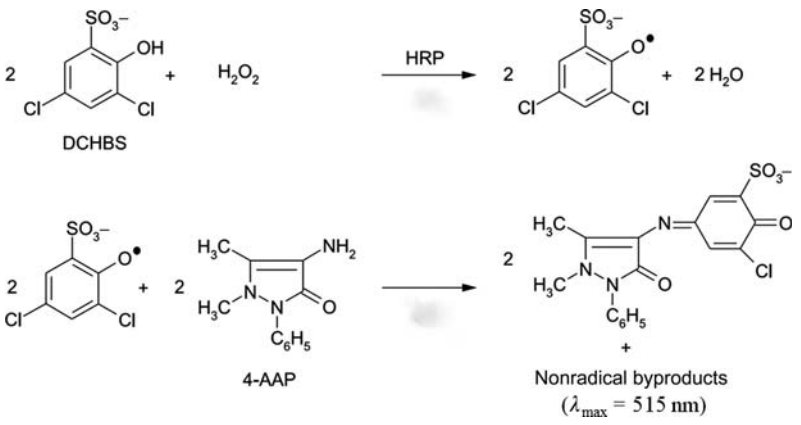


FIGURE 5.16 H_2O_2 can react with DCHBS and AAP in the present of peroxidase to generate a pink product.

3.2. Incubate for 2 h at 30°C in the dark.
3.3. Collect the incubation medium and centrifuge at 10,000 × g for 5 min.
3.4. Measure the absorbance at 515 nm.
3.5. Calculate H_2O_2 concentration using the molar extinction coefficient 2.6 × 10^4 M^{-1} cm^{-1}.

4. Precautions

4.1. To study the effect of environmental stress on H_2O_2 production, an appropriate amount of NaCl, sorbitol, and heavy metal is added to reaction mixture to carry out salt, osmotic, and heavy metal stresses. In addition, plant materials incubated in reaction mixture are submitted to high or cold temperature to explore the production of H_2O_2.
4.2. For the H_2O_2 specificity control, add 1.66 g KI to the reaction mixture before adjusting to the final volume.

5.6.5.2 In Vitro Method

2. Material

2.1. 100 mM K-phosphate buffer (pH 7.0): Mix 61 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 39 mL of 100 mM KH_2PO_4 (13.609 g/100 mL).
2.2. HRP (1 mg/mL) solution: Dissolve 1 mg HRP in 1 mL of 100 mM K-phosphate buffer (pH 7.0).
2.3. 10 mM DCHBS stock solution: Dissolve 26.5 mg DCHBS (MW = 265) in 10 mL of water and stored at 4°C.
2.4. 1 mM AAP stock solution: Dissolve 20.3 mg DCHBS (MW = 203) in 100 mL of water and stored at 4°C.
2.5. Spectrophotometer.

3. Methods

3.1. H_2O_2 is extracted from plant materials as in ABTS method.
3.2. Add 50 μL of HRP, 100 μL of 1 mM APP, and 100 μL of 10 mM DCHBS to 200 μL of the supernatant.
3.3. Add 100 mM K-phosphate buffer (pH 7.0) to final volume of 1 mL.
3.4. Measure absorbance at 515 nm using a spectrophotometer.
3.5. Calculate H_2O_2 content using the extinction coefficient of 2.6 × 10^4 M^{-1} cm^{-1}.

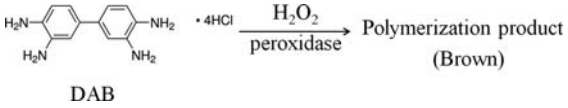


FIGURE 5.17 H_2O_2 reacts with DBA in the present of peroxidase to a brown polymerization product.

4. Precautions

This system can be used to determine the activity of enzymes involved in H_2O_2 production by adding enzymatic substances to reaction mixture, such as copper amine oxidase, glucose oxidase.

5.6.6 Method 6: DAB Method

1. Principle

H_2O_2 is usually detected in plant tissues by using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as substrate in the present of peroxidase. A marked brown and insoluble polymerization product is formed by the reaction of DAB with H_2O_2 (Fig. 5.17; Rodriguez and Taleisnik, 2012). The amount of brown product represents the concentration of H_2O_2 in plant tissues.

2. Materials

2.1. Staining solution: namely 1 mg/mL DAB solution: Dissolve 100 mg DAB in 100 mL of deionized water (adjust pH to 3.8 with 1 M HCl).
2.2. 96% (v/v) ethanol: Add 4 mL of deionized water to 96 mL of absolute ethanol.
2.3. Fixed solution: ethanol: lactic acid: glycerol (3:1:1).
2.4. Vacuum pump.
2.5. Camera.

3. Methods

3.1. Submerge the interested plant tissues in staining solution by vacuum infiltration 2−3 times (1−2 min per time) until the plant tissues are completely infiltrated.
3.2. Incubate for 5−6 h till brown precipitates are observed.
3.3. Chlorophyll can be removed by repeated washes with 96% (v/v) ethanol under heating at 40°C.
3.4. Stained plant tissues can be fixed with fixer solution.
3.5. Photograph will be taken with the help of a camera.

4. Precautions

4.1. A low pH of 3.8 is necessary for proper solubilization of DAB.
4.2. During vacuum infiltration, it is important to release the vacuum gently to enable better infiltration of plant tissues.

5.6.7 Method 7: $DCFH_2$-DA and DCF Fluorescence Methods

1. Principle

Fluorescence probe 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) is one of the most commonly used

dyes for determining total ROS (mainly H_2O_2 and $O_2^{\bullet -}$). Esterified $DCFH_2$-DA (nonfluorescent) can be permeable to cell membranes and enter freely the cells. Inside the cells, $DCFH_2$-DA is deacetylated by intracellular esterases to generate 2′,7′-dichlorofluorescein (DCF) that is trapped. The DCF can be oxidized by ROS molecules to a highly fluorescent substance with excitation at 504 nm and emission at 524 nm (Fig. 5.18; Rodriguez and Taleisnik, 2012; Park and Roubelakis-Angelakis, 2018). The intensity of fluorescence is proportional to the amount of ROS in plant tissues. Therefore, ROS can be monitored by fluorescence microscopy or fluorospectrophotometer using in vitro and in vivo methods.

5.6.7.1 In Vivo Method: ($DCFH_2$-DA Method)

2. Materials

2.1. 20 mM K-phosphate buffer (pH 6.0): Add 13.2 mL of 100 mM K_2HPO_4 (1.74 g /100 mL) to 86.8 mL of 100 mM KH_2PO_4 (1.36 g/100 mL). Autoclave at 121°C for 20 min. Store at room temperature. Before use, dilute with water from the concentrated stock to 20 mM K-phosphate buffer.

2.2. 10 μM $DCFH_2$-DA: Dissolve 1 mg of $DCFH_2$-DA (a pack, MW = 487) in 2 mL of DMSO to obtain 1 mM $DCFH_2$-DA. Storage in separate tubes at −20°C in a black box. Before experiments, dilute from 1 mM stock solution to 10 μM $DCFH_2$-DA with 20 mM K-phosphate buffer.

2.3. Confocal laser scanning microscopy.

3. Methods

3.1. Submerge the detach plant tissues (or whole seedlings) in 10 μM $DCFH_2$-DA for 30 min.

3.2. Rinse the samples thoroughly with water at least twice to eliminate any remaining fluorescent dye.

3.3. Visualize green fluorescence by confocal laser scanning microscopy with 504/524 nm (excitation/emission).

3.4. Photograph.

4. Precautions

4.1. $DCFH_2$-DA is sensitive to light. The stock solution should be covered with aluminum foil to prevent light exposure.

4.2. For the H_2O_2 specificity control, add 1.66 g KI to 10 μM $DCFH_2$-DA.

5.6.7.2 In Vitro Method: (DCF Method)

2. Materials

2.1. Dimethylsulfoxide (DMSO).

2.2. Extraction buffer: Namely 10 mM Tris−HCl (pH 7.2): Mix 50 mL of 200 mM Tris (2.42 g/100 mL) with 44.2 mL of 200 mM HCl (1.7 mL/100 mL). Make up to 1000 mL with deionized water.

2.3. 10 μM DCF: Dissolve 1 mg DCF (MW = 401) in 1 mL of DMSO to obtain 2.5 mM DCF. Dilute to final concentration of 10 μM DCF with 10 mM Tris−HCl (pH 7.2).

2.4. Bradford reagent (used for protein quantification).

2.5. Refrigerated centrifuge.

2.6. Fluorescence microscopy or fluorospectrophotometer.

3. Methods

3.1. Before starting the experiment, turn on the fluorescence microscope to warm up.

3.2. Plant tissues (100 mg) are ground in liquid nitrogen with mortar and pestle.

3.3. Homogenates are mixed with 1 mL of extraction buffer.

3.4. Centrifuge at 10,000 × g for 15 min at 4°C.

3.5. Add 100 μL of supernatant to 890 μL of 10 mM Tris−HCl (pH 7.2) [use 1 mL of 10 mM Tris−HCl (pH 7.2) as the blank].

3.6. Add 10 μL of 1 mM DCF (final concentration will be 10 μM) to sample. Vortex and incubate for 10 min in dark [use the mixture of 100 μL of plant extract and 900 μL of Tris−HCl (pH 7.2) as the control to remove the background fluorescence].

3.7. Visualize by fluorescence microscope or fluorospectrophotometer with excitation at 504 nm and emission at 524 nm.

3.8. Estimate protein concentration in samples using Bradford method.

3.9. The amount of ROS is expressed as relative fluorescence units mg^{-1} protein.

4. Precautions

4.1. DCF is sensitive to light. The stock solution should be covered with aluminum foil to prevent light exposure.

4.2. To get the specificity for H_2O_2, catalase (300 units/mL) should be added to reaction mixture to remove H_2O_2 before DCF is added.

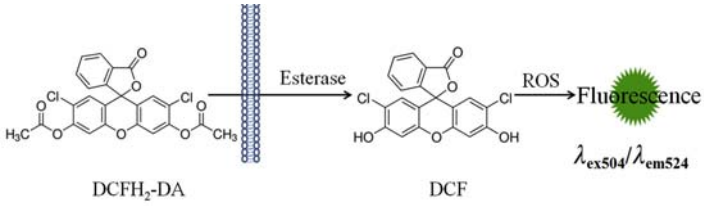


FIGURE 5.18 $DCFH_2$-DA, which can freely permeate into cytosol, is deacetylated by intracellular esterases to produce DCF, which can be oxidized by ROS to generate fluorescence.

4.3. This method can carry out in vitro measurement for H_2O_2 using 10 μM $DCFH_2$-DA instead of 10 μM DCF.

5.6.8 Method 8: AR and AUR Fluorescence Methods

1. Principle

Fluorescence probes10-acetyl-3,7-dihydroxyphenoxazine, also known as Ampliflu Red (AR, OxiRed probe) and Amplex UltraRed reagent (AUR), are highly sensitive and stable probes for H_2O_2. In the presence of horseradish peroxidase (HRP), the AR and AUR probes react in a 1:1 stoichiometry with H_2O_2 to produce highly fluorescent resorufin with excitation at 571 nm and emission at 585 nm for AR, as well as excitation at 568 nm and emission at 581 nm for AUR (Fig. 5.19; Rodriguez and Taleisnik, 2012; Park and Roubelakis-Angelakis, 2018). Because AUR cannot cross the cell membrane, but AR can, AUR is widely used to detect extracellular H_2O_2, while AR is used to detect intracellular H_2O_2. The intensity of fluorescence is proportional to the amount of H_2O_2. Therefore the content of H_2O_2 in plant samples can be assayed by colorimetric or fluorometric methods.

2. Materials

2.1. 50 mM sodium phosphate buffer (pH 7.4): Add 19 mL of 100 mM $NaH_2PO_4 \cdot H_2O$ ($NaH_2PO_4 \cdot H_2O$: 1.39 g/100 mL) to 81 mL of 100 mM Na_2HPO_4 ($Na_2HPO_4 \cdot 7H_2O_2$).

68 g/100 mL or $Na_2HPO_4 \cdot 12H_2O$ (3.59 g/100 mL). Autoclave at 121°C for 20 min. Store at room temperature. Before use, dilute to 50 mM sodium phosphate buffer with deionized water.

2.2. 50 μM AR working reagent: Dissolve 1.29 mg of AR (MW = 257.25) in 1 mL of DMSO to obtain 5 mM AR, which is stored at −20°C. Just prior to use, dilute to final concentration (50 μM) with 50 mM sodium phosphate buffer (pH 7.4).

2.3. 50 μM AUR reagent working solution: Dissolve 1 mg AUR (MW = 300) in 666 μL DMSO to obtain 5 mM AUR. Dilute to 1 mM AUR with DMSO and then store at −20°C. Just prior to use, dilute 1 mM AUR to final concentration of 50 μM with 50 mM sodium phosphate buffer (pH 7.4).

2.4. Fluorescence microscopy or fluorospectrophotometer.

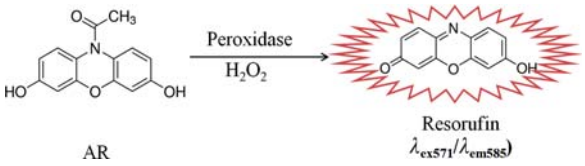


FIGURE 5.19 AR reacts with H_2O_2 in the present of peroxidase to generate resorufin.

3. Methods

See "$DCFH_2$-DA and DCF Methods." In this experiment, $DCFH_2$-DA and DCF should be replaced with AR and AUR.

3.1. Plant tissues are incubated in 50 μM AR or 50 μM AUR working solution for 30−60 min at room temperature in the dark.

3.2. Assay fluorescence by fluorescence microscopy with excitation at 571 nm and emission at 585 nm for AR, and excitation at 568 nm and emission at 581 nm for AUR.

3.3. Photograph or calculate H_2O_2 content according to standard curve with known concentrations of H_2O_2 from 0 to 50 μM.

4. Precautions

4.1. DMSO is hazardous. Reagents containing DMSO should be carefully handled. Avoid contact with skin and eyes.

4.2. This method can carry out in vitro measurement for H_2O_2 using 50 μMAR or AUR instead of 10 μM DCF (see DCF method).

4.3. For the H_2O_2 specificity control, add 1.66 g KI to 50 μM AR or 50 μM AUR.

5.6.9 Method 9: BES-H_2O_2-Ac and BES-H_2O_2 Fluorescence Method

1. Principle

Fluorescence probes 3′-*O*-Acetyl-6′-*O*-penta-fluorobenzenesulfonyl-2′-7′-difluorofluorescein (BES-H_2O_2-Ac) and 6′-*O*-penta-fluorobenzenesulfonyl-2′-7′-difluorofluorescein (BES-H_2O_2) are nonfluorescent. When they are oxidized by H_2O_2 they form highly fluorescent oxidized products (2′,7′-difluorofluorescein) with excitation at 485/530 nm (excitation/emission) (Fig. 5.20; Maeda et al., 2007; Rodriguez and Taleisnik, 2012; Park and Roubelakis-Angelakis, 2018). The intensity of green fluorescence is a direct correlation to the amount of H_2O_2 in plant samples. BES-H_2O_2-Ac can freely enter into the cells, which is usually used to explore intracellular H_2O_2; while BES-H_2O_2 cannot cross the cell membrane, extracellular H_2O_2 is commonly detected by fluorescence microscope.

2. Materials

2.1. 20 mM K-phosphate buffer (pH 6.0): Add 13.2 mL of 100 mM K_2HPO_4 (1.74 g /100 mL)to 86.8 mL of 100 mM KH_2PO_4 (1.36 g/100 mL). Autoclave at 121°C for 20 min. Store at room

temperature. Before use, dilute to 20 mM K-phosphate buffer with deionized water.

2.2. 50 μM BES-H_2O_2-Ac working reagent: Dissolve 1 mg BES-H_2O_2-Ac (MW = 640.44) in 310 μL of DMSO to obtain 5 mMBES-H_2O_2-Ac, which is stored at 4°C. Just prior to use, dilute to final concentration (50 μM) with 20 mM K-phosphate buffer (pH 6.0).

2.3. 50 μM BES-H_2O_2 reagent working solution: Dissolve 1 mg BES-H_2O_2 (MW = 598.40) in 334 μL of DMSO to obtain 5 mM BES-H_2O_2, which is stored at 4°C. Just prior to use, dilute to final concentration (50 μM) with 20 mM K-phosphate buffer (pH 6.0).

2.4. Fluorescence microscopy or fluorospectrophotometer.

3. Methods

See "$DCFH_2$-DA and DCF Methods." In this experiment, $DCFH_2$-DA and DCF should be replaced with BES-H_2O_2-Ac and BES-H_2O_2.

3.1. Plant tissues are incubated in 50 μM BES-H_2O_2-Acor 50 μM BES-H_2O_2 reagent working solution for indicated time at room temperature in the dark.

3.2. Detect fluorescence by fluorescence microscopy or fluorospectrophotometer with excitation at 485 nm and emission at 530 nm for BES-H_2O_2-Ac (for extracellular H_2O_2) and BES-H_2O_2 (for intracellular H_2O_2).

3.3. Photograph or calculate H_2O_2 content according to standard curve with known concentrations of H_2O_2 from 0 to 50 μM.

4. Precautions

4.1. DMSO is hazardous. Reagents containing DMSO should be carefully handled. Avoid contact with skin and eyes.

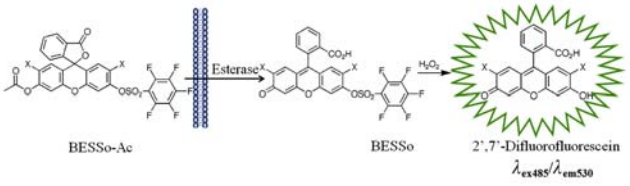
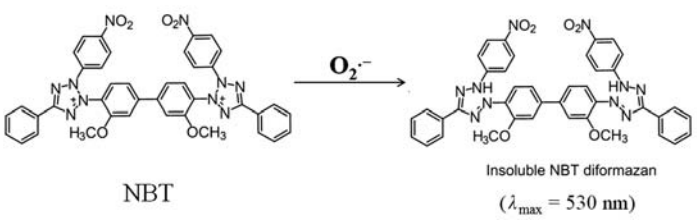


FIGURE 5.20 BESSo, which can freely permeate into cytosol, is deacetylated by intracellular esterases to produce BESSo, which can be oxidized by H_2O_2 to generate green fluorescence.



4.2. This method can carry out in vitro measurement for H_2O_2 using 50 μM BES-H_2O_2-Ac or BES-H_2O_2 instead of 10 μM DCF (see DCF method).

4.3. For the H_2O_2 specificity control, add 1.66 g KI to 50 μMBES-H_2O_2-Acor 50 μM BES-H_2O_2.

5.7 SECTION 6: SUPEROXIDE RADICAL MEASUREMENT

5.7.1 Method 1: NBT Method

1. Principle

The qualitative determination of $O_2^{\bullet -}$ with nitrotetrazolium blue chloride depends on the formation of a blue insoluble 1,3,5-triphenyltetrazolium formazan precipitate that results from the reaction between $O_2^{\bullet -}$ and NBT. The formazan has a maximal absorbance at 530 nm and the extinction coefficient of $1.28 \times 10^4\ M^{-1}\ cm^{-1}$ (Fig. 5.21; Rodriguez and Taleisnik, 2012; Li and Gong, 2014). The absorbance is positively proportional to the amount of $O_2^{\bullet -}$ in plant samples. Therefore, $O_2^{\bullet -}$ production can be detected by in vivo and in vitro methods.

2. Materials

2.1. 10 mM $MnCl_2$: Dissolve162 mg $MnCl_2$ (MW = 161.87) in 100 mL of deionized water.

2.2. 100 mM K-phosphate buffer (pH 7.0): Mix 61 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 39 mL of 100 mM KH_2PO_4 (13.609 g/100 mL).

2.3. 0.5% NBT solution: Dissolve 0.5 g NBT (MW = 817.64) in 100 mL of 100 mM K-phosphate buffer (pH 7.0).

2.4. Destaining solution: A mixture of ethanol and glycerin (9:1).

2.5. Vacuum pump.

2.6. Camera.

5.7.1.1 Spectrophotometric Method

3. Methods

3.1. Submerge the plant tissues (200 mg) in 0.5% NBT solution by vacuum infiltration 1−3 times (1 min each time).

FIGURE 5.21 NBT can react with $O_2^{\bullet -}$ to generate insoluble NBT diformazan.

3.2. Incubate for indicated time (such as 1 h) at room temperature in the dark.
3.3. Centrifuge at 8000 × g for 5 min at room temperature.
3.4. The supernatant is heated at 85°C for 15 min, and then cooled rapidly on ice.
3.5. Read absorbance at 530 nm with a spectrophotometer.
3.6. The $O_2^{\bullet -}$ content is expressed as the increase in absorbance g^{-1} FW or calculated using the extinction coefficient of $1.28 \times 10^4\ M^{-1}\ cm^{-1}$.

5.7.1.2 Histochemical Staining Method

3. Methods
3.1. Submerge the plant tissues (200 mg) in 0.5% NBT solution by vacuum infiltration 1−3 times (1 min each time).
3.2. Incubate for 2 h at 30°C in the dark.
3.3. Remove NBT solution and add destaining solution. Boil for 10 min in water bath until the green color has faded almost completely (this step can be ignored for nongreen tissues).
3.4. Photograph will be taken by using a camera.

4. Precautions
4.1. NBT can be replaced with cytochrome C, the extinction coefficient of formazan is $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ at 550 nm.
4.2. To study the effect of environmental stress on $O_2^{\bullet -}$ production, an appropriate amount of NaCl, sorbitol, and heavy metal is added to NBT solution to carry out salt, osmotic, and heavy metal stresses. In addition, plant materials incubated in NBT solution are submitted to high or cold temperature to explore the production of H_2O_2.
4.3. For the $O_2^{\bullet -}$ specificity control, add 0.5 mL of the $MnCl_2$ solution or SOD (50 μm/mL) before making up to the final volume of NBT.

5.7.2 Method 2: XTT Method

1. Principle

Tetrazolium compounds that form soluble formazans can be used to quantify $O_2^{\bullet -}$ production. The reaction between $O_2^{\bullet -}$ and Na, 3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium] (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) produces a soluble formazan, which has a maximal light absorption at 470 nm and the extinction coefficient of $2.16 \times 10^4\ M^{-1}\ cm^{-1}$ (Fig. 5.22; Rodriguez and Taleisnik, 2012; Li and Gong, 2014). The absorbance is proportional to the content of $O_2^{\bullet -}$ in plant samples.

2. Materials
2.1. 10 mM $MnCl_2$: Dissolve 162 mg $MnCl_2$ (MW = 161.87) in 100 mL of deionized water.
2.2. 100 mM K-phosphate buffer (pH 7.0): Mix 61 mL of 100 mM K_2HPO_4 (17.418 g/100 mL) with 39 mL of 100 mM KH_2PO_4 (13.609 g/100 mL).
2.3. 0.5 mM XTT: Dissolve 34 mg XTT (MW = 673.52) in 100 mL of 100 mM K-phosphate buffer (pH 7.0).
2.4. Vacuum pump.
2.5. Spectrophotometer.

3. Methods
3.1. Submerge the plant material completely in the XTT solution by vacuum infiltration for 1 min.
3.2. Incubate for 5 h at 30°C in the dark.
3.3. Collect the incubation medium and centrifuge at 10,000 × g for 5 min.
3.4. Measure the absorbance of the incubation medium at 470 nm.
3.5. Calculate the $O_2^{\bullet -}$ production using molar extinction coefficient of $2.16 \times 10^4\ M^{-1}\ cm^{-1}$).

4. Precautions
4.1. To study the effect of environmental stress on $O_2^{\bullet -}$ production, an appropriate amount of NaCl, sorbitol, and heavy metal is added to XTT solution to carry out salt, osmotic, and heavy metal stresses. In addition, plant materials incubated in XTT solution are submitted to high or cold temperature to explore the production of H_2O_2.
4.2. For the $O_2^{\bullet -}$ specificity control, add 0.5 mL of the $MnCl_2$ solution or SOD (50 μm/mL) before making up to the final volume of XTT.

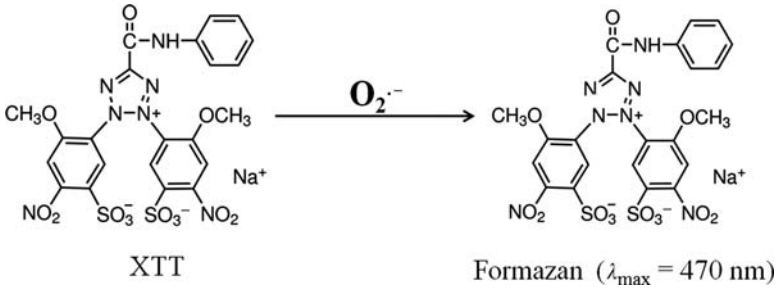


FIGURE 5.22 XTT can react with $O_2^{\bullet -}$ to generate soluble NBT formazan.

5.7.3 Method 3: BESSo-AM and BESSo Fluorescence Methods

1. Principle

Superoxide radical ($O_2^{\bullet -}$) fluorescence probes 3′-o-(4,5-dimethoxy-2-nitrobenzenesulfonyl)-2′,4′,5′,7′-tetrafluorofluorescein (BESSo) and acetylated BESSo (BESSo-AM) are nonfluorescent. BES-H_2O_2-AM can freely permeate into the cells in where it is deacetylated by intracellular esterase to form BESSo (Maeda et al., 2007; Rodriguez and Taleisnik, 2012; Park and Roubelakis-Angelakis, 2018). In addition, BESSo cannot cross the membrane into the cells. BESSo can be oxidized by $O_2^{\bullet -}$ to form 2′,7′-difluorofluorescein, which has highly fluorescent oxidized products with excitation at 505/544 nm (excitation/emission) (Fig. 5.23; Maeda et al., 2007; Rodriguez and Taleisnik, 2012; Park and Roubelakis-Angelakis, 2018). Therefore, BESSo is usually used to detect extracellular $O_2^{\bullet -}$; while BESSo-AM can determine intracellular $O_2^{\bullet -}$ in plant materials. Because the intensity of green fluorescence is direct correlation to the amount of $O_2^{\bullet -}$ in plant samples, the $O_2^{\bullet -}$ content can be detected by fluorescence microscope.

2. Materials

2.1. 20 mM K-phosphate buffer (pH 6.0): Add 13.2 mL of 100 mM K_2HPO_4 (1.74 g/100 mL) to 86.8 mL of 100 mM stock KH_2PO_4 (1.36 g/100 mL). Autoclave at 121°C for 20 min. Store at room temperature. Before use, dilute to 20 mM K-phosphate buffer with deionized water.

2.2. 30 μM BESSo-AM reagent working solution: Dissolve 1 mg BESSo-AM (MW = 721.54) in 462 μL of DMSO to obtain 3 mM BES-So-AM, which is stored at 4°C in a black box. Just prior to use, dilute to final concentration (30 μM) with 20 mM K-phosphate buffer (pH 6.0).

2.3. 30 μM BESSo reagent working solution: Dissolve 1 mg BES-So (MW = 649.48) in 195 μL of DMSO to get 3 mM BESSo, which is stored at 4°C in a black box. Just prior to use, dilute to final concentration (30 μM) with 20 mM K-phosphate buffer (pH 6.0).

2.4. Confocal laser scanning microscopy or fluorospectrophotometer.

3. Methods

See "$DCFH_2$ and DCF Method." In this experiment, $DCFH_2$ DA and DCF should be replaced with BESSo-AM and BESSo.

3.1. Plant tissues are incubated in 30 μM BESSo-AM or 30 μM BESSo reagent working solution for 30–60 min at room temperature in the dark.

3.2. Detect fluorescence by confocal laser scanning microscopy (for in vivo method) or fluorospectrophotometer (for in vitro method) with excitation at 505 nm and emission at 544 nm.

3.3. Photograph.

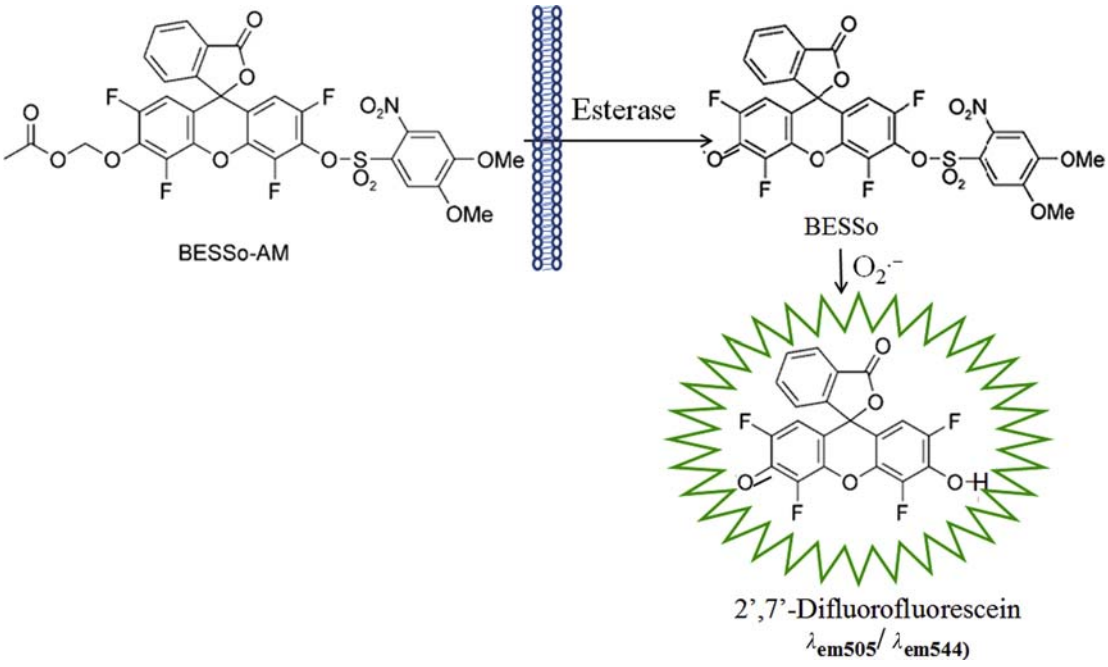


FIGURE 5.23 BESSo-AM, which can freely permeate into cytosol, is deacetylated by intracellular esterases to produce BESSo, which can be oxidized by $O_2^{\bullet -}$ to generate green fluorescence.

4. **Precautions**
   - **4.1.** BESSo-AM and BESSo working reagent should be freshly prepared and stored at 4°C in a black box to protect from light.
   - **4.2.** This method can carry out in vitro measurement for $O_2^{\bullet -}$ using 30 μM BESSo-AM or 30 μM BESSo instead of 10 μM DCF (see DCF method).
   - **4.3.** DMSO is hazardous. Reagents containing DMSO should be carefully handled. Avoid contact with skin and eyes.

5.8 SECTION 7: HO• QUANTIFICATION

5.8.1 Method 1: Benzoate Method

1. **Principle**

   Hydroxyl radical (HO•) production can be quantified by spectrofluorometry as the formation of hydroxyl benzoate (fluorescence can be produced at 407 nm emission after excitation at 305 nm) resulting from the reaction of $HO^{\bullet}$ and benzoate (BZ) (Fig. 5.24; Rodriguez and Taleisnik, 2012).

2. **Materials**
   - **2.1.** 20 mM K-phosphate buffer (pH 6.0): Add 13.2 mL of 100 mM K_2HPO_4 (1.74 g/100 mL) to 86.8 mL of 100 mM stock KH_2PO_4 (1.36 g/100 mL). Dilute to 20 mM K-phosphate buffer with deionized water.
   - **2.2.** 2.5 mM BZ: Dissolve 31 mg BZ (MW = 122) in 100 mL of 20 mM K-phosphate buffer (pH 6.0). Store at 4°C in a black bottle to protect from light.
   - **2.3.** Vacuum pump.
   - **2.4.** Spectrofluorometer.

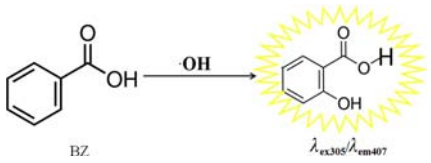


FIGURE 5.24 HO• can react with BZ to generate a fluorescence product hydroxyl benzoate.

3. **Methods**
   - **3.1.** Submerge the plant material completely in the BZ solution by vacuum infiltration for 1 min.
   - **3.2.** Incubate for indicated time (such as 7 h) at 30°C in the dark.
   - **3.3.** Collect the incubation medium and centrifuge at 10,000 × g for 5 min.
   - **3.4.** Measure the fluorescence using a spectrofluorometer with 305/407 nm (excitation/emission).
   - **3.5.** Run blanks without BZ in parallel to correct for unspecific fluorescence.
   - **3.6.** Prepare a BZ calibration curve by measuring a series of BZ dilutions (from 0 to 2.5 mM).
   - **3.7.** Transform the fluorescence values of the biological samples into •OH molar concentration using the calibration curve values.

4. **Precautions**
   - **4.1.** To study the effect of environmental stress on •OH production, an appropriate amount of NaCl, sorbitol, and heavy metal is added to BZ solution to carry out salt, osmotic, and heavy metal stresses. In addition, plant materials incubated in BZ solution are subjected to high or cold temperature to explore the production of H_2O_2.
   - **4.2.** Due to sensitivity of BZ to light, BZ solution should be freshly prepared and stored at 4°C in a black bottle to protect from light.

5.8.2 Method 2: 2-deoxy-D-Ribose Method

1. **Principle**

   Hydroxyl radical ($HO^{\bullet}$) can react with 2-deoxy-D-ribose (DDR) to generate malondialdehyde (MDA), which in turn reacts with 2-thiobarbituricacid (TBA) and produces a pink product. The product exhibits a maximal light absorption at 532 nm and the molar extinction coefficient of $1.55 \times 10^5\ M^{-1}\ cm^{-1}$ (Fig. 5.25; Schopfer et al., 2001). The absorbance is proportional to the content of $HO^{\bullet}$ in plant samples.

2. **Materials**
   - **2.1.** 20 mM K-phosphate buffer (pH 6.0): Add 13.2 mL of 100 mM K_2HPO_4 (1.74 g/100 mL) to 86.8 mL of 100 mM stock KH_2PO_4 (1.36 g/

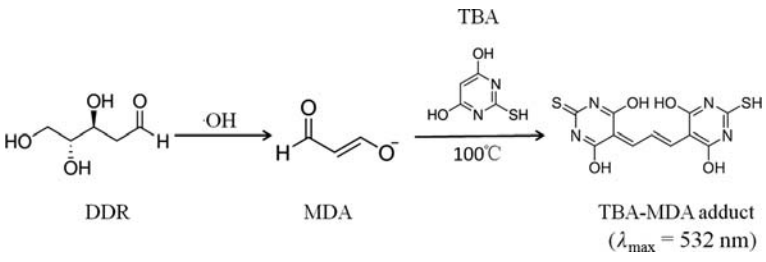


FIGURE 5.25 DDR is converted by •OH into MDA, which can react with TBA to generate a pink product.

100 mL). Dilute to 20 mM K-phosphate buffer with deionized water.

2.2. 20 mM 2-deoxy-D-ribose: Dissolve 268 mg 2-deoxy-D-ribose (MW = 134) in 100 mL of 20 mM K-phosphate buffer (pH 6.0).

2.3. 1% (w/v) TBA: Dissolve 1 g TBA (MW = 144) in 100 mL of 50 mM NaOH (0.2 g/100 mL).

2.4. 2.8% (w/v) trichloroacetic acid (TCA): Add 227 mL of deionized water to 500 g TCA (a pack, MW = 163) to obtain 100% (w/v) TCT, and then dilute to 2.8% TCA with deionized water.

2.5. Vacuum pump.

2.6. Spectrophotometer or spectrofluorometer.

3. Methods

3.1. Submerge the plant tissues completely in 20 mM 2-deoxy-D-ribose by vacuum infiltration for 1 min.

3.2. Incubate for an indicated time (such as 7 h) in darkness at 25°C on a shaker.

3.3. Collect the incubation medium and centrifuge at 10,000 × g for 5 min.

3.4. Mix 0.5 mL of centrifuged incubation medium with 0.5 mL of 1% TBA. Mix and then add 0.5 mL of 2.8% TCA.

3.5. Boil at 100°C for 10 min.

3.6. Measure the fluorescence using a spectrofluorometer (excitation: 532 nm, emission: 553 nm) or a spectrophotometer at 532 nm.

3.7. Calculate MDA content using the extinction coefficient of $1.55 \times 10^5\ M^{-1}\ cm^{-1}$ and convert into $HO^{\bullet}$ content.

4. Precautions

4.1. This method also can be used to determine MDA content in plant samples by spectrophotometry.

4.2. TAB should be freshly prepared.

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CHAPTER

6

Drought Tolerance in Plants: Molecular Mechanism and Regulation of Signaling Molecules

Pradyumna Kumar Singh[1,2], *Dipali Srivastava*[1], *Poonam Tiwari*[1], *Madhu Tiwari*[1], *Giti Verma*[1] *and Debasis Chakrabarty*[1,2]

[1]Council of Scientific and Industrial Research – National Botanical Research Institute (CSIR-NBRI), Lucknow, India
[2]Academy of Scientific and Innovative Research (AcSIR), New Delhi, India

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6.1 INTRODUCTION

The changing climatic conditions have enhanced disaster events like droughts, floods, and snowstorms. A decrease in the rainy season, but heavy rain in a short time, is observed worldwide, which creates flood conditions at one time and increases the risk of drought at the rest season (Trenberth, 2011). Drought stress negatively affects plant growth and soil fertility and is a major environmental threat to limit crop yield in the agriculture sector (Khan et al., 2015). The severity of the problem may be understood by the fact that nearly 60% of cereals grain production is dependent on the rainfed regions (Rosegrant et al., 2002), which plays a key role to ensure food security for people. The conditions may be worst for those areas dependent on high water−consuming crop production, for example, rice.

The impact of drought stress on the plant may depend on the duration of water deficit in the soil. Drought stress causes water loss from a cell, which leads to cellular dehydration, osmotic stress, and reactive oxygen species (ROS) production (Hasanuzzaman et al., 2018; Khan and Khan, 2017). Plants have evolved several morphological, physiological, and molecular adaptation mechanisms, such as reduction in water loss, enhancement in antioxidant activity, production of osmolytes, and modulation of hormonal and transcriptional regulation, to mitigate drought stress. However, the plant reduces water loss by limiting leaf surface area and stomatal opening. The increase in plant root surface area by the deep rooting system and lateral root growth is a strategy to maintain water balance and nutrient supply in drought stress (Hund et al., 2009). ROSs such as superoxide, peroxide, and hydroxyl radical damage the plant cell membrane and negatively affect the redox potential and photosynthesis apparatus. Drought stress increases the ROS production, stomatal closure, and dehydration, and disturbs the osmotic homeostasis of the plant. The antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and glutathione-*s*-transferase reduced the ROS content and maintained the reduced glutathione (GSH) pool to balance redox state of a plant cell in drought stress (Gill and Tuteja, 2010; Nahar et al., 2015). In addition, nonenzymatic antioxidants like ascorbic acid and GSH play an important role in detoxification of ROS and act as a photoprotectant in abiotic stress conditions including drought stress (Khan et al., 2012, 2016).

The primary strategy of drought tolerance in the plant is a continuation of osmotic balance to protect cellular structure and function during water deficit conditions (Bhargava and Sawant, 2013; Slama et al., 2015). Osmolytes are nontoxic, highly soluble, low molecular weight compounds that maintain the cellular protein function, membrane integrity, and physiological function of the plant in drought stress (Slama et al., 2015). A large group of compounds such as amino acid, sugar, and polyhydric alcohols act as osmoprotectants and ROS scavengers. The accumulation of osmolytes (proline, sucrose, trehalose, glycine betaine) in plants enhances drought tolerance (Slama et al., 2015; Masood et al., 2016; Per et al., 2017).

The classical plant hormones such as auxin (IAA), gibberellins (GA), cytokinins (CKs), abscisic acid (ABA), and ethylene regulate the growth and developmental processes in various stresses like drought, heat, cold, and salt (Wani et al., 2016). Besides, other signaling molecules' (nitric oxide, hydrogen peroxide, brassinosteroids, and salicylic acid) role in response to drought stress elucidates their importance in the key physiological processes (Wani et al., 2016; Duan et al., 2017). Plants enhanced the ABA biosynthesis in water deficit conditions, leading to stomatal closures inhibiting the water loss, but at the same time, also decreased the gaseous exchange and hydraulic conductance resulting in slower photosynthesis rate and plant growth (Bhargava and Sawant, 2013). Accumulation of ABA activates the ROS signaling, which leads to nitric oxide (NO) biosynthesis resulting in stomata closing (Yu et al., 2014). A higher level of IAA stimulated the expression of stress-related genes, modulated root architecture, and maintained the water homeostasis in the plant to cope with drought stress (Kazan, 2013). The GA deficient *Arabidopsis* plant showed higher tolerance to water stress but compromise the growth (Colebrook et al., 2014). Drought stress negatively affects the carbon and nitrogen metabolism. Higher levels of CKs enhance tolerance to drought stress by promoting the carbon and nitrogen metabolism in the plant (Reguera et al., 2013; Zwack and Rashotte, 2015). Plant hormones, NO, and salicylic acid (SA) also modulate drought adaptive physiological responses to water deficit stress. Induced level of SA enhances the ABA-independent stomatal closure and drought tolerance by influencing ROS production and the ascorbate−glutathione cycle (Miura et al., 2013; Miura and Tada, 2014). The SA mediated drought tolerance mechanism is not well known to date.

Drought tolerance response is the result of various molecular pathway cascades including the perception of water deficit stress, activation of signaling network followed by transcriptional, metabolic, and regulatory element response (Liu et al., 2014). Drought-responsive molecular mechanisms are tightly controlled by the regulatory elements such as transcription factors and protein kinases. The *MYB*, *NAC*, *bZIP*, *AP2/ERF*, and

AREB/ABF transcription factor (TF) family genes regulate the stomatal movement and expression of drought-responsive genes acting up- or downstream of a metabolic pathway (Kim et al., 2010; Joshi et al., 2016). Protein kinases, which add a phosphate group to the amino acid residue of the protein, are a key regulator of protein activity via post-translational modification. The role of mitogen-activated protein kinases (MAPKs), including calcium-dependent protein kinase (CDPKs) and CBL-interacting protein kinases (CIPKs) in drought tolerance has been elucidated in the plant by using transgenic approaches (Wang et al., 2016a,b). In response to drought stress plants undergo adaptive mechanisms at a molecular, physiological, and metabolic level, which subsequently activates stress-responsive mechanisms to reestablish homeostasis. Among these mechanisms, one category belongs to those that are directly involved in the protection of important proteins and membranes such as osmoprotectants, late embryogenesis abundant (LEA) proteins like dehydrins, free radical scavengers, and chaperones.

6.2 ROLE OF OSMOPROTECTANT REGULATORY GENES IN DROUGHT STRESS

At the metabolic level, plants decrease their cellular osmotic potential by the accumulation of compatible solutes or osmoprotectants. They help in the stabilization of membranes and proteins against abiotic stresses without disrupting plant metabolism (Giri, 2011). Many important crops cannot synthesize osmoprotectants that are present in various stress-tolerant organisms. Subsequently, engineering genes related to biosynthetic pathways of osmoprotectants can enhance tolerance to stress. Fig. 6.1 gives an overview of biosynthetic pathways of the significant osmoprotectants discussed in this chapter. This figure also highlights the expression of the transgene for plant genetic engineering, which subsequently resulted in drought and other abiotic stress tolerance. Osmoprotectants include amino acids (e.g., proline), polyol/sugars (e.g., mannitol, trehalose, fructans), and glycine betaine.

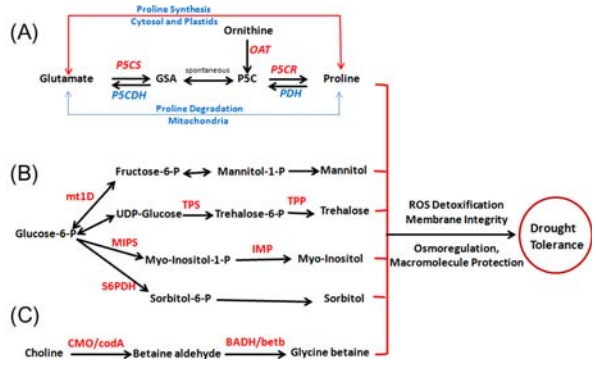

FIGURE 6.1 Overview of osmoprotectants' biosynthesis pathway and their role in drought stress tolerance.

6.2.1 Proline

In plants, accumulation of proline has been suggested to contribute to stress tolerance by acting as osmoprotectant, molecular chaperone, and as an antioxidant. Proline biosynthesis follows two pathways in plants, that is, glutamate and ornithine pathway. The glutamate is the major proline accumulation pathway, and it is biosynthesized from glutamate by Δ1-pyrroline-5-carboxylate synthetase (P5CS) further it is reduced to proline by Δ1-pyrroline-5-carboxylate reductase (P5CR) (Fig. 6.1A). In the alternative pathway, ornithine is transaminated to P5C by ornithine-δ-aminotransferase, which further follows the same pathway. As proline biosynthesis follows feedback inhibition, the understanding of proline catabolism becomes equally important. Proline catabolism occurs in mitochondria using the sequential action of proline dehydrogenase or proline oxidase (PDH or POX) on proline to produce P5C from proline and further P5C is converted to glutamate by P5C dehydrogenase (P5CDH). Both P5CS and PDH catalyze the first and the rate limiting steps of proline biosynthesis and degradation, respectively, and their activities could influence proline accumulation and plant growth under stress. Overexpression of moth bean *P5CS* in tobacco plants resulted in improved salt and drought tolerance (Kishor et al., 1995; Zhu et al., 1998). Overexpression of *Arabidopsis* and *Oryza sativa P5CS* conferred drought tolerance in petunia plants (Yamada et al., 2005). However, antisense-*AtP5CS* transgenic plants showed hypersensitivity to stress (Nanjo et al., 1999). Similarly, insertion mutation of *p5cs1* (Székely et al., 2008) also resulted in reduced osmotolerance. Overexpression of *Arabidopsis P5CR* (*AtP5R*) gene in soybean improved drought and heat stress (De Ronde et al., 2000, 2004). Overexpression of *PDH* did not confer osmotolerance in *Arabidopsis*, except in the presence of exogenously supplied proline. There are several reports that show that the *P5CS* expression in *Arabidopsis* and rice plants was upregulated during dehydration and the production of *PDH* was upregulated during rehydration (Oono et al., 2003; Nanjo et al., 2003). These findings highlighted the role of proline biosynthesis in plants against osmotic stress induced by drought and other abiotic stresses.

6.2.2 Polyols/Sugars

Overall carbon metabolism and the levels of specific sugars are severely affected by drought and other abiotic stress. Polyols such as glycerol, mannitol, D-ononitol, sorbitol, and trehalose are osmoprotectants in algae and certain halophytic plants. Overexpression of mannitol 1-phosphate dehydrogenase (*mtlD*), the gene for the biosynthesis of mannitol in transgenic tobacco and *Arabidopsis*, resulted in mannitol production and a salinity-tolerant (Thomas et al., 1995). The expression of *mtlD* and mannitol accumulation in the chloroplast conferred osmotic stress tolerance. It has also been reported that the ectopic expression of the *mtlD* gene in wheat improves tolerance to water stress and salinity (Abebe et al., 2003). Tobacco plants were transformed with *imtl* gene encoding myo-inositol-omethyl-transferase of ononitol biosynthetic pathway showed higher tolerance to salt and drought stress than wild-type control (Sheveleva et al., 1997). Several transgenic plants with genes of sorbitol biosynthesis have been reported. Transgenic tobacco plants expressing apple *Stpd1* (sorbitol-6-phosphate-dehydrogenase) demonstrated that the plants with lower sorbitol content showed normal growth while the one with higher content exhibited growth retardation (Sheveleva et al., 1998). Another example is of *Diospyros kaki* transformed with *Stpd1*, which also accumulated sorbitol but showed higher photosynthetic activity than control plants (Gao et al., 2001).

Trehalose is a non-reducing disaccharide and its pretreatment induces drought tolerance in radish (*Raphanus sativus* L.) plants (Akram et al., 2015). The overexpression of a fusion gene, trehalose-6-phosphate synthase/phosphatase (*TPSP*), containing the coding region of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) (Fig. 6.1B) improved abiotic stress tolerance in rice. The transgenic rice plants accumulated 3–10 times more trehalose than that of the control plants and showed high levels of tolerance to salt, drought, and low-temperature stresses as compared with the non-transformed plant (Garg et al., 2002). Another similar report shows a fusion of the drought-inducible promoter *StDS2* to trehalose-6-phosphate synthase (*TPS1*) in potato. The transgenic lines showed high stomatal conductance and a satisfactory rate of net photosynthesis (Stiller et al., 2008). In another study, *Arabidopsis* overexpressing *AtTPS1* accumulated trehalose only at low levels, but transgenic plants acquired desiccation tolerance. This study has also shown that *AtTPS1* has a role in the regulation of glucose and ABA signaling during vegetative development (Avonce et al., 2004). Apart from its known function of chaperone and osmolyte, the role of trehalose and trehalose-6P in signaling is of great importance (Ferndez et al., 2010).

6.2.3 Glycine Betaine

Glycine betaine (GB) is a methylated derivative of glycine and an important osmoprotectant in bacteria, animals, and plants. GB accumulation has been reported in plants when exposed to environmental stresses such as salt, drought, and extreme temperatures (Giri, 2011). It can be synthesized in transgenic plants using genes from plants that encode for either of the enzymes: choline monooxygenase (*CMO*), betaine aldehyde dehydrogenase (*BADH*) or alternatively betaine aldehyde dehydrogenase (*betB*) or choline oxidase (*codA*) genes from bacteria (Fig. 6.1C). GB accumulation leads to enhanced tolerance to drought and salt stress (Sakamoto and Murata, 2000). Targeted accumulation of GB in chloroplasts has been achieved by engineering a plastid-expressed *CMO* gene, leading to higher PSII activity. Thus the protection of PSII can lead to improved drought and salt tolerance in crops (Zhang et al., 2008). Rice, maize, and many crop plants lack the ability to accumulate GB naturally during abiotic stress (Giri, 2011); introduction of GB biosynthesis gene(s) into nonaccumulators by transgenic approach has been successfully used for improved abiotic stress tolerance in diverse plant species, for example, rice (Su et al., 2006), potato (Ahmad et al., 2008), and wheat (Wang et al., 2010).

6.2.4 Osmotin

Osmotin and osmotin-like proteins (OLPs) are a group of stress-responsive proteins that belong to the PR-5 pathogenesis-related group of proteins. Several studies reported the dual function of osmotin and osmotin-like proteins in plant defense against abiotic stress (Subramanyam et al., 2012) and osmotic stress (Chowdhury et al., 2017). Although the underlying mechanism still remains unclear, the overexpression of the osmotin gene in tobacco plants improved their tolerance to salinity, wounding, infection, and drought stress (Barthakur et al., 2001). It has been reported in wheat (Noori and Sokhansanj, 2008), tomato (Goel et al., 2010), and soybean (Subramanyam et al., 2012) that the overexpression of the osmotin gene conferred tolerance against various abiotic stresses.

6.2.5 Dehydrins

Dehydrins are one of the key components of dehydration tolerance, although the precise mechanism is

not clear. However, it has been proposed that they may carry out their function through membrane stabilization by acting as chaperones to prevent the aggregation and/or inactivation of proteins under dehydration (Yang et al., 2012). Several transgenic studies revealed that dehydrin gene expression has a positive effect on plant stress tolerance, including cold, drought, and salinity. It has been reported that *OsDHN1* overexpressing rice confers high tolerance to drought and salt stress (Kumar et al., 2014). Liu et al. (2015) also reported a positive correlation between *ShDHN* gene expression and drought tolerance in tomato (*Solanum habrochaites* L.). At the functional level, *OsjDHN* genes exhibited subfunctionalization, that is, different genes displaying differential expression profiling during various developmental stages and under PEG-induced drought stress (Verma et al., 2017). In another study, overexpressing dehydrin genes such as *ERD10*, *LTI30*, *RcDhn5*, and *DHN-5* in *Arabidopsis* plants showed tolerance to various abiotic stresses (Puhakainen et al., 2004; Brini et al., 2007; Peng et al., 2008). Similarly, the differential accumulation of 31- and 40-kDa dehydrins in the drought-sensitive and tolerant warm-season Bermuda grass indicated their contribution to drought or dehydration tolerance in warm-season Bermuda grass (Hu et al., 2010). A study from Verma et al. (2017) reported evolutionary conservation of dehydrins during the course of domestication/evolution in 11 *Oryza* species and subspecies, as they play an important role in combating dehydration stress. Recently, we found that rice transgenic lines overexpressing *OsDHN* gene have improved tolerance against drought, osmotic stress, exogenous ABA, and ABA + PEG treatment (unpublished).

6.3 REDOX REGULATORY MACHINERY OF PLANTS DURING DROUGHT CONDITIONS

One of the significant reasons for plant growth reduction on exposure to drought is the breakdown of redox homeostasis of the cell (Halliwell, 2006). Production of ROS causes extensive damage to DNA, proteins, membrane lipids, and other cellular components (Foyer and Noctor, 2005). The most common ROS comprise $O_2^{\bullet -}$(superoxide radical), $^1O_2$ (singlet oxygen), $^{\bullet}OH$ (hydroxyl radical), and H_2O_2 (hydrogen peroxide). ROS are being produced as a byproduct of electron transport chain in deferent cellular compartments like cytosol, chloroplasts, mitochondria, and peroxisomes under both favorable as well as unfavorable conditions. The antioxidant machinery of the cell plays a vital role in maintaining redox homeostasis by scavenging ROS for normal cellular functioning, and comprises two types of components: enzymatic and nonenzymatic. Enzymatic components include several antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (GPX; EC 1.11.1.7), glutathione peroxidase (GPx; EC 1.11.1.9), monodehydroascorbatereductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; GR, EC 1.6.4.2), and glutathione-S-transferase (GST; EC 2.5.1.18) whereas ascorbate (ASC), α-tocopherol (α-toc), and GSH fall into the category of nonenzymatic components. Both types of components are equally important and work in a coordinated manner to provide a significant defense from oxidative stress conditions.

6.3.1 Enzymatic ROS Regulation During Drought

The role of ROS scavenging enzymes has been elucidated by manipulation of antioxidant components through genetic engineering and can enhance the performance of plants under drought stress leading to increased amounts and activities of antioxidants (Cao et al., 2017). SOD, an antioxidant enzyme, is a metalloenzyme that forms the first line of defense against superoxide-induced damages under stress conditions. Overexpression of the *Arachis hypogaea AhCuZnSOD* gene in tobacco demonstrated that transgenic lines exhibited improved growth parameters in response to drought-induced osmotic stress (Negi et al., 2015). Likewise, transgenic cotton plants overexpressing *Tamarix albiflonum TaMnSOD* enhanced drought tolerance through improved plant growth development with reduced oxidative stress (Zhang et al., 2014). Interestingly, co-expression of *cytapx* (from *Pisum sativum*) and/or *cytsod* (from *Spinacia oleracea*) in tobacco plants not only increased the SOD and APX activity but also enhanced the POX and CAT enzyme activity resulting in a reduction of oxidative stress under drought conditions (Faize et al., 2011). CATs and APXs both are key components of the antioxidative machinery that regulate the H_2O_2 content in peroxisomes and in the cytosol, as well as in the chloroplast, respectively. Studies have suggested that catalase gene expression enhanced during drought and ABA treatment ultimately maintains the redox homeostasis in a plant cell by increasing the enzyme activity of CAT (Du et al., 2008; Nie et al., 2015). Moreover, drought tolerant plants were raised by simultaneous homologous overexpression of both cassava Cu/Zn superoxide dismutase (MeCu/ZnSoD) and catalase (MeCat1)

enzymes (Xu et al., 2013). On the other hand, exposure of drought stress increased the expression of APX gene and altered the cellular metabolism (Koussevitzky et al., 2008). Recently, Cao et al. (2017) reported that on the introduction of cytosolic APX gene from *Populus tomentosa* into tobacco plants, there were found to be decreased levels of malondialdehyde (MDA) and H_2O_2 levels with higher APX activity in the transgenic plants suggesting its role in drought tolerance. A study demonstrated that rice seedlings with loss of function in rice *OsAPX2* mutants show susceptibility to drought stress, and exhibit lower APX activity with higher H_2O_2 and MDA levels, while constitutive overexpression lines showed drought tolerance (Zhang et al., 2013). MDHAR and DHAR enzymes keep the redox state of the plant cell in drought conditions by retaining the cellular ASC pool by using NADPH and GSH respectively. Polyethylene glycol (PEG) induced osmotic stress increased the expression of MDHAR in the wild-type tomato. Further, a higher status of net photosynthesis rate, lower level of hydrogen peroxide, MDA, and a coordinated higher activity of the antioxidant enzymes served as the major determinants in a sense transgenic line for depicting PEG-induced osmotic stress tolerance compared with antisense lines and WT plants (Li et al., 2012). Similarly, enhanced tolerance to PEG stress has been developed by overexpression of *AtMDAR1* in transgenic tobacco (Eltayeb et al., 2007). Moreover, Eltayeb et al. (2006) also found that transgenic tobacco plants carrying cytosolic *DHAR* gene from *Arabidopsis thaliana* result in higher enzyme activity, which in turn results in improved photosynthesis protection and increased revival under drought stress.

GR maintains the glutathione pool of the cell by catalyzing the reduction of GSSG to GSH in normal as well as stress conditions. The deviation in cytosolic and dual targeted GR gene expression to drought stress was investigated in cowpea tolerant cultivar and a susceptible cultivar. Cytosolic GR gene was upregulated in both cultivars. However, upregulation of dual-targeted GR was found in the susceptible cultivars only under drought treatment (Contour-Ansel et al., 2006). As ABA is the biomarker of drought stress, the transcription of *B. campestris BcgGR1* is strongly induced by exogenous application of ABA suggesting its role in drought stress (Lee et al., 2002). *Arabidopsis* plants overexpressing *ApGPX2* and *PgGPx* (*Pennisetum glauccum*) genes showed tolerance under drought stress (Gaber et al., 2006; Islam et al., 2015). Plant GSTs are well-known enzymes for detoxification of xenobiotic compounds, but also, they play a role in response to drought-induced oxidative stress (Cicero et al., 2015; Liu et al., 2013). Several studies have revealed that heterologous expression of *GST* genes improves resistance to drought stress in plants by enhancing their ROS scavenging ability (Yang et al., 2014; Xu et al., 2016) (Table 6.1)

6.3.2 Nonenzymatic ROS Regulation During Drought

Apart from enzymatic antioxidants, plant cells possess other non-antioxidant compounds (ASC, GSH, and α-toc) that play a fundamental role in plant metabolism. These low−molecular weight antioxidants modulate the cellular redox homeostasis by interacting with ROS and affect the physiological and biochemical processes of plants to ameliorate the adverse effects during drought conditions. Several studies revealed that drought tolerance could be enhanced by the manipulation of key enzymes of biosynthetic pathways of antioxidant compounds and they maintain the intracellular redox environment of plants in stress conditions (Ma et al., 2014; Woo et al., 2014). Ascorbate has been abundantly found in reduced form in all cell compartments and acts as a principal ROS detoxifying compound in response to different types of stress conditions (Ahmad et al., 2010). In a study, an alfalfa GDP-mannose 3, 5-epimerase gene encoding the enzyme involved in ascorbate biosynthetic pathway was overexpressed in *Arabidopsis* and it was found that higher accumulation of ascorbate in transgenic plants positively correlated with drought stress tolerance (Ma et al., 2014). Similarly, Lim et al. (2012) introduced the rat L-gulono-γ-lactone oxidase (*GLOase*) gene into the genome of the tomato plant and developed transgenic plants with higher ascorbate and chlorophyll content as compared with wild-type plants. *Arabidopsis* ascorbate-deficient *vtc1* mutant exhibited drought-sensitive phenotype with reduced activity of antioxidant enzymes and an increased ratio of dehydroascorbate/total ascorbate.

In addition, a lipid-soluble antioxidant α-tocopherol is an important component of the plant defense machinery that not only scavenges the harmful radicals but also performs the quenching of singlet oxygen generated in the photosynthesis process (Espinoza et al., 2013). Transgenic plants overexpressing tocopherol cyclase coding gene from *Arabidopsis* and tobacco respectively showed higher tolerance to drought stress with better growth parameters and antioxidant activity than wild-type plants (Liu et al., 2008; Woo et al., 2014).

Glutathione is a tripeptide thiol that is abundantly found in its reduced form and scavenges reactive oxygen species (Foyer and Noctor, 2005). It synthesized in two steps involving two enzymes: glutamyl-cysteinyl synthetase (γ-ECS) and glutathione synthetase (GS).

TABLE 6.1 The Transgenic Intervention of Various Antioxidant Enzymes in Crop Plants to Improve Drought Tolerance

Antioxidant enzymes	Host	Target	Gene	References
APX	*Salicornia brachiata*	*Nicotiana tabacum*	SbpAPX	Singh et al. (2014)
	Eleusine coracana	*E. coli*	Ec-apx1	Bhatt et al. (2013)
	Populus tomentosa	*Nicotiana tabacum*	PcAPX	Cao et al. (2017)
	Oryza sativa	*Oryza sativa*	OsAPX2	Zhang et al. (2013)
DHAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	DHAR	Eltayeb et al. (2006)
GPX	*Pennisetum glaucum*	*Oryza sativa*	PgGPx	Islam et al. (2015)
	SynechocystisPCC6803	*Arabidopsis thaliana*	GPX-2	Gaber et al. (2006)
MDAR	*Arabidopsis thaliana*	*Nicotiana tabacum*	AtMDAR1	Eltayeb et al. (2007)
SOD	*Sedum alfredii*	*Arabidopsis thaliana*	SaCu/Zn SOD	Li et al. (2017)
	Arachis hypogaea	*Nicotiana tabacum*	CuZnSOD	Negi et al. (2015)
	Tamarix albiflonum	*Gossypium hirsutum L.)*	TaMnSOD	Zhang et al. (2014)
SOD/ APX	*Spinacia oleracea/Pisum sativum*	*Nicotiana tabacum*	cytsod/cytapx	Faize et al. (2011)
GST	*L. esculentum*	*Arabidopsis thaliana*	LeGSTU2	Xu et al. (2015)
	Pyrus pyrifolia	*Nicotiana tabacum*	PpGST	Liu et al. (2013)
	Arabidopsis thaliana	*Arabidopsis thaliana*	AtGSTU19	Xu et al. (2016)
	Tamarix hispida	*Arabidopsis thaliana*	ThGSTZ1	Yang et al. (2014)
	Citrus sinensis	*Nicotiana tabacum*	CsGSTU	Cicero et al. (2015)
SOD/ CAT	*Manihot esculenta*	*Manihot esculenta*	MeCu/ZnSOD and MeCat1	Xu et al. (2013)

Bhatt, D., Saxena, S.C., Jain, S., Dobriyal, A.K., Majee, M. and Arora, S. 2013. Cloning, expression and functional validation of drought inducible ascorbate peroxidase (*Ec-apx1*) from *Eleusine coracana*. Mol. Biol. Rep. 40, 1155-1165.
Li, Z., Han, X., Song, X., Zhang, Y., Jiang, J., Han, Q., Liu, M., Qiao, G. and Zhuo, R. 2017. Overexpressing the *Sedum alfredii* Cu/Zn superoxide dismutase increased resistance to oxidative stress in transgenic *Arabidopsis*. Front Plant Sci. 8, 1010.
Singh, N., Mishra, A. and Jha, B. 2014. Over-expression of the peroxisomal ascorbate peroxidase (*SbpAPX*) gene cloned from halophyte *Salicornia brachiata* confers salt and drought stress tolerance in transgenic tobacco. Mar. Biotechnol. 16, 321-332.

The proportion of GSH to GSSG (oxidized form) plays a central role in maintaining cellular redox state. Exogenous application of glutathione enhanced the antioxidant enzyme activities in drought-treated mung bean seedlings and improved their physiological activity and growth (Nahar et al., 2015). Some studies have revealed the advantage of increasing GSH level through overexpression of γ-ECS that enhanced resistance to drought and ABA-induced osmotic stresses (Sengupta et al., 2012; Wu et al., 2009).

6.4 HORMONAL REGULATION OF DROUGHT TOLERANCE IN PLANTS

Plant endurance during drought is governed by several pathways; among them, plant hormones play an inevitable role. Plant growth regulators, in spite of their biological roles, are central players during abiotic or biotic stresses. Advancement in transcriptome and mutant analysis provides a better lead towards an understanding of plant growth regulators during drought. The two hormones, ABA and ethylene, are well studied to date and proved to be activated and provide tolerance during low water deficit condition. All other major hormones like auxin, CKs, and GA are also involved significantly during drought but the molecular mechanisms related to these hormones are partially understood. Moreover, the other growth regulators such as brassinosteroids, SA, and jasmonic acid (JA) are also significantly involved to cope with drought.

6.4.1 Auxin

During drought, soil layers become dry and plant lateral root development and curvature is needed to maintain the effective uptake of water. Auxin is known to be responsible for lateral root development and maintaining the root architecture. YUCCA genes are the rate limiting step of auxin biosynthesis and are drought-induced, although molecular mechanism is

needed to decipher. *iaaM-OX* transgenic lines of *Arabidopsis* with higher endogenous IAA are drought resistant while *yuc1yuc2yuc6* triple mutants decreased stress resistance in comparison with WT plants (Shi et al., 2014). The plant overexpressing *MIZ1* (MIZU-KUSSEI1), a regulator of hydrotropism, has less lateral and more penetrating root and is drought sensitive, showed that it inhibits the auxin response in the pericycle cells. This inhibition is overcome by the exogenous auxin treatment in *Arabidopsis* (Moriwaki et al., 2011). Auxin-responsive factors (ARFs) are also a key regulator of lateral root development. Microarray analysis shows that drought-stressed plant having upregulated transcripts of ARFs. ARFs are also regulated at the posttranscriptional level via several miRNAs, like miR167 (Kinoshita et al., 2012) and miR164 (Guo et al., 2005) in *Arabidopsis*, and modulate root architecture, lateral root development. The miR167a is a negative regulator of *IAR3*. During drought conditions, miR167 is reported to be downregulated results in an increased *IAR3* level. Overexpressed *IAR3 Arabidopsis* plant having freer auxin accumulation and enhanced root architecture leads to drought tolerance impact (Kinoshita et al., 2012). miR393 is an osmotic stress-induced miRNA that degrades *TIR* and *AFB2* transcripts and inhibits the lateral root growth (Chen et al., 2012a,b). This miRNA is responsible for attenuating the auxin-mediated response. Auxin also upregulated the function of small auxin-upregulated RNAs (SAURs), which have been shown to be responsible for drought stress tolerance via more root development and upregulation of stress-related genes when overexpressed in *Arabidopsis* (Guo et al., 2017) but the molecular mechanism of drought tolerance in respect to auxin is still unknown.

6.4.2 Cytokinins

Cytokinins (CKs) are the phytohormones involved in cytokinesis and that promote cell division and differentiation. Cytokinin levels were reported to be downregulated during drought stress. This leads to low CK level in roots and leaves (Merewitz et al., 2010). Several plants like bentgrass, tobacco, and rice have been proved to be more drought tolerant when either exogenously supplied with CK compounds or overexpressed adenosine phosphate-isopentyl transferase (*IPT*) genes (Peleg et al., 2011). It has been suggested that drought mediated signaling hindered the CK production either at CK biosynthesis or at degradation level. Transcript analysis of IPT transgenic plant compared with WT plant was having less upregulated and more downregulated drought-responsive gene (Rivero et al., 2010). It suggested that CK regulates several metabolic processes drought-responsive genes. Interestingly, overexpression of cytokinin degradation enzymes cytokinin oxidase/dehydrogenase1 (CKX1) by the root-specific promoter WRKY6 showed more degradation and low level of CK in root which leads to enlarging the root system required for more water absorption in a water deficit environment (Macková et al., 2013). From these data, it can be hypothesized that in water deficit conditions, low level (WTKY6: CKX1) of cytokinins in roots while high IPT level in shoots (35S: CKX1) have more tolerant effects against drought.

6.4.3 Gibberellins

Gibberellins (GAs) are well known as a classical growth hormone. The exact pathway of GA metabolism and signaling during water deficit is yet to be unraveled but GA level has been reported to be downregulated in maize (Wang et al., 2008a,b). The three dioxygenases that participated in the metabolic pathway during GA biosynthesis, GA 20-oxidase, GA 3-oxidase, and GA 2-oxidase, have been overexpressed in *Arabidopsis* under 35S promoter and knockout lines also prepared and studied under drought. The results suggested that high GA concentration leads to drought sensitive response of plants while low GA concentration is drought tolerant (Colebrook et al., 2014). Upregulation of GA 2-oxidase inhibits the GA biosynthesis. The DELLA protein also plays a crucial role in the regulation of GA biosynthesis. DELLA protein, in the absence of GA, binds to GID1, which is a receptor for GA. GA binds to the GID1 and leads to the interaction with an SCF ubiquitin ligase to the DELLA protein. The subsequent 26S proteasomal degradation of DELLA protein results in GID1 import to the nucleus and further gene activation in response to GA signaling. Several proteins involved in different hormone signaling pathways interact through DELLA (Bai et al., 2012) suggesting that GA may cross-talk through different hormones. DELLA has also been reported as a positive factor for stress tolerance (Achard et al., 2006). An ethylene responsive factor ERF6 has been reported (Dubois et al., 2013) to participate in both ethylene and GA mediated responses. DELLA gene *RGL3* is also transcriptionally upregulated under JA signaling (Wild et al., 2012).

6.4.4 Abscisic Acid

The first hormone that comes to mind for drought stress is ABA, which is also thought to be a negative regulator of many developmental processes in plants. The NCED3 enzyme is a rate limiting factor of ABA

biosynthesis. Mutated nced3 have shown to be less tolerant to drought stress in several plant species while overexpression of NCED3 showed more water use efficiency and drought tolerance (Tung et al., 2008). Stomatal closure is the earliest response to reduce transpirational water loss in water stress and is tightly regulated by ABA-dependent or ABA-independent pathways. ABA bound to the plasma membrane-bound receptors like PYR, PYL, RCARs present in the guard cell and its downstream signaling involves the generation of secondary signals like H_2O_2, which induces Ca^{+2} release from the plasma membrane, and intrinsic (cytoplasmic) Ca^{+2} also increases via tonoplast and ultimately leads to stomatal closure via activating outwardly rectifying K^+ (Ma et al., 2009; Taiz and Zeiger, 2002). Several reports are available that suggest the direct role of ABA to close the inward rectifying K^+ channels that are responsible for stomatal opening. Biosynthesis and transport of ABA is enhanced during drought stress. ABA is sensed by receptors like pyrabactin resistance 1 (PYR), pyrabactin resistance1-like (PYL), and the regulatory component of abscisic acid receptor proteins (RCARs). ABA inhibits binding of phosphatase type 2Cs (PP2C) protein to sucrose nonfermenting 1-related protein kinase 2s (SnRK2). In a nonstress environment, SnRK2 is bound with PP2C-like (ABA insensitive-1 and 2, ABI1, and ABI2), which prevented the SnRK2 kinase activity. Once ABA bound to the PP2Cs, SnRK2 was released and free to phosphorylate many ABA-responsive factors (ABFs) (Fujii et al., 2009). ABFs are basically bZIP (basic leucine zipper) proteins and activate many ABA-responsive elements (ABREs) containing genes. These genes involved various transcription factors of stress-related genes, genes of osmolytes production, wax deposition genes, and senescence and dormancy related genes. ABA also inhibits the nonstomatal loss of water from cuticular deposition by enhancing wax gene synthesis.

Senescence is a physiological response that includes programmed cell death (PCD) of mature leaves and nutrient transfer from the senescing organ. ABA induces early senescence genes (premature leaves) during severe drought stress (Volaire and Norton, 2006). The *SWEET15* gene is reported to have an enhanced synthesis in senescence leaves having ABA receptor (PYL) overexpressed plants than the WT (Zhao et al., 2016). Another crucial event is dormancy, which is also regulated by ABA. Many bud dormancy related genes possess a CACGTGT motif in their promoters (González-Grandío et al., 2013), which are recognized by ABA-related bZIP TFs (Shen and Ho, 1995). ABA maintains the life of developing tissues (sink) and leads to the death of source tissues, which is crucial during extreme drought conditions.

6.4.5 Ethylene

Ethylene has been reported to increase during drought stress in several plant species like French bean, orange, avocado, *Vicia faba*, and in many other plant species. The 1-aminocyclopropane-1-carboxylic acid synthase (ACS) is a key enzyme of ethylene biosynthesis. C-terminal region of ACS protein has different phosphorylation sites. ACSs are divided into three types on the basis of different phosphorylation site. *Type I* ACSs have mitogen-activated protein kinase 3 and/or 6 (AtMPK3-6) as well as calcium-dependent protein kinase (AtCDPK2, CDPK, or CPK) phosphorylation domain, *type II* ACS proteins have only CPK phosphorylation site, and *type III* do not possess any phosphorylation site in the C-terminal domain. ACSs are not only regulated by phosphorylation but also by phytohormones and secondary messengers like Ca^{+2} (Arraes et al., 2015). Aminocyclopropane-1-acid carboxylic oxidase (ACO) is a final enzyme that converts ACC to ethylene. ACC moves through the xylem from the root to more distant plant parts and is converted to ethylene wherever it acquires the signal. In maize, it has been shown that ethylene acts as an inhibitor of leaf growth and ACC act as a signal transduction molecule under water deficit conditions (Sairam et al., 2008). *RD29A* and *RD29B* genes are upregulated during drought stress. The promoter of *RD29A* has two *cis*-acting elements; one is an ABA-responsive element (ABRE), activated via ABA, and the other one is a dehydration-responsive element (DRE), activated under osmotic stress. *RD29B* only has ABA-mediated activation and ABRE domain (Yamaguchi-Shinozaki and Shinozaki, 1994). Ethylene-responsive factor (ERFs) also binds to the DRE elements. *Arabidopsis* plant with a constitutive promoter, 35S: ERF1, proved to be drought tolerant and ERF protein bound directly to the DRE element of the RD29B promoter (Cheng et al., 2013). DRE element contains the core sequences A/GCCGAC, a *cis*-acting promoter element that is involved in regulation of gene expression under drought and leads to low yield and higher survival.

6.4.6 Brassinosteroids

Polyhydroxysteroids have been recognized as the sixth class of plant hormone, called brassinosteroids, and possess distinct growth-promoting ability (Bishop and Yokota, 2001). Brassinosteroids serve as a positive regulator during drought stress but the molecular mechanism is still unresolved. *Brassica napus* seedlings treated with epibrassinolide (EBR) during drought stress results in high antioxidant enzyme and reduction in reactive oxygen species leads to tolerance to drought stress (Kagale et al., 2007). A drought sensitive

variety of wheat coped well during drought via increased water uptake and reduced ion leakage after treatment with brassinosteroids (Sairam, 1994). These results indicate a significant role of brassinosteroids to cope with drought via upregulating different enzymes and antioxidants. Drought stress related marker genes were studied in EBR treated and nontreated *Arabidopsis* plants. The consistent accumulations of *RD29A*, *ERD10*, and *RD22* transcripts have been identified in EBR treated *Arabidopsis* plant. RD29A and ERD10 both have a chaperone-like function, and prevent aggregation of proteins during water deficit. There is a possible interaction between ABA and brassinosteroids, although the complete pathway has not yet been deciphered, but ABA-responsive genes like *LPT4* and ABA-marker gene *RD22* have been identified to be upregulated during BR treatments in the plant (Divi et al., 2010).

6.4.7 Salicylic Acid

Salicylic acid (SA), a phenolic phytohormone, mediates systemic acquired resistance during biotic stress and also provides tolerance against abiotic stresses. During water deficit, SA content increased twofold in barley root (Bandurska, 2005). Addition of SA prior to the drought treatment improves drought tolerance in various plants like wheat (Hamada, 2001), muskmelon seedlings (Korkmaz et al., 2007), tomato and bean seeds (Senaratna et al., 2000). Low levels of ROS accumulation have been reported when plants are pretreated with 0.1−0.5 mM of SA (Harfouche et al., 2008). ROS acts as a secondary messenger and leads to ROS mediated enzymatic antioxidant activation such as SOD, GPX, and HSPs (Shi et al., 2006) and many nonenzymatic antioxidants like glutathione, ascorbic acid, and carotenoids (Miyake and Asada, 1994). SA accumulating mutants of *Arabidopsis* plants like adr1, *myb96-1d*, *siz1*, *acd6*, and *cpr5* were drought tolerant when supplemented with SA (Miura and Tada, 2014) *LTI29* and *LTI30* genes upregulated during drought stress and SA treatment in microarray data. *LTI29* and *LTI30* overexpressed lines accumulated more dehydrin (Puhakainen et al., 2004). Dehydrin is an important player during drought stress in plants (Brini et al., 2007) so it may also be concluded that SA is involved in dehydrin accumulation and drought tolerance. MAP kinase cascade is also activated through SA and ROS as downstream signaling (Cristina et al., 2010). The phosphorylation of MPK6 in *Arabidopsis* roots is associated with the application of SA (Mockaitis and Howell, 2000). This led to the conclusion that at low concentration SA acts as a positive regulator of drought tolerance by activating secondary signals like ROS, and by upregulating dehydrins and MPKs.

6.4.8 Jasmonic Acid

Jasmonic acid also provides tolerance against drought, however, reports also suggested that JA has a negative impact on plants under drought, so till now, its exact role is under observation (Riemann et al., 2015). The controversial action of JA is also varied due to observations under different experimental setups such as developmental stage of the plant, the concentration of drought agents, etc. (Kim et al., 2009a,b). The plant exposed to drought accumulated more proline and antioxidants (either enzymatic or nonenzymatic). Exogenously supplied JA or MeJA to the plant enhanced antioxidant capacity (Nafie et al., 2011) and proline accumulation (Mahmood et al., 2012). Three ecotypes of *A. Thaliana* have been observed under drought and wound stress. Wounding activated both JA and 12-OPDA (biosynthetic intermediate of JA), but drought stress induced only 12-OPDA, which probably gives an idea that drought mediated signals block the conversion of 12-OPDA to JA, and 12-OPDA is a functional jasmonate during drought. Transgenic plants of *Arabidopsis* having the same level of ABA but the range of 12-OPDA generation indicated that more 12-OPDA is promising against drought stress with reduced stomatal opening (Savchenko et al., 2014). *Arabidopsis* seedlings defective functional JA-Ile biosynthesis (jar1-1) and JA insensitive lines (jai1 and coi1-16) were reported for more proline accumulation after exposure to dehydration showed that more proline accumulation is independent of ABA (De Ollas et al., 2015).

6.5 MOLECULAR MECHANISM OF REGULATORY ELEMENTS IN DROUGHT STRESS

Modern genetics and molecular advancement have improved our knowledge in deciphering the role of metabolic and regulatory proteins during water stress utilizing transcriptomics, proteomics, and metabolomics approaches (Hirayama and Shinozaki, 2010). The regulatory genes include TFs, protein kinases, and protein phosphatases, which play the crucial role in abiotic stress synchronizing signal perception and transduction to regulate downstream stress-responsive genes (Wani et al., 2013). Transcription factors act as the key ingredient of gene differential regulation and their expression. As a rule, the transcription factors possess a DNA binding domain and a transcriptional activation/repression domain, to regulate transcription

process of the targeted gene and vast reports are available emphasizing their role in abiotic stress particularly drought. Plant TFs widely known to play a role in water stress have been categorized into different subfamilies, for example, *MYB*, *AP2/ERF*, *bZIP*, Zn finger, and *NAC* (Riechmann et al., 2000).

6.5.1 MYB Transcription Factor Family

The *MYB* gene family is one of the immensely vast and functionally varied classes of TFs, and plays a central role in the control of plant-specific processes, including primary and secondary metabolism, cell fate and identity, and development response to abiotic and biotic stresses (Dubos et al., 2010). Predominantly, MYB proteins act as transcription factors and characterized by the presence of conserved MYB repeats (R) at N-terminus and the variable region at C-terminal. The conserved N-terminus region is responsible for DNA-binding and protein−protein interactions while the variable region is associated with harmonizing regulatory activity of the protein. In rice, it was reported that 65% of *MYB* genes expressed in seedlings were differentially regulated under drought stress (Katiyar et al., 2012). An even higher percentage was observed in *Arabidopsis thaliana* (L.) Heynh. Transcriptomic data collected in the GENEVESTIGATOR database (Zimmermann et al., 2004) showed that 51% of *AtMYB* genes were upregulated and 41% were downregulated in drought stress (Katiyar et al., 2012). For example, virus-induced gene silencing (VIGS) of *NbPHAN*, a member of MYB TFs family, showed altered leaf shapes, impaired tolerance against drought stress, and shallow expression of stress-related genes in *Nicotiana benthamiana* (Huang et al., 2013). It is also involved in regulation of lateral root growth via the ABA-auxin signaling network to impart drought tolerance. Overexpressing *AtMYB96* exhibited drought tolerant and reduced stomatal aperture while *myb96-1* mutant plants showed the reverse phenotype under drought and ABA treatment. The *MYB60* expression was enhanced by blue and white light while it was reduced in the presence of ABA and desiccation (Newman et al., 2004). Conversely, *MYB61* causes reticence of light-induced stomatal opening as its expression was upregulated in the dark (Suzuki et al., 2014). Two other *Arabidopsis* guard cell expressing genes *AtMYB44* (Jung et al., 2008) and *AtMYB15* (Ding et al., 2009) showed higher expression upon exposure to ABA and several abiotic stresses. The overexpression lines showed drought tolerant phenotype opposite to the *atmyb44* knockout mutant. *AtMYB44* acts as a negative regulator of ABA signaling.

MYB transcription factor gene *GbMYB5* conferred drought tolerance in cotton and transgenic tobacco by modulating the efficient ROS scavenging system, polyamine biosynthesis genes (*ADC1* and *SAMDC*), the LEA abundant protein-encoding gene *ERD10D*, and drought-responsive genes (*NCED3*, *BG*, *RD26*) (Chen et al., 2015). A unique example of *AtMYB21* expression under drought in flowers leads to maintenance of fertility. On the other hand *atmyb21* mutant plants' flowers delayed filament elongation recovery after drought treatment as compared with wild-type plants. It suggests that *AtMYB21* is a vital target gene that contributes to flower development as well as to drought tolerance possibly by involving cross-talk between JA/GA and ABA in drought response (Su et al., 2013). Thus we could summarize that *MYB* TFs play an important role in regulating and maintaining plant homeostasis during various abiotic stresses including drought.

6.5.2 Ethylene Response Element-Binding Factors (AP2/ERF) Family

Another important class of transcription factor is the APETALA2/ethylene response element-binding factors (AP2/ERF) family, which is involved in development along with biotic and abiotic stress responses (Xu et al., 2011; Sharoni et al., 2010). It has been characterized by the presence of conserved AP2/ERF DNA-binding domain (Song et al., 2013) specific to GCC box involved in ethylene-responsive transcription (Rashid et al., 2012). By number and similarity of the AP2/ERF domains, this family has been divided into four subfamilies: AP2 (Apetala 2), RAV (related to ABI3/VP1), DREB (dehydration-responsive element-binding protein), and ERF (Rashid et al., 2012; Sharoni et al., 2010). Among these four subfamilies, the most studied family of AP2/ERF in abiotic stress response are the AP2 and DREBs proteins.

In rice, six DREB2 family genes had been identified (Srivasta et al., 2010), while *DREB2A* and *DREB2B* were highly induced under drought stress (Nakashima et al., 2014) and resulted in improved osmotic tolerance of transgenic plants (Mizoi et al., 2013). A total of eight members of the DREB2 subgroup in *Arabidopsis*, *DREB2A*, and *DREB2B* expression were induced by dehydration, high salinity, and heat in an ABA-independent manner (Nakashima et al., 2000; Sakuma et al., 2006). Constitutive expression *OsDREB2B* in *Arabidopsis* plant improved tolerance towards water stress by targeting *DREB2A* target genes (Matsukura et al., 2010). Improved drought tolerance was visible in a variety of species overexpressing *DREB1/CBF* gene including tomato, soybean (De Paiva Rolla et al., 2014), rice (Nakashima et al., 2014), tobacco (Phuong et al., 2015), and wheat (Shavrukov et al., 2016). *Arabidopsis*

DREB1C gene in the transgenic rice plants improved drought stress in upland cultivar (Ishizaki et al., 2013). *OsDREB1F* homologous constitutive overexpression enhanced dehydration stress in rice via the ABA-mediated pathway (Wang et al., 2008a,b). Various other AP2/ERF-type like TFs were functionally characterized in rice to decipher their role in drought tolerance (Abogadallah et al., 2011).

6.5.3 Basic Leucine Zipper Transcription Family

The basic leucine zipper family is composed of basic nuclear localizing, DNA binding N-terminal conserved bZIP domain and leucine-rich motif at C-terminus for dimerization (Wang et al., 2015). Function of bZIP TF family is much diversified in plants (Liu et al., 2014; Pourabed et al., 2015). The bZIP role is also explored to understand its role in drought tolerance mechanism in crops (Llorca et al., 2014). Studies would help in portraying its functioning in dehydration stress, such as how *SlAREB* in tomato (*Solanum lycopersicum*) improved tolerance to drought and salt stress by regulating the stress-responsive gene in an ABA-dependent manner (Hsieh et al., 2010). Likewise, overexpression of *OsbZIP23*, *OsbZIP46*, and *OsbZIP16* in rice improved drought tolerance through ABA-mediated signaling (Xiang et al., 2008; Tang et al., 2012; Chen et al., 2012a,b).

A recent study in *Arabidopsis* revealed that overexpression of *TabZIP60* improved plant's tolerance against multiple stresses like drought, salt and freezing along with increased sensitivity to ABA (Zhang et al., 2015). In *Arabidopsis*, overexpression of *ABF4*/*AREB2*, *AREB1*, and *ABF3* TFs led to enhanced drought tolerance by reducing the transpiration rate of the transgenic line as compared with wild-type (Fujita et al., 2011). Moreover, *areb1 areb2 abf3* triple mutant laid out ABA hyposensitivity and lessened dehydration stress tolerance as compared with single and double *AREB*/*ABF* knockout mutants. This outcome suggested synergistic action between the three bZIP TFs help in shoring up the plants to withstand drought and osmotic stress (Yoshida et al., 2010).

6.5.4 Zn-Finger Transcription Factor Family

Zinc-finger proteins (ZFPs) also play a diverse role in plants, and express accordingly to the type and degree of different abiotic stresses (Sun et al., 2010). On the basis of number and sequence order of C and H residues in the secondary structure, DNA binding Zn fingers are divided into nine groups: C2H2, C8, C6, C3HC4, C2HC, C2HC5, C4, C4HC3, and C3H (Schumann et al., 2007; Gupta et al., 2012). Zinc-finger TFs are a large family of TFs that consist of 23 subfamilies, and WRKY is one of the most studied TFs in regards to biotic and abiotic stress (Li et al., 2014). Here, we emphasize its function in abiotic stress particularly in drought stress. Most of the time, WRKY transcription factors and ABA signaling work hand in hand. Zn finger TF, Zat10 knockout, and RNAi mutants were more tolerant to osmotic and salinity stress in *Arabidopsis*. *ZFP252* (TFIIIA C2H2 ZF) constitutive expression in rice enhanced the drought tolerance by enhancing proline accumulation, sugar content, and drought-responsive *OsDREB1A* gene expression (Xu et al., 2008). Transgenic tobacco, overexpressing *GhWRKY41*/*SpWRKY1*, regulated stomatal conductance and ROS levels to impart salt and drought tolerance (Chu et al., 2015; Li et al., 2015). *FcWRKY70* in *Fortunella crassifolia* accumulated a more significant amount of putrescine in comparison with wild-type (WT) and enhanced dehydration stress (Gong et al., 2015). Similarly, *SiWRKY066/082* (from *Setaria italica*) and *PgWRKY1* (from *Panax ginseng*) were involved in stress by regulating hormonal signaling (Muthamilarasan et al., 2015; Nuruzzaman et al., 2016). *AtWRKY46* plays an important role in hormonal regulation especially of ABA and auxin facilitated lateral root development under osmotic and salinity stress (Ding et al., 2015). Transgenic *Arabidopsis* harboring *HaWRKY76* from sunflower provided drought and flood tolerance (Raineri et al., 2015). Zinc-finger TFs help in coping with drought stress through regulation a number of factors like ROS scavenging (Baek et al., 2015) increasing the contents of proline, abscisic acid (ABA), chlorophyll, soluble sugars, and reducing the water loss rate (Luo et al., 2012; Wang et al., 2016a,b). Through detailed study and analysis (*TaZFPs*) from *Triticum aestivum*, ZFPs was considered to play an important role in environmental adaptation during drought stress (Cheuk and Houde, 2016).

6.5.5 NAC Transcription Factor Family

The NAC gene family is also one of the largest gene families of TFs, performing diverse functions from developmental processes, leaf senescence, and lateral root development to biotic and abiotic stress response (Nuruzzaman et al., 2012). NAM (for no apical meristem), *ATAF* 1 and 2 (*Arabidopsis transcription activation factor)*, and *CUC2* (for cup-shaped cotyledon) collectively form the NAC domain of the NAC gene family. Unlike other TFs, NAC proteins consist of conserved nuclear localizing DNA-binding N-terminal domain and variable C-terminal transcription regulatory (TR) region (Olsen et al., 2005). Multiple reports suggested that NAC genes are involved in drought stress

responses. Transgenic rice harboring the *OsNAC2/6* and *OsNAC10* genes improved the drought and salt tolerance (Nakashima et al., 2009; Jeong et al., 2010), and enhanced grain yield of plants overexpressing *SNAC1* gene under drought stress (Hu et al., 2006). NAC TF genes were induced by hormone application such as auxin, ethylene, and ABA (He et al., 2005; Sperotto et al., 2009). But the role of ABA was prominent in adaptation to drought stress as it regulated the lateral root development for efficient water use. Similarly, rice homologs *OsNAC071/OsNAC5/OsNAC009* and *OsNAC6* expression were induced by dehydration, high salinity, and ABA (Takasaki et al., 2010; Nakashima et al., 2007). The mechanism underlying ABA-mediated signaling under stress ensures the expression of drought-responsive genes like LEA protein, MYB, bZIP, and MYC, etc., to protect the vegetative and reproductive tissue from cellular damage on dehydration. *ANAC019* and *ANAC055* overexpression in *Arabidopsis* imparts drought tolerance by targeting JA/ABA-mediated pathway (Bu et al., 2008). Heterologous overexpression of *AhNAC2* gene in *Arabidopsis* improved drought and salt tolerance by targeting stress-responsive genes: *RD29A*, *RD29B*, *RAB18*, *ERD1*, *AtMYB2*, *AtMYC2*, *COR47*, *COR15a*, *KIN1*, *AREB1*, and *CBF1*. Moreover, rice harboring *ONAC045* gene enhanced drought and salt tolerance by targeting desiccation tolerant genes *OsLEA3-1* and *OsPM1* (Zheng et al., 2009). All these evident reports emphasized the major role of NAC TFs in abiotic stresses particularly drought and salinity through hormone regulation.

6.6 CONCLUSION AND FURTHER PROSPECTS

Drought stress causes membrane damage, ROS generation, protein dysfunction, and an imbalance in metabolic and redox homeostasis in the plant. Several molecular pathways for tolerance and adaptation to drought stress have been illustrated (Figs. 6.1 and 6.2). The cross-talk between the different signaling molecule and regulatory genes (MYB, WRKY, DREB/ERF, and NAC) suggested that drought tolerance in the plant is a multifaceted phenomenon, and is governed by a complex regulatory mechanism. So, to find a key player in drought stress might be a potential candidate for crop improvement.

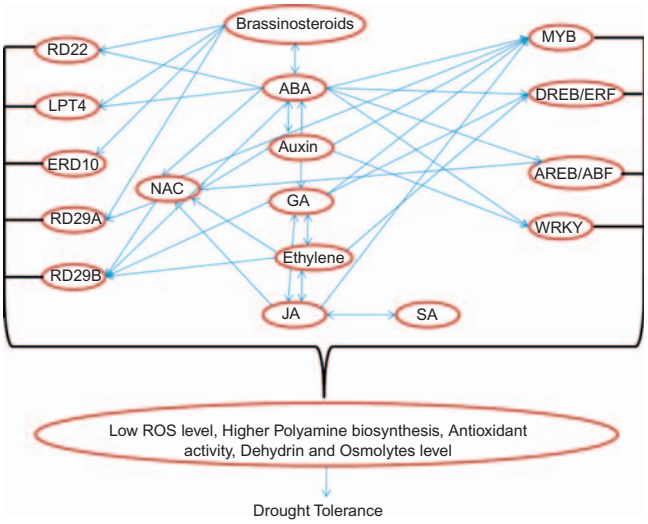

FIGURE 6.2 Cross-talk between the different signaling molecule and regulatory genes that take part in water deficit stress.

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CHAPTER

7

Crop Improvement of Cereals Through Manipulation of Signaling Pathways in Response to Drought Stress

Sunitha Mellacheruvu, Ashwini Talakayala and Mallikarjuna Garladinne

Plant Molecular Biology Laboratory, Agri Biotech Foundation, Hyderabad, India

OUTLINE

7.1 INTRODUCTION

Since plants are sessile organisms, they have to adapt continuously under adverse environmental stresses. These unfavorable conditions suppress plant growth and development and ultimately affect crop yield. Abiotic stresses like drought, salinity, cold, heat, and heavy metals are the major abiotic stress factors that impact agriculture productivity and threaten the global food security (Zhu, 2016; Pasala et al., 2016). The scenario of climate change and global warming has led to increase in intensity of extreme weather, alterations in cropping texture in terms of crop life cycle, increased water evaporation, and severe drought and salinity stress coupled with oxidative stress (Fedoroff et al., 2010). However, plants have developed intricate mechanisms to overcome these adverse conditions. Crop plants need to cope with adverse external

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pressure created by environmental and edaphic conditions with their intrinsic biological mechanisms. However, this affects growth and development and ultimately affects productivity (Meena et al., 2017). Plants sense the stress signals and try to adapt under these unfavorable environmental stress conditions through various evolutionary mechanisms like physiological and biochemical alterations connecting various signal transduction pathways and cascades of endogenous metabolic developmental activities (Budak et al., 2015; Khan and Khan, 2017; Phukan et al., 2017) (Fig. 7.1).

The process of signal perception and activation of signaling cascades involves a large number of factors and molecules belonging to diverse classes of gene families. In contrast to animals, plants harbor larger multigene families along with the occurrence of plant-specific ones. In this chapter, various abiotic stress-responsive genes were analyzed and categorized into different classes such as osmoprotectants (glycine betaine, proline), reactive oxygen species (ROS) scavengers (superoxide dismutase, SOD; ascorbate peroxidase, APX; dehydroascorbate reductase, DHAR; monodehydroascorbate reductase, MDHAR; and glutathione reductase, GR), late abundant (LEA) proteins), heat shock proteins, and transcription factors (TFs) such as C-repeat/dehydration-responsive element binding protein (CBF/DREB), myeloblastosis/myelocytomatosis (MYB/MYC), basic leucine zipper protein (bZIP), no apical meristem ATAF & cup shaped cotyledon (NAC), abscisic acid/ABA-responsive element (ABA/ABRE), etc. (Todaka et al., 2015; Per et al., 2017). These gene families are involved in regulation of stress signal transduction pathways in plants.

Improving plant tolerance against environmental stresses by manipulating the signaling pathways is a very important strategy for crop sustainability and enhancing productivity. The availability of several genetic approaches like overexpression of genes, silencing, and genome editing have made it easy to understand the role of signaling molecules and their pathways (Akpinar et al., 2015). Recent approaches like RNA deep sequencing, transcriptomic analysis have been employed at transcription level for identification of the genes involved in various processes

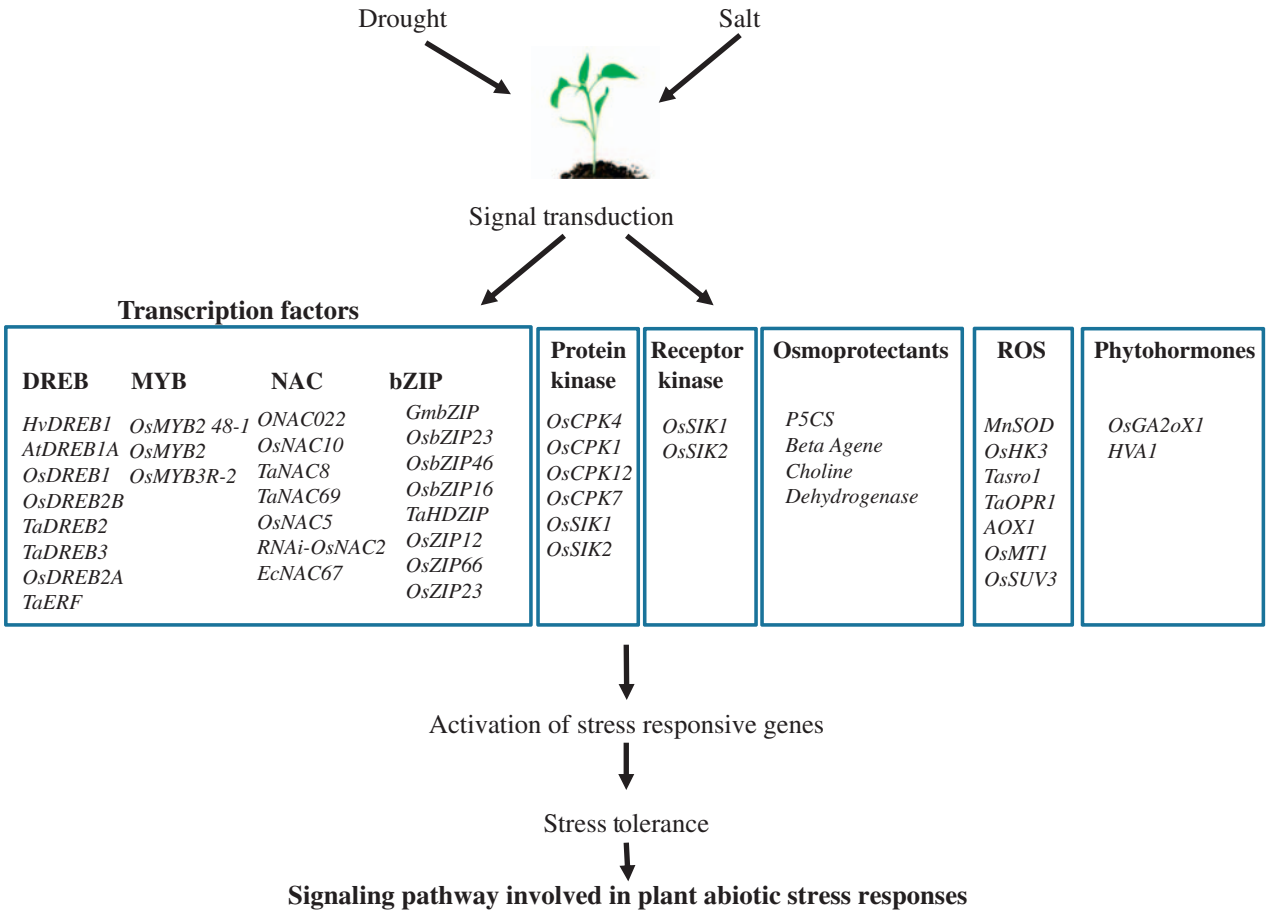

FIGURE 7.1 Schematic representation of signaling pathways during abiotic stresses. Upon stress signal perception, different transcription factors and signaling pathways get activated leading to differential gene expression for generating appropriate stress tolerance.

including signaling. *Agrobacterium tumefaciens* and particle bombardment methods of transformation have been extensively been used for generation of transgenic plants for achieving stress tolerant cereal crops. Significant progress has been made in the generation of drought and salinity tolerant transgenic crops.

7.2 TRANSCRIPTION FACTORS ASSOCIATED WITH SIGNALING MECHANISM

TFs act as master switches and trigger simultaneous expression of a large number of stress-response genes that contribute to the stress tolerance phenotype (Zandkarimi et al., 2015). TFs are key regulators that function in the upregulation of downstream genes by interacting with ABA, jasmonic acid, and salicylic acid in regulating the signaling pathways during drought stress (Nakashima et al., 2014; Per et al., 2018). Mostly the TFs belong to either ABA dependent or independent signal transduction pathways. These pathways were studied extensively in rice and *Arabidopsis* systems (Todaka et al., 2012). Different kinds of TFs including *DREB/CBF*, *ERF*, *MYB*, *AREB/ABF*, *NAC*, zinc fingers, etc. are known to be involved in regulation of signaling mechanism in response to drought and salinity stresses (Yamaguchi-Shinozaki and Shinozaki, 2009). Several research groups have developed transgenic plants by manipulating the TFs; these are listed in Table 7.1.

TABLE 7.1 Expression of Transcription Factors Involved in ABA-Dependent and -Independent Signaling Pathways Through Transgenic Approach

Item	Gene	Promoter	Plant	Phenotype of transgenics	References
DREB	*HVDREB1*	*CaMV35S*	Wheat	Increased salt stress tolerance	Xu et al. (2009)
	OsDREB2B	*CaMV35S rd29A*	Rice	Nonfunctional transcript in rice	Matsukura et al. (2010)
	TaDREB2 and *TaDREB3*	*Double 35S, Maize Rab17*	Wheat and barley	Constitutively expressing plants delayed in growth, flowering and grain yield; drought and frost tolerance	Morran et al. (2011)
	TaERF	*Ubiquitin*	Wheat	Proline was accumulated under drought stress and increased chlorophyll content in levels	Rong et al. (2014)
	OsDREB2A	*Rd29A*	Rice	Drought and salinity tolerance with higher number of panicles and more grain yield	Mallikarjuna et al. (2011)
	AtDREB1A/CBF3	*Rd29A*	Wheat	Substantial tolerance to water stress and delayed in wilting under drought stress	Pellegrineschi et al. (2004)
	AtDREB1A/CBF3	*35sCAMV*	Rice	Tolerance to abiotic stress without displaying stunted growth	Oh et al. (2005)
	OsDREB1/CBF	*CaMV35S*	Rice	Expression of cold responsive genes	Ito et al. (2006)
MYB	*OsMYB3R-2*	*CaMV35S*	Rice	Increased chilling tolerance	Ma et al. (2009)
	OsMYB48-1	*CaMV35S*	Rice	Drought and salinity tolerance. Reduced rate of water loss/lower MDA, higher proline content under stress conditions	Xiong et al. (2014)
	OsMYB2	*Ubiquitin*	Rice	Hypersensitive to shoot and root inhibition by exogenous ABA	Zhao et al. (2014)
NAC	*OsNAC10*	*CaMV35S*	Rice	Drought, salinity and low-temperature stress toleranceImproved in grain yield	Jeong et al. (2010)
	TaNAC8	*CaMV35S*	Wheat	Transcriptional gene regulation and showed biotic and abiotic stress tolerance	Xia et al. (2010)
	TaNAC69	*Drought-inducible promoter (HvDhn4s)*	Wheat	Drought tolerance with improvement in water use efficiency	Xue et al. (2011)
	OsNAC5	*CaMV35S*	Rice	Tolerant to drought and salt	Song et al. (2011)
	EcNAC67	*Rd29A*	Rice	Drought and salt tolerance with increase of high root and biomass	Rahman et al. (2016)

(*Continued*)

TABLE 7.1 (Continued)

Item	Gene	Promoter	Plant	Phenotype of transgenics	References
	ONAC022	*CaMV35S*	Rice	Accumulation of proline and minimized water loss, reduced stomatal open, high survival ratio during stress conditions	Hong et al. (2016)
	OsNAC2	*CaMV35S*	Rice	Silencing lines showed enhanced tolerance to high salinity and drought stress at both the vegetative and flowering stages	Shen et al. (2017)
bZIP	*OsbZIP23*	*Ubiquitin*	Rice	Drought, salt tolerance but sensitivity to exogenous ABA	Xiang et al. (2008)
	GmbZIP	*CaMV35S, rd29A*	Wheat	Drought tolerance without growth retardation	Gao et al. (2011a)
	OsbZIP46	*Ubiquitin*	Rice	Drought and salt tolerance. Hypersensitive to shoot growth inhibition by exogenous ABA	Tang et al. (2012)
	OsbZIP16	*CaMV35S, rd29A*	Rice	Drought and salt tolerance. Hypersensitive to shoot growth inhibition by exogenous ABA	Todaka et al. (2015)
	TaHDZIP	*CaMV35S*	Bread Wheat	Drought and frost tolerance with undesired features like reduced plant growth, delayed flowering and reduced biomass and yield	Yang et al. (2017)
	OsbZIP12	Cytochrome c	Rice	Improved drought tolerance and hypersensitivity to ABA.	Joo et al. (2014)
	OsbZIP66	GOS2 (rice eukaryotic translation initiation factor 1-like gene) and RCc3 (rice lipid transfer protein-like gene)	Rice	Enhanced drought tolerance	Yoon et al. (2017)

7.2.1 Dehydration Responsive Element Binding Proteins

The DREBs are novel TFs, belonging to the AP2 (apetala)/ERF (ethylene responsive element binding factors) family distinctive to plants (Agarwal et al., 2006). There are approximately 124 ERF proteins sharing a conserved 58–59 amino acid conserved region (ERF domain) that binds to two *cis*-acting elements, that is, C-repeat/dehydration responsive motif (CRT/DRE) (A/GCCGAC) found in the expression of cold and dehydration responsive genes and GCC box, found in many pathogenesis-related gene promoters conferring ethylene responsiveness (Agarwal et al., 2006; Zhang et al., 2004). The DREB TFs induce a set of downstream and abiotic stress related genes that help in plant survival during adverse environmental conditions. DREB TFs are dichotomized as DREB1 and DREB2, and are involved in signal transduction pathways under low temperature and dehydration, respectively (Agarwal et al., 2006). DREB1/DREB2-homologous genes have been isolated from several crop species such as rice, wheat, barley, maize, sorghum, etc. In important cereal crops like wheat and barley, a number of *CBF* homologs have been mapped to the Fr-2 chromosomal region (Skinner et al., 2005; Miller et al., 2006). A functional Fr-A1/VRN1 allele (winter hardiness/vernalization requirement) plays an important role in regulating the CBF-mediated *Cor/Lea* gene expression in wheat (Kobayashi et al., 2005).

The DREB TFs are potential genes that have been exploited for generation of transgenic cereals for enhancing drought and salt stress tolerance. The overexpression of *AtDREB1A/CBF3* driven by the stress-inducible *rd29A* promoter in transgenic wheat improved water stress tolerance, and delayed wilting under greenhouse conditions (Pellegrineschi et al., 2004). Similarly, the constitutive overexpression of *DREB1A/CBF3* under *35S* promoter in rice increased stress tolerance to drought and high salinity without any growth inhibition or phenotypic aberrations (Oh et al., 2005). Constitutive overexpression of *OsDREB1* showed improved tolerance against drought, high salt, and low temperature and also accumulated the osmoprotectants like proline and different soluble sugars in transgenic rice plants. However, stunted growth was observed in transgenic rice plants (Ito et al., 2006). Expression of *HvDREB1* gene in barley leaves was significantly

induced by salt, drought, and low-temperature stress (Xu et al., 2009). Similarly, maize DRE-binding proteins DBF1 and DBF2 were involved in *rab17* regulation through the drought-responsive element in an ABA-dependent pathway (Kizis and Pages, 2002).

Stress-inducible expression of *OsDREB2A* conferred drought and salinity stress tolerance in rice and also improved the number of panicles and seed weight in comparison to control plants (Mallikarjuna et al., 2011). The expression of *OsDREB2B* in transgenic rice and *Arabidopsis* plants led to transactivation of its target stress adaptive genes (Matsukura et al., 2010). Ectopic expression of cotton DREB leads to enhanced drought, salt, and cold stress tolerance due to soluble sugars and chlorophyll production in wheat (Gao et al., 2009). *TaDREB2* and *TaDREB3* TFs of wheat were expressed constitutively (*CaMV35S*) and also inducible expression (Maize *Rab17*) in wheat and barley showed significant frost tolerance. The constitutively overexpressing plants showed delay in growth and yield, but the plants expressing under inducible condition displayed superior growth performance than untransformed plants. The induced expression of these two genes led to upregulation of 10 other CBF/DREB TFs and also massive number of other stress responses like *LEA/COR/DHN* genes involved in protection from desiccation stress (Morran et al., 2011). The transgenic rice expressing *SbDREB2* under the control of *RD29A* promoter exhibited drought tolerance with normal seed set, whereas constitutive expression of the same gene under the regulation of *CaMV*35S promoter showed abnormal plant growth and seed set (Bihani et al., 2011). Overexpression of wheat *TaERF* (AP2/ethylene responsive factor) promoted drought tolerance by accumulating the proline and increased levels of chlorophyll levels in wheat (Rong et al., 2014). The heterologous expression of *TaCBF14* and *TaCBF15* led to elevated transcript of gibberellin catabolic *HvGA2ox5* gene through accumulation of DELLA protein, but dwarf phenotype was observed in transgenic barley (Soltész et al., 2013). Overall, the stress-inducible expression of DREB TFs significantly improves the stress tolerant phenotypes without growth penalty despite that constitutive expression possesses the abnormal plant growth and development.

7.2.2 MYB Transcription Factor

The MYB family of TFs is a large family and plays a fundamental role in response to biotic and abiotic stresses through ABA-dependent signal transduction pathway. *MYB* genes code for TFs with a characteristic of 52 amino acid MYB motifs. These TFs contain varying numbers from one to four MYB domains termed as R1, R2, R3, and R4 (Du et al., 2009). Each MYB domain has three regularly spaced tryptophan residues that are separated by 18 or 19 amino acid residues and each domain forms a helix-turn-helix fold that is crucial for MYB TF−DNA interaction (Saikumar et al., 1990). The MYB TFs play an important role in biological processes such as cell cycle regulation (Cominelli and Tonelli, 2009), cell proliferation (Xie et al., 2010), developmental processes (Komaki and Sugimoto, 2012), hormone signal transduction (Zhao et al., 2014a, b), and abiotic stress responses (Katiyar et al., 2012). In rice, the MYB-related TF *OsMYB48-1* acts in response to water stress by regulating the transcription of genes involved in early and late response to stress and also stress-induced ABA biosynthesis genes (Xiong et al., 2014). Rice R2R3 type MYB, OsMYB2 TF was overexpressed in rice, and conferred abiotic stress tolerance and also accumulated elevated levels of soluble sugars and proline in transgenic plants under salt stress. Moreover, there were improved levels of antioxidant enzymes and highly upregulated stress related genes like *OsLEA3*, *OsRab16A*, and *OsDREB2A* in transgenic rice plants compared with wild type (Yang et al., 2012). In another study, overexpression of *OsMYB3R-2* exhibited elevated proline content with enhanced cold tolerance in transgenic rice plants (Ma et al., 2009).

7.2.3 NAC Transcription Factor

NAC TFs are highly conserved and play a key role in flower development, formation of secondary walls, cell division, shoot apical meristem formation, and leaf senescence, as well as protective role to biotic and abiotic stresses (Olsen et al., 2005; Nakashima et al., 2012; Nuruzzaman et al., 2013; Banerjee and Roychoudhury, 2015). These proteins share a common structure with a conserved N-terminal NAC domain and a highly variable C-terminal domain. The NAC domain designation is derived from a conserved domain originally associated with the no apical meristem, ATAF1, ATAF2, and cup-shaped cotyledon genes from *Arabidopsis* (Aida et al., 1997). NAC family TF *OsNAP* is a transcriptional activator, induced by ABA, drought, salinity, and low-temperature treatments in transgenic rice plants. Interestingly, the *OsNAP* stimulated the expression of other stress related TFs like *OsDREB1A*, *OsMYB2*, *OsAP37*, and *Osap59* (Chen et al., 2014). The transgenic rice plants constitutively expressing *OsNAC022* exhibited enhanced drought tolerance, which was attained by accumulation of proline and soluble sugars. In addition, minimized water loss and reduced stomatal opening were observed in transgenic plants (Hong et al., 2016). Overexpression of *OsNAC1* and *OsNAC10* TF under control of two different constitutive

promoters such as *GOS2* (rice eukaryotic translation initiation factor 1-like gene) and root specific promoter *RCc3* (rice lipid transfer protein-like gene) enhanced drought, salinity, and low-temperature stress tolerance at the vegetative stage. However, root specific expression of *OsNAC10* in rice led to improvement in grain yield even under field drought conditions (Jeong et al., 2010, 2013; Redillas et al., 2012). *TaNAC8*, a novel NAC of wheat, responds to stripe rust pathogen infection and abiotic stresses (Xia et al., 2010). Similarly, overexpression of *TaNAC69* in transgenic wheat leads to enhanced dehydration tolerance and improvement of water use efficiency (Xue et al., 2011). Barley *HVA1* gene conferred drought and salt tolerance in transgenic maize (Nguyen and Sticklen, 2013). Also transgene pyramiding of the *HVA1* and *mtlD* conferred drought and salt tolerance with enhanced crop biomass in maize (*Zea mays* L.) (Nguyen et al., 2013).

The role of *OsNAC5* was investigated by generating knockdown and overexpressing in rice. The RNAi lines became sensitive to abiotic stresses, whereas overexpressing lines positively correlated with accumulation of higher content of proline, soluble sugars, and reduced levels of malondialdehyde (MDA) and H_2O_2. It suggested that the *OsNAC5* overexpressing lines possessed more tolerance to oxidative and salt stress (Song et al., 2011). *OsNAC2* overexpressing lines negatively correlated with downregulation of several ABA-dependent stress-related genes, whereas silencing of this gene showed elevated tolerance to high salinity and drought stress during vegetative and flowering stage (Shen et al., 2017). A NAC TF from finger millet, *EcNAC67* ectopically expressed in rice, exhibited robust root and shoot biomass with higher relative water content in comparison with wild type during drought and salinity stress at the greenhouse level. Moreover, the transgenic rice lines possessed reduced in spikelet sterility and higher grain yield (Rahman et al., 2016). The above results indicated that these NAC TFs are potential candidate genes for generating transgenic lines in other crops also.

7.2.4 Basic Leucine Zipper Protein Transcription Factor

bZIPs comprise a large family of TFs that contain a highly conserved bZIP domain with two structural features, that is, a basic domain responsible for sequence-specific DNA binding and an adjacent heptad leucine repeat domain referred to as a leucine zipper (Zhang et al., 2017). bZIPs are present throughout the plant kingdom and are involved in a variety of physiological processes, such as seed maturation, germination, flower development, fertility (Zou et al., 2008), plant senescence (Lee et al., 2006), photomorphogenesis, light signaling (Mallappa et al., 2006), responses to various abiotic stresses, and/or stress signaling (Hossain et al., 2010; Wang et al., 2010). Some bZIP family members, including *OsbZIP23*, *OsbZIP46*, *OsbZIP71*, and *OsbZIP16*, were positively upregulated and conferred tolerance to different abiotic stresses (Todaka et al., 2015).

Overexpression of *GmbZIP1* in wheat enhanced the responses of transgenic plants to ABA and improved tolerance to several abiotic stresses without any growth retardation of plants (Gao et al., 2011a). The stress-responsive *TaHDZip1-5* gene coding for homeodomain *leucine zipper class 1* TF is observed to play a key role in plant tolerance against frost and drought stresses. The constitutive and stress-inducible expression of *TaHDZip1-5* gene elevated the frost and drought tolerance in transgenic wheat plants. However, it showed negative impact on phenotypic features and reduced grain yield (Yang et al., 2017). The RING finger containing *E3 ligase*, that is, salt and drought induced ring finger 1 (*OsSDIR1*) expressing transgenic rice exhibited strong drought tolerance (Gao et al., 2011b). Another leucine zipper (*HD-ZIP*) from maize, that is, *Oshox22* acts as a strong transcriptional activator in rice, which is induced by ABA, salt, and polyethylene glycol through ABA-dependent signaling pathway (Zhang et al., 2012).

Expression of *Zmhdz10* confers enhanced levels of proline content and reduced electrolyte leakage and lower MDA content in transgenic rice, which contributed to drought and salt stress tolerance through ABA-dependent signaling pathway (Zhao et al., 2014). The transgenic rice plants overexpressing *OsbZIP23* TF showed elevated levels of drought and salt stress tolerance through ABA signaling mechanism in transgenic rice plants (Xiang et al., 2008). Similarly, overexpression of the *OsbZIP66* TF enhances drought tolerance of rice plants in an ABA-dependent manner (Yoon et al., 2017). Microarray analysis of the same plants reported to be involved in regulation of different downstream genes of *OsbZIP23* included protein kinases, LEA proteins, and several other stress related TFs (Xiang et al., 2008). *OsbZIP*12 is an important player in rice for conferring ABA-dependent drought tolerance and has high potential for use in the genetic improvement of stress tolerance (Joo et al., 2014).

7.3 GENETIC ENGINEERING OF KINASES FOR DROUGHT AND SALINITY TOLERANCE

7.3.1 Protein Kinases

Protein kinases are central integrators of plant abiotic stress signaling that link metabolic and physiological processes through regulation of ionic and osmotic

homeostasis in plant cells. Mitogen-activated protein kinases (MAPK) and sucrose nonfermenting related protein kinases (SnRK) are well-studied protein kinases in plants. SnRK1 is a subclass of protein kinases playing a key role in abiotic stress adaptation through the Salt Overly Sensitive (SOS) mechanism with regulation of ion homeostasis under salinity stress. Intriguingly, another SnRK2 kinase, *SAPK4* in rice, was involved in homeostasis through ionic and oxidative stress signaling pathways (Diédhiou et al., 2008). The SnRK2 family consisting of serine/threonine kinases was first reported to be involved in ABA signaling in wheat (Fujii and Zhu, 2012). Interestingly these proteins are involved in ABA-independent pathway in finger millet (Lata and Prasad, 2011).

Another class of protein kinases, MAPK mediated signal transduction cascades, has provided a pivotal role between physiological and cellular pathways in response to stresses. MAPK are highly conserved in all eukaryotes play an important role in response to biotic and abiotic stress stimuli. Overexpression of *MPK5* in rice was attributed a dual role as regulator of resistance against brown spot pathogen *Cochliobolus miyabeanus* and also as mediator of abiotic stress tolerance (Beckers et al., 2009).

Overexpression of calcium dependent protein kinase (*OsCPK4*) leads to enhanced drought and salt tolerance in overexpressing rice lines (Campo et al., 2014). In these transgenic lines, the oxidative stress-responsive genes like peroxidases, thioredoxin, glutathione, and laccase gene were upregulated in response to drought stress (Campo et al., 2011, 2014). In other studies, overexpression of *OsCDPK7* showed enhanced levels of drought and salinity stresses but not low-temperature stresses in rice, which indicated involvement in two different distinctive signal transduction pathways in rice (Saijo et al., 2000). Whereas the transgenic rice lines expressing *OsCIPK12* have been found to be involved in drought tolerance coupled with accumulation of osmoprotectants like proline and soluble sugars in transgenic plants (Xiang et al., 2007) (Table 7.2).

7.3.2 Receptor-Like Kinases

Plant receptor-like kinases (RLKs) are considered to be the most diverse super family of plant protection proteins involved in prevention of self-pollination, pathogen defense, hormone perception, adaptation to abiotic stresses, and increasing the crop yield (Bai et al., 2009). The plant cells often sense the environmental changes with the help of cell surface receptors on the membrane through activated RLK signaling pathways to trigger adaptive changes in the plants. RLKs are transmembrane proteins with amino terminal and carboxyl intracellular kinase domains with protein kinase activity. RLKs initiate the cellular signaling in plants. They are involved in different hormonal signaling pathways, growth, development, and plant pathogen interactions. *S-domain receptor-like kinases* (SRKs) are a highly polymorphic family of RLKs found in plants, play a key role in control of diseases, and improve the yield parameters in rice. The first plant *RLK* gene (*ZmPK1*) was identified and isolated from maize (Walker and Zhang, 1990) and then several RLKs were isolated from different plant species. Overexpression of LRR-RLK gene *LRK1* enhanced panicles and spikelets, which led to yield enhancement in rice (Zha et al., 2009). Another receptor-like protein, overexpression of *OsSIK2*, showed stress tolerance and delayed leaf senescence in transgenic rice plants (Chen et al., 2013). The transgenic expression of SRK, *OsLSK1* (large spike-like SRK 1) responded to exogenous application of growth hormones and to abiotic stresses. Overexpression of truncated *OsLSK1* increased plant height and number of panicles, which ultimately

TABLE 7.2 Expression of Kinases Involved in Signaling Pathways Through Transgenic Approach

Item	Gene	Promoter	Plant	Phenotype of transgenics	References
Protein kinase	*OsCPK7*	*CaMV35S*	Rice	Enhanced drought and salinity tolerance but not cold tolerance	Saijo et al. (2000)
	OsCPK12	*Ubiquitin*	Rice	Improved cold, drought and salt tolerance. Osmoprotectant, proline accumulated	Xiang et al. (2007)
	OsCPK1	*Ubiquitin*	Rice	Gene was negatively regulated and the silencing lines showed drought tolerance	Ho et al. (2013)
	OsCPK4	*Ubiquitin*	Rice	Drought, salt and oxidative stress tolerance. Peroxidase, thioredoxin, glutathione laccase genes upregulated	Campo et al. (2014)
Receptor-like kinases (RLKs)	*OsSIK1*	*CaMV35S*	Rice	Elevated drought and salinity tolerance. SOD, peroxidase, and catalase activity increased	Ouyang et al. (2010)
	OsSIK2	*CaMV35S*	Rice	Enhanced salt and drought stress tolerance	Chen et al. (2013)

TABLE 7.3 Expression of Genes Related to Phytohormones, Osmoprotectants, and ROS Signaling Genes

Item	Gene	Promoter	Plant	Phenotype of transgenics	References
Phytohormones	*OsGA2ox1*	*Actin*	Rice	GA synthesis in shoots during abiotic stress conditions	Sakamoto et al. (2003)
	HVA1	*Maize Ubiquitin*	Wheat	Improved water use efficiency, drought and salinity stress tolerance continuously for six seasons	Sivamani et al. (2000), Bahieldin et al. (2005)
Osmoprotectants	*P5CS*	*CaMV35S*	Wheat	Abiotic stress tolerance Showed increased levels of proline accumulation	Zhu et al. (1998)
	P5CS	*CaMV35S*	Wheat	Salt stress tolerance	Sawahel and Hassan (2002)
	Choline dehydrogenase	*CaMV35S*	Maize	Drought tolerance, higher grain yield, enhanced glycine betaine accumulation	Quan et al. (2004)
	Bet A	*CaMV35S*	Wheat	Salinity tolerance, higher germination rates	He et al. (2010)
ROS	*OsHK3*	*CaMV35S*	Rice	Crucial regulator of ABA signaling involved in antioxidant defense in rice	Wen et al. (2015)
	Tasro1	*Ubiquitin* and *CaMV35S*	Wheat and *Arabidopsis*	Increased salinity tolerance and activated AsA-GSH signaling pathway	Liu et al. (2014)
	TaOPR1	*Ubiquitin* and *CaMV35S*	Wheat and *Arabidopsis*	Induced salt stress tolerance coupled with ABA signaling pathway	Dong et al. (2013)
	AOX1	*CaMV35S*	Rice	Reduced oxidative stress under cold stress. Scavenging ROS	Li et al. (2013b)
	OsMT1	*OsActin*	Rice	Enhancement of antioxidative enzymes like catalase, peroxidase. and ascorbic acid	Yang et al. (2009)
	OsSUV3	*CaMV35S*	Rice	Reduction in lipid peroxidation and increase in antioxidative enzymes and salinity stress tolerance	Tuteja et al. (2013)

produced higher grain yield compared with nontransgenic plants (Zou et al., 2015) (Table 7.3).

7.4 MODULATION OF KEY GENES INVOLVED IN PHYTOHORMONE SIGNALING

Phytohormones are signaling molecules that regulate a variety of cellular processes, as well as growth and developmental activities in response to biotic and abiotic stresses in plants (Peleg et al., 2011). They work as chemical messengers to coordinate cellular activities and signal transduction pathways in higher plants (Vob et al., 2014). Although plant response to abiotic stresses depends on various factors, phytohormones are considered to be the most important endogenous substances for modulating physiological and molecular responses, a critical requirement for plant survival as sessile organisms (Fahad et al., 2015). They often rapidly alter gene expression by inducing or preventing the degradation of transcriptional regulators via ubiquitin proteosome system (Santner and Estelle, 2010). They are also involved in ion homeostasis and enhanced antioxidant defense pathway (Sreenivasulu et al., 2012). Their pivotal roles in promoting plant acclimatization to ever-changing environments by mediating growth, development, source/sink transitions, and nutrient allocation have been well established (Fahad et al., 2015). Phytohormones are auxin (IAA), cytokinins (CKs), ABA, ethylene (ET), gibberellins (GAs), salicylic acid (SA), brassinosteroids (BRs), and jasmonates (JAs). The strigolactones (SL) are relatively new phytohormones. Engineering of key genes of phytohormone signaling could be a perfect platform for biotechnologists to improve biotic/abiotic tolerant crops. Considering that phytohormones are key regulators of plant growth and development as well as mediators of the response to environmental stresses (Sreenivasulu et al., 2012; Khan et al., 2012, 2013, 2014, 2015a,b, 2016, 2017; Khan and Khan, 2014; Per et al., 2017), hormone metabolism and signaling processes are excellent targets of manipulation to obtain enhanced abiotic stress tolerance. However, maintenance of hormonal balance to minimize possible adverse effects on

growth and development is critical (Cabello et al., 2014; Khan and Khan, 2013).

Among various phytohormones, ABA is perhaps the most sought-after hormone for engineering abiotic stress tolerance in crop plants owing to its identity as a stress hormone and its vast array of functions under environmental stress conditions, particularly drought. As a result, many of the key ABA biosynthetic pathway enzymes have been investigated through genetic transformation of related genes for improved abiotic stress tolerance (Jewell et al., 2010). Park et al. (2008) overexpressed an ABA responsive stress related gene in *Arabidospsis* and the resulting plants exhibited greater osmotic stress tolerance. Constitutively expressing *HVA1* under the regulation of *maize ubiquitin* promoter conferred enhanced water use efficiency, drought, salt, and higher biomass production due to ABA signaling in wheat (Sivamani et al., 2000). Moreover, higher yields were recorded continuously for six seasons at multiple locations. However, it was not released for commercial use (Bahieldin et al., 2005). Overexpression of gene(s) involved in ABA biosynthesis or catabolic pathways resulted in increased drought tolerance, but led to impaired growth due to pleiotropic effects even with the use of inducible promoters (Hwang et al., 2010). To offset these unwanted growth anomalies, Zhang et al. (2013) overexpressed *CRK45*, a stress-inducible kinase involved in ABA signaling, and the resulting transgenic plants showed enhanced drought tolerance but with tighter control of ABA levels and signaling, indicating the role of *CRK45* in fine-tuning of ABA levels. Similarly, *IPT* was expressed under the control of stress-inducible promoters to avoid pleiotropic effects, leading to increased cytokinin content, antioxidant scavenging, and better root growth with overall improved grain yield under drought conditions in *Agrostis stolonifera* (Xu et al., 2016).

Arabidopsis YUCCA6 gene (a member of the *YUCCA* family of *Flavin monooxygenase-like proteins*), involved in tryptophan-dependent IAA biosynthesis pathway, was expressed in poplar under the control of stress-inducible *SWPA2* promoter and conferred abiotic stress tolerance (Wang et al., 2015). The transgenic lines displayed IAA overproduction morphological phenotypes, including rapid shoot growth and retarded tap root development with increased root hair formation. In addition, the plants had higher levels of free IAA and early expression of the IAA responsive gene transcripts. The transgenic lines showed tolerance to drought stress, associated with reduced levels of ROS (Wang et al., 2015). The rice mutant *CONSTITUTIVELY WILTED1* was deficient in the *YUCCA* homolog (Woo et al., 2007). Drought tolerance was achieved by overexpression of *AtYUC6* in potato and the plants were recovered after withholding water for 18 days (Kim et al., 2014). Constitutive overexpression of *OsGA2ox1* (*GA2 oxidase*) under regulation of *actin* promoter showed dwarf phenotype and failed to set grains during the flowering stage (Sakamoto et al., 2003). Attempts to engineer phytohormones for enhanced abiotic stress tolerance of plants are listed in Table 7.2.

7.5 ENGINEERING OF OSMOREGULATORY GENES

Osmoprotectants regulate the cellular osmotic adjustment, mitigate damaging risk caused by ROS, prevent membrane injury, and stabilize proteins and enzymes (Ashraf and Foolad, 2007). Many crop plants lack the ability to synthesize the special osmoprotectants that are naturally accumulated by stress tolerant organisms. Recently has been much emphasis on catalyzing biosynthetic pathways of osmoprotectants in crop plants. Water scarcity is a major abiotic stress that threatens the cereal productivity in subtropical regions. Genes involved in the synthesis of osmoprotectants—organic compounds such as amino acids (proline), a variety of sugars, sugar alcohols (mannitol, trehalose, and galactinol), quaternary, and other amines (glycine betaine and polyamines)—that accumulate during osmotic adjustment have been used to generate stress-tolerant transgenic plants (Vinocur and Altman, 2005; Masood et al., 2016; Per et al., 2017). Naturally, the glycine betaine (GB) accumulates in several plants like sugar beet, maize, spinach, and barley in response to abiotic stresses (Hurkman et al., 1991). Improvement in salt tolerance was observed in transgenic wheat by introducing *betA* gene for GB synthesis (He et al., 2010). Expression of GB1 in maize and soybean showed higher level of GB1 content in transgenic lines in comparison with wild type (Castiglioni et al., 2018). Transgenic rice transformed with bacterial *choline dehydrogenase* gene leads to accumulation of higher levels of glycine betaine, which showed improved tolerance to drought and chilling conditions (Quan et al., 2004). *Delta pyroline-5-carboylate synthetase*, a bifunctional enzyme involved in proline biosynthesis (Hu et al., 1992), was induced by high salt stress and dehydration (Delauney and Verma, 1993). Interestingly, the transgenic wheat plants carrying *Vigna aconitifolia pyrroline T carboxylate synthetase* (P5CS) gene exhibited the drought stress tolerance even after 15 days of water shortage. Proline was accumulated in these transgenic plants under drought stress conditions (Vendruscolo et al., 2007). Transgenic rice plants expressing *P5CS* gene showed increased levels of proline (Sawahel and Hassan, 2002). A rice gene, ornithine

delta-aminotransferase gene *OsOAT*, was overexpressed in rice conferring increased content of δ-OAT and proline levels coupled with enhanced drought, osmotic, and oxidative stress tolerance (You et al., 2012).

7.6 REACTIVE OXYGEN SPECIES SIGNALING DURING DROUGHT AND SALINITY STRESS

ROS are harmful byproducts of physiological changes and environmental stress responses in plants. ROS are known to be signaling molecules and secondary messengers involved in regulation of plant growth and development during biotic and abiotic stresses (Mittler et al., 2004; Khan and Khan, 2017). ROS are generated through incomplete reduction of molecular oxygen in plants (Miller et al., 2010). Excess production of ROS ultimately results in apoptosis of cells (Gill and Tuteja, 2010). ROS includes hydrogen peroxide (H_2O_2), superoxide anions (O_2•-), hydroxyl radical (OH•-), and singlet oxygen. ROS are precisely regulated by enzymatic and nonenzymatic antioxidant defense systems. Scavenging ROS are mediated through enzymatic reactions and regulating signaling pathways in crop plants (Baxter et al., 2013; Chakradhar et al., 2017). The plants have developed coherent antioxidant ROS scavenging machinery through enzymatic (SOD, APX, GR, MDHAR, and DHAR) and nonenzymatic reactions (ascorbic acid, reduced glutathione, tocopherols, carotenoids, flavonoids, etc.) (You and Chan, 2015). There is significant evidence that manipulating ROS provides an opportunity to enhance the abiotic stress tolerance in genetically engineered crop plants.

Several ROS scavenging enzymes have been studied in cereals. Plant *NADPH oxidase* is known as a homolog of *respiratory burst oxidase*, key signaling enzymes involved in ROS regulation and several signal transduction pathways (Marino et al., 2012). The activity of *NADPH oxidase* was induced by treatment with ABA and Ca^{2+} in leaves of maize seedlings (Jiang and Zhang, 2003). The *NADPH oxidase* genes were regulated by *histidine kinase* (*OsHK3*) in rice, which was involved in the production of H_2O_2 in ABA signaling (Wen et al., 2015). Transgenic rice plants overexpressing *Mn-SOD1* exhibited scavenging of mitochondrial peroxide free radicals during stress conditions (Li et al., 2013a). The transgenic rice expressing *OsDhn1* showed drought, salt, and methyl viologen tolerance coupled with enhancement of chlorophyll content, biomass, and also induced scavenging of free radicals (Kumar et al., 2014) The overexpression of *Ta-sro1* (similar to radical induced cell death) gene in wheat and *Arabidospsis* stimulated the scavenging of ROS and also upregulated the activity of AsA-GSH (ascorbic acid-glutathione reductase) and GPX (glutaredoxin) cycle enzymes, which helps in cellular redox homeostasis (Liu et al., 2014). Another ROS regulating enzyme, *TaOPR1* (wheat *oxophytodienoate reductase*), also exhibited tolerance to salt coupled with ABA signaling pathway in wheat and *Arabidopsis* (Dong et al., 2013). In another study, the *alternative oxidase 1 (AOX1)* gene overexpressed in rice conferring reduced the oxidative stress damage under cold stress by ROS scavenging (Li et al., 2013b). Overexpression of *OsMT1* (metallothionein) gene led to the enhancement of antioxidant enzyme activities of catalase, peroxidase, and ascorbic acid under drought stress and also *OsMT1* gene induced the expression of several zinc finger TFs by alteration of Zn^{2+} homeostasis (Yang et al., 2009). Overexpression of *OsSUV3* (a dual helicase) resulted in reduction of lipid peroxidation and H_2O_2 production coupled with increase in antioxidative enzymes and also the transgenic plants showed high salinity tolerance in rice (Tuteja et al., 2013). To improve abiotic stress in crop plants by exploiting ROS signaling pathway genes, key regulatory molecules need to be identified. Taken together these pathway genes can be utilized for the genetic transformation of different genetic backgrounds of cereals to enhance the abiotic stress tolerance in field conditions.

7.7 CONCLUSION AND FUTURE PROSPECTS

Climate change and severity of abiotic stresses are major threats that will impact basic fundamental processes, growth, and development of plants. These modulations ultimately affect the agriculture production and global food security.

In the present chapter, we highlighted the success stories of crop improvement through genetic engineering approaches by utilizing the genes related to abiotic stress signaling. Genetic engineering of TFs, phytohormones, osmoprotectants, protein kinases, and receptor kinases represents an important platform for abiotic stress tolerance, providing new opportunities to maintain sustainable crop production to feed the world under changing environmental conditions. Rapid development of genomic technology and efforts of many research groups have led to understanding of plant abiotic stress response. Still many challenges lie ahead to uncover and understand the complexity hidden in stress signal-transduction pathways. Among the greatest challenges that remain to be addressed are the development of stable engineered crops that produce staple foods such as rice, wheat, maize and corn. Recently, genome editing has become the most promising tool for facilitating the alterations or silencing the

gene of a particular locus. The CRISPR-Cas/Cpf tool is widely used for inducing knockin or knockouts of rice, wheat, maize, barley, etc. These applications may pave the way to improve yield and extend crop improvement or trait improvement such as biotic, abiotic, and nutritional value enrichment. It may resolve problems associated with stress response and adaptation pathways. Toward this goal, study should be focused on a combination of stress responses such as those in field environments, because different stresses are most likely to occur simultaneously under field conditions.

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CHAPTER

8

Role and Regulation of ROS and Antioxidants as Signaling Molecules in Response to Abiotic Stresses

Pooja Sharma[1], *Priyanka Sharma*[2], *Priya Arora*[1], *Vinod Verma*[3], *Kanika Khanna*[1], *Poonam Saini*[1] *and Renu Bhardwaj*[1]

[1]Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India
[2]Department of Biotechnology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, Maharashtra, India [3]Department of Botany, DAV University, Jalandhar, Punjab, India

OUTLINE

8.1 INTRODUCTION

Plants normally come across a combination of many abiotic stresses in their natural habitats. Abiotic stresses like drought, salt, cold, heat, and heavy metal cause a series of morphological, physiological, biochemical, and molecular changes that unfavorably affect plant growth, development, and productivity

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(Asgher et al., 2015; Khan et al., 2012, 2013, 2014, 2015, 2016; Pasala et al., 2016). These abiotic stresses modulate pathways of metabolism and boost expression level of transcription factors (TFs), which activate stress responsive genes. During plant stress response, reactive oxygen species (ROS) are produced and act as important molecules that activate downstream metabolic pathways (Chan et al., 2016). ROS interact with numerous metabolites and cause severe damage to proteins, DNA, and lipids and affect normal functioning of the cell (Apel and Hirt, 2004; Foyer and Noctor, 2005; Ashraf, 2009; Khan and Khan, 2017). ROS mainly includes singlet oxygen, hydrogen peroxide, superoxide radical, and hydroxyl radical. The excitation of O_2 results in the formation of singlet oxygen ($^1O_2$) (Triantaphylidès and Havaux, 2009). The transfer of one, two, or three electrons to O_2 forms a superoxide radical ($O_2^{\bullet -}$), H_2O_2 or a hydroxyl radical ($OH^{\bullet}$), respectively (Mittler, 2002). These are mainly synthesized by involvement of NADPH oxidases (termed respiratory burst oxidase homologs; RBOHs) and some oxidases and peroxidases, and in chloroplast, mitochondria, peroxisome, and other cellular compartments, via different pathways (Suzuki et al., 2011; Vaahtera et al., 2014; Gilroy et al., 2016; Mignolet-Spruyt et al., 2016). ROS generation (metabolically or for signaling purposes) and ROS scavenging is a continuous process and occurs in all cellular compartments of the cells. This process is controlled by the ROS gene network (Mittler et al., 2004). Overproduction of ROS causes oxidative stress. During oxidative stress the capacity of cellular defenses to remove these toxic species is lowered in comparison with their production. During stress, ROS generation is higher than ROS consumption (Ahmad et al., 2008, 2010a,b, 2011; Koyro et al., 2012). ROS also changes gene expression by modifying TFs. At lower or optimal concentrations, ROS function as secondary messengers in signaling cascades. Redox homeostasis in plants during stressful conditions is regulated by two mechanisms involving the enzymatic components comprising the superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione-S-transferase, peroxidase (POX) and catalase (CAT), and the nonenzymatic low molecular compounds like ascorbic acid (AA), reduced glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and proline (Gill and Tuteja, 2010; Miller et al., 2010).

8.2 REACTIVE OXYGEN SPECIES AS SIGNALING MOLECULES

ROS are accepted as central regulators of plant development and studies on tip growing systems indicated a main function for the NADPH oxidases in forming such developmentally significant ROS. Tip growing cells also revealed the role of cytosolic ROS in regulation of ion channel gating and are directly associated with ROS generation in the apoplast, where they act to alter the properties of the cell wall. The organization of ROS generation and their activities between compartments is budding as an imperative theme in understanding how growth and development programs are coordinated (Swanson and Gilroy, 2010). ROS plays an important function in the acclimation process of plants to abiotic stress conditions. They mainly function as signaling molecules that regulate various pathways during plant acclimation to stress (Choudhury et al., 2017). Abiotic stresses are coupled with the formation of chemical entities called ROS, that is, H_2O_2, O_2^-, OH^-, etc. They are able to induce cellular damages by breakdown of proteins, enzyme inactivation, changes in the gene, and inhibition in different pathways of metabolic significance. ROS behave as secondary messengers that signal important cellular roles, that is, cell proliferation, necrosis, and apoptosis (Choudhury et al., 2013). Under abiotic stress conditions, ROS production occurs that causes harm to normal functioning of plants. It also plays an important function in cellular damage and abiotic stress signaling. Plants contain various antioxidative mechanisms that deal with high ROS in the cells (Gautam et al., 2017). The participation of ROS in signal transduction indicates that there is a coordinated role of regulation networks to sustain ROS at nontoxic levels in a fragile balancing act between ROS generation and ROS-scavenging pathways, and to adjust ROS responses and following downstream processes (Mittler et al., 2004). Many studies from various plant species showed the ROS production and activity of different antioxidant enzymes enhanced during abiotic stresses (Damanik et al., 2010; Selote and Chopra, 2010; Tang et al., 2010; Turan and Ekmekci, 2011).

The ABA is the main regulator of abiotic stress resistance in plants, and regulates various stress responsive genes by a complex regulatory network so as to present tolerance to the environmental stresses (Cutler et al., 2010; Raghavendra et al., 2010). The induction of ABA stress tolerance is partly associated with activation of antioxidant defense systems, that is, enzymatic and nonenzymatic components, which provide protection to the plant cells against oxidative damage (Huang et al., 2012; Zhang et al., 2012, 2014a, b). Water stress-induced ABA enhancement and exogenous application of ABA inhibit the enhanced production of ROS, then leads to antioxidant system activation in crops (Jiang and Zhang, 2002; Ye et al., 2011). Various studies have showed that brassinosteroids (BRs) can activate antioxidant defense to enhance

stress tolerance in crop plants (Özdemir et al., 2004; Xia et al., 2009). Gibberellins (GAs) take part in the reaction of plants to abiotic stress and their action is linked with the management of growth by controlling cell elongation and division (Colebrook et al., 2014). GAs play their role through DELLA protein regulation, negative regulators of GAs signaling (Achard et al., 2006). The GAs binding with the nuclear receptor GID1 induces conformational variations in the proteins and favoring associations with DELLA proteins. As a result of this, DELLA is ubiquitinated and targeted for breakdown through 26S proteasome (Colebrook et al., 2014). The signaling of GA regulates stress tolerance by controlling the cellular redox homeostasis. Under water-deficit conditions the GA concentration was decreased in the maize and as a result there was enhancement in the activity of DELLA proteins that resulted in enhanced ROS quenching capacity and enhanced endurance (Wang and Li, 2008). Nitric oxide (NO) is the most important ROS and linked with various physiological processes in the plants (Niu and Liao, 2016). NO mediates the posttranslational amendments of marked proteins through S-nitrosylation and nitration. ABA induces the synthesis of NO and ROS under water-deficit conditions. Both NO and ROS form 8-nitro-cGMP and induce the closure of stomata (Joudoi et al., 2013). Auxin also induces the synthesis of ROS and NO, and both ROS and NO act in auxin-induced signaling (Yadav and David Bhatla, 2011; Farnese et al., 2016). In *Arabidopsis* Col-0 and gsnor1-3 (a mutant defective in protein denitrosylation), auxin signaling and auxin transport were correlated and Shi et al. (2015) reported that auxin signaling and polar auxin transport was decreased indicating the function of S-nitrosylation in auxin signaling. NO and ROS were studied to take part in osmotic tolerance of wheat seedlings by enhancing the biosynthesis of ABA and NO by protecting against oxidative damage (Misra et al., 2010).

ROS plays a significant function in development of plants and ROS that are formed by NADPH oxidases produce the O_2^{-2} (Segal and Abo, 1993). Application of inhibitors, that is, diphenylene iodonium (DPI), indicated that NADPH oxidases obtained by ROS controls development of cells in roots of maize showing that ROS helps in growth. DPI is a flavin enzyme inhibitor and when treated with DPI may also have influence in other protein activities in count to NADPH oxidases (Moulton et al., 2000; Liszkay et al., 2004). NADPH oxidases also play an important role in controlling the cell growth and there are some indications that these also participate in apical dominance and leaf shape (Sagi et al., 2004). The cell wall has an important function in the expansion of the cell, wall loosening, which allows the cells to enlarge, whereas cross-linking of wall reduces the expansion. There are facts that ROS take part in such processes. They are concerned with cell wall loosening in growing tissues and as the cell growth ceases and cells get differentiated the cell wall becomes hard (Gapper and Dolan, 2006).

ROS scavenging enzymes, that is, SOD, CAT, and APX play a significant role in the ROS-scavenging pathway. The existence of antioxidant enzymes and compounds in every cellular part indicates the significance of ROS detoxification for defense against stress conditions (Mittler et al., 2004). Small molecules, that is, Ca^{2+} and calmodulin (CaM), NO, and ROS have been observed to take part in the ABA-induced antioxidant defense system (Jiang and Zhang, 2003; Hu et al., 2007). In rice Ca^{2+}/CaM-dependent protein kinase, *OsDMI3* is significant for ABA-induced enhancement in the expression and SOD and CAT activities. ABA-induced H_2O_2 generation activates *OsDMI3* and their activation also increased H_2O_2 generation by enhancing the expression of NADPH oxidase genes (Shi et al., 2014). Moreover, it was also observed that *OsDMI3* plays a role upstream of *OsDMI1*, to regulate the antioxidant enzyme activities and in the generation of H_2O_2 in rice plants (Shi et al., 2014). ABA-induced H_2O_2 generation and ABA-induced activation of *OsMPKs* promotes the ZFP36 and ZFP36 expression and regulates the NADPH oxidase expression and MAPK genes and the H_2O_2 generation in ABA signaling (Zhang et al., 2014a,b). H_2O_2 molecules formed in the chloroplast can disperse outside the organelles and act as a signaling molecule in the cytoplasm, switching MAP-kinase cascade activation that activates nuclear genes, mainly the cytoplasmic APX encoding gene (Yabuta et al., 2004; Mubarakshina et al., 2010). It also participates in inducing the expression of light responsive genes. Therefore, treatment of plants with H_2O_2 enhanced expression of *APX2* (which encodes APX), and ZAT10 and ZAT12 (genes encoding TFs). However addition of CAT resulted in decreased *APX2* and *ZAT10* expression (Davletova et al., 2005).

Apart from chloroplasts, plant mitochondria are the major site for generation of ROS such as H_2O_2 and also the target of ROS (Rasmusson et al., 2004). Plant mitochondria have particular electron transport chain (ETC) components and roles in processes such as photorespiration. The mitochondrial ETC harbors electrons with high free energy for direct reduction of O_2, which is the inevitable main source of mitochondrial ROS production in aerobic respiration (Rhoads et al., 2006). Moreover, generation of ROS in mitochondria occurs under normal respiratory conditions but may be increased due to biotic and abiotic stress conditions. Complexes I and II are recognized as the site of $O_2^{\cdot-}$ generation and in aqueous solution $O_2^{\cdot-}$ is fairly reactive but it is reduced to H_2O_2 by SOD dismutation (Quan,

2008; Möller, 2001; Grene, 2002). Further H_2O_2 reacts with iron and copper to form highly toxic HO· and this uncharged HO· can enter through membranes and leaves the mitochondria (Rhoads et al., 2006). Abstraction of hydrogen atom by ROS, mainly by HO, starts peroxidation of mitochondrial membrane polyunsaturated fatty acid. The significance of this is the generation of cytotoxic lipid alkenals, aldehydes, hydroxyalkenals, etc. Nevertheless, plant mitochondria control production of ROS through energy dissipating systems and as result of these mitochondria play a key function in the adaptation of plants to abiotic stress conditions. Scarpeci et al. (2008) reported that methyl viologen induced production of O_2^- in *Arabidopsis thaliana* chloroplasts during active photosynthesis and indicate that O_2^- produced in photosynthetically active chloroplast leads to activation of genes that take part in the signaling pathways. C_3 and C_4 photosynthesis under salt stress conditions was reported and it was observed that amaranth plants unlike wheat plants were able to detoxify the O_2^- by SOD and amaranthine antioxidant and decreased the LPO intensity (Gambarova and Gins, 2008). At low concentrations H_2O_2 acts as a signaling molecule that takes part in acclamatory signaling inhibiting tolerance to biotic and abiotic stress conditions, and at high concentrations leads to programmed cell death (Quan, 2008). H_2O_2 also has been revealed to act as an important regulator in various physiological processes, that is, senescence, photorespiration, photosynthesis, stomatal movements, cell cycle, growth, and development (Bright et al., 2006; Peng et al., 2005; Mittler et al., 2004; Foreman et al., 2003; Noctor and Foyer, 1998). It is also accepted as a secondary messenger for the signals produced through ROS because of its comparatively long life and high permeability across the membranes (Quan, 2008).

8.3 REACTIVE OXYGEN SPECIES INVOLVED IN PLANT DEFENSE

Different types of biotic or abiotic stresses enhance the levels of ROS production, which further inactivates different antioxidants and enzymatic systems in plants. This causes oxidative stress in plants that illustrates the perquisite for initiation of the signaling response in plants for acclimation mechanisms (Jaspers and Kandasjarvi, 2010; Miller et al., 2010). ROS are defined as the group of different free radicals, ions, and molecules that play a crucial role as oxidation signaling molecules and are mainly derived from O_2 (Foyer and Noctor, 2009). On an estimate, around 1% of O_2 utilized by plants is directed for ROS production in different cell organelles such as peroxisomes, chloroplasts, and mitochondria (Sharma et al., 2012). Plants mainly possess three mechanisms for ROS production: (1) in ETC in mitochondria and chloroplasts; (2) different oxidases and peroxidases such as amine oxidase, lipoxygenase, NADH oxidase, xanthine oxidase, glycolate oxidase, and NADPH oxidase; and (3) chlorophyll containing molecules (Blokhina et al., 2003). In chloroplasts, photosynthetic products are the predominant ROS producers. For example, Rubisco enzyme catalyzing carboxylase−oxygenase reactions leads to both utilization and production of oxygen. Therefore, ETC in chloroplasts produces higher levels of oxygen, which leads to electron leakage that generates ROS. Moreover, chlorophyll coupled with photosensitizers also generates ROS by sunlight (Mittler, 2002). However, the principal producers of ROS are the mitochondria, which generate molecules such as H_2O_2 by reducing oxygen in aerobic respiration (Koyro et al., 2012; Ahmad et al., 2013). The signals initiated by ROS in these organelles promote transcriptional changes and reprogramming of cells, which either undergo cell death or cell protection (Foyer and Noctor, 2005; Fig. 8.1). Different types of ROS produced in the cells are hydrogen peroxide (H_2O_2), hydroxyl radical($OH^\bullet$), superoxide radical ($O_2^{\bullet -}$), and singlet oxygen ($^1O_2$), which are nontoxic until they react with the organic molecules to get activated (Apel and Hirt, 2004).

O_2 activation can occur by two different ways: (1) energy absorption to reverse the spin of unpaired electrons, or (2) reduction of monovalent ions in stepwise manner. By this act, the formation of $^1O_2$ takes place followed by its reduction to H_2O_2, OH and $O\bullet-_2$ (Apel and Hirt, 2004).

8.3.1 Process of Reactive Oxygen Species Signaling in Plants

As a consequence of abiotic stress a flux of calcium results in the cytosol of the plant cell. Calcium acts as a secondary messenger and directly activates RBOHs, and/or triggers a cascade of events that activate calcium-dependent protein kinases. These kinases phosphorylate and further activate RBOHs (Miller et al., 2009; Mittler et al., 2011; Dubiella et al., 2013; Gilroy et al., 2014). The activated RBOHs produce ROS at the apoplast, which is sensed by neighboring cells. It triggers the flux of calcium in these cells and results in the activation of their own RBOHs. ROS-derived calcium flux, coupled with activation of RBOHs by calcium, is then autopropagated from cell to neighboring cell throughout the entire plant, and triggers systemic responses to abiotic stress (Miller et al., 2009).

ROS generated as a result of environmental stress also results in the activation of mitogen activated protein kinases (MAPKs), which are highly conserved signaling pathway proteins that play an important role in

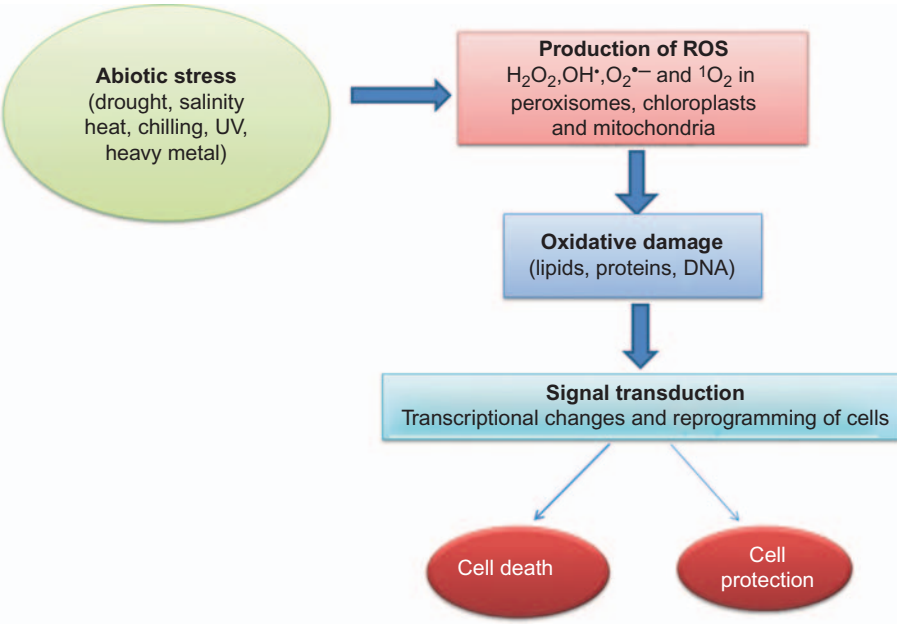

FIGURE 8.1 Abiotic stress induced ROS production and reprogramming of cells.

signal transduction under stress conditions. These are activated in response to abiotic stress such as drought, salt, and osmotic stress. MAPKs phosphorylate specific serine/threonine residues to regulate various cellular functions and activities (Fig. 8.2). In the MAPKs pathway, the MAPK cascade consists of three functionally interlinked protein kinases, MAPK kinase kinase [MAPKKK], MAPK kinase [MAPKK], and MAPK (Ho, 2015). The activated MAPKKK phosphorylates and activates MAPKK, which further phosphorylates and activates a MAPK, and results in the activation of specific signaling molecules such as TFs to induce cellular responses from the nucleus of a cell. These phosphorylation cascades work either upstream or downstream of ROS (Asai et al., 2002)

8.4 REACTIVE OXYGEN SPECIES-MEDIATED DAMAGE TO MACROMOLECULES

When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress." High levels of ROS can cause damage to lipids, proteins, and DNA (Valko et al., 2006) leading to aging and cell death (Ashraf, 2009).

8.4.1 Lipids

On exceeding threshold limit, the ROS increases lipid peroxidation in cell membranes ultimately affecting normal cellular function. Lipid peroxidation produces lipid-derived radicals leading to enhanced oxidative stress, which eventually damages proteins and nucleic acids. Plants growing under environmental stress reveal increased lipid peroxidation consecutively corresponding to aggravated levels of ROS. One of the final products of lipid peroxidation is malondialdehyde (MDA). Two common sites of ROS attack on the phospholipid molecules are the ester linkage between glycerol and the fatty acid and the unsaturated (double) bond between two carbon atoms. The polyunsaturated fatty acids (PUFAs) present in membrane phospholipids are predominantly susceptible to attack by ROS. A single •OH can result in peroxidation of many polyunsaturated fatty acids as the reactions involved in this process are part of a cyclic chain reaction (Sharma et al., 2012).

In general, the process of lipid peroxidation involves three discrete stages: (1) initiation, (2) progression, and (3) termination. The initial phase comprises activation of O_2, which is a rate limiting step. $O_2^{\bullet-}$ and •OH can react with methylene groups of PUFA forming lipid peroxy radicals, conjugated dienes, and hydroperoxides (Smirnoff, 1995).

1. Initiation step:

$$PUFA - H + X^{\bullet} \rightarrow PUFA + X - H$$
$$PUFA + O_2 \rightarrow PUFA - OO^{\bullet}$$

2. Progression step: The peroxy radical formed is highly reactive and propagates the chain reaction.

$$PUFA - OO^{\bullet} + PUFA - OOH \rightarrow PUFA - OOH + PUFA^{\bullet}$$

3. Termination step: The formation of conjugated diene occurs when free radicals attack the

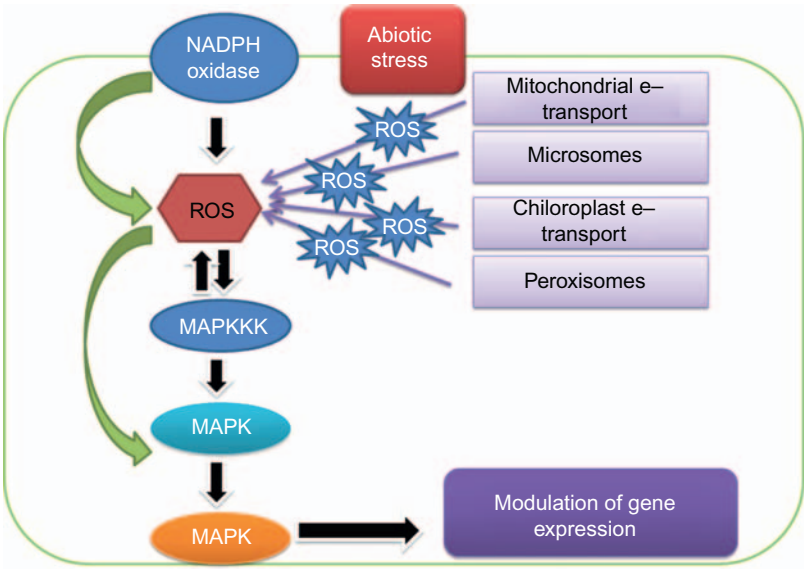

FIGURE 8.2 Mitogen activated protein kinases (MAPK) signaling pathway abiotic stresses. Source: *Adapted from Jalmi and Sinha (2015).*

hydrogens of methylene groups separating double bonds thereby, rearranging the bonds (Recknagal and Glende, 1984). The lipid hydroperoxides produced (PUFA-OOH) can undergo reductive cleavage by reduced metals (e.g., Fe^{2+}) as follows:

$$Fe^{2+}\text{complex} + \text{PUFA} - \text{OOH} \rightarrow \text{Fe3} + \text{complex} + \text{PUFA} - \text{O}^{\bullet}$$

Numerous reactive species such as lipid alkoxyl radicals, malonyldialdehyde, crotonaldehyde, acrolein, alkanes, lipid epoxides, and alcohols can be simply formed by the decomposition of lipid hydroperoxide (Davies, 2000). The lipid alkoxy radical produced (PUFA-O•) can initiate additional chain reactions (Buettner, 1993).

$$\text{PUFA} - \text{O}\bullet + \text{PUFA} - \text{H} - \rightarrow \text{PUFA} - \text{OH} + \text{PUFA}\bullet$$

Peroxidation of polyunsaturated fatty acid by ROS can cause chain breakage leading to increased membrane permeability and fluidity.

8.4.2 Proteins

ROS can aggravate reversible or irreversible alterations of proteins, consecutively causing modifications in the regulation of plant metabolism, as well as the activation of transcriptional regulatory networks. Due to augmented ROS production, fragmentation of the peptide chain, aggregation of cross-linked reaction products, site-specific amino acid modification, and increased susceptibility of proteins to proteolysis occur. Tissues damaged by oxidative stress generally contain increased concentrations of carbonylated proteins, which are extensively used as a marker of protein oxidation (Moller and Kristensen, 2004). Diverse stresses in plants cause enhanced modification of proteins (Tanoua et al., 2009a,b). The amino acids in a peptide differ in their propensity to attack by ROS. The most vulnerable residues to oxidation are the sulfur containing methionine and cysteine. The thiol of cysteine may be oxidized by hydroxyl radicals, superoxide, and hydrogen peroxide to a disulfide that can be readily reversible. Oxidation of methionine in numerous proteins has a minute effect on protein structure and function. An example of the reversible oxidation of methionine is the inactivation of the small heat shock protein in chloroplasts that is reactivated by thioredoxin in reaction catalyzed by methionine sulfoxide reductase (Gustavsson et al., 2002). Tyrosine oxidation can modify residue hydrophobicity with subsequent effect on protein structure. Oxidation of tryptophan is an irreversible protein modification (Rinalducci et al., 2008). Another example of irreversible protein modification is oxidation of iron–sulfur centers by $O_2^{\bullet -}$. It has been suggested that protein oxidation could predispose it to ubiquitination consequently making it a target for proteasomal degradation (Cabiscol et al., 2000).

8.4.3 DNA

ROS induces severe damage to DNA, including cross-links, base deletion, deoxyribose oxidation, strand breaks, pyrimidine dimers, and base modifications such as oxidation and alkylation (Tuteja et al., 2001). ROS can cause oxidative damage to nuclear, chloroplastic, and mitochondrial DNA. Moreover, changes in the nucleotides of one strand can cause mismatches with the nucleotides in the other strand

causing subsequent mutations. Any damage to the DNA can lead to changes in the encoded proteins, which may result in malfunctions or complete inactivation of the encoded proteins. Both the sugar and base moieties of DNA are susceptible to oxidation by ROS. It has been reported that $OH^{\bullet}$ is most reactive and can cause damage to all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose sugar backbone (Halliwell and Gutteridge, 1999). $^1O_2$ primarily attacks guanine; H_2O_2 and $O_2^{\bullet}$ do not react at all (Wiseman and Halliwell, 1996). DNA damage results in various physiological effects, such as cell membrane destruction, reduced protein synthesis, and damage to photosynthetic proteins, ultimately affecting growth and development of the whole organism (Britt, 1999). DNA damage can result either in arrest or induction of transcription, stimulation of signal transduction pathways, replication errors, and genomic instability (Cooke et al., 2003). The major type of DNA damage caused by exposure to UV-B is the formation of dimers between neighboring pyrimidines. UV photoproducts consist primarily of 6-4PPs dimers and cyclobutane pyrimidine dimers (Tuteja et al., 2009). Excessive changes caused by ROS lead to permanent damage to the DNA with potentially detrimental effects for the cell.

8.5 REACTIVE OXYGEN SPECIES PRODUCTION AND INTRACELLULAR PROTEIN OXIDATION

During photosynthesis and respiration process, ROS lead to protein oxidation of carbonyl (CO) groups. Among the 20 amino acids of the protein, several amino acids can be directly modified by means of side-chain reactions with ROS. Most susceptible amino acids are those having sulfhydryl groups and those with aromatic side-chain groups. The condition of aromatic side-chain amino acids, including tryptophan, phenylalanine, ROS-induced oxidation starts during an array of intermediates (Moller et al., 2007; Foyer and Noctor, 2009). For example, the oxidation of phenylalanine residues leads to the development of mono- and dihydroxy derivatives but tryptophan residues are transformed into numerous hydroxy derivatives. Furthermore, histidine residues can be oxidized to 2-oxohistidine and 4-OH-glutamate, however tyrosine residues are converted to a dihydroxy derivative, dopamine (DOPA), nitrotyrosine, chlorotyrosin, and a dityrosine derivative. The carbonyl groups (aldehydes and ketones) are also produced on protein side chains (especially of proline, arginine, lysine, and threonine) where they are oxidized. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins either by the α-amidation pathway or by oxidation of the glutamyl side chain. The nucleophilic side chains of Cys, His, and Lys residues may be formed when the CO group is introduced into proteins. The carbonyl group can further react with α-amino group of lysine residues, which leads to the formation of intra- or intermolecular cross-links promoting protein aggregation (Davies, 2000; Dietz, 2014; Waszczak et al., 2015).

Both Met and Cys play crucial roles in cell metabolism. Met is mostly found in globular protein in the interior membrane-spanning protein domains. These are liable to oxidation to MetO residues (Waszczak et al., 2015, Miki and Funaro, 2012). The Cys residues provide a lot of functions, such as catalysis, stabilization of protein arrangement during metal binding, disulfides, and regulation of protein role. Cys residues also focus on many posttranslational modifications (Fig. 8.3). Reversible oxidation of Cys thiols is known to participate in redox regulation of proteins by means of the formation of sulfenic acid intermediates (RSOH), inter- and intramolecular disulfide bonds (R−S−S−R), diverse disulfide bond with glutathione (R−S−SG), and overoxidation to sulfinic acids ($R\text{-}SO_2H$) (Miki and Funaro, 2012). These redox-derived changes in protein function can affect transcription, phosphorylation, and other important signaling events, and/or alter metabolic fluxes and reactions in the cell by altering enzymatic properties (Mittler, 2017). The studies of Gulyas et al. (2017) also provided evidence about the redox control of free amino acid profile in wild-type and ascorbate or glutathione deficient mutant *A. thaliana* plants before and after hydroponic treatment with various redox agents. As a consequence of excessive ROS production, site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge, and increased susceptibility of proteins to proteolysis occur. Tissues injured by oxidative stress generally contain increased concentrations of carbonylated proteins, which are a widely used marker of protein oxidation. Enhanced modification of proteins has been reported in plants under various stresses (Niu and Liao, 2016; Liu and He, 2017; Mittler, 2017).

8.5.1 Reactive Oxygen Species-Induced Posttranslational Modifications

Redox reactions on metabolic processes can alter a wide variety of downstream protein targets by influencing key regulators of distinct PTMs, such as phosphorylation, acetylation, and ubiquitination. Such modifications include components that control various metabolic rates, that is, AMP-activated protein kinase,

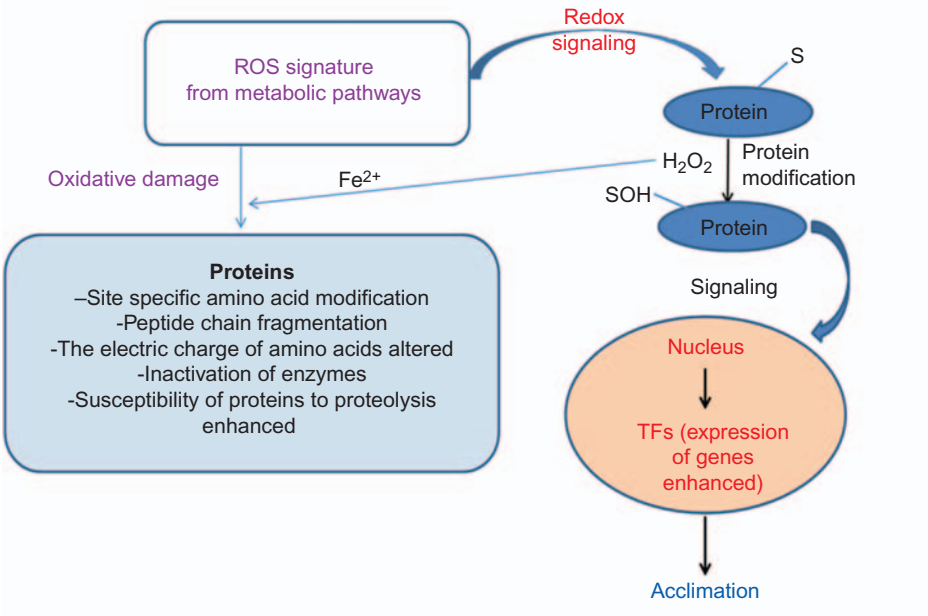

FIGURE 8.3 Oxidation of cysteine residues on proteins by H_2O_2, affecting their structure and function and triggering/regulating cellular signaling pathways (Mittler, 2017).

protein kinase C, adenylate kinase, and pyruvate kinase (Corcoran and Cotter, 2013). In particular redox modifications of cysteine (thiol) residues have been extensively studied and can result in reversible and irreversible modifications with effects on protein function (Fig. 8.3). Fortunately, a lot of developments have been made in the area of redox proteomics to enhance the determination of alterations to proteins under conditions of stress. At the same time the oxidized products can be important secondary signaling molecules, and in such cases damage and signaling are two sides of the same story, which can either coexist or cause severe imbalances (Fig. 8.3). The stress of ROS also causes modification of proteins in a variety of ways, that is, direct and indirect. In direct modifications, modulation of a protein's activity occurs through S-nitrosylations, carbonylation, disulfide bond formation (sulfonylation), and glutathionylation. However, by indirect process, proteins can be modified by interaction of conjugation with byproducts of fatty acid peroxidation (Choudhury et al., 2017).

8.5.1.1 S-Nitrosylation

The effects of nitric oxide (NO) on signaling and wide metabolic pathways are recognized as S-nitrosylation (or nitrosation) on key cysteine residues proteins (McDonagh, 2017). The formation of S-nitrosylation is the process when the covalent binding of NO to thiol groups of Cys protein occurs through $ONOO^-$ and there are also reports of transfer of nitrosyl or transnitrosylation by the actions of proteins, such as thioredoxins (Benhar, 2015). S-nitrosylation on target proteins is considered an important mechanism for NO signaling transduction, posttranslational modification that can regulate the function of some proteins during stress; however as this PTM is reversible and highly labile PTM, it has been suggested as an intermediate in the formation of disulfide bonds (Wolhuter and Eaton, 2017). Camejo et al. (2013) showed that different enzymes involved in respiration, antioxidation, and photorespiration were S-nitrosylated during salinity stress. In plants subjected to low temperatures, the main S-nitrosylated proteins were those related to C metabolism (Puyaubert et al., 2014). S-Nitrosylation of proteins is essential for metabolic reprogramming that is necessary to keep homeostasis under stress conditions. S-Nitrosylation also induces changes in some TFs, which affect their binding to DNA, as well as inactivate RBOH (Yun et al., 2011). For example, S-nitrosylation can act as a negative regulator of MYB TFs, which are essential regulators of abiotic stress responses (Tavares et al., 2014).

8.5.1.2 Protein Carbonylation

Protein carbonylation is an irreversible PTM associated with the oxidation of residues, such as Arg, His, Lys, Pro, and Thr (Choudhury et al., 2017). Carbonylation of proteins can also be induced by indirect reactions of lipoperoxidation products with Cys and His residues of proteins (Madian and Regnier, 2010) and even with glycation/glycoxidation (for Lys) process. A broad range of carbonylation proteins in plants and other organisms have been studied, and various quantification techniques developed in recent years are extensively reviewed (Fedorova et al., 2014; Rogowska-Wrzesinska et al., 2014). Choudhury et al. (2017) highlighted that mitochondrial enzymes such as aconitase, pyruvate dehydrogenase, and glycine

decarboxylase are sensitive to inactivation by oxidation and carbonylation, and these enzymes might be inhibited by an increase in ROS production that results in slowing down the Tricarboxylic acid cycle (TCA) cycle and consequently decreasing the energy status of the cell (Schwarzlander and Finkemeier, 2013; Camejo et al., 2015). However, some studies suggested that the pattern of (chloroplast) protein carbonylation in *Arabidopsis* is distinct from that in nonphotosynthetic eukaryotes, due to the mechanism that carbonylation first increases with age but drops abruptly prior to the vegetative-to-reproductive transition and does not coincide with senescence (Johansson et al., 2004). These findings propose that old leaves rid themselves of oxidized proteins prior to bolting. We speculate that this is due to the targeted removal of organellar content through a combination of chlorophagy, Rubisco-containing bodies, and senescence-associated vacuoles (Wang and Blumwald, 2014; Van Wijk, 2015; Xie et al., 2015). There are multiple protein carbonylation studies investigated with various plant species mainly focusing on chloroplasts and mitochondria in leaves as well as seed biology and fruit ripening (Lounifi et al., 2013). Carbonylated proteins have been detected especially in cytosol and mitochondria (Smakowska et al., 2014), and chloroplasts are involved in various metabolic pathways, protein folding, and other processes. However, within these pathways, carbonylation is not evenly distributed, and specific proteins appear preferentially targeted for this PTM. It was also proposed that there is cross-talk between ROS-induced carbonylation and reactive nitrogen species-induced protein nitrosylation, particularly under biotic and abiotic stress (Lounifi et al., 2013).

8.5.1.3 Sulhydryl Oxidations of Met and Cys (Sulfonylation)

Sulfonylation is one of the main mechanisms that cause the oxidation of sulfhydryl groups by H_2O_2 generating sulfenic acid (R-SOH) (Fig. 8.3). This process can initiate to the formation of disulfide (S−S) bonds between cysteine residues, which in turn results in conformational changes and protein/enzyme activity alteration. The "recovery" of a protein from this oxidized state is mainly mediated via thioredoxins (Trxs), peroxiredoxin (PRXs), and the glutathione (GSH) system in response to stress. The previous studies indicated that several enzymes of the Calvin cycle [Fru-1,6-bisphosphatase (FBPase)] are regulated, reduced, and are active in the light whereas they are oxidized and inactive in dark conditions (Scheibe et al., 2005; Balsera et al., 2014; Dietz and Hell, 2015). Choudhury et al. (2017) reviewed that regulation via reduced Trx prevents a waste of energy by activation of enzymes such as FBPase and seduheptulose−bisphosphatase in the reductive cycle and a parallel inactivation of the Glc-6-P dehydrogenase found in the oxidative cycle in the light. However, in the dark, Trx becomes oxidized and the opposite situation becomes predominant. The reduction state of Trx creates a conditional separation of metabolic fluxes within the same compartment.

In addition to the nonenzymatic oxidation of Cys, a small set of plant Cys oxidases (PCOs) is enzymatically oxidizing N-terminal Cys in the case of several ethylene response factor (ERF)-VII TFs, generating a sulfenic acid PTM (Weits et al., 2014). The PTM turns these TFs into a substrate for arginyl transferases, ultimately leading to polyubiquitination and proteasomal degradation (Van Dongen and Licausi, 2015). In contrast to Cys oxidation, another type of sufonylation is known for Met oxidation and its impact on plants. Nevertheless, a study in *Arabidopsis* identified and quantified hundreds of Met oxidation sites induced by H_2O_2 generated in situ in the peroxisomes through enhanced photorespiration and in a peroxisomal catalase mutant (Jacques et al., 2015). A family of Met sulfoxide reductase enzymes reduced the oxidation of Met by Trx, thus repairing these oxidized (damaged) proteins.

8.5.1.4 Sulfur Glutathionylation

Tripeptide glutathione (γ-glutamylcysteinyln-glycine, GSH) is one of the crucial low molecular weight nonprotein thiols that plays an important role in intracellular defense against ROS-induced oxidative damage. It gains a physiological importance in buffering the GSSG/GSH pool as well as having additional regulatory functions against oxidative stress and maintaining thiol homeostasis (Zaffagnini et al., 2012a; Rouhier et al., 2015). Due to its reducing power, GSH plays an important role in diverse biological processes. GSH can form a disulfide bridge with available free thiol on a protein and forms a protein named S-glutathionylation. Several plant enzymes have been shown to undergo glutathionylation, in particular in mitochondria and chloroplasts but also in the cytosol (Zaffagnini et al., 2012b). The most extensive glutathionylation proteomics study in photosynthetic organisms was done for *Chlamydomonas reinhardtii*, showing that many metabolic pathways (e.g., nucleotides, thiamine, chlorophyll, fatty acid, and respiration) are targets for this PTM (Zaffagnini et al., 2012a). Protein glutathionylation can be regarded a posttranslational modification mechanism with significant effects on the various enzyme activities and TFs. However, even with lot of these recent findings, protein glutathionylation in plants still remains as one of the poorly studied aspects in abiotic stress response.

8.5.1.5 Acetylation

Protein acetylation is a posttranslational regulatory mechanism that regulates gene expression under redox state. The previous studies revealed that protein acetylation occurs in two diverse forms: N-terminal acetylation and Lys acetylation. N-terminal acetylation is a nonreversible Nα-terminal modification that results in the loss of a positive charge and is carried out by N-terminal acetyl transferases (the NAT family). It emerges that these NATs can also acetylate the ε-amine of Lys (Starheim et al., 2012). It was proved that N-terminal acetylation is positively correlated to the levels of acetyl-CoA and involves cellular metabolic state to this PTM. The majority of proteins are N-acetylated and it was proved by Ferrandez-Ayela et al. (2013) that a NAT-A loss-of-function in *Arabidopsis* mutant showed pleiotropic developmental and growth defects in *Arabidopsis*, however a loss of function of NAT-C illustrated reduced plant growth and photosynthetic capacity (Pesaresi et al., 2011). Recently, Dinh et al. (2015) identified a chloroplast NAT enzyme (AtNAA70) in *Arabidopsis* and showed N-a-acetylation when expressed in *Escherichia coli*, in particular for Met, Ala, Thr, and Ser. However, Lys acetylation is reversible ε-amino group modification of lysine (K) by K acetyl transferases (KATs) and K deacetylases (KDACs) (Friso and Van Wijk, 2015). Histones are one of the target proteins of KATs and KDACs. Studies of crop acetylome were performed in rice, wheat, and soybeans (Nallamilli et al., 2014; Smith-Hammond et al., 2014; Zhang et al., 2016). It was highlighted that a wide range of cellular processes were affected by K acetylation from metabolic processes, signal transduction, RNA processing, protein translation, and stability. Proteins related to cell death were included among 44 proteins identified as acetylated proteins in rice. This was followed by a study in wheat, another important cereal crop (Zhang et al., 2016). As described above, protein acetylation controls the metabolic adaptations via modifying nonhistone metabolic enzymes. Furthermore, it participates in the shift of energy production via changing gene expression.

PTMs is the dynamic nature of the modifications in cell signaling and potential cross-talk between redox and nonredox dependent PTMs that regulate protein activity. Including an increased number of potential PTMs combined with targeted postanalysis would increase sensitivity and provide a comprehensive overview on the role of ROS-induced protein modifications and their role in stress acclimation in cellular signaling (Friso and van Wink, 2015; McDonagh, 2017; Choudhury et al., 2017).

8.6 ROLE OF ANTIOXIDANTS AND ITS SIGNALING IN ABIOTIC STRESS

Plants have a variety of constitutively expressed antioxidant defense mechanisms to scavenge the ROS generated during abiotic stress. The importance of the cellular antioxidant machinery in protection against various stresses is emphasized by many researchers (Dalton et al., 1999, Tuteja, 2007, 2009). Plants produce antioxidants, which can scavenge ROS. Antioxidants are of two types: enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants include catalase (CAT), SOD, peroxidase (POX), APX, monodehydroascorbate reductase (MDHAR or MDAR), dehydroascorbate reductase (DHAR or DAR), and glutathione reductase (GR) (Ahmad et al., 2008, 2010a,b, 2011). The nonenzymatic antioxidants are glutathione (GSH), ascorbate (AsA), carotenoids, tocopherols, flavones, and anthocyanins (Gupta et al., 2005). These enzymes are located in different plant cells and work together to detoxify ROS. Enzymatic antioxidants containing SOD, CAT, APX, POX, GR, and MDAR decrease the levels of O_2 and H_2O_2 in plants (Ahmad et al., 2013; Rasool et al., 2013). Under stress conditions, SOD forms the first line of defense against ROS-induced damages. The SOD removes $O^{\bullet-}_2$ by catalyzing its dismutation into O_2 and H_2O_2. APX reduces H_2O_2 to H_2O and (dehydroascorbate) DHA, using AA as a reducing agent. DHAR reduces dehydroascorbate (DHA) to AA by using reduced glutathione (GSH) as an electron donor and GSH is converted to its oxidized form (GSSG) (Eltayeb

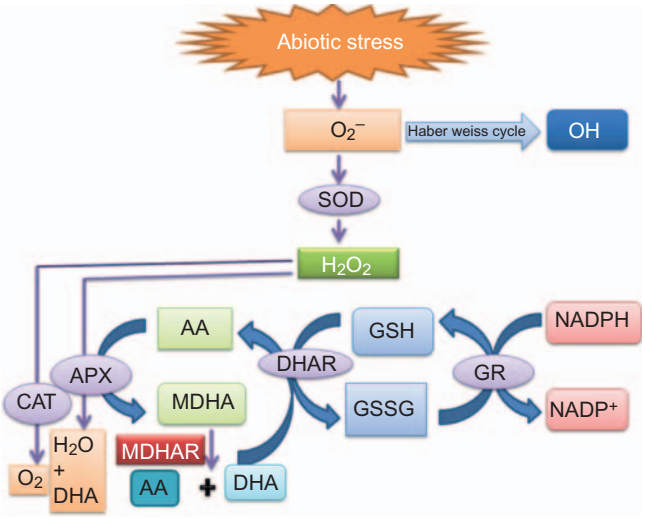

FIGURE 8.4 Antioxidant defense mechanism. Source: *Adapted from Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. doi:10.1016/j.plaphy.2010.08.016 and Choudhury, S., Panda, P., Sahoo, L., Kumar, S., Panda, S.K., 2013. Reactive oxygen species signalling in plants under abiotic stress. Plant Signal Behav. (8)4, e23681.*

et al., 2007). The organellar redox state is maintained by different enzymatic antioxidants like GR and MDHAR. MDHAR regenerates AA from the short-lived monodehydroascorbate (MDHA) and ultimately replenishes the cellular AA pool. GR reduces GSSG to GSH by using NADPH as a reducing agent (Fig. 8.4). The role of various antioxidants in plants during abiotic stress is given in Table 8.1.

TABLE 8.1 Antioxidant Defense Mechanism in Various Plant Species During Abiotic Stress

Plant	Stress	Stress imposition	Antioxidant activity	References
Solanum lycopersicum	Low temperature	15°C-Day 8°C-Night	Enhanced activities of SOD, APX, MDHAR, DHAR	Liu et al. (2018)
Passiflora edulis	Aluminum	0.2 mM Al^{3+} 0.2 mM Al^{3+} For 10 days	SOD activity increased	Preeti et al. (2018)
Zea mays	Drought	80% FC 60% FC 40% FC	SOD, CAT, and POX activity decreased with severity of drought	Anjum et al. (2017)
Nicotiana tobacum	H_2O_2, salt, and drought	1% H_2O_2, 300 mM NaCl, 30% PEG	Overexpression of DHAR	Chang et al. (2017)
Citrullus lanatus	Drought	30% FC for 12 days	SOD, CAT, APX, GR, and MDHAR	Mo et al. (2016)
Cicer arietinum	Salt	50–100 mM NaCl 25-day-old plant to 70-day-old plant	SOD, APX, CAT, GR activities increased	Ahmad et al. (2016)
Lycopersicon esculentum	Low temperature	10°C-Day 3°C-Night 12°C-Day 7°C-Night 20°C-Day 14°C-Night 25°C-Day 18°C-Night	SOD, CAT activity increased. Proline content also increased in leaves with decreasing temperature	Khan et al. (2015)
Triticium aestivum	Osmotic	PEG-6000 for 5 days	SOD, GR, APX activity increased	Naderi et al. (2014)
Limonium sinense	Salt	50 mL of 500 mM/L NaCl solution for 7 days	Increased activity of SOD, POX, CAT. MDA content increased in seedlings	Zhang et al. (2014a, b)
Triticum aestivum	Cold	5°C-Day 2°C-Night for 3 days	Increased activity of SOD, POX, GR, APX	Turk et al. (2014)
Eichhornia crassipes	Lead	Seedlings were treated with different concentrations of Pb $(NO_3)_2$ (100, 200, 400, 600, 800, 1000 mg/L) for 10 days	Increased activity of SOD, APX, POX. MDA content increased with increasing concentration of lead	Malar et al. (2014)
Vigna radiate	Lead and salt	Lead chloride 70 mM Lead nitrate 150 mM NaCl 225 mM	SOD, APX, GPX, GR activity increased	Siddiqui (2013)
Oryza sativa	Copper	8 μM copper for 3 days	Upregulation POX, APX, and DHAR	Song et al. (2013)

(*Continued*)

TABLE 8.1 (Continued)

Plant	Stress	Stress imposition	Antioxidant activity	References
Canna edulis	Drought	Drought stress for 35 days	MDA content increased, SOD, POX, CAT activities first increased and then decreased	Zhang et al. (2013)
Solanum tuberosum	Drought	Drought conditions maintained for 2 weeks	Increased activity of POX and SOD	Boguszewska et al. (2010)
Cassia auriculata	UV-B	50 and 100 min exposure to UV-B source	Enhanced SOD, CAT activity	Agarwal (2007)
T. aestivum	Cadmium	100 mg Cd/kg soil	Increased GR activity	Khan et al. (2007)

8.7 CONCLUSION

Various abiotic stresses lead to the overproduction of ROS in plants, which are highly reactive and result in oxidative stress. These species affect cell membrane properties and cause oxidative damage to nucleic acids, lipids, and proteins and make them nonfunctional. The plant cell and its organelles like chloroplast, mitochondria, and peroxisomes have antioxidant defense systems to overcome ROS-induced oxidative stress. The antioxidant defense mechanisms include enzymatic antioxidants and nonenzymatic antioxidants. ROS also acts as a signaling molecule and has capacity to regulate the downstream signaling pathway components and to impart a specific response toward a particular stress. Persistent research is required to understand the mechanisms regulating ROS signaling pathways and their interplay during abiotic stresses.

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CHAPTER

9

Role and Regulation of Plants Phenolics in Abiotic Stress Tolerance: An Overview

Mohd Irfan Naikoo[1], *Mudasir Irfan Dar*[1], *Fariha Raghib*[1], *Hassan Jaleel*[2], *Bilal Ahmad*[2], *Aamir Raina*[3,4], *Fareed Ahmad Khan*[1] *and Fauzia Naushin*[3]

[1]Plant Ecology and Environment Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India [2]Plant Physiology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India [3]Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India [4]Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

OUTLINE

9.1 INTRODUCTION

Plants are exposed to multifarious abiotic stresses in constantly changing environments that are unfavorable for growth and development (Zhu, 2016). These abiotic stresses include water (drought and flooding), heavy metals, salinity, excess or deficiency of nutrients, high and low temperatures (chilling and freezing), extreme levels of light (high and low), radiation (UV-B and UV-A; ultraviolet, UV), ozone, sulfur dioxide, mechanical factors, and other less frequently occurring stressors (Pereira, 2016). Plants being rooted in the environment they grow in have to adapt with the changing conditions due to abiotic stresses and the accumulation of phenolics in plant tissues are considered as an adaptive response of plants to these adverse environmental conditions (Pereira, 2016; Lattanzio, 2013).

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Plants synthesize an enormous number of chemicals categorized as primary and secondary metabolites. The primary metabolites, that is, sugars, fatty acids, amino, and nucleic acids being essential for plant growth and development are ubiquitously distributed in all plants (Fiehn, 2002; Wu and Chappell, 2008). Secondary metabolites being much more diverse than the primary metabolites structurally as well as chemically are the specialized compounds that are not directly essential for basic plant metabolism but are required by plants for survival in the environment.

Plant phenolics or polyphenols are the most widely occurring groups of secondary metabolites with substantial physiological and morphological importance in plants. They are aromatic compounds with one or more hydroxyl groups and emerge from shikimate/phenylpropanoid pathway or polyketide acetate/malonate pathway, producing monomeric and polymeric phenols and polyphenols (Randhir et al., 2004). Plant phenolics play an important role in plant growth, development, and reproduction, and a key role as defense compounds against abiotic stresses, such as high light, low temperatures, UV-B radiations, heavy metals and nutrient deficiency (Lattanzio, 2013), protection against pathogens and predators (Bravo, 1998), producing color and sensory characteristics of fruits and vegetables (Alasalvar et al., 2001), besides exhibiting other essential properties like antiallergenic, antimicrobial, and antioxidant activity (Balasundram et al., 2006). Plant phenolics or polyphenols are the most widely distributed secondary metabolites and predominate in the plant kingdom. Bacteria, fungi, and algae produce peculiar phenolic compounds whereas bryophytes are regular producers of polyphenols like flavonoids, but in the vascular plants a full range of phenolic compounds or polyphenols are found (Swain, 1975; Harborne, 1980). An estimation of about 2% of all the carbon photosynthesized by plants is converted into phenolic compounds (Robards and Antolovich, 1997). Several thousand phenolic compounds are known to be synthesized by higher plants and the characterization of these compounds is continuously increasing. Plant leaves contain esters; amides and glycosides of hydroxycinnamic acids (HCAs); glycosylated flavonoids, particularly flavonols; and proanthocyanidins and their derivatives. Lignin, suberin, and pollen sporopollenin are some other polyphenolic polymers. Some soluble phenolics like chlorogenic acid are widely distributed, whereas some are restricted to specific genera or families, thereby are handy biomarkers for taxonomic studies.

9.2 STRUCTURE AND CLASSIFICATION

Structurally, phenolic compounds contain an aromatic ring with one or more hydroxyl substituents attached to it, ranging from simple phenolic molecules to highly polymerized compounds, thereby showing great structural diversity, and are often referred to as polyphenols (Bravo, 1998). Most phenolic compounds naturally appear as conjugates with mono- and polysaccharides, coupled with one or more phenolic groups, and may also exist as functional derivatives like esters and methyl esters (Harborne, 1989; Harborne et al., 1999; Shahidi and Naczk, 1995).

Phenolics though a very large and diverse group of chemical compounds can be classified in a number of ways. Harborne and Simmonds (1964) classified them into different groups based on the number of carbons in the molecule (Table 9.1).

9.3 BIOSYNTHESIS OF POLYPHENOLS

Plant phenolics are biosynthesized in plants from a biosynthetic intermediate, phenylalanine and shikimic acid through the shikimic acid pathway (Fig. 9.1). The starting metabolites of the pathway are erythose-4-phosphate and phosphoenolpyruvate (PEP), which are intermediates of pentose phosphate pathway (PPP) and glycolysis, respectively.

The first step involves the conversion of glucose in the PPP to glucose-6-phosphate and then irreversibly to ribulose-5-phosphate by the aid of glucose-6-phosphate dehydrogenase (G6PDH). The PPP advances to produce erythrose-4-phosphate. Similarly from glycolysis, phosphoenolpyruvate is generated, which is then used together with erythrose-4-phosphate through the phenylpropanoid pathway to generate phenolic compounds after being channeled to the shikimic acid pathway to produce phenylalanine (Fig. 9.1).

9.4 PHENOLICS AND ABIOTIC STRESS TOLERANCE

The great degree of interactions between plants and their changing environments has been a major driving force behind the emergence of specific natural products (Lattanzio, 2013). In this regard, the accumulation of phenolics in plant tissues is considered as an adaptive response of plants to adverse environmental conditions, thereby expanding evolutionary fitness. This accumulation resulted due to the activity of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and other enzymes. Activity of PEP-carboxylase also increases, indicating a shift from sucrose production to the processes of defense and repair. Plant phenolics confer various physiological functions for survival and adaptation to environmental disturbances (Landolt et al., 1997; Andersen, 2003;

TABLE 9.1 Classification of Phenolic Compounds

Class	No. of C-atoms	Structure	Occurrence
Simple phenolics, benzoquinones	6	C6	Rare to common
Phenolic acids and related compounds	7	C6−C1	Common
Acethophenones, phenylacetic acids	8	C6−C2	Rare
HCAs, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	9	C6−C3	Rare to common
Napthoquinones	10	C6−C4	
Xanthones	13	C6−C1−C6	Rare
Stilbenes, anthraquinones	14	C6−C2−C6	Rare
Flavonoids, isoflavonoids	15	C6−C2−C6	Common
Betacyanins	18		Rare
Lignans, neolignans	18	$(C6-C3)_2$	
Biflavonoids	30	$(C6-C3-C6)_2$	Rare
Lignin		$(C6-C3)_n$	Common
Melanins	N	$(C6)_n$	
Condensed tannins (proanthocyanidins flavolans)		$(C6-C3-C6)_n$	

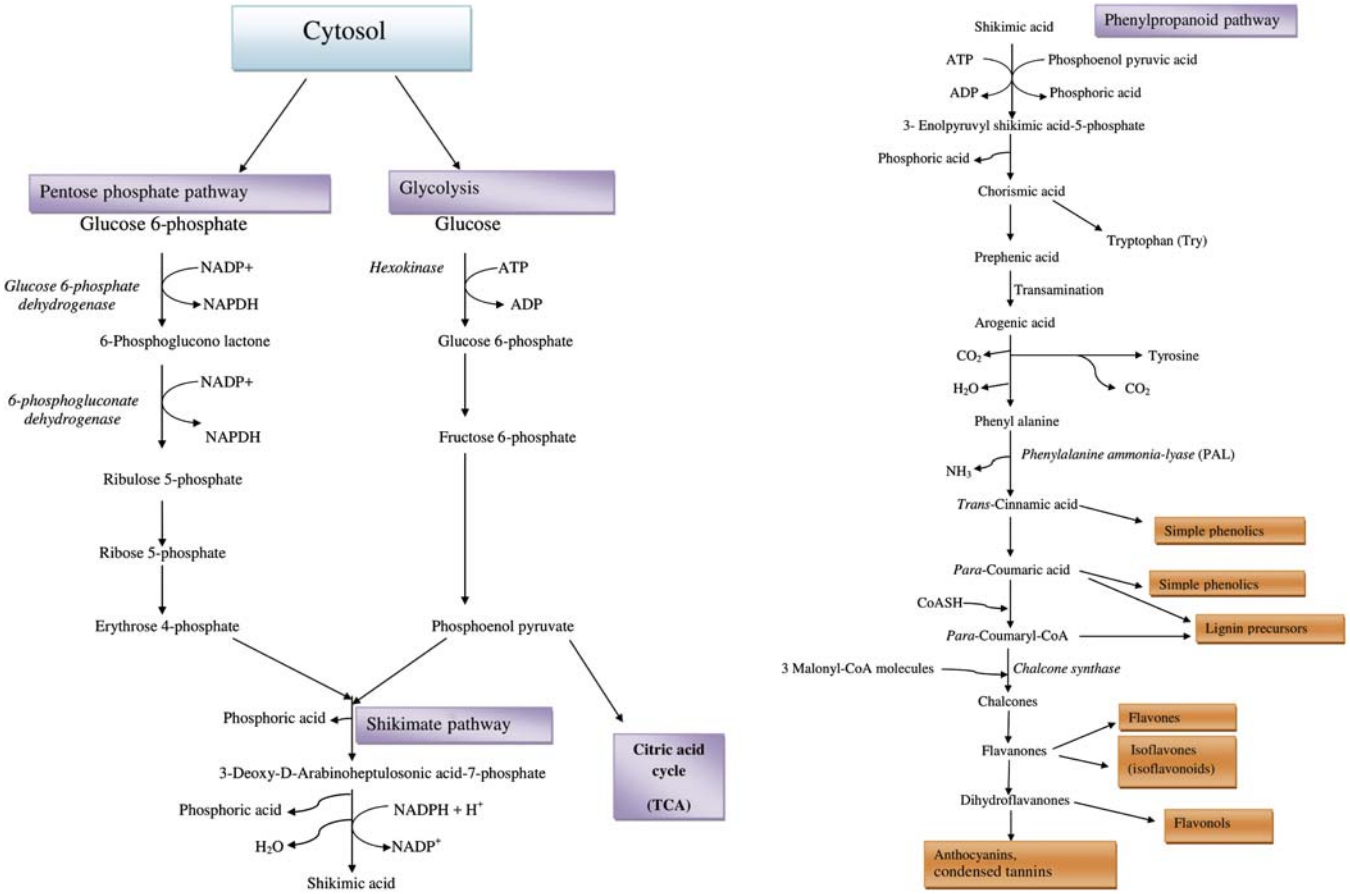

FIGURE 9.1 The phenolic compound biosynthesis pathway. A schematic representation of the biosynthesis of phenolic compounds in the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. Source: *Redrawn from Lattanzio, V., 2013. Phenolic compounds: introduction. In: Ramawat, K.G., Mérillon, J.M. (Eds.), Natural Products, Springer-Verlag: Berlin, Heidelberg. http://doi.org/10.1007/978-3-642-22144-6_57 and Lin et al. (2016).*

Lattanzio et al., 2009). Plant phenolics are generally considered pivotal defense compounds when environmental stresses, such as high light or UV radiation, low temperatures, pathogen infection, herbivores, heavy metals, and nutrient deficiency, lead to increased production of free radicals and other oxidative species in plants. Plants respond to these biotic and abiotic stress factors by increasing their capacity to scavenge reactive oxygen species (ROS; Khan and Khan, 2017). The induction of secondary metabolism gene expression by the abovementioned environmental stresses is often mediated by integrating signaling molecules such as salicylic acid, jasmonic acid, and their derivatives (Winkel-Shirley, 2002; Gould and Lister, 2006; Nascimento and Fett-Neto, 2010; Khan and Khan, 2013; Khan et al., 2013, 2014, 2015; Per et al., 2018).

Plants are exposed to various abiotic stresses during their life cycle. When a plant is subjected to abiotic stress, a number of genes are turned on or off, resulting in increasing levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of defense against these stresses (Ahmad et al., 2008; Jaleel et al., 2009; Tuteja et al., 2009). Abiotic stress promotes the production of damaging active oxygen species within the cells (Dar et al., 2017). Phenolics are varied secondary metabolites (flavonoids, tannins, hydroxycinnamate esters, and lignin) found abundantly in plant tissues and are actively involved in defense mechanisms against biotic and abiotic stress. As compared with nonstressed conditions, plants often produce a higher quantity of phenolic compounds under certain stress conditions (Selmar, 2008). Phenolic compounds performing as antioxidants terminate free radical chains and chelate redox-active metal ions that are capable of catalyzing lipid peroxidation (Schroeter et al., 2002). Phytophenolics, particularly polyphenols, function as antioxidants to support the primary ascorbate-dependent detoxification system as a backup defense mechanism of vascular plants contrasting to monophenols (Yamasaki et al., 1995, 1999). Polyphenols are more effective antioxidants in vitro than ascorbate and tocopherols and have an ideal structural chemistry for free radical-scavenging activity. Antioxidative features of polyphenols arise from the ability of the polyphenol-derived radical to steady and delocalize the unpaired electron (chain-breaking function), their high reactivity as hydrogen or electron donors and from their capability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans et al., 1997). Kagan and Tyurina (1997) reported that phenolics are univalently oxidized to their respective phenoxyl radicals when they function as antioxidants either by enzymatic or direct radical-scavenging mechanisms. Plants synthesize phenolic compounds to survive in stress conditions (UV radiation, drought, salt, metal, and low temperature stress). Most plants constitutively synthesize phenylpropanoids including flavonoids and HCAs. However, accumulation of phenolics in plants can be induced by abiotic and biotic stresses, for example, UV radiation, high light illumination, low temperatures, wounding, low nutrients, and pathogen attack (Dixon and Paiva, 1995; Yamasaki et al., 1995).

Certain secondary metabolic compounds are intensively synthesized under conditions of abiotic stress like drought where these act as antioxidants (Nascimento and Fett-Neto, 2010). Phenolic compounds accumulation in plant tissues is regarded as a distinctive plant stress characteristic. One among the largest three groups of secondary metabolites produced in plants, phenols have been alienated into five subgroups (coumarins, flavonoids, lignins, phenolic acids, and tannins) (Gumul et al., 2007), and are synthesized in plants via shikimic acid and chorismic acid pathways. Phenolic compounds have been regarded as metabolic alteration byproducts (Solecka, 1997). These not only serve a vital function of defense in plants but are also known to influence animals and humans that consume these phenol-enriched plant products (Franca et al., 2001; Amarowicz and Weidner, 2009). The expression of phenolic compounds has been, however, observed to be upregulated (Wróbel et al., 2005; Weidner et al., 2009a) or downregulated (Weidner et al., 2007, 2009b) in response to diverse environmental stresses, thus leading to the increased or decreased content of the phenolic compounds. A number of studies have demonstrated the increased production of phenols in different plant tissues under abiotic stress conditions (Dixon and Paiva, 1995; Wróbel et al., 2005; Weidner et al., 2009a). During water deficit and chilling stress conditions, Chung et al. (2006) have reported increased content of total phenolic compounds in *Rehmannia glutinosa*. Further confirmation has come from the studies of Posmyk et al. (2005) in soybean subjected to chilling stress. This accumulation is due to enhanced activities of enzymes, PAL, CHS, and other enzymes involved in their biosynthesis. Additionally, phosphoenolpyruvate (PEP)-carboxylase activity also increases, which suggests a shift from the production of sucrose to metabolic processes supporting defense and repair. Phenolics confer a variety of physiological functions to plants to survive and adapt to various environmental disturbances (Landolt et al., 1997; Andersen, 2003; Lattanzio et al., 2009). Phenolic acids are synthesized in response to abiotic stress through hydration, dehydration, and methylation of cinnamic acid (Dixon and Paiva, 1995). Many

secondary metabolism products in plants that exhibit antioxidant properties belong to this class of compounds (Oszmański, 1995). As antioxidants, these phenolic compounds scavenge ROS (Amarowicz et al., 2004, 2010; Caillet et al., 2006; Amarowicz and Weidner, 2009), catalyze oxygenation reactions by forming complexes with some metals, and hinder the activities of certain oxidizing enzymes (Elavarthi and Martin, 2010). As stated earlier, accumulation of phenols in plant tissues is a characteristic feature of several environmental stresses that divert considerable quantity of substrates from primary metabolism to the formation of secondary products leading to significant perturbations in the cellular homeostasis. Strong stimulation of mRNAs encoding G6PDH, a carbohydrate metabolism enzyme that provides shikimate pathway substrates, and 3-deoxyarabinoheptulosonate 7-phosphate synthase, which is a shikimate pathway enzyme required for phenylalanine biosynthesis has been observed in response to stress (Cheynier et al., 2013). Furthermore, accumulation of free proline in plants as a result of various biotic and abiotic stresses has been reported. Researchers have anticipated that a stress-stimulated enhancement in the shift of reducing equivalents into proline biosynthesis (cytosolic) as well as degradation (mitochondrial) cycle might be responsible for enabling sensitive regulation of redox potential in the cytosol (Logemann et al., 2000; Lattanzio et al., 2009; Verslues and Sharma, 2010). The abovementioned points imply that different environmental perturbations selectively induce the primary as well as secondary metabolic activities, which are directly and indirectly involved in the accretion of phenolic compounds (Cheynier et al., 2013; Lattanzio et al., 2009). A likely series of biochemical reactions occurring inside the cell, which convey a signal from the outside cell environment into the inside of the plant cell, leading to an effective physiological response, may thus be envisaged (Fig. 9.2) (Hare and Cress, 1997; Lattanzio et al., 2009). This signaling pathway proposes a connection between primary and secondary metabolism, which couples the accretion of proline (a stress metabolite) with the energy transfer toward the biosynthesis of phenylpropanoid through the oxidative PPP (Cheynier et al., 2013). Under various stress conditions, the plant undergoes forceful accumulation of ample amounts of free proline. It can be synthesized de novo or can be released by the protein degradation and is accompanied by NADPH oxidation. Enhanced $NADP^+$/NADPH ratio leads to increased activity of the oxidative PPP, which in turn provides precursors for biosynthesis of phenolic compounds via the shikimic acid pathway (Cheynier et al., 2013; Lattanzio et al., 2009).

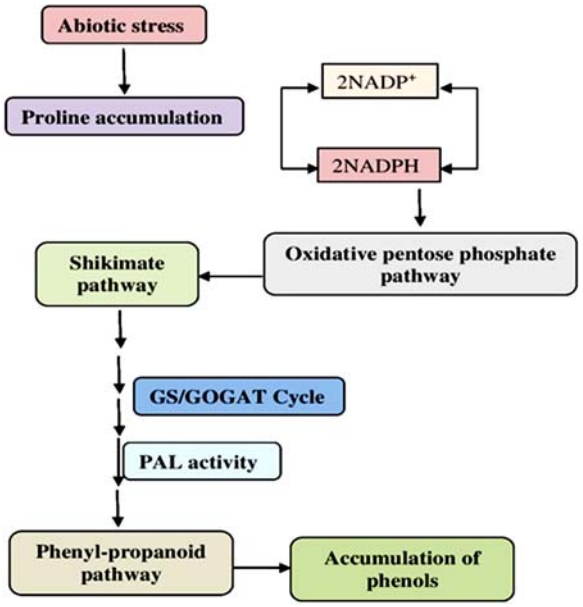

FIGURE 9.2 Showing the abiotic stress-mediated phenol biosynthesis in plants. Source: *Redrawn from Cheynier et al. (2013); Lattanzio V., Cardinali, A., Ruta, C., Morone Fortunato, I., Lattanzio, V.M.T., Linsalata, V., et al., 2009. Relationship of secondary metabolism to growth in oregano (Origanum vulgare L.) shoot cultures under nutritional stress. Environ. Exp. Bot. 65, 54–62.*

9.4.1 Phenolics as Ultraviolet Sunscreens

Light is a well-known physical factor that can affect the synthesis of metabolites in plants. Exposure of ambient solar UV-B radiation (280–320 nm) to plants in open fields adversely affects DNA, proteins, and membranes and alters metabolism through the generation of ROS. Plants synthesize phenolic compounds, which act as a screen inside the epidermal cell layer to defend themselves from this damaging radiation and by adjusting the antioxidant systems at both the cell and whole organism level thereby intercepting mutagenesis and cell death by dimerization of thymine units in the DNA, and possible photo destruction of coenzymes NAD or NADP (Daayf and Lattanzio, 2008). Flavonoids with their high absorptivity at 250–270 and 335–360 nm act as good UV screens (Lattanzio, 2013; Winkel-Shirley, 2002; Carletti et al., 2003). Flavonoids and other phenolic compounds play a significant role in UV protection (Li et al., 1993). Increased flavonoid synthesis was observed in plants radiated with UV light, which confirms the ability of flavonoids to absorb radiation of high energy with the maximum absorption at 250–270 and 335–360 nm (Michalak, 2006; Falcone Ferreyra et al., 2012; Winkel-Shirley, 2002; Liang et al., 2006). It is an observed fact that tropical and high-altitude plants have a higher percentage of flavonoids than temperate plants. The change in the

proportion of flavonoids composition of plant leaves is due to excess of light or UV radiation largely due the activation of flavonoid biosynthetic genes (Kolb et al., 2001). Several studies have confirmed that the excess of light or UV radiation changes the flavonoid composition of plant leaves (Olsson et al., 1998; Kolb et al., 2001). Plant defense mechanisms of polyphenols against UV radiation as direct shields has been regarded as a key biological function by several authors (Rozema et al., 2002; Burchard et al., 2000). Larcher (1995) revealed that phenolics, particularly anthocyanin, which accumulate in the epidermis can act as a darkening filter and protect the mesophyll from extreme radiation. Flavonoids (especially kaempferol derivatives), phenolic acid esters, or isoflavonoids and psoralens accumulate during stress and prevent UV-B from reaching the mesophyll (Stapleton, 1992). Flavones and flavonols, the two important groups of flavonoids found in flowers, accumulate in epidermal layers of leaves and stems and absorb light strongly in the UV-B region without interrupting visible (PAR) wavelengths, thus function to shield cells from UV-B radiation (Lake et al., 2009). Ryan et al. (2001) proved that flavonoids are essential in UV protection using mutants of *Arabidopsis*, UV-hypersensitive phenotypes that have a block in flavonoid production. Anthocyanin synthesis was stimulated by UV light from 280 to 320 nm synergistically when combined with red light in apples (Arakawa et al., 1985). Liu et al. (1995) revealed that flavonoid improved in barley and Kramer et al. (1991) also found increased content of polyamines in cucumber by UV-B radiation. Flavonols concentration also improved in Norway spruce (*Picea abies*) on UV-B exposure (Fischbach et al., 1999). Along similar lines, the increased production of the important anticancerous phenolic compounds, that is, vinblastine and vincristine, due to UV-B exposure in *Catharanthus roseus* has been reported by Bernard et al. (2009). Shiozaki et al. (1999) also revealed that flavonoids in the roots of pea plants was enhanced on UV (300−400 nm) exposure. Flavonols production was stimulated by UV-B in silver birch and grape leaves (Tegelberg et al., 2004). Moreover, photosynthetic pigments, condensed tannins were accumulated under six different daily doses of UV radiation (UV-A and UV-B), whereas its precursor, (+)-catechin, significantly decreased (Lavola et al., 2003). In a UV-tolerant rice cultivar, C-glycosylflavone contents increasingly appeared but were not found in a susceptible cultivar when exposed to different UV-B light levels (Markham et al., 1998). Furthermore, in several plant species, enhanced flavonoid levels have been measured at higher altitudes (Bachereau et al., 1998; Zidorn et al., 2005; Rieger et al., 2008; Spitaler et al., 2008; Murai et al., 2009). It has been proven in several plant species that the expression of *CHS* is transcriptionally activated by UV light, which is the first enzyme in the flavonoid biosynthesis pathway (Koes et al., 1989; Schulze-Lefert et al., 1989).

9.4.2 Plant Phenolics and Their Role in Heavy Metal Stress

Heavy metal toxicity is one of the important abiotic stresses that alter physiological and metabolic processes, thus leading to harmful effects in plants (Villiers et al., 2011). It has been reported that certain flavonoids exhibit the ability to provide heavy metal stress protection by transition metals chelation (e.g., Fe, Cu, Ni, Zn), which generates hydroxyl radical via Fenton's reaction (Mira et al., 2002;Williams et al., 2004). Kidd et al. (2001) revealed that the chelation of these metals in the soil may be an effective form of defense against the effects of high metals concentration toxicity. Michalak (2006) observed that the biosynthesis of phenolic compounds that are precursors of lignin intensifies under stress conditions, for example, in plants subjected to heavy metal stress. Research on corn plants (*Zea mays* L.) confirmed this further when grown on soil contaminated with aluminum ions and root exudates were found with high levels of catechin and quercetin. Winkel-Shirley (2001) reported that flavonoids are involved in plants' defense, growing in soils that are rich in toxic metals such as aluminum. The production of betalains in *Beta vulgaris* is stimulated by Cu^{2+} (Trejo-Tapia et al., 2001). The hairy roots were exposed to metal ions to improve betalaines production (Thimmaraju and Ravishankar, 2004). Red cabbage seedlings accumulated phenolic compounds, total antioxidant capacity, and increased PAL activity when treated with copper (Posmyk et al., 2009). Accumulation of betacyanins in callus cultures of *Amaranthus caudatus* is stimulated by Cu^{2+} (Obrenovic, 1990). Flavonoids accumulation was also observed in cell cultures of *Ginkgo biloba* treated with $CuSO_4$ as compared with untreated cells (Kim et al., 1999). Similarly, association between concentration of $CuSO_4$ and flavonoid level in cell cultures of *Digitalis lanata* was reported (Bota and Deliu, 2011). Nickel stress leads to significant decrease in anthocyanin levels as observed by Hawrylak et al. (2007). Michalak (2006) observed that plants with high content of tannins, such as tea, are able to tolerate high concentrations of manganese in a soil, as they are protected by the direct chelation of these ions. Lavid et al. (2001) reported the heavy metal ions binding with polyphenols in *Nympheae* where heavy metals (Hg, Pb, Cr) were chelating by the polyphenols rich methanol extract.

9.4.3 Plant Phenolics and Their Role in Drought Stress

Drought is the major abiotic stress that affects plant growth and development and causes losses in agricultural production. As has been reported by several studies, phenolics content increased in plants under water scarcity. Flavonoid accumulation is important to improve drought tolerance in wild-type and *Arabidopsis thaliana* mutants revealed by transcriptomic and metabolomic approaches (Nakabayashi et al., 2014). Ballizany et al. (2012) revealed that the quercetin (a flavonol) contents enhanced significantly in white clover under drought conditions, which was higher in the more drought-resistant genotypes. Kirakosyan et al. (2003) reported that under drought conditions, flavonols have been increased in other species also, such as *Crataegus laevigata* and *Crataegus monogyna*. Similarly, in *Cistus clusii* plants upon controlled drought treatments, and in plants collected from the field in summer, characterized by high temperatures and prolonged lack of rain in the Mediterranean climate, an increase in flavanol levels has been reported (Hernandez et al., 2004). Akula and Ravishankar (2011) reported that the defense mechanism against drought stress is triggered by bioactivity of leaf phenolic molecules. Phenolic acids and flavonoids as antioxidant accumulation and sunshields are involved in plants' response to drought stress (Nichols et al., 2015). Larson (1988) also reported the increased level of flavonoids and phenolic acids in willow leaves under drought conditions causes oxidative stress. In drought-resistant tomato cultivars kaempferol and quercetin (flavonoids) were enhanced while reduced in drought sensitive cultivars reported by Sánchez-Rodríguez et al. (2011). In red-hulled and black-hulled rice the radical-scavenging ability depended on the concentrations of proanthocyanidins and anthocyanins, respectively (Oki et al., 2002). Flavonoids and phenolic acids were synthesized in a large amount in wheat leaves and cell-damaging oxidants also generated under drought stress (Ma et al., 2014). Chalker-Scott (1999) reported that plant tissues containing anthocyanins are usually rather resistant to drought. For example, a purple cultivar of chili resists water stress better than a green cultivar revealed by Bahler et al. (1991). In *Chenopodium quinoa* saponins content decreased from 0.46% to 0.38% dry weight (dw) in plants growing under low water deficit and in high water deficit conditions, respectively (Soliz-Guerrero et al., 2002). Phenolic compounds concentration was 10% improved in *Hypericum brasiliense* grown under drought stress as compared with control plants (De Abreu and Mazzafera, 2005). Similarly, phenolic compound were also increased in pea plants (*Pisum sativum*) when grown under drought conditions (Nogue's et al., 1998). In both leaves and flowers of *Tridax procumbens* significant increases of total phenolic content were observed under drought stress (Gnanasekaran and Kalavathy, 2017). Antioxidant capacity of phenolic acids was reflected by change in their contents during the process of finger millet malting (Subba Rao and Muralikrishna, 2002). Nichols et al. (2015) reported that the high levels of flavonols, quercetin, and kaempferol contents were related with improved stress tolerance capacity of white clover under drought conditions.

9.4.4 Plant Phenolics and Their Role in Cold Stress

It has been observed that nonfreezing low temperatures enhance phenolic metabolism in plants (Akula and Ravishankar, 2011). Phenolic metabolism is stimulated at a critical low temperature, which is the threshold temperature at which chilling injury is also induced (Janska et al., 2010). This low temperature results in cold-induced stimulation of the PAL activity (EC 4.3.1.5) as well as other enzymes necessary for phenolic biosynthesis, leading to increased phenolic production and modified plant development either independently or by interaction with known plant growth promoters, especially ethylene (Lattanzio et al., 1994, 2001). Cold stress increases phenolic production into the cell wall either as suberin or lignin (Griffith and Yaish, 2004). Lignification and suberin deposition increase resistance to cold stress. These cell wall thickenings protect the plant from freezing stress. An increase in cell wall thickening could reduce cell collapse during freezing-induced dehydration and mechanical stress, thus providing freezing resistance of the plant (Chalker-Scott and Fuchigami, 1989). Apple trees are found to be associated with high levels of chlorogenic acid as an adaptive measure to cold climate (Perez-Ilzarbe et al., 1997). Christie et al. (1994) reported the anthocyanins accumulation during cold stress and Pedranzani et al. (2003) reported that cold and water stresses initiated changes in endogenous jasmonates in *Pinus*.

9.4.5 Plant Phenolics and Their Role in Nutrient Stress

Plant growth depends on the supply of recycled nutrients. External nutrient supply and nutrient mineralization by soil microorganisms contribute the nutrient requirements. The factors regulating nutrient cycle include climate, substrate (litter) quality, and decomposer organisms. Polyphenols influence the supply

and flow of inorganic and organic soil nutrients available for plants and/or microbes. Phenolic compounds find their way to the soil as leachates from the above- as well as below-ground parts of plants and/or within above and belowground plant litter (Hättenschwiler and Vitousek, 2000). Polyphenols affect the composition and activity of decomposers thus influencing the rates of decomposition and nutrient cycling (Lattanzio et al., 2006).

Phenolic compounds show a sensitive response to nutrient deficiency, thus providing a method for diagnosing nutrient disorders prior to the appearance of visible symptoms. Deficiencies of N, P, K, and S usually result in increased concentrations of phenolic compounds, and abundant N generally inhibits phenolic accumulation (Gershenzon, 1913; McClue, 1977). Visual symptoms of N or P deficiency are red or purple tints of the leaves due to accumulation of anthocyanins (Hewitt, 1963). Nutrient stress increased threefold anthocyanidins level and doubled quercetin-3-O-glucoside in tomato (Bongue-Bartelsman Phillips, 1995). Osmotic stress by sucrose and other agents regulate anthocyanin production in *Vitis vinifera* cultures (Tuteja and Mahajan, 2007).

9.5 CONCLUSION AND FUTURE PROSPECTS

Plant phenolics are the most common and widespread secondary metabolites, comprising a large reservoir of natural chemical diversity with a huge range of compounds and enzymes and a wide spectrum of mechanisms of gene regulation, and transport of metabolites and enzymes. Plants accumulate phenolic compounds in their tissues as an adaptive response to adverse environmental stresses including wounding, pathogen attack, mineral deficiencies, and temperature stress. Polyphenols modify the developmental status of the plant independently or by interacting with plant growth promoters like ethylene. Furthermore, these compounds, the precursors for lignin and suberin, are polymerized into the cell wall. These cell wall thickenings protect the plant from freezing stress. An increase in cell wall thickening could reduce cell collapse during freezing-induced dehydration and mechanical stress, thus providing freezing resistance of the plant (Chalker-Scott and Fuchigami, 1989). Polyphenols influence the supply and flow of inorganic and organic soil nutrients available for plants and/or microbes. They also show a sensitive response to nutrient deficiency, thus providing a method for diagnosing nutrient disorders prior to the appearance of visible symptoms.

Despite a handful of studies on biosynthesis of phenolic compounds and their accumulation as an adaptive response against abiotic stresses, studies on the proper mechanism of their accumulation and their interactions with other cell metabolites are desperately needed to develop a complete understanding of their increased expression and imparting tolerance under such circumstances.

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CHAPTER

10

Bioactive Molecules as Regulatory Signals in Plant Responses to Abiotic Stresses

Giulia Franzoni[1], *Alice Trivellini*[2], *Roberta Bulgari*[1], *Giacomo Cocetta*[1] *and Antonio Ferrante*[1]

[1]Department of Agricultural and Environmental Sciences, Università degli Studi di Milano, Milano, Italy [2]Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa, Italy

OUTLINE

10.1 INTRODUCTION

The bioactive compounds in plants are a wide range of molecules that are produced under suboptimal growing conditions. The biosynthesis of these molecules is aimed to enhance crop tolerance to abiotic and biotic stresses and overcome stressful conditions or to avoid attack from animal and pathogens. Most bioactive compounds in plants belong to the secondary metabolites, but there are also compounds coming from primary metabolism. These molecules have also several beneficial effects in humans and animals, if eaten, and they are of great interest in the diet. The primary role of these compounds is played in plants because they produce them for their benefit in certain environmental conditions. Plants have modified their structure and metabolism for adaptation to different environments. Their adaptation ability has an energy cost that represents part of the energy invested in their defense strategies. Plants based on their adaptation strategies have colonized stressful environments characterized by drought (predesert and desert zones),

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salinity (coastal areas), high or low temperatures, high or low light intensities, polluted areas, etc.

The physiological, biochemical, and molecular changes behind the plant's adaptation ability are extremely important to exploit in the agricultural systems and extend the arable lands in poor environments.

10.2 THE ROLE OF ASCORBIC ACID, CAROTENOIDS, AND FLAVONOIDS IN STRESS SIGNALING

Being sessile organisms, plants have developed several strategies to counteract stressful environmental conditions. Most of these strategies are promptly activated and can involve different classes of molecules, which play specific roles in the signaling network. Moreover, different species can respond to the same stimulus in a different way and the molecules involved in the response and signaling can vary even for a specific variety within a species. The stress response can involve a large array of different compounds that are constitutively synthetized by plants or specifically produced in response to an external stimulus or environmental condition. These last compounds are classified as plant secondary metabolites, they can belong to various chemical classes of molecules and can be involved in different metabolic pathways. Many studies have been conducted on the role of plants' secondary metabolites in defense responses and signaling related to biotic stress factors (Akula and Ravishankar, 2011; Nakabayashi and Saito, 2015). However, more recently new functions in plant signaling in response to abiotic stresses have been also discovered, especially for ascorbic acid, flavonoids, carotenoids, and their derivatives.

Ascorbic acid (AsA) is a very important molecule in plants, being involved in several physiological processes tightly connected to both primary and secondary metabolism (Davey et al., 2000), including stress responses. Its biosynthesis can occur via different independent pathways (Smirnoff et al., 2001). Ascorbate is not often considered as a signaling molecule in plants and for several years, its importance as an antioxidant has been largely underlined. AsA can, in fact, actively quench the oxidative cascade and the consequent accumulation of reactive oxygen species (ROS), being oxidized to monodehydroascorbic acid (MDHA) and to dehydroascorbic acid (DHA). Also, its antioxidant activity can be efficiently mediated by the combined action of a series of enzymatic reactions, known as the ascorbate–glutathione cycle (or Halliwell–Asada cycle) (Chew et al., 2003; Khan and Khan, 2017). There is a large plethora of studies produced in the last few years on this topic and on the practical implications in both plant stress metabolism (Cocetta et al., 2014; Pandey et al., 2017) and development (Cocetta et al., 2012; Alós et al., 2014; Wang et al., 2015). It should be considered that ROS are among the key players in plant stress signaling (Suzuki et al., 2012; Baxter et al., 2013), thus the ability of AsA in maintaining ROS homeostasis could indicate an active role in signaling for this molecule too. However, the importance of AsA is due to several other biological functions in which it is actively involved. A protective role of melatonin has been proposed in tomato subjected to heat and salt stress. The authors showed that melatonin treatment altered the expression of genes encoding for the enzymes involved in AsA recycling (Martinez et al., 2018). More recently, it has been suggested that AsA can have a role in cytosolic Ca^{2+} signals, by activating Ca^{2+} influx in *Arabidopsis* roots (Makavitskaya et al., 2018). AsA is also involved in hormone signaling. *Arabidopsis* mutants with low levels of AsA showed an increment in ethylene production and reduced biomass accumulation. The authors observed also that low AsA altered the expression of genes involved in the hormone pathways that control growth (Caviglia et al., 2018).

Carotenoids are a large group of molecules covering several roles in plants. They act as antioxidants, pigments involved in photoassimilation and in plant–animal interactions (Nisar et al., 2015), and they are the precursors of abscisic acid, one of the most important plant hormones (Walter and Strack, 2011). Recently, new functions for carotenoids have been discovered, including the role in modulating gene expression in response to stress. In fact, ROS-induced oxidation of certain carotenoids (mainly β-carotene) determines the accumulation of carotenoid derivatives, which are potential signal molecules (Havaux, 2014). Among those molecules, β-cyclocitral or β-ionone were reported to increase in response to photooxidative stress and to stimulate the expression of genes related to cellular defense against stress in *Arabidopsis* (Ramel et al., 2012). Isoprene, which is the constituent unit of carotenoids, could also have a role in stress signaling (Havaux, 2014). In fact, different abiotic stressful conditions have been reported to stimulate the emission of large amounts of isoprene-oxidation products (Jardine et al., 2013). Also, in a previous study, volatile isoprenoids have been proposed as important actors able to modulate oxidative stress induced by various factors (Vickers et al., 2009). It always important to consider that different metabolic routes or responses can be activated at once and no one of the considered responses act separately from the others. For example, it has been shown that an impairment in carotenoids and porphyrins (chlorophyll precursors) biosynthesis obtained by exogenous treatments with chemical

inhibitors, can be sensed by stress signaling molecules, such as hydrogen peroxide, ascorbate, glutathione, and salicylic acid, and can in turn activate enzyme-mediated antioxidant responses in rice (Khan et al., 2016; Park and Jung, 2018).

Flavonoids are a subgroup of phenolic compounds that have been reported to have several biological activities in both plants and animals. The efficacy of flavonoids as antioxidants is well known and has been largely documented, also in relation to response to biotic and abiotic stresses (Brunetti et al., 2013; Rejeb et al., 2014). However, it is also known that these molecules can have other important biological functions, being involved in transport and signaling transduction pathways (Peer and Murphy, 2006). The presence of flavonoids in the nucleus has been demonstrated in different plant species, and the potential target of flavonoid regulation are transcription factors, kinases, and ABC (ATP-binding cassette) transporters. However, most of these interactions are thought to be related to developmental processes more than stress response metabolism. For example, heavy metal stress has been reported to induce an increment in both ROS and flavonoids (Khan and Khan, 2014). In this sense flavonoids can alter the ROS-mediated signal transduction by acting as antioxidant and metal chelators (Skórzyńska-Polit et al., 2010; Brown et al., 1998), but the presence of a specific signaling mechanism involving them has not been demonstrated. More recently, it has proposed that the interaction of flavonoids with protein kinases could also suggest an important role for flavonoids in photoprotection other than in plant development. The authors concluded that flavonoids could affect MPAK signaling by directly binding to the active sites of the proteins or by modulating their activation through ROS-scavenging activity (Brunetti et al., 2013). Important changes in flavonoids accumulation were observed in *Arabidopsis* in response to drought stress, but the exact mechanisms of signaling and regulation are still unclear (Nakabayashi et al., 2014).

Glucosinolates are a class of sulfur-containing secondary metabolites, typically from the *Brassicaceae* family. These compounds are particularly appreciated as components of functional foods and, at the same time, they have an important role in plant stress responses, together with their degradation products (such as isothiocyanates and nitriles) (Halkier and Gershenzon, 2006). Recently, great attention has been paid to the cross-talk between some of the physiological processes in stress response and glucosinolate metabolism, and it has been proposed that glucosinolates may act as signals themselves (Martínez-Ballesta et al., 2013). However, this aspect need to be further explored.

In the near future, the application of innovative techniques of investigation like transcriptome analysis, gene silencing, proteomics, metabolomics, as well as the use of mutants, will help to better understand the mechanisms of stress sensing, signaling, and regulation and perhaps to discover novel functions or molecules involved in this complex network.

10.3 AMINO ACIDS AND DERIVATES UNDER STRESS

Amino acids are molecules made up of two functional groups: amine ($-NH_2$) and carboxylic acid ($-COOH$), and a specific side-chain (R), which is responsible for the differences in chemical properties. They can be classified in many ways depending on their polarity, pH level, or functional groups location. According to the structure and the chemical characteristics of the R group, amino acids are arranged in six classes: aliphatic, hydroxyl or sulfur containing, cyclic, aromatic, basic, acidic, and their amides (Anjum et al., 2014).

Amino acids have commonly been considered as precursors and constituents of proteins, but they are also building blocks for several other nitrogen containing compounds such as nucleic acids or numerous metabolites such as hormones, cell wall components, chlorophyll, and secondary metabolites, and are present in the cell in a free form too. Moreover, they act as regulatory and signaling molecules improving plant stress tolerance and regulating the timing and developmental progression of leaf senescence (Less and Galili, 2008; Watanabe et al., 2013; Araújo et al., 2015; Planchet et al., 2015). Amino acids also have an important role in plant nutrition, for example, glutamine synthetase is a key enzyme involved in the assimilation and transport across the cell membrane of nitrogen (Moe, 2013; Anjum et al., 2014). Due to this biological significance amino acids are commonly used as fertilizers or components in several products such as biostimulants.

Plants are often exposed to suboptimal environmental conditions that are unfavorable for growth, development, and can reduce agronomic yield. The prevalent abiotic stresses limiting crop productivity are usually related to water amount (drought, flooding), salinity, temperature (cold, heat), nutrient deficiency, chemical toxicity, and pollutants. Defense and adaptation mechanisms of plants are dynamic, complex, and affect several metabolic pathways according to the nature of the stress, plant species, tissue, or developmental stage (Planchet et al., 2015; Araújo et al., 2015). However, plants exhibit shared responses that are in common to different stresses. Some of these

lead to an alteration both the amino acid spectra and their derivates (Rai, 2002; Less and Galili, 2008; Gill and Tuteja, 2010; Hussain et al., 2011; Araújo et al., 2015). Moreover, a bunch of studies suggested that exogenous application of amino acids and polyamines such as putrescine, spermine, and spermidine results in abiotic stress tolerance in various plants (Gill and Tuteja, 2010).

It is widely assumed that the amino acid pool is much induced during abiotic stress. Indeed, an overall increase of several defense molecules commonly referred to as osmoprotectants or compatible osmolytes (peptides, amines, amino acids, proteins, betaines, sugars) is a part of the plant mechanism to cope with osmotic stress, in particular due to drought, salinity, and flooding (Joshi et al., 2010), allowing the maintenance of cell turgor. Among these, specific amino acids such as proline, alanine, arginine, glycine, amides such as glutamine and asparagine, nonprotein amino acids like γ-aminobutyric acid, pipecolic acid, citrulline, and ornithine have been reported for their enhancement in plants under these conditions (Skirycz et al., 2010; Obata and Fernie, 2012; Anjum et al., 2014).

An important increase in the branched-chain amino acids (isoleucine, valine, and leucine) and aromatic amino acids (tryptophan, tyrosine, phenylalanine) has been observed also under less severe stress conditions, acting as alternative respiratory substrates (Araújo et al., 2011).

To cope with flooding and anaerobiosis, cytosolic pH commonly decreases and amino acids level changes. In those stressful conditions it has been observed that γ-aminobutyric acid, alanine, glycine, serine, and proline increase to limit cytoplasmic acidosis, while aspartic acid and glutamine decrease (Reggiani et al., 1988; Roberts et al., 1992).

Several studies have attributed a nonenzymatic antioxidant activity to proline and polyamines, suggesting ROS scavenging role and singlet oxygen quencher (Smirnoff and Cumbes, 1989; Mohanty et al., 2001; Matysik et al., 2002). Proline can also have an indirect action in protecting and stabilizing ROS scavenging enzymes such as peroxidase, glutathion-S-transferase, superoxide dismutase, and catalase, activating alternative detoxification pathways (Hare and Cress, 1997). However, there is contrasting evidence about this role. Indeed, Signorelli et al. (2013) demonstrated that proline does not play any role in quenching singlet oxygen.

Polyamines are also involved in these kinds of responses, working as antioxidants, free radical scavengers, or as membrane stabilizers (Velikova et al., 2000).

Amino acids such as asparagine and proline, but mostly polyamines bind to metals such as zinc, cadmium, and copper by forming a metal complex useful for protecting the activity of some enzymes against the effects of toxic heavy metals (Rai, 2002; Planchet et al., 2015).

There is a large amount of information on amino acids involvement in plant stress responses as reported above; otherwise currently very little is known about their signaling function especially under unfavorable conditions. Indeed, whereas in the past only hormones were considered as signal molecules, data of recent years suggest that this function may be performed also by other compounds.

Proline is one of the most important amino acids and plays several roles in plants both in stressed and unstressed conditions. Different studies have suggested an extra role as a signaling molecule involved in regulation of gene expression under stress (Szabados and Savoure, 2010; Hayat et al., 2012).

Maggio et al. (2002) proposed that proline may act as regulatory molecule in salt-induced vacuolation. This cellular strategy has been reported in yeast (Bone et al., 1998) and plants (Chang et al., 1996). It has been observed that signal transduction system involved with perception and adaptation to osmotic stress is associated with accumulation of increased intracellular proline. Some branched-chain amino acids (BCAAs) such as serine, proline, and leucine have been shown to have a direct activity as signaling molecules and others are the precursors for a variety of secondary metabolites or phytohormones with signaling function (Nambara et al., 1998; Urano et al., 2009; Hildebrandt et al., 2015). Examples of regulation of transduction signal induced by amino acids involve target of rapamycin (TOR) kinase. Through this pathway, functioning as regulatory integrators of environmental signals, BCAAs, in particular leucine, act to modulate global RNA (Kimball and Jefferson, 2006).

Hannah et al. (2010) designed a large experiment to investigate the response of *Arabidopsis* to different conditions. They applied transcriptomics and metabolomic analysis to monitor the changes induced by environmental perturbation. Interesting variation in leucine has been observed suggesting a possible role as regulator of gene expression. Based on the results obtained from the experiment they attributed to leucine an important role as mediator molecule. Indeed, a high correlation between gene expression analysis and transgenic *Arabidopsis* plant overproducing leucine has been observed. This leucine function has not been described in plants but in other organisms where leucine probably activates the TOR pathway stimulating protein synthesis (Dann and Thomas, 2006). It is very likely that a similar mechanism exists also in vegetable tissues (Dinkeloo et al., 2017).

Furthermore, Häusler et al. (2014) recently focused on some amino acids' role as metabolic intermediates/end-products and as signal molecules, comparing medical and plants fields. They suggested that the amino acid serine has a role in signaling. Both L-serine and D-serine act as metabolic signal in the communication between male gametophytes and pistil by regulating a glutamate receptor-like Ca^{2+} channel in pollen tubes. This mechanism looks like what happens in other organisms, where serine functions as neurotransmitter and regulates the activity of a nonselective ion channel. As well as leucine, serine may be also related to the TOR pathway even if there is no direct evidence to prove it.

Gamma-aminobutyric acid (GABA), a four-carbon nonprotein amino acid, is a significant component of the free amino acid pool. It accumulates rapidly in plant tissues as a response to biotic and abiotic stresses, but its physiological significance and the specificity of the response are largely unclear. Different roles have been proposed, such as the regulation of cellular pH and osmolarity, maintaining C/N balance, scavenging of ROS and defense against insect attack (Shelp et al., 1999; Bouche and Fromm, 2004; Mekonnen et al., 2016). Its fast increase may be related with the glutamate decarboxylase (GAD) activity and the interaction with Ca^{2+}/calmodulin. Indeed, different stress conditions trigger a transduction pathway that leads to an increase in cytosolic Ca^{2+}, which activates GAD resulting in GABA synthesis from glutamate (Shelp et al., 1999).

Different studies have also reported a strong link between the TCA cycle and GABA shunt, the pathway involving GABA synthesis and breakdown (Shelp et al., 1999; Bouche and Fromm, 2004). It has been reported that changes in the TCA cycle upstream of succinate production enhanced the flux through the GABA shunt, and vice versa, a mutation of the GABA pathways impact the accumulation of TCA cycle intermediates, since GABA is a significant source of succinate in this network (Gilliham and Tyerman, 2016).

Furthermore, Mekonnen et al. (2016) proposed that GABA acts as a signal molecule suggesting that its accumulation under drought conditions is a stress-specific response and this phenomenon induces the regulation of stomatal opening thereby preventing loss of water. The control of stomatal movement may be regulated by the interaction between GABA and ALMT (aluminum-activated malate transporter) channel and through the modulation of the activity of H^+-ATPase via 14-3-3 proteins. Under drought stress GABA concentration increases leading to a deactivation of the flux of anions into vacuole binding to ALMT6 protein. This implicates ALMT proteins as GABA receptors in plants acting as metabolic sensors that are able to translate environmental changes into physiological outputs. GABA has been proposed to be able to affect Ca^{2+} channel activity via provoking changes in membrane potential (Ramesh et al., 2015). The regulation of membrane potential is known to constitute a signal in several cell types.

Additionally, GABA represses the expression of 14-3-3 proteins, which are involved in light-induced stomata opening (Lancien and Roberts, 2006). All this speculation on GABA's signaling role results also from the similarities between its mechanisms in plant and mammalian cells where GABA is described as a neurotransmitter (Chebib and Johnston, 1999).

Recent studies have suggested an interplay between the GABA shunt and the TCA cycle through various bypasses (Fait et al., 2008). Although further analysis of these metabolic pathways is required, our metabolome analysis allows us to speculate that reduction of proline and agmatine as members of the glutamate family, and methionine, lysine, and saccharopine as members of the aspartate family, caused activation of the GABA shunt and the TCA cycle in nc3-2 under dehydration.

Polyamines are critical for plant growth and development, and play an important role in controlling downstream stress responses by modulating gene expression and regulating different transporters (Tuteja and Sopory, 2008). Transcriptomic analysis shows that spermine and spermidine act as anabolic regulators, while putrescine acts as a catabolic regulator in tomato fruit. Moreover it is supposed that there is a connection between TOR and polyamines due to their linkage with anabolic processes and C:N signaling in tomato (Handa and Mattoo, 2010). Spermine is also involved in defense signaling against plant pathogens by regulating a subset of defense-related genes and leading to hypersensitive response and to programmed cell death (Takahashi and Kakehi, 2009).

Polyamines have been positively correlated with plant responses to abiotic stresses for a long time. They accumulate under several unfavorable conditions such as salinity, drought, chilling, heat, hypoxia, heavy metals, and UV radiation (Alcázar et al., 2010; Gill and Tuteja, 2010; Gupta et al., 2013; Mattoo et al., 2015). Polyamines act in different ways to help plant tolerance as free radical scavengers and keep cellular ionic balance affecting K^+/Na^+ homeostasis and regulating ion channels (Zhao et al., 2007). Several studies have reported that polyamines act as signal molecules in the ABA-regulated stress response pathway and through the production of H_2O_2. Indeed, has been shown that salt stress and treatment with ABA induce polyamine transport in the apoplast (Gupta et al., 2013). Metabolome and transcriptome analysis have also revealed that polyamines and ABA modulate each

other's biosynthesis under stress to increase plant adaptive potential (Urano et al., 2009; Pal et al., 2015). Moreover, H_2O_2 produced during polyamines catabolism leads to the synthesis of ROS, which triggers a downstream signal cascade upregulating various stress responses proteins. Yamasaki and Cohen (2006) reported evidence of cross-talk of polyamine also with NO, a small gaseous molecule functioning as an intra- and intercellular messenger, but the mechanism for this action is still not understood and more experiments are needed. NO accumulation has been reported after spermidine and spermine treatments in *Arabidopsis*. This linkage between NO and polyamines could explain several physiological effects of polyamines under stress conditions (Pal et al., 2015). Polyamines play an important role in the regulation of ion channels during stress through the induction of signaling molecules like ROS and NO. Several studies also reported a role in the regulation of the vacuolar Ca^{2+} concentration and during the sequestration of Na^+ in salt stress conditions (Yamaguchi et al., 2006; Kusano et al., 2007; Liu et al., 2014). A lot of experiments have shown that amino acids and polyamines act as an elicitor of plant defense responses under stress. Much progress has been made by developing amino acids or polyamine deficient mutants, or by a multidisciplinary approach. However, the exact mechanism of action is not fully understood.

10.4 POLY- AND OLIGOSACCHARIDES AND PLANT RESPONSES

Oligosaccharides and polysaccharides are carbohydrate polymers made up of units of simple sugars called monosaccharides interconnected by glycosidic linkages. Generally, the first contain three to ten sugar residues; the latter are a long chain of monosaccharides that can reach up to a molecular weight of 100,000 daltons and comprise much of the biomass on the planet.

Carbohydrates have a pivotal role as sources of energy and carbon skeleton for organic compounds and as storage components. Cellulose, chitin, and pectin are the most abundant carbohydrates present in plant cells. High sugar level generally stimulates expression of genes involved in growth, storage of protein, and biosynthesis of starch and fructans (Gupta and Kaur, 2005). Their concentration and metabolism alter both during development and in response to different environmental signals. For example, sugar accumulation is stimulated by nitrogen limitation and high-intensity light. Under unfavorable conditions, soluble sugars, sugar alcohols, cyclic carbohydrates, and raffinose family oligosaccharides (RFOs) function as osmolytes to maintain cell turgor.

In addition, an important regulatory function as signaling molecules involved in plant growth and stress resistance has been recently observed. Studies on carbohydrates revealed that fragments from plant cell walls might act as signaling molecules to activate plant defensive genes and change plant growth and development (Ryan, 1987). In general, this metabolic network is linked with plant hormones. Indeed, several experiments have shown that a cross-talk occurs between glucose and ABA signaling (Rook and Bevan, 2003; Rolland et al., 2006; Bolouri-Moghaddam et al., 2010; Elsayed et al., 2014). Moreover, stress-induced ABA can upregulate different transcription factors that activate both galactinol synthase and raffinose synthase (Valluru and Van Den Ende, 2011). Interaction between sugars and ABA signaling might control the induction of leaves' senescence during drought stress (Wingler and Roitsch, 2008). This evidence suggests an involvement of carbohydrates in a process called "sweet immunity," stimulating the immune system of plants and abiotic stress responses (Zhang et al., 2009; Bolouri Moghaddam and Van Den Ende, 2013). This concept is commonly related to small sugars (mono-, di-, and some small oligosaccharides) such as sucrose, raffinose, or galactinol, that are able to activate plant defenses against pathogen attacks. Several observations suggested that also fructans (fructose-based oligo- and polysaccharides) may be involved in sweet priming processes, counteracting biotic and abiotic stresses.

Kafi et al. (2003) observed that levels of fructans increased in salt-stress wheat reaching higher levels in tolerant cultivars compared with sensitive ones. These molecules are the major storage sugars in cereals and cool zone grasses and are involved also in freezing and drought tolerance mechanisms. Indeed, they accumulate during stress conditions because they are highly soluble in water and resistant to crystallization at subzero temperatures (Kerepesi et al., 2002; Kawakami et al., 2008; Joudi et al., 2012; Van Den Ende and El-Esawe, 2014). Fructans have also an important role stabilizing membranes by inserting into the lipid headgroup region. This helps prevent leakage when water is removed from the system both during freezing and drought (Livingston et al., 2009). Their role is also confirmed by the higher expression of both defense-related TFs and fructans biosynthetic genes (Gaudet et al., 2011). Moreover, the introduction of fructan biosynthesis genes in nonfructan accumulator cultivars confers abiotic stress tolerance (Bie et al., 2012; Van Arkel et al., 2013). Wang et al. (2009) observed that after spraying a preparation containing fructan on tobacco leaves, the resistance against

tobacco mosaic virus was increased. They suggested an involvement of salicylic acid–dependent signaling pathways. This preparation increased the content of lignin and enhanced the activity of defense-related enzymes such as peroxidase or superoxide dismutase in the same leaves. Similar results have been obtained in tomato fruit treated with the same preparation in postharvest diseases (Sun et al., 2013). This treatment increased also the mRNA level of genes encoding various pathogenesis-related proteins and phenylanaline ammonialyase (PAL) gene.

Nishizawa et al. (2008) obtained similar results after overexpression of genes involved in biosynthesis pathway of RFOs. Van den Ende and Valluru (2008) hypothesized that RFOs, galactinol, and fructans might function as transportable stress signals mediating different stress responses. It's known that galactinol acts as a signal molecule in pathogen-induced resistance (Kim et al., 2008); however, the signal role of these molecules under abiotic stress is still unclear (Elsayed et al., 2014).

10.5 NITRATE ACCUMULATION AND STRESS REGULATION

Nitrogen (N) is an essential macronutrient required for the synthesis of vital molecules, such as proteins, nucleic acids, and chlorophyll, and it is a fundamental nutrient for cellular metabolism (Parker and Newstead, 2014). Its absorption at the root level determines plant growth and consequently crop productivity (Krapp et al., 2014; O'Brien et al., 2016).

In plants, several processes, including N uptake and assimilation, are known to be adversely affected by abiotic stresses, such as salinity, drought, and extreme temperatures (Goel and Singh, 2015). It is a well-established fact that high salt concentration inhibits nitrate reductase (NR) activity; this enzyme catalyzes the first enzymatic step in nitrate assimilation in plants, involving reduction of nitrate to nitrite. Several metabolic and environmental signals regulate NR activity (Kaiser et al., 1999). The reduced activity of the enzyme in the leaves of salt-stressed plants is attributed to salinity inhibited nitrate transport to the shoot, which in turn is due to interference with nitrate uptake and xylem loading (Cramer et al., 1995). The uptake of nitrogen, its translocation, and also the assimilation has been found to be affected by high salinity in cowpea plants, as reported by da Silveira et al. (1999). Nitrogen use efficiency was also reported to be reduced significantly with increased salinity levels (1.5, 4.5, and 6.5 dS m^{-1}) in chile pepper (Huez López et al., 2011). The high salinity has been shown to inhibit the activity of many of the key enzymes involved in nitrogen assimilation in maize plants, *Brassica juncea*, and tomato seedlings (Khan and Srivastava, 1998; Debouba et al., 2007; Goel and Singh, 2015). Likewise, under drought stress conditions, the activity of nitrate reductase and glutamine synthetase were found to be reduced in barley plants, as reported by Robredo et al. (2011). Effect of drought stress on *Brassica juncea* was reported by Singh et al. (2009) in a work that showed that photosynthesis, transpiration, water use efficiency, and other morphophysiological characters were adversely affected by this stressful condition (Goel and Singh, 2015). Increasing concentration of NaCl, polyethylene glycol, and metal salts on rice seedlings showed a marked inhibitory effect on the activity of NR (Sharma and Dubey, 2005). An important signaling molecule with diverse physiological functions in plants is nitric oxide (NO), which leads to the expression of stress response genes under various abiotic/biotic stressful conditions (Arasimowicz and Floryszak-Wieczorek, 2007 and references therein; Qiao and Fan, 2008). An enzymatic source of NO in plants is NR. Production of NO, dependent on NR activity, was recorded in several plant species, like cucumber (de la Haba et al., 2001), sunflower, spinach, maize (Rockel et al., 2002), *Arabidopsis* (Desikan et al., 2002), wheat, and tobacco (Planchet et al., 2005, 2006), and its production was demonstrated in vitro (Yamasaki, 2000) and in vivo (Rockel et al., 2002). This synthesis was strictly dependent on nitrite and nitrate content in the plant tissue (Yamasaki et al., 1999; Kaiser, 2001).

10.6 MELATONIN BIOACTIVE MOLECULE IN THE REGULATION OF ABIOTIC STRESS

Melatonin is a tryptophan derivative compound (*N*-acetyl-5-methoxytryptamine) that was discovered in the late 1950s in bovine pineal tissue (Lerner et al., 1958). Initially, melatonin was considered only to occur in animals, but further evidence emerged confirming that this compound is ubiquitously present in almost all organisms from primitive photosynthetic bacteria, red and green algae, fungi, and plants to humans (Poeggeler et al., 1991; Tilden et al., 1997; Yilmaz et al., 2014; Hattori et al., 1995; Dubbels et al., 1995).

In animals this signaling molecule is considered a hormone, acting as a neurotransmitter and influencing sleep, body temperature, mood, the retina, and sexual behavior through the circadian clock present in animals (Reiter et al., 2010). Anyway, this wide range of melatonin's functions in animals were acquired later during evolution (Reiter et al., 2010; Tan et al., 2010).

Several studies in unicellular organisms, plants and animals have confirmed the primary function of this phylogenetically ancient molecule, as a free radical scavenger and antioxidant to protect organisms from a variety of environmental and internal oxidative stresses (Galano et al., 2011; Manchester et al., 2015; Shi et al., 2015; Arnao and Hernandez-Ruiz, 2015; Zhou et al., 2015). Since the discovery of melatonin in plants around two decades ago (Hattori et al., 1995), several aspects have been investigated and driven by what is known in animals. These studies can be summarized in three main focus areas: (1) modulation of circadian rhythms and photoperiod-dependent processes; (2) medicinal and nutritional features (as nutraceutical value), with the aim of identifying plants species containing particularly high levels of melatonin; and (3) as a scavenger of free radicals thus preserving the integrity of plants under a variety of stressful conditions.

Thus, the data summarized below primarily consider the roles of melatonin related with diverse aspects linked to its protective function against abiotic stressors in plants. In particular, the melatonin levels in plants under stress conditions and the possibility of melatonin priming against multiple abiotic stresses are discussed.

10.6.1 Melatonin Biosynthesis and Its Level Under Stressful Conditions

Plant melatonin is synthesized via similar biosynthetic pathways to those in animals and comprises four consecutive enzymatic steps (Kang et al., 2011). The first step is the decarboxylation of tryptophan into tryptamine by tryptophan decarboxylase (TDC). The second step is the hydroxylation of the amine by the cytochrome P450 enzyme tryptamine 5-hydroxylase (T5H) to form serotonin (Fujiwara et al., 2010). Serotonin, in both plants and animals, is converted to N-acetyl serotonin in an enzymatic reaction catalyzed by serotonin N-acetyltransferase (SNAT), which is then O-methylated by hydroxyindole-O-methyltransferase (ASMT, formerly known as hydroxymethyl O methyltransferase, HIOMT) resulting in the formation of melatonin (Nawaz et al., 2016).

The rate limiting enzyme in the biosynthetic pathway of melatonin is ASMT (Liu and Borjigin, 2005). Interesting, recent works on subcellular localization of the final enzyme in this pathway (ASMT) show that this enzyme is localized in both mitochondria (Wang et al., 2017) and chloroplast (Zheng et al., 2017), thus representing the primary sites for melatonin biosynthesis. In fact, these plant organelles are major sources of free radical generation, such as ROS and RNS, and thus, they require strong protection against oxidative stress. Melatonin, produced in high level in plants by both chloroplast and mitochondria (Arnao and Hernández-Ruiz, 2014), is a potent antioxidant and free radical scavenger that provides onsite protection against the byproducts of energy metabolism and preserves the physiological functions of these important organelles, working as a powerhouse at photosynthetic sites (Tan et al., 2013).

Many studies show that endogenously produced melatonin increases significantly when plants are exposed to rapid change in the environmental conditions perceived by the plants as stress clues. These include hot or cold temperatures, salinity, drought, overwatering, ultraviolet radiation, high irradiation, and chemical or metal pollutants in water and soils. In barley roots (Arnao and Hernández-Ruiz, 2009) and lupin plants (Arnao and Hernández-Ruiz, 2013) the changes in endogenous levels of melatonin under natural or artificially perturbations were measured. A remarkable observation from these studies was that nearly all stress factors, such as drought, anaerobic, pH, and cold stress as well as using $ZnSO_4$, NaCl, and H_2O_2 as chemical stress agents, upregulated melatonin biosynthesis (Arnao and Hernández-Ruiz, 2009, 2013). The elevated melatonin production associated to different forms of environmental perturbation may serve as an adaptive reaction of plants to tolerate adverse environmental conditions due to its well-known antioxidant properties. Moreover, exposure to heavy metals is associated with toxic effects on biological macromolecules leading to oxidative damage. The significant rise in melatonin content observed in green microalgae *Ulva* sp. exposed to cadmium, lead, and zinc (Tal et al., 2011), and the mitigation of heavy metals-induced stress by melatonin exogenously applied, suggest the involvement of melatonin in adaptation to environmental stress.

Increases of melatonin were also reported to be a consequence of light intensity and spectral quality. Comparing plants grown indoors with plants grown under field conditions subjected to more variable conditions and sunlight showed that melatonin concentrations were increased more than threefold in plants cultivated in the field (Tan et al., 2007). Pepper nonshaded fruits contained more melatonin content than that of shaded fruit (Riga et al., 2014). In addition, Conti et al. (2002) reported that Alpine and Mediterranean species that normally are exposed to high natural light and UV intensities contained enhanced levels of melatonin compared with the same or related species from other areas. Similar results were obtained in *Glycyrrhyza uralensis* grown under high intensity UV-B radiation compared with those grown under low intensity radiation, showing increase melatonin levels in roots for plants exposed to high

UVB radiation (Afreen et al., 2006). Therefore, the upregulation of melatonin biosynthesis may protect the plant from large quantities of free radicals generated during photosynthesis, especially under stressful conditions such as exposure to excessive light intensity and UV radiations.

10.6.2 Priming Plants With Melatonin Against Abiotic Stresses

A key role for melatonin as a potent antioxidant appears to be a fundamental stress resistance mechanism in plants, supporting their resistance to different forms of stress (Zhang et al., 2015; Wang et al., 2013). As in the above-discussed studies, there are also many recent investigations that reveal a causal relationship between environmental stresses and induction of oxidative stress and damage, which are mitigated by exogenous treatments of melatonin. Melatonin pretreatment significantly increases the tolerance of both drought-tolerant *Malus prunifolia* and drought-sensitive *M. hupehensis* plants (Li et al., 2015) by the downregulation of genes involved in abscisic acid (ABA) catabolic route leading to a lower ABA content, and the enhancement of antioxidant enzyme activities, which directly scavenge H_2O_2 (Li et al., 2015). In Bermuda grass pretreated with melatonin and then exposed to different cold stress treatments a positive effect was observed of this indoleamine molecule toward cold resistance by maintaining cell membrane stability, increasing antioxidant enzymes activities, and improving the photosynthetic process (Fan et al., 2015). The role of melatonin in the alleviation of photoinhibition in tomato seedlings exposed to moderate light during chilling was recently reported to be dependent on its dual mode of action, as a direct scavenger of ROS and as a trigger of antioxidant responses, both actions leading to reduced level of ROS (Ding et al., 2017). Using exogenously applied melatonin Arora and Bhatla (2017) reported an interaction between this molecule and nitric oxide signaling pathways that is involved in the regulation of seedling growth leading to an attenuation in both oxidative and nitrosative stress under salt stress in sunflower. Furthermore, melatonin has been shown to contribute to extending leaf longevity while enhancing stress tolerance. Long-term pretreatment with melatonin significantly reduced chlorophyll degradation and suppressed the upregulation of senescence-associated gene 12 (*SAG12*) and helped maintain photosystem function under drought and dark-induced leaf senescence in apple (Wang et al., 2013). Similar results were obtained in rice where treatments with melatonin significantly reduced chlorophyll degradation, suppressed the transcripts of senescence-associated genes, delayed the leaf senescence, and enhanced salt stress tolerance (Liang et al., 2015). These authors, using a high-throughput RNA sequencing, reported a complex molecular network under the control of transcriptional activators and repressors (such as *bZIP*, *NAC*, *MYB TFs*, *DREBs*, and *HSFs* transcription factors), which consequently modulates the expression levels of genes involved in oxidation−reduction, chlorophyll biosynthesis, stress responses, nutrient metabolism and remobilization processes, and finally delays leaf senescence and enhances salt stress tolerance (Liang et al., 2015). These results indicate also that melatonin is an essential feature closely related to the delay of plant senescence.

10.7 CONCLUSION

Crop plants are subjected to multiple abiotic and artificial-induced stresses during their life span that greatly reduce productivity and also the quality of these commodities during postharvest. Recent research reviewed in Savvides et al. (2016) suggests that plants can be primed by chemical compounds to better tolerate different environmental perturbations. The evidence indicates that melatonin exogenously applied as a priming agent can prepare plant stress response due to its multifaceted action (Arnao and Hernández-Ruiz, 2014; Zhang et al., 2015; Wang et al., 2013). Given the promising potential of melatonin as a direct antioxidant molecule, as a trigger of antioxidant responses in plants, and its beneficial effects on senescence and cell death processes, it could represent an effective strategy to employ in crop management to both enhance tolerance against multiple abiotic stressors and to prolong shelf life of harvested leafy vegetables and flowers.

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CHAPTER

11

Biochemical and Molecular Regulation of Phenylpropanoids Pathway Under Abiotic Stresses

Alessandra Francini[1], *Andrea Giro*[2] *and Antonio Ferrante*[3]

[1]Institute of Life Sciences, Sant'Anna School of Advanced Studies, Pisa, Italy [2]DAFNAE—Department of Agronomy Animals Food Natural Resources and Environment, University of Padua, AGRIPOLIS—University Avenue, Legnaro, Italy [3]Department of Agricultural and Environmental Sciences, Universià degli Studi di Milanot, Milano, Italy

OUTLINE

11.1 INTRODUCTION

The phenylpropanoids pathway is very important in plants, since leads to the biosynthesis of important molecules with signaling and protection functions. The phenylpropanoids are ubiquitous compounds in plants and their biosynthesis is enhanced under stress conditions (Weaver and Herrmann, 1997). Indeed, plants have to cope with adverse environmental conditions by increase their tolerance against the different biotic and abiotic stresses, thus the potential and diversification ability of plants to produce functional molecules

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is essential for plant adaptation (Dixon et al., 2002). In agriculture, the understanding of plant adaptation is very important for reducing the quality and yield losses in crops during the adverse seasons, because the yield losses due to abiotic stress conditions can reach 70% of the total (Boyer, 1982; Mariani and Ferrante, 2017). Therefore, the biochemical and molecular studies of the biosynthesis of phenylpropanoids pathway can provide useful information for improving the yield in different agricultural areas in the world and leading the crop breeding and selection toward cultivars with different tolerance.

Furthermore, the phenylpropanoids pathway is the main sources of secondary metabolites in plants that are important because they have also antioxidant skills and the accumulation of these molecules inside fruits and vegetables can provide beneficial effects in the human diet.

The term "secondary" metabolites means that these compounds do not participate in the essential vital processes of the plant. However, contrary to this first definition these compounds play a very important role in the defense mechanism. In the past, these were considered as excretion products or final products of metabolism because they were not always present in the plants. Therefore, they were considered not essential. However, the phenylpropanoids are still classified as secondary metabolites as they are less important than compounds belonging to the primary metabolism.

The regulation of the biosynthesis of the different branches of this pathway can be exploited for improving either the nutraceutical value of food or the plant tolerance against the environmental stresses. Horticultural crops rich in flavonoids and anthocyanins are appreciated for their beneficial effect in counteracting stress and age-related diseases. The accumulation of phenolic compounds in plants under stress is well known, even if the phenolics composition of plant tissues under different abiotic stresses conditions has not easy to study. Indeed, the phenylpropanoids pathway is the main stream of thousands of molecules and the biosynthesis of them is regulated at several molecular and biochemical levels. Changes of the gene expression or biochemical activation of different enzymes are often tightly correlated with environment and stress factors. The defense mechanism of most phenylpropanoids is due to irritant, toxic, or repellent properties. Phenols are compounds with at least an aromatic ring with one or more hydroxy groups. The main classes of phenols in plants are reported in Table 11.1 (Balasundram et al., 2006; Pereira et al., 2009).

Phenylpropanoids derive from cinnamic acid and the main compounds are reported in Fig. 11.1.

TABLE 11.1 Classes of Phenolic Compounds in Plants and Carbon Number

Class	Structure	C number
Simple phenolics, benzoquinones	C6	6
Hydroxybenzoic acids, phenolic acids	C6–C1	7
Acethophenones, phenylacetic acids	C6–C2	8
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C6–C3	9
Napthoquinones	C6–C4	10
Xanthones	C6–C1–C6	13
Stilbenes, anthraquinones	C6–C2–C6	14
Flavonoids, isoflavonoids	C6–C3–C6	
Lignans, neolignans	$(C6–C3)_2$	18
Biflavonoids	$(C6–C3–C6)_2$	22
Lignins	$(C6–C3)_n$	n
Condensed tannins (proanthocyanidins or flavolans)	(C6–C3–C6)	

11.2 ABIOTIC STRESS AND PHYSIOLOGICAL CHANGES IN THE PHENYLPROPANOID PATHWAY

The phenylpropanoids pathway includes several enzymes such as phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5), chalcone synthase (CHS, E.C. 2.3.1.74), chalcone isomerase (E.C. 5.5.1.6), dihydroflavonol reductase (E.C. 1.1.1.219), and anthocyanidin synthase (ANS, E.C. 1.14.11.19). These enzymes induce the accumulation of purple pigments in the plants. The ANS is involved in anthocyanidins biosynthesis and last step of biosynthesis is catalyzed by flavonoid glycosyltransferases. Anthocyanins are water-soluble flavonoids that are produced in the cytoplasm and transferred inside the vacuole. These enzymes are responsible for the flavonoid biosynthesis before the accumulation in the vacuole (Holton and Cornish, 1995; Tanaka et al., 1995; Winkel-Shirley, 2001; Vogt and Jones, 2000).

The phenylpropanoids structure can be simple with C6–C3 carbon skeleton compounds and are biosynthesized from cinnamic acid. These compounds can have origin from the phenylalanine and undergo acylation, condensation, cyclization, glycosylation, hydroxylation, methylation, prenylation, and dehydration reactions. These include caffeic, ferulic, *p*-coumaric, and sinapic acids. The free acids are not accumulated in the cells and are often conjugated with organic acids and

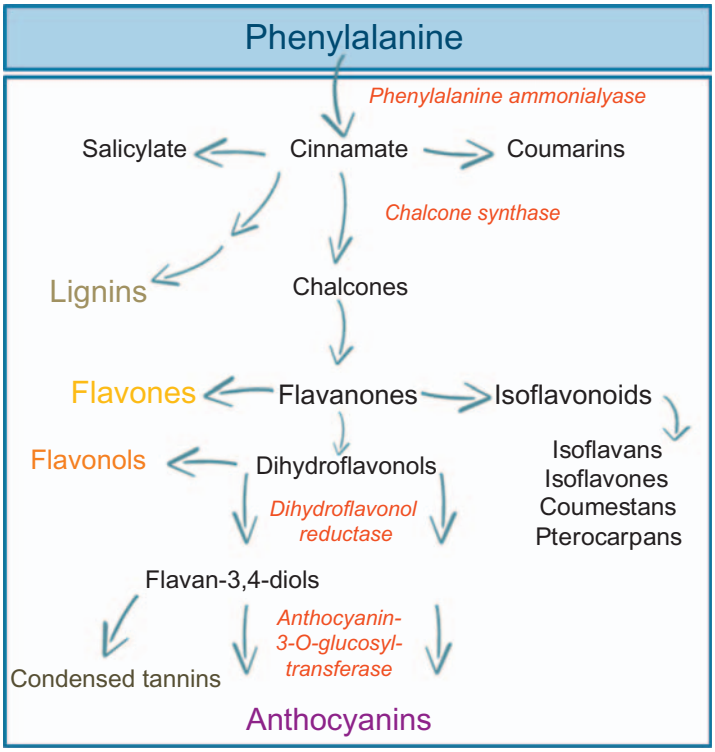

FIGURE 11.1 Phenylpropanoids pathway key enzymes involved in the biosynthesis of the main phenolic compounds. Most of these compounds are accumulated under stress conditions and provide defense mechanism.

carbohydrates. However, compounds with higher carbon skeleton with C15 are flavonoids that are biosynthesized from chalcone synthetase (Table 11.1). This enzyme condenses *p*-cumaryl-CoA and three molecules of malonyl-CoA. These C15 flavonoid skeleton compounds are the most stress-induced phenylpropanoids and lead to flavones, flavanones, anthocyanins, and 3-deoxyanthocyanins (Dixon and Paiva, 1995).

Several studies have reported that the large number of phenolic compounds can change the spectra in relation to the copigments, pH, and cell shape (Forkman, 1991). The pH usually is responsible for the color formation. Different species are not able to accumulate specific phenolic compounds indicating mutations or variation of the biosynthetic pathway. Molecular biology tools and plant transformation allowed obtaining plants with a modified the phenylpropanoids biosynthetic pathway. This strategy can be also developed in the plant's biofactory. A review highlights studies on transgenic plants with pigment pathways altered, including petunia, torenia, and carnation by the overexpression of heterologous flavonoid biosynthetic genes and/or the downregulation of endogenous genes (Tanaka, 2006).

Phenylpropanoids have an antioxidant activity and they can scavenge the reactive oxygen species (ROS) increasing tolerance to stressful conditions (Grace and Logan, 2000). ROS play a dual role as toxic byproducts of normal cell metabolism and as regulatory molecules in biotic and abiotic stress perception and signal transduction. Therefore, crops that are able to increase the biosynthesis and the accumulation of these compounds usually had better tolerance to the stress conditions.

11.3 WATER STRESS

The water stress usually increases the phyenylpropanoids biosynthesis in different plant species (Nogués et al., 1998; Parida et al., 2007). Water stress or drought reduces the plant metabolism, lowering the transpiration and photosynthesis activity. All physiological processes that consume water are drastically reduced. Unfortunately, the reduction of water has as consequence an increase of plant temperature with increase of respiration and the reduction of photosynthesis leading leaves to stress conditions for an excess of light. These conditions lead to accumulation of ROS and cell damage. The phenylpropanoids are involved in the detoxification and protection mechanisms. The induction of phenylpropanoids biosynthesis and accumulation has been observed in different plant species and different plant organs under water stress conditions.

In *Scrophularia striata* seedlings the exposure of seedling to water stress increased PAL and tyrosine ammonia-lyase (EC 4.3.1.23) enzymes activities and were correlated with the biosynthesis of secondary metabolites (Falahi et al., 2018). In stressed roots of

S. striata the main phenolic acids and flavonoids accumulated were salicylic acid, ferulic acid, cinnamic acid, coumaric acid, caffeic acid (phenolic acids), genistein, myricetin (flavonoids), and resveratrol (stilbenoid).

The accumulation of total phenols under water stress occurs in all plants and in both shoots and roots. Experiments carried out on soybean under water stress demonstrated that at the apical region of roots in the elongation zone an increase of isoflavone content was observed (Fig. 11.2). CHS proteins were highly accumulated in this root region (Yamaguchi et al., 2010). *Hypericum brasiliense* plants exposed to water stress showed an increase of phenols but with difference in terms of compounds between shoots and roots. Rutin and quercetin increased in shoots while these were not detected in roots (de Abreu and Mazzafera, 2005), indicating a different phenolics composition and distribution in the plant. The increase of quercetin and rutin under water stress has been also observed in other wood plants such as *Ligustrum vulgare* (Guidi et al., 2008) and *Crataegus monogyna* (Kirakosyan et al., 2004). These compounds can be associated to water stress and can be used as a marker for screening plant's tolerance to this abiotic stress.

In cherry tomatoes, it has been demonstrated that cultivars with higher biosynthesis of phenylpropanoids and flavonoids had higher tolerance to water stress. Cherry tomatoes under moderate water stress in the most tolerant cultivar significant changes were observed among phenolic acids and in quercetin-3-api-rut, kaempferol-3-api-rut, and kaempferol-3-rut (Sánchez-Rodríguez, et al., 2011). Medicinal plants that are usually rich in bioactive compounds exposed to water stress accumulated higher amounts of phenols. In *Echinacea purpurea*, the drought can induce an increase of accumulation of total phenol up to 67% (Gray et al., 2003).

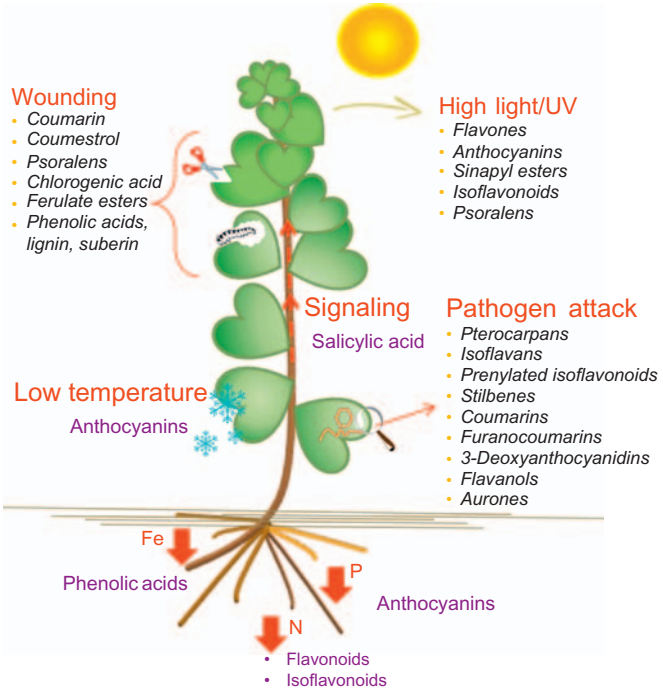

FIGURE 11.2 Environmental stresses affecting the biosynthesis of bioactive molecules able to protect the plants and induce adaptation strategies for coping with adverse conditions.

11.4 COLD STRESS

The low temperatures can induce damage to different plant organs acting at plasma membrane level. The cold injury usually acts by reducing the membrane stability and can disrupt the phospholipids. The tissue damage can be monitored by measuring the lipid peroxidation or electrolyte leakage (Campos et al., 2003; Ferrante et al., 2008). Chilling injury can also induce an increased respiration with accumulation of ROS (Suzuki and Mittler, 2006), which can be an accumulation from the stress, but they can also act as signaling molecules. The plants in response to cold can induce the biosynthesis of several compounds such as soluble sugars, sugar alcohol, and compounds derived from nitrogen such as proline. Among the defense mechanism, also the phenylpropanoid compounds play a role as scavengers of ROS (Sharma et al., 2012). Low environmental temperatures increase the PAL activities with a consequent accumulation of phenols. This behavior has been found in tomato and watermelon plants grown at 15°C compared with 25°C (Rivero et al., 2001). Results confirm that the accumulation of phenolic compounds can be a strategy of plants to increase tolerance and improve adaptation to suboptimal environmental temperature conditions.

11.5 SALINITY STRESS AND PHENYLPROPANOIDS ACCUMULATION

Plants subjected to stress conditions such as salinity respond by activating tolerance mechanisms at multiple levels of organization (anatomical, morphological, and molecular). The salt-stressed plants usually undergo damages from an excess of sodium accumulation in the cells. Most sodium is translocated and accumulated in the vacuoles, but when increased in the cytoplasm many physiological processes are compromised. From a biochemical point of view, the complex way of the phenylpropanoids pathway is usually activated in response to salt stress. This pathway is a key point for production of many secondary metabolites inducing scientists to investigate the mechanism of

regulation of their biosynthesis under salt stress conditions. In fact, phenylpropanoid pathway is, considered in the same way, a model for understanding metabolites flux control and a target for biotechnological manipulation to improve molecule accumulation.

Regarding salinity effects on phenolic compounds in olive plants, Wiesman et al. (2004) showed that the high polyphenol contents recorded in oils of saline irrigated plants can be explained by the acceleration of maturation of the olives, which could account for the higher levels of phenols.

The use of saline water in Chemlali olive plants irrigation induces phenol accumulation in oil produced (Behn et al., 2009). Rossi et al. (2016) demonstrated that phenolic compounds in Frantoio leaves increased under salinity stress in new and old leaves. On the other hand, in Leccino, where the translocation of sodium to leaf was even higher than in Frantoio, the phenolic compounds remained stable under salinity stress.

The phenylpropanoids pathway in cumin seems to be stimulated under salinity (Bettaieb Rebey et al., 2017). The total polyphenol content was higher in treated seeds and salinity improved the amount of individual phenolic compounds. The accumulation of phenolic compounds in response to NaCl treatment was primarily caused by an increase in the concentrations of the two major compounds, *p*-coumaric acid and luteolin, despite the fact that the profile of minor compounds was changed (Bettaieb Rebey et al., 2017). Mild salt stress (80 mmol/L NaCl) improves strawberry fruit quality by increasing the phenylpropanoids content. The increase of total phenolic content in strawberry was most related to the increase in the content of (+)-catechin. Since catechins and epicatechins have strong antioxidant properties, the increase in these phenolic acids implies an improvement on the functional properties of strawberry fruit.

11.6 LIGHT STRESS

Light is an essential factor for plant growth and development and each species has specific requirements to complete their physiologic life cycle. Light quantity (intensity and duration) and quality (spectral composition) affect fundamental plant physiological, morphological, and anatomical processes (Devlin et al., 2007). Furthermore, the quality and intensity of light is essential for the photosynthesis, the development, and reproduction. Leaves are organs that have the light harvesting complexes (chlorophylls, carotenoids) and the photoreceptors. However, the excess of light compared with the photosynthetic capacity of the plants can damage leaves' cells. The excess of light can be dissipated as fluorescence and heat by photosystems, but the excitation energy and electrons can generate ROS and accumulation in tissues, leading to cell damage. Plants for defense activate several detoxification enzymes and metabolites such as anthocyanins that are able to scavenge the ROS to reduce the damages of these radicals (Niyogi, 1999). This explains why phenols and in particular anthocyanins are accumulated under high intensity light stress. Anthocyanins accumulation under high-light conditions has been proven to act as a photoprotection strategy of the plants (Chalker-Scott, 1999; Field et al., 2001). The accumulation usually occurs in the upper part of the leaf epidermal layer, protecting the leaf pigments from photodamage. The protection function of anthocyanins has been proved by treating the plants with an inhibitor of PAL enzyme, the as 2-amino-indan-2-phosphonic acid (AIP). The treated plants showed higher leaf damage after the exposure to UV-B than the control, demonstrating the importance of phenylpropanoids in photoprotection (Gitz et al., 1998). The UV light induces in plants the biosynthesis of phenylpropanoids as a protection strategy. In fact, UV-A and UV-B can induce the activation of phenylpropanoids pathway (Mariz-Ponte et al., 2018). In particular, the short exposure to UV-A/B lights increased the phenolic compounds, without increasing the antioxidant systems. This means that the phenolic compounds are able to counteract the light damage without inducing any further protection systems.

11.7 SENESCENCE

Senescence is a genetically and highly programmed process that represents the last stage of organ or organism development (Rogers, 2013; Iqbal et al., 2017; Khan et al., 2017; Zentgraf et al., 2018). Leaves and flowers are plant organs that undergo senescence for specific function, such as the renewal of the leaves or after pollination in flowers. The leaf senescence in many deciduous plants during the autumn season is characterized by anthocyanins biosynthesis (Hoch et al., 2001), turning the leaf color dark purple. The accumulation of anthocyanins seems to be correlated with leaf protection from excessive light when during senescence other protecting pigments decline. This strategy can be associated with nutrient recycling of deciduous plants and the anthocyanins are responsible for the leaf functionality in this last stage of development (Field et al., 2001). The accumulation of anthocyanins occurs before the chlorophyll degradation and they should act as scavengers for ROS that increase during leaf and flower senescence (Chalker-Scott, 1999; Trivellini et al., 2017).

The anthocyanins have been considered to protect senescing leaves from excessive irradiance, substituting for the carotenoids' function. However, another hypothesis is the antioxidant function for removing the ROS formed during senescence. It can represent the last chance for avoiding cell death. The accumulation of anthocyanins prior to senescence has been observed in several plant species. In *Nicotiana mutabilis* the petal senescence is associated with the accumulation of anthocyanins (Macnish et al., 2010). The application of flower senescence inhibitors such as ethylene inhibitor delayed the anthocyanins accumulation, demonstrating the relationship between senescence and anthocyanins. In petunia, the higher anthocyanins content in different cultivars was also associated with longer flower life and treatments with AOA, an inhibitor of ethylene biosynthesis, reduces the anthocyanins biosynthesis (Ferrante et al., 2006).

11.8 NITROGEN DEFICIENCY AND PHENYLPROPANOIDS

Nutrient deficiency reduces growth and induces physiological disorders in plants. The most stressful nutrient deficiency can be observed in plants under nitrogen starvation. Nitrogen (N) is an essential element for plant growth and yield (Rubio-Wilhelmi et al., 2011). N is one of the main elements involved in amino acids biosynthesis (Fukushima and Kusano, 2014). Many metabolic changes occur in plants under low availability of nitrogen because its assimilation is strictly related into a complex network with primary metabolites (Fritz et al., 2006). Furthermore, the flux of primary metabolites, such as sugars and amino acids, is transferred into secondary metabolism by PAL inside the phenylpropanoids pathway. Lignin, flavonoids, and anthocyanins are biosynthesized through this pathway, so their accumulation in plants is related to PAL activity. PAL regulates the initial step of phenylpropanoids synthesis removing nitrogen from phenylalanine (Olsen et al., 2008). Therefore, low N content triggers the biosynthesis of phenols, because in response to the lack of nitrogen, plants increase PAL activity, to recycle amine from phenylalanine to counteract the reduced availability of free amine for the synthesis of new amino acids. As a consequence of the removal, ammonium ions released by PAL can be assimilated in different ways, for example, by GS/GOGAT system; hence the resulting nitrogen-free carbon skeletons of t-cinnamate can be shunted into different pathways of secondary metabolism. However, removing the amine group from the phenylalanine is a futile cycle that is transiently activated by plants. Structural genes such as PAL, chalcone synthase (CHS), and flavanone 3-hydroxylase (F3H) are overexpressed in response to low-N availability (Olsen et al., 2008; Sun et al., 2017). Moreover, other enzymes involved in the subsequent pathway of anthocyanins biosynthesis such as ANS are positively and negatively regulated by *MYB* transcription factors triggers by N-deficiency (Soubeyrand et al., 2014) (Sun et al., 2017). Therefore, using a subdeficient fertilization it is possible to enhance accumulation of phenols and anthocyanins in plants, improving their nutraceutical content.

11.9 MOLECULAR CHANGES OF THE GENES INVOLVED IN THE PHENYLPROPANOID PATHWAYS

The amount and the type of phenylpropanoid-derived compounds vary among the different plant species and also depend on the stress sensitivity of plants and the biosynthetic ability of the tissues. In *Arabidopsis* model plants, phenylpropanoids biosynthesis and metabolism have been widely studied and confirmed in different agricultural crops. Rocket plants exposed to preharvest (salinity, heat, and nitrogen starvation) and postharvest stresses (cold, dehydration, dark, wounding) showed several differentially expressed genes belonging to phenylalanine metabolism (Cavaiuolo et al., 2017).

A transcriptome approach analysis carried out on *Begonia semperflorens* studied the differential expressed genes and the phenylpropanoid biosynthesis genes under high-light and low-temperature stress conditions (Table 11.2). Results indicated that begonia activates the same defence strategy at both high-light and low temperature conditions, inducing the same phenylpropanoids related genes. Although, the phenilpropanoid metabolites were different under the two stressful conditions. Anthocyanins were biosynthesized under high-light conditions, while under low temperature lignin, proanthocyanidins, and anthocyanins were mainly accumulated (Dong et al., 2018).

11.9.1 Phenylalanine Ammonia-Lyase and Chalcone Synthase Transcriptional Induction

Among the abiotic stresses, the suboptimal environmental temperatures induce the expression of genes encoding for enzymes involved in the phenylpropanoids biosynthetic pathway. Low temperatures in correlation with light intensity regulate the phenylpropanoids pathway and particularly the key enzymes PAL and CHS (Leyva et al., 1995). Differently, microarray studies of *Arabidopsis* exposed to water stress revealed among the differential expressed genes the phenylpropanoid enzymes (Bray, 2002).

TABLE 11.2 Genes Encoding for Phenylpropanoid Enzymes Involved in the Stress Response in Plants

Species	Gene	Stress	References
Antirrhinum majus, *Medicago sativa*	CHS	$CuCl_2$, Wounding, UV	Junghans et al. (1993), Lipphardt et al. (1988)
Arabidopsis thaliana	PAL, CHS, ANS	Low temperature, UV-B, UV-A, blue light, high-lights intensity, salicylic acid, ethylene, MeJ	Leyva et al. (1995), Fuglevand et al. (1996), Hartmann et al. (1998), Wade et al. (2001), Feinbaum and Ausubel (1988)
Brassica rapa	ANS	Cold, freezing	Ahmed et al. (2015)
Petroselinum crispum	CHS	UV	Schmelzer et al. (1988), Schulze-Lefert et al. (1989)
Petunia hybrida	CHS	UV, low temperature	Koes et al. (1989), Shvarts et al. (1997)
Picea glauca	CHS	Wounding, JA, MeJ	Richard et al. (2000)
Pinus sylvestris	CHS	UV-B	Schnitzler et al. (1997)
Solanum tuberosum	PAL, CHS, flavanoid monooxygenase, flavone 3β-hydroxylase, chalcone-flavone isomerases, and dihydroflavonol 4-reductase	Drought	Schafleitner et al. (2007)
Vitis vinifera	CHS	Wounding, UV-C	Vannozzi et al. (2012)

At the molecular level, the stress conditions induce always the gene expression of PAL that is the key enzyme of phenylpropanoids. The lettuce plants treated with AIP, a specific inhibitor of PAL, showed lower tolerance to water stress and slower growth (Oh et al., 2009).

However, in cold stressed plants or low-temperature adapted plants activate the biosynthesis of many others phenylpropanoids compounds, in particular anthocyanin. The biosynthesis of these molecules has been reported in several plants in young, mature, or senescing leaves exposed to cold temperature. Moreover, maize seedlings, exposed to low temperature, showed as well as the increase of mRNA abundance of PAL, CHS, and ANS leading to anthocyanins accumulation (Christie et al., 1994). The CHS is regulated by different abiotic stress such as UV light, bacterial, or fungal infection (Dao et al., 2011) and the increase of this enhances the accumulation of flavonoids for reducing the stress of plants.

Water stress analogously as observed for other stress induces the gene expression of phenylpropanoid genes in different tissues. In roots of maize under water stress two cinnamoyl-CoA reductase 1 and 2 genes were enhanced the lignin biosynthesis as a defense mechanism strategy (Fan et al., 2006).

Salinity stress also affects the phenylpropanoids pathway. *Lotus japonicus* exposed to salt stress reduced PAL activities in roots, but in the tissue the quercetin glycosides increased while others phenylpropanoids decreased (Mrázová et al., 2017). Microarray analysis in *Vitis vinifera* showed that *VvCHS* gene family was upregulated by wounding and UV-C (Vannozzi et al., 2012).

11.9.2 Anthocyanidin Synthase Transcriptional Changes

Maize seedlings were exposed to low temperature for studying the molecular chances of the phenylpropanoid genes. The exposure of the seedlings at 10°C for 24 h induced the expression of genes involved in the anthocyanins accumulation. The anthocyanins genes were upregulated by 7- to 10-fold (Christie et al., 1994). Crops with high accumulation of anthocyanins during fruit ripening, such as grapevines, show earlier ripening when exposed to water stress. The water limitation increases the expression of the phenylpropanoid genes leading to higher accumulation and anticipated fruit ripening (Castellarin et al., 2007).

Brassica rapa plants exposed to cold or freezing temperature respond by activating four SNS genes (Ahmed et al., 2015). The expression of ANS genes was variable among species and the highest mRNA abundance was observed after 8 h of cold stress.

In potato plants after 23 days of drought upregulation *PAL*, *CHS*, *flavanoid monooxygenase*, *flavone 3β-hydroxylase*, *chalcone-flavone isomerases*, and *dihydroflavonol 4-reductase* genes were observed (Schafleitner et al., 2007). This induction can be a defense strategy to control the oxidative stress induced by the drought conditions.

11.10 REGULATION AND PHENYLPROPANOIDS PATHWAY

MicroRNAs (miRNAs) are gene products composed by 20–21 nucleotides that are able to regulate the transcription of target genes (Lee et al., 2002). The regulation of phenylpropanoids by microRNAs (miRNA) under abiotic stresses has been demonstrated in *Arabidopsis*. In particular, the *miRNA156* seems to play an important role in the regulation of plant tolerance (Cui et al., 2014). The overexpression of *miRNA156* induced an increase of stress tolerance to salt and drought, while the reduction of the expression increased the sensitivity. This study demonstrated the active role of miRNAs in plant adaptation under environmental stress conditions. In a study carried out on poplar and *Arabidopsis* a miRNA, the ptr miR408, was found to be associated with the tissue lignification as response to pathogen attack (Lu et al., 2005). Since, miRNAs are short sequences of RNA they can be synthetized and used for function studies. The application of artificial miRNAs can be exploited for the regulation of phenylpropanoids pathway and enhance the secondary metabolites accumulation and increase the crop tolerance (Sreekumar and Soniya, 2018).

11.11 CONCLUSION

The phenylpropanoids pathway has a pivotal role in the regulation of plant physiology and metabolism under stress conditions. These compounds induce crop tolerance by reducing the oxidative stress of tissues and increasing the plant recovery poststress events. All tissues under stress can induce the accumulation of phenylpropanoids under stress conditions. The most important organs of the plants, such as leaves, flowers, and roots, are able to accumulate high amount of phenylpropanoids. The identification of plant with high tolerance to abiotic stresses has to be searched among those that have high accumulation of phenylpropanoids. Further studies can be useful for leading genetic improvements with the aim to increase plant adaptation or in agricultural crops leading to higher yields even under stressful conditions.

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CHAPTER

12

Role and Regulation of Glucose as a Signal Molecule to Salt Stress

Péter Poór[1], *Zalán Czékus*[1,2] *and Attila Ördög*[1]

[1]Department of Plant Biology, University of Szeged, Szeged, Hungary [2]Doctoral School in Biology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

OUTLINE

12.1 INTRODUCTION

Environmental stresses can disrupt cellular structures and impair physiological functions of plants leading to growth perturbation, reduced fertility, premature senescence, and yield loss (Khan and Khan, 2013; Pasala et al., 2016). To counteract stressful changes and grow successfully, plants launch resistance mechanisms by reprogramming metabolism and gene expression to the stressful conditions, and by acquiring a new equilibrium between growth and development or defense (Morkunas and Ratajczak, 2014). Soluble sugars, the products of photosynthesis (glucose, fructose, and sucrose) interacting with phytohormones play an important role in maintaining the overall cell structure, in growth and development and in regulating stress responses in a complex manner (Couée et al., 2006; Chaves et al., 2009; Rosa et al., 2009; Wind et al., 2010; Per et al., 2018). Salt stress is one of the most harmful environmental stresses in arid and semiarid regions, which can also disrupt the homeostasis of plant cells (Khan et al., 2012, 2014), that is, accompanied by changes in sugar metabolism (Ashraf and Harris, 2004; Sami et al., 2016). Moreover, there is a strong correlation between changes in soluble sugar concentration and development of successful stress tolerance in plants. In this review we summarize the current knowledge about the effects of salt stress on glucose concentration, as well as the physiological, and the molecular aspects of glucose metabolism. Cross-talk between sugars and several phytohormones under salt stress has also been discussed (Fig. 12.1).

Plant Signaling Molecules.
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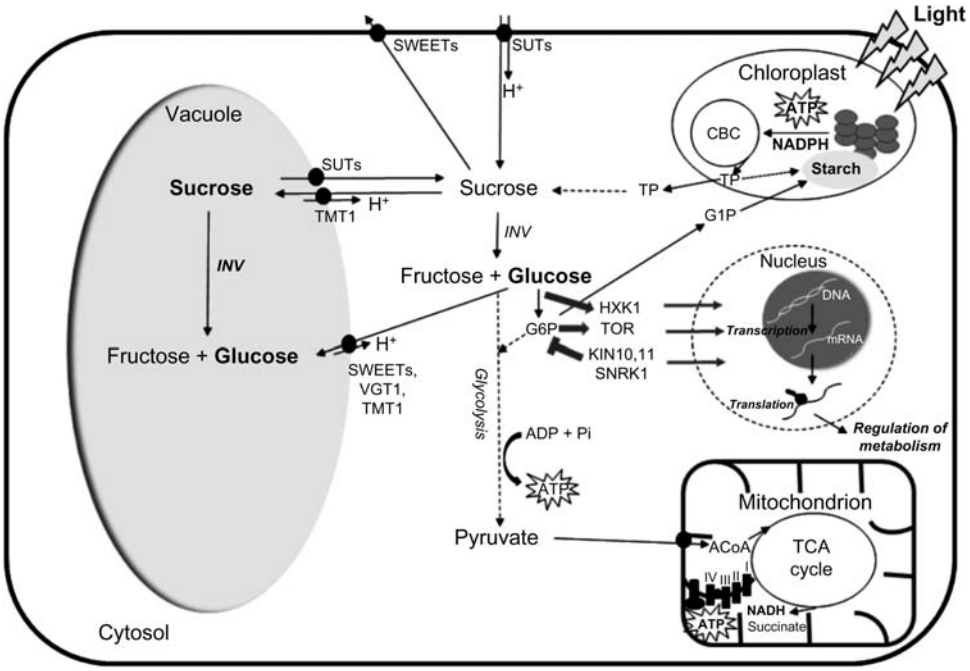

FIGURE 12.1 Schematic representation of glucose metabolism, transport, storage, and signaling in plant cells.
ACoA, Acetyl coenzyme A; *CBC*, Calvin–Benson cycle; *G1P*, glucose 1-phosphate; *G6P*, glucose 6-phosphate; *HXK1*, hexokinase 1; *INV*, invertase; *KIN10,11*, *Arabidopsis* protein kinase 10,11; *SUTs*, sucrose transporters; *SWEETs*, SUGARS WILL EVENTUALLY BE EXPORTED sugar facilitators; *TCA cycle*, tricarboxylic acid cycle; *TMT1*, glucose, fructose, sucrose-H^+ exchanger; *TOR*, target of rapamycin; *TP*, triose-phosphate; *VGT1*, vacuolar glucose transporter 1.

12.2 SALT STRESS EFFECTS ON PLANTS

Salt stress is one of the most important environmental stress factors that decreases the yield and productivity of crops (Munns, 2002; Munns and Tester, 2008; Pasala et al., 2016). Munns and Tester (2008) defined three distinct responses of plants to high salinity. Firstly, it induces osmotic stress in plants and reduces water uptake by the roots leading to a reduction in relative water content of the tissues and to decreased water potential, which results in growth inhibition, stomatal closure, and a decrease in photosynthesis and transpiration. Secondly, salt-specific ionic stress disrupts the optimal K^+/Na^+ ratio in the cells, where the high K^+ and low Na^+ concentrations in the cytoplasm play a primary role in the protection of cellular functions (Zhu, 2002). The supraoptimal concentration of Na^+ in the leaf tissues results in premature senescence of older leaves, decreased biomass production, or it may induce programmed cell death of plants (Shabala, 2009). Finally, salt stress can cause deregulation, overflow or even disruption of the electron transport chain in mitochondria and chloroplasts and thus induces high and rapid production of reactive oxygen (ROS) and nitrogen species in plant tissues, which can destroy membranes, proteins, and macromolecules (Gémes et al., 2011; Khan and Khan, 2017; Poór et al., 2015a).

Plant responses under salt stress depend not only on the concentration of NaCl but also on the duration of the stress and on the age and sensitivity of the plants (Munns and Gilliham, 2015). Plants have evolved two strategies to vary the harmful degree of salt stress: excluding Na^+ and Cl^-, particularly from photosynthesizing leaves, and synthetizing organic compounds for osmotic adjustment ("ion exclusion"); or accumulating Na^+ and Cl^-, but having strict ionic regulation in various cell compartments ("tissue tolerance") (Roy et al., 2014; Munns and Gilliham, 2015). Firstly, osmotic adjustment can be achieved by the accumulation of compatible solutes such as proline, glycine betaine, mannitol, or soluble sugars (glucose, fructose, and sucrose) (Sakamoto and Murata, 2002; Mahajan and Tuteja, 2005; Krasensky and Jonak, 2012; Khan and Khan, 2013; Khan et al., 2014; Sami et al., 2016; Takács et al., 2017; Per et al., 2017). Secondly, the maintenance of the optimal K^+/Na^+ ratio, the high cytosolic K^+, and low Na^+ concentrations are of primary importance to protect cellular functions (Zhu, 2003). The precise control of K^+/Na^+ is mediated by several K^+ and Na^+ transporters and H^+ pumps (H^+-ATPases, H^+-PPases, SOS1, HKTs, and NHXs) in shoot and root tissues (Rodríguez-Rosales et al., 2009; Yamaguchi et al., 2013; Shabala, 2013; Hamamoto et al., 2015; Almeida et al., 2017). Both osmotic and

ionic effects of salt stress induce the accumulation of ROS, which negatively affects cellular structures and metabolism. Thus, the generation and detoxification of ROS by various antioxidant systems can also determinate the survival of plants under salinity stress (Miller et al., 2010; Kocsy et al., 2013; Poór et al., 2015b; Tari et al., 2015). However, the role of glucose in the regulation of ROS scavenging in plant tissues under salt stress has not been examined in detail (Hu et al., 2012).

12.3 GLUCOSE SENSING, TRANSPORT, AND SIGNALING IN PLANTS

Sugars, interacting with phytohormones, play a pivotal role in the life cycle of plants, not only as the main carbon and energy source but also as signaling molecules. The photosynthetic tissues as a source can produce their own sugars while the nonphotosynthesizing tissues act as only a sink. Thus, the control of production, transport, storage, and utilization is crucial for controlling growth, development, and defense of plants (Sheen, 2014; Daloso et al., 2016; Aguilera-Alvarado and Sánchez-Nieto, 2017). Glucose is one of the hydrolytic products of sucrose by invertase (INV) and it acts as a major sugar signaling metabolite (Rolland et al., 2006; Sheen, 2014). Namely, it regulates cellular activity at multiple levels, from transcription and translation to protein stability and activity (Rolland et al., 2006). There were found three glucose-modulated master regulators in *Arabidopsis* (Sheen, 2014; Li and Sheen, 2016). Hexokinase (HXK1) is the most characterized direct glucose sensor mediating multiple functions in the glucose repression and promotion of gene transcription (Moore et al., 2003; Cho et al., 2006). At the same time, HXK catalyzes the first step of glycolysis, the conversion of glucose to glucose 6-phosphate (Granot et al., 2013). Control of glucose abundance and signaling by HXK contributes not only to the regulation of plant development and growth, but also to the defense mechanisms in accordance with several phytohormones, like ethylene or abscisic acid (ABA) (Claeyssen and Rivoal, 2007; Cho et al., 2010; Granot et al., 2013; Li and Sheen, 2016; Aguilera-Alvarado and Sánchez-Nieto, 2017). Besides the direct sensing of glucose by HXK1, intracellular sugar levels can be perceived as metabolic input by other energy sensing regulators thus coordinating plant metabolism and stress signaling (Li and Sheen, 2016). The evolutionarily conserved energy sensor TOR (target of rapamycin) protein kinase is also activated by glucose (Xiong and Sheen, 2014). While KIN10/11 protein kinases are repressed by glucose, which provides catalytic activity in the evolutionarily conserved energy sensor complex SNRK1 (Baena-González et al., 2007).

Not only sensing, but also carbohydrate partitioning, the process of assimilating, transporting and distributing sugars from source to sink tissues, is fundamental to plant growth and development, as well as to acclimatization under environmental stress stimuli (Rosa et al., 2009; Lemoine et al., 2013; Julius et al., 2017). Sucrose (SUTs) and monosaccharide (MSTs) transporters are the main components of this process and can mediate long-distance transport of sugars from source to sink organs. Two families of transporters are involved in the loading and unloading of plant monosaccharides across the plasma membrane. The first family members are MSTs, which are distributed to other seven subfamilies according to their substrate specificity, like the glucose transporting sugar transport protein (STP) and vacuolar glucose transporter (VGT). The second family are the SWEETs, which were initially characterized also as glucose uniporters (Chen et al., 2010; Julius et al., 2017; Martinoia, 2018).

It is well known that glucose plays role in controlling photosynthesis by repressing of chlorophyll *a*/*b* binding protein (CAB) and Rubisco small subunit (RBCS) genes (Krapp et al., 1993). Several experiments suggested that increasing sugar levels delayed seed germination and stimulated the induction of flowering or senescence in various plant species (Gibson, 2005). It was also observed that the senescence-associated gene 12 (*SAG12*), whose expression is highly senescence-specific, was induced several hundred-fold by growth on glucose in combination with low nitrogen supply (Wingler et al., 2005). These results suggest the important role of the timing, the amount, and tissue-specific accumulation of glucose during stress stimuli in plants.

12.4 ROLE OF GLUCOSE IN SALT STRESS RESPONSES

Various salt treatments can influence sugar production, transportation, or accumulation in many plant species and plant organs in a time- and concentration-dependent manner (Table 12.1). Glucose accumulation was observed in roots (Hartzendorf and Rolletschek, 2001; Anjum, 2008; Mišić et al., 2012; Siahpoosh et al., 2012; Gashaw et al., 2014; Swain et al., 2017), root exudate (Sacchi et al., 2000), stem (Hu et al., 2000; Kerepesi and Galiba, 2000), leaves (Hassine and Lutts, 2010; Rubio et al., 2010; Cao et al., 2011; Theerawitaya et al., 2012; Grzesiak et al., 2013; Lee et al., 2016), fruits (Navarro et al., 2005; Rubio et al., 2009; Yin et al., 2009; Kataoka et al., 2017), cotyledon (Ruffino et al., 2010), seedling (Morsy et al., 2007), calli (dos Santos and Caldeira, 1999; Liu and Van Staden, 2001), and cell suspension cultures (Mahasal et al., 2011) after various

TABLE 12.1 Effects of Salt Stress on Glucose Contents

Plants	Organs	Dose of NaCl exposure	Duration of NaCl exposure	Plant responses	References
Atriplex halimus	Leaf	160 mM	10 days	NaCl induced the accumulation of nonreducing sucrose, glucose, and fructose. Exogenous abscisic acid (ABA) treatment (50 μM) had no impact on glucose and fructose levels under salinity stress	Hassine and Lutts (2010)
Bruguiera sexangula	Cell suspension	150 mM	24 h	Glucose level was significantly reduced in salt-treated cells. Together with the reduced glucose level, the pool sizes of glucose-6-phosphate and fructose-6-phosphate were also reduced in salt-stressed cells	Suzuki et al. (2005)
Capsicum annuum	Leaf	30 mM	3 days	Salinity increased the fructose, glucose, and sucrose levels. Water-soluble carbohydrates increased with decreasing K^+ concentration	Rubio et al. (2010)
Capsicum annuum	Fruit	30 mM	184 days	Glucose and fructose increased in green and red fruits of pepper plants under saline condition	Rubio et al. (2009)
Catharanthus roseus	Leaf	250 mM	3 weeks	Treatment with NaCl led to a significant decrease in the contents of fructose and glucose during the 3-week course observed. In this case, sucrose content increased at the second and third week of treatment. Exogenous trehalose treatment (10 mM) elevated the contents of these sugars under salinity stress	Chang et al. (2014)
Chenopodium quinoa	Seed (embryonic axes, cotyledon)	400 mM	4, 6, 10, and 14 h	Reduced glucose and fructose contents were found in embryonic axes in the presence of NaCl. However, the glucose and fructose contents did not differ significantly in the cotyledons. Levels of sucrose were higher in NaCl-treated cotyledons than in untreated seeds	Prado et al. (2000)
Chenopodium quinoa	Cotyledon	250 mM	6, 12, and 21 days	The concentration of total soluble sugars, sucrose, and glucose were higher but the level of fructose was lower in salt-treated than in control cotyledons	Ruffino et al. (2010)
Citrus limon	Leaf	80 mM NaCl	60 days	Sucrose was dramatically reduced, while leaf fructose concentration was significantly increased and glucose content did not change under salt stress	Tsabarducas et al. (2015)
Citrus reshni, Poncirus trifoliata x Citrus sinensis	Root, leaf	40 and 80 mM	12 weeks	The concentrations of fructose, glucose, and sucrose in the leaves of Cleopatra mandarin and both in the leaves and roots of Troyer citrange decreased with the increase of salinity level. However, the concentration of sugars in the roots of Cleopatra mandarin increased with the increase in salinity level	Anjum (2008)
Glycine max	Calli	100 mM	1, 4, 8, 12 days	Glucose and fructose concentrations increased in the callus after NaCl treatments	Liu and Van Staden (2001)
Gossypium hirsutum	Root exudate	100 mM	9 days	Higher net exudation of glucose and fructose was observed from roots of cotton plants grown under saline condition	Sacchi et al. (2000)
Helianthus annus	Calli	50 and 100 mM	30 days	Higher concentration of NaCl increased glucose and fructose contents, but the moderate concentration of NaCl did not change significantly glucose and sucrose contents in calli	Dos Santos and Caldeira (1999)
Lolium perenne	Leaf protoplast	250 mM	6 h	The survival rate of the protoplasts increased when xylitol, cellobiose, 1-kestose, maltose, maltotriose, raffinose and trehalose were introduced, while no changes occurred when fructose, fucose, galactose, glucose, inositol, mannitol, mannose, rhamnose, sorbitol, sorbose, fructobiose, lactose, and sucrose were introduced	Unno and Maeda (2008)

(*Continued*)

TABLE 12.1 (Continued)

Plants	Organs	Dose of NaCl exposure	Duration of NaCl exposure	Plant responses	References
Lotus japonicus	Nodule	50 mM	10 weeks	Sucrose and glucose decreased in nodules exposed to NaCl stress. Salt and trehalase inhibitor validamycin A (30 μM) increased total soluble carbohydrates of nodules but not those such as starch, sucrose, and glucose	López et al. (2006)
Lupinus albus	Leaf	50 and 150 mM	12 days	Glucose content decreased with salt stress, sucrose content was almost three times higher in plants treated with 150 mM NaCl and fructose content did not change significantly	Fernandes et al. (2004)
Olea europaea	Leaf	75 and 125 mM	5 months	Glucose content increased after 75 mM, but decreased after 125 mM NaCl treatment. There is a close relationship between oleuropein concentration and glucose, which is characterized by a significant negative correlation and an opposite response of both substances as salinity increased. Oleuropein acts as a glucose-reservoir for osmoregulation or high energy-consuming processes required for plant adaptation to salinity	Petridis et al. (2012)
Oryza sativa	Seedling	100 mM	4 days	In chilling-sensitive rice genotype, galactose decreased or was unchanged while glucose and fructose increased in response to salt stress. In tolerant seedlings, these parameters did not change significantly, but sucrose decreased	Morsy et al. (2007)
Oryza sativa	Leaf	150 mM	24 h	Salt stress induced the accumulation of glucose, fructose, and saccharose in the leaves of wild-type rice. In *OsGMST1* knockdown rice seedlings, a mutant in the Golgi-localized monosaccharide transporter is hypersensitive to NaCl stress, as the NaCl-induced accumulation of glucose and fructose is impaired in knockdown plants, and the accumulation of sucrose was also different from the wild-type plants	Cao et al. (2011)
Oryza sativa	Cell suspension	100 mM	1, 3, 6, 10 and 14 days	Increased expression level of *OsMST3* (*Oryza sativa* L. monosaccharide transporter 3) and high glucose concentration at the early period of NaCl treatment was observed in suspension cells	Mahasal et al. (2011)
Oryza sativa	Leaf	150 mM	3 and 7 days	Glucose, fructose, and total soluble sugar contents in the flag leaf tissues of salt stressed Pathumthani 1 (PT1; salt-sensitive) rice were significantly enhanced especially after 3 days of salt exposure. In Homjan (HJ; salt-tolerant) rice, the soluble sugar level in the flag leaf was unchanged	Boriboonkaset et al. (2012)
Oryza sativa	Root	100 mM	13 days	Decreased pool sizes (glucose) were found in the sensitive rice cultivars upon exposure to salt stress. The salt-tolerant Pokkali cultivar showed increased pools of glucose, fructose, inositol, galactinol, and raffinose in the root. Leaves accumulated these carbohydrates after salt treatment	Siahpoosh et al. (2012)
Oryza sativa	Leaf	200 mM	1, 4 and 8 days	Glucose, fructose, and total sugar content increased as a function of time in both salt-tolerant (Pokkali) and salt-sensitive (IR29) genotypes of rice	Theerawitaya et al. (2012)
Oryza sativa	Root, leaf	16.6 dS m^{-1}	10 and 15 days	Soluble sugars, including sucrose, glucose, and fructose in leaf blade and leaf sheath were enriched in 0.1 mM N-2-(chloro-4-pyridyl)-*N*-phenyl urea (CPPU) pretreated plants subsequently exposed to NaCl. The patterns of accumulation of glucose and fructose in the root tissues were similar	Gashaw et al. (2014)

(*Continued*)

TABLE 12.1 (Continued)

Plants	Organs	Dose of NaCl exposure	Duration of NaCl exposure	Plant responses	References
Oryza sativa	Root, shoot	200 mM	24 h	The rice G-protein γ subunit (T_2*RGG1)* overexpressing transgenic plants accumulated almost twice the amount of glucose and 3.5-fold more fructose in both their roots and shoots as weighed against the WT plants during salinity stress	Swain et al. (2017)
Pennisetum clandestinum	Leaf	50, 100, 150 and 200 mM	15 days	The sucrose content was increased at 50 and 100 mM NaCl, but decreased at 150 and 200 mM NaCl in kikuyu grass. Accumulation of hexoses (glucose and fructose) was observed after treatments with 150 and 200 mM NaCl and a lower activity of glucokinase (GK), phosphoglucoisomerase (GPI), pyruvate kinase (PK), and glucose-6-phosphate dehydrogenase (G6PDH) was also detected	Muscolo et al. (2003)
Phragmites australis	Root, rhizome, leaf	1.5 and 10 ‰	16 days	The contents of sucrose, glucose, and fructose were higher in leaves > rhizomes > roots. Sugar contents increased up to 3.5-fold from 1.5‰ to 10‰ salinity level, but were lower at 1.5‰ versus the control in the rhizome. Sugar contents were the lowest in the roots and the highest in the leaves at 1.5‰ salinity. The sugar composition did not vary significantly except for leaves where the fraction of sucrose decreased, but glucose and fructose increased with increasing salinity level	Hartzendorf and Rolletschek (2001)
Schenkia spicata	Root cultures	50, 100, and 200 mM	4 weeks	In salt tolerant roots of *S. spicata*, glucose and fructose contents significantly increased with increasing salt concentration up to 100 mM NaCl, and a decrease was observed under severe salinity. In the roots of the salt sensitive genotype, sugar levels did not change	Mišić et al. (2012)
Solanum lycopersicum	Fruit	60 mM	During the Spring-Summer growing season	NaCl treatment increased glucose and fructose contents in tomato fruit juice. Treatments with Ca^{2+} and/or NH_4^+ or HCO_3 in combination with NaCl increased sugar concentrations compared to the control	Navarro et al. (2005)
Solanum lycopersicum	Fruit	50 mM	21, 28, 35, 42, and 49 days after flowering	Salinity stress strongly promoted the accumulation of sucrose, but did not elevate significantly glucose and fructose contents in tomato fruits	Saito et al. (2008)
Solanum lycopersicum	Fruit	160 mM	10, 18, 26, 34, and 42 days after flowering	Sugar levels of salinity-stressed tomato fruits were unchanged at 34 days after flowering, but had increased substantially by 42 days. Only sucrose had been kept at a similar level during this period even under saline conditions. Salinity stress enhanced the accumulation of glucose by 2.43 times, fructose by 2.05 times, sucrose by 7.87 times, and total sugars by 2.27 times at 42 days after flowering compared with those of the control. In contrast, starch content decreased during this period	Yin et al. (2009)
Solanum lycopersicum, Solanum pennellii	Root, old leaf, young leaf	70, 140, and 210 mM	8 weeks	Salt-stressed tomato plants showed lower concentration of glucose in roots and leaves, but no difference between NaCl treatments could be detected	Hunsche et al. (2010)

(*Continued*)

TABLE 12.1 (Continued)

Plants	Organs	Dose of NaCl exposure	Duration of NaCl exposure	Plant responses	References
Solanum lycopersicum	Root, leaf	100 mM	1 week	Salt stress decreased sucrose and glucose levels in the leaves and roots of tomato plants. Growth in 10^{-4} M salicylic acid (SA)-containing solution led to accumulation of soluble sugars in both leaf and root tissues, which remained higher in both plant parts during salt stress at 10^{-4} M SA. The activity of hexokinase (HXK) with glucose, but not fructose, as substrate was reduced by SA treatment in leaf and root samples, leading to accumulation of glucose and fructose in leaf tissues. HXK activity decreased further under high salinity in both plant organs	Poór et al. (2011)
Solanum lycopersicum	Fruit	50 mM	21 days	Salinity treatment caused a significant increase in sucrose, glucose, and fructose contents in tomato fruits. There was no effect from chemical or organomineral fertilizer on the increasing sugar concentration under the salinity condition, except for sucrose and glucose in the summer cropping, when accumulation of these sugars was higher	Kataoka et al. (2017)
Triticum aestivum	Stem	200 mM	4 days	Drought- and salt-tolerant wheat genotypes (Sakha-8; Kobomugi) accumulated more soluble carbohydrates (glucose and sucrose) than did the sensitive ones (Chinese spring; Regina) after the NaCl treatment	Kerepesi and Galiba (2000)
Triticum aestivum	Stem	120 mM	Continuously 3 d after emergence of leaf 4^{th}	The distribution of carbohydrates along the leaf axis showed distinct patterns that were altered by salinity and time in the photoperiod. Glucose and fructose concentrations were low at the base of the elongation zone and increased sharply up to the end of the leaf elongation zone after the salt treatment	Hu et al. (2000)
Triticum aestivum	Leaf	0.4 mol dm^{-3}	2 days	NaCl stimulated the accumulation of glucose and saccharose in leaves of different wheat genotypes	Grzesiak et al. (2013)
Eutrema/ Thellungiella spp. accessions	Leaf	25, 50, 100, 200, 300, 400, 500, 600, and 700 mM	26 days	The accumulation of glucose, fructose, and saccharose was positively correlated with the increasing salt stress levels	Lee et al. (2016)
Vicia faba	Pod	50 and 100 mg/L	5 times at 10 day intervals (foliar-sprayed)	Under normal watering the two NaCl concentrations induced loss of sugars (glucose, fructose, and sucrose) both to immature (pods harvested 10 days before the end of the experiment) and mature pods	Ouzounidou et al. (2014)

NaCl treatments. In contrast, glucose content did not change or decreased after NaCl treatments in leaves (Muscolo et al., 2003; Fernandes et al., 2004; Hunsche et al., 2010; Poór et al., 2011; Boriboonkaset et al., 2012; Petridis et al., 2012; Chang et al., 2014; Tsabarducas et al., 2015), fruits (Saito et al., 2008; Ouzounidou et al., 2014), seed (Prado et al., 2000), nodule (López et al., 2006), callus (dos Santos and Caldeira, 1999), cell suspension culture (Suzuki et al., 2005), and leaf protoplasts (Unno and Maeda, 2008) of various plant species (Table 12.1). These results suggested that in not only plant organs but also plant species/genotypes, the concentration and the duration of salt exposure could determine the intracellular glucose levels.

First of all, the genotypes of the given crops seemed to be crucial in salt stress responses. There were several important investigations with rice plants (*Oryza sativa* L.) to detect the effects of salt stress on different plant genotypes. Boriboonkaset et al. (2012) observed that glucose, fructose, and total soluble sugar contents in the flag leaf of salt stressed Pathumthani 1 (PT1; salt-sensitive) rice were significantly enhanced, especially on the third day of the salt exposure. In contrast, soluble sugar levels did not change in the flag leaf of Homjan (HJ; salt-tolerant) rice after treatment with 150 mM NaCl. At the same time, glucose, fructose, and total sugar contents increased as a function of time in leaves of both salt-sensitive (IR29) and salt-tolerant

(Pokkali) rice genotypes (Theerawitaya et al., 2012). In contrast, in the roots, glucose decreased in the sensitive cultivars, but it increased in the salt-tolerant Pokkali cultivar after 100 mM NaCl treatment (Siahpoosh et al., 2012). In roots of other plant species, glucose concentration also increased upon salinity stress. Glucose and fructose contents significantly elevated in salt-tolerant root culture of *Schenkia spicata* (L.) after 100 mM NaCl treatment, but sugar levels did not change in the salt-sensitive genotype (Mišić et al., 2012). Similar tendencies were observed in wheat (*Triticum aestivum* L.) cultivars. Drought- and salt-tolerant wheat genotypes (Sakha-8; Kobomugi) accumulated more soluble carbohydrates (glucose and sucrose) after 4 days in the stem than the sensitive ones (Chinese spring; Regina) after 200 mM NaCl treatment (Kerepesi and Galiba, 2000). In contrast, Morsy et al. (2007) investigated chilling-sensitive and chilling-tolerant rice genotypes after salt stress and they found that glucose and fructose concentrations increased in response to 100 mM NaCl, but only in the chilling-sensitive rice genotype. However, sugar concentration did not change significantly (only sucrose decreased) in the chilling-tolerant seedlings after the 4-day-long salt exposure. Based on these investigations we can conclude that glucose accumulation is common phenomenon in salt or drought (but not in chilling) tolerant crop genotypes in various plant organs (leaf, stem, and root).

Nevertheless, there can be significant differences in the glucose levels in different organs or in different parts of the organs. The concentrations of fructose, glucose, and sucrose decreased in the leaves of Cleopatra mandarin (*Citrus reshni* Hort. ex Tan), but at the same time the concentration of these sugars elevated in the roots after treatment with 80 mM NaCl (Anjum, 2008). In contrast, sucrose, glucose, and fructose increased in leaf blade and leaf sheath of rice and similar patterns of the accumulation of glucose and fructose were measured in roots after the salt exposure (Gashaw et al., 2014; Swain et al., 2017). However, 100 mM NaCl decreased sucrose and glucose levels both in leaves and roots of tomato (*Solanum lycopersicum* L.) plants (Poór et al., 2011). In addition, dynamic source–sink relations can be found between the various plant organs (fruit–leaf–root) or between differently aged organs. Hunsche et al. (2010) investigated the concentration of soluble sugars in old as well as in young leaves of different tomato genotypes. They found lower concentration of glucose in roots and leaves in salt-stressed plants upon 70, 140, and 210 mM NaCl treatments (Hunsche et al., 2010). Glucose levels can be different in fruits depending on their ripening stages. Rubio et al. (2009) observed elevated glucose and fructose levels in green and red fruits of pepper plants under saline condition. Moreover, contents of sucrose, glucose, and fructose were higher in leaves > rhizomes > roots of *Phragmites australis* (Cav.) Trin. ex Steud after different salt exposures (Hartzendorf and Rolletschek, 2001). In addition, the distribution of carbohydrates along the leaf axis can show distinct patterns, which can be altered by salinity and time in the photoperiod. It was provided that glucose and fructose concentrations were low at the base and increased sharply up to the end of the leaf elongation zone of wheat stem after salt treatment with 120 mM NaCl (Hu et al., 2000). It can be concluded that distribution of glucose between the different plant organs or inside the organ can be crucial in responses against high salinity. From this aspect, not only the production and accumulation of glucose but also the regulation of glucose transport can be crucial in case of the salinity stress. There are only a few investigations in rice plants, where the role of monosaccharide transporters was examined upon salt stress. Cao et al. (2011) found that in *OsGMST1* knockdown rice seedlings, a mutant in the Golgi-localized monosaccharide transporter is hypersensitive to 150 mM NaCl, as the NaCl-induced accumulation of glucose and fructose is impaired in knockdown rice leaves. Moreover, the potential role of monosaccharide transporters was confirmed in rice suspension cells, where 100 mM NaCl caused significant increase in the expression level of *OsMST3* (*O. sativa* L. monosaccharide transporter 3) and induced high glucose concentration at the early period of the salt treatment (Mahasal et al., 2011).

Changes in glucose metabolism can be dependent on the dose of NaCl treatments. Fortunately, many authors investigated effects of different concentration of NaCl on the selected plant species. Based on these observations, it can be concluded that glucose contents can significantly elevate with the increasing amount of salt up to the lethal salt concentration and a decrease can be observed under severe salinity. This tendency was confirmed by dos Santos and Caldeira (1999) in sunflower (*Helianthus annus* L.) calli after 50 and 100 mM NaCl treatments, by Muscolo et al. (2003) in leaves of kikuyu grass (*Pennisetum clandestinum* Hochst) after 50, 100, 150, and 200 mM NaCl treatments, by Petridis et al. (2012) in leaves of olive (*Olea europaea* L.) after 75 and 125 mM NaCl treatments, by Mišić et al. (2012) in *S. spicata* root cultures after 50, 100, and 200 mM NaCl treatments and by Lee et al. (2016) in leaves of *Eutrema/Thellungiella* spp. accessions after 25, 50, 100, 200, 300, 400, 500, 600, and 700 mM NaCl treatments. However, lower concentration of glucose was detected in roots and leaves of tomato plants after 70, 140, and 210 mM NaCl treatments but there were no differences between the glucose levels after 8 weeks (Hunsche et al., 2010). Nevertheless, others

found that higher concentration of NaCl (>250 mM) can induce cell death in tomato plants (Poór et al., 2012, 2013). 400 mM of NaCl decreased also glucose and fructose contents in seeds of *Chenopodium quinoa* (Prado et al., 2000) and 100 mM NaCl caused similar effects on glucose levels in pods of *Vicia faba* (Ouzounidou et al., 2014). Interestingly, both 50 and 150 mM concentrations of NaCl also decreased glucose content in leaves of *Lupinus albus* (L.) after 12 days (Fernandes et al., 2004).

The time and duration of salt exposure can be also crucial in glucose metabolism and plant stress responses. High glucose concentration was observed at the early period of 100 mM NaCl treatment after few days in rice suspension cells (Mahasal et al., 2011) and in soybean (*Glycine max* L.) calli (Liu and Van Staden, 2001). Increase in glucose content can be observed as a function of days in leaves of rice (Theerawitaya et al., 2012) and in cotyledons of *C. quinoa* (Ruffino et al., 2010), but it can be detected after several hours in *C. quinoa* seeds (Prado et al., 2000). In contrast, glucose levels elevated only several weeks after the salt exposure in fruits of tomato (Yin et al., 2009).

The accumulated glucose can play a role in several signaling and metabolic processes in the salt stressed tissues. Application of exogenous glucose attenuated the effects of salt stress in a dose-dependent manner of glucose (0.1, 0.5, and 50 mM) in leaves of wheat plants. Pretreatment with glucose showed significant reversal of salt stress caused by 200 mM NaCl in chlorophyll decay, loss of water and dry weight, shortening of root length, and accumulation of proline. The glucose-induced salt stress resistance was associated with enhanced intracellular K^+ and higher K^+/Na^+ ratio in wheat leaves. Moreover, pretreatment with glucose activated antioxidant enzyme activities (superoxide dismutase, peroxidase, catalase), thus decreasing lipid peroxidation in wheat seedlings based on thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) contents (Hu et al., 2012). In contrast, others found that in in vitro cell culture, treatment with 25 mM glucose together with 80 mM NaCl increased the ion leakage, TBARS, superoxide production, superoxide dismutase activity, and hydrogen peroxide (H_2O_2) contents after 2 days in rice suspension cells. However, these changes were lower or not significant after 4 days compared with the NaCl treated samples (Zhang et al., 2013). These findings suggested that glucose can modulate key antioxidant enzymes, thus can decrease the lipid peroxidation, reduce the membrane permeability and maintain the optimal K^+/Na^+ ratio. Rubio et al. (2010) also observed that water-soluble carbohydrates increased with decreasing K^+ concentration in the nutrient solution induced by salinity stress. Thus, the loss of K^+ can be an important signal to sugar accumulation and induction of defense mechanism.

Interestingly, several chemicals can induce glucose accumulation under salt stress. Chang et al. (2014) found that exogenous trehalose treatment (10 mM) elevated the contents of soluble sugars in leaves of *Catharanthus roseus* under salinity stress, but trehalase inhibitor validamycin A (30 μM) increased the total soluble carbohydrates of nodules in *Lotus japonicus*, but had no effect on starch, sucrose, and glucose contents (López et al., 2006). Chemical or organomineral fertilizer was also effective in the accumulation of glucose in fruits of tomato under salt stress (Kataoka et al., 2017) and treatments with Ca^{2+} and/or NH_4^+ or HCO_3 in combination with NaCl increased the sugar concentrations of tomato fruits (Navarro et al., 2005). Accumulated glucose can contribute to higher energy consumption in the stressed plant organs and can play a crucial role in osmoregulation (Hunsche et al., 2010; Petridis et al., 2012). Unfortunately, the enzymatic regulation of carbohydrate metabolism and in parallel the analysis of the coding sequences of these enzymes under salt stress was detected only in a few studies in more detail such as in case of the glycolysis (Muscolo et al., 2003), the role of hexokinases (Poór et al., 2011), and starch metabolism (Boriboonkaset et al., 2012).

12.5 GLUCOSE AND PHYTOHORMONES UNDER SALT STRESS

Glucose is a potent modulator of synthesis and the actions of several phytohormones (Sheen, 2014). Price et al. (2003) observed that exogenously applied glucose increased the expression of genes (*ABA2*, *ABI1*, and *ABI4*) involved in the biosynthesis of ABA in *Arabidopsis* seedlings. In addition, it was found that the antagonistic interaction between glucose and ethylene is mediated partly through ABA biosynthesis and signaling (León and Sheen, 2003). Moreover, glucose enhances the degradation of ETHYLENE-INSENSITIVE3 (EIN3), a key transcriptional regulator of ethylene signaling, through HXK1 (Yanagisawa et al., 2003).

Thus, regulation of glucose levels can be mediated by several phytohormones under salt stress. Hassine and Lutts (2010) observed that exogenous ABA treatment (50 μM) had no impact on glucose and fructose levels under salt stress in the leaves of *Atriplex halimus*. In addition, Yin et al. (2009) observed that salinity induced carbohydrate accumulation was also independent of ABA in tomato fruits. In contrast, exogenous pretreatment with salicylic acid (SA) led to the accumulation of soluble sugars in both leaf and root tissues of tomato plants under salinity stress (Poór et al., 2011).

Effects of other phytohormones, like ethylene in glucose metabolism under salt stress, will likely lead to new discoveries.

12.6 CONCLUSION AND FUTURE PERSPECTIVES

Despite the fact that the role of glucose has been intensively studied in the past 20 years, there are many gaps in comprehending the production, transport, storage, and utilization of glucose under salt stress. In this review, we highlighted the current state of the physiological and molecular aspects of NaCl-modulated glucose levels in different plant species, genotypes, and organs. Moreover, the regulation of glucose levels by phytohormones under salt stress were also mentioned.

Based on the revised works we can conclude that glucose accumulation is a common phenomenon of salt tolerant genotypes in various plant organs (leaf, stem, fruits and root). At the same time, there can be significant differences in the glucose levels in the different plant species, organs, or parts of the organs. Namely, distribution of glucose along the leaf axis can show distinct patterns, which can be altered by salinity in a time-dependent manner. Moreover, not only the production and accumulation of glucose but also the regulation of glucose transport can be crucial in the case of salt stress. The changes in glucose levels in the roots or in fruits can be also crucial to survive the severe salinity stress. Changes in glucose metabolism can be dependent on the dose and duration of NaCl treatments. Unfortunately, only a few authors have investigated the effects of different concentrations of NaCl on selected plant species. Based on other studies, glucose contents were increased up to lethal salt concentration and decreased under severe salinity. Moreover, most of the authors determined glucose accumulation only at one time-point after the salt exposure, although the rise may vary from some hours to a few days or weeks. The accumulated glucose can play a role in several signaling and metabolic processes mediated by various phytohormones in the salt stressed tissues, in which salicylic acid can be a significant component. Accumulated glucose can contribute to higher energy consumption in the salt stressed plant organs and can play a role in osmoregulation.

In the future, accurate description of the role of the key enzymes in the glucose metabolism and the role of glucose transport and glucose utilization mediated by various phytohormones would provide new insights into converging and diverging signaling pathways under different salt conditions. Understanding the mechanism that can regulate glucose levels at the cellular, tissue, organ or whole plant levels is an important problem in current plant biology as well as in agriculture. Precise investigation of salt tolerant genotypes may be used to increase the yield and salt stress tolerance under today's changing environment.

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CHAPTER

13

Role of Sugars in Abiotic Stress Signaling in Plants

Iffat Zareen Ahmad

Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India

OUTLINE

13.1 INTRODUCTION

Sugars are the most vital biomolecules since they are present in every cell of the body, and are rich in energy. They play an important role in primary as well as secondary metabolism of plants and animals (Lloyd and Zakhleniuk, 2004; Zhang et al., 2014). In plants, they are the product of photosynthesis and are a component of most of the biologically active compounds (Pego et al., 2000). Recently, the function of sugars as critical signaling molecules in relation to both the metabolic status in cells and response to different abiotic stresses has been shown (Lastdrager et al., 2014; Rolland et al., 2006). The diverse and complex networks of sugars warrant a detailed comprehension of their impact on regulatory and metabolic processes at the cellular and the whole plant levels (Lastdrager et al., 2014). Sugar signaling research will remain an exciting area of investigations for many years to come (Smeekens et al., 2014).

Sugars can be classified as monosaccharides, disaccharides, and polysaccharides on the basis of the number of monomers present. The functional groups they possess include aldoses and ketoses. Monosaccharides, mainly glucose, play the role of an important sensory metabolite. Disaccharides (sucrose, trehalose), raffinose, and fructans are three important categories of water-soluble sugars that primarily play a role in plant stress responses and might interact with reactive oxygen species (ROS) signaling pathways. Besides, oligosaccharides and some kinases have also been reported to contribute significantly to signaling at the cellular level (Fig. 13.1). Regardless of the expected complexity of sugar sensing and signaling in photosynthetic

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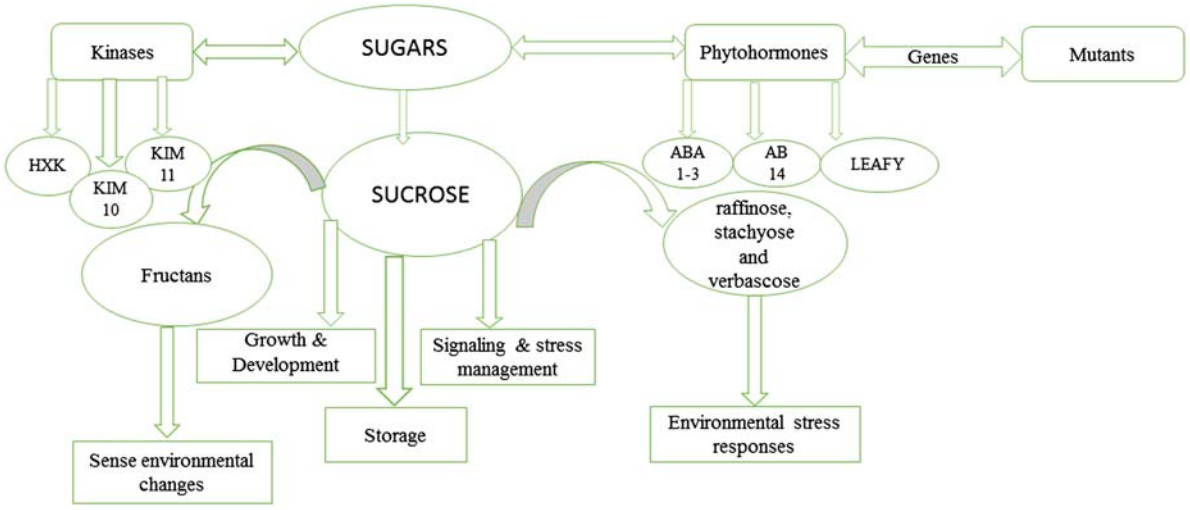

FIGURE 13.1 An overview of sugar signaling in plants in response to stress.

plants, stimulating progress has been made in the past 2 years. Some of the important sugars as signaling molecules are listed below:

1. Sucrose is the most commonly studied sugar in gene regulation development (Gibson and Graham, 1999; Halford et al., 1999; Jang et al., 1997; Koch, 1996; Lalond et al., 1999; Roitsch, 1999; Smeekens and Rook, 1997). It is the major form of translocated sugars in plants. Its role in growth, development, storage, signaling, and stress acclimation makes it the most studied molecule (Salerno and Curatti, 2003).
2. Sucrose combines with galactosyl moieties to give rise to raffinose, stachyose, and verbascose in the cytosol (Schneider and Keller, 2009). Their accumulation, gene expression, and metabolism are greatly associated with environmental stress responses (Nishizawa et al., 2008).
3. Fructans, fructose polymers derived from sucrose, are present in around 15% of angiosperms. There are many plants for which fructans are the main storage form of carbohydrates (Suzuki et al., 1993). These plants are very important parts of ecosystems in which there are frequent environmental changes (Albrecht et al., 1997).
4. Oligogalacturonides (OGs) (Ferrari et al., 2013) illustrate an excellent example of a sugar being both a metabolite and a signaling molecule.
5. Hexokinase (HXK) is a double-function enzyme and glucose sensor, the first to be documented in plants (Graham et al., 1994; Jang et al., 1997; Pego et al., 1999; Smeekens and Rook, 1997; Umemura et al., 1998).

Sugar signaling can be thrust area of research, and a compilation of the present data on this also can be very useful for those researchers who are working on this topic (Table 13.1). The present chapter aims at providing insight into the known information on sugars as signaling molecules under stressful and optimal environments.

13.1.1 Sugars as Signaling Molecules

The growth and development of the plant must be in accordance with its metabolism, particularly with the rate of photosynthesis and the nutrient uptake. This synchronization involves local association between hormonal response and metabolic state, as well as long-distance networks between shoot and root tissues. Recently, numerous mechanisms at the molecular level have been given to elucidate the association of sugar signaling with hormone pathways. In a study, DELLA and PIF proteins were developed as hubs in sugar–hormone cross-regulation networks (Ljung et al., 2015). The studies on mutants *stimpy* or *ramosa3* have proposed the role of sugars as signaling molecules for controlling various facets of plant development. In the developmental activities, it was shown that mutant phenotypes are modulated by sugars and they also play a putative role (e.g., trehalose-6-phosphate) in inflorescence development. Because of the plasticity in nature of plant growth and development they are greatly influenced by environmental and dietary conditions (Eveland and Jackson, 2012).

Sugars may control expression of hundreds of genes in plants. A novel biological system was developed that was based on stem cell–like *Arabidopsis* suspension culture. The cells were maintained in a hormone-free medium and were provided xylose as

TABLE 13.1 The Effects of Sugars and Enzymes on Different Physiological Parameters and Signaling

S. no.	Sugar/enzyme	Effects	Metabolites/genes affected	References
1.	Sucrose	Gene regulation	Maize sucrose synthase and soluble invertase	Koch (1996)
2.	Hexokinase	Gene regulation	Antisense hexokinase (AtHXK) genes	Jang et al. (1997)
3.	Sucrose & glucose	Gene regulation metabolism	Sucrose phosphorylase, enzymes of glycolysis	Smeekens and Rook (1997)
4.	Sucrose	Signaling	Source sink regulation	Roitsch (1999)
5.	Hexokinase	Sugar sensor	Sugars	Halford, Purcell, and Hardie (1999), Jang and Sheen (1997), Smeekens and Rook (1997)
6.	Galactinol and raffinose	Gene expression	GolS1 or GolS2	Nishizawa et al. (2008)
7.	Fructans	Sugar accumulation in hypoxia, flood	Sugars	Albrecht et al. (1997)
8.	Sucrose	Gene expression	Cucumber malate synthase (MS) and isocitrate lyase (ICL)	Graham et al. (1994)
9.	Sugars	Sugar-mediated plant growth regulation	Trehalose-6-phosphate, target of rapamycin kinase, and Snf1-related kinase 1 regulatory systems	Lastdrager et al. (2014)
10.	Mannose	Germination of *Arabidopsis* seeds	Hexokinase expression	Pego et al. (1999)
11.	Trehalose-6-phosphate	Inflorescence		Eveland and Jackson (2012)
12.	Sugars	Fungal pathogen	Increased resistance	Morkunas (2014)
13.	Sucrose	Sugar signaling, phosphate deficiency	Increased size of roots	Hammond and White (2011)
14.	Raffinose, sucrose, and starch	Gene expression	TOR kinase	Dobrenel et al. (2013)
15.	Invertase	Plant development	Phenotypic alterations in carrot embryo	Tang et al. (1999)
16.	Sucrose	Sugar signaling	Circadian rhythms	Moghaddam and Van den Ende (2013)
17.	Suc itself, glucose (Glc) and trehalose-6-phosphate (T6P)	Sugar signaling, growth and development	Sugar-mediated plant growth regulation	Dahiya et al. (2017)
18.	Oligogalacturonides (OGs)	Defense response and development	Auxin	Ferrari et al. (2013)
19.	Hexokinase	Sugar sensor and metabolism	Snf1-related kinases (SnRKs), extracellular sugar sensors, and trehalose metabolism	Rolland et al. (2006)
20.	Hexokinase	Sugar sensing and signaling, physiology, and development	KIN10 and KIN11 protein kinases, bZIP transcription factors	Hanson and Smeekens (2009)

the only carbon source. Using functional genomics 290 sugar-responsive genes were identified, responding rapidly (within 1 h) and specifically to low concentration (1 mM) of glucose, fructose, and/or sucrose. The exact nature of the signaling sugar molecules and sites of their perception were elaborated using nonmetabolizable sugar analogs for selected genes. The expression of selected sugar-responsive genes was not restricted to a specific tissue or cell type, as shown in transgenic and wild-type *A*rabidopsis *thaliana* seedlings. It was further proved that response was associated with photoperiod-related changes in sugar availability (Kunz et al., 2014). Studies indicated that sugars can act as signaling molecules and can control gene expression and developmental processes in plants in a manner similar to plant hormones.

Uncoupling of glucose signaling from its metabolism provided substantial proof in favor of this. Glucose sensors have been identified and isolation and characterization of mutants and other regulatory components have been done in plant sugar signal transduction pathways (Sheen et al., 1999).

Sugars play osmoprotective roles and their levels increase to avoid degradation of enzymes and to decrease the amount of ROS. In the presence of drought stress, synthesis of different sugar molecules, such as mono-, di-, and oligosaccharides, accelerates to give osmoregulation. Another approach to osmoregulation is by balancing the low molecular sugars by phloem transport and sucrose metabolism. Other than osmoregulation, soluble sugars also act as signaling molecules to modulate the sensitivity of plants and thus help in cell responses. The sensor proteins are needed for sensing the level of sugars in cells and lead to signal transduction cascades. The exogenous application of various sugar molecules (trehalose, mannitol, sorbitol, etc.) has been found to be an effective approach to increase the resistance against various abiotic stresses including drought stress. Plants that are grown under abiotic stress are under distress, which results in the increased accumulation of ROS (Khan and Khan, 2014). This buildup of ROS invokes both injurious as well as signaling effects at the organellar and cellular levels. The consequence of an environmental trial is mostly dependent on the subtle equilibrium between the synthesis of ROS and their scavenging by both enzymatic and nonenzymatic antioxidants (Choudhry et al., 2016; Khan and Khan, 2014; Khan et al., 2014; You and Chan, 2015). Nevertheless, this classical knowledge needs greater and renewed understanding, since it has become gradually more clear that soluble sugars such as disaccharides, raffinose family oligosaccharides, and fructans—next to their associated metabolic enzymes—are intensely associated with stress-induced collection of ROS in plants. There are several examples of such signaling sugar molecules that are based on their location within the cell, as distinct organelles seem to use different mechanisms. Interestingly, the vacuole seems to be a most significant component in the network involved in ROS signaling of plants. The explicit understanding of the connection between the mechanisms regulating ROS signaling under abiotic stress will help in clarifying and taking up mechanistic approaches to improve crop tolerance to different environmental conditions due to abiotic stresses and the mitigation of ROS produced due to this (Choudhry et al., 2016; Keunen et al., 2013; You and Chan, 2015). The role of exogenous application of sugars under drought stress is also studied. Drought has significant effects on plants at the cellular and metabolic levels. The amount of osmoprotectants increase, which prevents degradation of enzymes and scavenges ROS. One of these protective compounds is sugar. To provide osmoregulation under drought conditions, the levels of mono-, di-, and oloigosaccharides is enhanced. Sugars also act as signaling molecules and initiate signal transduction cascades. Even the exogenous application of some sugars like trehalose, mannitol, sorbitol, etc. has been shown to improve drought resistance (Bhardwaj et al., 2016). This book chapter reviews the metabolism of sugar as well as its role in stress signaling. Considerable information has been presented in the last 2 years on important groups in sugar-mediated plant growth regulation, with particular stress on trehalose-6-phosphate, target of rapamycin kinase, and Snf1-related kinase 1 regulatory systems. The synthesis of protein has been regulated by sugars and this aspect of metabolism is fundamental in controlling the growth of the plant, and many research efforts to understand this regulation of translation by sugars have been carried out (Lastdrager et al., 2014).

Sugars are involved in many metabolic and signaling pathways in plants. Sugar signals may also contribute to immune responses against pathogens and probably function as priming molecules leading to pathogen-associated molecular patterns-triggered immunity and effector-triggered immunity in plants. These putative roles also depend greatly on coordinated relationships with hormones and the light status in an intricate network. Although evidence in favor of sugar-mediated plant immunity is accumulating, more in-depth fundamental research is required to unravel the sugar signaling pathways involved. This might pave the way for the use of biodegradable sugar-(like) compounds to counteract plant diseases as cheaper and safer alternatives for toxic agrochemicals (Moghaddam and den Ende, 2012). In most cases in which fungus affects a plant, an increased amount of sugars in plant tissues improves the resistance of plant. Many different theories have been suggested by various researchers working in this area over time to explore the mechanistic approach of this "high-sugar resistance." Sugars act as an important substrate that releases energy and also provides the building blocks for defense responses in plants, while they may also play a role as signal molecules in networking with the hormonal signaling systems, which regulate the immune system of the plant. Sugars increase the oxidative burst during the initial phases of infection, resulting in increased lignification of cell walls, stimulation of the flavonoid synthesis, and induction of some proteins. Some sugars act as grooming agents that stimulate and induce greater resistance of the plant to pathogens (Morkunas, 2014).

The studies carried out during the last decade have revealed the understanding behind the sensing signal by plants and their response towards the availability

soil phosphorus (P) (Amtmann et al., 2006; George et al., 2011; Nilsson et al., 2010; Vance, 2010; White and Hammond, 2008; Yang and Finnegan, 2010). Earlier studies have shown the potential of shoot-derived carbohydrate signals to induce acclimatory responses in roots in low P condition. In this perspective, these carbohydrates act as systemic plant growth regulators (Hammond and White, 2011). Photosynthate is transported mainly to sink tissues as sucrose via the phloem. In low P availability, plants store sugars and starch in their leaves. Enhanced loading of sucrose to the phloem in conditions of P starvation mostly works to relocate carbon resources to the roots, which results in the increase in their size in comparison with the shoot (Hermans et al., 2006). The translocation of sugars via the phloem also has the ability to initiate sugar signaling cascades that modify the expression of genes responsible for plant responses to low P availability. These comprises of improving biochemical changes in root to acquire soil P, by increasing expression and activity of inorganic phosphate (Pi) transporters, the secretion of acid phosphatases and organic acids to release P from the soil, and the optimization of internal P use (Hammond and White, 2011).

13.1.1.1 Glucose

Glucose has been shown to be a positive regulator of TOR kinase enzyme and the explanation is given about its effect on different processes including biosynthesis of the stress-related sugar raffinose, sucrose, and starch as well as glycolysis (Dobrenel et al., 2013).

Glucose has the characteristics of a plant hormone and its specific effects on the level of gene expression and developmental plans are a feature of plant hormone actions (Koch, 1996; Lalond et al., 1999). Moreover, a study showing that HXK acts as a specific glucose sensor, and that the action of nonmetabolizable glucose signals is mediated through unknown sensors/receptors, further proved the behavior of glucose as a plant hormone with dual functions of a signaling molecule and a metabolite. The latest research on transgenic plants (Herbers et al., 1996; Jang et al., 1997; Tang, Luscher & Sturm, 1999) and sugar response mutants (Dijkwel et al., 1997; Mita et al., 1997; Zhou et al., 1998) has revealed close cross-talk between glucose and other plant hormone signaling pathways. The characterization of transgenic carrot plants recently with antisense cell wall invertase or vacuolar invertase constructs showed malformed embryos and bushy shoots that can be corrected by hexoses (Tang et al., 1999). These phenotypes resulted due to lack of hexose signaling, which might be responsible for maintaining the balance between the plant hormones auxin and cytokinin. In gin2 mutant, which has been isolated recently, the lack of AtHXK1 resulted in changed sensitivity to auxin and cytokinin.

13.1.1.2 Sucrose

A different characteristic regarding sugars as signaling molecules has been given in articles reporting the assimilation of sucrose-mediated signaling pathways in cellular networks (Moghaddam and Van den Ende, 2013). This explained the interaction of sugar signals with other critical signaling systems of the cell including the circadian clock and phytohormones, in monitoring defense responses and developmental activities such as flowering (Moghaddam and Van den Ende, 2013). In the same way, the regulatory steps that combine diurnal signals with downstream cellular responses may occur at the sugar uptake step. The work has been reported on the sucrose transporter 4 (SUT4) from potato (Chincinska et al., 2013). Another instance where sugars function as regulatory molecules has been done on RAPAMYCIN (TOR) kinase complexes (Dobrenel et al., 2013). These complexes combine with additional partner proteins to bring about and involve a broad range of cellular responses, including metabolism, mRNA processing, and autophagy, frequently along with nutrient signaling.

Two aspects of sugar biochemistry that have been extensively studied as the characteristic features of many sugars, mainly sucrose, are their controlled distribution within the cell and transport over long distances from sinks to sources. The metabolism of sucrose at the cellular level is dependent on, and regulated by, the activities of sucrose synthase and sucrose-phosphate synthase (SPS). Researchers have provided understanding on two rice SPS paralogs, *OsSPS1* and *OsSPS11*, and their particular expressions in response to diurnal factors and carbohydrate availability (Yonekura et al., 2013). The long-distance transport of sucrose is assisted by the activities of specialized transport proteins. Scientists explored the functioning of these transporters as checkpoints to forward information on metabolic fluxes to initiate cellular responses (Chincinska et al., 2013).

Sucrose metabolism plays crucial roles in plant development mainly by generating a range of sugar signaling molecules such as Suc itself, glucose, and trehalose-6-phosphate. Sugars not only fuel cellular carbon and energy metabolism but also play pivotal roles as signaling molecules. Sugars have a central regulatory function in steering plant growth.

13.1.1.3 Oligogalacturonides

The information given by a researcher in a review article on OGs demonstrated an outstanding example of a sugar being both a metabolic and a signaling

molecule (Ferrari et al., 2013). OGs comprise of α-1,4-linked galacturonosyl residues and are essential constituents of the cell wall. Biotic stress conditions lead to the release of hydrolytic enzymes by the cell wall, which is either stimulated by fungal growth or by mechanical damage imposed by herbivores. The OGs released due to this stimulus then work as signaling molecules to produce a defense response in the particular plant cell and adjacent tissues (Ferrari et al., 2013).

An improved understanding of the regulation of developmental processes and allelopathic and defense-inducing characteristics of "oligosaccharin" signaling carbohydrates gives opportunity not only for better knowledge of plant biology, but also offers better opportunities for commercial exploitation.

13.2 KINASES AS ENZYME SENSORS

The latest evidence suggested that even a millimolar range of signaling molecules can stimulate sensing and signaling by applying sugar binding enzymes, proteins, or transporters. Even though multiple sugar sensors/receptors probably exist, HXK as a dual-function enzyme and glucose sensor was the first to be reported in plants (Graham et al., 1994; Jang and Sheen, 1997; Jang et al., 1997; Smeekens and Rook, 1997; Umemura et al., 1998). Sucrose is the main form of translocated sugars in plants and is the most commonly studied plant sugar with respect to gene regulation and development (Gibson and Graham, 1999; Halford et al., 1999; Jang et al., 1997; Koch, 1996; Lalond et al., 1999; Roitsch, 1999; Smeekens and Rook, 1997).

HXK acts in a dual manner and it functions both as enzyme and sensor. HXK is a dimeric cytosolic enzyme that is required for glycolysis. It is a matter of debate whether HXK is a glucose sensor and it senses the intracellular glucose signals, whether the catalytic and regulatory functions of HXK are separable, and whether the ATP/AMP (adenosine triphosphate and adenosine monophosphate) ratio is the actual signal. Manipulation of glucose metabolites in a leaf cell showed that sugar phosphates, the changes in ATP levels, phosphate depletion, and other metabolites cannot substitute for the glucose signal (Jang and Sheen, 1997). The inhibition of *Arabidopsis* seed germination by mannose could occur without affecting ATP or inorganic phosphate levels in seeds, and could be overcome by a HXK inhibitor (Pego et al., 1999). These data gave support in favor of the uncoupling of glucose signaling and glucose metabolism. In genetically modified *Arabidopsis* plants and yeast, different regulatory functions in glucose repression but similar catalytic activities were exhibited by plant and yeast HXK. Therefore, glucose metabolism alone cannot explain many of the glucose responses. The function of HXK as a glucose sensor seems to be conserved in plants, as overexpression of the *Arabidopsis* HXK1 gene stimulated glucose hypersensitivity in transgenic tomato and in transformed maize leaf cells (Sheen, unpublished data). *Arabidopsis* HXK1 mutants lacking glucose repression have been identified recently, with no corresponding effects on sugar metabolism.

Sugars act as an important signaling molecule in plants. Different sugar signals are being generated to monitor growth, development, and stress response. The study at the gene level showed extensive interactions between sugar and plant hormone signaling, and a main role for HXK as a conserved glucose sensor. Varied sugar signals stimulate multiple HXK-dependent and HXK-independent pathways and utilize diverse molecular mechanisms to regulate transcription, translation, protein stability, and enzymatic activity. Significant and complicated roles for Snf1-related kinases (SnRKs), extracellular sugar sensors, and trehalose metabolism in plant sugar signaling are now also developing (Rolland et al., 2006). Sugar sensors have been documented and proposed that include the most studied glucose sensor HXK1, besides sucrose and trehalose-6-phosphate. The diversity and complexity of sugar sensing and signaling and their effects on many physiological and developmental processes and integration with other signaling pathways make their study quite interesting. Notably, KIN10 and KIN11 protein kinases are fundamental in coordinating several of the responses to sugars and stress. bZIP transcription factors play an important role and facilitate effects of sugar signaling on gene expression and metabolite content (Hanson and Smeekens, 2009).

The perception of sugars by the cell is another very significant aspect in sugar research. The most significant indication on cellular sugar sensing systems presently comes from hexose kinases, which phosphorylate glucose (hexokinase) and fructose (fructokinase). HXKI from *Arabidopsis* has been involved in the early steps (Jang et al., 1997; Moore et al., 2003), and two related review articles provided comprehensive information on HXK and fructokinases in plants (Granot et al., 2013; Tiessen and Padilla-Chacon, 2013), along with other sugar metabolizing enzymes such as invertases, sucrose synthases, and SPS (Tiessen and Padilla-Chacon, 2013). Literature that has been produced on various proteins with respect to their gene families, their subcellular localization, and particular metabolic activities, as well as effects on developmental activities and association with signal transduction events. Further, regulatory steps involved in sugar and stress-related signal transduction are dependent primarily on the activity of SnRK1-protein kinases. These kinases are multienzyme complexes and

cystathionine-β-synthase (CBS) domain-containing proteins belong to this group. The two subunits of these complexes, AtPV42a and AtPV42b, are misregulated in histone acetyltransferase 1 (hac1) mutants (Heisel et al., 2013). The hac1 mutants showed abnormal sugar responses and fertility defects, which could be partly clarified by the altered levels of AtPV42a and AtPV42b expression. It was strongly shown that there is the participation of microRNAs in SnRK1-protein kinase-dependent processes (Confraria et al., 2013). Sugar response regulation also requires mRNA processing steps, as shown by Funck et al. (2012). They were identified by map-based cloning of a sugar response mutation as ESP1, a CstF64-like putative RNA processing factor. There is a role of ESP1 in mRNA 3′-end formation, and the work involves RNA maturation as a crucial factor for normal sugar response (Funck et al., 2012).

13.3 SUGAR SIGNALING AT GENE LEVEL

Sugars can behave as signaling molecules as they have the tendency to be global regulators of gene expression. For example, mimicking hormones for transforming nutrient status to regulate growth and the floral development process (Koch, 1996, 2004; Ohto et al., 2001; Price et al., 2004; Rolland et al., 2002, 2006; Smeekens, 2000; Smeekens et al., 2010; Wobus and Weber, 1999). In this manner, sugar-dependent regulation of the gene reveals an excess of carbohydrate or its exhaustion (Koch et al., 1996; Koch, 2004; Rolland et al., 2002). The interplay between nutrient status and regulation of transcription permits the plant to regulate growth, both at the level of the complete plant and at the cellular level, which is dependent on a particular tissue or cell so as to strongly manage developmental processes with existing carbohydrate. In conditions of low sugar levels, genes involved in photosynthesis, carbohydrate transport and storage, and nitrogen metabolism are upregulated. On the other hand, the excess of sugar stimulates the usual sink organ activities including import of carbohydrate, consumption, and storage, and the biosynthesis of starch and anthocyanin.

Several genes that are involved in sugar sensing and signaling have been recognized in mutants for transformed responses to exogenous sugars during germination of seed and early growth of seedling in *Arabidopsis* (Gibson, 2005; Rolland et al., 2002, 2006; Smeekens, 2000). For example, *glucose insensitive* (*gin*) mutants don't undergo growth arrest under inhibitory concentration of glc, demonstrating usual hypocotyl elongation, cotyledon greening, and expansion. Screening of mutants related to other sugar response phenotypes showed that certain *sucrose uncoupled* (*sun*), *sugar insensitive* (*sis*), and/or *impaired sucrose induction* (*isi*) mutations were allelic to *gin* loci, giving an idea that these genes may function at the border of various sugar signaling pathways (Gibson, 2005; Rolland et al., 2002; Smeekens, 2000; Zhou et al., 1998). Such mutants could lead to secondary responses to hexose products produced from sucrose-dependent stimulation of sucrose-splitting enzymes, for instance extracellular invertases. Moreover, the disaccharide trehalose, and its intermediary metabolites, trehalose-6-phosphate (T6P), are involved in regulating some growth responses (Eastmond and Graham, 2003; Paul, 2008; Smeekens et al., 2010)

13.4 SUGAR SIGNALING AND PLANT METABOLISM

The regulation of metabolism and specific growth responses tend to be triggered and/or moderated on the basis of the nature of the sugar signal. For example, the transport form of sugar in plants, that is, sucrose, can be recognized as a signal directly (Chiou and Bush, 1998) or, otherwise, a signal can arise through its hexose cleavage products, glucose (glc) or UDP-glc and fructose (Li et al., 2011; Price et al., 2004; Rolland et al., 2002).

As reported, the generation of sugar signals depends on either the concentration or the relative ratios to other metabolites, for example, C:N (Coruzzi and Bush, 2001; Palenchar et al., 2004), or through sugar-specific sensors and/or transporters (Buttner, 2010; Lalonde et al., 1999, 2004; Vaughn et al., 2002; Williams et al., 2000). The latest addition to this is the possible role of metabolic enzymes as dominant members of transcriptional regulatory complexes; HXK represent the best example. The abovementioned study is an example of remarkable cross-talk between metabolic pathways and/or sensors, and gene regulation. Further, one more sensing mechanism has been reported involving cell surface receptors, such as *RGS1*, which negatively regulate G-protein signaling (Chen et al., 2003; Chen and Jones, 2004).

In general, signaling potential of hexoses tends to have greater effect on promoting organ growth and cell proliferation, whereas sucrose is normally associated with differentiation and maturation (Borisjuk et al., 2002; Koch, 2004). Relative ratios of hexoses to sucrose are sensed and retained by sucrose metabolic enzymes, which function in a spatiotemporal manner to synchronize growth during important developmental phases (Koch, 2004; Xu et al., 1996). The role of sugar metabolic enzymes and transporters is to establish sugar gradients within tissues (Weschke et al., 2000, 2003). Earlier work on developing legume embryos revealed that differential glc concentrations along a spatial gradient can

be related to enhanced mitosis (Borisjuk et al., 1998, 2003), proposing a connection between hexoses and the cell cycle. In accordance with this, sugars have been reported to control cell division through modulation of cyclinD (CycD) gene expression (Gaudin et al., 2000; Riou-Khamlichi et al., 1999, 2000).

13.5 SUGAR SIGNALING AND PHYTOHORMONES

Sugars can also cross-talk with existing phytohormone signaling networks to regulate important growth processes like embryo development, seed germination, and seedling and tuber growth (Gazzarrini and McCourt, 2001; Gibson, 2004, 2005; Leon and Sheen, 2003; Rolland et al., 2002, 2006). There is also evidence to suggest that sugars can regulate meristem maintenance and identity at the genetic level (Satoh-Nagasawa et al., 2006; Wu et al., 2005). Indeed, Pien et al. (2001) observed spatiotemporal expression of genes of carbohydrate metabolism in the tomato shoot apical meristem (SAM) and developing leaf primordia. In *Arabidopsis*, misexpression of a specific extracellular invertase, in the SAM resulted into alterations in flowering time and inflorescence structure (Heyer et al., 2004). In addition, sucrose can salvage flowering time mutant phenotypes, by controlling meristem identity genes, such as *LEAFY* (Ohto et al., 2001). The latest research has also revealed that exogenous sucrose can compensate for regulators of meristem maintenance in the shoot (Wu et al., 2005) and root (Wahl et al., 2010).

It has been shown that plants defective in abscisic acid (ABA) and/or ethylene sensitivity and signaling display changed sugar response phenotypes. Therefore, research has revealed a huge overlap between sugar, ABA, and ethylene signals in regulating processes such as seed germination and seedling growth (Gazzarrini and McCourt, 2001; Gibson, 2004, 2005; Leon and Sheen, 2003; Rolland et al., 2002, 2006). A number of mutants identified in sugar response screens also showed defect in ABA metabolism, which proved a cross-talk between them. For example, certain ABA biosynthesis (*aba*) and ABA-insensitive (*abi*) mutants are insensitive to high glc (Arenas-Huertero et al., 2000; Brocard et al., 2002; Dekkers et al., 2008; Leon and Sheen, 2003). Rigorous cross-talk between sugar and ABA signaling pathways has been described for various aspects of plant development and metabolism (Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002; Gibson, 2004, 2005); for example, embryo growth, transition in phase from rapid cell division to cell enlargement and storage of reserves (Finkelstein and Gibson, 2002; Wobus and Weber, 1999a,b). ABA increases sucrose stimulation of starch biosynthetic genes (Rook et al., 2001). On the other hand, ABA and glc act antagonistically during seed germination and initial growth of seedlings, where exogenous glc enables wild-type *Arabidopsis* seeds for germination on otherwise inhibitory ABA levels (Leon and Sheen, 2003). A direct association between sugar signaling and hormone biosynthesis was shown by characterization of ABA biosynthetic genes, *ABA1*−*ABA3*, which have been also individually isolated as *gin* mutants (Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001). Exogenous glc can enhance both expression of these ABA synthesis genes and, subsequently, endogenous ABA levels (Cheng et al., 2002). An important association between sugars and ABA perception is demonstrated by *ABI4*, which encodes an AP2 domain transcription factor involved in sugar response during germination and seedling growth (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). In maize seeds, *ABI4* is controlled by sugar in the developing embryo, and binds regulatory elements for both ABA and sugar (Niu et al., 2002). Further, a recent study recognized a splicing factor, *SR45*, as a negative regulator of sugar signaling during early seedling growth. *SR45* is involved in the repression of glc-induced ABA accumulation, and downregulation of genes for ABA biosynthesis and signaling (Carvalho et al., 2010).

Ethylene signaling pathways are also closely connected with sugar and ABA sense networks (Gazzarrini and McCourt, 2001; Leon and Sheen, 2003). For example, ethylene mutants, *ethylene receptor 1* (*etr1*) and *ethylene insensitive 2* and *3* (*ein2* and *ein3*), are glc hypersensitive, while *constitutive triple response 1*(*ctr1*), a negative regulator of ethylene signaling, is glc insensitive (Gibson et al., 2001; Yanagisawa et al., 2003; Zhou et al., 1998). ABA levels are improved in the *ein2* mutant, and wild-type seedlings treated with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) phenocopy *gin* mutants (Ghassemian et al., 2000).

CONCLUSION

Sugars are known to have enormous and diversified roles in the organism. Besides being an important energy-giving primary metabolite, now the focus of research has shifted towards sugars' function as regulatory and signaling molecules. The literature in this particular area of research emphasizes the variety of processes related to sugars and their requirement in the cell, and also the regulatory networks to which they are associated. The role of sugar signaling in circadian rhythm, and particular developmental programs has also been discussed. Interestingly, sugar signaling works at the gene level and also in

association with various phytohormones. The function of sugars as signaling molecules becomes obvious when the plant is under biotic or abiotic stress. In such conditions, plants produce osmoprotectants, mainly sugars, for the maintenance of homeostasis and to scavenge ROS. Sugars provide protection to enzymes from degradation and also initiate the signaling pathways to combat stress. Despite the existing research on this topic, there remains a dearth of knowledge, as many major issues would be addressed if we could understand the regulatory complexity and the components involved in sugar homeostasis, (sub)cellular allocation, and long-distance transport. Sugar signaling study will continue to be a fascinating area of research for many years to come.

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CHAPTER

14

Methylglyoxal: A Novel Signaling Molecule in Plant Responses to Abiotic Stresses

Zhong-Guang Li[1,2,3]

[1]School of Life Sciences, Yunnan Normal University, Kunming, P.R. China [2]Engineering Research Center of Sustainable Development and Utilization of Biomass Energy, Ministry of Education, Kunming, P.R. China [3]Key Laboratory of Biomass Energy and Environmental Biotechnology, Yunnan Province, Yunnan Normal University, Kunming, P.R. China

OUTLINE

14.1 INTRODUCTION

Oxygenated short aldehyde methylglyoxal (MG) containing α,β-dicarbonyl is a side-product of many metabolic processes, such as glycolysis, photosynthesis, fatty acid degradation, and the oxidation of amino acids and proteins. The generation of MG is spontaneous and unavoidable in the life activity of all

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organisms (Kaur et al., 2014a,b; Hossain et al., 2011, 2016; Singh and Dhaka, 2016). Due to high reactivity of MG with protein, DNA, RNA, and lipid, for a long time, it has been considered to be a toxic metabolite (Kaur et al., 2014a,b; Hossain et al., 2011, 2016; Singh and Dhaka, 2016). Nowadays, MG is emerging as a novel signaling molecule at low concentration, which takes part in the regulation of seed germination, plant growth, development, reproduction, and response and adaptation to adverse environment (including biotic and abiotic stress), similar to other signaling molecules such as Ca^{2+}, hydrogen peroxide (H_2O_2), nitric oxide (NO), and hydrogen sulfide (H_2S) (Kaur et al., 2015a,b; Hossain et al., 2011, 2016; Khan et al., 2016; Li, 2016; Singh and Dhaka, 2016). Under normal physiological conditions, MG in plant cells can be maintained a low and nontoxic physiological level, approximately 25−75 μM in many plant species such as (Yadav et al., 2005a,b; Hossain et al., 2009; Khan et al., 2016; Li, 2016; Singh and Dhaka, 2016). Under stress conditions, endogenous MG levels in plant cells can be dramatically triggered and produce a MG "burst" or "wave." This burst has become a common stress signaling or marker, followed by regulating the formation of plant abiotic stress tolerance or inducing MG stress (Hossain et al., 2016, 2017; Hoque et al., 2016). MG stress, also known as carbonyl stress, an analogue to oxidative stress due to overaccumulation of reactive oxygen species (ROS), refers to the rapid reaction of MG with protein, nucleic acid, and lipid (Kaur et al., 2014a,b; Hossain et al., 2011, 2016; Khan and Khan, 2017; Singh and Dhaka, 2016). In plants, enzymatic and nonenzymatic pathways all can produce MG during the process of cellular metabolism, but the latter is the major contributor, especially under stress conditions (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016). In general, approximately 0.05%−0.3% glucose can be spontaneously converted into MG in glycolysis and this ratio increases under stress conditions (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016).

To maintain MG homeostasis in plant cells, plants have evolved a unique and effective detoxification system composed of a glyoxalase system and nonglyoxalase system. The former refers to glyoxalase I (Gly I), glyoxalase II (Gly II), and glyoxalase III (Gly III), which is the key player in MG scavenging, eliminating 99% MG. Nonglyoxalase system mainly includes aldose/aldehyde reductase (ALR), aldo-keto reductase (AKR), MG dehydrogenase (MGDH), MG reductase (MDR), and aldehyde dehydrogenase (ADH). They convert MG into lactaldehyde or pyruvate using reduced nicotinamide adenine dinucleotide (NADH) from glycolysis or tricarboxylic acid cycle as cofactor (Kaur et al., 2014a,b; Hossain et al., 2016; Singh and Dhaka, 2016; Hasanuzzaman et al., 2018). Nonglyoxalase system only eliminates less than 1% MG, it is a minor route under normal conditions unless the glyoxalase system is blocked (Kaur et al., 2014a,b; Hossain et al., 2016; Singh and Dhaka, 2016).

As a novel signaling molecule, the MG metabolic pathway and its physiological function has been extensively studied in the field of plant biology and huge progress has been made (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016). In this chapter, based on the current knowledge on MG and its detoxification system, the generation and elimination of MG, MG signaling triggered by adverse environments, environment and chemical priming-induced abiotic stress tolerance related to MG, and signaling crosstalk of MG with other signaling molecules such as Ca^{2+}, H_2O_2, NO, and H_2S have been highlighted. This chapter helps researchers to further understand MG homeostasis in plant cells and its physiological functions, laying a foundation for crop improvement related to abiotic stress tolerance.

14.2 GENERATION OF MG IN PLANTS

Like signaling molecules Ca^{2+}, H_2O_2, NO, and H_2S, multiple pathways of anabolism and catabolism are the important criteria of signaling molecules (Neill et al., 2002; Dodd et al., 2010; Li et al., 2016; da-Silva and Modolo, 2018). Multiple metabolic pathways can rapidly produce signaling molecules when needed, but are effectively eliminated when not needed by plants (Neill et al., 2002; Dodd et al., 2010; Li et al., 2016; da-Silva and Modolo, 2018). In plants, MG is commonly synthesized in cytoplasm, mitochondria, chloroplast, and nucleus and maintains homeostasis in plant cells via multiple metabolic pathways, discussed as follows.

14.2.1 Nonenzymatic Pathway: A Key Player

Glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), collectively known as triosephosphate (TP), are important intermediate products of glycolysis and photosynthesis. Due to its instability, the phosphoryl group of TP, especially DHAP, is easy to remove and produce MG (Kaur et al., 2014a,b; Hossain et al., 2016; Min et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). G3P and DHAP can interconvert via the catalyzation of isomerase (TPI). Therefore, DHAP is the major contributor of MG production by nonenzymatic pathway, and TPI plays a key role (Kaur et al., 2014a,b; Hossain et al., 2016; Min et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). In general, during glycolysis, about 0.05%−0.3% glucose can be spontaneously

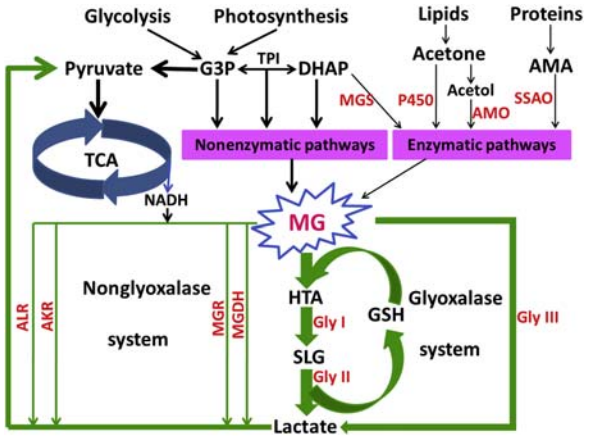

FIGURE 14.1 MG homeostasis in plants. MG can maintain homeostasis in plant cells by generating from enzymatic and nonenzymatic pathways as well as eliminating via glyoxalase and nonglyoxalase systems in plants. Source: *Adopted from Wang, Y., Zhou, Z. H., Ye, X.Y., Li, Z.G., 2018. Methylglyoxal: a new signaling molecule in plants. Plant Physiol. J. 54, 10–18 (Wang et al, 2018).*

converted into MG by nonenzymatic pathway, which is enhanced under stress conditions (Kaur et al., 2014a, b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016). Therefore, the nonenzymatic pathway is a key player of MG biosynthesis in plants.

14.2.2 Enzymatic Pathways: A Secondary Way

In addition to nonenzymatic pathways, plants can also actively synthesize MG by the following four enzymatic pathways. Firstly, DHAP can be converted into MG by the catalyzation of MG synthase (MGS). Secondly, cytochrome P450 can catalyze the conversion of acetone produced from fatty acid metabolism to MG. Thirdly, acetol from acetone can be transformed into MG by the catalyzer acetol monooxygenase (AMO). Fourthly, aminoacetone (AMA) originated from protein metabolism is able to convert into MG via the catalysis of semicarbazide-sensitive amine oxidase (SSAO) (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016; Fig. 14.1). Enzymatic pathways have been found in many organisms including animals, yeast, and fungus, but they are not completely clear in plants (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Min et al., 2016; Singh and Dhaka, 2016).

14.3 ELIMINATION OF MG IN PLANTS

Due to its toxic effect at high concentrations, plant cells maintain homeostasis of MG via production and scavenging system. Similar to its generation, MG has both glyoxalase system and nonglyoxalase system to scavenge excessive MG in plants, listed as follows.

14.3.1 Glyoxalase System: The First Defense Line

The glyoxalase system, also known as the MG detoxification system, is composed of glyoxalase I (Gly I), glyoxalase I (Gly II), and glyoxalase III (Gly III) (Kaur et al., 2014a,b, 2016; Hossain et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). During the process of detoxification, MG spontaneously reacts with reduced glutathione (GSH) and forms hemithioacetal (HTA), which is then converted to S-D-lactoylglutahione (SLG) by Gly I. Afterwards, Gly II catalyzes the conversion of SLG to D-lactate and regenerates GSH, which is recycled back into the glyoxalase system or regulates the redox homeostasis in plant cells (Kaur et al., 2014a, b; Hossain et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). For Gly III, it can directly convert MG into D-lactate in a single step without the help of any cofactor including GSH, referred to as the shorter route (Kaur et al., 2014a,b; Hossain et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). Commonly, more than 99% MG produced from enzymatic and nonenzymatic pathways are eliminated by the glyoxalase system (Kaur et al., 2014a,b; Hossain et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). Therefore, the glyoxalase system is the first line of defense against MG in plants.

14.3.2 Nonglyoxalase System: A Minor Route

In addition to the glyoxalase system, plants can scavenge MG by the nonglyoxalase system. The nonglyoxalase system mainly includes aldose/aldehyde reductase (ALR), aldo-keto reductase (AKR), MG reductase (MDR), and MG dehydrogenase (MGDH) (Kaur et al., 2014a,b; Hossain et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). These enzymes use reduced nicotinamide adenine dinucleotide (NADH) produced from glycolysis and/or tricarboxylic acid cycle (TCA) as cofactor to convert MG into lactaldehyde and pyruvate; the latter goes back to TCA, which in turn regulates energy metabolism and redox homeostasis (Kaur et al., 2014a,b; Hossain et al., 2016; Singh and Dhaka, 2016; Fig. 14.1). As the elimination of MG by nonglyoxalase system is less than 1%, this pathway is a minor route for scavenging MG in plants (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016).

14.4 MG SIGNALING TRIGGERED BY ENVIRONMENTAL STRESS IN PLANTS

As mentioned above, under normal physiological conditions plant cells contain a small amount of MG, from 25 to 75 μM, varying in concentration in different organs and plant species (Kaur et al., 2014a,b; Hossain

et al., 2016; Li, 2016; Singh and Dhaka, 2016). However, under abiotic stress such as salt, drought, heavy metal, extreme temperature, and so forth, MG can be rapidly produced and increases two to sixfold, reaching 150–450 μM, triggering MG signaling "burst" (Kaur et al., 2014a,b; Hossain et al., 2016; Li, 2016; Singh and Dhaka, 2016; Fig. 14.2). This burst has become a common response and/or adaptation signaling of plant to abiotic stress, followed by inducing the formation of plant abiotic stress tolerance or leading to MG stress.

In rice (*Oryza sativa*) seedlings, NaCl stress with different concentrations (150 and 200 mM) increased endogenous MG level to 160% and 290% respectively, and this increase was enhanced with increasing stress strength and duration (Mostofa et al., 2015a,b; Rahman et al., 2016d). Similar results were observed in mustard (*Brassica campestris*) seedling by Hossain et al. (2013a). Our previous results also showed that NaCl stress could rapidly induce an increase in endogenous MG level in wheat (*Triticum aestivum*) seedlings, increasing 1.6-fold compared with the control (Li et al., 2017a,b). In addition, under moderate and severe drought stress simulated with 5% and 25% PEG-6000, MG content rapidly increased in mung bean (*Vigna radiata* L.) seedlings and severe stress seedlings accumulated more MG (39 μmol/g FW) than that of moderate stress seedlings (15 μmol/g FW) (Nahar et al., 2015b, 2017a). In *Brassica juncea* seedlings, a significant accumulation of endogenous MG was recorded under drought stress (Reddy and Sopory, 1999). Also, under heavy metal stress such as 0.1 mM copper (Cu), 0.5 and 1.0 mM arsenic (As), as well as 0.25, 0.30, and 0.5 mM cadmium (Cd), the content of endogenous MG was markedly increased in stress-rice seedlings (Mostofa et al., 2015c; Rahman et al., 2015, 2016a, c). In mung bean seedlings, Cd stress (1.0 and 1.5 mM) more significantly increased the accumulation of endogenous MG (Nahar et al., 2016c, 2017a). In addition to these abiotic stresses, extreme temperature can also induce an increase in endogenous MG level in several plant species. For example, when *Ficus concinna* seedlings were subjected to high temperature stress at 35°C and 40°C, the accumulation of endogenous MG was quickly activated (Jin et al., 2015). Similar results were reported in mung bean seedlings under heat stress by Nahar et al. (2017a). Furthermore, cold stress at 6°C also increased the level of endogenous MG in mung bean seedlings from 14 (control) to 26 μmol/g FW (cold stress for 2 days), and this increase was strengthened with increasing cold stress duration from 26 (2 days) to 35 μmol/g FW (3 days) (Nahar et al., 2015d). Similarly, in tea (*Camellia sinensis*) seedlings, a rapid increase in MG was observed under cold stress at 4°C, rising from 170 (cold stress for 4 h) to 350 μmol/g FW (cold stress for 142 h) (Kumar and Yadav, 2009).

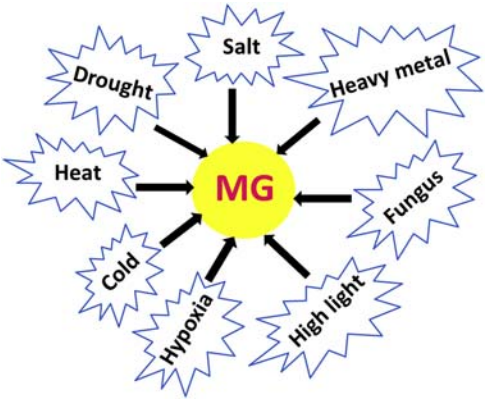

FIGURE 14.2 Adverse environments can trigger MG signaling in plants. MG signaling can be induced by adverse environments such as salt, drought, heavy metal, heat, cold, high light, and hypoxia in plants. Source: *Adopted from Wang, Y., Zhou, Z.H., Ye, X.Y., Li, Z.G., 2018. Methylglyoxal: a new signaling molecule in plants. Plant Physiol. J. 54, 10–18.*

14.5 ABIOTIC STRESS TOLERANCE RELATED TO MG IN PLANTS

In general, protein (including enzyme) denaturation, loss of biomembrane integrity, and oxidative, osmotic, and MG stresses are the common traits of abiotic stress such as salt, drought, heavy metal, and extreme temperature in plants (Golldack et al., 2014; Iqbal et al., 2016; Li et al., 2016; Min et al., 2016; Hasanuzzaman et al., 2013, 2017d). Correspondingly, the acquisition of plant abiotic stress tolerance is usually implicated in synthesis of stress proteins, repair and reestablishment of biomembrane integrity, enhancement of antioxidant system, osmotic adjustment, and activation of glyoxalase system (Golldack et al., 2014; Li et al., 2016; Hasanuzzaman et al., 2013, 2017d). Environmental stresses can trigger second messengers including MG signaling, which in turn stimulates the glyoxalase system, followed by inducing the formation of plant abiotic stress tolerance (Fig. 14.2; Table 14.1).

14.5.1 Salt Tolerance

Overaccumulation of salt such as NaCl, Na_2SO_4, $NaCO_3$, and $NaHCO_3$ in soil leads to salt stress. Salt stress commonly results in MG, oxidative, osmotic, and ion stresses (Zhu, 2002; Céccoli et al., 2015; Hoque

TABLE 14.1 Chemical Priming-Induced Abiotic Stress Tolerance and Its Relation to Glyoxalase System in Plants

Priming reagents	Glyoxalase	Abiotic tolerance	Plant species	References
Put, Sp, and Spd	Gly I↑, Gly II↑	Salt	*V. radiata*	Nahar et al. (2016a)
Pro and GB	Gly I↑, Gly II↑	Salt	*V. radiata*	Hossain and Fujita (2010)
Ca^{2+}	Gly I↑, Gly II↑	Salt	*O. sativa*	Rahman et al. (2016d)
Tre	Gly I↑, Gly II↑	Salt	*O. sativa*	Mostofa et al. (2015a)
H_2S	Gly I↑, Gly II↑	Salt	*O. sativa*	Mostofa et al. (2015b)
MG	Gly I↑, Gly II↑	Salt	*T. aestivum*	Li et al. (2017a)
HS	Gly I↑, Gly II↑	Salt and drought	*B. campestris*	Hossain et al. (2013a, 2013b)
Se	Gly I↑, Gly II↑	drought	*B. napus*	Hasanuzzaman and Fujita (2011)
SNP	Gly I↑, Gly II↑	PEG	*B. napus*	Hasanuzzaman et al. (2017a)
MG	Gly I↑, Gly II↑	Cd	*T. aestivum*	Li et al. (2017b)
Spd	Gly I↑, Gly II↑	Al	*V. radiata*	Nahar et al. (2017b)
Spm	Gly I↑, Gly II↑	Cd	*V. radiata*	Nahar et al. (2016b)
Put and NO	Gly I↑, Gly II↑	Cd	*V. radiata*	Nahar et al. (2016c)
Ca^{2+}	Gly I↑, Gly II↑	Cd, As	*O. sativa*	Rahman et al. (2015, 2016a)
Si	Gly I↑, Gly II↑	Cd	*B. napus*	Hasanuzzaman et al. (2017b)
H_2O_2	Gly I↑, Gly II↑	Cd	*B. napus*	Hasanuzzaman et al. (2017c)
GABA	Gly I↑, Gly II↑	Cr	*B. campestris*	Mahmud et al. (2017a)
MA	Gly I↑, Gly II↑	Cr	*B. campestris*	Mahmud et al. (2017b)
CA	Gly I↑, Gly II↑	Cd	*B. campestris*	Mahmud et al. (2018)
SA and SNP	Gly I↑, Gly II↑	Zn	*C. tinctorius*	Namdjoyan et al. (2017)
Mn	Gly I↑, Gly II↑	Drought and Cd	*O. sativa*	Rahman et al. (2016b,c)
Spm	Gly I↑, Gly II↑	Heat and drought	*V. radiata*	Nahar et al. (2017a)
GSH	Gly I↑, Gly II↑	Heat, drought, and salt	*V. radiata*	Nahar et al. (2015a,b,c)
Se	Gly I↑, Gly II↑	Heat	*B. napus*	Hasanuzzaman et al. (2014)
Pro and GB	Gly I↑, Gly II↑	Chilling	*C. sinensis*	Kumar and Yadav (2009)
Spm	Gly I↑, Gly II↑	Chilling	*V. radiata*	Nahar et al. (2015d)

CA, citric acid; Ca^{2+}, calcium ion; GABA, gamma-aminobutyric acid; GB, glycine betaine; GSH, glutathione; H_2O_2, hydrogen peroxide; H_2S, hydrogen sulfide; HS, heat shock; MA, maleic acid; MG, methylglyoxal; Mn, manganese; NO, nitric oxide; Pro, proline; Put, putrescine; SA, salicylic acid; Se, selenium; Si, silicon; Sp, spermidine; Spd, spermine; Tre, trehalose; SNP, sodium nitroprusside. Arrow (↑) indicates increase.

et al., 2016; Singh and Dhaka, 2016). Therefore, the improvement of salt tolerance in plants is closely associated with the induction of the glyoxalase system and antioxidant system, synthesis of osmolytes (also known as compatible solutes), and ion homeostasis (Zhu, 2002; Céccoli et al., 2015; Hoque et al., 2016; Singh and Dhaka, 2016).

In mung bean (*V. radiata* L.) seedlings, salt stress led to the toxicity of Na, a decrease in mineral nutrient uptake such as K, Ca, Mg, and Zn in roots and shoots; oxidative stress (as reflected in increase in lipid peroxidation, H_2O_2, and $O_2^{\bullet -}$); and MG stress (as indicated in an increase in MG level) (Nahar et al., 2016a). Pretreatment of bean seedlings with putrescine (Put), spermidine (Spd), and spermine (Spm) alone increased the activities of antioxidant enzymes (dehydroascorbate reductase, DHAR; glutathione reductase, GR; catalase, CAT; and glutathione peroxidase, GPX) and glyoxalase enzyme (Gly I and Gly II), and the contents of GSH and ascorbate (AsA), which in turn weakened salt-induced oxidative and MG stresses (Nahar et al., 2016a). In addition, combination of exogenous Put, Spd, and Spm could increase their endogenous levels, followed by reducing cellular Na

accumulation and maintaining nutrient homeostasis (Nahar et al., 2016a). Similarly, under salt stress, exogenously applied proline (Pro) and glycine betaine (GB) to mung bean seedlings resulted in an increase in GSH content, which in turn maintained a high GSH redox state and higher activities of Gly I, Gly II, GPX, glutathione-S-transferase (GST), and GR, followed by decreasing the accumulation of MG, H_2O_2, and $O_2^{\bullet -}$ compared with the control seedlings (Hossain and Fujita, 2010). In addition, salt stress significantly improved the contents of endogenous MG, malondialdehyde (MDA), H_2O_2, $O_2^{\bullet -}$, Pro, GSH, and oxidized glutathione (GSSG), and reduced AsA content and the GSH/GSSG ratio in mung bean seedlings (Nahar et al., 2015a). In addition, the activities of Gly I, Gly II, monodehydroascorbate reductase (MDHAR), DHAR, and CAT declined in salt-stressed seedlings; while the activities of APX, GR, SOD, GST, and GPX increased (Nahar et al., 2015a). Interestingly, salt-stressed mung bean seedlings treated with exogenous GSH improved the activities of Gly I, Gly II, APX, MDHAR, DHAR, GR, SOD, CAT, GPX, and GST, as well as AsA and GSH content and GSH/GSSG ratio, compared with treatment with NaCl alone, which in turn decreased the MG, MDA, H_2O_2, and $O_2^{\bullet -}$ contents, followed by improving the resistance of the mung bean seedlings to the salt stress (Nahar et al., 2015a). These data suggest that polyamines (PAs), Pro, GB, and GSH have a protective action against salt-induced MG and oxidative stresses by enhancing MG detoxification and antioxidant defense systems in mung bean seedlings.

In rice (*O. sativa*) seedlings, salt stress resulted in oxidative stress (an increase in lipoxygenase activity and the accumulation of $O_2^{\bullet -}$, H_2O_2, and MDA), MG stress (MG accumulation), and osmotic stress (an increase in Pro and a decrease in relative water content), which in turn led to stunted growth, severe yellowing and leaf rolling (Mostofa et al., 2015a,b). On the other hand, pretreatment with trehalose (Tre) and NaHS (H_2S donor) alone dramatically depressed the activity of lipoxygenase (LOX) and the accumulation of $O_2^{\bullet -}$, H_2O_2, MDA, and Pro; considerably increased the contents of AsA, GSH, relative water content (RWC), and Chl, as well as redox status; and remarkedly stimulated the activities of Gly I, Gly II, SOD, GPX, APX, GST, MDHAR, DHAR, and GR in salt-stressed rice seedlings, which in turn significantly addressed salt-induced toxicity symptoms (Mostofa et al., 2015a,b). In the same way, salt stress led to destruction of ion homeostasis (through Na^+ influx and K^+ efflux), loss of mineral nutrient uptake, and oxidative, and MG stresses, and finally caused growth inhibition, chlorosis, and water shortage in the salt-stressed rice seedlings (Rahman et al., 2016d). Adversely, the salt-stressed seedlings supplemented with exogenous Ca^{2+} improved ion homeostasis by inhibition of Na^+ influx and K^+ leakage, and increased the capacity of ROS and MG detoxification by activating the antioxidant defense and glyoxalase systems, followed by recovering from water loss, chlorosis, and growth inhibition (Rahman et al., 2016d).

14.5.2 Drought Tolerance

The primary effects of drought stress are osmotic, oxidative, and MG stresses. The strategies that can alleviate these stresses can improve plant drought stress tolerance. Chemical priming is a very important and effective approach that increases plant abiotic stress tolerance including drought tolerance (Antoniou et al., 2016; Hossain et al., 2017). Chemical priming commonly induces the enhancement of the antioxidant system and glyoxalase system and the accumulation of osmolytes such as Pro, GB, Tre, soluble sugar, and γ-aminobutyric acid (GABA), which in turn improve plant drought tolerance (Antoniou et al., 2016; Hossain et al., 2017).

In rapeseed (*Brassica napus*) seedlings, drought stress simulated using polyethylene glycol (PEG) resulted in reduction in RWC and the activities of Gly I and Gly II, while it increased Pro, H_2O_2, and MDA contents (Hasanuzzaman et al., 2017a). In contrast, exogenous NO (using sodium nitroprusside (SNP) as donor) improved the levels of nonenzymatic antioxidant pool (GSH and AsA) and the activities of antioxidant enzymes (APX, MDHAR, DHAR, GR, GPX, GST, and CAT) and glyoxalase system (Gly I and Gly II), which in turn successfully alleviated oxidative stress (as reflected by a decrease in H_2O_2 and MDA) and MG accumulation (Hasanuzzaman et al., 2017a). Likewise, drought stress mimicked by PEG caused a significant increase in the contents of H_2O_2, MDA, GSH, GSSG, and AsA, as well as the activities of MDHAR, GR, GST, GPX, and Gly I; a decrease in Gly II and CAT; but was unchanged in APX in rapeseed seedlings (Hasanuzzaman and Fujita, 2011). On the other hand, further increase in the contents of AsA and GSH and the activities of APX, DHAR, MDHAR, GR, GST, GPX, CAT, Gly I, and Gly II was observed in selenium (Se)-pretreated seedlings under drought stress, which in turn maintained a higher GSH/GSSG ratio and lower ROS and MG levels, and ultimately increased the tolerance of the rapeseed plants to drought stress (Hasanuzzaman and Fujita, 2011).

In mung bean seedlings, Nahar et al. (2015b) reported that drought stress resulted in oxidative stress (as evidenced by H_2O_2, $O_2^{\bullet -}$, and MDA);

increased the MG, GSSG, GSH, and Pro levels and the activities of APX, GST, Gly I, and Gly II; and decreased leaf succulence, leaf chlorophyll (Chl), RWC, AsA, and GSH/GSSG ratio. In contrast to drought stress alone, exogenous GSH further enhanced the components of the antioxidant and glyoxalase systems in drought-affected mung bean seedlings except for AsA, Pro, Gly I, and DHAR. Similarly, in *Brassica* species seedlings, drought stress significantly increased the LOX activity and the levels of MDA, H_2O_2, Pro, oxidized ascorbate (DHA), and GSSG, which in turn reduced seedling biomass, Chl content, and RWC. In contrast, spraying drought-stressed seedlings with JA increased Gly I, Gly II, DHAR, GR, and GPX activities (Alam et al., 2014).

Interestingly, heat shock (HS) as environment priming can induce cross-tolerance in plants (Hossain et al., 2017). In mustard seedlings, as compared with the control, HS (42°C, 5 h) positively modulates the activities of APX, DHAR, GR, GST, GPX, CAT, Gly I, and Gly II, and maintained lower levels of GSSG, H_2O_2, MDA, and MG (Hossain et al., 2013a). These results showed that HS (previous stress exposure) protects the mustard plants from salt- and drought-induced oxidative stress by a coordination of antioxidant and glyoxalase systems.

14.5.3 Heavy Metal Stress Tolerance

With the advance of industrialization, urbanization, and agriculturalization, heavy metal pollutants are more and more severe, which significantly affects crop productivity and human heath (Hossain et al., 2012). The excessive accumulation of MG and ROS is a common consequence of heavy metal toxicity in plants, which leads to MG and oxidative stresses, that is, lipid peroxidation, protein oxidation, enzyme inactivation, and DNA damage (Hossain et al., 2012). Plants can acquire the tolerance against heavy metal stress by sophisticated glyoxalase and antioxidant defense systems to scavenge excessive MG and ROS (Hossain et al., 2012).

In *B. juncea* seedlings, Cd stress led to its endogenous accumulation in the roots and shoots in a dose-dependent manner. This accumulation resulted in oxidative damage (as reflected in elevated MDA, H_2O_2, $O_2^{\bullet -}$, and LOX activity), MG stress (as indicated in accumulated MG), and downregulating of the antioxidant defense and glyoxalase systems, which in turn reduced growth, biomass, water status, and Chl content (Khan and Khan, 2014; Mahmud et al., 2018). However, treatment with citric acid (CA) enhanced the pool of AsA and GSH and the activities of the antioxidant enzymes (APX, MDHAR, DHAR, GR, GPX, SOD, and CAT) and the glyoxalase system (Gly I and Gly II), and the phytochelatin (PC) content. Therefore, CA reduced oxidative damage, increased leaf RWC, Chl, and the content of Cd in the root and shoot, as well as Cd translocation from the roots to the shoots in a dose-dependent manner (Mahmud et al., 2018). These data suggest for the first time that CA plays a dual role in mustard seedlings by increasing phytoremediation and upregulating the antioxidant defense and glyoxalase systems (Mahmud et al., 2018). Similarly, accumulation of chromium (Cr) increased in a dose-dependent manner, which in turn disrupted antioxidant defense and glyoxalase systems and led to an increase in H_2O_2, $O_2^{\bullet -}$, MG, and Chl contents, RWC, and LOX activity, followed by reducing mustard seedling growth (Mahmud et al., 2017a,b). Furthermore, pretreatment with GABA and maleic acid (MA) alone reduced Cr uptake and upregulated the contents of AsA and GSH and the activities of APX, MDHAR, DHAR, GR, GPX, SOD, CAT, Gly I, and Gly II, which in turn reduced oxidative damage and MG stress. In addition, GABA increased leaf RWC and Chl content, but decreased Pro and PC levels, and finally restored mustard seedling growth (Mahmud et al., 2017a,b).

In rice seedlings, Cd stress increased the levels of MG and ROS, while Ca^{2+} treatment significantly increased the content of AsA and the activities of Gly I, Gly II, SOD, CAT, GST, MDHAR, and DHAR; and reduced Cd uptake, which in turn reversed overproduced MG and ROS, thereby reducing Cd toxicity (Rahman et al., 2016a). Likewise, exogenously applied Ca^{2+} significantly decreased As and ROS levels, increased AsA content and the activities of Gly I, Gly II, MDHAR, DHAR, CAT, GPX, and SOD, restoring water loss and plant growth, compared with rice seedlings exposed to As only (Rahman et al., 2015). In rapeseed (*B. napus* L.) seedlings, Cd stress increased the contents of H_2O_2, MDA, and GSSG, as well as the activities of LOX, APX, and GST; and decreased the contents of AsA and GSH, GSH/GSSG ratio, as well as their redox pool. In addition, Cd declined the activities of MDHAR, DHAR, and CAT, but was unchanged in Gly I and Gly II. Inversely, exogenous application of Si and H_2O_2 alone reduced H_2O_2 and MDA contents and increased the AsA and GSH pools and activities of APX, MDHAR, DHAR, GR, CAT, Gly I, and Gly II (Hasanuzzaman et al., 2017b,c). Therefore, the enhancement of the nonenzymatic and enzymatic antioxidants and glyoxalase system helped to decrease the oxidative damage and MG stress, followed by improving the resistance of rapeseed seedlings to Cd toxicity.

14.5.4 Heat Tolerance

High temperature deleteriously affects the whole plant life cycle from seed germination, seedling establishment, growth, development, to senescence, and even survival (Wahid et al., 2007; Khan et al., 2013a,b; Hemmati et al., 2015). High temperature commonly leads to direct injury, namely loss of biomembrane integrity and protein denaturation and indirect injury also known as secondary injury, that is, oxidative, osmotic, and MG stresses. These injuries result in physiological disorder, metabolic inordinance, and loss of crop productivity (Wahid et al., 2007; Hemmati et al., 2015). Plants have correspondingly developed a series of complex strategies to perception, response and adaptation to high temperature at the morphological, anatomical, physiological, biochemical, and molecular levels (Wahid et al., 2007; Hemmati et al., 2015). Understanding the mechanism of damage and adaptation of plants to high temperature stress is fundamental for the development of tolerant plant species.

Application of PAs plays a key role in conferring abiotic stress tolerance in plants. In mung bean seedlings, pretreatment with Spm upregulated the activities of antioxidant enzymes (SOD, CAT, GPX, DHAR, and GR) and glyoxalase system (Gly I and Gly II); and maintained a higher levels of AsA and GSH, which in turn reduced oxidative stress (a decrease in H_2O_2, $O_2^{\bullet -}$, LOX activity, and MDA) and MG stress (a lower MG accumulation) under high temperature stress (Nahar et al., 2017a). In addition, exogenous PAs could increase its endogenous content, as osmolyte, which carried out osmoregulation and restoration of plant water status under high temperature stress, followed by preventing the degradation of photosynthetic pigments and improving seedling growth parameters (Nahar et al., 2017a). Similarly, high temperature stress markedly reduced the activities of MDHAR, DHAR, GPX, CAT, and Gly I, followed by triggering oxidative stress, as reflected in an increase in H_2O_2, $O_2^{\bullet -}$, and MDA, but increased Gly II (Nahar et al., 2015c). However, pretreatment of mung bean seedlings with exogenous GSH increased the activities of APX, MDHAR, DHAR, GR, GPX, GST, CAT, Gly I, and Gly II; improved endogenous GSH content and the GSH/GSSG ratio; and reduced GSSG content, compared with the control seedlings (Nahar et al., 2015c). Therefore, GSH improved the glyoxalase and antioxidant systems, which in turn reduced MG and oxidative stresses, followed by exhibiting better physiological performance under high temperature stress (Nahar et al., 2015c).

Selenium, a minor element, plays a crucial role in the acquisition of plant abiotic stress tolerance (Hasanuzzaman and Fujita, 2011; Hasanuzzaman et al., 2014; Khan et al., 2015). Exogenously applied Se to rapeseed seedlings improved the activities of MDHAR, DHAR, GR, GPX, CAT, Gly I, and Gly II, and the contents of Pro, AsA, and GSH, as well as the GSH/GSSG ratio under high temperature stress (Hasanuzzaman et al., 2014). The activation of antioxidant and glyoxalase systems by Se further alleviated oxidative damage and MG toxicity, as indicated by a decrease in MDA, H_2O_2, and MG, as compared with heat-treated seedlings without Se supplementation (Hasanuzzaman et al., 2014). Similarly, in rice seedlings, high temperature stress led to oxidative stress, as indicated by an increase in LOX activity, MDA, and H_2O_2, which in turn decreased fresh weight (FW) and Chl content (Mostofa et al., 2014). In addition, high temperature stress also significantly increased the activities of Gly I and Gly II (Mostofa et al., 2014). In contrast, foliar spray with Spm alleviated an increase in MDA and H_2O_2, but improved the contents of AsA, GSH, FW, Chl and AsA, as well as GSH redox status (Mostofa et al., 2014).

14.5.5 Chilling Tolerance

Chilling injury refers to the damage of chilling-sensitive plants from tropic and subtropic regions by low temperature (above ice point) but below the temperature that they normally experience (Ruelland et al., 2009; Zhu, 2016). Chilling stress commonly triggers membrane transition, that is, conversion of liquid-crystal state to solid state, which leads to loss of biomembrane integrity (Ruelland et al., 2009; Zhu, 2016). In addition, oxidative, osmotic, and MG stresses also can be induced by chilling stress in many plant species (Ruelland et al., 2009; Zhu, 2016). Therefore, the strategies, including environment or chemical priming, that can maintain liquid-crystal state of biomembrane and remit oxidative, osmotic, and MG stresses all can improve the tolerance of chilling-sensitive plants to chilling stress. In *Jatropha curcas* seedlings, chilling shock (5°C, 4 h) or chilling hardening (12°C, 2 h) could improve the chilling tolerance by a coordination of osmolytes and antioxidant system (Ao et al, 2013a,b; Li et al., 2013). Similarly, in mustard seedlings, salt, and drought stress abruptly increased H_2O_2 and MDA levels, producing oxidative stress (Hossain et al., 2013b). In addition, both stresses significantly improved the contents of GSH and GSSG, and the activities of MDHAR and Gly I; but declined CAT and Gly II activities. However, chilling shock (6°C, 5.5 h) maintained a higher level of AsA and GSH contents and GSH/GSSG ratio; higher activities of APX, DHAR, GR, GST, GPX, CAT, Gly I, and Gly II; and lowered the levels of GSSG, H_2O_2, and

MDA in mustard seedlings, as compared with the control (Hossain et al., 2013b). These adaptive changes in the antioxidant system and glyoxalase system induced by chilling shock are the basis for the acquisition of abiotic stress tolerance in chilling-sensitive plants.

In mung bean seedlings, chilling stress led to oxidative stress, as indicated in an increase in H_2O_2 and MDA contents, by modulating the nonenzymatic (AsA and GSH) and enzymatic components (APX, GR, DHAR, and MDHAR) of AsA-GSH (Nahar et al., 2015d). Also, chilling stress reduced RWC, Chl content, and seedling growth. On the other hand, exogenous pretreatment of mung bean seedlings with Spd significantly increased the contents of AsA and GSH, and the ratios of AsA/DHA and GSH/GSSG; and decreased DHA and GSSG contents under chilling stress (Nahar et al., 2015d). Similarly, Spd pretreatment also increased APX, MDHAR, DHAR, and GR activities in mung bean seedlings under chilling stress, which in turn reduced the oxidative stress and MG toxicity, followed by improving seedling growth (Nahar et al., 2015d). In the same way, chilling stress led to MG stress (MG accumulation) and oxidative stress (an increase in MDA) in tea bud (Kumar and Yadav, 2009). However, exogenous application of Pro and GB alleviated a decrease in Gly I and Gly II activities and an increase in MG level under chilling stress (Kumar and Yadav, 2009). In addition, exogenous Pro activated GST and GR activities, but GB only increased GR under chilling stress (Kumar and Yadav, 2009). Interestingly, involvement of Gly I and Gly II in the acquisition of chilling tolerance was identified using proteomics protocol in arabidopsis (*Arabidopsis thaliana*), rice, and onion (*Allium cepa*) (Goulas et al., 2006; Lee et al., 2009; Chen et al., 2013).

14.6 ABIOTIC STRESS TOLERANCE IMPROVED BY OVEREXPRESSING GLYOXALASE GENES

As mentioned above, plant abiotic stress tolerance is closely connected with the glyoxalase system. A number of studies have found that transgenic plants overexpressing glyoxalase genes (*Gly I*, *Gly II*, or *Gly II*) alone or in combination can improve multiple abiotic stress tolerance such as salt, drought, heavy metal, heat, and chilling tolerance (Li, 2016; Hasanuzzaman et al., 2017d; Hossain et al., 2017). For example, transgenic rice plants overexpressing *GLY I* gene (from *Brassica*) and *GLY II* gene (from *O. sativa*) increased the activities of Gly I, Gly II, and TPI; and reduced the levels of G3P, DHAP, pyruvate, GSH, and MAD under salt, drought, and heat stresses (Gupta et al., 2018). In addition, transgenic plants alleviated the oxidative damage of chloroplast and mitochondrial ultrastructure by ROS and maintained photosynthetic efficiency under these stresses (Gupta et al., 2018). Finally, plants overexpressing *GLY I* combined with *GLY II* imparted tolerance to abiotic stresses like salinity, drought heat, and provided resistance to the sheath blight fungus (Gupta et al., 2018).

Similarly, under normal conditions, in rice seedlings, a gene *OsGly I* encoding Gly I was ubiquitously expressed in different organs such as root, stem, leaf, leaf sheath, and spikelet with varying abundance (Zeng et al., 2016). Under NaCl, $ZnCl_2$, and mannitol stresses, *OsGly I* was markedly upregulated in different organs of rice seedlings (Zeng et al., 2016). In addition, transgenic rice seedlings overexpressing *OsGly I* increased Gly I activity, which in turn reduced the accumulation of MG and MDA, followed by improving the tolerance of transgenic seedlings to NaCl, $ZnCl_2$, and mannitol stresses, compared with wild-type plants (Zeng et al., 2016). Also, transgenic plants performed higher seed setting rate and yield (Zeng et al., 2016). Both *Gly I* and *Gly II* genes, known as *JcGLYI* and *JcGLYII* from *J. curcas*, were heterologously expressed in *Escherichia coli* and yeast individually, which conferred the resistance to PEG (5%), NaCl (200 mM), and MG (5 mM) stresses (Mudalkar et al., 2017). Furthermore, in *B. juncea* seedlings, overexpression of *Gly I* improved the tolerance to several stresses such as salinity, heavy metal, and drought stress as compared with untransformed control plants, indicating that the overexpression of the *gly I* gene is a better option for improving salt, drought, and heavy metal stress tolerance in transgenic plants (Rajwanshi et al., 2016).

In addition, the transgenic tobacco overexpressing *Gly I* and *Gly II* alone or in combination increased the activities of GR, GST, GPX, and APX, GSH level, and GSG/GSSG ratio; and reduced the accumulation of endogenous MG and MDA, which in turn improved the growth of transgenic plants under salt stress as compared with the nontransgenic plants (Yadav et al., 2005b). In addition to glyoxalase, AKR is another enzyme involved in MG detoxification in plants. Its expression level can be greatly induced by abscisic acid (ABA), H_2O_2, NaCl, and mannitol treatments (Turoczy et al., 2011). Furthermore, the transgenic tobacco overexpressing *OsAKR1* from rice exhibited higher AKR activity and less accumulation of MG in leaves than the wild-type plants under normal and heat stress conditions, which in turn increased the resistance to high temperature stress at 44°C (Turoczy et al., 2011).

14.7 MG PRIMING-INDUCED ABIOTIC TOLERANCE

Many signaling molecules such as Ca^{2+}, H_2O_2, NO, and H_2S priming can improve the abiotic stress tolerance in various plant species; the acquisition of plant abiotic stress tolerance is involved in MG and ROS detoxification systems, osmotic adjustment, synthesis of stress proteins, and so forth (Antoniou et al., 2016; Li et al., 2016; Zhou et al., 2017; Hossain et al., 2017). A lot of studies have reported that MG priming can increase the resistance of plants to a wide range of abiotic stresses, further supporting the signaling role of MG. For example, in *Brassica rapa* L. seedlings, MG priming could stimulate the activities of MG detoxification system (Gly I and Gly II) and ROS detoxification enzymes (SOD, APX, CAT, GR, and APX), reduced the oxidative damage and MG toxicity, as reflected in a decrease in the accumulation of H_2O_2, $O_2^{\bullet -}$, and MG, which in turn promoted seed germination and subsequent seedling growth of seedlings under zirconium stress (Bless et al., 2017). In addition, NaCl stress reduced seed germination and seedling growth, as indicated in seedling height, root length, fresh weight, and dry weight (Li et al., 2017a). However, the toxic effects of NaCl were remitted by exogenous MG treatment, but worsen by MG scavenger *N*-acetyl-L-cysteine (NAC), illustrating that MG could improve the tolerance of wheat to salt stress (Li et al., 2017a). Further experiments showed that MG activated Gly I, Gly II, SOD, CAT, APX GR; increased GSH and AsA levels; and decreased oxidative stress indicated by decrease in $O_2^{\bullet -}$, H_2O_2, and MDA levels in wheat seedlings under NaCl stress (Li et al., 2017a). In addition, MG also accumulated osmolytes such as Pro and GB soluble sugar, thereby enhancing the capacity of osmotic adjustment in wheat seedlings under NaCl stress. The above positive effects of MG were weakened by NAC, further verifying the signaling role of MG (Li et al., 2017a).

Similarly, under Cd stress, wheat seedlings showed reduced seedling height, root length, FW, and DW in a concentration dependent manner (Li et al., 2017b). Interestingly, the negative effects of Cd on wheat seedlings were alleviated by exogenous MG, suggesting that MG could improve Cd toxicity in wheat (Li et al., 2017b). Further investigations have illustrated that MG increased the activities of GR and gamma-glutamylcysteine synthetase (γ-ECS), a key enzyme in GSH biosynthesis, and GSH level in Cd-stress wheat seedlings compared with the control seedlings without MG treatment (Li et al., 2018). In contrast, γ-ECS and GR activities and GSH level were weakened by NAC, DHBA (2,4-dihydroxy-benzylamine), and BCNU (1,3-bischloroethyl-nitrosourea), specific inhibitors of GR, and BSO (buthionine sulfoximine), a specific inhibitor of GSH biosynthesis (Li et al., 2018). Moreover, MG increased the activities of Gly I and Gly II and declined endogenous MG level in Cd-treated seedlings (Li et al., 2018). On the other hand, an increase in Gly I and Gly II activities and a decrease in MG level were reversed by NAC, IAS (isoascorbate), and SA (squaric acid), specific inhibitors of Gly I (Li et al., 2018). Furthermore, MG alleviated an increase in H_2O_2 and MDA in Cd-treated wheat seedlings (Li et al., 2018).

14.8 SIGNALING CROSSTALK BETWEEN MG AND CA^{2+}, ROS, NO, AND H_2S

In general, the acquisition of plant abiotic stress tolerance can be classified into four steps: stress perception by sensors, signal transduction and signal network formation, gene expression, and physiological and biochemical change (Antoniou et al., 2016; Li et al., 2016; Hossain et al., 2017). As mentioned above, environmental stress can trigger second messengers such as Ca^{2+}, H_2O_2, NO, H_2S and MG, while exogenous signaling molecule priming can increase their endogenous levels, which in turn switches on plant abiotic stress tolerance via signaling crosstalk (Antoniou et al., 2016; Li et al., 2016; Hossain et al., 2017).

In prokaryote *E. coli*, $[Ca^{2+}]_{cyt}$ also was induced by exogenous MG in a concentration dependent manner, and this induction was blocked by la^{3+}, a plasma membrane Ca^{2+} channel blocker (Campbell et al., 2007; Hoque et al., 2016). Similarly, in eukaryote *Saccharomyces cerevisiae*, exogenous MG treatment could activate a high osmolarity glycerol-mitogen-activated protein kinase cascade, which in turn increased cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) through stimulating Ca^{2+} channels, followed by triggering the calcineurin-mediated Ca^{2+} signaling pathway (Maeta et al., 2005; Hoque et al., 2016). These results suggest that Ca^{2+} transient requires the entry of extracellular Ca^{2+} into cells across the plasma membrane. In *Brassica oleracea*, calmodulin (CaM) inhibitors, trifluoperazine (TFP), and chlorpromazine (CPZ), alone inhibited the activity of Gly I (Bagga et al., 1987; Hoque et al., 2016). Likewise, Gly I activity in *B. juncea* was activated by Ca^{2+}/CaM, but blocked by TFP (Deswal and Sopory, 1999; Hoque et al., 2016). These data indicate that the calcium messenger system (mainly Ca^{2+} and CaM) may activate Gly I, which in turn regulates MG homeostasis in cells, further verifying the interaction of MG and Ca^{2+}/CaM.

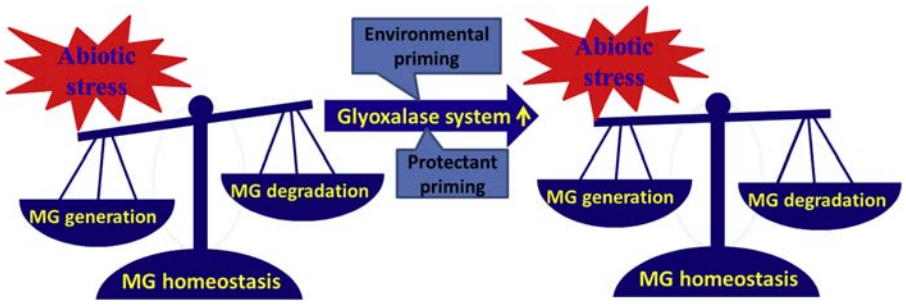

FIGURE 14.3 Key role of glyoxalase system in MG homeostasis in plants. Adverse environments induce MG excessive accumulation, while environment (hardening) or chemical (protectant) priming restores MG homeostasis, followed by switching on abiotic stress tolerance in plants.

In guard cells of *Arabidopsis*, MG could increase the activity of salicylhydroxamic acid (SHAM)-sensitive peroxidases (POD), which in turn induced extracellular ROS (H_2O_2 and $O_2^{\bullet -}$) production, followed by inducing $[Ca^{2+}]_{cyt}$ oscillations in guard cells, and ultimately led to stomatal closure (Hoque et al., 2012c). Similarly, MG also could inhibit inward-rectifying potassium channels, reducing the accumulation of K^+ in guard cells, followed by inhibiting light-induced stomatal opening in *Arabidopsis* (Hoque et al., 2012a). In addition, MG could catalyze the photoreduction of O_2 at photosystem I (PS I), leading to $O_2^{\bullet -}$ production, which is converted into H_2O_2 by CAT or APX, further exerting the signaling role of H_2O_2 and $O_2^{\bullet -}$ (Saito et al., 2011; Li, 2016). Many studies also found that MG could induce the accumulation of H_2O_2 and $O_2^{\bullet -}$ by inhibiting mitochondrial electron transfer chain, complex III and antioxidant enzymes (Chang et al., 2005; Wang et al., 2009; Desai et al., 2010; Hoque et al., 2016).

Interestingly, in animal cells, many studies have found crosstalk between MG and NO (Su et al., 2013), ROS (Chang et al., 2005), and H_2S (Yang et al., 2014; Chang et al., 2010).

14.9 CONCLUSION AND FUTURE PROSPECTS

Signaling molecule MG, similar to other second messengers such as Ca^{2+}, H_2O_2, NO, and H_2S, can be rapidly triggered by adverse environments (including salt, drought, heavy metal, heat, chilling, and high light stresses) or chemicals (like ABA, H_2O_2, and 2,4-dichlorophenoxyacetic) via coordinating action of its production (mainly nonenzymatic pathways) and scavenging (principally glyoxalase system) systems in plants (Hossain et al., 2009; Fig. 14.2). Owing to the dual role of MG, excessive MG has to be eliminated by glyoxalase and nonglyoxalase systems (Fig. 14.1) and maintain a low and nontoxic physiological level to exert its signaling role in seed germination, plant growth, development, reproduction, and response to abiotic stress. The acquisition of plant abiotic stress tolerance (such as salt, drought, heavy metal, heat, and chilling tolerance) induced by environment or chemical (protectant) priming is closely related to MG and its detoxification system, especially glyoxalase system plays a key role (Table 14.1 and Fig. 14.3).

Recently, the research on MG and its detoxification system has extensively attracted attention and obtained great progress in seed germination, plant growth, development, reproduction, and adaptation to abiotic stress (Hasanuzzaman et al., 2017d; Hossain et al., 2017; Sankaranarayanan et al., 2017). However, some open questions have to be settled urgently:

1. With the development of genomics, transcriptomics, proteomics, and metabolomics, an in-depth understanding of MG homeostasis and its signaling roles will be expounded in various plant species using omics approach in the future.
2. MG priming-induced abiotic stress tolerance and its mechanisms are waiting for answer at the physiological, biochemical, and molecular level.
3. In animal cells, signaling crosstalk between MG and Ca^{2+}, ROS, NO, and H_2S has gotten much attention (Maeta et al., 2005; Campbell et al., 2007; Chang et al., 2005, 2010; Su et al., 2013), but their crosstalk is not completely clear in plants.
4. MG can lead to glycation of proteins, which in turn regulates protein secondary structure and activity (Gomord and Faye, 2004). However, whether glycated proteins are the receptors of MG needs to be further investigated.
5. MG as a novel signaling molecule, its concentration in cells, or compartmentalization requires precise and timely quantitative determination in vivo and in vitro. Shaheen et al. (2014) used fluorogenic probes, DAF-2 (4,5-diaminofluorescein) and DAR-1 (4,5-diaminorhodamine), to detect the content of endogenous MG in living animal cells, while these probes have nonspecificity. Therefore, specific fluorescence methods must be further explored in the response of plants to abiotic stress and signaling crosstalk in plants in future.

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CHAPTER

15

Role of Trehalose and Regulation of its Levels as a Signal Molecule to Abiotic Stresses in Plants

Silvia Morgutti[1], *Noemi Negrini*[1], *Chiara Pucciariello*[2] *and Gian Attilio Sacchi*[1]

[1]Department of Agricultural and Environmental Sciences—Production, Landscape, Agroenergy, University of Milan, Milan, Italy [2]Institute of Life Sciences, Sant'Anna School of Advanced Studies, Pisa, Italy

OUTLINE

15.1 INTRODUCTION

Other than directly supplying C skeletons and energy for biosynthetic and metabolic processes, sugars play an important role in signaling cellular energy status and triggering responses to sustain growth and developmental processes, as well as respond to biotic and abiotic stresses (Lastdrager et al., 2014; Wingler, 2018). The ability to sense continuously the sugar levels and thus control the energy status is crucial for the cell survival. In plants, sugar sensing varies during life in relation to developmental and environmental factors. Several studies on the role of sugar signaling in plant development have been conducted that mostly focus on specific developmental processes (O'Hara et al., 2013). The cellular levels of the disaccharide sucrose (Suc) and the hexoses glucose (Glu) and fructose (Fru) indirectly regulate the expression of different genes involved in

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the control of plant growth and development, as well as in the responses to stresses. This chapter focuses on the nonreducing disaccharide trehalose (Tre) and its phosphorylated precursor, trehalose-6-P (T6P). For these sugars roles in signaling the cell energy status, including the response to abiotic stresses, are to date emerging.

15.2 TREHALOSE IN PLANTS

Tre high solubility and chemical nonreactivity allow the accumulation of this sugar at high cellular concentrations in all major groups of organisms except vertebrates without significant interference with metabolism (Figueroa and Lunn, 2016). Due to these properties, Tre has been reported to play roles as osmolyte, osmoprotectant, and C storage. In higher plants, this role was attributed to Suc, since for a long time it was supposed that in angiosperms only some resurrection plants were able to synthesize Tre that at high concentrations was thought to contribute to cell membrane stabilization in the desiccated state. Several studies demonstrated that the Tre biosynthetic pathway is instead widespread in the plant kingdom and that the intermediate T6P plays unexpected functional roles, including the regulation of plant development and growth, relationships with other organisms, and plant responses to several stresses (Fernandez et al., 2010).

Tre is formed by an α,α-1,1-glucoside bond between two α-Glu units. In plants, Tre biosynthesis is a two-step process that occurs in the cytosol and involves the enzymes T6P synthase (TPS) and T6P phosphatase (TPP). These enzymes catalyze the condensation reaction of uridine diphosphate glucose (UDPG) and Glu6P to T6P and the subsequent dephosphorylation of T6P to Tre, respectively. Finally, Tre is hydrolyzed by trehalase (TRE) into two Glu molecules (Fig. 15.1).

In *Arabidopsis*, 11 *TPS* genes, four homolog to yeast *TPS1* (*AtTPS1-AtTPS4*, grouped as class I) and seven homolog to yeast *TPS2* (*AtTPS5-AtTPS11*, grouped as class II), were identified. Recently, it was demonstrated that, together with AtTPS1, other TPS isoforms have TPS activity. The successful complementation of *tps1* yeast mutants with AtTPS1, AtTPS2, and AtTPS3 demonstrates the catalytic activity of these isoforms (Delorge et al., 2015). For the other proteins of the family, and in particular for those of class II, which possess both synthase and phosphatase domains but are devoid of TPS activity (John et al., 2017), the function still needs to be clarified and currently a regulatory activity as sensors for the level of T6P has been suggested (Vandesteene et al., 2010; Zang et al., 2011). Ten *TPP* genes encoding active enzymes when expressed in yeast or in *Escherichia coli* have been identified in *Arabidopsis thaliana* (Vandesteene et al., 2012). Excluding the TPP catalytic phosphatase box domains, they do not show sequence homology with the related microbial genes. The presence of multiple *TPP* genes strongly suggests the need for a fine regulation, at different histological and/or cellular levels, of the substrate (T6P) and/or the product (Tre) of the encoded activities. The presence of active TPSs and TPPs is not limited to *A. thaliana*, but is widespread among all the major plant taxa including monocots (Avonce et al., 2006; Lunn, 2007; Pramanik and Imai, 2005; Shima et al., 2007; Zang et al., 2011).

Only one *TRE*-encoding gene has been identified in *A. thaliana*, as well as in other higher plants among which are rice and soybean (Frison et al., 2007; O'Hara et al., 2013; Paul et al., 2008).

The regulation of TPS and TPP activities in plants is poorly understood. TPS1 proteins possess an autoinhibitory N-terminal domain, which regulates the catalytic activity; upon removal of that domain, the enzyme catalytic activity is increased. The region responsible for the inhibition corresponds to the R/L-rich region located at the N-terminal site of the protein, which is present in several orthologs from different plant genera and species. The autoinhibitory domain is absent in the AtTPS2-isoforms. AtTPS1 can be phosphorylated on S^{252} by Ca^{2+}-dependent protein kinases (Lunn et al., 2014) even if, to our best knowledge, no report on the effect of this regulation on activity is available. In the resurrection plant *Selaginella lepidophylla*, SlTPS1 is activated by K^+, Mg^{2+}, and Ca^{2+} but is insensitive to Tre or Suc (Valenzuela-Soto et al., 2004); nevertheless, it should be considered that SlTPS1 kinetic properties may be peculiar for this species. Class II AtTPSs may also be phosphorylated by a sugar-dependent mechanism, possibly also involved in protecting them from ubiquitin (UQ)−proteasome system degradation (Lunn et al., 2014).

Other regulatory mechanisms of this pathway likely exist, possibly involved in the fine tuning of the Tre/T6P ratio (see later, Section 15.3). Moreover, Tre levels have been suggested to regulate activity of TRE (John et al., 2017).

15.3 TREHALOSE AS A PROTECTANT AGAINST ABIOTIC STRESSES

Similarly to other sugars and, more in general, to compatible solutes, direct roles in tackling the effects of stressful environmental conditions (drought, salt, extreme temperatures, chemicals, flooding) have for a long time been assumed for Tre when it accumulates inside the cell. This hypothesis has been largely investigated in higher plants by experimentally modifying the cellular levels of Tre through exogenous treatments

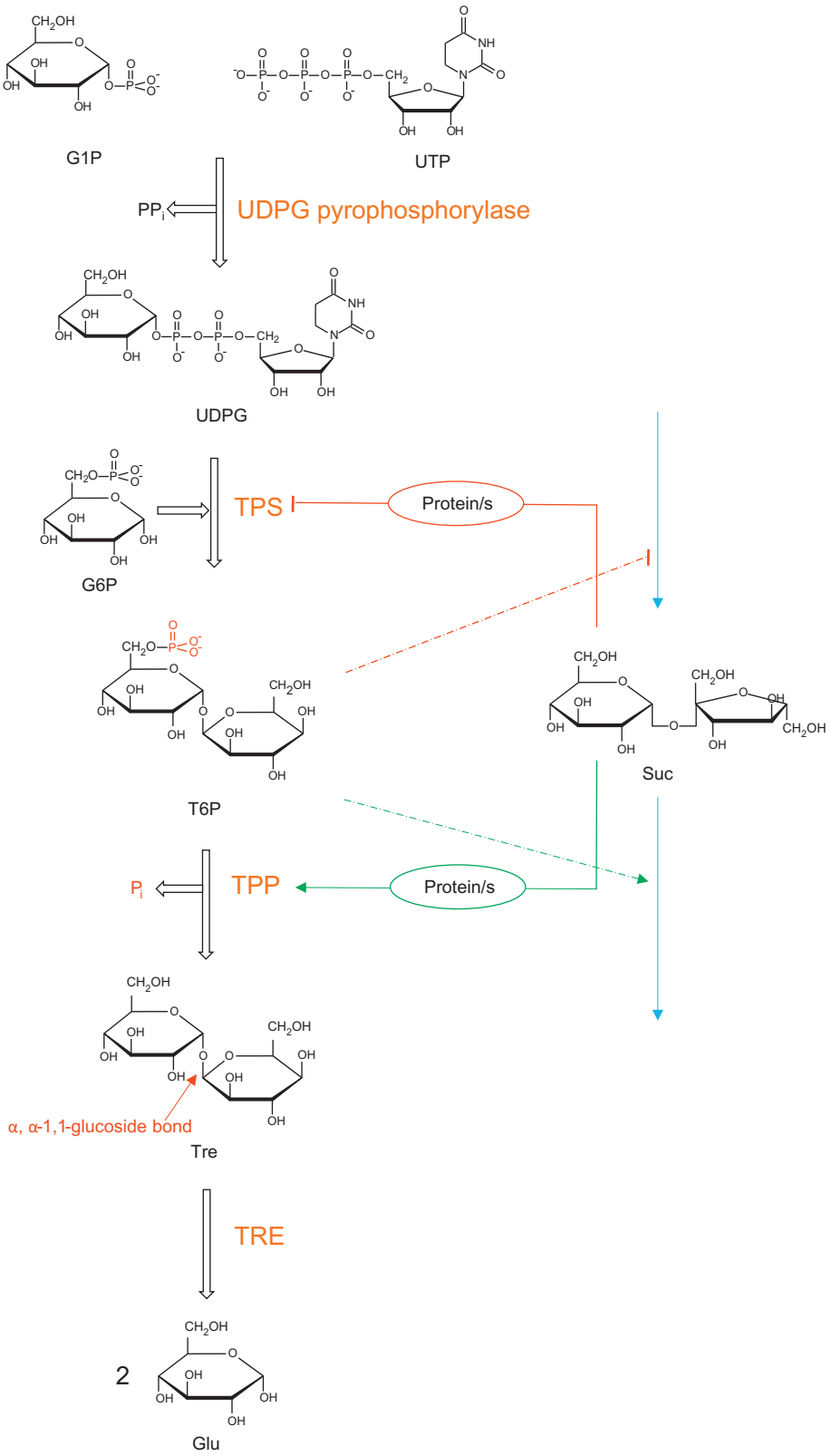

FIGURE 15.1 The trehalose (Tre) metabolic pathway in plants and its possible regulation. Red or green lines indicate negative or positive regulation, respectively. Dashed lines summarize the existence of multiple-step processes.

and/or manipulation of its metabolism using chemical inhibitors or transgenic approaches.

On the other hand, plants may face Tre-dependent effects upon contact with pathogens like bacteria and fungi, and larvae and adults of herbivorous insects that contain high Tre amounts. In these cases, Tre can be perceived as an elicitor of the plant response to biotic stress (Tayeh et al., 2014). To our best

knowledge, to date specific Tre transporters have not yet been described in plant tissues (Griffiths et al., 2016). Nevertheless, in *A. thaliana* an ERD6 sugar transporter, homolog to the TRET1 Tre transporter of insect cells (Kikawada et al., 2007), has been identified (Schluepmann and Paul, 2009).

Some evidence has shown that Tre treatment enhances the plant's tolerance to different abiotic stresses since it directly or indirectly induces the plant antioxidant system(s), scavenging the overproduction and accumulation of reactive oxygen species (ROS) generated as a secondary stress factor by virtually all environmental primary stresses (Aldesuquy and Ghanem, 2015; Sharma et al., 2012).

Intracellular ROS levels are kept within the range of tolerability by a vast array of antioxidant enzymes and chemical compounds (Hasanuzzaman et al., 2012). Sugars (Suc, raffinose family oligosaccharides, and fructans) also contribute to abiotic stress tolerance being involved in the cellular redox status balance, although high sugar levels may correspond to both activation and decrease in specific ROS-producing pathways, and both high and low sugar levels may result in an enhancement of ROS antioxidant/signaling responses (Keunen et al., 2013). Tre has been proposed to act against the damage produced by oxygen radicals (Elbein et al., 2003), scavenging ROS in vitro (Stoyanova et al., 2011) and in vivo (Nery et al., 2008). In both monocots and dicots, the effects of Tre treatment are often accompanied by upregulation of genes encoding antioxidant enzymes and accumulation of antioxidants eventually leading to the enhancement of enzymatic and nonenzymatic antioxidant defense mechanisms (Ali and Ashraf, 2011; Nounjan et al., 2012; Sadak, 2016). Cluster analysis of expression profiling data in Tre-fed *A. thaliana* seedlings showed induction of genes encoding activities that suggest a link between Tre metabolism and oxidative stress responses (Bae et al., 2005a; Schluepmann et al., 2004). Nevertheless, an opposite effect of the Tre treatment on antioxidant systems has been observed concerning the expression of an *APR1* gene (Bae et al., 2005a) that encodes a 5′-adenylylsulfate reductase of the plant SO_4^{2-} assimilation pathway involved in the synthesis of antioxidant thiols (Leustek et al., 2000). Tre treatment induced in *A. thaliana* cells the expression of detoxification and stress response proteins (Bae et al., 2005b), like a cytosolic dehydroascorbate reductase1 (DHAR1) concomitant with an increase in the Tre levels in the cell. Consistently, in *A. thaliana* seedlings, exogenous Tre counteracted the increased ROS levels induced by salt treatment (Yang et al., 2014).

Tre ameliorates the effects of oxidative stress independently from the primary stressing cause. Indeed, in winter wheat under heat stress, low amounts of Tre supplied in the growth medium increase the internal levels of Tre and diminish electrolyte leakage (a parameter linked to membrane damage), levels of malondialdehyde (MDA), the product of lipid peroxidation, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and lipoxygenase activity (Luo et al., 2010). In drought-affected wheat, Tre treatment significantly increases the activities of the detoxifying enzymes ascorbate peroxidase and peroxidase, and of phenylalanine ammonia lyase, with consistent effects also on the levels of nonenzymatic antioxidant compounds (Aldesuquy and Ghanem, 2015; Ibrahim and Abdellatif, 2016). In drought-stressed maize, fenugreek, *Brassica juncea*, and radish seedlings, application of Tre increases compounds and enzymatic activities related to oxidative stress resistance, and decreases the levels of oxidative stress-related parameters, with positive effects also on growth, photosynthetic pigments, and proteins (Akram et al., 2016; Alam et al., 2014; Ali and Ashraf, 2011; Sadak, 2016; Shafiq et al., 2015). When stress is induced in barley by acid rain, Tre treatment limited membrane damage at the root level (reduction in MDA levels and increase in H^+ extrusion) and also increases catalase and peroxidase activities and general antioxidant capacity (Ding et al., 2018). Tre application is also effective in reducing oxidative stress in wheat callus, where the beneficial effect is ascribed to nonenzymatic mechanisms (Ma et al., 2013), and in salt-stressed rice (Mostofa et al., 2015a; Nounjan et al., 2012).

Heavy metals pollution can also generate ROS (Jalmi et al., 2018). In rice, Tre treatment prior to plant exposure to excessive Cu amounts improves ascorbic acid content, redox status, and the activities of major antioxidant enzymes (Mostofa et al., 2015b). In the aquatic plant *Lemna gibba*, Tre increases upon Cd exposure, and application of external Tre increases the enzymatic and nonenzymatic antioxidant activity-related parameters whereas it decreases the levels of oxidative stress-related compounds (Duman et al., 2011).

Changes induced in Tre metabolism may also interfere with oxidative stress effects and responses. Transgenic tobacco overproducing AtTPS1 shows lesser oxidative damage when exposed to Cd and excess Cu (Martins et al., 2014). Al, which induces oxidative stress and oxidative stress-related genes in *A. thaliana* (Richards et al., 1998) and inhibits energy supply in plants, increases Tre levels and reduces TRE activity in mycorrhized roots of *Pinus massioniana* (Tan et al., 2005).

Cell ROS levels increase dramatically upon ultraviolet (UV) radiation exposure (Saini et al., 2018). Although the existing literature on a possible relationship between UV stress and Tre metabolism in plants is scanty, in silico expression profiling of all *AtTPS* and *AtTPP* genes revealed that *AtTPS4* is specifically induced by UV-B treatment, whereas *AtTPS3*, *AtTPPB*, and *AtTPPI* are repressed (Iordachescu and Imai, 2008).

ZAT10 is a zinc finger of *A. thaliana* transcription factor (TF) whose mRNA abundance is regulated by several stimuli, among which are ROS. Its constitutive expression results in the increase of different ROS-response transcripts (Mittler et al., 2006). *Arabidopsis* plants overexpressing *ZAT10* show enhanced tolerance to both high light- or H_2O_2-induced oxidative stress. A *TPS* gene has been identified among those upregulated in *ZAT10*-overexpressing plants (Rossel et al., 2007). It may then be hypothesized that there is a possible connection between *ZAT10* expression and Tre metabolism in the framework of the responses to oxidative stress.

Photosynthesis and photorespiration are major sources of ROS (Foyer and Noctor, 2005). Exposure to excess light increases ROS generation, resulting in photooxidative damage. In vitro, Tre prevents the inactivation of isolated photosystem II (PSII) complexes probably affecting the extent of hydration, favoring optimal protein conformation and cyclic electron flow around PSII against photo- and heat-induced inactivation (Mamedov et al., 2015). In vivo, in Tre-treated *A. thaliana* seedlings, the levels of DHAR1 were increased (Bae et al., 2005b), increasing the possibility to protect PSII from photooxidative damage.

High temperatures (HT) induce ROS synthesis (Ruelland and Zachowski, 2010). Wheat seedlings treated with Tre prior to exposure to HT showed enhanced PSII activity and higher levels of D1 protein (Luo et al., 2018). In vitro, Tre scavenges heat stress-generated free radicals, and protects superoxide dismutase (SOD) activity from heat inactivation (Luo et al., 2008).

Most of the effects cited earlier, rather than to significant Tre accumulation, appear correlated to changes in the ratio between Tre and its precursor T6P. The exact mechanism underlying the Tre-induced increase in T6P has not yet been clarified (Yadav et al., 2014), although it has been suggested that Tre inhibits TPP activity (Delatte et al., 2011). Moreover, the protective effect should be carefully evaluated because of the presence of the apoplastic-oriented TRE that may degrade Tre (Frison et al., 2007). Tre may thus function essentially as an elicitor and induce specific signal transduction pathways (Delorge et al., 2014).

15.4 TREHALOSE-6-P IN PLANT METABOLIC AND PHYSIOLOGICAL ACTIVITIES

Alterations in the Tre biosynthetic pathway causing fluctuations in the level of T6P lead to a plethora of effects on plant metabolic, physiological, and developmental processes, like photosynthesis, sucrose utilization, starch metabolism, cell division, cell wall synthesis, inflorescence architecture, embryo and leaf development, seedling and adult plant biomass, and tolerance/sensitivity to different stresses (Griffiths et al., 2016; O'Hara et al., 2013; Paul et al., 2008; Ponnu et al., 2011).

T6P is a low abundance molecule whose levels are determined by the relative rates of biosynthesis (TPS) and dephosphorylation (TPP), controlled by transcriptional and posttranslational mechanisms, and related to the cell Suc status (Lunn et al., 2006; Yadav et al., 2014). A few elements are recognized toward the feasibility of T6P as a signaling molecule (Paul, 2008; Paul et al., 2008), with particular regard to its low amounts, lack of participation in major metabolic fluxes, and observation that the genes involved in its metabolic pathway converge on the regulation of T6P levels.

It is now largely accepted that T6P contents are correlated to those of Suc: T6P accumulates when Suc in the cell is high, acting as a specific signal for Suc availability. On the other hand, C starvation depletes T6P levels (Figueroa and Lunn, 2016; Nunes et al., 2013a). This conclusion is supported by evidence from several experiments: (1) in C-starved *A. thaliana* seedlings the level of T6P collapses, but when they are treated with exogenous Suc, in a very short time the level of T6P dramatically increases, more than those of other soluble sugars (Lunn et al., 2006); (2) the rapid described restoration of T6P levels is not observed when other sugars that cannot be metabolically converted to Suc are supplied as C-source (Yadav et al., 2014); (3) the diurnal phase shifts of T6P overlap with those of Suc in *Arabidopsis* shoots (Martins et al., 2013); (4) in P- or S-starved plants no response concerning the T6P levels is observed, whereas in the case of N-starvation fluctuations in T6P levels do occur, but they are concomitant with the fluctuations of Suc consequent to changes in the N-metabolism (Yadav et al., 2014), reinforcing the idea that T6P specifically monitors cell C-availability.

These observations, originally obtained in the model plant *A. thaliana*, have been confirmed in other plant species including wheat, maize, tomato, and cucumber (for extensive references, see Figueroa et al., 2016).

The exact mechanism(s) by which Suc fluctuations are transduced in those of T6P still have to be completely elucidated. Any mass action effect of the T6P precursors G6P and UDPG has been experimentally excluded (Yadav et al., 2014), whereas little knowledge is to date available about the existence of direct kinetic effects of Suc on TPS and TPP activities. When Suc is supplied to C-starved plants in the presence of the transcriptional inhibitor cordycepin, the Suc-dependent T6P increase is observed, whereas it does not take place if protein synthesis is inhibited by cycloheximide, indicating dependence of the process from de novo protein biosynthesis (Yadav et al., 2014).

The link between Suc and T6P is mediated by one or more not yet identified protein/s in turn acting stimulating the activity of TPS and/or reducing that of TPP (Fig. 15.1). Nevertheless, recent results obtained by immunoblotting experiments and polysome-loading analyses appear to exclude that the target is the TPS enzyme. Overexpression in *A. thaliana* of *E. coli otsA* or *otsB* genes encoding TPS and TPP, respectively, suggests the existence also of a feedback mechanism by which T6P is able to control, directly or indirectly, the Suc level reducing its synthesis and/or stimulating its metabolic consumption (Yadav at al., 2014; Fig. 15.1).

The magnitude of the T6P/Suc ratio values seems critical, contributing, in specific cell types and plant developmental stages, to the regulation of Suc availability (Yadav et al., 2014). In plants, developmental transitions and growth are supported by time- and organ-specific metabolic changes that reprogram the source−sink relationships. The sensing of adequate availability of C sources, and the consequent trigger of signaling cascades that lead to specific metabolic changes, must be highly coordinated to ensure that the C sink is capable to sustain a developmental program (Wingler, 2018).

Adverse environmental conditions result in a decline in the cell energy levels. Stress response strategies adopted by plants include perception of unbalanced C metabolism induced by the stress condition and the onset of metabolic changes aimed at restoring an adequate energy level even at the cost of a growth slowdown.

15.5 SUGAR SIGNALING SYSTEMS IN PLANTS

In plants, two regulatory networks related to the sugar signaling system are involved in the response to changes in nutrient and energy status (Wingler, 2018): the high-C- and the low-C-availability signaling pathways. In the former one, accumulation of hexose is sensed by hexokinase-1 (Granot et al., 2014) and the protein kinase target of rapamycin (TOR; Lastdrager et al., 2014), acting through two independent pathways (Xiong et al., 2013). Accumulation of Suc is reflected in a consequent increase in T6P levels, triggering changes in the energy metabolism (O'Hara et al., 2013). In plants the latter one is centered around the activities of (1) the Snf1-related protein kinase 1 (SnRK1), homolog of animal AMP-activated protein kinase (AMPK) and yeast sucrose nonfermenting 1 (SNF1) kinase (Wurzinger et al., 2018); (2) the C/S1 group of the bZIP (basic leucine zipper) TFs; and (3) the miRNAs dependent repression of specific genes (Wingler, 2018).

T6P represents a cross-point between the high-C and the low-C signaling pathways. Indeed, although the regulation of SnRK1 is complex, it is important to stress that its activity is downregulated when T6P is high, and the contrary happens when the level of T6P is low and a starvation response (energy saving program) is triggered (Lastdrager et al., 2014; Nunes et al., 2013a; Zhang et al., 2009). Despite their common function in promoting growth in response to C-availability, no direct interaction between T6P and TOR signaling has been identified (Figueroa and Lunn, 2016).

In *Arabidopsis* leaves T6P also acts on plastidial metabolism. It promotes the redox activation of ADP-glucose pyrophosphorylase (AGPase) in response to cytosolic sugar levels enhancing starch synthesis (Kolbe et al., 2005). T6P also affects the SnRK1-mediated transcriptional regulation of genes encoding enzymes involved in starch biosynthesis and degradation (Zhang et al., 2009).

As shown in *A. thaliana* plants expressing the *E. coli T6P synthase* gene (*otsA*), increased T6P levels may also determine a transient decrease in Suc as a consequence of posttranslational activation of nitrate reductase (NR) and phosphoenolpyruvate carboxylase. The final effect would be that of diverting photoassimilates from Suc and generate C skeletons and fixed N for amino acid synthesis (Figueroa et al., 2016).

15.6 TREHALOSE/TREHALOSE-6-P/ SNRK1 SYSTEM

The evolutionarily conserved SnRK1 is the plant homolog of yeast SNF1 and animal AMPK (Wurzinger et al., 2018). These kinases, altogether known as the SNF1/AMPK/SnRK1 family, regulate the cell energy homeostasis activating catabolic energy-producing pathways and inhibiting the anabolic energy-consuming ones when intrinsic or environmental factors impact with the overall cell energy availability. The mechanisms by which these orthologous enzymes sense the cell energetic status in different organisms, thus triggering the adaptive responses aimed at reestablishing the energy homeostasis, are quite different. In this section details about the plant SnRK1 system will be furnished, whereas for SNF1 and AMPK updated information can be found in dedicated reviews (Margalha et al., 2016).

The SNRK1 complex, like its orthologs, is a heterotrimeric protein consisting of one catalytic and two regulatory subunits. In the different plant species several isoforms have been identified for each subunit type, suggesting the existence of different isoform-specific functions and/or tissue/subcellular localizations (Emanuelle et al., 2016).

The SnRK1 system has been most largely investigated in the model plant *A. thaliana*, but increasing evidence is available also for several other plant species (Margalha et al., 2016). In *A. thaliana* two isoforms of the catalytic subunit (α1 and α2, also known as KIN10 and KIN11 or SnRK1.1 and SnRK1.2), three (β1, β2, and β3) of the first noncatalytic subunit, and one (βγ) of the second one have been identified as constituents of the SnRK1 complexes. In *Arabidopsis* the existence of a further gene has been reported, encoding a third type of catalytic subunit (α3, KIN12 or SnRK1.3), but it is poorly expressed in most plant tissues (Margalha et al., 2016). Finally, a subunit (γ) whose ortholog is present and active in SNF1 and AMPK complexes has been observed in plants, but it does not seem to be part of the SnRK1 complex and no functional role has been clearly demonstrated (Emanuelle et al., 2015). Different combinations of all subunits result in the presence, in *A. thaliana*, of six different SnRK1 isoenzymes; hormones and metabolic and environmental factors differently modulate the expression of the specific SnRK1 subunits. All the α subunits present an activation T-loop containing a specific Thr (T^{175} and T^{176} in α1 and α2, respectively) whose phosphorylation by three possible kinases (geminivirus kinase 1 and 2 (GRIK1 and GRIK2), and Ca^{2+}-activated calcineurin B-like interaction protein kinase15 (CIPK15)), is decisive for activity. Dephosphorylation of $T^{175/176}$ by two alternative upstream phosphatases, abscisic acid (ABA) insensitive1 (ABI1) and protein phosphatase 2CA (PP2CA), inactivates SnRK1 (Emanuelle et al., 2015). The function of the β subunits is probably to act as a scaffold between the α and the βγ subunits. Sequence analyses show that the β1 and β2 subunits of SnRK1 contain a carbohydrate-binding motif (CBM) that, nevertheless, does not seem to be decisive for activity; activity persists even when the complex includes the β3 subunit that lacks CBM. Interestingly, the plant typical βγ subunit harbors a CBM domain fused with the N-terminal sequence of the γ subunit that shares with other eukaryotes (Viana et al., 2007). This peculiarity makes βγ able to complement the *snf4* yeast mutant, carrying a modified CBM sequence on the β subunit that makes it unable to bind carbohydrates (Emanuelle et al., 2015). Other than phosphorylation/dephosphorylation of $T^{175/176}$ in the α subunit, several other posttranslational modifications control the activity of SnRK1, among which are ubiquitination of the different subunits, sumoylation of the α subunits and successive degradation by the UQ−proteasome system, and myristoylation of the β1 and β2 subunits with consequent changes in their subcellular localization (for a review, see Margalha et al., 2016). SnRK1 activity is regulated also by its redox status: a simulated oxidative burst leads to changes in α1 activity involving a C residue. This seems a novel regulatory mechanism for modulation of SnRK1 activity regardless of phosphorylation state (Wurzinger et al., 2017). Differently from SNF1 and AMPK, the activity of SnRK1 is not allosterically regulated by the cellular AMP/ATP and ADP/ATP ratios, due to lack, in the βγ subunit, of the cystathionine beta-synthase domain that binds the adenosyl groups (Emanuelle et al., 2015).

Several experiments have shown that sugars indirectly and/or directly finely modulate the activity of SnRK1, supporting the idea of its involvement in sugar sensing/signaling. This hypothesis has been progressively corroborated demonstrating that activation of SnRK1 upon exposure to several stresses inducing cellular energy deficiencies can be removed by addition of Glu or Suc (Baena-González and Hanson, 2017). Moreover, it is now increasingly clear that the transcript, protein, and metabolite profiles observed in plants under starvation largely overlap with those induced by the activation of the SnRK1 pathway, and are widely different from those observed in Glu- or Suc-fed plants (Cookson et al., 2016). Under low-energy conditions, SnRK1 becomes active triggering a cascade of regulative events that repress anabolic pathways limiting developmental and growth processes. Therefore, the activation of SnRK1 regulates transcription and metabolism in response to energy deprivation and, by slowing down energy-consuming processes, allows the cell to survive C shortage/stress conditions through the modulation of the activity of key enzymes in N, C, or fatty acid metabolism, and/or massive transcriptional reprogramming (Harthill et al., 2006; Kulma et al., 2004; Mair et al., 2015; Tomé et al., 2014).

Using SnRK1 complexes extracted from *A. thaliana* leaves (Zhang et al., 2009), wheat grain (Martínez-Barajas et al., 2011), or potato tubers (Debast et al., 2011), it has been shown that T6P, Glu1P, and Glu6P have inhibitory effects on this kinase. The *Ki* values of the different phosphorylated sugars are consistent with their physiological concentrations in the cell (Nunes et al., 2013a).

Microarray data showed opposite effects of T6P on SnRK1-regulated genes: genes downregulated by SnRK1 and involved in reactions of anabolic importance (amino acid, protein, and nucleotide synthesis) are upregulated by T6P, whereas genes upregulated by SnRK1 and involved in degradation processes are downregulated by T6P. This result further supports the inhibitory effect of T6P on SnRK1 in growing tissues, likely requiring a protein factor not present in mature tissues (Zhang et al., 2009). Also ribose-5-P (R5P), an intermediate of the oxidative pentose-5-P (OPP) pathway, strongly allosterically inhibits SnRK1

in wheat grain (Piattoni et al., 2011). R5P may indicate the operativeness of the anabolic OPP pathway: therefore, the R5P inhibition of SnRK1 appears functionally consistent with the C status of the cell (Nunes et al., 2013b).

From the earlier statements it becomes clear that SnRK1 is a regulator that confers to the cell the ability to adequate and integrate, at multiple levels, the metabolism to the needs imposed by internal (developmental) processes and/or external (environmental) conditions. The effects of SnRK1 activity on the cell transcriptional profiles are realized through (1) direct regulation of TFs (Mair et al., 2015; Puranik et al., 2012); (2) SnRK1 direct binding to the chromatin; and (3) miRNAs and transcript turnover rates. Translational regulation involves ribosome biogenesis and the initiation factor of protein synthesis. Targets of posttranslational SnRK1 protein phosphorylation are key metabolic enzymes, actors of mitogen-activated protein kinase (MAPK) cascades and, interestingly, class II TPS proteins suggesting a feedback regulatory loop on the Tre metabolic pathway (Broeckx et al., 2016; Margalha et al., 2016).

The S1/C group of bZIP TFs plays a key role within the SnRK1 signaling pathway in relation to C-availability. In fact, SnRK1α1/SnRK1α2 and Suc exert opposite effects on the transcription/translation of S1 group bZIPs: SnRK1α1/SnRK1α2 enhance their transcription, whereas Suc inhibits translation of their mRNAs (Baena-González, 2010; Baena-González et al., 2007; Kang et al., 2010; Lastdrager et al., 2014; Weltmeier et al., 2009). The specific components bZIP1, bZIP11, and bZIP53 participate to the metabolic response to low energy supply (Dietrich et al., 2011; Ma et al., 2011). Specifically, overexpression of bZIP11, while inducing growth inhibition, is positively related to the levels of *TPP5*/*TPP6* expression and TRE activity, with diminished T6P levels (Ma et al., 2011). The *Arabidopsis* bZIP63 also plays a key role in regulating the starvation response (Baena-González et al., 2007). Its phosphorylation by SnRK1 is suggested to induce an altered interaction with other bZIP proteins, eventually leading to changes in gene expression (Mair et al., 2015; Nukarinen et al., 2016). Moreover, bZIP63 seems to integrate the sugar and ABA signals, since repression of its translation requires the ABA biosynthetic pathway, suggesting an interesting crosstalk with stress conditions (Matiolli et al., 2011). This hypothesis is supported by the observation that other bZIPs, like AREBP/ABF (ABA response element-binding protein/factor) contain highly conserved SnRK1 target sites and are phosphorylated by SnRK1 (Broeckx et al., 2016). Some TFs belonging to NAC (NAM (nonapical meristem)-ATAF-CUC (cup-shaped cotyledon)) families can also interact with SnRK1 by regulating the expression of downstream genes in response to physiological/developmental processes and abiotic stresses (Liu et al., 2014; Pinheiro et al., 2009). In particular, the *Arabidopsis* ATAF1 (*Arabidopsis* transcription activation factor) binds the α1 and α2 catalytic subunits, possibly functioning as a component of complexes regulating the transcriptional activity (Kleinow et al., 2009). *TRE1* expression is enhanced by overexpression of *ATAF1*, parallel to reduced T6P levels and sugar starvation metabolome (Garapati et al., 2015). *ATAF1* and closely related *NAC*s are upregulated by different stresses and plants overexpressing *ATAF1* are more tolerant to drought (Wu et al., 2009). Overall, the action of bZIP/NAC TFs appears to mimic that of starvation/stress conditions by mechanism(s) at different steps of the regulatory Tre/T6P/SnRK1 system.

Several other TFs interact with SnRK1 and are involved in different physiological processes (Broeckx et al., 2016; Margalha et al., 2016). SnRK1 has been reported to control, through protein complexes involving other TFs, the expression of specific genes. In *Oryza sativa* and *A. thaliana*, under submergence-induced hypoxia, α1 enhances the expression of genes encoding the two enzymes of anaerobic fermentation, *alcohol dehydrogenase1* and *pyruvate decarboxylase1*, through chromatin binding (Cho et al., 2012, 2016).

In *Arabidopsis*, SnRK1α1/2 affect the expression of more than 1000 genes, repressing the ribosomal protein genes and eventually inhibiting the translation process (Baena-González, 2010; Baena-González et al., 2007, 2008). Genes upregulated by SnRK1α1 or repressed by addition of exogenous sugars seem thus involved in the switch into catabolic pathways that provide metabolic energy. Conversely, those involved in biosynthetic pathways are, with few exceptions, repressed by SnRK1α1 and starvation (Baena-González, 2010; Baena-González et al., 2008).

SnRK1 appears to be involved in an antagonistic crosstalk between the Glu and the ethylene signaling pathways: in Glu-fed *A. thaliana* protoplasts, overexpression of *OsSnRK1* and *SnRK1α1* suppresses the expression of the *ethylene responsive factor1* (*ERF1*), a marker gene of the ethylene transduction pathway (Cho et al., 2012).

MicroRNAs, which posttranscriptionally control specific cell processes also involving stress responses, are involved in the SnRK1 signaling cascade. In *A. thaliana*, upon dark-induced starvation or *SnRK1α1* overexpression, the transcript levels of genes encoding miRNAs precursors decrease (Confraria et al., 2013). In wheat, the grain development-associated *miR1211* has been suggested to target the regulatory subunit of SnRK1 (Meng et al., 2013).

Phosphorylation by SnRK1 may reversibly inactivate enzymes involved in biosynthetic/assimilatory

pathways, like NR and sucrose phosphate synthase. SnRK1 also phosphorylates 6-phospho-fructo-2-kinase/fructose 2,6-bisphosphatase, decreasing its activity (Nukarinen et al., 2016) and altering the levels of the important regulator Fru 2,6-bisphosphate. Phosphorylation by SnRK1 of the key glycolytic enzyme cytoplasmic pyruvate kinase (Beczner et al., 2010) seems to target the enzyme to the UQ/26S proteasome pathway for degradation (Tang et al., 2003). As a whole, these results appear to strengthen the view of a SnRK1-mediated redirection of C flux toward a starvation program (Cho et al., 2016).

Phosphoproteomic analyses in SnRK1-mutant *Arabidopsis* plants under extended darkness and hypoxia linked specific phosphorylation events and T6P metabolism. SnRK1 was confirmed to phosphorylate in vivo, and inactivate, the class II TPSs 5/7/8 (Cho et al., 2016; Nukarinen et al., 2016).

The T6P/SnRK1 system may impact on the levels of expression of genes including components of other signaling cascades (like Ca^{2+} modulators) capable to further amplify its signal (Baena-González et al., 2008). In *Arabidopsis* plants with increased T6P levels increased expression of a gene encoding a calmodulin-like calcium binding protein was detected, suggesting a cross-talk with Ca^{2+} signaling/transduction pathway(s) (Schluepmann et al., 2004). Moreover, in rice, the regulation of the SnRK1-dependent pathway by OsCIPK15 suggests a possible relationship of Ca^{2+}-dependent regulation mechanism(s) with the energy-sensing network (Lee et al., 2009) (Fig. 15.2).

15.7 TREHALOSE/TREHALOSE-6-P/SNRK1 SYSTEM IN SPECIFIC STRESS RESPONSES: HYPOXIA AND COLD

15.7.1 Hypoxia

Changes in O_2 availability often occur during the plant life due to environmental conditions and developmental processes. Several worldwide areas are susceptible to water flooding events, when located close to rivers or exposed to heavy rains. Plants can experience low O_2 conditions in organs and tissues characterized by steep O_2 gradients due to low gas diffusion rate or high metabolic activity (van Dongen and Licausi, 2015).

Plants cannot survive low O_2 conditions for a long period. Oxygen is required in the electron transport chain in mitochondria to produce ATP, and O_2 shortage eventually leads to an energy crisis that can culminate with death (Perata and Alpi, 1993). A metabolic reconfiguration is the primary hallmark of O_2 limitation. This includes the shift from aerobic respiration to degradation of carbohydrates via glycolysis and subsequent fermentation, which partially compensate the drop in ATP availability.

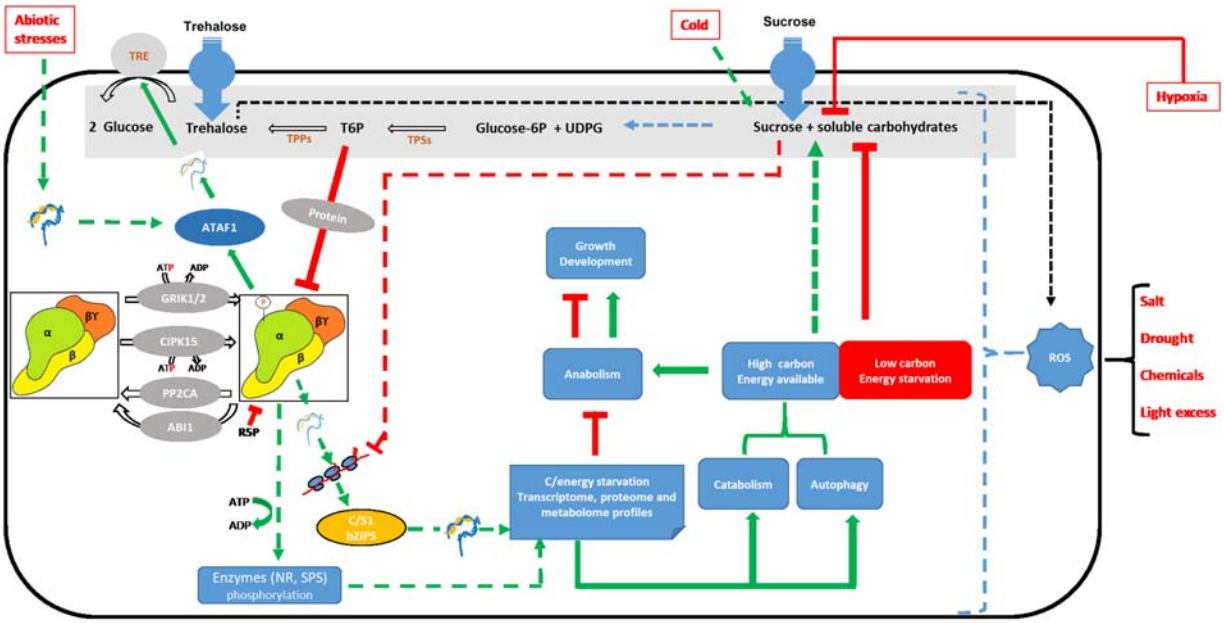

FIGURE 15.2 Proposed model of the Tre/T6P/SnRK1 system and its regulation. Red or green lines indicate negative or positive regulation, respectively. Dashed lines summarize the existence of multiple-step processes. ABI1, ABA insensitive1; CIPK15, calcineurin B-like interaction protein kinase15; GRIK, geminivirus kinase; NR, nitrate reductase; PP2CA, protein phosphatase 2CA; R5P, ribose-5-P; ROS, reactive oxygen species; SPS, sucrose phosphate synthase; TPS, T6P synthase; TPP, T6P phosphatase; TOR, target of rapamycin; Tre, trehalose; T6P, trehalose-6-P; UDPG, uridine diphosphate glucose.

Under energy starvation, sugar mobilization for a proper reallocation of consumption is crucial, and communication between source and sink organs has a pivotal role in determining the plant survival. In this context, rice (*O. sativa* L.) is one of the few crops that can cope with the energetically critical low O_2 condition. This semiaquatic crop has been the focus of many studies, since it is well adapted to different types of flooding and displays several mechanisms of adaptation (Singh et al., 2017).

Germination and seedling establishment are critical phases for rice survival under low O_2 (Miro and Ismail, 2013). At this stage, source to sink communication through signaling has a major role in the mobilization of nutrients from the endosperm (source) to the elongating embryonic axis (sink; Yu et al., 2015). In cereal grains, starch is the major reserve in providing a C-source to generate energy and metabolites for coleoptile and radicle growth until the achievement of the photoautotrophic stage. Therefore, the regulation of α-amylases hydrolytic enzymes gene expression and activity to degrade starch in the endosperm is of primary importance. Among major cereals, only rice harbors the α-amylase subfamily of enzymes necessary to the endosperm starch mobilization to readily fermentable sugars under O_2 deprivation (Perata et al., 1992, 1993; Guglielminetti et al., 1995). In cereal grains, SnRK1 has a key role in source to sink communication at germination and seedling developmental stage, since it converges the crosstalk among sugar and nutrient demand, and hormone and abiotic stress signal to regulate nutrient mobilization (Yu et al., 2015).

Recently, the major quantitative trait locus *qAG-9-2* for rice anaerobic germination capability has been identified in the landrace Khao Hlan On (KHO; Kretzschmar et al., 2015). The genetic determinant for the low O_2 tolerance was recognized to be the *OsTPP7* gene, whose product converts T6P into Tre. In KHO rice, TPP7 activity likely modifies the T6P/Suc homeostasis to reduce the repressive action of T6P on SnRK1. In rice, the SnRK1 family members SnRK1A and SnRK1B are analogous to the mammalian and yeast counterparts. During anaerobic rice germination, SnRK1A acts through the sugar starvation−responsive MYB1 (MYBS1) complex to activate the expression of the starvation-induced α-amylase *Ramy3D* gene and the α-amylase activity for the endosperm hydrolysis of starch. The MYBS1 TF interacts with the key TA-box sugar responsive element located on the sugar responsive complex of the α-amylase promoter region (Lu et al., 2002, 2007). As consequence, rice varieties that do harbor the *OsTPP7* gene are suggested to be more effective in activating starch hydrolysis in a fast and continuous way, therefore increasing the ability to elongate the coleoptile and establish the plantlet under O_2 shortage. In this context, CIPK15 has been found to play a critical role in the SnRK1-dependent pathway under low O_2. In fact, a *cipk15* knockout rice mutant is impaired in anaerobic germination and has a reduced activity of α-amylases hydrolytic enzymes, probably because of a posttranscriptional regulation of SnRK1A by CIPK15 phosphorylation (Lee et al., 2009). CIPK15 was identified as the hub of both low O_2 and sugar starvation signals, where the former involves the presence of a Ca^{2+} signal likely perceived by the Ca^{2+}-sensing calcineurin B-like CBL4 (Ho et al., 2017) and relayed through CIPK15. Notably, *MYBS1* and *CIPK15* transcripts have been found to be upregulated in AG-1 rice plants expressing *OsTPP7* under the control of its native promoter in the IR64 variety, which lacks the native *OsTPP7* gene (Kretzschmar et al., 2015).

Only a few investigations have focused on the T6P-related signaling in adult rice plants under submergence. In deepwater rice, the striking internode elongation under water, finalized to reach the air surface to restore aerobic respiration, is positively regulated by the ERFs SNORKEL1 and SNORKEL2 (Hattori et al., 2009). A recent transcriptomic analysis of shoots from those plants revealed that the expression of genes related to Tre biosynthesis, *OsTPS12*−*OsTPS14* and *OsTPP1* and *OsTPP11* (corresponding to *OsTPP7*), is strongly upregulated under submergence in the deepwater cv C9285 in comparison with to the nondeepwater one Taichung 65. This suggests that T6P and Tre accumulate in deepwater rice and are possibly involved in the adaptive mechanism (Minami et al., 2018).

Similarly, a fine regulation of C metabolism is a key element in the rice quiescence strategy regulated by the ERF-VII *Submergence1A* (*Sub*1A) gene under submergence (Fukao et al., 2006). Sub1A possibly acts repressing α-amylases under low O_2 to preserve C for the recovery phase. A detailed transcriptome and metabolome comparison between the M202 cv and its near-isogenic *Sub*1A introgressed line M202(Sub1A) revealed that *Sub*1A presence steadily dampens *CIPK15* mRNA under submergence, consistent with the observed modest increase in α-amylases transcripts (Locke et al., 2018). However, *Sub*1A presence does not clearly affect the T6P content, despite the strong differences observed in the regulation of transcripts related to the T6P metabolism. The levels of T6P dramatically decline in rice shoots during submergence in both M202 and M202(Sub1A), suggesting a possible release of SnRK1 activation to signal sucrose limitation.

In rice seedlings under abiotic stresses, the SnRK1A-interactive negative regulators SKINs dampen SnRK1A

and MYBS1 migration to the nucleus where they activate enzyme expression for nutrient mobilization (Lin et al., 2014). Under submergence, the overexpression of *SKIN1* and *SKIN2* results in an arrest in shoot elongation that is recovered by a sugar supply. However, it is not yet known whether the T6P pathway is involved in this additional mechanism of SnRK1 regulation.

Under low O_2 the activation of Tre metabolism is suggested to be a common mechanism among different organisms. Indeed, *TPS* and *TPP* genes have been found to be expressed upon O_2 shortage in some bacteria, fungi, and plants other than rice, among which is *Arabidopsis* (Mustroph et al., 2010). However, in *Arabidopsis* little is known about the regulation of this pathway under O_2 shortage. In this plant, the presence of a molecular mechanism that senses the O_2 variations, and which relies on a direct sensor of O_2, is suggested to be at the basis of the plant acclimation. Studies on *A. thaliana* showed that ERF-VII TFs are stabilized under hypoxia and can drive the transcription of hypoxia-related genes, including those involved in fermentative metabolism which can provide a certain level of ATP to preserve the plant basal metabolism (Gibbs et al., 2011; Licausi et al., 2011). Recent results showed that limited carbohydrate sources under O_2 shortage dampen the induction of anaerobic genes. This likely occurs independent of the stabilization of ERF-VII but upstream of their interaction with the anaerobic genes promoters, suggesting a further mechanism of regulation (Loreti et al., 2018). However, when testing the role of the SnRK1.1 protein, the use of SnRK1.1-overexpressing plants and of a SnRK1.1 dominant negative mutant ruled out a possible involvement of a Suc sensing mechanism activated through this way (Baena-González et al., 2007).

Investigations on key components in the regulation of plants' source to sink communication via the T6P/Suc balance under low O_2 are only relatively recent. Indeed, a full understanding of the molecular mechanism(s) that regulate(s) sugar mobilization through that way may suggest key strategies for crop improvement and enhanced tolerance to flooding.

15.7.2 Cold

To date, in Europe rice is increasingly sown on dry soil since this water-saving practice also requires lower labor costs compared with the traditional sowing on flooded soil (Mazza et al., 2016). In these conditions, however, rice seeds and plantlets face some risks concerning, amongst others, cold stress, due to the low or drastically dropped temperatures during early sowing in the absence of the thermal buffer effect of water.

Exposure to extreme temperatures is one of the most common risks for plant growth and crop yield (Yadav, 2010). Concerning low temperatures, in the term "cold stress" are included two distinct stresses, chilling and freezing, encountered by plants species originating in temperate areas when exposed to temperatures below 20°C or 0°C, respectively (Miura and Furumoto, 2013).

Plants are generally most sensitive to low temperature at the seedling and reproductive stages. However, at different developmental phases cold induces evident phenotypic symptoms of suffering like poor germination, stunted seedling establishment, growth retardation, reduced leaf expansion, chlorosis and necrosis, and pollen sterility. In a relatively short time, chilling induces alterations of membrane fluidity and integrity, and a general functional decline of transport and intracellular compartmentalization with consequent negative effects on metabolic activities. Ultrastructural changes in a wide range of cell components are observed, followed by severe effects on reduction and impairment of photosynthesis, protein synthesis, and general metabolic processes. Freezing is first of all characterized by the onset of structural injuries at the cell and tissue levels due to the early formation of ice crystals in the apoplast (Ruelland and Zachowski, 2010; Takahashi et al., 2013; Theocharis et al., 2012; Yadav, 2010).

Following the perception of low temperatures, plants react by adjusting cellular metabolism to enhance tolerance mechanisms according to acclimation programs. In some cases, whenever freezing conditions are preceded by progressive chilling periods, the acclimation processes can lead the plant to the acquisition of freezing tolerance (Miura and Furumoto, 2013; Rihan et al., 2017).

In major detail, membrane rigidification/fluidification and/or cytoskeleton depolymerization could be responsible for sensing the altered temperature and trigger signaling responses that involve altered levels of intracellular free Ca^{2+} and activation of phospholipase D and consequent phospholipid degradation (Ruelland and Zachowski, 2010). Changes of enzyme activities have an impact on energy metabolism. In particular, concerning the photosynthetic machinery, cold reduces the release of Pi and determines the accumulation of phosphorylated intermediates with consequent low regeneration of ribulose bisphosphate and overall photosynthesis inhibition (Ruelland and Zachowski, 2010). The slowdown of metabolic reactions alters the balance between photochemistry (light energy input) and metabolic reactions and growth (energy use), and of the plastoquinone redox state,

inducing the production of ROS, whose accumulation at low temperatures is also favored by reduced activities of ROS-scavenging enzymes (Ruelland and Zachowski, 2010). Plant metabolism is thus redirected toward the synthesis of osmo- and cryoprotectant molecules like soluble sugars (Suc, raffinose, stachyose, Tre), sugar alcohols (sorbitol, inositol), and low-molecular weight N compounds (proline, glycine betaine). These compounds, together with dehydrins, cold-regulated (COR) proteins, and others, contribute to stabilization of membrane components and cytoplasmic proteins, maintenance of hydrophobic interactions and ion homeostasis, and ROS scavenging. Vacuolar fructans are released by a vesicle-mediated transport into the apoplast, where they stabilize membranes and indirectly contribute to osmotic adjustment upon freezing and dehydration by the release of hexose sugars. Therefore, both symplastic and apoplastic soluble sugars contribute to membrane stabilization (Krasensky and Jonak, 2012). Cold acclimation is also accompanied by increases in the levels of antioxidant compounds and in the activity of ROS-scavenging enzymes. The increase in the proportion of unsaturated fatty acids in phospholipids and the suppression of phospholipase D help to maintain membrane functionality and reduce phospholipid degradation (Janská et al., 2010).

All these events are under hormonal (particularly ABA) control, and are mediated by Ca^{2+} and some metabolites, including sugars, that act as signals (Janská et al., 2010; Yadav, 2010; Zhang et al., 2014). The different signal transduction pathways converge toward expression of target genes encoding CORs, in turn responsible for the accumulation of regulatory and functional proteins and metabolites leading to stress tolerance. Many intermediates, like the components of the complex systems of protein kinases (Ca^{2+}-dependent protein kinases, MAPKs cascades), as well as different TFs, like ICEs (inducer of C-repeat binding factor (CBF) expression), CBF/DREBs (dehydration-responsive-element-binding), MYC/MYBs, are involved (Janská et al., 2010; Miura and Furumoto, 2013; Rihan et al., 2017; Theocharis et al., 2012).

In *E. coli* a cold shock (rapid decrease from 37°C to 16°C) induces an increase in the levels of both *ostA* and *ostB* mRNAs as well as of Tre (up to eightfold) accompanied by enhanced cell viability at the lowest temperature, suggesting that Tre metabolism is involved in chilling tolerance in this organism (Kandror et al., 2002).

Also in plants increasing evidence reports on the effects of cold stress in enhancing Tre/T6P metabolism. A metabolomic study in *A. thaliana* aimed at identifying metabolite temporal dynamics associated with acclimation-induced freezing tolerance indicates that the Tre levels increase by eightfold 4 days after cold exposure (Kaplan et al., 2004). In both *japonica* and *indica* rice, *OsTPP1* and *OsTPP2* genes have been found to be differentially expressed under chilling other than drought and salt; the *OsTPP1* transcripts are induced very early (1−2 h) by chilling (Ge et al., 2008; Nakamura et al., 2011; Pramanik and Imai, 2005; Shima et al., 2007). The accumulation of *OsTPP1* transcripts is accompanied by increases in TPP activity and Tre contents; however, the increase in Tre is transient and too low to account for a function as stress protectant, suggesting again the possible role of Tre in the regulation of metabolism, with particular regard to the C one, under cold stress (Pramanik and Imai, 2005). Moreover, *OsTPP1* overexpression enhances cold tolerance and is accompanied by upregulation of *OsTPS1* and of a few cold stress-induced genes, like the *low-temperature-induced protein* genes *Lip5* and *Lip9*, and the TF *DREB1B* (Ge et al., 2008). *OsTPS1* is constitutively expressed and not upregulated upon abiotic stress. Nevertheless, its overexpression induces cold tolerance and Tre accumulation associated to upregulation of genes involved in Tre/T6P metabolism (*OsTPP1*, *OsTPP2*, and *OsTRE1*). Transformed lines overexpressing individually class II *OsTPS*s genes also show improved tolerance to cold (Li et al., 2011). As reported in Section 15.2, the role of class II TPSs is not yet completely clarified, even if it has been proposed that they modulate TPS1 activity through the formation of protein complexes (Zang et al., 2011).

In a chilling-tolerant rice CT6748-8-CA-17 cv the levels of Tre are constitutively higher than those reported in the sensitive one, INIAP12. Upon cold stress, Tre contents decrease in the tolerant cultivar that accumulates higher levels of sugars (Suc, raffinose, galactose) and shows strongly reduced levels of oxidative products (MDA and H_2O_2) compared with the sensitive one. In the tolerant cultivar, Tre may contribute to membrane stabilization and protection from stress-generated ROS. However, it cannot be excluded that the constitutive presence of higher Tre levels may act in advance as a priming factor in response to the stress (Morsy et al., 2007). A similar behavior was recently reported in *A. thaliana* (Nunes et al., 2013a).

In grapevine (*Vitis vinifera* L.), exposure to low temperature induces Tre accumulation; in parallel, the levels of expression of a *VvTPP* gene and of *VvTRE* are up- and downregulated, respectively, whereas the levels of *VvTPS1* transcripts remain essentially unchanged. In this system, the increase in T6P levels

upon cold exposure is parallel to the observed chilling-induced accumulation of Suc, reinforcing the hypothesis of a possible action of T6P as a signal molecule possibly involving SnRK1 also in response to cold (Fernandez et al., 2012). A comprehensive transcriptomic profiling with meta-analysis of data to discriminate the transcriptomic changes related to general or specific cold-responses was conducted concerning the combined effects of severe cold and circadian rhythm variations in maize seedlings (Jończyk et al., 2017). Alteration of circadian regulation of gene expression appears to be one of the main targets of severe cold and the transcriptome changes largely precede the physiological response. Of particular interest is the link between the massive repression of photosynthesis-related genes during the day and photoinhibition, and the participation of Tre and stachyose in low-temperature signaling as suggested by the transcript profiles of several genes related to stachyose and Tre biosynthesis/degradation, that appear to be dramatically and specifically altered by cold.

A few studies focused on the interplay between Tre metabolism and signaling pathways activated by cold. In particular, in rice a few components (ICEs, MYBS3, NAC5) of TF systems appear to be involved in a complex crosstalk with the Tre/T6P system related to the upregulation of *OsTPP1* and *OsTPP*2 (Nakamura et al., 2011; Song et al., 2011; Su et al., 2010). Moreover, an *OsmiRNA319* gene encoding a miRNA that responds to cold stress has been individuated. Its overexpression enhances cold tolerance through the downregulation of two plant-specific TFs (*OsPCF6* and *OsTCP21*, involved in the control of cell proliferation in growing tissues), and induces upregulation of a few cold stress-responding genes among which is *OsTPP1/2* (Wang et al., 2014). This evidence suggests the possible involvement of a different mechanism in the modulation of cold stress response in relation to the Tre/T6P/SnRK1 system (Confraria et al., 2013; Tomé et al., 2014).

Chilling stress induces a condition of limited growth due to a reduced sink capacity even in the presence of high sugar availability. In *Arabidopsis* seedlings fed with Suc or grown under sink-limited conditions induced by low temperature, the T6P levels always correlate closely with Suc and both compounds increase under low temperature (in vitro SnRK1 activity is poorly affected by temperature). As expected, the increase in T6P levels in the sink-limited condition upregulates a few marker genes (in particular *TPS5*) normally downregulated by SnRK1, and downregulates the SnRK1-upregulated marker genes. Interestingly, plants with reduced T6P contents for overexpression of *E. coli otsB*, or overexpressing *SnRK1α1*, when transferred from cold (characterized by high Suc levels) to warm (characterized by low Suc), recover growth less promptly than their isogenic lines, suggesting that T6P is necessary to allow growth restart when the stress origin is removed, independent of overall Suc availability. Under sink-limited conditions T6P seems not directly related to growth rate but changes in gene expression induced by T6P are related to the plant recovery of growth when stress is removed (Nunes et al., 2013a). Moreover, at low temperature the relationship between endogenous Suc levels and expression of *TPS1* is not linear, suggesting that factors other than Suc may regulate *TPS1* expression and T6P content under low temperature (Nunes et al., 2013c).

15.8 CONCLUDING REMARKS

Global climate change, which is to date exacerbating extreme events in frequency and intensity, has potentially dramatic consequences for crops, with a negative impact on food availability on a worldwide scale. Water shortage, temperature-related stress, flooding, and salinization of lands are all factors capable to jeopardize overall crop yield. In this context, studies on the plant's capability to tolerate such abiotic stresses in harsh environments and/or marginal lands, and the development of tolerant crop varieties will surely, in the long term, have positive consequences on the well-being of a large part of the world's population. The understanding of the basis mechanisms that regulate the energy metabolism in the context of the sink−source relationships both during plant development and in response to stress conditions is of paramount importance. The finely tuned Tre metabolic pathway is suggested to play a key role in the regulation, together with the SnRK1 system, of such plant processes. This network involves a plethora of actors at several cell/tissue/organ levels therefore appearing extremely complex during both normal and stress conditions.

Although further studies are necessary to elucidate all the steps (at the molecular, biochemical, and physiological levels) of this network, promising perspectives can be foreseen for the possibility to exploit this knowledge with the aim of obtaining crop species and cultivars with improved characteristics of stress tolerance and resistance in the field (Table 15.1).

TABLE 15.1 Trehalose Metabolism and Abiotic Stresses

Stress type	Plant material	Treatment/condition	Response/effect	References
TREATMENT WITH EXOGENOUS TREHALOSE				
None	*Arabidopsis thaliana* (L.) Heynh., Columbia ecotype (Col-0); seedling liquid culture	30 or 100 mM Tre supplemented in liquid culture	Induction of different stress response genes and proteins; repression of an *APR1* gene of thiol synthesis	Bae et al. (2005a,b), Schluepmann et al. (2004)
Drought	*A. thaliana*, Col-0 ecotype, seedlings and protoplasts	0.5, 1, 5 mM Tre supplemented to hydroponic medium	Enhanced SOD and peroxidase (POD) activity	Yang et al. (2014)
	Maize (*Zea mays* L., cvs Agaiti-2002 and EV-1098) leaves	Foliar spray (30 mM Tre)	Enhanced enzymatic and nonenzymatic antioxidant systems; decreased levels of stress-related compounds	Ali and Ashraf (2011)
	Wheat (*Triticum aestivum* L., cvs Gemmieza-7 and Sahel-1) leaves	Foliar spray (1.5 mM Tre)	Enhanced enzymatic and nonenzymatic antioxidant systems; decreased levels of stress related compounds	Aldesuquy and Ghanem (2015)
	Wheat (*T. aestivum*, cv Giza 168) leaves	Foliar spray (10 mM Tre)	Increase in nonenzymatic antioxidants and APX activity	Ibrahim and Abdellatif (2016)
	Wheat (*T. aestivum*, cv Zhoumai 18) callus	50 mM Tre supplemented to callus growing medium	Decreased levels of stress related compounds; increased levels of nonenzymatic antioxidants and SOD levels/activity; decreased POD, CAT levels/activity	Ma et al. (2013)
	Brassica spp. (*B. napus* L., cv BARI Sharisha 13; *B. campestris* L., cv BARI Sharisha 9; *B. juncea* L., cv BARI Sharisha 11) seedlings and leaves	5 mM Tre supplemented to seedling growing medium	Enhancement of enzymatic and nonenzymatic antioxidant systems; decreased levels of stress related compounds and enzymatic activities	Alam et al. (2014)
	Fenugreek (*Trigonella foenum graecum*, cv Giza 30) leaves and seeds	Foliar spray (500 μM Tre)	Enhanced growth, photosynthetic pigments, antioxidant compounds and total antioxidant activity	Sadak (2016)
	Radish (*Raphanus sativus* L., cvs Manu and 40-day) leaves	Seed presoaking (25 mM Tre)	Increased growth, chlorophyll *a* content, water-use efficiency, SOD activity	Akram et al. (2016)
	Radish (*R. sativus*, cvs Manu and 40-day) roots	Seed presoaking and foliar spray (25 and 50 mM Tre)	Enhancement of enzymatic and nonenzymatic antioxidant systems; decreased levels of stress related compounds	Shafiq et al. (2015)
Salt	*A. thaliana* Heynh., Col-0 ecotype, seedlings and protoplasts	0.5, 1, 5 mM Tre supplemented to hydroponic medium	Enhanced SOD and POD activity	Yang et al. (2014)
	Rice (*O. sativa*, cv KDML105) seedling leaves	10 mM Tre supplemented to hydroponic medium	Upregulation of genes encoding antioxidant enzymes, increased APX activity	Nounjan et al. (2012)
	Rice (*O. sativa*, cv BR 11) leaves	10 mM Tre supplemented to hydroponic medium	Increased nonenzymatic and enzymatic antioxidant systems; reduced ROS accumulation,	Mostofa et al. (2015a)
Acid rain	Barley (*Hordeum vulgare* L. cv Gairdner) roots and leaves	Seed soaking (5, 10, 15 mM Tre)	Enhanced enzymatic and nonenzymatic antioxidant systems; decreased levels of stress related compounds; increased H^+-ATPase activity	Ding et al. (2018)
Heavy metals	Rice (*O. sativa*, cv BRRI dhan29) leaves	10 mM Tre supplemented to hydroponic medium prior to exposure to Cu	Enhanced ascorbic acid content, redox status, activities of major antioxidant enzymes	Mostofa et al. (2015b)

(*Continued*)

TABLE 15.1 (Continued)

Stress type	Plant material	Treatment/condition	Response/effect	References
	Duckweed (*Lemna gibba* L.) fronds	0.5, 1, 2, 5 mM Tre supplemented to hydroponic medium together with Cd	Enhanced enzymatic and nonenzymatic antioxidant systems; decreased levels of stress-related compounds	Duman et al. (2011)
Heat	Wheat (*T. aestivum*) seedlings	1.5 mM Tre supplemented to hydroponic medium	Increased levels of enzymatic and nonenzymatic antioxidant systems and related genes; decreased levels of oxidative stress-related compounds and enzymes; increased membrane functionality; enhanced PSII activity; higher levels of D1 protein	Luo et al. (2010, 2018)
EFFECT OF STRESS CONDITIONS ON TREHALOSE METABOLISM				
Hypoxia	Rice (*O. sativa*, landrace Khao Hlan On) embryos and coleoptiles	Submersion in water for up to 21 days	Activated starch hydrolysis, modified T6P/Suc homeostasis	Kretzschmar et al. (2015)
	Rice (*O. sativa*, deepwater cv C9285; nondeepwater cv Taichung 65, ssp. *japonica*) shoots and leaves	Submersion in water for up to 24 h of seedlings at the leaf stages 4LS and 6LS	Increased expression of *OsTPS12-OsTPS14* and *OsTPP1* and *OsTPP11* in deepwater rice	Minami et al. (2018)
Cold	*A. thaliana*, Columbia ecotype, seedlings	Exposure of 2-week-old plants to 4°C up to 8 days	Increased Tre levels	Kaplan et al. (2004)
	A. thaliana, Col-0 ecotype, seedlings	Exposure of 7-day-old seedlings to 10°C up to 72 h	Increased T6P levels and enhanced *TPS5* expression	Nunes et al. (2013a)
	Rice (*O. sativa*, ssp. *japonica*, cv Yukihikari) roots and shoots	Exposure of 7-day-old seedlings to 12°C and 4°C up to 48 h	Early induction of *OsTPP1* transcription; enhanced TPP activity and increased Tre levels	Pramanik and Imai (2005)
	Rice (*O. sativa*, ssp. *japonica*, cv Yukihikari) roots and shoots	Exposure of 7-day-old seedlings to 12°C up to 48 h	Early induction of *OsTPP1* and *OsTPP2* transcription	Shima et al. (2007)
	Rice (*O. sativa*, cvs Nona Bokra (ssp. *indica*) and Nipponbare (ssp. *japonica*)) seedlings	Exposure of 2-week-old seedlings to 8−6°C up to 72 h or to 4°C up to 24 h	Early induction of *OsTPP1* transcription	Ge et al. (2008), Nakamura et al. (2011)
	Rice (*O. sativa*, cvs CT6748-8-CA-17, chilling-tolerant, and INIAP12, chilling-sensitive) seedlings	Exposure of S3 (prophyll emergence stage) plantlets to 13−10°C for 4 days	Constitutive presence of higher Tre levels and decreased levels of oxidative stress-related compounds in the chilling-tolerant cv	Morsy et al. (2007)
	Maize (*Z. mays*, spp. *indentata*, inbred line CM109) leaves	Exposure of V3 (fully developed third leaf) stage plantlets to 8−6°C up to 24 h	Increased levels of *TPS*, *TPP*, and *TRE* transcripts	Jończyk et al. (2017)
	Grape (*Vitis vinifera* L., cv Chardonnay clone 7535) roots, stems and leaves from in vitro cultured plants	Exposure of 6-week-old plants to 4°C up to 24 h	Enhanced Tre accumulation and expression of *VvTPPA* gene; reduced expression of *VvTRE*	Fernandez et al. (2012)
BIOTECHNOLOGICAL INTERVENTIONS ON TREHALOSE METABOLISM IN RELATION TO THE RESPONSE TO STRESS CONDITIONS				
Hypoxia	*OsTPP7*-overexpressing rice (*O. sativa*, cv IR64) seedlings	Submersion in water for up to 21 days	Upregulation of hallmark genes (*MYBS1* and *CIPK15*) for anaerobic germination in plants expressing *OsTPP7*	Kretzschmar et al. (2015)
Cold	*OsTPP1*-overexpressing rice (*O. sativa*, cv Nipponbare) seedlings, shoots	Exposure of 2-week-old seedlings to 8−6°C for 18 days	Enhanced cold tolerance; upregulation of *OsTPS1* and of cold stress-induced genes (*Lip5*, *Lip9*, *DREB1B* TF)	Ge et al. (2008)

(*Continued*)

TABLE 15.1 (Continued)

Stress type	Plant material	Treatment/condition	Response/effect	References
	Class I *OsTPS1*- and class II *OsTPSs*-overexpressing rice (*O. sativa*, cvs Nipponbare and ZH11) seedlings, shoots	Exposure of 2-week-old seedlings to 4°C for 5 days	Increased Tre levels, upregulation of *OsTPP1*, *OsTPP2*, *OsTRE1*; enhanced cold tolerance	Li et al. (2011)
	OsNAC5-overexpressing rice (*O. sativa*, ssp. *japonica*, cv Zhonghua 10) seedlings	Exposure of 4-week-old seedlings to 4°C for 6 days	Enhanced expression of *OsTPP1* and *OsTPP2*	Song et al. (2011)
	MYBS3-overexpressing rice (*O. sativa*, cv Tainung 67) seedlings	Exposure of 10-day-old seedlings to 4°C for 24 h	Enhanced expression of *OsTPP1* and *OsTPP2*	Su et al. (2010)
	Osa-miR319b-overexpressing rice (*O. sativa*, cv Kong Yu 131) seedlings	Exposure of V3 (fully developed third leaf) stage seedlings to 4°C for 7 days	Enhanced expression of *OsTPP1* and *OsTPP2*	Wang et al. (2014)
Drought	*ATAF1*-overexpressing *A. thaliana* (Col-0 ecotype) seedlings	Exposure of 17-day-old seedlings to irrigation interruption up to symptom appearance	Increased drought tolerance; enhanced *TRE1* expression and TRE activity; reduced T6P levels and sugar starvation metabolome.	Garapati et al. (2015), Wu et al. (2009)
Heavy metals	*AtTPS1*-overexpressing tobacco (*Nicotiana tabacum* L., cv Petite Havana SR1) plants, leaves	Exposure to Cd/excess Cu	Decreased levels of oxidative stress-related compounds; increased activities of detoxifying enzymes	Martins et al. (2014)

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CHAPTER

16

Sugar Regulates Plant Growth and Development Under In Vitro Conditions

Durdana Shah[1], *Nasreena Sajjad*[2], *Rohaya Ali*[2], *Nazish Nazir*[1], *Sumaya Hassan*[2] *and Snober Shah*[3]

[1]Plant Tissue Culture Laboratory, Centre of Research for Development, University of Kashmir, Srinagar, Jammu and Kashmir, India [2]Department of Biochemistry, University of Kashmir, Srinagar, Jammu and Kashmir, India [3]Cytogenitics and Molecular Biology Laboratory, Centre of Research for Development, University of Kashmir, Srinagar, Jammu and Kashmir, India

OUTLINE

16.1 INTRODUCTION

Plants as photoautotrophic organisms are able to produce the carbohydrates they require and have developed mechanisms to coordinate carbohydrate production and its metabolism (Rook et al., 2003) Carbohydrate-derived signals regulate the expression of genes involved in both photosynthesis and metabolism, and control carbohydrate partitioning. Genetic screens for sugar-response mutants have shown the close interaction between sugar and hormone-signaling pathways (Rook et al., 2003). Sugars produced from plant photosynthesis play a central role to support and integrate the functions and actions of

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internal and external regulatory signals in driving diverse biological processes from embryogenesis to senescence. Although the knowledge on how plants produce, transport, metabolize, store, and sense diverse sugar signals has been significantly advanced, the spectrum of sugar signals, sensors, and molecular mechanisms mediating primary signaling remain to be fully explored (Granot et al., 2014). Many informative review articles presented recent progress on broad aspects of sugar-related research in plant biology, encompassing source–sink communication; sugar–hormone interactions; new sugar transporters and their functions; sugar regulation of plant development; chloroplast–nuclear signaling; sucrose, starch, and trehalose metabolism and signaling; clock–sugar connections; as well as sugar and stress. The accumulated knowledge will provide an excellent and comprehensive platform for future research, especially on elucidating the molecular, cellular, and biochemical basis of sugar sensing and signaling underlying the plasticity and potential in plant growth and development. Emphasis in this review is placed on the emerging understanding of the dynamic, primary, and integrated sugar signaling mechanisms and transcriptional networks triggered by direct and indirect sugar signals via sugar, energy, and metabolite sensors.

Sugars play a very essential role in growth and developmental processes. An adequate supply of carbohydrates is needed both to energize metabolism and provide the "building blocks" to make cellular structures and cell walls. Plant hormones may integrate metabolism with developmental programs by directing the carbon and determining its use. The pivotal role of sugars as signaling molecules is well illustrated by the variety of sugar sensing and signaling mechanisms discovered in free-living microorganisms such as bacteria and yeast (Ramon et al., 2008). For such unicellular organisms, nutrient availability is the main extracellular factor controlling growth and metabolism. The role of nutrients as regulatory molecules has come to be appreciated only recently in mammals despite extensive previous research on glucose homeostasis and diabetes. In plants, sugar production through photosynthesis is a vital process, and sugar status modulates and coordinates internal regulators and environmental cues that govern growth and development. Although the regulatory effect of sugars on photosynthetic activity and plant metabolism has long been recognized, the concept of sugars as central signaling molecules is relatively novel. Recent progress has begun to reveal the molecular mechanisms underlying sugar sensing and signaling in plants, including the demonstration of hexokinase (HXK) as a glucose sensor that modulates gene expression and multiple plant hormone-signaling pathways (Rolland et al., 2002a,b,c). Analyses of HXK mutants will provide new evidence for distinct signaling and metabolic activities. Diverse roles of Snf1-related protein kinases (SnRKs) in carbon metabolism and sugar signaling also are emerging. In addition, Suc, trehalose, and other HXK-independent sugar sensing and signaling pathways add more complexity in plants (Rolland et al., 2006). Biochemical, molecular, and genetic experiments have supported a central role of sugars in the control of plant metabolism, growth, and development and have revealed interactions that integrate light, stress, and hormone signaling and coordinate carbon and nitrogen metabolism (Ramon et al., 2008). A number of reviews have appeared in the past few years emphasizing different aspects of sugar signaling and its interactions with other plant signal transduction pathways (Cho et al., 2010; Ehlert et al., 2006; Rolland et al., 2002a,b,c; Gazzarrini and McCourt, 2001).

16.2 ROLE OF SUGARS IN PLANT GROWTH AND DEVELOPMENT UNDER IN VITRO CONDITIONS

In plants, growth usually is a complex molecular network and irreversible change in size involving cell division and cell elongation. These networks must continuously adapt to an ever-changing environment (Gonzalez et al., 2012; Powell and Lenhard, 2012). Plants have played a crucial role in the evolution of life on earth through the making of energy-rich sugar molecules and oxygen by photosynthetic carbon fixation. Sugars are the most important carbon and energy source to cells, and also have important regulatory functions in controlling metabolism, growth, and development of plants. Sugars operate both as immediate substrates for intermediary metabolism and as effective signaling molecules. Plant sugar regulation is mediated by sugar signals, which are generated at different locations depending on environmental conditions and developmental stage. Sucrose (Suc) transport and hydrolysis play key regulatory roles in sugar signal generation (Ruan, 2014) In plants, sensing and signaling pathways have been described for different sugars (Hanson and Smeekens, 2009), but only for glucose is detailed information on sensing and signaling mechanisms available (Ramon et al., 2008). Over the past decade, various cellular, chemical, genetic, proteomic and genomic approaches in the reference plant Arabidopsis thaliana have begun to unravel the surprisingly broad range of functions and actions of three glucose-modulated master regulators, HXK1,KIN10/11, and TOR, which control the expression of thousands of plant genes involved in a wide spectrum of cellular functions (Dobrenel et al., 2016).

In the recent years important progress has also been made in identifying the dominant plant growth controlling regulatory systems that receive input from sugars and sugar derived metabolic signals. These systems are either growth promoting or growth inhibiting. Systems that have a promoting role on growth are the hexokinase glucose sensor, the trehalose-6-phosphate (T6P) signal, and the Target of rapamycin (TOR) kinase system in response to high sugar levels. Growth and floral transition halted in the absence of either T6P or TOR kinase. Systems that have an inhibitory effect on growth are the plant SNF1-related protein kinase1 (SnRK1), homolog of the animal AMP-activated protein kinase (AMPK) and yeast sucrose nonfermenting 1 (SNF1) kinase, and C/S1 bZIP transcription factor network in response to low sugar level. Therefore, all phases of plant life cycle from seed germination to hypocotyl elongation, cotyledon expansion, adventitious root formation, true leaf formation, flowering, and senescence are dependent on sugar (Rolland et al., 2006). The environmental conditions affect the availability of photosynthetic carbon in the form of sucrose, which in turn controls plant growth and development. The most common sugar translocated in plants is sucrose and out of all translocated materials in plants sucrose represents over 95% of the dry weight (Zimmermann and Ziegler, 1975). In plant cell, tissue, and organ culture the most common carbon source used is sucrose. In tissue culture medium sucrose is the most widely used carbohydrate in most of the plants, like *Curcuma*, the *Phaseolus*, and *Hyoscyamus* genus. MS media (Murashige and Skoog, 1962) is the most commonly used media in plant tissue culture, which constitutes about 3% of sucrose. However, the photosynthetic efficiency of cultured plants in the presence of high sucrose concentration in the media is restricted by reducing the levels of chlorophyll, key enzymes for photosynthesis, and epicuticular waxes promoting the formation of structurally and physiologically abnormal stomata. The light conditions, carbon in the media, and their interaction have an important effect on growth and development of plants. For sustaining photomixotrophic metabolism, ensuring optimal development in the plant tissue culture sucrose acts as a fuel source, it has more recently been emphasized that sucrose has other significant roles such as carbon precursor or signaling metabolite (Müller, 2011). Sucrose is metabolized in plant cell and tissue culture for every metabolic process that the cell will conduct to provide energy and carbon skeletons. The maintenance of osmotic potential and the conservation of water in cells are also supported by sucrose. Maltose is one of the other carbon sources used in culture media, in cereal anther culture, and it has resulted in significant enhancement of callus and plant regeneration frequencies, including wheat (Orsinky et al., 1990), barley (Karsai et al., 1994), and rye (Deimling et al., 1992).

Every aspect of plant growth and development including seed germination, cell proliferation and death, cell expansion and elongation, primary root length, seedling growth and development, root gravitropism, lateral roots, carbon and nitrogen metabolism and stress responses, root hairs, shoot meristem maintenance, reproduction, senescence, photosynthetic gene expression, crop yield and product quality are influenced by glucose, which acts as signaling molecule (Eveland and Jackson, 2012).

Trehalose-6-phosphate (T6P), which is a significant signaling metabolite, is the precursor of trehalose in the biosynthetic pathway and is tangled in the regulation of plant growth and development in response to carbon availability. The essential aspects of carbon consumption and metabolism in cell and tissue cultures have yet to be fully understood even though carbohydrates are of chief importance for cell growth, maintenance, and differentiation under in vitro conditions. In cell suspension cultures, where sucrose is promptly broken down for energy and carbon skeleton, most sucrose metabolism research has been performed by using tissue from plants growing in environmental conditions where the synthesized sucrose is for translocation and storage. For the enzymatic cleavage of sucrose there are two pathways. First, in what is called the alternative pathway, sucrose synthase conserves the energy of the glycosidic bond by cleaving sucrose into fructose and UDP glucose. UDP and pyrophosphate are components on which this pathway is dependent (Xu et al., 1998) and in 1986 it was first proposed to operate in plants. In this pathway the reaction is catalyzed by UDP glucose pyrophosphorylase, and generated UDP glucose feeds glucose 1-phosphate directly into glycolysis. Second, in what is called the classic pathway, with the loss of the glycosidic energy bond the invertase reaction produces glucose and fructose. Prior to subsequent metabolization these products must be phosphorylated by glucokinase and fructokinase. One alternative that is described as an adaptative pathway is catalyzed by a readily reversible pyrophosphate-dependent phosphofructokinase (PFP) and through the utilization and synthesis of pyrophosphate an equilibrium reaction conserves energy. The second, which is described as maintenance reaction, is catalyzed by the irreversible nucleotide triphosphate-dependent phosphofructokinase (PFK). So through UDP glucose pyrophosphorylase PFP when working in the gluconeogenic direction could produce PPi to drive sucrose breakdown (Xu et al., 1998).

16.3 SUGAR SIGNALING: PHYSIOLOGICAL, MOLECULAR, AND GENETIC APPROACHES IN PLANTS

Sugars play a central role in metabolism but they can also act as signaling molecules. During various stresses, that is, biotic or abiotic, the sugar concentration changes dramatically (Smeekens and Hellmann, 2014). The main target of sugar signaling is carbon metabolism and regulation of photosynthesis. As we now know sucrose is the main photosynthetic product, therefore, there are many sugar signaling effects on growth and metabolism. Sugars act as regulatory signals that control the expression of various genes involved in many processes. There are various sugar signaling pathways that regulate gene expression. Discovery of unique and global repression of photosynthetic genes by glucose in *Arabidopsis* led to identification of HXK1 as the first plant glucose sensor, which mediates glucose repression or promotion of gene transcription and plant growth. Following various signal transduction pathways which occur in plants, for example, HXK1-dependent pathways,that is related with the HXK1-mediated signaling function and the major effect exerted by this pathway is repression of photosynthetic gene expression signal transduction pathways occurring in plants, for example, HXK1-dependent pathways, gene expression, which is related with the HXK1-mediated signaling function and the major effect exerted by this pathway is repression of photosynthetic gene expression (Xiao et al., 2000). Another pathway is glycolysis-dependent, that is, glucose induction of PR1 and PR5 gene expression. Finally, there are HXK1-independent signaling pathways (Fig. 16.1). The presence of sugar is sensed by sugar receptors, which then results in downstream signaling events (Jang and Jen Sheen, 1997). The group of bZIP transcription factors that link sugar availability to growth. bZIP transcription factors are transnationally regulated in response to sugar signals. Various numbers of physiological, biochemical, and molecular approaches have been used to study sugar responses in plants (Kang et al., 2010). *Arabidopsis* is the genetic model system of choice for the isolation of sugar-response mutants. The sugar status of plant cells is sensed by sensor proteins (Rook et al., 2003). The signaling pathways involve mitogen-activated protein kinases, protein phosphatases, Ca^{2+} and calmodulins, resulting in appropriate gene expression. Depending on the status of soluble sugars a number of genes are either induced or repressed. Further various stresses like abiotic stresses to plants result in alterations in sugar status and therefore affect the expression of various genes by down- and upregulating their expression.

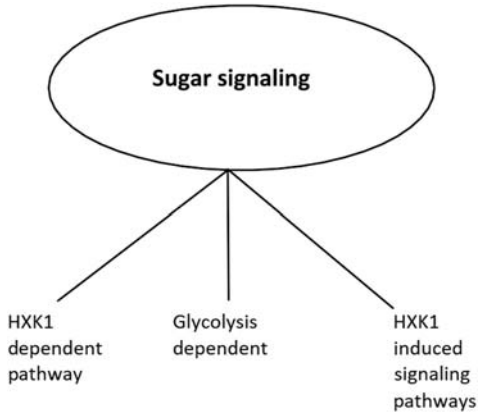

FIGURE 16.1 Various sugar signaling pathways that regulate gene expression in plants. HXK1-dependent pathways gene expression, which is related with the HXK1-mediated signaling function. HXK1-independent signaling pathways and glycolysis-dependent, that is, glucose induction of PR1 and PR5 gene expression.

16.4 PHYSIOLOGICAL APPROACHES OF SUGAR SIGNALING IN PLANTS

For all living organisms on this planet survival is dependent on photosynthesis, that is, fixation of carbon and light energy from the sun. As the sugar molecule is the prime carbon and energy source, sugars have acquired important regulatory functions in different organisms early on in evolution. Plants are photosynthetic, sugar-producing, and sessile, where maintaining homeostasis requires a complex and flexible regulatory machinery. Hence, sugar signaling plays an important role in controlling metabolism, stress resistance, growth, and development (Rolland et al., 2006). The regulation is controlled at numerous levels in plants, for example, by allosteric regulation of metabolic enzymes, and tissue-specific or temporal-specific expression of genes (Ehlert et al., 2006) It has been reported previously that expression of genes involved in starch and fructan metabolism, among other pathways, is regulated by sugars (Sun et al., 2003). Generally, in higher plants, high sugar levels stimulate expression of genes involved in sink function, such as growth, storage of proteins, and biosynthesis of starch and other carbohydrates, whereas low sugar levels promote photosynthesis and mobilization of energy reserves, such as breakdown of storage starch or lipids. Sugar signaling can be dissected into three steps, sugar sensing, signal transduction, and target gene expression. Further complicating matters is the dual function of sugars as nutrients and signaling molecules and also the interaction between sugar signaling and hormonal networks. Hexoses, sucrose, and

trehalose might serve as elicitors of plant sugar signaling (Smeekens and Rook, 1998; Müller et al., 2001).

Sugars are essential to the fundamental processes required for plant growth. Therefore, carbohydrate production, metabolism, and use must be carefully coordinated with photosynthate availability, environmental cues, and timing of key developmental programs. Sugars in general can act as signaling molecules and as global regulators of gene expression, for example, acting like hormones and translating nutrient status to regulation of growth and the floral transition. Therefore, sugar-responsive gene regulation reflects carbohydrate abundance or depletion. The translation of nutrient status to transcriptional regulation allows the plant to modulate growth, both at the whole plant level and locally, in tissue- or cell-specific patterns, potentially to coordinate developmental programs with available carbohydrate. In response to sugar depletion, genes involved in photosynthesis, carbohydrate remobilization and export, and nitrogen (N) metabolism tend to be upregulated. Alternatively, sugar abundance induces typical sink organ activities such as carbohydrate import, utilization, and storage, and starch and anthocyanin biosynthesis. Specific growth and metabolic responses tend to be activated and modulated based on the nature of the sugar signal. For example, sucrose, the primary transport sugar in plants, can be sensed as a signal directly (Chiou and Bush, 1998) or, alternatively, a signal can arise via its hexose cleavage products, glucose or UDP-glc and fructose.

Sugar signaling has been involved in carbon and nitrogen assimilation and transport. Regarding carbon metabolism, one case that has been long studied is the induction of fructan (polymers of Fru) synthesis in grasses. Although in nature fructan metabolism is mainly induced during periods of low temperature, the effect of cold is not direct but through its role in increasing cell Suc concentration due to lower carbon utilization. It has been shown that, at warm temperature, light induces fructan accumulation in detached leaves of different grass species, and that Suc mimics the light effect. Although glucose supply (and other sugars as well) can also induce fructan synthesis, the efficiency of these sugars is much lower than that of Suc. This fact, together with results from the application of various sugar analogs, led to the conclusion that in nature, Suc is most likely the molecule that initiates the signaling cascade leading to the induction of fructan synthesizing enzymes (Wiese et al., 1999). Sucrose also appears to act as a signaling molecule that initiates/activates starch synthesis. Sucrose regulates nitrogen and respiratory metabolism after feeding tobacco with different sugars. Nitrogen transport also appears to be regulated by Suc. A case in which the ammonium transporter gene, CitAMT1, is specifically induced by Suc has been reported for citrus plants. Besides, it has been shown that the *A. thaliana* nitrate and ammonium transporter genes are induced after the addition of Suc; however, it is uncertain whether Suc is acting as a signal molecule in this response since hexoses are effective as well. A particularly important protein in carbon and nitrogen metabolisms is PII, which coordinates the regulation of nitrogen assimilation in response to nitrogen, carbon, and energy availability. The expression of the gene (GLB1) that encodes PII protein is induced by light and Suc in dark-adapted *A. thaliana* plants. This effect is not triggered by mannitol or nonmetabolizable carbon source. Regarding other mineral nutrients, the expression of genes encoding for ion transporters for phosphate, sulfate, and potassium may be upregulated by Suc. Suc appears to modify the expression of a number of genes related to P starvation, which leads to an altered root physiology. Sucrose also plays an important role in control of copper homeostasis through sugar-responsive miRNAs in *A. thaliana* (Smeekens, 2000).

16.5 MOLECULAR AND GENETIC APPROACHES OF SUGAR REGULATION IN PLANTS

Sugar signaling transduction pathways are categorized in three distinct types in plants, based on the role of HXK1. In the first HXK1-dependent pathway, gene expression is correlated with the HXK1-mediated signaling function (Loreti et al., 2008).A major effect of this pathway is the repression of photosynthetic gene expression. Target genes in this pathway can now be defined genetically by the gin2 mutants and catalytically inactive alleles. A second pathway is glycolysis-dependent and can also be sustained by heterologous yeast Hxk2 activity. An example is the glucose induction of PR1 and PR5 gene expression. Finally, there is evidence for HXK1-independent signaling pathways. Glucose induction of CHS, PAL1 and genes encoding AGPase as well as glucose repression of ASN1 are observed independent of sense and antisense overexpression of *Arabidopsis* HXK1 or overexpression of yeast Hxk2 (Cho et al., 2010). Transcriptional responses to nonphosphorylated glucose analogs have been observed in *Chenopodium* cell suspension cultures, *Chlorella*, and transgenic *Arabidopsis*, although one should be cautious about possible nonspecific effects of such chemicals. The glucose analog 3-OMG, not perceived as a sugar signal, is still phosphorylated by HXK with low catalytic efficiency, and the product, 3-OMG-6-phosphate, accumulates in these cells; see Fig. 16.2. Conversely, none of the 200 glucose-responsive *Arabidopsis* genes

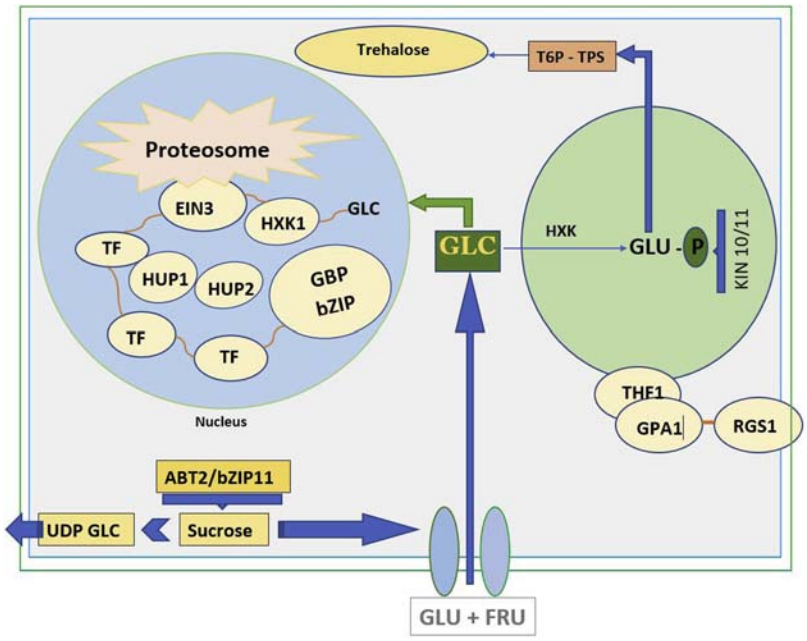

FIGURE 16.2 Glc and Fru are transported or mobilized from cytosolic and vacuolar Suc and plastid starch. Glc then enters metabolism after HXK-catalyzed phosphorylation. The HXK sugar sensor, as a cytosolic protein or associated with mitochondria or other organelles, then could activate a signaling cascade through HXK-interacting proteins (HIPs) or affect transcription directly after nuclear translocation.

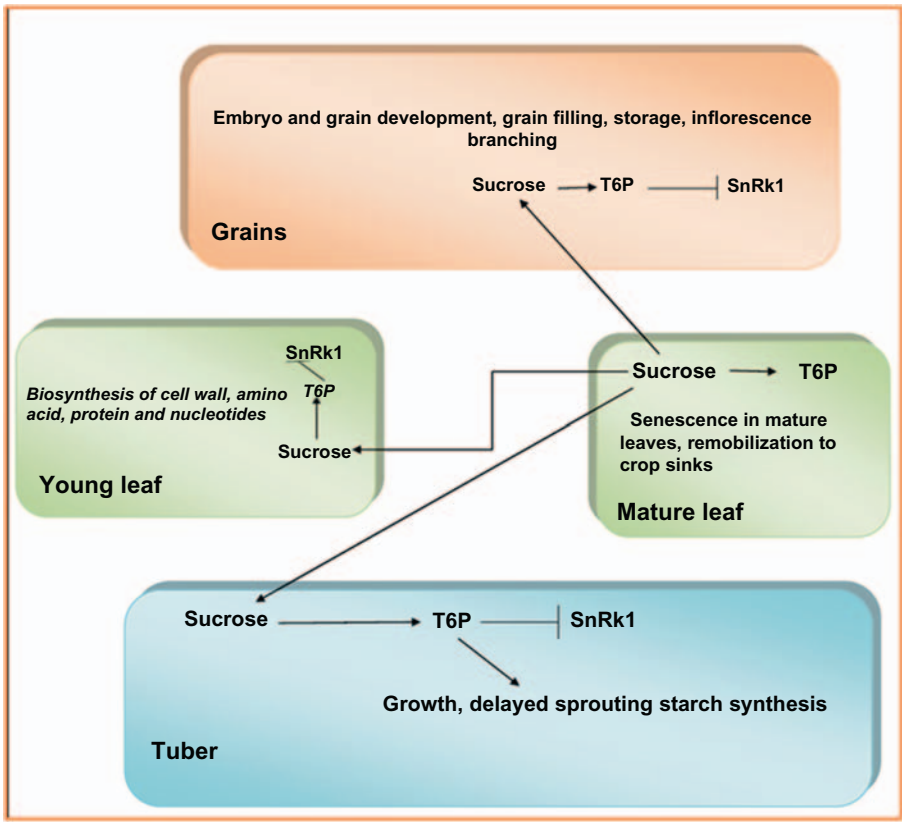

FIGURE 16.3 Sucrose metabolism in various tissues of plants.

identified in a recent study responds to 3-OMG or 6DOG (Dekkers et al., 2004).

There are a number of selected genes, for example, a gene that encodes a sugar beet proton sucrose symporter, whose expression is regulated by sucrose (see Fig. 16.3) but not glucose or fructose; this observation points to an HXK-independent, sucrose-specific signaling pathway. Interestingly, nonmetabolizable

sucrose analogs such as palatinose and turanose can also affect carbohydrate metabolism and gene expression. This observation suggests the existence of a disaccharide sensing system at the plasma membrane. However, such analogs again have no physiological relevance and can elicit distinct responses consistent with their perception as stress-related stimuli.

Over the years, a large number of plant genes have been found to be transcriptionally regulated by sugars, consistent with the coordinated regulation of source and sink activities. Importantly, several genes that encode metabolic proteins involved in sugar signal generation undergo transcriptional feedback regulation by their own products. Repression of photosynthesis gene promoters, for example, has been studied in mesophyll protoplasts and transgenic seedlings. As well as photosynthesis genes, the INV and SUS genes are also extensively regulated by sugar availability. Also, when sugar levels are high, carbohydrate storage through starch synthesis is upregulated by the induction of genes that encode AGPase (Riou-Khamlichi et al., 2000).

Many sugar-regulated genes and promoters have been used to screen for *Arabidopsis* mutants with potential defects in transcription control. A screen using the regulatory sequences of the sugar-inducible AGPase large subunit (APL3) gene fused to a negative selection marker has identified several impaired sucrose induction (isi) mutants. Another screen based on the activity of a luciferase (LUC) reporter gene under the control of the APL3 promoter yielded high sugar-response (hsr) mutants that exhibited elevated LUC activity and APL3 expression in response to low sugar concentrations. The screen using sugar-regulated expression of an *Arabidopsis* β-amylase generated low beta amylase (lba) and high beta amylase (hba) mutants with altered sugar regulation of a subset of genes. *Arabidopsis* reduced sugar response (rsr) mutants were selected using the sucrose activated promoter of patatin, a potato tuber storage protein. Molecular analysis of the mutants will bring new information on the mechanisms underlying sugar-mediated transcription control. The new microarray technologies now enable genome-wide expression analyses of *Arabidopsis* sugar and starvation responses. *Arabidopsis* phosphorus-deficient3 (pho3) mutant plants are used specifically to study the genomic response to sugar accumulation. This mutant is affected in the sucrose transporter2 (SUC2), and therefore accumulates soluble sugars, starch, and anthocyanins. High expression levels of genes that encode sucrose phosphate synthases (SPS), the plastid glucose G6P/phosphate translocator (characteristically expressed only in heterotrophic tissues), and the AGPase large subunits are consistent with the starch accumulation in the mutant. Also consistent with the phenotype, there is a large increase in the expression of TFs and enzymes involved in anthocyanin biosynthesis. Apparently, secondary metabolism is also an important target for transcription regulation by sugars. Using a more comprehensive approach, the short term effects of glucose and nitrogen in global gene expression in the dark have been studied in liquid-grown *Arabidopsis* seedlings. The use of the protein synthesis inhibitor cycloheximide shows that glucose repression is a more direct process than glucose induction, which often requires de novo protein synthesis (Moore et al., 2003). TFs with sugar-regulated expression profiles are likely regulators of the broad transcriptional response to sugars. Several global gene expression studies have been published on sugar starvation responses. Using cDNA microarrays and seedlings grown in the presence or absence of sucrose, a small number of (mostly carbohydrate and amino acid metabolism) genes were shown to be upregulated in concert during sugar depletion. A more detailed analysis of nutrient mobilization in response to sucrose starvation in *Arabidopsis* cells cultured in suspension has been carried out using the ATH1 gene chip. Consistent with extensive nutrient recycling for cell survival, genes that were upregulated are involved in carbohydrate, amino acid, protein and lipid catabolism, and autophagy. Although these cultures were nonphotosynthetic, several photosynthesis associated genes were also upregulated upon starvation. Genes that were downregulated are involved in metabolism (biosynthesis), protein synthesis, and cell division. Similar expression profiles were observed in the responses of *Arabidopsis* rosettes to an extended night period and a starchless pgm mutant at the end of the night. These studies also introduce the use of MAPMAN, a practical and informative tool to display complex genomic data in diagrams of metabolic and regulatory pathways. Interestingly, the molecular events in dark-induced senescence of *Arabidopsis* leaves (analyzed using a combination of cDNA microarray and biochemical analyses) exhibited extensive similarities with the sugar starvation response. Many TF genes were identified as putative regulators. However, a comparative microarray study reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence. Extended dark treatment causes a starvation condition that overrides the transcriptional regulation by circadian rhythm. However, in addition to energizing sugar production and (re)setting the clock, light can also directly affect gene expression through light-specific mechanisms. In a recent study, the effects of both light and sugar were examined. The results reveal that the majority of affected genes are coregulated by both stimuli. More extensive time-course gene expression analyses using wild-type and the pgm mutant plants under a 12 h photoperiod

provide a clear picture of the essential roles of sugar signals for a large set of circadian regulated genes (Price et al., 2004).

The large genomic datasets generated in microarray experiments provide an excellent opportunity to identify conserved DNA elements in the promoters of coregulated genes. Currently, most information on regulatory *cis*-elements involved in sugar signaling comes from a few selected genes, encoding sweet potato tuber and cereal seed proteins, and proteins involved in maize photosynthesis (Ohto et al., 2001). Studies on sugar activation of sweet potato tuber class I patatin, SUS, sporamin, and β-amylase promoters identified several sucrose-responsive *cis*-elements, including the sucrose-responsive element (SURE), A and B-boxes, the TGGACGG element, an SP8 motif, and an SP8-binding protein, SPF1. SPF1 is a WRKY-type sucrose-repressed negative regulator with putative orthologs in other species, including *Arabidopsis*. These factors typically bind to (T) TGAC (C/T) Wboxes, also found in defense-related gene promoters. A sugar-induced WRKY-type TF, SUSIBA2, that is, expressed in barley endosperm binds to the SURE and W-box, but not the SP8a element, to activate the barley isoamylase1 (iso1) promoter. In addition, a novel DNA-binding protein, designated STOREKEEPER (STK), specifically recognizes the B-box motif to control sucrose induced patatin expression in potato tubers. A more recent dissection analysis involved the sugar/ABA-induced sweet potato sporamin. A promoter in transgenic tobacco has yielded a minimal promoter (Spomin) that contains negatively acting regions and two carbohydrate metabolite signal responsive elements (CMSRE), CMSRE-1 (TGGACGG) and CMSRE-2, in addition to the SP8a motif. The most recent and fruitful studies of transcription control have been obtained by analyzing the sugar-inducible promoter of a sporamin gene that encodes the most abundant protein in sweet potato storage roots. Two putative TFs, WRI1 (activator of Spomin::LUC1; ASML1) and a novel CCT domain protein (ASML2), were isolated recently by enhancer activation-tagging of an *Arabidopsis* line carrying the LUC reporter under control of a short, minimal sugar/ABA inducible sporamin promoter. Several sugar-regulated genes, including βAMY and, in the case of ASML2, APL3, are activated in the transgenic lines. Both TF genes are also specifically induced by high sugar concentrations. Apparently, the WRI1 TF plays an important role in directing the carbon flow toward storage when sugar levels are high. The *his2* mutant displays high Spomin: LUC1 reporter activity even in noninducing conditions and is deficient in a novel B3 domain transcriptional repressor (Gomez et al., 2006).

Sugars also modulate hormone signaling at the transcriptional level (Riou-Khamlichi et al., 2000). Most obviously, glucose induces ABA and ABI gene expression as a core mechanism of its signal transduction. A detailed analysis of three factors involved in sugar signaling, ABI4, ABI5, and CTR1, documents their specific and developmental stage. Glucose repression of several ethylene biosynthesis and signal transduction genes suggests that interactions between sugar and ethylene signaling take place in part at the transcriptional level. Studies with several maize photosynthetic gene promoters suggest the involvement of different regulatory elements in sugar repression and negative control of positive *cis*-elements. Extensive studies of sugar repression and starvation induction of transcription have also been carried out on the promoters of rice genes that encode α-amylases (αAMY), involved in seed starch degradation. In a study with a minimal αAMY3 promoter, a sugar-response sequence (SRS) was identified with three essential elements for high sugar starvation-induced expression: the GC-box, the G-box, and the TATCCA element. Interestingly, three novel MYB proteins with a single DNA-binding domain (OsMYBS1-3) specifically bind to the TATCCA element to regulate αAMY expression. The identification of G-box *cis*-elements provides a link between nutrient stress and other environmental stress responses. The G-box motif (CACGTG) is, for example, involved in phytochrome-mediated light control of gene expression and is very similar to ABRE (CCACGTGG). The ABRE-binding factors ABF2, ABF3, and ABF4 have also been implicated in sugar signaling. Analysis of a conserved minimal light-responsive module (CMA5) recently revealed an ABI4 dependent sugar and ABA repression mechanism involving a novel element conserved in several RBCS promoters. This S-box element (CACCTCCA) is an ABI4-binding site and is typically closely associated with the G-box in light-regulated promoters. Novel bioinformatics and experimental approaches will be required to use fully the large number of publicly available microarray data to uncover new regulatory elements and TF functions in sugar regulation (Paul and Foyer, 2001; Schluepmann et al., 2003).

16.6 IMPORTANCE OF SUGAR INTERACTION WITH PHYTOHORMONES IN REGULATION OF GROWTH AND DEVELOPMENT UNDER IN VITRO CONDITION

Plant growth regulators include naturally occurring plant hormones such as indole acetic acid (IAA), gibberellins, zeatin, abscisic acid (ABA), and ethylene, and also a number of synthetic chemicals that affect or

control growth and development in plants (Khan et al., 2012, 2013, 2014, 2015, 2016; Khan and Khan 2014; 2017). These compounds are important regulators of plant growth and mediate responses to both biotic and abiotic stresses. In the future, a major challenge will be to understand how the information conveyed by these simple compounds is integrated during plant growth (Table 16.1). Plant biologists have been fascinated by the regulatory capacity of phytohormones since the time of their discovery, and the notion that hormone levels or responses could be manipulated to improve desired plant traits has long been an area of intense interest (Sasaki et al., 2002).

Each type of plant growth regulator has a wide range of physiological effects in different plants. Phytohormones have been shown to increase growth and yield of plants (Nazar et al., 2014). These effects are determined by the kind of growth regulator, its concentration, the presence or absence of other growth regulators, and by the genetic makeup and the physiological status of the target tissue. The same physiological response in different tissues even of the same plant may require different growth regulator(s) or different combinations of growth regulators. The growth and development of plants under varied environmental conditions determine agricultural production. The growth, development, and senescence of plant's organs can influence crop production by modulating photosynthesis, nutrient remobilization efficiency, and harvest index (Jing et al., 2005; Khan et al., 2012). Every stage of the plant's life cycle is regulated by plant hormones. In general, plant biological activity is manipulated by more than one hormone, thus the biological phenomenon often reflects the combined interplay of several different hormones. Meanwhile, unlike animals, which can escape from harsh environments, plants can only survive through adjusting various biological activities when encountering biotic and abiotic stresses. During these situations, plant hormones also cooperate to modify biological responses for the formation and maintenance of plant stress tolerance.

Sugar production and distribution has been found to play vital role in plant physiology. Plant growth and development in plants is modulated through sugar production by photosynthesis and also organizes internal regulators and environmental signals (Smeekens, 2000). The effect of carbon allocation on organ and on whole plant architecture is illustrated most dramatically by carbohydrate storage and the concomitant cell expansion in reserve organs such as roots, fruit, seed, and tubers. Sugars are the most important carbon and energy source to cells, and also have important regulatory functions in controlling metabolism, growth, and development of plants under in vitro conditions (Ramon et al., 2008). Sugars operate both as immediate substrates for intermediary metabolism and as effective signaling molecules. Sugars as signaling compounds have intense effects in all stages of the plant's life cycle from germination and vegetative growth to reproductive development and seed

TABLE 16.1 Phytohormones and Their Functions in Growth and Development Under In Vitro Conditions

Auxins	• Auxins are synthesized in young leaves and buds. They move mainly from apical to the basal end (basipetally) in excised coleoptile sections. Usually the transport is unidirectional, called polar transport, but recently it has been reported that it has been transported acropetally in phloem • IAA, IBA are naturally occurring auxins • 2-4-D, Dicamba and NAA are synthetic auxins Functions under in vitro conditions • Elongates cell • Regulates apical dominance • Forms lateral and adventitious roots • Delays the onset of leaf abscission • Regulates lateral floral bud development • Promotes fruit development
Cytokinins	• Cytokinins move to the leaves from roots, keeping root and shoot growth in balance • Occur in both free and bound forms • Zeatin is the most abundant naturally occurring free cytokinin; butdihydrozeatin (DZ) and isopentenyl adenine (iP) are found in higher plants and bacteria • Root tip is important for its synthesis; however, cambial tissue and developing seed are also site synthesis Functions under in vitro conditions • Promotes chloroplast development • Promotes cell division • Promotes cell expansion in leaves and regulates growth of stem and roots • Modifies apical dominance and promotes lateral bud growth

The auxin and cytokinin ratio regulates morphogenesis and callus formation under in vitro conditions.

formation (Smeekens, 2000). Plant sugar regulation is mediated by sugar signals, which are generated at different locations depending on environmental conditions and developmental stage. Sucrose (Suc) transport and hydrolysis play key regulatory roles in sugar signal generation (Ruan, 2014). In plants, sensing and signaling pathways have been described for different sugars (Hanson and Smeekens, 2009) but only for glucose detailed information on sensing and signaling mechanisms is available (Grigston et al., 2008).

16.7 FUNCTION OF PHYTOHORMONES UNDER IN VITRO CONDITIONS

Significant evidence of associations between sugar and phytohormones response and other metabolic pathways have also been provided by recent studies (Gibson, 2005). However, no systematic study has been done to explore the molecular basis of interaction between the two signaling molecules, that is, sugar and auxin, although both regulate similar processes and are so fundamental to plants (Mishra et al., 2009). Resistance toward exogenous auxin application is exhibited by glucose insensitive mutant *gin2*, which is mutated in glucose sensor HXK gene (Moore et al., 2003). Sugar and auxin regulate a number of common responses vis-à-vis root and shoot development, vascular morphogenesis, cell division and expansion, embryogenesis, immunity, chloroplast and anthocyanin biogenesis, de novo organogenesis, lateral root formation, leaf morphology and senescence, stress responses, immunity, nodule organogenesis, and photomorphogenesis (Kieber and Schaller 2010; Müller 2011; Brenner et al., 2012; Gupta and Rashotte 2012; Hwang, Sheen and Müller 2012; Shi and Rashotte 2012; Spíchal 2012). Sugars and cytokinin (CK) can control similar responses, through a two-component signaling cascade. Cytokinin (CK) signaling modulates sugar-induced anthocyanin biosynthesis via regulating sugar-inducible structural and regulatory genes (Das et al., 2012). Cyclin D3 (CycD3) expression is induced by sucrose alone and in combination with cytokinin (Kushwah and Laxmi, 2014). However, CK does not induce CycD3 expression in the absence of sucrose (Riou-Khamlichi et al., 1999). In cytokinin (CK) metabolism and signaling, the several genes involved can be regulated by glucose (Kushwah and Laxmi, 2014). Physiologically, hypocotyl length in dark could be regulated by both glucose and cytokinin. CK-regulated gene expression may also be affected by glucose via nontranscriptional pathways. Sugar and CK signaling is linked in antagonistic manner in the delayed leaf senescence phenotype found in glucose receptor *gin2* mutant (Moore et al., 2003).

16.8 CONCLUSION AND FUTURE PROSPECTS

Sugar plays an important role in the metabolism of plants. They are now recognized as regulatory molecules with signaling functions in plants and other organisms. Yeast genetics has enabled the rapid and detailed elucidation of diverse sugar sensing and signaling pathways, plant sugar signaling has proven more difficult to study due to the complexity of source−sink interactions, responses to diverse sugar signals and metabolites, and the intimate integration of a web-like signaling network governed by plant hormones, nutrients, and environmental conditions (Rolland et al., 2006). The use of different experimental systems, including isolated cells, excised tissues, cell cultures, whole plants, and mutants under different environmental and nutrient conditions at various developmental stages is critical in dissecting the plethora of sugar responses and their connections in plants. Microarray and clustering analysis are new, powerful genomic tools to provide a global view on the transcript dynamics controlled by different sugar responses and identify novel regulatory components and target genes. The sharing of massive datasets is beginning to provide new insights into the extent and mechanisms of sugar-regulated gene expression and interactions with other signals. The molecular details of signal transduction pathways and their crosstalk with other pathways will be revealed by using a combination of genomic proteomic and genetic approaches. Current technology limits the ability to visualize and quantify the precise location and concentration of various sugar molecules and metabolites in living cells. Novel molecular sensors and fluorescence resonance energy transfer-based imaging will hopefully circumvent this limitation and provide critical information to facilitate the elucidation of intracellular sugar signal transduction pathways.

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CHAPTER

17

Role of Mineral Nutrients in Abiotic Stress Tolerance: Revisiting the Associated Signaling Mechanisms

Mohammad Abass Ahanger[1] *and Parvaiz Ahmad*[1,2]

[1]Department of Botany, Govt PG College Rajouri, Rajouri, Jammu and Kashmir, India
[2]Department of Botany and Microbiology, Faculty of Science, King Saud University, Riyadh, Saudi Arabia

OUTLINE

17.1 INTRODUCTION

On one hand, world population is expected to cross 8.3 billion in the next two decades (FAO, 2010) and rapid urbanization and industrialization, on the other hand, have resulted in considerable shrinkage of the arable land area all over the globe, thereby raising the grave issue of sustainable food security. No doubt, it has been widely accepted that most of the food crops have shown apparent increase in productivity during the past few years. Therefore maintaining such positive effects for meeting the needs of the increasing population in future needs the integration of scientific methods with the conventionally employed agricultural practices (Fan et al., 2012; Ahmad and Wani, 2014; Ahanger et al., 2017a). In nature normal growth and development of plants is regulated by several environmental factors affecting the sustainability of crop productivity. Sustainable crop production is primarily a function of several biotic and abiotic environmental stress factors, and also is directly linked with the mineral status of soil (Sogbedi et al., 2006). It is interesting to point out that both biotic as well as abiotic stresses adversely affect the soil mineral status

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and the access of plant roots to the existing minerals in the rhizosphere, therefore affecting growth and development at the plant level (Turkan and Demiral, 2009; Khan et al., 2015; Ahanger and Agarwal, 2017a,b). Increasing magnitude of stresses in future is expected to impose considerable loss of fertile land, for example, high soil salinity impedes plant productivity by triggering ionic and osmotic effects leading to restricted mineral uptake and altered assimilation (Fatma et al., 2014; Ahanger and Agarwal, 2017a). Contrary to this, water deficit impedes nutrient access to plant roots (Osakabe et al., 2014) while metal(loid)s restrict the uptake of mineral ions by potentially reducing the activity of ion transporters (Tamas et al., 2014). Individually stresses are not so lethal to plants when compared with their combined effects, and cumulative exposure of crops to biotic and abiotic stresses can reduce productivity by 75% (Noman et al., 2017). Loss in productivity is due to the negative effects of the stress-triggered alterations in the key metabolic pathways leading to anomalies in the developmental progression. Among the key negative regulators are included the reactive oxygen species (ROS), which are continuously generated in cellular organelles like chloroplast, mitochondria, peroxisomes, etc. Among the key consequences of ROS are included the inhibition of photosynthetic and mitochondrial electron transport systems, and metabolic dysfunction leading to damage to cellular structures and offering premature senescence (Petrov et al., 2015; Rogers and Munne-Bosch, 2016). However, reports are available that advocate the beneficial role of ROS via their involvement in the stress signaling (Camejo et al., 2016; Mittler, 2017; Khan and Khan, 2017). In addition there are other adversaries triggered by stress exposures that are more or less regulated by the availability of sufficient concentrations of different mineral elements. Availability of mineral elements controls the growth and protects metabolism under extreme conditions (Ahanger et al., 2017b) and has been observed to bring integration of several signaling events either through their direct involvement or by inducing improved synthesis of key signaling molecules (Iqbal et al., 2015; Khan et al., 2015; Ahanger et al., 2015). Besides this having effects on the expression of genes controlling growth and yield, mineral elements strongly affect the distribution of plant species (John et al., 2007; Barbosa et al., 2014). Since environmental stresses are potential threats for sustainable agricultural productivity, the present review focuses on the current understandings about the stress-triggered deleterious effects on the uptake, transport, and assimilation of key mineral elements making interpolation of relevant reports and identifying the key future targets for overcoming such effects. In addition the role of nutrients in mediating signaling individually and combinedly for elicitation of stress tolerance mechanisms has also been discussed.

17.2 MINERAL NUTRIENTS AND STRESS TOLERANCE

17.2.1 Nitrogen

Nitrogen (N) is a vital plant nutrient considered as a major determining factor required for growth and yield productivity of major crops. It makes up nearly 4% of total dry matter and is a key component of important molecules like proteins and nucleic acids. Sufficient availability of N throughout the plant life cycle or growing season is important for attaining the optimum growth and yield. Nitrogen availability has been reported to affect proteins and many other compounds essential for plant growth processes including chlorophyll and enzyme activities (Iqbal et al., 2015). Besides imparting growth promotion through its own effects, N availability also affects the uptake and utilization of other key elements like phosphorous, potassium, sulfur, zinc, boron, etc. causing significant enhancement in the yield and yield associated attributes (Dash et al., 2015). Therefore, it can be inferred that N availability has direct influence on the uptake as well as assimilation of other key minerals. However lack of sufficient quantities of these nutrients also influences N uptake.

Nitrogen availability profusely contributes to biomass accumulation by influencing photosynthesis and synthesis of proteins and nucleic acids, and impeded uptake and assimilation affects growth at the whole plant level (Masclaux-Daubresse et al., 2010). Ammonium (NH_4^+) and nitrate (NO_3^-) are the abundant forms of N taken up by plants and NH_4^+ is released from some metabolic processes including amino acid deamination during seed germination, biosynthesis of amino acids, and photorespiration. This NH_4^+ must be reassimilated to meet the cellular nitrogen needs so that any possibility of NH_4^+ toxicity can be avoided. For maintaining the beneficial consequences of NH_4^+ the plant must maintain optimal activity of glutamine synthetase (GS, EC: 6.3.1.2), glutamate synthase (GOGAT, EC: 1.4.1.14), and glutamate dehydrogenase (GDH, EC: 1.4.1.2) enzymes. Contrary to this for NO_3^- assimilation nitrate reductase (NR, EC: 1.6.6.1) and nitrite reductase (NiR, EC: 1.7.2.1) form key mediators to contribute to the formation of NH_4^+. The assimilated nitrogen moiety is fixed in the form of organic acids by aminotransferases resulting in biosynthesis of amino acids (Masclaux-Daubresse et al., 2010; Ahanger et al., 2017b). It has been proved that plant species maintaining optimal functioning of nitrogen

metabolizing enzymes exhibit apparent increase in amino acids involved in growth regulation and stress tolerance (Pandey et al., 2004; Iqbal et al., 2015; Ahanger et al., 2017b). At root plasma membrane levels the transport proteins functioning for the nitrate uptake have been identified as low affinity (LA) and high affinity transport system (HATS) and their expression has been observed to show considerable variation with respect to available nitrate concentration, that is, LATS and HATS act when external nitrate concentration is high and low respectively (Masclaux-Daubresse et al., 2010). Several such transport proteins have been isolated from plants like *Arabidopsis* and their functional characterization has been done, and their role in long distance transport of nitrate has been studied and confirmed using mutant lines (Tsay et al., 1993; Huang et al., 1999; Filleur et al., 2001; Chiu et al., 2004; Almagro et al., 2008). Chopin et al. (2007) have demonstrated that overexpressing *AtNRT2.7* accumulates greater nitrate, making the seeds less dormant. The availability of nitrate induces the development of lateral roots in *Arabidopsis* (Zhang and Forde, 1998). Exposure of *Brassica juncea* to abiotic (osmotic, salinity, high and low temperatures) stresses downregulates the expression of key genes coding for nitrate and ammonium transport proteins and their assimilatory enzymes (Goel and Singh, 2015).

It has been shown that the availability of nitrogen affects the synthesis of nitrogen containing metabolites leading to regulation of several signaling events by modulating the expression of key molecules like transcription factors (TFs) in addition to their involvement in such processes. N availability affected the synthesis of glutamine in rice and supplementation of exogenous glutamine reversed the ill effects by affecting the expression important TFs including *LBD37*-like genes involved in the regulation of nitrogen metabolism and *DREB1A*, *IRO2*, and *NAC5* TF genes mediating regulation of stress responses (Kan et al., 2015), reflecting in the involvement of glutamine in amplification and subsequent modification of signals, and crosstalk with key molecules for bringing growth regulation under stress. Iqbal et al. (2011; 2015) have demonstrated that increasing N application optimizes the photosynthetic efficiency of *Brassica juncea* and the involvement of exogenously applied ethylene was shown to regulate N-use efficiency.

Nitrogen is primarily transported by amino acids and therefore identifying the QTL preferably controlling the N uptake and the subsequent incorporation into amino acids can be worthwhile for improving the nutrient use efficiency (NUE) in plants. For achieving this integrating plant physiology and genetics to reflect in clear understanding of plant performance under optimal and suboptimal N supplementation and the identification of several QTL controlling transport, assimilation, and metabolism of N have been identified (Han et al., 2016). Among these identified genes some have been worked out for their role in N metabolism, for example, transgenic *Pisumsativum* overexpressing amino acid transporter *AMINO ACID PERMEASE1* (*AAP1*) showed greater allocation of N via the vasculature to the shoot and seeds thereby producing greater biomass and yield (Perchlik and Tegeder, 2017).

It has been demonstrated that *Saccharomyces cerevisiae* Snf1 protein kinase of the Snf1/AMP is directly involved in nitrogen signaling. Its involvement in pseudohyphal differentiation depends on the stimulatory phosphorylation of Snf1 at Thr210 and N limitation induces improved Thr210 phosphorylation besides the negative regulation of Snf1 by rapamycin-sensitive TOR (target of rapamycin) kinase actively involved in signaling nitrogen and amino acid availability (Orlova et al., 2006). In yeast cells, the TOR pathway has been implicated in regulating cellular responses to nutrients, including proliferation, translation, transcription, autophagy, and ribosome biogenesis. It has been reported that overexpressing Tap42-Sit4, a protein phosphatase regulatory subunit, restores the pseudohyphal growth in cells exposed to rapamycin. Possible involvement of Tor protein kinases and Tap42−Sit4 protein phosphatase interactions have been proposed to regulate N nutrient-sensing pathway involving the activation of the MAP kinase or cAMP pathways (Cutler et al., 2001). In addition, of the molecular regulation the biochemical regulation of N assimilation has been worked out. Nitrate has been confirmed as the main primary signal molecule triggering activation of the transcription of key genes controlling nitrate assimilation and related genes, which further directly affects the processes like photosynthesis, cell cycle control, and other related translational events (Takei et al., 2002).

17.2.2 Sulfur

Sulfur (S) is another key nutrient actively regulating the plant growth and development. Fourth in importance after N, P, and K, S forms an integral part of several metabolically important compounds like vitamins, phytohormones, coenzymes, etc., which affect plant growth and vigor considerably. S is considered to potentially regulate the growth and development of plant under normal and stress environments (Asgher et al., 2014; Fatma et al., 2016). Limited S availability restricts yield and quality of product, and adequate S nutrition enhances photosynthesis and growth in plants (Fatma et al., 2014). In addition, S shows a growth regulatory interaction with other minerals like

N assimilation (Scherer, 2008). Deficiency of S hampers the process of chlorophyll biosynthesis, N assimilation, photosynthesis, protein synthesis (Lunde et al., 2008). Once taken up by a plant, S gets incorporated into some key organic molecules, for example, thiol (−SH) groups in cysteine residues or nonprotein thiols like glutathione. Among these S containing compounds, mostly reduced glutathione is sensitive to oxidized environment and has been recognized for its potential role in modulating the plant responses to a range of stresses (Tausz et al., 2004; Szalai et al., 2009; Khan et al., 2015). These S containing compounds like glutathione are involved in the removal of excess ROS thereby preventing the oxidative damage to cells, and maintaining the concentration of ROS for integration of several beneficial roles (Rausch and Bucher, 2002). In addition, optimal availability and assimilation of S regulates the biosynthesis of a nonprotein thiol leading to maintenance of the homeostasis between the reduced (GSH) and oxidized (GSSG) forms of glutathione thereby maintaining the signaling of stress response proteins and hence the oxidative stress amelioration. Since stresses pose deleterious effects on the metabolism of minerals including S, therefore a prerequisite requirement for mitigating the ill effects on growth and productivity will be the understanding of the involvement of S in regulation of different signaling pathways under stress.

Sulfur supplementation regulates growth of plants under a range of environmental stresses. It has been observed that S supplementation restricts the generation of excess ROS thereby preventing the oxidative damage (Fatma et al., 2014; Asgher et al., 2014; Khan et al., 2015). Recent reports have suggested the positive influence of applied S on the regulation of endogenous levels of phytohormones like ABA, JA, NO, and ethylene (Khan et al., 2014a,b; Fatma et al., 2016; Per et al., 2017, 2018). Sulfur is involved in the maintenance of K/Na ratio (Abdelhamid et al., 2013) and restricting the accumulation of toxic ions like cadmium (Matraszek et al., 2016). In addition to restricting the generation of ROS, S supplementation improves the synthesis of Rubisco thereby enhancing photosynthetic efficiency and the yield productivity (Lunde et al., 2008), however the detailed mechanisms at the physiological and molecular levels are largely unknown.

Several S containing compounds have been demonstrated to protect plants from the stress induced deleterious effects, however, very little is known about the molecular mechanisms involved in regulating the assimilation of S by stresses. Though reports are available, conferring the demand driven assimilation of S for promoting its greater incorporation in protective compounds (Fatma et al., 2014; Khan et al., 2015) nevertheless it should be noted that excess S can prove repressive in regulation of growth and development of plants. For mediating scavenging of ROS plants upregulate S assimilation by improving rate of transport as well as reduction. A reduction in the concentrations of GSH induces the activity of 5-phosphosulfate reductase reflecting in the demand driven synthesis of S containing compounds for stress amelioration. S transport mediated by ATP-sulfurylase and subsequent reduction by 5-phosphosulfate reductase are the key regulatory steps in S metabolism and increased levels of mRNA coding for S transporters like ATP-sulfurylase and 5-phosphosulfate reductase have been observed reflecting in the availability dependent regulation at the transpirational level (Nikiforova et al., 2003). Many researchers are of the opinion that O-acetyl serine accumulation during stressful conditions may probably serve as signal for inducing the regulation at transcriptional levels (Ohkama et al., 2002; Hubberten et al., 2012). In *Arabidopsis thaliana*, Hubberten et al. (2012) have identified six different genes whose expression was correlated with the accumulation of OAS through the OAS mediated changes at transcriptional levels of their gene expression. Under S deficiency the expression of S responsive genes is also regulated by the availability of growth hormones, for example, in *Arabidopsis thaliana* exogenously supplied cytokinins, *trans*-zeatin, and *trans*-zeatin riboside upregulated the expression of sulfur-deficiency responsive element (beta SR), a beta subunit gene of beta-conglycinin of seed storage protein (Ohkama et al., 2002). Additionally products of S assimilation like GSH have been proposed to act as long distance signal in bringing coordination between the synthetic precursors and their assimilatory pathways (Lappartient et al., 1999). A kinase, GCN2, has been identified to act as a sensor of the carbon/nitrogen precursor availability, and S limitation precursor, and is transduced to TOR by downregulation of the glucose metabolism and downregulation of TOR activity causes reduction in translation and meristematic activity, while elevating autophagy (Dong et al., 2017). The role of S in regulation of MAPK signaling cascades is not available and the identification of key signaling events triggered by S deficiency or availability needs to be evaluated. Assimilation of S is proved to be under direct control of phytohormones including SA, ethylene, auxins, etc. The involvement of three sulfur-starvation responsive TFs, *IAA13*, *IAA28*, and *ARF-2* (*ARF1*-binding protein), related to auxin signaling has been observed to regulate developmental events in plants. Overexpressing or loss of function of *IAA28* imparted no major visible morphological changes, whereas *IAA13*- and *ARF1-BP*-overexpressing lines exhibited much slower growth than the wild ones and it was confirmed that the maintenance of steady-state metabolite levels and

expression of pathway-relevant genes under normal and sulfate-deficient conditions has no exclusive link with S availability. Instead, it has been observed that the up- or downregulation of the specific TFs mediate metabolic changes, which in turn affect sulfur metabolism (Faljenberg et al., 2008).

Decreased S inputs have increased the incidence of S-deficiency in crops, causing decline in yield and yield associated attributes. Ability of crops to respond to S-deficiency stress shows considerable variation between crops and such gaps can be eliminated by introducing the genetic improvement of S-utilization efficiency. Focus of any genetic or molecular study should be improvement in the capture of available S resources followed by subsequent accumulation of S reserves and the remobilization of accumulated reserves. Inability of plant species to overaccumulate S and remobilize S-reserves restricts S-use efficiency. For improving this genetic manipulation of the transporters and their expression can be a key factor (Hawkesford, 2000; Lee et al., 2016).

Howarth et al. (2009) have identified a sulfate deficiency-induced gene, *sdi1*, in *Triticum aestivum* L. and it has been demonstrated that knocking down of sdi1 in *Arabidopsis thaliana* improved the endogenous sulfate concentrations under S deficient conditions. Another high-affinity sulfate transporter *SULT1;*1 has been seen to be highly regulated in the epidermal and cortical cells in *Arabidopsis* roots under S deficiency, and its induction under the control of promoter sulfur responsive element (SURE) has been demonstrated to be regulated by improving external S supplementation like GSH and cysteine (Maruyama-Nakashita et al., 2005).

Controlled gene expression leads to limited uptake of excess S and optimizes subsequent assimilatory pathways with endogenous expression of sulfate transporters being regulated by applied S where reduced S-containing compounds act as negative regulators and *O*-acetylserine as positive regulators. Contrarily constitutive expression of the transporters will eliminate this regulation paving the accumulation of sulfate reserves so that the accumulated sulfate in the vacuoles and other reduced sulfur pools like glutathione or protein may be remobilized under S-limiting conditions. Targeting the remobilization of vacuolar sulfate can revert the deleterious changes induced by S deficiency ultimately affecting uptake from the soil solution and subsequent delivery to the site of reduction in chloroplast or plastids (Hawkesford, 2000; Kataoka et al., 2004). For further insights about this, the identification of gene family and their phylogenetic relationships and differences in spatial expression to unlock their functional roles remains to be evaluated.

17.2.3 Potassium

Potassium is a third important element for plants and is mostly absorbed in the form of ions as K^+. Most of the available K in soil is either dehydrated or coordinated to oxygen atoms, and thereby is rendered unavailable for absorption (Ahanger et al., 2017b,c; Tittal et al., 2017). K availability to plants is sternly affected by the soil nutrient dynamics, soil K pool, physiochemical properties, and the type of the existing soil. Depending on its availability and accessibility to plants K has been identified to exist in four different pools, that is, (1) water soluble K, (2) exchangeable K, (3) nonexchangeable K, and (4) structural K. Water soluble K is easily available to plants in addition to the exchangeable K pool that is represented by the electrostatically bound K to clay minerals and organic or humic substances. Both these available K pools are replenished by crops for their optimal physiological performance, even though they form a very small fraction of the whole K soil pool. In comparison, nonexchangeable K obtained from the weathering of mica rich rocks is not easily available to plants while the structural pool existing as micas, muscovite, biotite, and other feldspars constituting a major part of soil K is totally unavailable for plants (see Agarwal et al., 2009; Zorb et al., 2014). However it shall be noted here that the available K pool (water soluble K) is often leached if unabsorbed by the plant roots. K application has a direct effect on the structure and properties of soil. Enhancement in the water holding capacity of soil and maintenance of the structural stability of soil particularly the sandy soils has been reported (Holthusen et al., 2012). K or mineral induced soil stability may be ascribed to increased shear resistance contributing to enhanced water retention. Mineral induced stability in soil structure is ascribed to increase in electrolyte concentration causing flocculation subsequently resulting in precipitation in the form of salt crystals (Van Olphen, 1977). In addition to K, Mg^{2+} and Ca^{2+} are also effective cations for mediating the stabilization of soil structure because of their high relative flocculating power (Rengasamy and Sumner, 1998). Increasing water retention potential or water holding capacity can be very beneficial for crop plants in areas with limited water availability.

As an abundant inorganic constituent of cells and cation in the cytosol, K has a significant role in regulation of several physiological functions including protein synthesis, membrane polarization, and osmotic potential hence controlling osmotically driven functions like cell movements, stomatal functioning, and phloem transport (Zorb et al., 2014; Ahanger et al., 2017c). Therefore, enough quantity of K must be present in soil solution to ensure significant absorption,

transport, and allocation to various organs. However K in soils displays considerable fluctuations making the fulfillment of demand driven needs of existing crops difficult. Plants have developed certain mechanisms to show efficient uptake of K so as to ensure maintained growth under limited K availability. Improved capacity for K uptake, efficient redistribution of absorbed K between cytosol and the vacuole for cellular homeostasis, and modification of root system are the key strategies. Such processes require precisely regulated and controlled signaling cascades and mechanisms (Ashley et al., 2006; Cherel et al., 2014). In addition to the complex soil dynamics, K availability is also influenced by the root−soil interactions. Expression of high affinity K transporters and the associated signaling cascades are initiated under low K availability and in this connection ROS and certain phytohormones including auxin, ethylene, JA are believed to mediate the sensing of K deficiency (Ashley et al., 2006; Agarwal et al., 2009; Ahanger et al., 2017c). Through molecular and electrophysiological studies, certain putative transport proteins are identified for controlling the uptake as well as compartmentalization of K ions (Osakabe et al., 2013). Transporter proteins, mainly operating at the soil root, xylem, phloem, and tonoplast levels, can be seen elsewhere, however it shall be noted that K starvation leads to release of vacuolar K (Nieves-Cordones et al., 2014) and it has been shown that expression of transporter genes is increased for ensuring homeostasis in plant tissues under water stress and K deficiency (Song et al., 2015).

Deficient K leads to growth retardation, accumulation of simple carbohydrates, low chlorophyll content, altered photosynthetic activity, and eventually to reduction in growth and yield (Sharma et al., 2006; Jatav et al., 2014; Ahanger et al., 2015), and can increase sensitivity of plants to biotic as well as abiotic stresses (Zorb et al., 2014) in comparison with sufficient K that has been reported to maintain growth and yield of most crop plants by inducing positive changes in metabolism (Tiwari et al., 1998; Sharma and Agarwal, 2002; Sharma et al., 2006; Tomar and Agarwal, 2013; Jatav et al., 2012, 2014; Ahanger et al., 2015, 2017b; Ahanger and Agarwal, 2017a,b). Presence of high K reduces pest and disease incidence through improved synthesis of high molecular weight compounds including proteins, starch, and cellulose (Perrenoud, 1990; Marschner, 1995). Any minor variation in K induced regulation of biotic infestation reflects the alterations in K nutritional status at a whole plant level and the form of K salt applied (Amtmann et al., 2008; Wang et al., 2013). Additionally K has a critical role as inorganic osmotica as well as the synthesis of other compatible osmolytes affecting functioning like stomatal movements and water relations. K maintains photoassimilate loading and the activity of key metabolic enzymes (Ahanger et al., 2014; Erel et al., 2015; Ahanger et al., 2015, 2017b). Sufficient availability of K enhances carboxylation and overall photosynthetic efficiency leading to optimal yield production under normal as well as stressed conditions (Tiwari et al., 1998). It has been observed that under K deficiency mediated reduction in photosynthetic rates are mainly due to the limited access to the CO_2 due to stomatal closure (Jin et al., 2011) and osmotic regulation by K brings the expansion of leaves and cells. Such reduction in CO_2 assimilation causes increase in excited energy dissipations through nonphotochemical quenching (Erel et al., 2015). Improved photosynthetic performance due to K is related to the alterations in stomatal and gas exchange characteristics, and regulation of enzymes like Rubisco (Erel et al., 2015). In addition, K deficient plants show increased photodamage. Photosynthetic arrest under stressful conditions mediated by nonstomatal limitations includes inhibition of chlorophyll biosynthesizing system (Xiao-guang et al., 2015) and K application induces positive influence on the chlorophyll synthesis (Tiwari et al., 1998), chloroplastic CO_2 concentrations, and ETS (Xiao-guang et al., 2015).

Due to heterogeneous distribution of the developmental resources, plant growth gets affected, and sensing these changes for optimizing growth is very important. Therefore, it is important to elicit shoot to root or root to shoot signaling processes for coordinating the different root and shoot developmental events for maintaining the overall plant growth. Active involvement of K in regulating the key physiological responses like stomatal movements together with the hydraulic conductivity can be monitored with the timely examination of the available K and its subsequent flow through xylem and phloem tissues (Kudoyarova et al., 2015).

Additionally, reduced ion concentrations in xylem can change volume of pectin pit membranes leading to alteration of hydraulic conductivity (Zwieniecki et al., 2001). Cations like K^+ bring modifications in the pore dimensions of pit membranes thereby modulating the resistance to water flow through the xylem (Wheeler et al., 2005). K supplementation increases hydraulic conductivity as well as leaf specific conductivity due to its interaction with the pectic matrix of vessels. However, K-mediated enhancement in hydraulic conductivity benefits the plant by maintaining cell turgor, stomatal functioning, and gas exchange parameters (Oddo et al., 2012). Under nutrient deficient conditions, biosynthesis of major energy reductants is reduced due to reduced photosynthesis and hence results in perturbed carbon and energy metabolism. Moreover specific tolerance proteins are differentially

expressed in plants exposed to nutrient starvation including the proteins having a role in protecting the important plant cellular processes like primary and secondary metabolism, energy metabolism and defense, signal transduction, transcription, synthesis, targeting and storage of protein (Deng et al., 2014).

The dominant role of K ions in maintenance of turgor and water homeostasis is obvious from its evident role in the processes like pressure driven solute transport in xylem and phloem, vacuolar accumulation of K^+ and K^+ flux that mediate plant movement. For example, K-induced changes in stomatal movements through its uptake and release greatly affects plant water relations and photosynthetic efficiency (Mahouachi et al., 2006). Adequate K concentrations improve the oxidative damage–averting potential of plants by regulating stomatal functioning, osmoregulation, and water use efficiency (Shabala and Pottosin, 2014). Under field conditions, reports communicating the beneficial role of supplementing relatively higher doses of K under water stress conditions are well available (Tiwari et al., 1998; Sharma and Agarwal, 2002; Jatav et al., 2012, 2014; Ahanger and Agarwal, 2017a,b).

In stress-exposed plants, efflux of K and calcium (Ca) occurs from the leaf mesophyll cells, which ultimately alters the stomatal characteristics and the photosynthetic efficiency. Drought induced efflux of K and Ca from the leaf mesophyll has been correlated with other growth parameters including height, biomass accumulation, and chlorophyll contents as well as photosynthetic attributes like intracellular CO_2 concentration, net CO_2 assimilation, stomatal conductance, and transpiration rate. Effluxed K and Ca from the mesophyll cells sense the intensity of stress (Britto et al., 2010). Stress induced K leakage is because of the increased expression of K efflux channels caused by the overproduction of ROS, and K starvation further aggravates this situation by reducing the activity of root aquaporins and the hydraulic conductivity (Kanai et al., 2011). Stresses generate excessive ROS leading to lipid peroxidation and intensifying the K leakage.

Addition of K can enhance tissue K^+/Na^+ ratio and hence the stress tolerance of plants. Supplying sufficient K reduces the oxidative damage by enhancing the activities of antioxidant enzymes (Soleimanzadeh et al., 2010; Soledad et al., 2015; Ahanger et al., 2015; Ahanger and Agarwal, 2017a,b). Under salinity stress, adequate K^+ in plant tissues is maintained through selective uptake and efficient compartmentalization of K^+ and Na^+ ions (Munns et al., 2006) leading to maintenance of structural and functional integrity of the cell. It is believed that cytosolic K^+/Na^+ ratio not the tissue ratio is the key determinant of saline stress withstanding potential (Shabala and Cuin, 2007). However, identifying the key targets for improving ion sequestration capability of crop plants shall prove very promising in improving stress tolerance.

Plant species showing greater discrimination between K^+ and Na^+ during absorption from the soil solution as well as transport to the upper tissue shoot preferably accumulate K^+ and sequester Na^+ into the vacuole or apoplast (Wu et al., 2013). Selectivity in K^+ and Na^+ during xylem loading and the potentiality of plants to redirect Na^+ back from leaves to roots impart greater tolerance to saline stress (Attia et al., 2009). The Na^+ concentrations are maintained well below the toxic levels and selective uptake of Na to upper plant parts has been reported quite often (Sharma et al., 2006; Attia et al., 2009, 2011; Tomar and Agarwal, 2013; Jatav et al., 2012, 2014; Iqbal et al., 2015; Ahanger and Agarwal, 2017a). Root tissues sense and transduce K deficiency to cell cytosol leading to initiation of several biochemical and physiological events for short as well as long term responses. It has been suggested that factors including membrane potential, concentration of ROS and phytohormones regulate short term responses to K deficiency (Wang and Wu, 2013; Wang et al., 2013). Studies have reported the induction of genes like TF and mitogen activated protein (MAP) kinase after one hour of mineral starvation (Wang et al., 2002). At transcriptional levels, K deficiency induces regulation of gene expression of proteins coding for metabolic processes, cation binding, ion transporters, and the genes associated with jasmonic acid, defense response, and K transporters (Ruan et al., 2015). Low K status triggers high affinity K transporters and activates signaling events similar to other stresses like wounding and molecules like ROS and phytohormones like ethylene, auxin, and jasmonic acid. In addition, several developmental responses in root tissues are initiated due to K deficiency (Ashley et al., 2006). It has been reported that K starvation mediated induction of various genes varies with the plant species and the genotype, for example, genes coding for jasmonic acid, ROS, Ca^+, and receptor-like signaling, lignin synthesis etc., exhibited different pattern of expression in two cultivars of watermelon suggesting that gene repression in response to stress can lead to less energy consumption for better root growth mediation for improving K uptake and tolerance to K deficiency (Fan et al., 2014).

Transcriptomic studies have revealed that K starvation reduces growth in rice by triggering the downregulation of TFs like MYB, zinc finger, helix lope helix and bZIP, and transporters involved in metal, lipid, and ions like K, Na, P, etc., and several signaling associated molecules like MAPK, phosphatases, and calcium sensors (Shankar et al., 2013). So it could be suggested that K deprivation mediated activation of

genes and gene networks acting as concert sensors to external K availability and its subsequent distribution as well as adaptation. Therefore interplay between both up- and downregulated genes in response to K concentration variations can be a determining factor for averting the alteration in developmental and physiological stages. It is believed that calcium-mediated CBL-CIPK signaling is involved in regulating the shaker family K^+ channels *AKT1* and *AKT2*, however exact mechanisms mediating K deficiency are largely not known (Luan et al., 2009; Tokas et al., 2013; Fan et al., 2014). Research confirming the molecular mechanisms of K^+ sensing, uptake, distribution, and homeostasis in crop plants is in its infancy therefore extensive work is needed to understand the exact mechanisms underlying K nutrition and signaling. Nath and Tuteja (2016) have suggested the involvement of microRNAs in sensing and signaling nutrient deficiency in plants. Recently, Song et al. (2017) have demonstrated the involvement of nitric oxide in K starvation sensing and signaling in tobacco; however further studies are required to unravel the exact mechanisms.

17.3 PHOSPHOROUS

Phosphorus (P) is one of the abundant macronutrients in plant tissues; however its low availability in the soil is often a growth-limiting factor. P ranges from 0.1% to 1% of the total plant dry matter thereby making it among the three most abundant and much needed mineral nutrients for plants (Marschner, 2012). P is actively implemented in several growth and developmental aspects of plants, for example, formation of membrane phospholipids and nucleic acids, ATP generation, and its subsequent exchange, in the regulation of cellular processes through mediation of phosphorylation and dephosphorylation of key metabolites, etc. In plants, specifically, some processes like photosynthesis, photorespiration, or the growth patterns like the complex relations between the source and sink of photoassimilates resulting in modular growth makes studying the effect of available P on plant physiology of special interest. Chiefly acquired as inorganic phosphate (Pi), it remains in equilibrium as H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-} anions (Hernandez and Munne-Bosch, 2015). However it should be noted here that under physiological pH, that is, 5–6, $H_2PO_4^-$ is the chief form of Pi absorbed by roots compared with other forms thereby contributing enormously to the whole P content of the plant (Schachtman et al., 1998). Pi can easily be immobilized in the soil after the formation of insoluble complexes with organic matter or several other mineral cations including Al^{3+} or Fe^{3+} and Mg^{2+} or Ca^{2+} in acidic and calcareous soils respectively (Manning, 2008). Instead of having abundant occurrence in soils the availability of Pi is rather scanty for absorption to plants therefore it is emerging as the one of the most important mineral nutrients limiting normal plant growth, development, and yield (Hernandez and Munne-Bosch, 2015). Most Pi is obtained exclusively from mining, however only 20% of the Pi supplemented to plants is absorbed, making the need for P fertilization to be kept in check with the productivity rate (Mishima et al., 2003). Additionally the leached Pi causes serious environmental pollution leading to eutrophication after entering into fresh water bodies (Carpenter, 2005). The demand for increased crop productivity for serving the world's population and the advent of bioenergetic crops often force agriculturalists to use excessive P rather to cut down its annual fertilization rates. Therefore in this context it has been suggested that in the future Pi sources may get exhausted or dwindle. Therefore it becomes important to understand the responses of crop plants to fluctuating Pi status or sources for improved productivity so that the genetic potential of crops can be exploited.

Uptake and transport of Pi are precisely regulated so as to ensure optimal phosphorous use efficiency (PUE). For piercing the plant root P may prefer either symplastic or apoplastic pathway to reach the vascular bundle (Rausch and Bucher, 2002). Pi moves actively into the plants against gradient and its incorporation into the symplast or apoplast involves several key transport proteins like high affinity P/H + cotransporters phosphate transporter 1 (PHT1), which acts as symporter and is responsible for P uptake in angiosperms. Contrarily another protein, phosphate transporter B (PTB), is hypothesized as a Na+/Pi symporter catalyzing Pi uptake in chlorophytes (Lopez-Arredondo et al., 2014; Bonnot et al., 2017). Transport mediated by PHT1 is coupled to extrusion of H^+ by ATPases pumping leading to prevention of alkalinization of extracellular medium, however, small concentration of Pi is maintained in the root cells to meet the Pi requirements whilst the remainder is loaded into the xylem for long-distance transport. Within the xylem, loading has been observed to be regulated by another phosphate transporter protein PHO1 in *Arabidopsis thaliana* L (Nussaume et al., 2011). Under Pi deficiency its redistribution is mediated by the phloem transport. It has been reported that the *PHT1* family is localized mainly in root epidermal cells; however other members like *PHT2*, *PHT3*, and *PHT4* are localized in the inner membrane of plastids, inner mitochondrial membrane, and Golgi apparatus respectively (Chen et al., 2011; Lopez-Arredondo et al., 2014). It has been postulated that differential distribution of

PHT transporter families is responsible for controlling the distribution of Pi among organelles, cells, and tissues (Poirier and Bucher, 2002; Raghothama and Karthikeyan, 2005; Nussaume et al., 2011; Gu et al., 2016). However, little is known about the genetic manipulation of Pi transport proteins for improving PUE and subsequently understanding the regulatory mechanisms at transcriptional, posttranscriptional, translational, and posttranslational levels (Gu et al., 2016). Most *PHT1* members are involved in direct Pi uptake, which might be dominated by arbuscular mycorrhizal Pi uptake pathway solely relying on the AM-inducible *PHT1* genes (Smith et al., 2011). However under P starvation several P starvation genes are induced and most of them are regulated at transcriptional levels thereby pointing to their regulation via P starvation signaling as well as the P starvation response transcription factors (PHR TFs) (Bustos et al., 2010; Goel and Singh, 2015). These PHF TFs belong to the MYB-CC family and have been observed to regulate the expression of genes after binding with palindromic sequences available at the proximal promoter regions (Bustos et al., 2010; Wu et al., 2013). In addition the TFs from belonging to other families have also been reported to regulate the P transport and Pi starvation signaling (Jain et al., 2012).

Genes encoding the signal molecules like AtIPS1/At4, miRNAs, SPXs, biosynthetic genes of sulfolipids and galactolipids, Pi transporters and purple acid phosphatases acting downstream of PHR1 are directly or indirectly involved in regulating Pi transport (Wu et al., 2013; Gu et al., 2016). Among these molecules SPXs have been widely reported to be involved in sensing and transport of Pi in both plants as well as yeasts (Secco et al., 2012). Based on the type as well as presence or absence of secondary domain at C terminus major SPXs have been identified as EXS (*ERD1/XPR1/SYG1*), MFS (major facilitator superfamily), or RING (really interesting new gene) domain (Secco et al., 2012). *Arabidopsis* PHO1 encoding a protein of the SPX-EXS subfamily localized to the endomembranes of Golgi and the trans-Golgi network in root pericycle cells has been reported to control loading of Pi into xylem and transport to shoot tissue (Arpat et al., 2012). Further, it has been suggested that AtPHO1 acts as Pi exporter effluxing Pi out of cells into the xylem vessel (Arpat et al., 2012). Nevertheless *PHO1:GFP* fusion has been reported to get relocated to the plasma membrane in leaves infiltrated high Pi concentrations (Arpat et al., 2012). Therefore further investigating the probable roles of AtPHO1-localized organelles on Pi transport and distribution will be interesting, exploiting the data recently generated describing its topology and domain-specific functions (Wege et al., 2015). It has been revealed that it is the membrane-spanning EXS domain not the SPX domain of AtPHO1 that regulates the Pi export activity, specifying the localization to the Golgi and trans-Golgi network (Wege et al., 2015; Gu et al., 2016). Stefanovic et al. (2007) have observed that as many 10 genes in *Arabidopsis* encode the close homologs of *AtPHO1*, and *AtPHO1;H1* but not others (*AtPHO:H2-H10*) partially rescue the phenotype of the pho1 mutant. Khan et al. (2014a,b) have demonstrated that a SPX-EXS member in *Arabidopsis*, *AtPHO1;H3*, is involved in suppression of root-to-shoot Pi transfer and in rice only three SPX-EXS genes have been found to be phylogenetically associated with *AtPHO1* (Secco et al., 2010).

Additionally the vacuole is a key reservoir of many ions including Pi, however, the proteins responsible for the transport of Pi across the tonoplast and the underlying regulatory mechanisms are largely unidentified. Wang et al. (2012) have identified the involvement of two rice genes belonging to the SPX-MFS subfamily, *OsSPX-MFS1* and *OsSPX-MFS2*, that can serve as key targets in Pi starvation-induced miRNA, osa-miR827. All these members of SPX-MFS are localized to the tonoplast (Wang et al., 2015; Gu et al., 2016; Ham et al., 2018) where *OsSPX MFS1* and *2* are involved respectively in the facilitation of import and export of Pi across the tonoplast, while OsSPX-MFS3 has been shown to mediate Pi efflux from vacuole into cytosol (Wang et al., 2015). By and large the actual roles of *OsSPX-MFSs* and their protein products in Pi transport are still not fully understood. Liu et al. (2015) have shown that vacuolar phosphate transporter 1 (VPT1), an ortholog of *OsSPX-MFS3* in *Arabidopsis*, mediates the transport of Pi into the vacuole (Liu et al., 2015). Under Pi-sufficient and -deficient conditions, the phosphorylation and dephosphorylation of PHT1 regulates its interaction with PHF1 therefore resulting in regulation of Pi concentration in various cellular compartments (Ham et al., 2018).

During P deficiency plants display mechanisms for improving the uptake and distribution among aboveground tissues so that use efficiency is increased, however, focus has been mainly on the roots for unraveling the role of mechanisms increasing Pi availability. It has been observed that Pi availability to roots is increased by secreting the acid phosphatases, nucleases, or organic acids, therefore modifying the root architecture either through biotechnological approaches or exploitation of beneficial microbes like plant growth promoting rhizobacteria (PGPR) or arbuscular mycorrhizal fungi (AMF), and fine tuning it with optimized uptake to its aboveground parts by enhancing the expression of transport proteins can be a worthwhile future strategy (Cabugao et al., 2017; Battini et al., 2017). Molecular and genetic studies have shown the prime mechanisms regulating uptake

and utilization of P, and the influence of transporters, regulators, root architecture, metabolic adaptations, quantitative trait loci, hormonal signaling, and microRNAs. In connection to this, for acquiring improved PUE transition from the molecular mechanisms and plant architecture, modification to practical strategies is needed. Therefore, it can be proposed that introducing PGPR and AMF symbioses for efficient solubilization and uptake of P, intercropping with suitable crop species for maintaining P activation and its subsequent mobilization in the soil and improving the expression of homologous genes with advantageous agronomic properties for attaining greater PUE in plants under normal as well stressful conditions. In addition breeding for phosphorus-efficient varieties and introgression of key quantitative trait loci can be handy in achieving improved crop PUE (Veneklaas et al., 2012; van deWiel et al., 2016; Hasan et al., 2016). Several microRNA molecules have been identified to regulate the sensing and signaling of P deficiency under stress conditions, and their involvement in triggering mechanisms for controlling P assimilation needs to be studied (Kumar et al., 2017).

Low P uptake and redistribution leads to imbalance in the ability of plants to process light energy intensifying the chances of photooxidative stress. Xing and Wu (2014) reported declined carbon assimilation due to P deficiency. In chloroplasts, Pi is essential for photosynthesis and several reports advocate that Pi deficiency leads to reduced net CO_2 assimilation by largely affecting stomatal functioning (Singh et al., 2014; Zhang et al., 2014). Additionally biochemical limitations including reduced generation as well as protection of ribulose-1, 5-bisphosphate or Rubisco or mesophyll limitations to CO_2 diffusion are key effects of reduced P (Rao and Terry, 1995; Singh et al., 2014; Zhang et al., 2014). Besides this, the activity of phosphoenolpyruvate carboxylase, the enzyme responsible for shuttling CO_2 from the mesophyll into the bundle sheath, is reduced under low Pi availability (Schlüter et al., 2013). Research is needed for comprehensive understanding of the implication of antioxidants and other defense molecules in the protection of the photosynthetic apparatus under Pi starvation. In Pi starved plants, Okazaki et al. (2013) has observed the replacement of phospholipids by galactolipids in biomembranes and the implication of new plant lipid (glucuronosyldiacylglycerol) has been reported. Moreover roots are the main organs involved in sensing and signaling P homeostasis in leaves; however, exact mechanisms are not fully known and involvement of Ca^{2+}, ROS, sucrose, membrane proteins, etc. have been proposed (Lei et al., 2011). In addition understanding the modulation of phytohormone concentrations with response to changing P homeostasis and the subsequent integration of signaling cascades in bringing tolerance at the whole plant level can be interesting.

17.4 CALCIUM

Ca is involved in processes like cell division, cell elongation, cell differentiation, cell polarity, cytoplasmic streaming, gravitropism, photomorphogenesis, plant defense, and stress responses. As a divalent cation (Ca^{2+}) acts as an intracellular messenger in the cytosol and has a key role in maintaining the structural stability of cell wall and cell membranes, and in addition acts as a counter cation for anions in the vacuoles (White and Broadley, 2003). Ca provides structural rigidity by forming cross-links within the pectin polysaccharides (Easterwood, 2002). Mostly existing as calcium oxalate crystals in plastids the structural integrity as well as the quality of fruit produced is strongly coupled to Ca^{2+} availability. It has been reported to activate several enzymes like ATPase, phospholipases, amylase, succinate dehydrogenase, and antioxidants (Pliethand Vollbehr, 2012; Ahanger et al., 2014; Ahmad et al., 2015; He et al., 2015). Ca availability has been observed to affect the stomatal closure, hydraulic conductivity, sap flow, and uptake of ions like K^+, Mg^{2+}, etc. (Cabot et al., 2009; Ahmad et al., 2015). It is believed that in the absence of an external stimulus plant cells tend to maintain low cytosolic Ca^{2+} concentration, however, a rapid increase in cytosolic concentrations is achieved when exposed to external stimuli like light, touch, hormones, and biotic and abiotic stresses resulting mainly due to Ca^{2+}/H^+ antiporter and Ca^{2+} pumps mediated efflux of Ca (Bush, 1995). Among the common signaling pathways elevating the concentration of cytosolic Ca is the phospholipase C (PLC) pathway, which is regulated by cell surface receptors, including G protein coupled receptors and receptor tyrosine kinases activating PLC enzyme leading to hydrolysis of the membrane phospholipid PIP2 resulting in the generation of two secondary messengers, that is, 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG leads to activation of protein kinase C while IP3 diffuses into the endoplasmic reticulum binding to its receptor (IP3 receptor, which is a Ca^{2+} channel) thereby leading to release of Ca^{2+} from the endoplasmic reticulum. Such stimulus mediated increase of Ca in cytoplasm is called Ca signature and it is believed that in addition to IP3, cyclic ADP ribose also has significant influence on Ca^{2+} channels therefore it could be said that increased Ca concentration in cytosol is due to the cumulative effect of different stimulatory molecules on the activity of Ca channels (Guse et al., 1999; Pottosin and Schonknecht, 2007; Noh et al., 2015). More importantly the transduction of Ca

signatures into the biochemical and morphological responses are rather complex and a number of factors have been identified to regulate the specificity of Ca towards a particular response (Guse et al., 1999; Zhang et al., 2014). In addition to this the internal Ca concentration and Ca signature integrate the external stimuli with key intracellular responses, and spatial and temporal changes in Ca^{2+} determine the fate of a particular physiological response and its dynamics and amplitude (Allen et al., 1999; Zhu, 2016). Ca binding proteins have a critical role in decoding and subsequently transducing the Ca signatures and the activation of target as well as metabolic pathway (Bagur and Hajnoczky, 2017).

Increased concentration of cytosolic Ca leads to the activation of several Ca^{2+} sensor proteins thereby converting the signals into a biochemical change. Among these sensors are included calmodulin (CaM), calcium dependent protein kinases (CDPK), and calcineurin B-like (CBL) having a crucial role in the abiotic stress signaling in plants (Das and Pandey, 2010). Conformational changes are triggered in the sensor molecule due to binding of Ca^{2+} and hydrophobic pockets get exposed leading to interactions of a sensor and target proteins (Wilkins et al., 2016). Kinases inhibit autophosphorylation thereby increasing substrate phosphorylation. Ca and its sensors like calmodulin (CaM) regulate the expression of structural and regulatory genes by acting as TFs or increased Ca^{2+} may directly bind TFs modulating their activity hence regulating the gene expression (Yael et al., 2010). Usually Ca^{2+}/CaM complex interacts with TFs and modulates either their DNA binding or transcriptional activities or indirectly regulates transcription by associating with the multicomponent transcriptional machinery consisting of Ca^{2+}/ CaM complex, transcription factor binding protein (TFBP), and TFs. Finally, Ca^{2+}/ CaM complex leads to regulation of gene expression by modulating the phosphorylation status of TFs. This indirect regulation is achieved by a CaM binding protein kinase and a CaM binding protein phosphatase (Kim et al., 2009). It has been reported that plants exhibiting overexpression of these protein kinases show greater stress tolerance, for example, transgenic rice overexpressing *OsCDPK7* increases drought and salinity tolerance by improving the induction of stress responsive genes (Saijo et al., 2000). Overexpression of CBL5 protein in *Arabidopsis* enhanced the tolerance to salt and drought stress (Cheong et al., 2010).

Ca^{2+} addition under drought stress has been reported to enhance water conservation and improve the hydrophobicity of cellular membranes while lowering its permeability through its interaction with phosphates, carboxyl of phosphatides, and proteins in cellular membranes, thus strengthening their stability (Shao et al., 2008). Ca^{2+} alters the hydration status of membranes and improves the cohesive property of cell walls leading to enhancement of the protoplasm viscosity and hence imparting more resistance to cellular dehydration. It can be concluded that Ca^{2+} brings stabilization of plant cells by direct positive effects on the structural components or basis of drought resistance (Ma et al., 2009; Ahanger et al., 2014). Addition of Ca has been reported to increase contents of several amino acids like alanine and γ-aminobutyric acid, polyamines including putrescine and spermidine, in addition to chlorophyll content *Picearubens* exposed to low temperature stress (Schaberg et al., 2011). Ca supplementation significantly mitigated the negative effects of drought stress on fresh and dry weight, chlorophyll, and relative water content concomitant with reduced membrane leakage in *Vicia faba* (Abdel-Basset, 1998). Ca mediated heat stress tolerance has been attributed to increased antioxidant potential and the maintenance of tissue water content (Jiang and Huang, 2001). Xu et al. (2013) has demonstrated Ca-induced upregulation of antioxidant system to influence photosynthetic efficiency of plants under water stress. Such effects of Ca on metabolism have been shown to involve the Ca-mediated regulation of ABA signaling and antioxidant system (Wang et al., 2017). Upregulation of the antioxidant system by Ca protects the functioning of PSII and associated photosynthetic components by checking the ROS concentrations (Sakhonwasee and Phinkasan, 2017).

Similar to other macroelements, Ca^{2+} as secondary signal plays an important role in integrating extracellular signals and environmental cues including light and stress factors eliciting changes in the cellular Ca levels, termed as calcium signatures. The concentration of Ca^{2+} is precisely maintained by the Ca^{2+} stores like vacuoles, endoplasmic reticulum, mitochondria, and cell wall. Ca^{2+} is present in mM concentrations in the cell wall and vacuoles and is released whenever required by the cell. It has been demonstrated that organelles including mitochondria, chloroplasts, and nuclei have the potential to generate calcium signals on their own (Xiong et al., 2006).

Ca^{2+} is an important secondary signaling molecule and makes a convergence point for several signaling pathways. Plant cells tend to reprogram the cellular setup by initiating a network of signaling events from perception to response. It shall be noted here that only cytosolic Ca^{2+} is involved in diverse signaling pathways and responds to numerous stimuli. Stress mediated increase of Ca concentration activates calmodulin, which in turn regulates the expression of several TFs and kinase and phosphatase proteins thereby integrating the signal perception with the expression of stress-specific genes in the nucleus

(Virdi et al., 2015). Therefore, identification of stress specific TFs and their subsequent manipulations through genetic and molecular approaches can prove helpful in unraveling the hidden mechanisms of Ca mediated stress tolerance.

Ca^{2+}signaling pathway also regulates a K^+ channel for low-K response in *Arabidopsis*. Ca forms an essential component of the sucrose signaling pathway that leads to the induction of fructan synthesis (Martinez-Noel et al., 2006), in addition also to regulating cell cycle progression in abiotic stress exposed plants. Ca^{2+} competes with other cations both for these sites and for the uptake from the soil. High levels of Ca^{2+} ameliorates the uptake and negative effects of toxic ions (Cd, Al and Na) while maintaining the higher levels of other cations like K, Mg, P, etc., and in certain cases it has been observed that uptake of Ca is affected by the presence of essential elements in the soil solution (Sanders et al., 2002; Sakhonwasee and Phinkasan, 2017). Ca deficiency occurs due to low base saturation of soils or greater acidification or competition with other cations or restricted transpiration bringing down the xylem flow mediated supplementation of Ca to growing tissues (Zhang et al., 2014). Calcium deficiency results in stunted root growth and altered leaf appearances (Ahmad et al., 2015). Under drastic deficiency there are symptoms like reduced cell membrane integrity and appearance of bitter pit, blossom end rot, and tip burn in apple fruit, tomato, and lettuce respectively.

17.5 CONCLUSION AND FUTURE PROSPECTS

Stresses alter the uptake as well as metabolism of key nutrient elements by affecting the expression of transport and metabolism proteins. Resource variability due to the heterogeneous distribution intensifies the effects on plant development. Therefore, sensing the change in mineral concentrations may be beneficial for optimizing growth via the integrative mechanisms at root and shoot levels. Responses of plants elicited in response to limited nutrient availability may have some similarity with some stresses, and identification of genes and gene products that are up- or downregulated in response to the availability of a particular nutrient and the subsequent modulations through various biotechnological interventions can be helpful in understanding the actual mechanisms involved. Focus should be on dissecting the molecules regulating the root-to-shoot and shoot-to-root signaling events for bringing coordination between root and shoot under optimal, suboptimal, and supraoptimal availability of nutrients. Moreover, identification and studying the possible involvement of phytohormones, TFs, metabolites, etc. in sensing the nutrient availability and triggering the downstream events for bringing the modulations in metabolism is required. Developing a defined set of markers for enhancing the uptake, allocation, and metabolism of nutrients and hence the use-efficiency can help in understanding the genus or species specific responses to nutrient availability. Using "omics" techniques the identification of key signaling components regulating physiological responses at the whole plant level and the associated tolerance adaptation.

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CHAPTER

18

Sulfur Availability Potentiates Phytohormones-Mediated Action in Plants

Badar Jahan[1], *Zebus Sehar*[1], *Asim Masood*[1], *Naser A. Anjum*[1], *M. Iqbal R. Khan*[2] *and Nafees A. Khan*[1]

[1]Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India [2]Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India

OUTLINE

18.1 INTRODUCTION

Macroelements are ubiquitously present in soil and serve as a major source of mineral nutrients for plants. Amongst these elements, sulfur (S) is one of the foremost indispensable plant nutrient elements like nitrogen (N), phosphorus (P), and potassium (K) (Marschner, 1995). Deficiency of any of the elements in soil significantly impacts plant metabolism, which in turn affects nutritional quality, plant susceptibility to pests, and decreases protein biosynthesis and photosynthetic functions resulting in diminished crop yield (Mazid et al., 2011; Capaldi et al., 2015). In particular, S serves as a key nutrient universally found in plants

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to mediate plant growth and development and plant metabolism. The importance of S as a plant nutrient has been accepted for a long time, but dynamic research started when extensive S deficiencies were reported. The deficiency of S was reported to impair plant metabolism (Honsel et al., 2012), decrease chlorophyll content and photosynthesis (Kastori et al., 2000), and affect photosynthetic efficiency and the activity of ribulose1, 5 bisphosphate carboxylase (Rubisco) (Lunde et al., 2008). These aspects have been discussed in recent reports in plants under both optimal as well as stressful environments (Dubousset et al., 2010; Iqbal et al., 2013; Khan et al., 2013; Nazar et al., 2014a,b). Sulfur is present in nature both in inorganic and organic forms (Takahashi et al., 2011). The inorganic S is converted to S-containing compounds such as cysteine (Cys) and methionine (Met) and to secondary products such as sulfoxides and glucosinolates through a cascade of enzymatic steps (Leustek et al., 2000; Saito, 2004; Koprivova et al., 2008; Abdallah et al., 2010). These compounds alter several physiological processes and alleviate salt tolerance (Khan et al., 2014a,b). Reports indicate that S is also found in vitamins (biotin and thiamine), active ingredients of a large range of imperative metabolites, cofactors [CoA and S-adenosyl−methionine (SAM)], peptides (glutathione and phytochelatins), many coenzymes, and prosthetic groups (Mazid et al., 2011; Takahashi et al., 2011; Iqbal et al., 2012; Khan et al., 2012a,b, 2013, 2014a,b; Nazar et al., 2012, 2013). Notably, several studies reveal that photosynthetic organisms synthesize a wide variety of S-compounds, using sulfate as a primary S source (Leustek et al., 2000; Saito, 2004). Furthermore, S is used in the form of sulfate by root (Fatma et al., 2016). Sulfate assimilation is an elementary biological pathway of primary metabolism that provides the plant with S (Koprivova and Kopriva, 2016). Sulfur assimilation pathway is also associated with the synthesis of ethylene through Cys and Met. ATP−sulfurylase (ATP-S) and serine acetyl transferase (SAT), the two enzymes of S-assimilation, play a crucial role in Cys synthesis. In addition, ATP-S catalyzes the activation of sulfate, and SAT is dependable for the entry step from serine metabolism to Cys biosynthesis (Fatma et al., 2013). Consequently, the accessibility of sulfate and its uptake and assimilation are vital for plant growth and development, cellular metabolism, and response to a range of biotic and abiotic stresses (Leustek et al., 2000; Saito, 2004; Rausch and Wachter, 2005). In fact, a plant's exposure is inevitable to varied stress factors including salinity, drought, temperature, ozone, soil acidification, and heavy metal toxicity (Bulbovas et al., 2014; Medici et al., 2014; Nogueirol et al., 2015). Nevertheless, plants possess very proficient resistance pathways that tolerate the scavenging of reactive oxygen species (ROS), shielding the cells from oxidative damage (Gratão et al., 2005; Khan and Khan, 2017). Sulfur-containing compounds are involved in ROS metabolism and reduction of oxidative stress (Fatma et al., 2013; reviewed by Anjum et al., 2015). S-assimilation and cellular level of Cys, Met, and reduced glutathione (GSH) can be controlled to improve plant's stress tolerance capacity (Khan et al., 2008; Anjum et al., 2010, 2015). A vast amount of literature is available on the involvement of phytohormones in plant growth and development and also in plant responses to varied stress factors (Khan et al., 2012a,b). Additionally, the involvement of phytohormones such as ethylene in S-mediated improved stress tolerance has been reported in abiotic stressed plants (Asgher et al., 2014; Iqbal et al., 2013; Masood et al., 2012). Further, a large array of phytohormones such as auxins (AU), gibberellins (GA), cytokinins (CK), ethylene (ET), abscisic acid (ABA), brassinosteroids (BR), salicylic acid (SA), and nitric oxide (NO) were shown in crosstalk with S in maintaining plant metabolism (Ohkama et al., 2002; Maruyama-Nakashita et al., 2003; Khan et al., 2015a,b). Herein, based on recent reports on the subject, S-assimilation and its role in plant metabolism are overviewed, involvement of S-assimilation in the synthesis of major hormones is highlighted, a crosstalk is presented between S and other phytohormones, and major aspects so far least explored in the current context are listed.

18.2 OVERVIEW OF SULFUR ASSIMILATION AND ITS ROLE IN PLANT METABOLISM

18.2.1 Sulfur Assimilation

Pathways involved in S uptake and assimilation have been extensively studied (Saito, 2000, 2004; Rausch and Wachter, 2005; Kopriva, 2006). S-assimilation is highly regulated in a demand-driven way (Lappartient and Touraine, 1996; Leustek et al., 2000; Kopriva and Rennenberg, 2004; Kopriva, 2006; Davidian and Kopriva, 2010; Nazar et al., 2011a,b). For the proper growth and development of plants S uptake- and assimilation-pathway is generally induced (Kopriva and Rennenberg, 2004). S is available in the form of sulfate in roots to uptake (Davidian and Kopriva, 2010); whereas under limited S supply, plants are adapted to use foliar absorbed H_2S as S source for the growth (Koralewska et al., 2008). The sulfate uptake by roots and its transport to shoots are stringently restricted and worked as prime points of regulation of S-assimilation. In addition, sulfate reduction occurs in leaf chloroplasts and produces sulfide. The regulatory steps of sulfate assimilation include the transport of sulfate into the cells using ATP-S and the reduction of adenosine 5′-phosphosulfate (APS) to sulfite by APR (Vauclare et al., 2002). Additionally, with the help of ferredoxin-dependent sulfite reductase (SiR), S is further reduced to sulfide and sulfide is then

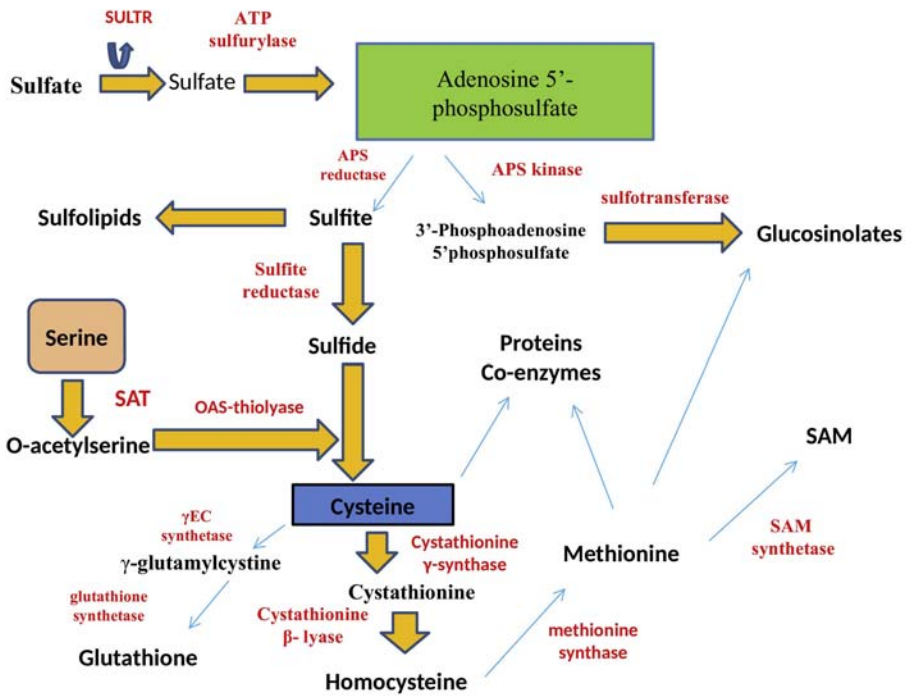

FIGURE 18.1 Schematic demonstration of sulfate assimilation. APS is a branching point as it can enter a reductive assimilation via APS reductase and be phosphorylated by APS kinase. In the reductive branch of sulfate assimilation, a key enzyme is amino acid skeleton of O-acetylserine (OAS) to form cysteine, which can be incorporated into peptides and proteins or serve as a donor of reduced sulfur for methionine and coenzyme syntheses. SULTR, sulfate transporter; APS, adenosine-5′-phosphosulfate; SAT, serine acetyl transferase; γ-EC, γ-glutamylcysteine; OAS, O-acetylserine; CoA, acetyl coenzyme A; SAM, S-adenosylmethionine (S-AdoMet).

integrated into the amino acid frame of *O*-acetylserine (OAS) to form Cys. However, APS can also be phosphorylated by APS kinase (APK) to form 3-phosphoadenosine 5-phosphosulfate (PAPS), a donor of activated sulfate for many sulfation reactions. Both plants and microorganisms are able to reduce sulfate into sulfide and incorporate it into organic metabolites (Takahashi et al., 2011). The S-assimilation pathway involves the enzymes, ATP-S, APS kinase, PAPS reductase or APS reductase, and SiR to synthesize cysteine (Krueger et al., 2010; Khan et al., 2013; Herrmann et al., 2014;Anjum et al., 2015; Fig. 18.1). Moreover, in sulfide assimilation, two different enzymes are also involved, that is, SAT, which produces OAS from Ser and acetyl-CoA, and O-acetylserine/O-acetylhomoserine sulfhydrylase, which is involved in the transfers of sulfide at the β-position of OAS. Both of these enzymes are constrained in the cytosol, chloroplast, and mitochondria, contrasting to those of the reductive phase (Saito, 2000, 2004; Hell et al., 2002). Notably, S homeostasis and response to trace element exposure are linked to plants. Treatment of plants with trace elements leads to increases in both sulfate uptake (Nocito et al., 2002, 2006; Van Hoewyk et al., 2008) and induction in the activities of enzymes involved in S-assimilation (Cobbett, 2000; Lafaye et al., 2005; Herbette et al., 2006; Weber et al., 2006). In contrast, S-assimilation is too stoutly consistent with the assimilation of nitrate as well as carbon (reviewed by Kopriva and Rennenberg, 2004; Nero et al., 2009).

18.2.2 Sulfur Transport Mechanism in Plants

S is present in plants chiefly in the oxidized form of inorganic sulfate. S is taken up by the roots as sulfate into plants cells via sulfate transporters (SULTRs) SULTR 1;2 (sulfate-H^+ co-transporters M) and is motivated by the electrochemical gradient established by proton motive force created by ATPase (Leustek et al., 2000; Saito, 2000; Takahashi et al., 2000; Hawkesford, 2000, 2003). A number of genes encoding sulfate transporters were reported to be cloned and characterized in various plant species (Smith et al., 1997; Takahashi et al., 1997, 2000; Bolchi et al., 1999; Vidmar et al., 1999, 2000; Rae and Smith, 2002; Shibagaki et al., 2002; Yoshimoto et al., 2002, 2003; Howarth et al., 2003; Hopkins et al., 2004). Approximately 12−16 genes are reported to encode SULTR in plant species. According to their protein sequence similarities SULTR proteins can be classified into SULTR 1−5 (for a review: Buchner et al., 2004; Davidian and Kopriva, 2010; Hawkesford, 2003). Furthermore, 12 SULTRs, classified into four different groups (SULTR1, SULTR2, SULTR3, and SULTR4) were reported from *Arabidopsis thaliana* (Takahashi et al., 2012). Their functions in plants and tissue localization have been widely studied (Davidian and Kopriva, 2010; Takahashi et al., 2011). Besides this, the high-affinity transporters SULTR1;1, SULTR1;2, and SULTR1;3 consist of groups 1 sulfate transporters. SULTR1;1 and SULTR1;2 were reported to be expressed in the epidermis and cortex of roots and facilitating the initial uptake of sulfate from the soil (Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). Additionally, it was stated by Yoshimoto et al. (2003) that SULTR1;3 is limited to a small area in the phloem and mediates source-to-sink translocation of sulfate; besides this the group 4 sulfate transporters SULTR4;1 and SULTR4;2 are constrained to the tonoplast and are concerned in remobilization of vacuolar sulfate collection (Kataoka et al., 2004a). There are two ATP-S

isoforms present in plants. A minor ATP-S form is localized in the cytoplasm and a major ATP-S form is localized in plastids. Moreover, the enhanced in steady-state levels of mRNAs for high-affinity sulfate transporters, ATP-S, and APR upon S undernourishment is detected by Northern analysis (Takahashi et al., 1997; Yamaguchi et al., 1999) or cDNA arrays (Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003).

18.2.3 Role of Sulfur and its Compounds in Plant Metabolism

Sulfur metabolism has been described both at the biochemical and molecular levels, and was shown to play crucial roles in plant metabolism and physiology (Schmidt and Jager, 1992; Leustek et al., 2000; Hell et al., 2002; Hesse and Hoefgen, 2003) (Fig. 18.2). S-metabolism and its metabolites play a significant role in modulating plant stress responses (Rausch and Wachter, 2005; Khan et al., 2013). Furthermore, S-metabolism in plants is a highly regulated mechanism that adjusts the production of S-containing metabolites on demand, availability of S, and also changes in the environmental conditions and in response to prevent accumulation of toxic substances (Takahashi et al., 2011). Sulfur may up- or downregulate mechanism of plant growth and development through its control on diverse metabolic activities; glutathione (GSH) acts as an important antioxidant under stress and is also the main nonprotein source of S to the plants (Kopriva et al., 2004; Ghelfi et al., 2011; Rennenberg and Herschbach, 2012; Seth et al., 2012). Furthermore, GSH is a universal molecule, which acts as an imperative part of plants related to cellular defense, signal transduction, redox status, and detoxification processes (Noctor et al., 2012). GSH controls S-assimilation and plays crucial roles in various plant processes including cell differentiation, cell death, balance in redox status, defense against biotic and abiotic stresses, protein folding, as the precursor of phytochelatins, and in detoxification of xenobiotics (Foyer et al., 2001; Mullineaux and Rausch, 2005). The key enzyme in S-metabolism is the OAS-TL that catalyzes the formation of Cys via OAS (Youssefian et al., 2001). The production of GSH against biotic and abiotic stresses is the key preventive step in Cys biosynthesis that can be regarded as the elite function of S reduction in plants (Youssefian et al., 2001; Mera et al., 2014). In GSH biosynthesis, the availability of Cys is an additional vital thing, but a supportable supply of glutamate and glycine is also important (Kopriva and Rennenberg, 2004).

Szalai et al. (2009) reported that GSH maintains Cys, homocysteine, enzymatic proteins, ascorbate (AsA) in active form and may standardize the thiol/disulfide ratio in proteins and defend cell membrane against H_2O_2 and free radicals. Additionally, in plant metabolism, SAM is one of the most imperative S-compounds (Azevedo et al., 2006). It is mainly a methyl donor involved in transmethylation of nucleic acids, proteins, polysaccharides, and fatty acids (Ma et al., 2003). However, glucosinolates (GS) are secondary metabolites derived from amino acids, consisting of a thioglucose moiety, a sulfonated aldoxime, and a side chain obtained from aliphatic or aromatic amino acids (Halkier and Gershenzon, 2006).

18.3 CROSSTALK BETWEEN SULFUR AND PHYTOHORMONES

Evidence from recent literature reveals that phytohormones are vital for regulation of S nutrition (Ohkama

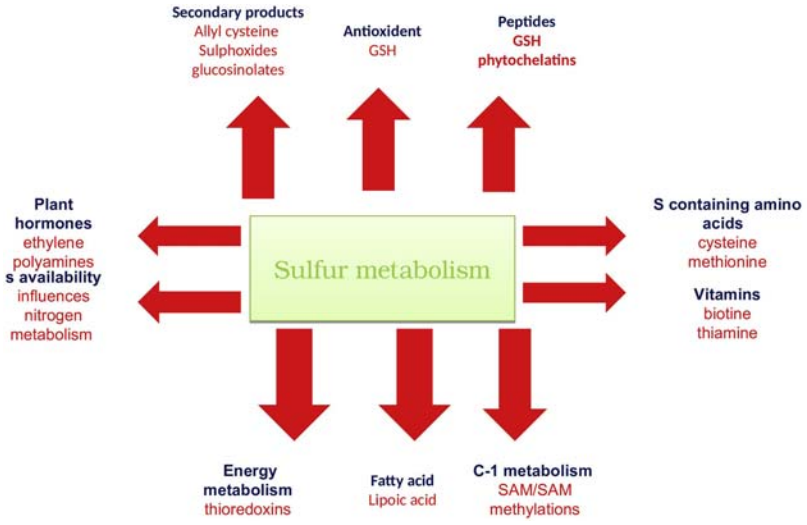

FIGURE 18.2 Diagram represents the involvement of S in the biosynthesis of plant metabolites or physiological functions.

et al., 2002; Maruyama-Nakashita et al., 2003). Besides this, some studies have reviewed the significance of the association between S-assimilation and phytohormones (Maruyama-Nakashita et al., 2004, 2005; Kopriva, 2006). According to Peleg and Blumwald (2011) phytohormones are crucial for plant acclimation and adaptation to environmental changes. Moreover, in the plant developmental processes and metabolism and plant defense pathways, efficient nutrient use is linked to signaling pathway of phytohormones (Fatma et al., 2013).

Additionally, phytohormones can interrelate with mineral nutrients under both stress and normal conditions, performing a vital role in salt stress control and disturbing plant growth recovery, germination, cell division, and seed production, still when applied exogenously (Fatma et al., 2013; Khan et al., 2015b; Per et al., 2018). At the same time as phytohormones have a large impact on S-metabolism, some of them require these pathways for their synthesis. A good example is ET; according to Sauter et al. (2013) the synthesis of ET is securely linked with Met metabolism. The substrate for ET biosynthesis, SAM, the activated form of Met is capable of synthesizing the 1-aminocyclopropane carboxylate (ACC). In a Yang cycle the coproduct of ACC synthesis, 5 methylthioadenosine, is cast-off to Met and at the same time as it serves plants as a single source of S it also inhibits ET production (Sauter et al., 2004). Undoubtedly, among plant hormones, ET is most closely connected with S-metabolism. Studies have shown (Brenner et al., 2005; Kopriva, 2006) CK is well known to be connected to the numerous processes of micronutrients, such as N cycle and its metabolism, and P assimilation. It seems that CK has a common role in the assimilation of the above nutrients, including sulfur. In addition, CKs were reported to decrease the expression of genes encoding macronutrient transporters, that is, nitrate, and phosphate and sulfate transporters. Besides this, CKs were reported to reduce the expression of SULTR1;1 and SULTR1;2, resulting in a reduction of sulfate uptake in roots (Maruyama-Nakashita et al., 2004). In general, polyamines are not considered as phytohormones, but are required to be present in higher concentrations for their action. Besides this, there is another group of growth promoting factors, called small signaling peptides, that act more like phytohormones (Czyzewicz et al., 2013).

For the synthesis of one more phytohormone, ABA, S is indirectly involved and is consequently affected by S deficient conditions. In comparison with ET, the relationship between S-assimilation and ABA synthesis is not quite direct; for the metabolic reactions S is not required (Cao et al., 2014). According to Cao et al. (2014), sulfate deficiency leads to reduced rate of Cys synthesis, resulting in lower tolerance to abiotic stress and steady-state levels of ABA. GA was reported to enhance the effects of salt stress by regulating the availability of other phytohormones in soybean plants Capaldi et al. (2015). Phytohormones, for instance, JA, ABA, SA, and ET play vital roles in the accumulation of GSH under stressful conditions. On the other hand, ET and SA regulate S-metabolism to induce salt tolerance (Kopriva and Rennenberg, 2004; Iqbal et al., 2013; Nazar et al., 2013; Asgher et al., 2014). Additionally, both SA and NO were reported to require GSH for their action. S-nitrosoglutathione (formed when NO is conjugated to GSH) on other hand is indispensable for its transport and to facilitate nitrosylation of proteins (Stamler, 1994). However, evidence for direct regulation of S-metabolism is insufficient. Therefore, we can say that sulfate assimilation pathway is not only required by phytohormones for their actions, it is also crucial for the synthesis of at least some.

18.3.1 Auxin

Auxin is among the most versatile plant hormones and is found to play vital roles in growth and development of plants, and was reported to act as a signaling molecule under sulfate limitation (Nikiforova et al., 2003). Even as AU interacts with diverse aspects of S-metabolism, direct regulation of the pathway by this hormone is not well understood. Additionally, the first link is the production of indole 3-acetic acid (IAA), and indole-3 acetaldoxime, indolic glucosinolates are common intermediates between AU and S (Mikkelsen et al., 2004). On the other hand, alterations in indolic glucosinolates synthesis are frequently associated with interruption of AU homeostasis (Barlier et al., 2000; Douglas Grubb et al., 2004; Reintanz et al., 2001). Interestingly, the next chief link between AU and S is directly associated with the function of AU in control of root growth (Koprivova and Kopriva, 2016). Several studies reveal S starvation upregulated those genes that are expressed of AU inducible such as IAA18, At1g51950, tryptophan synthase βchain, At5g38530, putative AU-regulated protein, At2g33830 (Nikiforova et al., 2003; Hirai and Saito, 2004), and indole-3-acetonitrile to IAA is converted by NIT3 nitrilase, and powerfully increased by S starvation (Kutz et al., 2002). According to López-Bucio et al. (2003) AU production may result in an increased in lateral root density in *Arabidopsis* under sulfate-limited conditions. Furthermore, in wild-type, *Arabidopsis* suppresses lateral root development by S deficiency, whereas this response is compromised in the axr1−3 mutant (Dan et al., 2007). In sulfate starvation, microarray results revealed the expression of tryptophan metabolism and some AU responsive genes (Nikiforova et al., 2003; Koprivova and Kopriva, 2016). Based on the

transcriptome and metabolomic facts AU influx and the IAA28-mediated AU signaling circuit play vital role in modulating S response (Nikiforova et al., 2005a,b). Under the exogenous application of AU, S deficiency-activated BGLU28 (β-glucosidase 28) expression is downregulated. In several of the studies, it is hypothesized that BGLU28 acts by releasing S from glucosinolate, which is possibly a major S derived compound in plant cell vacuoles (Maruyama-Nakashita et al., 2003, 2006; Dan et al., 2007). AU signaling component AXR1, a component of the 26S proteasome, was also found to be involved in the S starvation (Dan et al., 2007).

18.3.2 Cytokinin

CKs are recognized to use authority on the gaining of several macronutrients, such as N, P, and S. Particularly, CKs decreased the expression of genes encoding multiple macronutrient transporters, such as nitrate transporters, sulfate transporters, and phosphate transporters (Brenner et al., 2005). Whereas jasmonate, ET, and ABA can be considered as stress hormones, the participation of CKs in the regulation of S-metabolism is probably the most observable. Additionally, CKs are well known to act together with mineral nutrient plants status (Collier et al., 2003; Franco-Zorrilla et al., 2005; Nam et al., 2012; Sakakibara et al., 2006). Studies have shown (Leustek et al., 2000) that APR1 is a fundamental enzyme in S-metabolism and its upregulation by CKs also suggests that S-assimilation is also regulated by CKs. However, the direct link of CKs and S has been recognized when CKs were initiated to apply a negative effect on expression of SULTR1;1 and SULTR1;2, resulting in sulfate uptake by roots being reduced (Maruyama-Nakashita et al., 2004). Generally, the reduction in transcript levels was accompanied by a reduction in sulfate uptake and was alleviated in *cre1* mutants (Maruyama-Nakashita et al., 2004). Apart from this, CKs are also involved in upregulation of APR and SULTR2;1 genes S limitation in *Arabidopsis* (Ohkama et al., 2002).

18.3.3 Abscisic Acid

ABA appears to be the phytohormone that responds more quickly and has the biggest impact on sulfate assimilation. In plants under drought stress, ABA biosynthesis and expression of genes induced by ABA resulted in stomatal closure (Peleg and Blumwald, 2011). Under exogenous ABA application the upregulation of SULTR3;1 transcription in roots indicates that it is essential for a significant coregulation of plastid sulfate uptake (Cao et al., 2013). Sulfate has been reported to affect ABA root-to-shoot signaling that affects stomatal closure during early stages of water stress in maize plants (Ernst et al., 2010). Conversely, in some circumstances, mostly in salt and drought stress, ABA is important for signaling and has a great impact on sulfate assimilation as these conditions are accompanied by increased production of ROS. Hence, they reduced S and form a higher demand for GSH. Most enzymes of sulfate assimilation such as APS reductase, ATP-S, SAT, sulfite reductase, cytosolic isoform of OAS-TL, and sulfate transporters SULTR1;2, 3;1, 3;4 and 4;1 are induced by salts (Barroso et al., 1999; Ruiz and Blumwald, 2002; Koprivova et al., 2008; Cao et al., 2014). According to Zhu (2002), salt stress signaling includes both ABA-dependent and ABA-independent pathways. ABA was reported to suppress the growth of lateral root to enable the growth of primary root to access water deep inside the ground during drought (Xiong and Fei, 2006). Calderwood and Kopriva (2014) showed that many studies were conducted on the role of hydrogen sulfide as a new gas signal in plants during the last few years, both in humans and animals. The well-known function of H_2S is in the regulation of stomata opening (García-Mata and Lamattina, 2010; Lisjak et al., 2010; Scuffi et al., 2014). The regulation of stomata opening is intermittent in mutants in the enzyme producing H_2S (Scuffi et al., 2014), Furthermore, stomata are closed in the *cad2* mutant having high Cys level and low GSH level (Okuma et al., 2011; Noctor et al., 2012), therefore for correct ABA function a steady supply of Cys is essential. It seems that for ABA there is an interconnection between other phytohormones and sulfate assimilation rather than a unidirectional regulation. However, in the ABA biosynthesis pathway, the conversion of an abscisic aldehyde to ABA is catalyzed by abscisic aldehyde oxidase (EC 1.2.3.1, AAO3), which requires the sulfurated molybdenum cofactor (Mendel and Hänsch, 2002; Schwarz and Mendel, 2006). Sulfuration of Moco is essential for ABA biosynthesis and has been demonstrated by mutating ABA3 (AT1G16540) resulting in ABA deficiency (Xiong et al., 2001). In response to environmental stresses, ABA induces mRNA accumulation of cytosolic OASTL. Accumulation of ABA induces negative role of AtGSTUI7 in stress tolerance by affecting GSH pool (Chen et al., 2012a,b) and indicating that it has more profound effect on control of S-metabolism under stress conditions (Kopriva, 2006). In *Arabidopsis*, ABA plays an important role in maintaining the redox state by increasing GSH level (Jiang et al., 2010).

18.3.4 Gibberellic Acid

The exogenous application of GA enhances photosynthesis, growth, and utilization of N in mustard. However there is a high requirement of S in mustard

and the assimilatory pathway of S is synchronized with the N. It has been studied that the application of GA_3 to mustard enhances the photosynthetic production with the higher efficiency of N utilization, which in turn increases the S-use efficiency (SUE) of crops (Khan and Mobin, 2005).

It has been studied that the application of GA upregulates the transcript level of APR1 and APR2 in *Arabidopsis* (Koprivova et al., 2008). The GA_3 application to plants accelerates the uptake of N and S, however, their utilization is interlinked (Khan et al., 2007). It has been suggested that the utilization of N is impossible in mustard grown in S deficient soil (Khan and Mobin, 2005).

18.3.5 Ethylene

The crosstalk between S-assimilation and ET signaling in plants is considered important because of the close link between ET synthesis and S-metabolism through the Met, a S-containing amino acid that is a substrate for S-adenosyl methionine synthase (SAMS) dependable for the production of SAM or AdoMet (Fontecave et al., 2004; Roje, 2006; Khan et al., 2017). Met and SAM used for polyamines (PA), nicotianamine (NA), and ET biosynthesis are recycled in the Met salvage cycle (known also as a Yang cycle). Significantly, soluble Met seems to be a rate-limiting metabolite of ET biosynthesis (Katz et al., 2006; Bürstenbinder et al., 2007). Noteworthily, sulfate limitation 1 (SLIM1) is the key regulator of sulfate deficiency responses, which is the considerable tie between S and ET signaling. Maruyama-Nakashita et al. (2006) have reported that SLIM1 is constituent of a family of five ethylene insensitive 3-like (EIL) transcription factors. EIN3 also acts as a transcription factor that controls a bulky number of ET-responsive genes (Chao et al., 1997). Additionally, EIN3 regulated the many S related genes (Chang et al., 2013). Interestingly, in *Brassica napus* investigation for genes' fundamental variation in nutrient homeostasis two genes of ET signaling, EIN2 and EIN5, were shown by associative transcriptomics to be associated with a deviation in sulfate and phosphate levels (Koprivova and Kopriva, 2014). ET signaling is crucial not only for the SLIM1 facilitated response of the sulfate assimilation pathway and managing S metabolite pools, but also for nutrient homeostasis. The interconnection of ET and S has been very freshly reviewed in great detail (Wawrzynska et al., 2015). Recent reports suggested that the combined application of ethephon and S to cadmium (Cd) treated mustard synergistically enhanced the photosynthetic performance in comparison with Cd-treated plants by reduction in oxidative stress and diminishing the glucose and ET sensitivity with an increase in Met, Cys, GSH (Khan et al., 2016). In *Arabidopsis*, the application of ET leads to increase in mRNA level of APR1 and APR3 (adenosine 5′-phosphosulfate reductase), which plays a pivotal role in S-assimilation (Koprivova et al., 2008).

18.3.6 Jasmonic Acid

The primary and secondary S-metabolism are coordinately affected by jasmonates and during sulfate deficiency jasmonate synthesis is induced (Jost et al., 2005; Nikiforova et al., 2003). JA is an endogenous growth regulator reported to induce a range of physiological and developmental responses in many plant species (Engelberth et al., 2001) and may also act as a stress modulator by enhancing or suppressing the stress responses of plants (Agrawal et al., 2002). Additionally, several studies have revealed that jasmonate or methyl jasmonates (MeJA) regulated those genes that are involved in sulfate reduction, such as Cys content, GSH synthesis, and glucosinolate metabolism at the transcriptional stage (Xiang and Oliver, 1998; Harada et al., 2000; Jost et al., 2005; Sasaki-Sekimoto et al., 2005; Shan and Liang, 2010; Gfeller et al., 2011). In *Arabidopsis thaliana* treated with MeJA, the levels of mRNA increased consequently to many genes concerned in S-assimilation and GSH synthesis, without affecting the SO_4^{-2} transporters with associated mRNA levels and content of S metabolites (Kopriva, 2006). Hence, JA has a signaling role for suggesting S-assimilation below S shortage (Sasaki-Sekimoto et al., 2005; Srivastava et al., 2012). Analysis using cDNA macroarrays of jasmonate regulated metabolic pathways in *Arabidopsis* showed that jasmonates activate the expression of factors involved in nine metabolic pathways belonging to two functionally associated groups: (1) biosynthesis of indole glucosinolate, a defense compound occurring in the Brassicaceae family; and (2) glutathione and ascorbate metabolic pathways, which are important in defense responses to oxidative stress (Sasaki-Sekimoto et al., 2005). Again the S deficiency responsive APR1 gene is also reported induced under JA treatment (Sasaki-Sekimoto et al., 2003).

18.3.7 Salicylic Acid

While acting as an endogenous signaling molecule SA was found to play crucial roles in plant defense mechanisms, by regulating both physiological and biochemical processes (Gunes et al., 2007; Joseph et al., 2010). SA indirectly assimilates the inorganic S by activating the S assimilatory pathway enzymes' SAT and

GSH content and increased the heavy metal resistance (Iqbal et al., 2015). It has been found that the SA accelerates the S uptake and metabolism by regulating the biosynthesis of GSH in ozone exposed *Arabidopsis* (Yoshida et al., 2009). It has been suggested that various phytohormones like JA, ABA, and SA upregulate the expression of genes that are related to S-metabolism, and hence play a pivotal role in plant stress defense by increasing cellular S-containing compounds like Cys, GSH, thiols, phytochelatins, glucosinolates, etc. (Kutz et al., 2002; Rausch and Wachter, 2005). It has been found that the various S-containing secondary metabolites like thionines, glucosinolates, and GSH were increased by the exogenous application of SA under various abiotic stresses in *Brassica napus* (Kumar et al., 2015; Kiddle et al.,1994). Nazar et al. (2011b) have shown that the application of 0.5 mM SA increases the inorganic S content in salt-treated mung bean and this inorganic S is easily incorporated into the amino acid Cys through the cascade of enzymatic process. However, the Cys acts as a key regulatory amino acid in the C-terminal region of the transactivation domain of SA receptor NPR1 (Rochon et al., 2006).

18.3.8 Nitric Oxide

NO is a gaseous, signaling, small reactive molecule that interacts with diverse cellular compounds, including other radical species (Correa-Aragunde et al., 2015), and participates in multiple biological processes in plants (Neill et al., 2003; Wendehenne et al., 2004; Crawford and Guo, 2005). It has been suggested that NO triggers the induction of seed germination and reduction of seed dormancy (Beligni and Lamattina, 2000; Libourel et al., 2006; Zhang et al., 2009), induction in cell death (Pedroso and Durzan, 2000), and regulation of plant metabolism and senescence (Ya'acov et al., 1998; Guo and Crawford, 2005). Furthermore, it is also involved in responses to abiotic and biotic stresses, and apoptosis (Delledonne et al., 1998; Garcıa-Mata and Lamattina, 2002; Zhao et al., 2004; Zhang et al., 2006).

NO requires GSH for its action. GSH reacts with NO and NO-derived peroxynitrite to produce nitrosoglutathione (GSNO) (Barroso et al., 2006), which is crucial for enhanced S requirement of plants for better survival (Wang et al., 2015). In addition, this metabolite can be converted by the enzyme GSNO reductase (GSNOR) into oxidized glutathione (GSSG) and NH_3 (Leterrier et al., 2012). Besides this GSNOR regulates the cellular levels of GSNO maintaining NO homeostasis, which is fundamental for transient cell signaling (Malik et al., 2011). Moreover, according to Frungillo et al. (2014) GSNO also regulates the NO concentration in the cell via inhibition of the N assimilation pathways. In the process of nitration, NO also activates diverse biochemical pathways and interacts with metals to fabricate metal proteins, and with sulfhydryl groups and nitro groups to provide resistance against salt stress (Leterrier et al., 2012). Feng et al. (2013) proposed that NO negatively regulates CK signaling by restrictive phosphorelay activity via S-nitrosylation. Furthermore, NO acts in a chief role for a salt-induced increase in APR whereas expression of all three APR isoforms and the enzyme activity are induced by salicylate (Koprivova et al., 2008). Additionally, NO regulate stomata aperture dependent on hydrogen sulfide (Scuffi et al., 2014). Noticeably more research is desired to discover how NO is involved in S-metabolism.

18.3.9 Brassinosteroids

BRs are the ubiquitous steroidal component considered as a new phytohormone in the plant kingdom; they have pleiotropic effects, which impact various developmental processes like rhizogenesis, seed germination, flowering, abscission, and maturation (Rae and Smith, 2002). In addition to the developmental process, BRs play an important role in abiotic stress (Kapoor et al., 2014). Foliar spray of 24-epiBL has shown a steep rise in enzymatic and nonenzymatic components of the defense system during abiotic stress (Ramakrishna and Rao, 2013; Kapoor et al., 2014). However, there is no interplay link found between S-metabolism and BR hormones.

18.4 CONCLUSION

Sulfur has emerged as a requisite nutrient for plant growth and survival owing to the role of S assimilates in structural components of essential cellular molecules and signaling networks. Sulfur-containing compounds (amino acids, vitamins, thioredoxin system, glutathione, lipoic acid, and glucosinolates), play significant roles in plant metabolism and stress response. It has become evident from the literature that S regulates various plant processes and also is the requirement for the activity of almost all the phytohormones-mediated responses.

The involvement of phytohormones in plant growth and development is well accepted. These phytohormone-mediated responses are influenced by several factors. Physiological and biochemical insights are available on the outcomes of the crosstalk of S-assimilation with phytohormones such as auxin, gibberellins, cytokinin ethylene, abscisic acid jasmonates, salicylic acid, and nitric oxide. However, it has not been emphasized

how the availability of sulfur regulates the action of phytohormones. The present chapter has emphasized the importance of S in regulation of phytohormones-mediated responses. It appears that S is the essential requirement for phytohormones to deliver their best. It would be interesting to get more insights into the molecular genetics of the aforesaid aspects. If done, the outcomes of these studies can help us in sustainably improving plant health and productivity under abiotic stress conditions.

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CHAPTER

19

Role and Regulation of Plant Hormones as a Signal Molecule in Response to Abiotic Stresses

Palak Bakshi[1], *Neha Handa*[1], *Vandana Gautam*[1], *Parminder Kaur*[1], *Shelja Sareen*[2], *BilalAhmad Mir*[3] *and Renu Bhardwaj*[1]

[1]Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India
[2]Department of Biotechnology, BBK DAV College for Women, Amritsar, Punjab, India
[3]Department of Botany, School of Life Sciences, Satellite campus Kargil, University of Kashmir, Srinagar, Jammu and Kashmir, India

OUTLINE

19.1 INTRODUCTION

Plants are multicellular organisms that sense the variations in environment and communicate and coordinate with various signaling cues. Various environmental factors that affect plant growth and productivity include drought, temperature, salinity, flooding, UV radiation, heavy metal (HM) toxicity, etc. Due to anthropogenic activities the pace of plants to get exposed to various abiotic stresses during their complete life cycle has been increased. However, plants adapt themselves to ever-changing environmental conditions by rapidly detecting and responding to external changes, which shows the plasticity of phenotype and represents their dynamic nature. They effectively evaluate and regulate internal as well as external signals and respond accordingly.

Plant growth regulators (PGRs) are synthesized at very low concentrations and act as effective chemical messengers, communicating and coordinating the

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cellular activities in plants. They include auxin (AUX), cytokinin (CK), abscisic acid (ABA), gibberellins (GA), ethylene, jasmonic acid (JA), salicylic acid (SA), nitric oxide (NO), and brassinosteroid (BR). These plant growth regulators are derived from plant biosynthetic pathways and manage to play a dual function in plants. They not only play an essential role in growth and development of plants but also act as signal molecules in response to various biotic and abiotic stress conditions (Zhao et al., 2016). ABA, a 15-carbon compound synthesized by mevalonic acid pathway in chloroplast and other plastids is considered as an effective signal molecule in plant defense against various abiotic stress conditions (Keskin et al., 2010). SA, a phenolic nature plant growth regulator synthesized from shikimate pathway has a well-established role in abiotic stress by redox regulation, synthesis of heat shock proteins, and osmotic balance (Khan et al., 2015). In plants, SA enhances the resistance against salt, drought, and temperature stress (Yuan and Lin, 2008). CKs are adenosine substituted compound synthesized by two distinct pathways, the de novo biosynthetic pathway and t-RNA pathway, playing an effective role in various plant developmental processes as well as in stress responses by interacting with other PGRs (Zwack and Rashotte, 2015). GAs are terpenoid compounds synthesized by methyl erythritol phosphate pathway in plastids and also play an important role in various developmental process of plants, that is, growth, germination, dormancy, flowering, senescence, as well as in defense against abiotic stress conditions by enhancing or reducing expression of GA genes such as *GA2ox* and DELLA (Sharan et al., 2017). JA, an organic compound synthesized by octadecanoid pathway from linolenic acid known to have role in various physiological processes as well as significant role in plants to withstand stress conditions. ABA, ethylene, SA acts as important mediator along with mitogen activated protein kinase (MAPKs) in JA signaling and synthesis (Ahmad et al., 2016). Brassinosteroids (BRs), a class of steroidal PGR, play an essential role in enhancing stress resistance by upregulating the expression of stress marker genes along with plant growth and development (Krishna et al., 2017). Ethylene, olefinic gaseous PGR synthesized from methionine, shows increase in its biosynthesis in plants under various abiotic stress conditions, which shows its pivotal role in stressed conditions as well (Thao et al., 2015; Khan and Khan, 2014; Khan et al., 2014). These plant growth regulators act as effector molecules that perceive the signals, respond downstream by cascading effects, and are involved in ubiquitin proteasomal processes, which trigger the stress response by altering the gene expression (Santner and Estelle, 2010). Expression of different antioxidative enzymes like superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), dehydroascorbatereductase (DHAR), and monodehydroascorbate reductase (MDHAR) get modulated in response to various abiotic stress conditions (Khan and Khan, 2017). However, these PGRs also modulate synthesis or response of various other plant hormones, which show they are interrelated with each other either synergistically or antagonistically (Peleg and Blumwald et al., 2011).

In the present chapter, an attempt has been made to understand the recent advances on PGRs as defense responsive molecules. It also covers signal transduction of PGRs, their crosstalk, and defensive responses under various abiotic stresses.

19.2 PERCEPTION AND TRANSDUCTION OF SIGNALS

Perception of external changes and internal signaling helps the plant to respond accordingly leading to diverse signaling pathways, which initiates various stress responses. However, the process of the signal transduction pathway is regulated by a sensitive network in which each component, such as sensors, secondary messengers, phosphoprotein cascades, transcription factors, phytohormones, and stress-responsive genes plays an essential role. Eventually the sudden and quick response will be triggered to defend plants from damages in a large sense. Signal transduction depends on various cellular activities and their coordination, which is a complicated process as most of the steps take place in a time- and space-dependent manner (Todaka et al., 2017).

Plant hormones, besides playing an important role in plant growth and development, are also considered as phytomolecules of prime importance in intercellular regulation and transduction of signals (Busch and Benfey, 2010). The generation of signal transduction response through phytohormones and secondary messengers is generated after the primary response like increase in cytosolic calcium. In recent years, regulation and functions of various phytohormones like auxin, cytokinin, ABA, jasmonic acid, ethylene, and brassinosteroid have been reported (Fig. 19.1). Production, accumulation, and gene upregulation of these phytohormones like synthesis of ABA in response to stress have been well reported.

Phytohormones act as systemic signal molecules and transfer information to different parts, for example, ABA can be transported at various distant sites away from where it is produced and plays important physiological roles (Sauter et al., 2001). Various plant cells respond differently even for the similar hormone signals and even different hormones interact with each

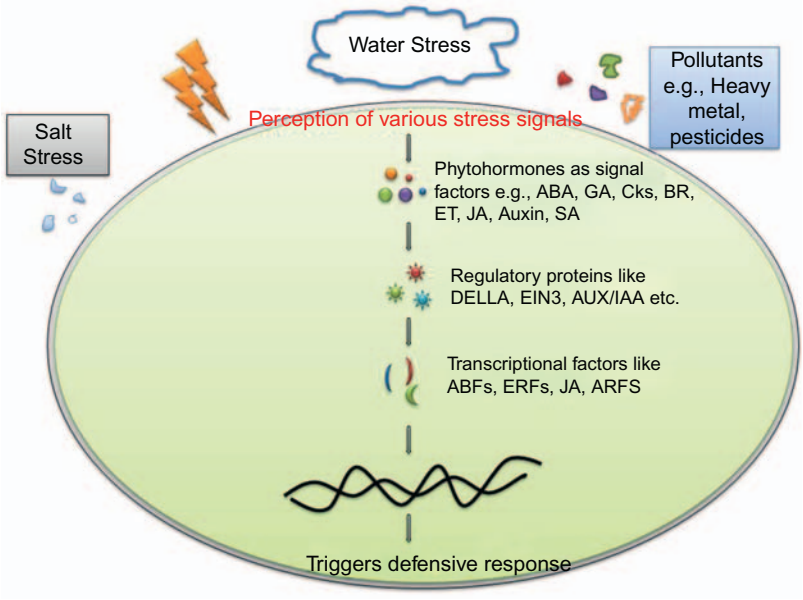

FIGURE 19.1 Signal transduction of plant growth regulators in plants.

other for coherent response to cells. The response to these signals occurs at the molecular level in the form of gene regulation, which could be perceived and studied using transcription (Goda et al., 2008).

19.3 REGULATION OF VARIOUS ABIOTIC STRESSES BY PLANT HORMONES

19.3.1 Water Stress (Deficit or Flooding)

Water stress causes severe loss in plant development. Both water deficit and excess water conditions end up in decreased crop production. Excess water leads to flooding, which directly affects the supply of oxygen to plant root cells. Inhibition of oxygen supply results in distortion of root functions such as nutrient absorption and uptake. However, under deficit conditions shortage of water availability results in damage of membranes. This is due to the increased porosity in the cell membrane, which disrupts the bilayer structure of the cell wall. Water logging and deficit conditions cause various anatomical, physiological, and molecular changes in plants. Plants face stunned growth due to the deficit supply of oxygen under waterlogged conditions (Sairam et al., 2008). Decline in photosynthesis, respiration due to hindrance in gas diffusion in flooding situation were observed (Amador et al., 2012). Similarly water deficit conditions result in decreased crop and fruit production, reduction in growth, shoot length, and area of leaf as well as decreased gas exchange (Deblonde and Ledent, 2001; Bourtsoukidis et al., 2014). Various factors control the plant response to water stress, such as opening and closing of stomata, which is mainly controlled by ethylene and ABA (Shinozaki and Yamaguchi Shinozaki, 2000). Response to dehydration in the content of plant hormones in *Arabidopsis* wild-type (WT) plants and genes regulating biosynthesis of ABA given dehydration treatment was observed. Increase in the content of ABA was observed in early phases of water deficit conditions (Urano et al., 2017).

19.3.1.1 Role of Abscisic Acid Under Water Stress

ABA has been reported to have an important role in water stress by stimulating sudden responses in plants like closing and opening of stomata and control of ion homeostasis (Pandey et al., 2017). ABA also affects the long term effects in plants due to water stress by regulating the genes responsible for stress. Water scarcity or flooding induces ABA production in leaves of the various plant species (Jiang and Zhang, 2002; Nayyar et al., 2005; Satisha et al., 2005). Destressing of plants resulted in reversed levels of ABA content in plants. Enhanced ABA levels restrict plant water loss via transpiration through stomata by inducing closure of stomata, which increases osmotic balance of plants and results in increased root hydraulic conductivity (Thompson et al., 2007; Zandalinas et al., 2018).

The amount of ABA content accumulated in guard cells depends upon the various factors like plant species, leaf age, climatic conditions, plant nutrient status, etc. ABA also upregulates the expression of various genes under water stress such as *ZEAXANTHIN*

EPOXIDASE gene (*ZEP*), *LOW EXPRESSION OF OSMOTIC STRESS-RESPONSIVE* gene (*LOS6*), and *ALDEHYDE OXIDASE* gene (*AAO3*). Under water deficit conditions, the concentration of ABA increases abruptly to a level that induces efflux of ion and disruption of uptake of sugars and reduction in aperture size of the stomata. Reports of enhancement of ABA content by 30 times under drought stress have been recorded (Outlaw, 2003).

Exogenous application of ABA has been reported to enhance plant tolerance to water stress (Marcinska et al., 2013; Javid et al., 2011). The increase in the production of ABA during drought is due to the upregulation of genes encoding enzyme NCED3 (9-*cis*-epoxycarotenoid dioxygenase). It was reported by Thompson et al. (2000) that plants given dehydration conditions result in instant increase in *NCED3* level significantly after 15–30 min. Induction of genes *AtNCED3*, *AtZEP* (Zeaxan thin epoxidase), and *AtAA03* (ABA-aldehyde oxidase) in *Arabidopsis* and overexpression of *OsNCED3* in rice was observed after dehydration (Cheng et al., 2002; Ye et al., 2011).

During drought, the key role of ABA in plants is the ion homeostasis and inhibition of intracellular water loss by stimulating root–leaf signaling and ultimately inducing stomatal closure (Wilkinson and Davies, 2002; Tuteja, 2007). ABA accumulation induces the efflux of K^+ ion by decreasing the turgor pressure in the guard cells (Anjum et al., 2011). The ion channel responsible for exchange of various anions and K^+ ions, that is, S-type anion channels and K^+ channels (*SLAC*1 and *KAT1*), is regulated by the sucrose nonfermenting-1-related-2-protein kinases (SnRK2). The kinase SnRK2 increases the activity of S-type anion channels and restricts the KAT1. Activation of S-type anion channels in guard cells by Ca^{2+} and ABA in different plant species has been reported by Raschke et al. (2003), Roelfsema et al. (2004), and Mori et al. (2006).

Enhanced activity of SLAC1 causes efflux of anions, which leads to depolarization of the membrane as a result of phosphorylation by SnRK. The activation of membrane depolarization results in efflux of K^+ cations from the cell via guard cell outward-rectifying K^+ channel (GORK), also known as K^+ efflux channel (Jeanguenin et al., 2008). Under water deficient conditions the accumulation of ABA as well as the S-type anion channel and KAT1 is deactivated by the SnRKs.

Inhibition of KAT1 activity due to accumulation of ABA and Ca^{2+} ion in the cytoplasm has been reported (Grabov and Blatt, 1999) through phosphorylation regulated by SnRK, which in turn results in the restricted entry of K^+ ions into the guard cells (Hubbard et al., 2010). Efflux of K^+ and other anions from the guard cells cause dehydration in cells, which leads to loss of turgor pressure and closure of stomata in response to ABA.

19.3.1.2 Role of Jasmonic Acid Under Water Stress

Jasmonate biosynthesis is activated during stress conditions (Wasternack, 2007) and many of the genes involved in JA signaling are controlled by drought stress (Huang et al., 2008). The significant role of JA during drought stress in closure of stomata has been reported by Suhita et al. (2003, 2004) and Munemasa et al. (2007). Exogenous application of JA or methyl JA (MeJA) results in its conversion to JA-Ile (iso-Jasmonyl-L-isoleucine), a bioactive form of JA. It binds to the ^{SCF}COI complex, which comprises the coronatine insensitive 1 (COI 1) F-box protein (Sheard et al., 2010).

The coordination of components in a complex results in degradation of the JAZ repressor protein (Jasmonate ZIM-domain) proteasome. The CPK6 (Ca^+ dependent protein kinase 6) is identified as a positive regulator of MeJA and assist in ABA signaling in the guard cells. Similar to ABA, MeJA activates the S-type anion channels and interacts by increasing the entry of Ca^+ ions, which activates the CDPK and results in series of events eventually leading to stomatal closure during drought conditions (Munemasa et al., 2007).

19.3.1.3 Role of Ethylene Under Water Stress

Increased biosynthesis of ET under drought stress has been reported by Upreti et al. (1998). The induction of synthesis of ethylene is mainly done by enhancing the synthesis of ACC synthase and ACC oxidase (enzyme responsible for synthesis of ethylene). Accumulation of ethylene helps plants to reduce water loss by increasing plant senescence and inhibited growth. The content of ET accumulation is controlled by various factors and high dose of drought stress tends to downregulate the activity of ACC synthatase enzyme.

Under flooding conditions ET promotes adventitious root formation, which helps plants in growth by regulating nutrient and water uptake (Sairam et al., 2008; Pistelli et al., 2012). During drought direct relationship between ACC expression and biosynthesis of ethylene has been reported in citrus (Gómez-Cadenas et al., 1996). The action of ethylene in repression of ABA induced stomatal closure. The *Arabidopsis* plants exposed to gaseous ethylene tend to inhibit the closure of stomata even after application of ABA (Tanaka et al., 2005). ABA accumulation reduces the synthesis of ethylene, which results in sudden increase of ABA concentration during water stress and leads to reduced content of ethylene.

19.3.1.4 Role of Auxin and Cytokinin Under Water Stress

These phytohormones, when present inside plants during water stress, induce opening of stomata whereas when at higher concentrations they result in stomatal closure. Auxin plays a major role in controlling the movements (H^+-ATPase) across the plasma membrane (PM) in the guard cells. Efflux of H^+ from the guard cells promotes the influx of K^+, which is regulated by the hyperpolarization of the membrane. The exit of K^+ ion results in opening of stomata when auxin is present in lower content and high concentration of it leads to efflux of K^+ ions and restricts the influx leading to stomatal closure (Daszkowska-Golec and Szarejko, 2013). CKs work in association with ABA and mostly inhibit the closing of stomata (Tanaka et al., 2006).

19.3.1.5 Role of Brassinosteroids Under Water Stress

BRs work in association with ABA and promote closing of stomata to inhibit water loss through transpiration. The same have been studied in *Vicia faba* where application of EBL (epibrassinolide) indicates the stomatal closure and restricts their opening in epidermal cells (Haubrick et al., 2006). Resistance to water scarcity in *Arabidopsis thaliana* and *Brassica napus* given exogenous application of EBL causes drought resistance (Fariduddin et al., 2014). Similarly 24-EBR has been reported to promote closing of stomata in *A. thaliana* through ethylene signaling transduction pathway by activation of Gα protein, hydrogen peroxide (H_2O_2), and nitric oxide (NO) production (Shi et al., 2015).

19.3.2 Temperature Stress

Temperature stress is one of the most hazardous stresses amongst abiotic stresses and an increase by 0.2°C in temperature per decade has been predicted leading to global temperature rise by 1.8−4.0°C by the year 2100 (IPCC, 2007). Plants, in particular, are at a primary risk and face several adverse effects of stress caused due to temperatures beyond optimum levels. Heat stress especially alters growth and development, various physiochemical processes, and crop quality and yield in plants (Hasanuzzaman et al., 2013; Khan et al., 2013; Khan and Khan, 2013). Similarly, low temperatures lead to poor reproductive development, slow growth, low yield, and have adverse effects on the macromolecules present in the cells, which in turn slows down metabolic pathways and impairs membrane functions due to solidification (Smith and Stitt, 2007; Jewell et al., 2010). Many methods have been employed to enhance the tolerance of plants against temperature stress and the role of plant hormones in enhancing tolerance against temperature extremes is proving highly beneficial. Table 19.1 highlights the various hormones that, when supplied exogenously, aid in improving resistance of plants against temperature stress by modulating various physiological processes and important biomolecules.

19.3.3 Salinity Stress

Salinity is high concentration of soluble salt in the water body; it is one of the abiotic stress factors and a primary cause of concern for the human population, as it affects overall agricultural practices (Shrivastava and Kumar, 2015). Out of total land surface about 10% of the global area is affected by salt stress (Pessaraki and Szabolcs, 2010). FAO's database Aquastat shows that in some countries the severity of damage caused by salinity can be as high as 50% of the areas fully equipped for irrigation (Food and Agriculture Organization, UN, 2018 http://www.fao.org/soils-portal/soil-management/management-of-some-problem-soils/salt-affected-soils/more-information-on-salt-affected-soils/en/ (accessed on 6.04.18)) (Table 19.2).

19.3.4 Metal Stress

HMs, natural constituents of Earth's crust, lately due to prominent accumulation have become a major problem in terms of both nutrition and environment. HMs are nonbiodegradable chemical species that have genotoxic, cytogenic, and mutagenic effects on living beings (Emamverdian et al., 2015). They can enter the food chain as a result of uptake by plants (Gangwar et al., 2014). Their accumulation in plants also induces the formation of ROS. Plants have hormone regulators that regulate growth and development of plants. From time to time various research studies have shown that exogeneous applications of these plant regulators induce protection against metal induced stress and improve overall yield and biomass (Krishnamurthy and Rathinasabapathi, 2013; Srivastava et al., 2013) (Table 19.3).

19.3.5 UV Radiation

Radiation falling between the wavelength spectrums of 10−400 nm is called ultraviolet (UV) radiation. It is a component of solar radiation, shorter than visible radiation but longer than X-rays; electromagnetic in nature, and about 10 percent of solar radiation is comprised of UV radiation (Andrady et al., 2006). Ultraviolet radiation is further divided into different types according to their wavelength ranges. In recent times, as a consequence of the stratospheric ozone

TABLE 19.1 Effect of Various Hormones in Regulation of Temperature Stress

S. no.	Plant species	Stress	Hormone treatment	Response	References
1.	*Dendrobium officinale*	Chilling stress (0°C for 6 & 12 h)	SA (1.5 mM/L sprayed after 7 days of chilling pretreatment)	Improved chlorophyll content, photosynthetic rate, photochemical efficiency, and superoxide dismutase (SOD) activity; reduced content of malondialdehyde (MDA)	Huang et al. (2016)
2.	*Brassica juncea*	High temperature (44°C for 3 days)	HBL (28-homobrassinolide) presoaking for 8 h in 10^{-9} M	Reduced contents of MDA, H_2O_2, and NO; enhanced activities of SOD, catalase (CAT), glutathione peroxidase (GPOX), and ascorbate peroxidase (APOX)	Sirhindi et al. (2017)
3.	*Cucumis sativus*	Chilling stress (8°C for 3 days)	SA (50 μM foliar spraying after 4 h)	Enhanced expression of phenylalanine ammonia lyase and benzoic acid-2- hydroxylase; reduced content of H_2O_2	Dong et al. (2014)
4.	*Cynara cardunculus*	High temperature (30°C)	ET (30 μ/ML)	Increase in early root growth, root hair density, root area, and lateral roots	Shinohara et al. (2017)
5.	*Ficus concinna*	High temperature (35°C and 40°C for 48 h)	EBR (24-epibrassinolide) (0.25 μM EBR conc. at the rate 15 mL per plant)	Enhanced activities of superoxide dismutase (SOD), glutathione-S-transferase (GST), GPX, APOX, monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), dehydroascorbate reductase (DHAR), glyoxalase I (Gly I) and glyoxalase II (Gly II); increase in contents of reduced glutathione (GSH) and ascorbate (AsA); reduced contents of reactive oxygen species (ROS), malondialdehyde (MDA), and methylglyoxal (MG)	Jin et al. (2015)
6.	*Hordeum vulgare*	Low temperature (7/5°C day/night temperature for 3 days)	SA (0.1 mM applied to 7-day-old plant)	Reduced MDA with SA in both cultivars; H_2O_2 decreased in cv. Akhisar. enhanced activities of POD and SOD in both cultivars; CAT activity enhanced in Tokak cultivar	Mutlu et al. (2016)
7.	*Lycopersicon esculentum*	High temperature (40/30°C day/night temperature for 8 days)	EBR (0.01,0.1,1.0 mg/L applied 6 weeks after germination)	Net photosynthetic rate, stomatal conductance, maximum carboxylation rate of RuBisCo enhanced with hormone treatment; activities of SOD, APOX, CAT, and guaiacol peroxidase (GPOD) increased; reduction in H_2O_2 and MDA production	Ogweno et al. (2008)
8.	*Lycopersicon esculentum*	High temperature (38/30°C day/night temperature)	Plants produced 1.7−2.4 fold high spermidine and spermine	Reduced MDA, improved activities of APOX, CAT, GPOD, and SOD; improved CO_2 assimilation	Cheng et al. (2009)
9.	*Lycopersicon esculentum*	Low temperature (4°C for 28 days)	SA (1 mM for 15 min before storage)	Reduced chilling injury; increased expression of GA biosynthetic gene (*GA3ox1*), increased GA_3 levels and DELLA proteins degradation enhanced; enhanced activities of antioxidative enzymes and reduced ROS accumulation	Ding et al. (2016)
10.	*Malus domestica*	Sun exposed, not exposed	Endogenous levels of IAA, ABA, JA, SA, and ET were estimated	ABA, SA, and JA increased significantly in exposed tissues indicating the role of these hormones in modulating defense responses to photooxidative damage; IAA was not found to be related to injury development	Torres et al. (2017)
11.	*Solanum melongena*	High temperature (43/38°C day/night temperature for 8 days)	EBR (0.1 μM conc. to 6-week-old plant)	Enhanced growth, chlorophyll content, net photosynthetic rate, stomatal conductance, transpiration rate, maximum quantum efficiency of PSII, potential photochemical efficiency, the quantum efficiency of PSII; increased activities of SOD, POD, CAT, and APOX; enhanced levels of GSH, ascorbate (AsA), proline, soluble sugar, and proteins; reduced levels of superoxide anions, H_2O_2 and MDA	Wu et al. (2014)
12.	*Triticum aestivum*	Low temperature (5/2°C day/night temperature for 3 days)	1 mM SA with sodium nitroprusside 0.1 mM for 12 h	Reduced levels of superoxide anions, H_2O_2, and MDA; increased activities of SOD, CAT, and POD	Esim and Atici (2015)
13.	*Vitis vinifera*	High temperature (140°C)	SA (150 μM)	Accumulation of *PAL* mRNA and phenolics	Wen et al. (2008)
14.	*Vitis vinifera*	High temperature (43°C for 5 h)	SA (100 μM)	Improved net photosynthesis rate and ribulose-1,5-bisphosphate carboxylase (RuBisCo); heat shock protein 21 (HSP) immune signals increased in SA treated plants subjected to heat stress	Wang et al. (2010)

TABLE 19.2 Effect of Various Hormones in Regulation of Salinity Stress

S. no.	Plant species	Stress	Hormone treatment	Response	References
1.	*Brassica juncea L.*	NaCl (100 and 150 mM)	GA_3 (75 mg/Lonce a week for 45 days)	Exogenous application of GA_3 found to enhance growth and yield The significant rise in activity of SOD, CAT, POD, GR, and APX was observed	Ahmad (2010)
2.	*Medicago sativa*	NaCl (200 mM)	SA (0.1 & 0.5 mM applied 49 days after sowing)	The heightened activity of POX, SOD, APX, DHAR, and GR was reported CAT activity was reduced by 40% The improved photosynthetic efficiency and nitrogen fixation capacity was observed	Palma et al. (2013)
3.	*Medicago sativa*	200 mM NaCl	ABA (1 & 10 μM conc. applied 2 days before salt treatment)	The significant rise in SOD, CAT, and GR was reported; reduced MDA was also observed	Palma et al. (2014)
4.	*Medicago sativa L.*	13.6 dS/m NaCl solution	Brassinolide (5 μM/L)	Significant increase in shoot dry weight, fresh weight, and root dry weight in Victoria and golden empress and increase in root fresh weight in Victoria and Victor Enhanced activities of SOD, POD & CAT observed in Victoria & Victor seedlings. Significant reduction in MDA accumulation was noticed.	Zhang et al. (2007)
5.	*Pistacia vera* L.	(0, 30, 60, or 90 mM) NaCl	SA (0, 0.10, 0.50, or 1.00 mM applied 3 weeks after emergence)	Various physiological parameters like water content, leaf chlorophyll content, photosynthetic efficiency, and chlorophyll fluorescence ratio were found to be increased; SA applications at 0.50 and 1.00 mM were effective in reducing proline content and electrolyte leakage	Bastam et al. (2013)
6.	*Pusa Basmati-1*	NaCl (75, 100, 125 mM)	EBL (10^{-11}, 10^{-9}, 10^{-7} M presoaking for 8 h)	Enhanced growth was observed in rice seedlings; there was a significant rise in proline and protein content Reduced content of MDA was recorded	Sharma et al. (2013)
7.	*Ricinus communis L.*	NaCl (100 Mm)	GA_3 (seed presoaking in 250 μM)	The activity of SOD, POD, and CAT and proline content was increased Plant height, leaf area, root, stem, leaf weight was also increased with application of GA_3	Zhou et al. (2014)
8.	*Solanum melongena L.*	NaCl (90 mM)	EBR (0, 0.025, 0.05, 0.10, and 0.20 mg/dm^3 during fourth or fifth true leaf stage)	The enhanced activity of APX, SOD, CAT, POD, and reduced content of GSH was observed The reduction in production of MDA and H_2O_2 was significant	Ding et al. (2012)
9.	*Triticum aestivum L.*	NaCl (150 mM)	EBR (0, 0.052, 0.104, 0.156 L/M applied on 14-day stage)	The improvement in growth parameters were observed only in S-24 The enhanced photosystem II efficiency in both the cultivars was reported There was no change in SOD activity, whilst POD and CAT activities were increased in S-24 only	Ali et al. (2008)
10.	*Vigna radiata L.*	NaCl (50 mM)	SA (0.5 mM sprayed 15 days after sowing)	The remarkable increase in nitrogen, sulfur assimilation, GSH content, and increase in photosynthesis was observed The activity of APX and GR was higher in Pusavishal than T44; whilst activity of SOD was higher in T44 Overall, the effect of SA was prominent in Pusavishal as compared with T44	Nazar et al. (2011)
11.	*Vigna sinensis*	NaCl (25, 50, 100 and 150 mM)	Brassinolide (0.05 ppm after 25 and 32 days from sowing)	High saline conditions significantly affected the morphological features like shoot and root length, number of leaves, leaf area, etc.; elevated levels of TBARS content was reported, which was decreased with exogenous applications of brassinolide There was a significant increase in soluble protein content, tocopherol content, and GSH The enhanced activity of antioxidant enzymes PPO, POX, and SOD was also reported	El-Mashad and Mohamed (2012)
12.	*Zea mays*	NaCl (25, 50, 75, and 100 mM)	HBL (10^{-8}, 10^{-6}, 10^{-4} mM for 12 h)	The enhanced activities of SOD, POD, CAT, and APOX was observed Oxidative damage was reduced with the application of 28-homoBL.	Arora et al. (2008)

TABLE 19.3 Effect of Various Hormones in Regulation of Metal Stress

S. no.	Plant species	Stress	Hormone treatment	Response	References
1.	*Solanum lycoperscium*	Cadmium (Cd) (3, 9 mg/kg)	6-furfurylaminopurine (artificial cytokinin) (10 μM)	Increase in ascorbate peroxidae (APX), glutathione reductase (GR)	Singh et al. (2018)
2.	*Brassica juncea*	Lead (Pb) (0.25, 0.50 & 0.75 mM)	EBL $10-7$ M and SA, 1 mM for 8 h	Proline, trehalose, glycine betaine, glutathiones, ascorbic acid, and tocopherol content was increased The enhanced activities of guaiacol peroxidase, catalase, glutathione reductase, and glutathione-s-transferase were observed	Kohli et al. (2018)
3.	*Brassica juncea*	Manganese (Mn) (0, 3, 6, or 9 mM)	SA (10 μM)	The metal induced stress effects were reversed with the application of SA; improved growth, better photosynthetic traits, proline accumulation, heightened activity of antioxidant enzymes, which is believed to be a contributing factor in stress tolerance in mustard plants	Parashar et al. (2014)
4.	*Brassica juncea*	Copper (Cu) (50, 100, & 150 mg/kg sand)	HBL (10^{-10}, 10^{-8}, and 10^{-6} M for 8 h)	The improved activities of CAT, SOD, and POX along with rise in proline content was observed; significant improvement in growth characteristics and photosynthetic parameters were also recorded after 28-HBL applications	Fariduddin et al. (2009)
5.	*Poa pratensis*	Cd (0, 5, 10, or 50 μM)	SA (500 μM for 7 days)	Enhanced activities of APX and SOD were observed; CAT activity was decreased; chlorophyll content and mineral nutrients content, for example, K, Ca, Mg, and Fe were also increased Reduction in H_2O_2 and MDA was reported	Guo et al. (2013)
6.	*Pisum sativum*	Chromium VI (Cr VI) (50, 100, & 250 μM)	IAA (10 & 100 μM)	IAA applications protect pea plants against Cr by regulating oxidative stress and Cr deposition There is noticeable decrease in nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase (GOGAT) activities, and increase in glutamate dehydrogenase activity was observed	Gangwar and Singh (2011)
7.	*Triticum aestivum*	$Cd(CH_3COO)_2$ (1 mM)	SA (50 μM)	The improved growth characteristics and poststress recovery was observed after SA applications; PAL, the enzyme involved in lignin biosynthesis, was activated and contributed in carrier functioning of cell walls; significant decline in MDA accumulation and electrolyte leakage were also observed	Shakirova et al. (2016)
8.	*Triticum aestivum*	Cadmium chloride ($CdCl_2$) (500 or 1000 μM Cd)	Indole-3-acetic acid (IAA) (500 μM) or SA (500 μM)	Significant rise in antioxidant activities of SOD, POX, and CAT attenuated the Cd induced stress in wheat seedlings	Agami and Mohamed (2013)
9.	*Vigna radiata*	Aluminum (Al) (0.0, 1.0, or 10.0 mM)	24-epibrassinolide (EBL) or 28-homobrassinolide (HBL) (10^{-8} M)	The increased activities of antioxidative enzymes CAT, POX, and COD was reported in aluminum stressed plants, which were further enhanced after 24- EBL and 28-HBL applications; various morphological and physiological parameters were also improved	Ali et al. (2008)
10.	*Vigna radiata*	Nickel (Ni) (0, 20, 40, and 60 mg/kg)	GA_3 (10^{-4} M)at 15, 30, and 45 days after germination	Enhanced growth parameters pertaining to length, shoot, and root dry weight as well as overall yield were recorded; reduced nickel concentration was also reported	Ali et al. (2015)
11.	*Brassica juncea*	Cd	Castasterone (10^{-11} M, 10^{-9} M, and 10^{-7} M for 8 h)	Partial regain of biomass as well as enhanced ascorbate peroxidase (APOX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), glutathione peroxidase (GPOX), and glutathione-s-transferase	Yadav et al. (2018)

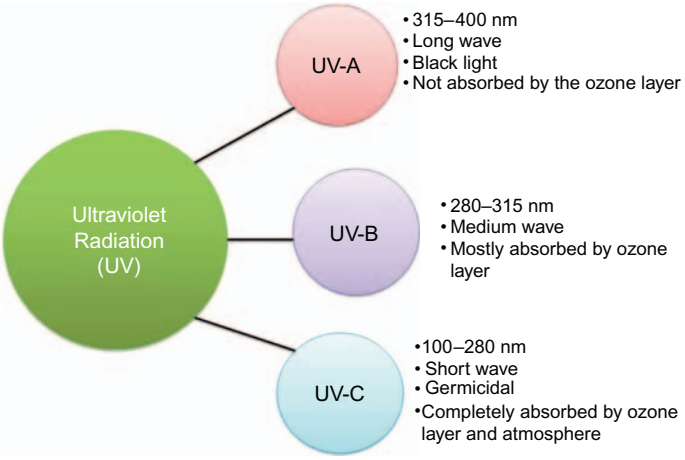

FIGURE 19.2 Different types of ultraviolet radiation and their wavelength range. Source: *Modified after Mainster, M.A., 2006. Violet and blue light blocking intraocular lenses: photoprotection versus photoreception. BJO. 90(6), 784–792 (Mainster, 2006).*

layer depletion by anthropogenic activities, increased concentrations of UV light has entered into the atmosphere (Gill et al., 2015; Khan et al., 2015). Different types of ultraviolet radiation and their wavelength ranges are given in (Fig. 19.2).

Being nonmotile and needy for sunlight, it is impossible for plants to avoid contact with ultraviolet light. Increase in the UV-B radiation's intensity disturbs the electron transport in PS II; inhibits the normal production and functioning of nucleic acids, photosynthetic pigments, and other metabolites; and injures the membranes, DNA, and proteins, and therefore, causes considerable damage to the crops in terms of productivity (Mohammed and Tarpley, 2011). Plants protect themselves from UV stress by producing UV shielding secondary metabolites. The efficiency of the process depends upon the proper organization and utilization of those metabolites, which is managed by the different plant hormones.

Exogenous application of salicylic acid controlled the UV-B stress impacts in *Glycine max* plants by increasing the water use efficiency and improving photosynthetic function (Li et al., 2014). Ranceliene and Vyšniauskiene (2012) reported that salicylic acid is capable of decreasing the chromosome aberrations caused by UV-B. Improvement in oil yield and growth of *Thymus daenensis* Celak and *Thymus vulgaris* L. plants under UV stress was observed after the foliar spray of salicylic acid (Yadegari, 2017). In the UV-B stressed plants of *Oriza sativa*, salicylic acid mediated the stress amelioration by increasing the PS II activity, total phenolic content in leaves, pollen viability, and yield (Mohammed and Tarpley, 2013). Auxin plays a vital signaling role in preventing UV-B radiation from causing downward curling of *A. thaliana* leaves (Fierro et al., 2015). Auxin also regulates the ultraviolet radiation induced morphogenesis and flavonoid accumulation (Hectors et al., 2012).

ABA activates the plant's guard against UV radiation stress (Berli et al., 2010). It also increases the concentrations of UV-B radiation absorbing metabolites like kaempferol, flavonols, and quercetin in the plants (Mazid et al., 2011; Tossi et al., 2012). ABA enhances Ca^{2+} concentration in cytosol; causes alkalinization of the cytosol; increases hydroxycinnamic acid, ferulic acid, and caffeic acid concentrations; and modifies membrane properties, thereby, bringing on the radiation stress tolerance in plants (Berli et al., 2010).

19.3.6 Crosstalk of Different Hormones

The defense responses against stresses are regulated by interaction between signaling pathways of several hormones like ABA, SA, JA, and ethylene (Verma et al., 2016). Also, studies have shown that growth promoting hormones, that is, auxins, cytokinins, and gibberellins too have a stress tolerance role (Verma et al., 2016). A study on rice seedlings has shown that the gene *TLD1/OsGH3.13* for indole-3-acetic acid (IAA)-amidosynthetase was involved in imparting drought tolerance by upregulating the expression of late embryogenesis abundant (*LEA*) genes (Zhang et al., 2009). In addition to this, it was also found that ethylene was involved in regulating the many genes for auxin synthesis, perception, and action (Stepanova and Alonso, 2009). Enzymes for auxin biosynthesis (*ASA1/WEI2/TIR7*, *ASB1/WEI7*, *TAA1/SAV3/WEI8*), auxin responsive factors (*ARF2*, *ARF1*), and auxin transporters (*PIN1*, *PIN2*, *PIN4*, *AUX1*) are the genes that are specifically regulated by ethylene (Li et al., 2004a; Stepanova et al., 2005; Ruzicka et al., 2007; Stepanova et al., 2008). However, in lateral root development, a negative regulation of auxin transport via ethylene was reported (Negi et al., 2010). Also, in *Arabidopsis*, an analog of SA called benzothiadiazole-S-methyl ester was reported to downregulate the expression of TRANSPORT INHIBITOR RESISTANT 1 (TIR1)/AUXIN SIGNALING F-BOX (AFB) genes (Wang et al., 2007). This response increased the stability of auxin repression protein (AUX/IAA), which further led to inhibition of auxin responses (Wang et al., 2007). Therefore, it was suggested that suppression of auxin signaling during stress resistance becomes necessary because onset of systemic acquired resistance (SAR) led to reduced auxin responsive gene expression and also, auxins promote susceptibility (Verma et al., 2016).

Cytokinins have also been reported to regulate defense responses synergistically with SA. It was reported that interaction between two transcription factors, that is,

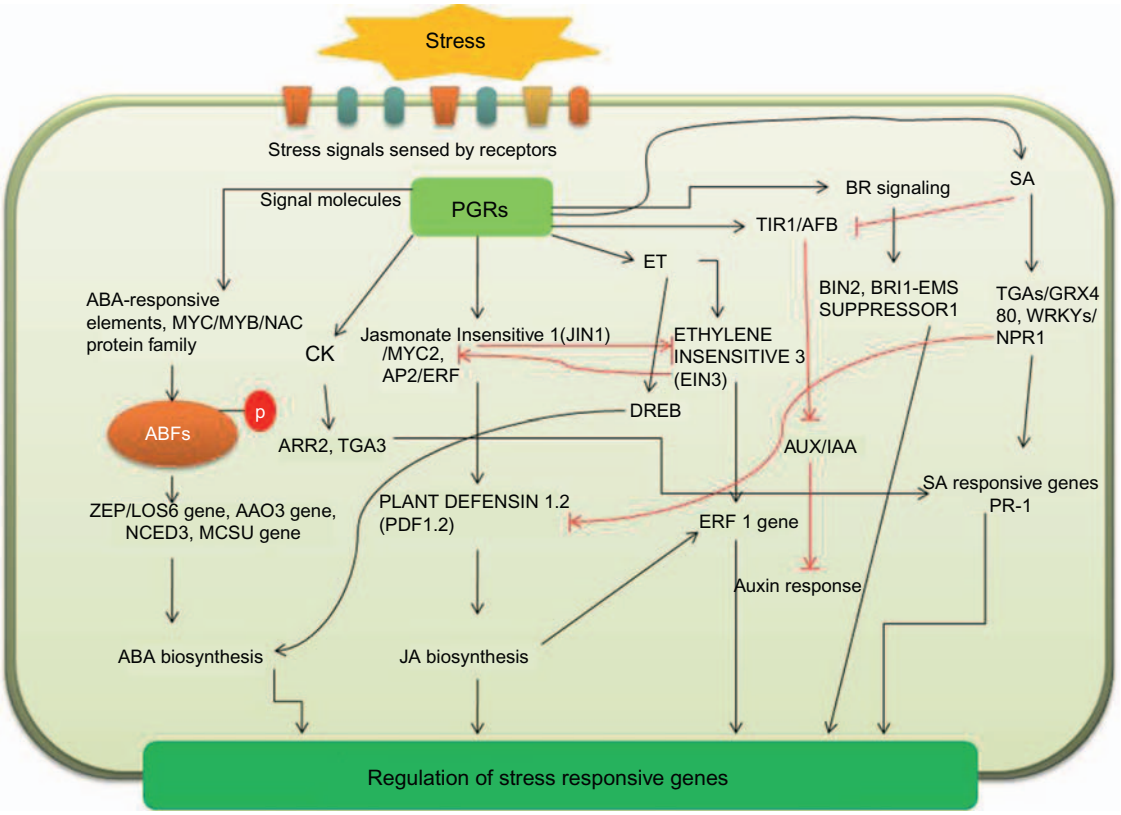

FIGURE 19.3 Plant growth regulators' signaling and their crosstalk.

ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2), which gets activated by cytokinins, and TGA3, a bZIP-type transcription factor, triggers SA dependent defense response (Choi et al., 2010). In rice, the interaction between OsNPR1 and WRKY45 indicated synergistic behaviors of cytokinins and SA leading to resistance against *Magnaporthe oryzae* (Jiang et al., 2013). ABA has been known to suppress cytokinin biosynthesis (Nishiyama et al., 2011), but studies on cytokinin receptors like *Arabidopsis* histidine kinases (AHK1, AHK2, AHK3) suggested that AHK1 aided in ABA signaling and provided resistance against drought and salinity stresses. AHK2 and AHK3 were reported to negatively regulate both ABA signaling and osmotic stress (Tran et al., 2007).

An antagonistic interaction between SA and JA has been reported in case of biotic stresses (Bari and Jones, 2009). It was established by Doherty et al. (1988) that aspirin, an acetyl derivative of SA, inhibited the JA-mediated response to wounding. The NON-EXPRESSOR OF PR (pathogen related) GENE 1 (NPR1) is regulated by SA, and this NPR1 suppresses *LIPOXYGENASE 2* (*LOX2*), *VEGETATIVE STORAGE PROTEIN* (*VSP*), and *PDF1.2*; these genes are responsive to JA. Therefore, NPR1 is chiefly responsible for SA-JA antagonism (Spoel et al., 2003). Also, a transcription factor called WRKY70 increased the expression of SA responsive genes (PR genes), while suppressing the expression of JA responsive genes (*PDF1.2*), thus playing a role in SA-JA antagonism (Li et al., 2004b). Ethylene and JA, however, have been reported to act synergistically in defense responses against biotic stresses (Verma et al., 2016). Ethylene as well as JA aid in either inducing or stabilizing a transcription factor ETHYLENE INSENSITIVE3 (EIN3) that leads to improved tolerance to necrotrophs and root hair formation (Zhu et al., 2011). *PR* genes were also reported to be activated by ETHYLENE-RESPONSIVE FACTOR1 (ERF1) for which both ethylene and JA are required (Lorenzo et al., 2003). Ethylene induces another transcription factor, dehydration responsive element (DRE)-BINDING PROTEIN (DREB), that interacts with ABA when plants are exposed to abiotic stresses (Arc et al., 2013). ABA induces seed dormancy and ethylene helps in breaking dormancy and inducing seed germination (Arc et al., 2013) (Fig. 19.3).

19.4 CONCLUSION AND FUTURE PROSPECTS

The present study emphasizes the role of phytohormones in ever-changing environmental conditions.

Molecular analysis of signal cascade of phytohormones and their interactions play an effective role in understanding the key role of phytohormones in defense. Crosstalk among different hormones also opens new insight in the research field. Molecular level understanding of different pathways helps to form transgenic plants that can withstand extreme stress conditions, and opens up an important aspect in a different area of research.

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CHAPTER

20

Role and Regulation of Auxin Signaling in Abiotic Stress Tolerance

Veena Pandey, Indra Dutt Bhatt and Shyamal Kumar Nandi

G. B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, Uttarakhand, India

OUTLINE

20.1 INTRODUCTION

Plant hormones play a crucial role in regulating growth and development. Among all hormones, auxin (indole-3-acetic acid (IAA)) has a special place since it was the first growth hormone discovered in plants. Auxin, along with cytokinins, differs from other plant hormones in the fact that they are required for viability. No mutants lacking either auxin or cytokinin have been found, suggesting that mutations that eliminate them are lethal. IAA biosynthesis in plants occurs by tryptophan-dependent and tryptophan-independent manner. Four pathways for tryptophan-dependent IAA biosynthesis include IAM (indole-3-acetamide) pathway, IPA (indole-3-pyruvic acid) pathway, TAM (tryptamine) pathway, and IAOX (indole-3-acetaldoxime) pathway (Mano and Nemoto, 2012). Although free IAA is the biologically active form, most of the auxins in plants are found in a conjugated form, covalently bonded to glucose or amino acid. The primary sites of IAA biosynthesis include shoot apical meristems, young leaves, and developing fruits or seeds. Auxin synthesized in the aerial part is transported to other plant parts through polar transport. It is the only plant hormone known to be transported polarly (Taiz and Zeiger, 2002). Polar auxin transport (PAT) includes influx carriers such as AUX1/LAX1 (Auxin Resistant 1/Like Aux1) and auxin efflux carriers such as the PIN (PIN-FORMED), ATP binding cassette type B (ABCB), and the multidrug resistant proteins or P-glycoproteins (MDR-PGPs) (Kazan, 2013). More recently another class of auxin carriers, called PILS (PIN-LIKES), has been identified that regulates intracellular auxin transport and compartmentalization in plants (Barbez et al., 2012).

The physiological role of auxin in many plant developmental processes like phototropism, gravitropism, apical dominance, cell elongation, vascular differentiation,

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parthenocarpy, fruit development, abscission, and adventitious root formation is well known (Taiz and Zeiger, 2002; Zhao, 2010). In recent years, the role of auxin under various abiotic stresses has also been studied. This chapter provides an overview of the role of auxin signaling in various abiotic stress responses of plants.

20.1.1 Auxin Signaling in Plants

Auxin signaling involves auxin perception by receptors like ABP1 (auxin-binding protein) (Taiz and Zeiger, 2002), TIR1 (transport inhibitor response 1), and AFB (Auxin F-Box) proteins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Kazan, 2013). Auxin binding to its receptor leads to degradation of Aux/IAAs (Auxin/IAA) repressor by the 26S proteasome. Aux/IAA proteins repress auxin response factors (ARFs), a class of transcription factors (TFs) that bind to auxin responsive elements (ARE) found in the promoter region of auxin-induced genes (Kim et al., 1997). Degradation of Aux/IAAs releases ARFs from suppression, which then bind to the ARE and regulate auxin dependent gene expression (Kazan, 2013).

The two large TF families, that is, the ARFs and the Aux/IAAs, are mainly involved in transcriptional regulation of auxin responsive genes. The expression of these genes responds to environmental signals (Guilfoyle and Hagen, 2007). Due to the potential applications of ARF TFs for the development of improved stress tolerant transgenic crop plants, many ARF families have been identified in a number of crop species like *Oryza sativa* (Song et al., 2009; Jain and Khurana, 2009; Shen et al., 2010), *Sorghum bicolor* (Wang et al., 2010), *Solanum lycopersicum* (Wu et al., 2011), *Zea mays* (Xing et al., 2011; Wang et al., 2012), *Brassica rapa* (Mun et al., 2012), and *Glycine max* (Van Ha et al., 2013). Likewise, transcriptional control of Aux/IAA genes plays a central role in the establishment of the auxin-signaling pathways that regulate plant response to environmental variables. Various TFs that regulate the Aux/IAA genes belong to DREB/CBF family (which functions in stress tolerance) and directly promote transcription of these genes in response to abiotic stress (Shani et al., 2017).

Recent findings suggest that auxin biosynthesis pathway involving *YUCCA* gene (encoding a flavin monooxygenase, and belonging to the tryptophan-dependent auxin biosynthetic pathway) may be exploited to alter plant responses to the environment (Kim et al., 2013; Lee et al., 2012; Park et al., 2013). Plant response to high temperature was found to be mediated through the expression of *YUC2* and *YUC6* genes in *Arabidopsis thaliana* and *Hordeum vulgare* (Cheng, 2006; Sakata et al., 2010). Moreover, *Arabidopsis* plants overexpressing *YUC6* or transgenic poplar expressing *Arabidopsis YUC6* under control of stress-inducible SWPA2 promoter exhibited improved drought and oxidative stress resistance (Cha et al., 2015; Ke et al., 2015). Elevated auxin positively modulated the expression levels of multiple abiotic stress–related genes (*RAB18*, *RD22*, *RD29A*, *RD29B*, *DREB2A* and *DREB2B*) and increased antioxidant enzyme activities resulting in higher tolerance to the variable environment (Shi et al., 2014).

20.1.2 Auxin Signaling Under Abiotic Stresses

The role of auxin in plant development is well known; however, its possible function in response to various stresses is poorly understood. Several recent studies demonstrate a novel role of auxin signaling and transport in plant tolerance to abiotic stress (Krishnamurthy and Rathinasabapathi, 2013). Various abiotic stresses that involve auxin-mediated response in plants are represented in Table 20.1. The following section summarizes the role and signaling of auxin under different abiotic stresses.

20.1.2.1 Drought Stress

Auxin plays an important role in plant responses to drought stress. Auxin biosynthesis, distribution via changes in auxin transport, or conjugation with amino acids or sugar molecules all are regulated under abiotic stresses (Shibasaki et al., 2009). Members of an auxin-responsive GH3 gene family, encoding auxin conjugating enzymes (IAA-amido synthetases) have been shown to be also involved in drought-stress responses. *OsGH3.13* has been reported to enhance the expression of LEA (late embryogenesis abundant) genes, which increased the drought tolerance of rice seedlings (Zhang et al., 2009a). Overexpression of *OsGH3-2* in rice resulted in IAA-deficient phenotype with reduced free IAA, carotene, and ABA, which caused greater stomatal apertures causing increased water loss and more hypersensitivity to drought (Du et al., 2012). In rice, IAA level was reduced to 72% after 3 days of drought stress (Du et al., 2013a). Auxin positively modulates root biomass and branching, which might improve water uptake efficiency and hence, provide drought stress resistance (Shi et al., 2014).

Transcriptome analysis indicated that under dehydration, the expression levels of many auxin-related genes are changed. Furthermore, auxin partly participates in the positive regulation of drought stress resistance through the regulation of root architecture (Shi et al., 2014). In *Arabidopsis*, *MYB96* TF regulates lateral root (LR) meristem activation under drought conditions, possibly through an ABA–auxin signaling crosstalk, and the MYB96-knockout mutant was more susceptible to drought stress (Seo et al., 2009).

TABLE 20.1 Abiotic Stresses Which Encompass Auxin-Mediated Signaling Response in Plants

Abiotic stress	Crops	Major findings	References
Drought stress	*Arabidopsis, Oryza sativa*	Auxin level decrease under drought stress; however, higher auxin concentration improves drought tolerance by increasing compatible solutes, antioxidants, and root branching	Shibasaki et al. (2009), Zhang et al. (2009a), Seo et al. (2009), Du et al. (2012), Lee and Luan (2012), Du et al (2013a), Shi et al. (2014), Sharma et al. (2015)
Salinity stress	*Arabidopsis*	Regulation of auxin level helps to maintain ion homeostasis under salt stress; plants reduce auxin concentration and signaling to cope with salinity stress	Bao and Li (2002), He et al. (2005), Park et al. (2007), Sun et al. (2008), Popko et al. (2010), Ding et al. (2009), Wang et al. (2009), Iglesias et al. (2010, 2014), Jung and Park (2011), Liu et al. (2015)
Temperature stress	*Arabidopsis, Phaseolus vulgaris*, Tomato, pepper	High/low temperature stress reduces auxin concentration by converting active IAA to inactive conjugated form; however, application of exogenous auxin might help to reduce stress-induced plant injury	Morris (1979), Kuo and Tsai (1984), Franco et al. (1990), El-Abd et al. (1986), Ofir et al. (1993), Fukaki et al. (1996), Huberman et al. (1997), Wyatt et al. (2002), Shibasaki et al. (2009), Sakata et al. (2010), Oshino et al. (2007, 2011), Du et al. (2013b)
Nitrogen deficiency	*Arabidopsis*	Many nitrogen uptake transporters also mediate auxin transport in plants and they help plants to alter root architecture under nitrogen deficiency	Zhang et al. (2007), Gifford et al. (2008), Krouk et al. (2010), Vidal et al. (2010), Kiba et al. (2011), Gojon et al. (2011), Bouguyon et al. (2012)
Phosphorus deficiency	*Arabidopsis, Lupinus albus*	Phosphorus deficiency induces auxin-mediated lateral root formation in plants and increases plant sensitivity to auxin	Gilbert et al. (1998, 2000), Neumann et al. (2000), Lopez-Bucio et al. (2002), Hammond et al. (2003), Nacry et al. (2005)
Potassium deficiency	*Arabidopsis*, cotton, rice, tobacco, maize	Many potassium transporters are crucial for auxin transport and severe potassium deficiency reduces auxin concentration/transport and root growth; however, exogenous auxin application may increase lateral root formation and elongation under potassium deficiency	Cao et al. (1993), Philippar et al. (1999), Vicente-Agullo et al. (2004), Armengaud et al. (2004), Ashley et al. (2005), Zhang et al. (2009b), Ma et al. (2012), Song et al. (2015)
Sulfur deficiency	*Arabidopsis*	Plants increase auxin accumulation under sulfur deficiency, so as to induce more root growth and to gain more access to sulfur	Kutz et al. (2002), Nikiforova et al. (2003), Kopriva and Rennenberg (2004), Nikiforova et al. (2005), Maruyama-Nakashita et al. (2005), Kasajima et al. (2007), Falkenberg et al. (2008), Lewandowska and Sirko (2008), Frerigmann and Gigolashvili (2014)
Iron deficiency	*Arabidopsis, Phaseolus vulgaris, Cucumis sativus, Trifolium pratens, Malus xiaojinensis, Pyrus betulifolia*	Fe-deficiency increases auxin level in roots and induces formation of branched root hairs; auxin mediated response under Fe deficiency involves the interplay of ethylene and nitric oxide	Landsberg (1984), Romheld and Marschner (1986), Kim et al. (1992), Schmidt et al. (2000), Li et al. (2000), Schmidt and Schikora (2001), Zheng et al. (2003), Muller and Schmidt (2004), Li and Li (2004), Lombardo et al. (2006), Chen et al. (2010), Bacaicoa et al. (2011), Romera et al. (2011), Giehl et al. (2012), Wu et al. (2012), Li et al. (2016)
Aluminum toxicity	*Arabidopsis*, maize, *Triticum aestivum*	Aluminum toxicity causes reduction in cell expansion and inhibition of root growth by disruption of auxin transport	Hasenstein et al. (1988), Kollmeier et al. (2000), Shen et al. (2008), Sun et al. (2010), Yang et al. (2011)
Lead toxicity	*Arabidopsis, Oryza sativa, Triticum aestivum*	Lead-induced plant injury is associated with increased IAA-oxidase activity and reduction in IAA levels	Mukherji and Maitra (1977), Lane et al. (1978), Liu et al. (2009)
Cadmium toxicity	*Arabidopsis, Pisum sativum*	Cadmium stress causes enhanced IAA-oxidase activity, auxin degradation, and reduction in plant growth	Hasenstein et al. (1988), Hagen et al. (1988), Moons (2003), Chaoui and El Ferjani (2005), Ganesan (2008), Mei et al. (2009), Ding and Zhu (2009)
Copper toxicity	*Arabidopsis, Pisum sativum*	Copper toxicity inhibits primary root elongation by mediating auxin redistribution in plants	Chaoui and El Ferjani (2005), Yuan et al. (2013)
Arsenic toxicity	*Arabidopsis, Oryza sativa*	Exogenous supply of IAA can improve arsenic tolerance in plants	Krishnamurthy and Rathinasabapathi (2013), Pandey and Gupta (2015)

Activation of the *YUCCA7* gene of auxin biosynthesis resulted in elevated endogenous auxin levels and enhanced drought resistance in *Arabidopsis* (Lee and Luan, 2012). Auxin also positively modulated the accumulations of compatible solutes such as multiple sugars and sugar alcohols. Additionally, auxin positively regulated the activities of four enzymatic antioxidants (superoxide dismutase, catalase, peroxidase, glutathione reductase) under drought stress condition, thus conferring effective ROS (reactive oxygen species) detoxification to improve drought stress resistance (Shi et al., 2014).

A crosstalk between IAA and other hormones is thought to mediate drought responses of plants. Abscisic acid (ABA) mediated inhibition of LR growth is controlled by reducing PAT. *ABA insensitive3* (*ABI3*) interacts with ARF or Aux/IAA proteins and mediates LR growth (Shkolnik-Inbar and Bar-Zvi, 2010). ABA can alter auxin transport to mediate proton secretion at root tips and control root growth under moderate water deficit (Sharma et al., 2015). Auxin also plays an important synergistic and antagonistic role in the biosynthesis of other stress related hormones like ethylene (Li et al., 2004; Stepanova and Alonso, 2005; Ruzicka et al., 2007; Stepanova et al., 2007) and their crosstalk might be responsible for mediating auxin mediated response under stress.

20.1.2.2 Salinity Stress

Regulation of auxin biosynthesis and transport is required to mediate the response of plants to salt stress. Salt stress in *Arabidopsis* induced the expression of IAA biosynthesis genes (*nitrilase 1* and *nitrilase 2*), suggesting that the level of IAA might increase in response to salinity stress (Bao and Li, 2002). Salinity-induced expression of NAC-type TF (*AtNAC2*) mediates the process of plant LR development under salinity stress through auxin signaling pathways (He et al., 2005). Overexpression of *AtNAC2* in transgenic *Arabidopsis* plants resulted in the promotion of LR development. Jung and Park (2011) found that a membrane-bound NAC TF (*NTM2*) mediates the signaling crosstalk between auxin and salt stress via the *IAA30* gene during seed germination in *Arabidopsis*. Germination of the NTM2-deficient *ntm2-1* mutant seeds exhibited enhanced resistance to high salinity, while *ntm2-1* mutant overexpressing the *IAA30* gene reduced salt resistance.

SOS pathway (which maintains ion homeostasis under salt stress) modulates root response by regulating *PIN2* protein and auxin asymmetric distribution (Sun et al., 2008). The different levels of salt stress determine the degree of auxin biosynthesis in the shoot apex and the subsequent gradients of auxin in roots, resulting in changes in the number of elongated LRs and the rates of elongation (Wang et al., 2009). High salinity induced the expression of *AtGH3.5* gene, which encodes IAA amido-conjugate synthases. Overexpression of *AtGH3.5* resulted in decreased free auxin concentration and increase in auxin conjugate like IAA-Asp (Park et al., 2007). Free auxin content in the developing xylem of *Populus* declines during abiotic stress, while auxin conjugates increase in response to plant adaptation to stress (Popko et al., 2010).

Adaptation to salinity in *Arabidopsis* is mediated in part by an auxin/redox interaction. Auxin receptor double mutant (*tir1 afb2*) showed increased tolerance against salinity as measured by chlorophyll content, germination rate, root elongation, and enhanced antioxidant enzymes compared with wild-type plants (Iglesias et al., 2010). miRNA microarray hybridization analysis revealed that salt stress−induced differential expression of miRNAs in maize roots, some of which were involved in auxin signaling (Ding et al., 2009). Salt stress triggers miR393 expression, which leads to a reduction in the levels of the *TIR1* and *AFB2* auxin receptors. Salt stress also stabilized Aux/IAA repressors, which lead to downregulation of auxin signaling. It was also reported that miR393 is involved in repression of LR initiation, emergence, and elongation during salinity, since the *mir393ab* mutant showed reduced inhibition of LR growth upon NaCl treatment (Iglesias et al., 2014). Nitric oxide (NO) is involved in repressing PIN expression (thereby reducing auxin levels), stabilizing *IAA17* (thereby repressing auxin signaling) and mediating salt-induced inhibition of root meristem growth (Liu et al., 2015). All these findings suggest that the downregulation of auxin signaling might be a plant strategy to enhance tolerance to salinity stress.

20.1.2.3 Temperature Stress

Increasing evidence suggests a potential link between low- and high-temperature stress and auxin signaling. Morris (1979) showed that temperature affects the velocity of exogenous auxin transport in plants. High and low day/night temperature (35/24°C and 10/5°C) was found to reduce fruit set, pollen grain viability, and IAA levels in tomato (El-Abd et al., 1986). Also, a short exposure to 38°C to young fruits of tomato caused reduction in auxin-like substances (Kuo and Tsai, 1984). Similarly, in bean (*Phaseolus vulgaris*), heat stress significantly reduced the diffusion of endogenous IAA from reproductive organs into agar, possibly due to the direct inhibitory effect of the high temperatures (32/27°C) on the transport capacity of related tissues (Ofir et al., 1993). High temperatures (day/night regimes of 32/27°C, 40/27°C) reduced IAA levels and particularly auxin transport capacity in the reproductive organ in pepper (*Capsicum annuum*).

In addition, the reduction of auxin transport capacity was found to be the major mechanism of high temperature induced abscission of the reproductive organ (Huberman et al., 1997). Basipetal auxin transport in wild-type *Arabidopsis* was stopped at 4°C; but auxin transport was restored when plants were reintroduced to room temperature (Wyatt et al., 2002). However, Du et al. (2013b) reported that under the cold stress of 3 days, IAA level increased 1.6-fold, while it was increased by 1.3-fold after 6 h of heat stress, as compared with the control.

Cold stress inhibits the inflorescence gravity response in *Arabidopsis*, which is regulated by an asymmetric distribution of auxin (Fukaki et al., 1996; Wyatt et al., 2002). Cold stress affected the polar transport of auxin by selectively inhibiting the intracellular trafficking of auxin efflux carriers PIN2 and PIN3 (Shibasaki et al., 2009). For transport of auxin towards the shoot, the polar deployment of PIN2 protein and the constitutive cycling of this protein from the membrane to endosome are required for its functionality. Thus, reduced intracellular cycling functionality affects the PINs resulting in decreased shootward transport of auxin and inhibits the capability of roots to form an auxin gradient (Paciorek et al., 2005; Sukumar et al., 2009).

In *Arabidopsis*, many auxin-responsive genes respond to cold stress (Jain and Khurana, 2009). Likewise, many *OsGH3* genes (*OsGH3-2*, *OsGH3-5*, *OsGH3-6*, *OsGH3-7*, *OsGH3-9*, *OsGH3-11*, *OsGH3-13*), which convert active IAA to an inactive form, were found to be downregulated by heat stress in rice (Franco et al., 1990). High temperature reduced endogenous auxin in developing anthers by reducing expression of the *YUCCA* auxin biosynthesis and caused male sterility in barley and *Arabidopsis*. However, application of auxin completely reversed male sterility in both plant species (Sakata et al., 2010). Compromised auxin biosynthesis/IAA level under high temperature condition resulted in nuclear and organellar DNA proliferation arrest due to cotranscriptional alteration, however, exogenous auxin application promoted the expression of DNA replication-related genes, which induce anther cell proliferation under heat stress (Oshino et al., 2007, 2011). This suggests that auxin reduction is the primary cause of high temperature induced injury, and the exogenous auxin application may help to reduce plant injury and to sustain steady yields of crops.

20.1.2.4 Nutrient Deficiency Stress

Plants in nature encounter deficiency of a wide variety of essential mineral elements, which suppresses plant growth. The ability of plants to sense and respond appropriately to the soil nutrient availability is of fundamental importance for their adaptation. Auxin signaling and transport play important roles in regulating plant developmental responses to deficiency of various soil nutrients. Kazan (2013) suggested that a rapid alteration in plant root architecture in response to nutrient deficiency is achieved through auxin signaling.

Nitrogen: Auxin mediates plant response to nitrogen deficiency. *Arabidopsis* seedlings grown in low nitrogen conditions contain higher levels of auxin in roots as compared with seedlings grown in high nitrogen conditions (Kiba et al., 2011). However, an early report reveals that the inhibitory effect of high nitrate on LR growth of *Arabidopsis* is not alleviated by exogenous application of auxin, indicating that the auxin content is not the only factor regulating LR development (Zhang et al., 2007).

Using a cell sorting technique, Gifford et al. (2008) identified a nitrogen-inducible auxin response factor (ARF8), which is expressed in pericycle cells. They provided genetic evidence that the nitrogen signal regulates auxin signaling through the action of microRNA (miR167a) to control LR initiation. Recently, the auxin receptor gene *AFB3* was also found to be induced by nitrate (Vidal et al., 2010). Another link between nitrate and auxin signaling is provided by Krouk et al. (2010), who showed that NRT1.1/CHL1, which is a nitrate transporter and sensor (Ho et al., 2009), also facilitates cell-to-cell auxin transport. Under low NO_3^- concentrations, nitrate uptake transporter (NITRATE TRANSPORTER1.1 or NRT1.1) promotes basipetal auxin transport (from the LR tip towards shoot) to inhibit auxin accumulation in LR initials. At high NO_3^- levels, NRT1.1-dependent auxin transport out of the LRs is inhibited, leading to the accumulation of auxin in LR initials and promotion of LR growth (Krouk et al., 2010; Gojon et al., 2011; Bouguyon et al., 2012).

Phosphorus: Phosphorus starvation−induced cluster root formation in *Lupinus albus* involved the signaling of endogenous phytohormones like auxins and cytokinins (Gilbert et al., 1998, 2000; Neumann et al., 2000). The auxin-transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and N-(1-napthyl) phtalamic acid (NPA) were found to inhibit the formation of LRs under phosphorus deficiency in *Lupinus albus* (Gilbert et al., 2000). Phosphorus starvation in *Arabidopsis* potentially downregulated expression of some auxin regulated genes, suggesting a link between phosphate deficiency and auxin (Hammond et al., 2003). *Arabidopsis* plants growing under limiting (1 mM) phosphorus concentration were more sensitive to auxins in terms of the inhibition of primary root elongation and increase of LR density, suggesting that changes in auxin sensitivity play an important role in the effect of phosphorus deprivation on root architecture (Lopez-Bucio et al., 2002). Moreover, phosphorus deficiency was found to change root system architecture via modifying local auxin

concentrations within the root system, through changes in auxin transport rather than auxin synthesis (Nacry et al., 2005).

Potassium: Several reports suggesting links between potassium and auxin transport/signaling are available (Cao et al., 1993; Philippar et al., 1999; Vicente-Agullo et al., 2004; Ashley et al., 2005; Zhang et al., 2009b). The growth of maize coleoptiles was found to be mediated via auxin-regulated expression of *ZMK1*, an inwardly rectifying potassium channel (Philippar et al., 1999). TRH1 potassium transporter, which regulates root hair development and root gravitropic behavior in *Arabidopsis*, was found crucial for auxin transport (Vicente-Agullo et al., 2004). Evidence for the role of auxin-dependent processes in acclimation to K^+ deficiency was provided by demonstrating that the *CYP79B2* and *CYP79B3* genes involved in the tryptophan-dependent auxin biosynthesis were downregulated upon K^+ resupply to K^+-starved roots (Armengaud et al., 2004). Recent transcriptome analyses of rice roots subjected to K^+ deficiency have identified a large number of auxin-related genes (Ma et al., 2012), which is further evidence for auxin regulation of root responses to K^+ deficiency. More recently, it was found that potassium deficiency reduced root growth, auxin concentration, [^{3}H] IAA transport, and the expression levels of *PIN* genes in tobacco plants. However, application of exogenous auxin (NAA) under potassium deficiency increased LR formation and elongation (Song et al., 2015).

Sulfur: Sulfur deficiency increased accumulation of nitrilase (*NIT3*) gene, which encodes an enzyme that catalyzes the transformation of indole-3-acetonitrile to IAA in *Arabidopsis*. High *NIT3* expression leads to high auxin production and increased root growth, thus allowing the root system to penetrate more to gain access to sulfur (Kutz et al., 2002). Upregulation of genes involved in auxin biosynthesis (including myrosinase and nitrilase) and auxin response under sulfur deficiency was also reported by Nikiforova et al. (2003). Induction of genes involved in auxin synthesis upon S-starvation was also reported by Kopriva and Rennenberg (2004) and Lewandowska and Sirko (2008). Sequences named sulfur-responsive elements (SURE), which are very similar to the auxin response factor (ARF) binding sites, were identified in the promoter of many sulfur-responsive genes (Maruyama-Nakashita et al., 2005). Nikiforova et al. (2005) suggested that auxin signaling under sulfur deficiency is highly regulated. Sulfur deficit causes a surplus increase of auxin and the activation of auxin-induced genes. The altered auxin content triggers changes in free calcium levels in plant cells, which are sensed by calmodulin. Activated calmodulin influences the expression of the *IAA28* gene, which in turn represses the transcription of auxin-induced genes, thus providing the feedback control of the auxin signaling pathway (Nikiforova et al., 2005).

A genomic study showed that the *BIG* gene, which encodes a protein necessary for the polar transport of auxin, is involved in regulation of sulfur deficiency−responsive genes in *Arabidopsis thaliana* (Kasajima et al., 2007). Many auxin-relevant TFs like *IAA13*, *IAA28*, and *ARF-2* were found to serve as coordinators of the metabolic shifts driving sulfur homeostasis (Falkenberg et al., 2008). The role of various *MYBs* TFs in the regulation of sulfur assimilation enzymes, and their involvement in mobilization of auxin and LR formation under S deficiency, was confirmed by Frerigmann and Gigolashvili (2014).

Iron: Auxin plays a key role in altering root system architecture under variable iron availability. The induction of Fe deficiency stress responses has been linked to an increase of auxin in root tips (Landsberg, 1984; Romheld and Marschner, 1986). Possibly, the effect of auxin could be through ethylene, since high levels of auxin promote ethylene production by inducing the synthesis of ACC synthase (Kim et al., 1992). Low Fe availability frequently leads to the formation of branched root hairs (Muller and Schmidt, 2004) through a signaling cascade that involves auxin and ethylene (Schmidt et al., 2000; Schmidt and Schikora, 2001).

Several studies revealed that application of exogenous auxin analogs promoted the induction of root ferric chelate reductase (FCR) activity in *Phaseolus vulgaris* (Li et al., 2000), *Cucumis sativus* (Li and Li, 2004), and *Trifolium pratense* (Zheng et al., 2003), which enhanced Fe uptake under Fe deficient condition. Chen et al. (2010) concluded that NO acts downstream of auxin to activate root FCR activity under Fe deficiency. NO-mediated root hair development in *Arabidopsis* and lettuce in response to auxins were also reported by Lombardo et al. (2006). Fe-deficiency increased auxin and NO levels in *Arabidopsis* and the increase was greater when exogenous auxin was applied, but suppressed by an inhibitor of PAT (Chen et al., 2010). It was concluded that Fe deficiency responses involve the interplay of auxin, ethylene, and NO; however, auxin acts upstream of ethylene and NO (Romera et al., 2011).

Localized availability of iron upregulated *AUX1* gene to accumulate auxin in LR apices and induced LR elongation (Giehl et al., 2012). Removal of shoot apex in sunflower, bean plants, and *Malus xiaojinensis* arrested the rhizospheric acidification induced by Fe deficiency (Landsberg, 1981, 1984; Wu et al., 2012). However, exogenous application of auxin to the detopped shoots of *Pyrus betulifolia* recovered the Fe deficiency response (upregulation of citrate synthase (*PbCS1*) expression, which is involved in the synthesis

of citric acid that acts as a chelate substance for the long-distance transmission of Fe in the root system). In contrast, NPA (auxin transport inhibitor) application to the shoot tips arrested upregulation of the *PbCS1* expression. Thus, it was suggested that auxin is involved in mediating Fe-deficient response through regulation of the *PbCS1* expression (Li et al., 2016).

20.1.2.5 Heavy Metal Stress

Heavy metals are required in trace amount for the proper growth of the plant, but in excess concentration, they adversely affect the plant normal metabolic and developmental functions (Gill and Tuteja, 2010; Khan and Khan, 2014, 2017; Pandey and Gupta, 2015; Khan et al., 2016). Response of plants to heavy metal stress is mediated in part via auxin signaling. Heavy metal–induced decrease of cell expansion growth in wheat (Lane et al., 1978) and maize (Hasenstein et al., 1988) is attributed to alteration of auxin metabolism and transport. Auxin-mediated inhibition of cell elongation arises either from direct effects of heavy metals on auxin or due to their effect on proton extrusion (Barceló and Poschenrieder, 1990). Auxin signaling was found to be involved in heavy metal induced curvature of the root (away from metal ions) in *Zea mays*. Treatment of maize roots with auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) or naphthylphthalamic acid (NPA) prevented curvature in response to unilateral application of these ions, indicating the significance of auxin transport and distribution (Hasenstein et al., 1988). Link between auxin signaling and heavy metal stress has been studied by many researchers using different metal ions.

Aluminum: Aluminum toxicity is a major problem in acidic soil (Foy, 1988), which causes rapid inhibition of root growth (Doncheva et al., 2005). This aluminum-induced inhibition of root growth is associated with disruption of auxin transport and/or signaling in *Zea mays* (Kollmeier et al., 2000) and *Arabidopsis* (Sun et al., 2010). It was reported that Al^{3+} upregulated the expression of *PIN2* (a key component for mediating basipetal auxin transport) and inhibited transport of *PIN2* vesicles from plasma membranes to endosomes in *Arabidopsis*, leading to reductions in auxin concentration in root apical cells (Shen et al., 2008). Likewise, transcriptional levels of *AtAUX1* (gene encoding auxin influx protein) were also enhanced when exposed to Al^{3+}, thus indicating that Al^{3+} may target the *PIN2*- and *AUX1*-mediated auxin transport system, leading to disruption of auxin distribution in roots and inhibition of root elongation (Sun et al., 2010). Yang et al. (2011) showed that wheat plants cope with aluminum toxicity through the efflux of organic acids (malic acid), which is mediated through auxin.

Lead: Mukherji and Maitra (1977) reported that lead-induced reduction in germination and growth of rice is associated with increased IAA-oxidase activity. Moreover, the application of IAA was found to be partially capable of relieving lead toxicity. Lane et al. (1978) reported that high lead concentration caused reduction in the rate of cell division and elongation in *Triticum aestivum*, which was mediated through the action of auxin. Microarray study in *Arabidopsis* showed that lead treatment activated many genes involved in IAA biosynthesis, suggesting a possible role of auxin in heavy metal induced responses (Liu et al., 2009).

Cadmium: Crosstalk between cadmium stress signaling and auxin signaling pathway has been suggested by many researchers (Hagen et al., 1988; Moons, 2003; Ganesan, 2008). Cadmium (20 and 100 μM) caused oxidative stress, lipoperoxidation, and reduced growth in *Pisum sativum* seedlings, which is ascribed due to an elevation in the activities of IAA-oxidase and lignifying peroxidases. Enhanced activity of IAA-oxidase contributed to auxin degradation and reduced plant growth under heavy metal stress (Chaoui and El Ferjani, 2005). At the molecular level, cadmium stress was found to induce a number of miRNAs in rice (Huang et al., 2009) that function in auxin signaling (Ding and Zhu, 2009). Cation exchanger H^+/cation antiporter is required to induce auxin-mediated root growth in *Arabidopsis* under cadmium stress (Mei et al., 2009).

Copper: Copper toxicity (100 μM) induced a reduction in the growth of *Pisum sativum* seedlings and this reduction was found to be mediated through the enhanced activity of IAA-oxidase (Chaoui and El Ferjani, 2005). Yuan et al. (2013) reported that copper-mediated inhibition of primary root elongation is due to copper-mediated auxin redistribution in *Arabidopsis* seedlings. Genetic and physiological analysis demonstrated that *PIN1*, but not *PIN2* or *AUX1*, regulated this process (Yuan et al., 2013).

Arsenic: Auxin also plays an important role in plant tolerance to arsenic-induced oxidative stress (Krishnamurthy and Rathinasabapathi, 2013). Auxin transporter mutants *aux1*, *pin1*, and *pin2* were significantly more sensitive to As(III) than the wild-type plants. However, exogenous supply of IAA improved As(III) tolerance of *aux1* mutant via reactive oxygen species (ROS)-mediated signaling (Krishnamurthy and Rathinasabapathi, 2013). Pandey and Gupta (2015) concluded that application of selenium and auxin alone or in combination were very effective in lowering the As (III) induced stress in rice. Improved arsenic tolerance in the presence of auxin involves physiological, biochemical, and molecular interaction in a synergistic or additive way.

20.1.3 Crosstalk of Auxin With Other Hormones

Auxin interacts with other phytohormones to mediate various signaling responses under stress conditions. One such response includes changes in root architecture, which is mediated by interaction of auxin with other hormones like ABA (Saini et al., 2013), ethylene, and cytokinins (Harrison, 2012). Several miRNAs that are associated with auxin signaling (miR167 and miR168) and are positive regulators of adventitious root development in *Arabidopsis* contain ABREs, indicating that they are regulated by ABA signaling (Liu et al., 2008).

Arbona and Gómez-Cadenas (2008) found that under prolonged soil waterlogging, plants increased their IAA levels and reduced ABA levels; thereby promoted LR growth. Xu et al. (2013) found that under moderate drought stress, endogenous ABA accumulation in root tips of *Arabidopsis* and rice stimulated transport of auxin into the root apex and induced root growth. The transcript levels of AUX1 and PIN2 increased in the root tips under ABA stimuli, suggesting the interaction of auxin and ABA during stress-induced root growth (Xu et al., 2013). Similarly, the increased tolerance to cold stress is due to the combined effects of IAA and ABA. The change in auxin homeostasis affected ABA synthesis under drought and cold stress in rice, and the resulting balance of auxin and ABA homeostasis played a crucial role in stress responses (Du et al., 2012; Du et al., 2013b). Taniguchi et al. (2010) suggested the involvement of ABA in hydrotropic response of roots through modulation in auxin.

Stress response in plants is also dependent on interaction of auxin with ethylene. In a study, adventitious root development in response to flooding was found to be regulated by auxin mediated ethylene production (Vidoz et al., 2010; Muday et al., 2012). Flooded tomato plants treated with the aminoethoxyvinylglycine (ethylene biosynthesis inhibitor) and 1-naphthylphthalamic acid (auxin transport inhibitor) were unable to form adventitious roots. Auxin accumulation in stem upon waterlogging triggers de novo ethylene synthesis, which stimulates the transport of auxins towards the flooded parts of the plant and induced the root development under stress (Vidoz et al., 2010). Ethylene also stimulates enhanced sensitivity to auxins under temporary submergence (Park et al., 2011).

O'Brien and Benková (2013) suggested the possible antagonistic role of cytokinin and auxin in plant defense responses. Dynamic and complementary actions of auxin and cytokinins pathways, along with their crosstalk, regulate a plethora of developmental processes under stress (Bielach et al., 2017). Rowe et al. (2016) suggested that root growth under osmotic stress is regulated by a hormonal network of auxin, cytokinin, ABA, and ethylene. It was shown that PIN1 levels were reduced under osmotic stress in an ABA-dependent manner, overriding ethylene effects. Recently, Liu et al. (2017) found that hormonal crosstalk between auxin, cytokinin, and ethylene regulate root development in *Arabidopsis*.

20.2 CONCLUSION

Auxin is an important phytohormone that plays a crucial role in regulating many developmental processes in plants. Its amount is modulated in plants in

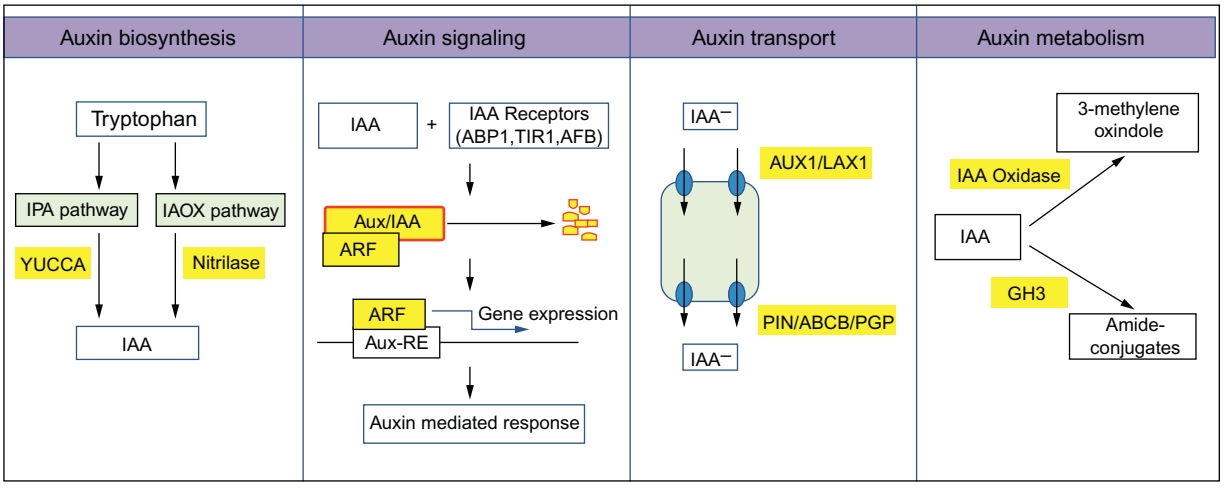

FIGURE 20.1 Components (shown in yellow) of auxin biosynthesis, signaling, transport, and metabolism, which can be targeted for enhancing abiotic stress tolerance in plants. *ABP1*, auxin-binding protein; *AFB*, auxin F-Box; *ARF*, auxin response factor; *Aux-RE*, auxin responsive elements; *IAOX*, indole-3-acetaldoxime; *IPA*, indole-3-pyruvic acid; *TIR1*, transport inhibitor response 1.

response to different environmental signals and provides developmental plasticity to plants. It is an essential signaling hormone and regulates plant growth and development under various abiotic stresses like heavy metal stress, nutrient deficiency, drought, salinity, and temperature stress. Roots, which are a key component for water and nutrient absorption, are also involved in sensing environmental signals. The role of auxin in mediating LR development is quite critical under stress conditions. Thus, modulation of auxin concentration in tissue specific manner can be a potential strategy to change root system architecture and to induce stress tolerance. Various auxin biosynthesis (*YUCCA* gene), metabolism (IAA-oxidase), conjugation (IAA-amidosynthetases), transport (PIN, AUX1/LAX1, ABCB carriers), or signaling (Aux/IAA, ARF) components have been identified in plants, which can be good candidate genes for improving abiotic stress tolerance in plants (Fig. 20.1). However, many auxin-signaling components and mechanisms are yet to be unraveled to increase auxin mediated tolerance in plants. A major challenge that remains to be addressed is the development of stable auxin-engineered crops that perform well under stressed as well as unstressed conditions. Towards this goal, study should be focused on development of transgenic crop plants that exhibit enhanced stress tolerance with no penalty on yield under unstressed conditions.

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CHAPTER

21

The Regulatory Signaling of Gibberellin Metabolism and Its Crosstalk With Phytohormones in Response to Plant Abiotic Stresses

Aditya Banerjee and Aryadeep Roychoudhury

Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India

OUTLINE

21.1 INTRODUCTION

The rapidly expanding human population demands a substantial increase in the global agricultural productivity. However, a major part of crop production is negatively affected by hostile environmental conditions. Such abiotic stresses manifested by salinity, drought, cold, heat, heavy metal toxicity, etc. induce physiological, biochemical, and molecular damages in plants (Tilman et al., 2011; Khan et al., 2012, 2013, 2014, 2015, 2016; Per et al., 2017). In spite of such constraints, global productivity has to be increased by almost 70% to feed an additional 2.3 billion people by 2050 (Tilman et al., 2011). Thus, novel strategies should be planned for generating the stress tolerant phenotype in susceptible plant species to boost crop yields even under suboptimal conditions.

Phytohormones are diverse signaling molecules with the ability to act as chemical messengers and promote cellular communication even at infinitesimally low concentrations (Roychoudhury and Banerjee, 2015). Phytohormones like auxins, cytokinin (CK), gibberellic acids (GAs), abscisic acid (ABA), and ethylene act as traditional phytohormones. Among these,

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the universal stress hormone ABA performs several important stress responsive functions by suppressing the amounts of GAs (germination and internodal growth promoting phytohormone) (Roychoudhury et al., 2015). The regulation of GA signaling and metabolism is a crucial decisive factor for the stress susceptible/tolerant phenotype. GAs modify the physiological metabolism in plants via their effects on photosynthesis and sink formation (Iqbal et al., 2011). These plant growth regulators promote phloem loading by inducing the activities of fructose-1,6-bisphosphatase and sucrose phosphate synthase. GA-mediated signaling stimulates the maintenance of an efficient source and sink. Such integration of source−sink relations is required for tolerance towards suboptimal conditions and poststress recovery (Iqbal et al., 2011). Hence, it is necessary to understand the functional significance of GAs in plants exposed to unfavorable environmental conditions. The current chapter presents an overview of the frontiers of GA research in plant abiotic stress responses.

21.2 GIBBERELLIC ACID METABOLISM IN PLANTS

GA constitute a large group of tetracyclic diterpenoid carboxylic acids, of which mainly GA_1 and GA_4 predominantly function as PGRs (Colebrook et al., 2014). GAs are positive regulators of germination, leaf expansion, stem elongation, initiation of flowering and trichome and reproductive developments (Claeys et al., 2012). GAs trigger growth-stimulatory functions and facilitate developmental phase transitions. It has been observed that *Arabidopsis* seedlings exposed to osmotic stress require the interaction of GAs with other phytohormones to exhibit stress responses (Munteanu et al., 2014).

The methylerythritol phosphate pathway operating in the plastids synthesizes GAs from *trans*-geranylgeranyl diphosphate. Thus, GAs share the common precursor of ABA (Banerjee et al., 2016; Banerjee and Roychoudhury, 2016a). The biosynthesis is facilitated by the sequential catalysis of two plastid localized terpene cyclases. The formed products are oxidized on the endoplasmic reticulum by cytochrome P450 monooxygenase and then by soluble 2-oxoglutarate-dependent dioxygenases (Hedden and Thomas, 2012). The GA-20-oxidase (GA20ox), GA3ox isozymes, and GA2ox comprises the family of dioxygenases. Abiotic stresses induce the expression of GA-catabolic *GA2ox* genes. These along with specific biosynthetic paralogues of *GA20ox* and *GA3ox* maintain cellular homeostasis by reducing GA accumulation (Wani et al., 2016).

The pleiotropic action of GAs during plant development is mediated by the degradation of DELLA protein (belonging to the GRAS family of proteins) (Colebrook et al., 2014). The conformational change in the nuclear receptor, GID1, is induced upon association with GA. As a result, the receptor interacts with the conserved N-terminal domain of the DELLA protein and promotes their association with an SKP, CULLIN, F-BOX CONTAINING (SCF) ubiquitin ligase. Thus, DELLA is ubiquitinylated and degraded by the 26S proteasomal machinery (Banerjee and Roychoudhury, 2016b) (Fig. 21.1). It has been opined that the DELLAs might activate or suppress gene expression by acting as transcriptional activators or in complexes with other transcription factors (TFs) (Hirano et al., 2012). Similarly they also act as inhibitors by sequestering gene

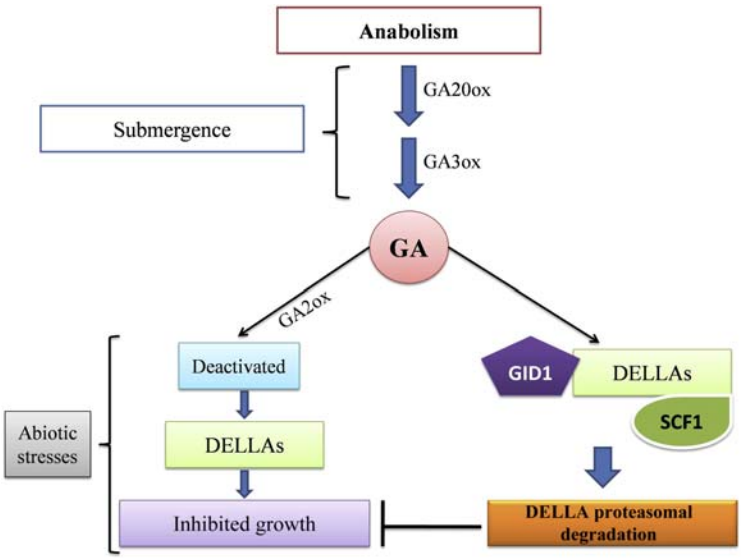

FIGURE 21.1 Gibberellic acid (GA) biosynthesis and its associated equilibrium during abiotic stress responses. Submergence promotes the expression of GA anabolic genes, *GA20 oxidase* (*GA20ox*) and *GA3ox*. However, under other abiotic stresses like salinity, drought, cold etc., the catabolic gene *GA2ox* is activated, which degrades GA and activates DELLAs. Thus, plant developmental growth is suppressed. Under natural conditions, GA stimulates the proteasomal degradation of DELLAs via polyubiquitinylation and regulates shoot and root growth (Colebrook et al., 2014).

activating TFs (Feng et al., 2008). This opens up potential avenues of GA signaling crosstalk with other pathways. DELLAs have been observed to interact with the molecular components of other phytohormonal cascades (Bai et al., 2012). Exploring the putative associations of DELLAs with basic leucine zipper (bZIP) or WRKY TFs can be adopted as future perspectives (Banerjee and Roychoudhury, 2017a; Banerjee and Roychoudhury, 2015).

21.3 REGULATORY SIGNALING OF GIBBERELLIC ACIDS DURING ABIOTIC STRESSES

The TF encoding gene, *Submergence 1A* (*Sub1A*) [family: APETALA2/Ethylene Response Factor (AP2/ERF); subfamily: ERF] regulates GA metabolism in *Arabidopsis* plants exposed to abiotic stress (Dubois et al., 2013). Salt and cold stresses in these plants suppress GA biosynthesis via two TFs of the DEHYDRATION-RESPONSIVE ELEMENT BINDING 1/C-REPEAT BINDING FACTOR (DREB1/CBF) family and one TF of a subfamily of the AP2/ERF family. The DWARF AND DELAYED FLOWERING 1 (DDF1) TF upregulated the expression of *AtGA2ox7* during salt stress (Magome et al., 2008). Mutation of *AtGA2ox7* slightly increased primary root length compared with the wild-type seedlings under saline treatment. However, no change in the extent of salt tolerance could be determined in the *ga2ox7* or *ddf1* mutants (Colebrook et al., 2014). Plants exposed to cold stress exhibit reduced accumulation of bioactive GAs via the DREB1B/CBF1 mediated upregulation of *AtGA2ox3* and *AtGA2ox6*. Lowering of cellular GA content resulted in the DELLA-dependent root growth inhibition during low temperatures (Achard et al., 2008a). Dubois et al. (2013) showed that ERF6 controls the expression of *GA2ox6* during mild osmotic stress. Interestingly, only *RGL3* (out of the five *DELLA* genes in *Arabidopsis*) was upregulated by stress. The other homologues had to be stabilized only by a low cellular GA content (Colebrook et al., 2014). It is well known that the ABA-independent pathway usually regulates the DREB1/CBFs (Roychoudhury et al., 2013). Association of the ethylene-dependent TF, ETHYLENE INSENSITIVE 3(EIN3) with the *DREB1/CBF* promoters might integrate these transcriptional regulators in the GA signaling pathway (Shi et al., 2012).

The DELLAs have been implicated in regulating the antioxidant system in *Arabidopsis* plants experiencing stress conditions (Achard et al., 2008b). The reactive oxygen species (ROS) are usually generated in uncontrolled amounts in almost all kinds of abiotic stresses. They trigger lipid peroxidation in membranes and degrade essential proteins within the cell. ROS can even oxidize nucleic acids and promote mutational effects that are cytotoxic (Banerjee and Roychoudhury, 2017b). DELLAs delay ROS-induced necrosis by inhibiting their accumulation during salt stress (Achard et al., 2008b). ROS controls the GA-mediated root growth. Thus, ROS might be the key regulatory component in the growth and stress tolerance effects exhibited by DELLAs (Achard et al., 2008b). In rice, the DELLA protein, SLENDER LIKE-1 (SLR1) ameliorated oxidative stress in the *Sub1A* lines separately exposed to drought and to dehydration after flooding (Fukao et al., 2011). Distinct roles of DELLA have been observed in *Arabidopsis* plants under control and stressed environments. DELLA regulated cellular proliferation under normal conditions. However, when the plants were exposed to osmotic stress, high accumulation of DELLA triggered mitotic exit and reduced the overall cell number compared with the control sets (Claeys et al., 2012). This partly illustrates the basis of DELLA-mediated growth arrest in plants under suboptimal environments.

21.4 THE SIGNALING CROSSTALKS BETWEEN GIBBERELLIC ACIDS AND RELATED PHYTOHORMONES

GA signaling is closely integrated with the traditional PGRs, ABA, and ethylene (Roychoudhury and Banerjee, 2017). Exogenous application of ABA resulted in inhibited root growth and corresponding accumulation of DELLAs in the *Arabidopsis* seedlings. The inhibition was however significantly reduced in the quadruple *della* mutants (Achard et al., 2006). The ABA-mediated accumulation of DELLA is related to decreased levels of bioactive GAs. Plants with mutated ABA receptor, *ABA Insensitive 1-1* (*ABI1-1*) did not exhibit the accumulation of DELLA (Colebrook et al., 2014). This shows that DELLA-associated GA signaling operates downstream to the ABI1 mediated cascade. ABI5 is a bZIP TF regulating seed germination and early seedling development by participating in the ABA signaling pathway (Banerjee and Roychoudhury, 2017a). It has been observed that ABI5 acts as an ABA analogue and integrates the components of GA, auxin, CK, jasmonate, and brassinosteroid (BR) signaling cascades (Skubacz et al., 2016). Liu et al. (2016) observed that three NUCLEAR FACTOR-Y C (NF-YC) homologues, NF-YC3, NF-YC4, and NF-YC9, regulate the GA and ABA-mediated germination of seeds. The NF-YCs associate with the DELLA protein RGL2. The module binds to the CCAAT *cis* elements in the *ABI5* promoter. ABI5 in turn regulates the expression of crucial GA- and ABA-responsive genes. Thus, the NF-YC-RGL2-ABI5 module

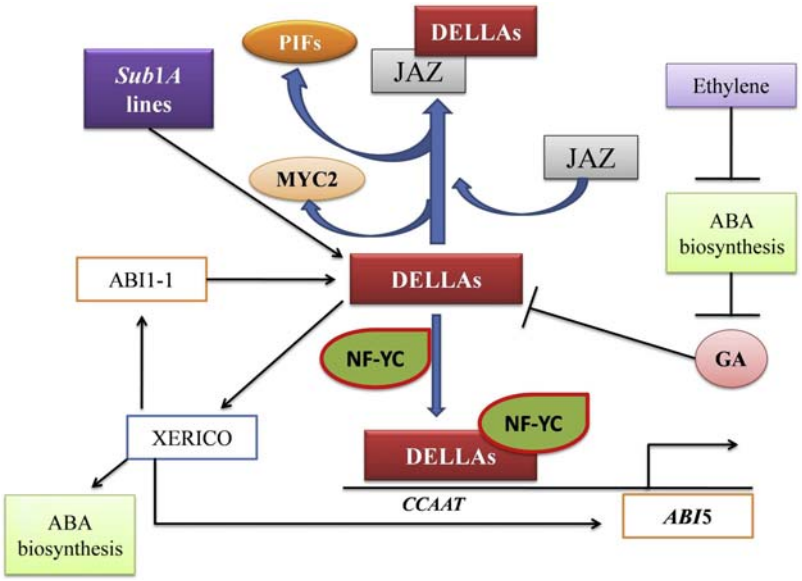

FIGURE 21.2 DELLA interaction with diverse signaling components mediates gibberellic acid crosstalks with other phytohormone signaling pathways. Signal transduction through ABSCISIC ACID INSENSITIVE1-1 (ABI1-1) stimulates the accumulation of DELLAs, which in turn promote abscisic acid biosynthesis by inducing the expression of *XERICO*. DELLAs also associate with NUCLEAR FACTOR-Y Cs to bind to the *cis* acting element, CCAAT in the *ABI5* promoter. This transcriptionally activates *ABI5*. The *Submergence1A* lines also maintain elevated levels of DELLA proteins. DELLAs often outcompete MYC2 to interact with JASMONIC ACID ZIM-domain proteins, which in turn compete with PHYTOCHROME INTERACTING FACTORs to bind with DELLAs. Elevated levels of ethylene during submergence triggers GA accumulation by repressing ABA synthesis. High cellular GA content degrades DELLA proteins and promotes escape responses during submergence.

mediates a GA-ABA crosstalk during germination (Liu et al., 2016) (Fig. 21.2). Zentella et al. (2007) showed that DELLA triggers the accumulation of XERICO, which represses a negative regulator of ABA biosynthesis (Fig. 21.2).

Analysis of the *constitutive triple response 1* (*ctr1*), *gibberellic acid insensitive-t6*(*gai-t6*) and *repressor of gibberellic acid* (*rga-24*) mutants revealed reduced salt tolerant phenotype, which indicates a possible integration of the ethylene signaling with DELLA (Achard et al., 2006). Ren and Wang (2016) identified two GA-responsive elements and an auxin-responsive element in the promoter of the auxin receptor gene *TIR1* in *Solanum lycopersicum*. Whereas naphthalene acetic acid (NAA) and ABA downregulated *SlTIR1*, exogenous application of GA upregulated the gene. It was observed that plants overexpressing *SlTIR1* failed to form reproductive seeds as *SlTIR1* promoted stenospermocarpy (abnormal seed development) (Ren and Wang, 2016). Ethylene accumulation promotes leaf hyponastic responses to submergence; whereas the redistribution of auxin, indole-3-acetic acid (IAA) from the leaf blade dictates the differential growth of the petiole in *Rumex palustris* (Cox et al., 2006). The petiolar ABA content is reduced in the presence of ethylene, which induces the expression of *GA3ox* so that the GA content is increased (Benschop et al., 2006). GA promotes escape response by bending the leaves upwards and stimulating petiole and leaf blade elongation to avoid anoxia during submergence (Polko et al., 2011).

Ethylene accumulation in submerged tissues of rice plants stimulates ABA catabolism via increased ABA-8′-hydroxylase activity (Jackson, 2008). The *Sub1* locus regulates the quiescence strategy of rice varieties acclimatized to short-lived, deep floods. Jung et al. (2010) highlighted that the ERF subfamily protein, SUB1A, controlled the expression of a variety of transcripts regulated by GA, ABA, ethylene, and CK. Under submergence, the *Sub1A* lines maintained elevated expression of *GA2ox* to restrict GA levels, stabilize the DELLA protein SLR1, and promote quiescence (Fukao et al., 2011). As a result of low GA content, the ABA levels were high, which effectively ameliorated the dehydration stress during desubmergence by inducing the expression of ABA-dependent drought-responsive transcripts. The plants thus maintained high relative water potential and exhibited low accumulation of ROS (Colebrook et al., 2014).

Jasmonate or jasmonic acid (JA) actively synthesized during cold stress interacts with phytohormones like GA, auxin, and ethylene to regulate leaf senescence and abiotic stress tolerance (Hu et al., 2017; Per et al., 2018). The DELLA proteins compete with MYC2 TF to bind to the JA ZIM-domain (JAZ) proteins (Hou et al., 2010). Yang et al. (2012) reported that even the JAZ proteins compete with the growth promoting PHYTOCHROME INTERACTING FACTORs (PIFs) to bind to DELLAs (Fig. 21.2). JA upregulated the DELLA gene *RGA-like 3* (*RGL3*) and its promoter was targeted by MYC2. RGL3 was found to interact with JAZ1 and JAZ8, of which JAZ8 is resistant to JA-mediated degradation (Wild et al., 2012). Thus, MYC2 released from JAZ1 (degraded by JA) in presence of JA triggers the expression of *RGL3*. Association of RGL3 with non-JA-degradable JAZ8 further promotes MYC2-dependent JA responses (Wild et al., 2012).

21.5 GIBBERELLIC ACIDS IN PLANT ABIOTIC STRESS: A RECENT UPDATE

In the rainfed lowland areas, submergence stress is considered to be a limiting factor for the growth of rice plants. GA is regarded as a negative regulator of submergence tolerance in rice. Treatment of plants separately with GA and its inhibitor paclobutrazol (PB) led to the identification of 3936 differentially expressed genes (DEGs) among the treated sets and the control. The PB treated plants exhibited submergence tolerance by maintaining photosynthetic capacity and reducing nutrient metabolism (Xiang et al., 2017). Liu et al. (2017) showed that GRAS40 in *S. lycopersicum* was upregulated by multiple abiotic stress treatments including D-mannitol, NaCl, and H_2O_2. It was found that SlGRAS40 interacted with GA and auxin signaling pathways during the vegetative and reproductive growth phases in tomato plants. The transgenics overexpressing *SlGRAS40* exhibited increased salt and drought tolerance compared with the control plants (Liu et al., 2017). Genes involved in JA, salicylic acid (SA) synthesis, and SA and BR signaling were downregulated, whereas the genes associated with GA degradation were upregulated in the transgenic plants overexpressing the *C-repeat binding factor 2* (*CBF2*) and *CBF3*, both involved in generating cold tolerance (Li et al., 2017).

In a recent study, several tissue specific GA dioxygenases have been identified as *CsGA20ox2*, *CsGA3ox2*, *CsGA3ox3*, *CsGA2ox1*, *CsGA2ox2*, and *CsGA2ox4* in *Camellia sinensis*. These genes have been proposed as candidate marker genes for abiotic stress tolerance breeding in tea plants (Pan et al., 2017). An AP2/ERF family protein, DREB2, from physic nut was found to be induced by ABA, but repressed by GA and salt exposures (Tang et al., 2017). Overexpression of the *DREB2* gene in rice resulted in dwarf and GA-deficient phenotype. Normal growth could however be restored by exogenous treatment with GA_3. Expression of biosynthetic genes like *OsGA20ox1*, *OsGA20ox2*, *OsGA20ox4*, and *OsGA3ox2* was significantly reduced in the plants that also showed elevated sensitivity to salt stress (Tang et al., 2017). Khalloufi et al. (2017) reported that exogenous application of 10^{-6} M GA_3 supplemented by inoculation with arbuscular mycorrhyzal fungi alleviated salt-induced damages in tomato plants by elevating the levels of CK, IAA, and ABA. The plants also maintained a high K^+/Na^+ ratio during stress (Khalloufi et al., 2017). Casein kinase II (CKII) is an evolutionarily conserved and crucial Ser/Thr kinase involved in regulating GA signaling during abiotic stress response in plants (Yuan et al., 2017). Analysis of the β-subunit of CKII (CKB1) was performed by creating *Arabidopsis* T-DNA mutants (*ckb1-1* and *ckb1-2*) and *CKB1* overexpressing plants. The mutants exhibited low sensitivity to ABA during germination and initial growth along with increased stomatal aperture, leaf water loss, and proline (compatible solute) accumulation. However, the *CKB1* overexpressing plants showed opposite results via alteration in a number of ABA and GA-regulatory genes. The research proposed CKB1 to be an ABA signaling–associated protein that positively regulates ABA signaling by establishing a parallel influence on GA metabolism (Yuan et al., 2017).

GA accumulation negatively regulates stress tolerance. Hence, attenuation of endogenous GA level by ectopic expression of *GA2ox6* increased the yield and promoted drought tolerance in rice (Lo et al., 2017). *GA2ox6* regulates the architecture and function of rice plants. The transgenics exhibited a thorough reprogramming in their transcriptomes along with reduced shoot height, more productive tillers, expanded root system, higher water use efficiency, and increased photosynthetic rate. Field trials using the transgenics increased the yield by 10%–30% compared with the wild-type plants under drought stress (Lo et al., 2017). GA reportedly regulates redox homeostasis by eliciting electron mobilization in the living aleurone layers of *Hordeum vulgare* cv. Himalaya grains (Mark et al., 2016). Maintenance of such redox equilibrium is essential for acclimatizing with suboptimal environments.

21.6 CONCLUSION AND FUTURE PERSPECTIVES

GA is a germination-promoting phytohormone that also stimulates internodal growth during seedling development. On evidential grounds, the current belief is that abiotic stress represses GA accumulation and its associated signaling cascades. Under such circumstances, cellular content of DELLA proteins is increased and these establish regulatory crosstalks with multiple phytohormones (Fig. 21.2). GA regulates the ROS levels in the cell. Furthermore, essential TFs like MYCs, DREBs, CBFs, JAZ-domain proteins, and PIFs participate in the GA signaling pathway during plant exposure to stress. Such rapid yet intricate interaction among phytohormones complicates the ultimate GA-mediated physiology under stress.

Identification of the epigenomic landscape in plants is an important aspect of understanding the context-dependent molecular physiology (Banerjee and Roychoudhury, 2017c). Epigenetic alterations at the global scale have been reported in abiotic stresses like salt, drought, and cold (Banerjee and Roychoudhury, 2017d; Banerjee et al., 2017). There is no literature available regarding GA-induced epigenetic dynamism

in plants. Thus, identifying such alterations in the epigenomic landscape under stress can be a novel endeavor. Transgenic rice plants overexpressing the GA catabolic gene, *GA2ox*, have succeeded in producing high yielding phenotypes with enhanced stress tolerance (Lo et al., 2017). Further, genome-wide studies must be conducted to identify more of such GA catabolic loci, which can be effectively mapped for stringent screening of high yielding, stress tolerant cultivars. Successful field trials followed by cultivation of such transgenic plants will ensure global food safety and a significant surge in the agricultural economy.

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CHAPTER

22

Abscisic Acid, a Principal Regulator of Plant Abiotic Stress Responses

Sushma Sagar and Amarjeet Singh
National Institute of Plant Genome Research, New Delhi, India

OUTLINE

22.1 INTRODUCTION

Plants are routinely troubled by various abiotic stresses such as high salinity, dehydration, and low temperature during their life span. These abiotic stresses have detrimental effect on plant development, longevity, and productivity. Plants have evolved with a sessile nature, and unlike animals, they cannot move away from adverse growth conditions. Rather, they are destined to combat these stress conditions in their stationary habitat. Therefore, plants have devised an adaptive mechanism that entails the activation of several signal transduction pathways, leading to diverse molecular, cellular, and physiological changes (Singh et al., 2016, 2018). Most signal transduction pathways triggered in response to biotic or abiotic stresses are mediated by one or more plant hormones. Therefore, plant hormones are a crucial player in regulating plants' response to various environmental cues (Iqbal et al., 2017; Khan et al., 2015a,b; Khan and Khan, 2014; Kazan, 2015; Per et al., 2018). Generally, phytohormones like salicylic acid (SA), jasmonic acid (JAs), and ethylene (ET) are implicated in plant response to pathogens, wounding, and other biotic stresses, whereas gibberellins (GAs), auxins (IAAs), brassinosteroids (BRs), and cytokinins are known to regulate plant development. However, recent advancements in plant stress related research have shown that all plant hormones could control multiple plant processes and are involved in crosstalk of signaling pathways.

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For example, SA, JA, and ET, apart from biotic stresses, are also involved in plant development and responses to abiotic stresses. Similarly, auxins and GA are crucial in abiotic and biotic stress responses (Colebrook et al., 2014; Kazan, 2013; Khan and Khan, 2013; Santino et al., 2013).

Abscisic acid (ABA) is the key hormone that primarily regulates plants' responses to various abiotic stresses; however, like other phytohormones ABA is also known to regulate plants' response to biotic stress and development (Singh et al., 2016). The discovery of the vital phytohormone ABA dates way back to the 1960s. Several independent and convergent experiments carried out by various research groups led to the discovery of ABA (Cracker and Abeles, 1969). However, the earliest and most convincing was the discovery of ABA in cotton, where it was involved in fruit abscission and dormancy (Li et al., 2017). As time and research progressed, newer functions of ABA were unveiled, including adaptation to various stresses, stomatal closure, sugar accumulation, seed development, etc. Due to its crucial role in abiotic and biotic stresses, ABA is known as a "stress hormone". Plenty of research on ABA accumulated ample information on its biosynthesis, storage, catabolism, site of action, and its possible targets. During the last decade, the ABA receptors and their crystal structures have been elucidated (Ma et al., 2009; Park et al., 2009). This information has provided a clear cut paradigm of the ABA signal transduction pathway. Moreover, recruiting the combinations of different key players such as PP2C phosphatase and SnRK2 kinases has helped to understand the signal transduction pathway. Recent studies have provided newer insights into the functional roles of the ABA signaling cascade in various aspects of plant growth and development. In this chapter, we discuss different facets of ABA in plants, including its biosynthesis, catabolism, ABA signaling pathway and various signaling components, and the role of ABA in abiotic stresses and plant development.

22.2 ABA BIOSYNTHESIS

ABA is synthesized via direct and indirect pathways. In fungi, ABA is synthesized directly from farnesyl diphosphate; on the other hand, in plants ABA is synthesized indirectly from carotenoids (Li et al., 2017). ABA synthesis in plants occurs in plastids and cytosol. A five carbon biological isoprene unit, isopentenyl diphosphate (IPP), is the precursor for the formation of ABA. Three molecules of IPP combine with dimethylallyl diphosphate to form a C_{20} molecule geranylgeranyl diphosphate (GGPP). In the next step, phytoene synthase catalyzes the condensation of two GGPP molecules together to produce a C_{40} molecule, phytoene (Kirby and Keasling, 2009). This is the first committed step for the biosynthesis of carotenoids. Further, desaturation and isomerization reactions lead to the formation of intermediates, such as lycopene. Lycopene is cyclized and hydroxylated to produce the first oxygenated carotenoid called zeaxanthin, by the enzyme β carotene hydroxylases. In the next step, zeaxanthin by the action of the enzyme zeaxanthin epoxidase (ZEP) is converted to all-*trans*-violaxanthin, which is further converted to 9′-*cis*-neoxanthin or 9-*cis*-violaxanthin by isomerization reaction with the help of the enzyme *cis*-isomerase. Subsequently, 9′-*cis*-neoxanthin or 9-*cis*-violaxanthin is oxidatively cleaved to produce the first C15 intermediate, xanthoxin (Li et al., 2017). This is the first committed step in the ABA biosynthetic pathway. All the reactions mentioned till now occur in the plastid. Xanthoxin is then transported to the cytosol by some unknown transporter where it is acted upon by short chain dehydrogenase reductase-like enzyme (SDR) to produce abscisic aldehyde. Lastly, abscisic aldehyde oxidase (AAO) encoded by *Arabidopsis ABA2* gene converts abscisic aldehyde to ABA (Fig. 22.1). AAO requires molybdenum as a cofactor for its activity. The *ABA3* gene in *Arabidopsis* encodes a sulfurase, which is capable of producing a cofactor MoCo. This cofactor is pivotal for ABA synthesis since *aba3* mutants are defective in ABA synthesis due to faulty AAO activity (Bittner et al., 2001). Hormones act optimally within certain concentration range. Too high or too low concentration poses a problem and can have severe consequences, therefore, the amount of hormone should be effectively regulated. The amount of active ABA available in a particular cell is determined by the rate of its biosynthesis, catabolism, and transport. The sensitivity of the plant cell towards ABA concentration is critical for ABA mediated response. During stress conditions there is a need for excess of ABA, thus, genes involved in ABA biosynthesis and various transcription factors are upregulated and activated (Banerjee and Roychoudhury, 2017). It is well known that zeaxanthin epoxidase (ZEP) has a regulatory role in ABA biosynthesis. Lower level of *ZEP* expression in etiolated tissue led to reduction in carotenoids, whereas overexpression of *ZEP* in tobacco resulted in elevated seed dormancy (Frey et al., 1999). Interestingly, the *Arabidopsis* genome encodes a single *ZEP* gene, however, even the most severe ZEP mutants do not completely lack ABA, suggesting the existence of an alternate minor pathway for ABA biosynthesis (Barrero et al., 2005). Moreover, the *zep* mutants are deficient in ABA and showed wilty phenotype and produced nondormant seeds (Finkelstein, 2013). Several reports have shown the upregulation of *AtZEP* gene in both shoot and root

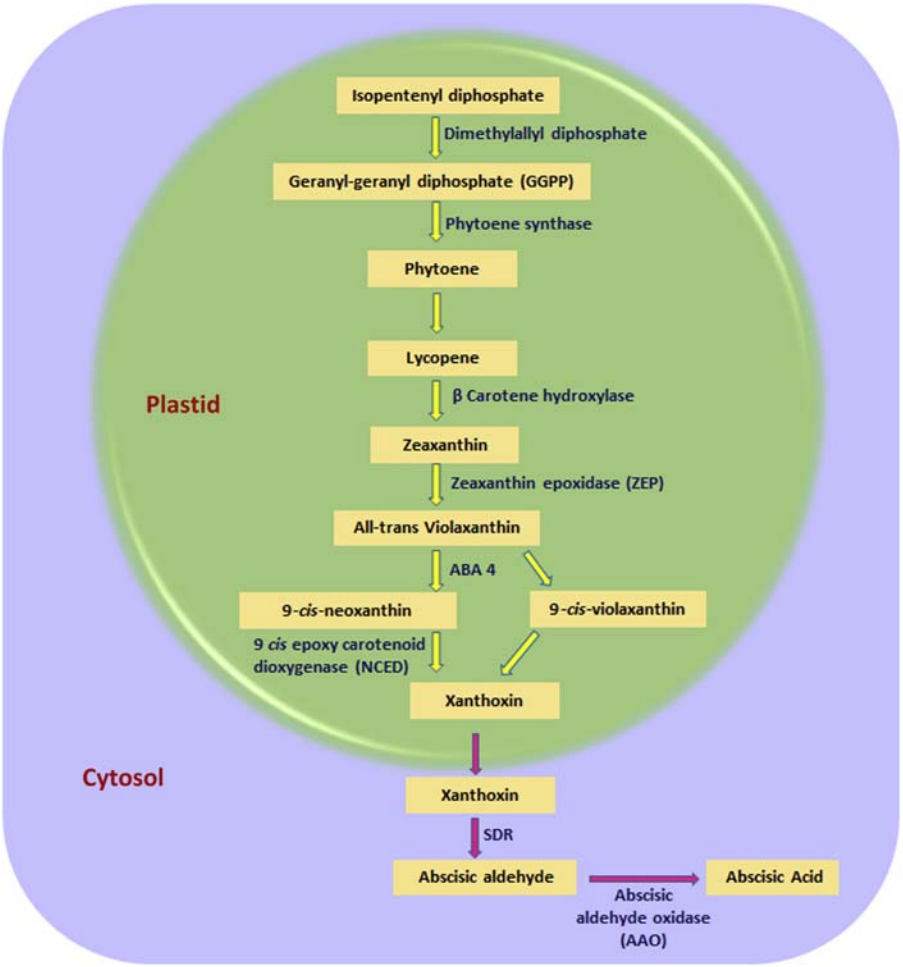

FIGURE 22.1 **Schematic representation of abscisic acid biosynthesis pathway.** Most of the reactions of ABA biosynthesis occur in the plastid. In the first step isopentenyl diphosphate is converted to a C_{20} molecule geranylgeranyl diphosphate (GGPP) by the enzyme dimethylallyl diphosphate. Through a series of reactions GGPP is converted to xanthoxin in the plastid. Xanthoxin is transported to the cytosol by an unknown mechanism where it is converted to abscisic aldehyde by the enzyme short chain dehydrogenase/reductase-like (SDR1). Finally, abscisic aldehyde is converted to abscisic acid by the action of the enzyme abscisic aldehyde oxidase (AAO).

when subjected to osmotic stress conditions (Li et al., 2017). Upregulation of *ZEP* gene also occurs on onset of drought stress *in Nicotiana plumbaginifolia*, *Arabidopsis*, and *Lycopersicon* roots (Audran et al., 1998, 2001). Hence, these findings support the key role of ZEP in regulating ABA synthesis in plants. Another crucial gene, 9-*cis*-epoxy-carotenoid dioxygenase (*NCED*), has a key role in ABA biosynthesis regulation. It has been observed that with the onset of stress, increase in level of ABA accompanies upsurge in NCED mRNA and protein, and whenever ABA level decreases, there is a fall in the level of *NCED* expression (Finkelstein, 2013). Rapid conversion of xanthophyll to ABA has been observed, especially during drought stress and xanthophyll is oxidatively cleaved to form ABA (Vishwakarma et al., 2017). This was reported in *Phaseolus vulgaris* where PvNCED1 accumulates under water deficit conditions, prior to ABA accumulation (Qin and Zeevaart, 1999). In avocado (*Persea americana*), higher expression of *PaNCED1* and *PaNCED3* were correlated with high ABA level during fruit ripening (Chernys and Zeevaart, 2000). Several independent researchers have witnessed such an increase in ABA level upon overexpression of *NCED* gene, for example; *AtNCED3* in *Arabidopsis* (Audran et al., 2001), *LeNCED1* in tomato and *PvNCED1* in tobacco (Schwartz and Zeevaart, 2010). Additionally, enzymes involved in the final step of ABA synthesis have a crucial role in regulating ABA level. However, lack of studies limits our knowledge on the role of this gene in the regulation mechanism of ABA synthesis.

22.3 CATABOLISM OF ABA

Under normal (unstressed) conditions, the amount of ABA in the cell must be reduced, thus, excess amount of ABA present needs to be neutralized. Catabolism and conjugation are important mechanisms for regulating the level of ABA (Nambara and Marion-Poll, 2005). Hydroxylation is a common reaction to catabolize ABA, and there exist three different ways of ABA hydroxylation in plants. It can be either hydroxylated at the C-7′, C-8, or at C-9′ position. However, hydroxylation at the C-8′ position is the predominant pathway (Li et al., 2017). ABA is inactivated through

hydroxylation at the 8′ position by the action of the enzyme ABA-8′ hydroxylase, which is a cytochrome P450 (Okamoto et al., 2011). Since 8′ABA is unstable, it is rapidly converted to phaseic acid, which is further converted to dihydrophaseic acid. In another mechanism, ABA is converted to 9′ hydroxyl ABA by 9′ hydroxylation. Whether both 8′ and 9′ hydroxylation are carried out by the same enzyme is not known and demands investigation. *In planta* regulation of ABA levels by hydroxylation has been displayed in *Arabidopsis* during water stress, salinity, and osmotic stress conditions, where abundance of multiple CYP707A transcripts and proteins were observed (Kushiro et al., 2004). In adzuki bean (*Vigna angularis*), ABA-glucosyltransferase (*AOG*) gene expression was rapidly increased under drought conditions (Xu et al., 2002). AOG catalyzes the transfer of a glycosyl group from a donor to an acceptor. Another way of checking and catabolizing higher level of ABA is by conjugating it with glucose ester to form ABA-GE, which is eventually stored in the vacuole. This form of ABA is inactive and can be considered as a storage form. Low permeability of biomembranes for ABA-GE suggests that it may be involved in long distance transport. ABA may be transported as ABA-GE and upon reaching the destined organ or tissue it may be cleaved to produce a functionally active form.

22.4 ABA SIGNALING PATHWAY

Generally, a signal transduction pathway involves initiation of signal by a stimulus, perception by the receptors, and transduction of signal by effectors and secondary messengers, leading to activation or inactivation of downstream components to generate the desired response. The core components of ABA signal transduction pathway include ABA receptors (ABAR), a group of START proteins called Pyrabactin resistance/Pyrabactin resistance like/Regulatory component of ABA receptor (PYR/PYL/RCAR), protein phosphatase 2C (PP2C), and sucrose non-fermenting 1 (SNF1) related kinase 2 (SnRK2) group of serine/threonine kinases (Singh et al., 2016). In the absence of ABA, ABARs remain unbound and inactive, whereas PP2C physically interacts with the SnRK2 and masks its kinase activation loop, thereby blocking the downstream signaling cascade. In the presence of ABA, as in case of stress and developmental trigger, ABAR binds to ABA, undergoes a conformational change and exposes its surface to interact with PP2C. Upon binding of PP2C with the receptors, inhibition of SnRK2 activity is removed. After unmasking, SnRK2 undergoes autophosphorylation and becomes active. Activated SnRK2 phosphorylates and regulates the activity of set of downstream genes including transcription factors, ion transport channel and ROS generating enzyme (Fig. 22.2). Function of activated or repressed downstream components ultimately lead to adaptive response (Sheard and Zheng, 2009; Sirichandra et al., 2009; Umezawa et al., 2010). A few of the identified targets of SnRK2s include inward rectifying K^+ CHANNEL IN *Arabidopsis thaliana* (KAT1) (Sato et al., 2009), SLOW ANION CHANNEL ASSOCIATED1 (SLAC1) (Geiger et al., 2009; Lee et al., 2009a), ROS generating enzyme RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF) (Sirichandra et al., 2009) and bZIP transcription factor (Nakashima et al., 2009).

22.5 ABA RECEPTORS

ABARs are the key components in ABA perception and were discovered recently by screening for mutants that were unable to grow in the presence of an ABA agonist Pyrabactin (Park et al., 2009). PYR/PYL/RCAR proteins are the members of soluble ligand binding START-domain protein superfamily (Ma et al., 2009; Park et al., 2009). The proteins of this family are comprised of a characteristic helix-grip fold and form a ligand binding pocket in the center. PYR/PYL/RCAR family proteins contain a START domain or Bet v fold, named after a major allergen in pollen of white birch (*Betula verrucosa*) (Radauer et al., 2008). PYR/PYL/RCARs contain an N-terminal alpha helical segment that is absent in Bet v fold. Also, they have a helix-grip fold consisting of seven stranded beta sheets flanked by two alpha helices. Crystal structures of PYL1 (Melcher et al., 2009) and PYL2 (Melcher et al., 2009; Yuan et al., 2010) have been delineated, both in free and ABA bound form. The structures of these receptors are highly similar, however there are some dissimilarities in the receptor binding pocket. Pyrabactin is an agonist of PYR1 and PYL1, however, it is an antagonist of PYL2 (Melcher et al., 2009; Peterson et al., 2010). Generally, the ABA binding pocket is buried deep within the receptor. The chemical structure of various functional groups of the ABA molecule complement with the walls of this pocket, thus allowing ABA to fit well inside the pocket. The hydroxyl, carboxylic, and ketone groups of ABA molecule interact with the polar side chain of PYR1. Whereas, the isoprene moiety and the cyclohexane ring of ABA molecule form hydrophobic interaction with apolar side chain of the PYR1 molecule (Santiago et al., 2009). A total of 14 genes encoding 159–211 amino acid–long PYR/PYL/RCAR protein are identified in the *Arabidopsis* genome (Santiago et al., 2012). PYR/PYL/RCAR proteins are highly conserved at the level of amino acid sequence. The function of PYR/PYL/RCAR proteins as ABA receptors has been validated in plants. The triple

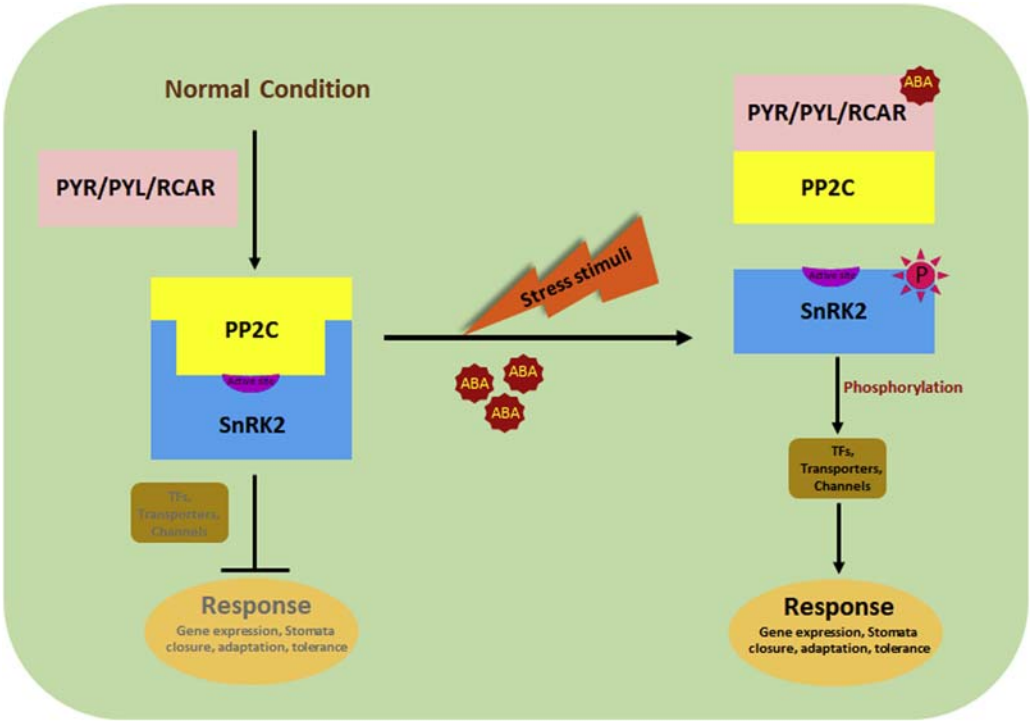

FIGURE 22.2 **ABA signal transduction pathway in plants.** In normal (unstressed) conditions due to absence or low level of ABA, ABAR (PYR/PYL/RCAR) remains unbound. Whereas, PP2C physically interacts with the SnRK2 and masks its kinase activation loop, thereby blocking the downstream signaling cascade. Perception of stress stimuli results in accumulation of ABA in the cell. In the presence of ABA, ABAR binds to ABA and undergoes a conformational change and interacts with PP2C. Interaction of ABAR with PP2C unmasks the inhibition from SnRK2, and the free SnRK2 undergoes autophosphorylation and becomes active. Activated SnRK2 phosphorylates and regulates the activity of a set of downstream genes including transcription factors, ion transport channel and ROS generating enzyme to generate an adaptive response, leading to stress tolerance.

mutant *pyr1/pyrl1/pyrl4* and the quadruple mutant *pyr1/pyrl1/pyrl2/pyrl4* are less sensitive to ABA and have abnormal root growth and germination (Gonzalez-Guzman et al., 2012). Moreover, quadruple mutants showed abnormal stomata closure even in presence of ABA (Nishimura et al., 2009; Santiago et al., 2009). On the other hand, overexpression of PYL8 and PYL9 enhanced transpiration rate in *Arabidopsis* (Ma et al., 2009; Saavedra et al., 2010). The rice and the poplar (*Populus trichocarpa*) genomes also encode for 14 members of the PYR/PYL/RCAR family (Tian et al., 2015; Yu et al., 2017a). These ABA receptors have been shown to regulate abiotic stress, particularly drought stress tolerance in rice, *Arabidopsis*, and tomato (González-Guzmán et al., 2014; Kim et al., 2014; Zhao et al., 2016). In a recent study, overexpression of poplar *PtPYL1* and *PtPYL5* in *Arabidopsis* conferred hypersensitivity to ABA and drought tolerance (Yu et al., 2016). Thereafter, overexpression of *PtPYL1* and *PtPYL5* also enhanced drought tolerance in hybrid poplar (*Populus davidiana X Populus bolleana*). Moreover, overexpression of *PtPYL1* and *PtPYL5* in hybrid poplar resulted in increased leaf size, which could be a strategy of the plant to retain more water under drought stress (Yu et al., 2017b). This genetic evidence confirmed the role of PYR/PYL/RCAR as ABA receptor, their importance in ABA signaling and their key role in mediating stress tolerance in plants.

22.6 PROTEIN PHOSPHATASE 2C

Protein phosphatases 2C (PP2C) are the largest group of serine/threonine protein phosphatase in plants and comprised of 80, 90, and 91 members in *Arabidopsis*, rice, and tomato, respectively (Singh et al., 2016). In rice and *Arabidopsis*, the PP2C family is subdivided into 11 subclades, from A to K (Singh et al., 2010). PP2Cs consist of a catalytic domain, which can be located either at the N-terminus or at the C-terminus (Schweighofer et al., 2004). This contributes to their structural variability and functional diversity. PP2Cs are the monomeric enzymes and they require metal ions such as Mn^{2+} or Mg^{2+} as cofactors for their activity (Singh and Pandey, 2012). Recent identification of novel ABA receptors in plants have placed PP2C at the center stage of the entire ABA signaling pathway. Several studies have shown that group A PP2Cs are

majorly involved in ABA related function, and act as negative regulators of ABA signaling (Bhaskara et al., 2012; Komatsu et al., 2009; Merlot et al., 2001; Saez et al., 2004; Singh et al., 2015). PP2Cs localize both in the cytosol and the nucleus, however they form a complex with ABARs mainly in the nucleus (Moes et al., 2008). *Arabidopsis* PP2C group A is comprised of nine members including ABI1, ABI2, HAB1, HAB2, AHG1, AHG3, HAI1, HAI2, and HAI3 (Singh et al., 2015) ABI1 is the first PP2C to be cloned (Meyer et al., 1994) followed by its closest homolog ABI2. Group A PP2Cs of rice, *Arabidopsis*, maize, and tomato are highly inducible under ABA, drought, salinity, and cold stress (Singh et al., 2010, 2015; Sun et al., 2011; Wei and Pan, 2014). In *Arabidopsis*, group A PP2Cs are critical in regulating ABA signaling under abiotic stresses. *Arabidopsis* ABI1 and ABI2 are the most extensively studied PP2Cs in plants and established as the major player of ABA signaling under abiotic stresses and during development (Fuchs et al., 2013; Singh and Pandey, 2012). Elimination of group A PP2Cs allows survival of mosses in complete desiccation conditions, suggesting that group A PP2Cs are important regulators of desiccation tolerance (Komatsu et al., 2013). Besides regulating function of SnRK2s in ABA signaling, PP2Cs also control abiotic stress triggered MAPK signaling (Danquah et al., 2014). PP2Cs dephosphorylate and deactivate MAPKs, thereby blocking the downstream signaling cascade (Smékalová et al., 2014). PP2Cs are also recognized as important regulators of plant development. They control and regulate plant development at various stages, including panicle stage, leaves, roots, inflorescence, etc. (Kamenetsky et al., 2015; Xue et al., 2008). Changes in the transcript abundance of various PP2C genes during reproductive development (seed and panicle) were observed in rice (Singh et al., 2010).

22.7 SNF1-RELATED PROTEIN KINASE 2 (SNRK2)

In *Arabidopsis*, 39 SnRK protein kinases are present that are divided into three different families: SnRK1 (3 members), SnRK2 (10 members), and SnRK3 (26 members) (Hrabak et al., 2003; Yan and Chen, 2017), based on the sequence similarity. SnRK2s have been recognized as the key players of ABA signaling pathway. Several members of SnRK2 family, such as SnRK2.1, 2.2, 2.3, and 2.6 are implicated in ABA related function in *Arabidopsis* (Fernando and Schroeder, 2016; Nakashima et al., 2009). The first SnRK2 shown to be involved in ABA signaling pathway was PKABA1, which is transcriptionally upregulated by ABA and suppresses gibberellic acid induced gene expression in aleurone layers of barley (Gómez-Cadenas et al., 1999). Later on, in-gel kinase assay showed that AAPK (ABA activated serine−threonine protein kinase) regulates ABA induced stomata closure in *Vicia faba* (Kwak et al., 2006). OST1 (Open stomata 1; SnRK2.6) is an ortholog of AAPK in *Arabidopsis* and SnRK 2.2, 2.3 are other members of the kinase family that are closely related to OST1. Double mutant *snrk2.2/2.3* exhibited ABA insensitive phenotype, in terms of inhibition of seed generation and root growth, and reduction in expression of ABA inducible genes (Fujii et al., 2007). Furthermore, triple mutant *snrk2.2/2.3/2.6* showed severe defects in ABA signaling and related responses, suggesting that SnRK2s are the positive regulators of ABA signaling (Nakashima et al., 2009). Interestingly, all SnRK2s were activated by osmotic stress except SnRK2.9, and *Arabidopsis* mutants defective in all 10 SnRK2s show defect in gene regulation and ABA accumulation under osmotic stress (Fujii et al., 2011). Hence, SnRK2s are indispensable for ABA dependent osmotic stress response in plants. Recently, overexpression of poplar *PtSnRK2.5* and *PtSnRK2.7* in *Arabidopsis* led to enhanced salt stress tolerance (Song et al., 2016). Furthermore, *PtSnRK2s* exhibited differential expression pattern in response to ABA treatment and different stress conditions, in an organ specific manner (Yu et al., 2017a). Interestingly, heterologous expression of *Arabidopsis SnRK2C* resulted in enhanced osmotic and salt stress tolerance in poplar (Yu et al., 2017b). Apart from SnRK2, members of the SnRK3 family are also implicated in ABA signaling. For instance, PKS3/CIPK15 interacted with calcium binding protein SCaBP5/CBL1, leading to insensitivity in seed germination and stomata movement, thereby negatively regulating the ABA signaling pathway (Yu et al., 2006). The *cipk23* null mutants showed decreased transpirational water loss and stomata opening, thus CIPK23 was recognized as a negative regulator of ABA signaling and response (Cheong et al., 2007; Zhang et al., 2014).

22.8 ABA-DEPENDENT GENE EXPRESSION

ABA induces the expression of various genes, especially transcription factors. About 10% of the protein coding genes are regulated by ABA in *Arabidopsis* (Fujita et al., 2011). Essentially, ABA dependent gene expression is regulated by two main families of bZIP transcription factors, AREB/ABF during vegetative stages and ABI5 in the seeds. During osmotic stress when the plant is still in the vegetative stage, the wide range of the ABA-mediated gene expression is governed by the AREB/ABF pathway (Bartels and Souer,

2004; Shinozaki and Yamaguchi-Shinozaki, 2007). One essential feature of genes regulated by ABA is that they contain G-box (CACGTG), ABA responsive *cis*-element (ABRE: PyAC GTGG/TC) in their promoters (Gómez-Porras et al., 2007). These elements have an ACGT central core that is recognized by the bZIP transcription factors in the plants (Choi et al., 2000). Transcription factors of the AREB family are another major group of genes that mediate ABA-dependent gene expression. Analysis of transgenic *Arabidopsis* plants overexpressing *AREB1/ABF2*, *AREB2/ABF4*, or *ABF3* showed involvement of these transcription factors in stress responsive ABA signaling (Kim et al., 2004). Further, the ectopic expression of *ABF2* in transgenic *Arabidopsis* plants could not activate target genes such as *RD29B* (Fujita et al., 2005) and ABF2 requires ABA for full activation (Yoshida et al., 2010). These transcription factors are considered as the master regulators of ABA-mediated gene expression under osmotic stress. This is supported by the observation that *areb1/areb2/areb3* triple mutants are ABA insensitive and showed significantly lower drought tolerance as compared with the single and double mutants (Yoshida et al., 2010). AP2/ERFs are another important group of transcription factors controlling ABA-mediated gene expression (Nakano et al., 2006). DREB is the most widely studied AP2/ERF and DRE/CRT binding protein namely, DREB1, has an important role in abiotic stress signaling. These transcription factors bind to the DRE/CRT *cis*-acting motif (A/GCCGAC), which is crucial for induction of many stress related genes (Agarwal et al., 2006). Recently, overexpression of *AtERF15* and *AtERF19* conferred drought tolerance in the transgenic *Arabidopsis* plants (Lee et al., 2015; Scarpeci et al., 2016). RAP2.1 and RAP2.6, the two AP2/ERF transcription factors that are identified as subregulons of DREB/CBF (Fowler and Thomashow, 2002). RAP2.6 interacts with *cis*-element CE1 (TGCCACCGG) or GCC (AGCCGCC) and acts as a positive regulator of osmotic stress in an ABA dependent manner (Zhu et al., 2010). Whereas, RAP2.1 acts as a negative regulator in ABA signaling pathway during cold and drought stress (Dong and Liu, 2010). Another AP2, ADAP (ARIA-interacting double AP2 domain protein) is considered a positive regulator of ABA response. The *adap* knockout mutant plants showed reduced drought tolerance and partial insensitivity to ABA (Lee et al., 2009b). Plant MYB transcription factors belong to the R2R3-type MYB family. In *Arabidopsis*, 126 R2R3-MYB genes are present and few of them act as mediators of ABA-dependent gene expression (Dubos et al., 2010). Transgenic *Arabidopsis* plants overexpressing *MYB2* and *MYC2* exhibited enhanced osmotic stress tolerance and sensitivity to ABA as compared with the wild type plants (Bhattacharjee and Jain, 2013). MYB 96 mediates ABA-dependent expression of *RD22* gene to confer drought stress tolerance via auxin signaling pathway (Seo et al., 2009). Hence, it can be inferred that MYB96 converges the ABA and auxin signaling pathway at some point. Furthermore, transcript level of MYB transcription factors, like MYB108 and MYB44, was upregulated upon treatment with various hormones and stress conditions like drought and salinity (Jung et al., 2007, 2008). All these findings by and large confirm that R2R3 MYB transcription factors control ABA-mediated gene expression in various stress conditions by targeting different signaling pathways. In *Arabidopsis*, 74 WRKY TFs are present and form a large gene family (Rushton et al., 2010). The major role of WRKY transcription factors is the transcriptional reprogramming in diverse cellular processes. WRKY63 controls the expression of stress related genes, such as *ABF2* and *RD29A*, thereby mediating plants responses to ABA, drought, and salt stress (Jiang et al., 2014; Ren et al., 2010). The expression of *AtWRKY40*, *AtWRKY18*, *AtWRKY60*, and *AtWRKY63* was increased upon ABA treatment (Rushton et al., 2012). Overexpression of wheat (*Triticum aestivum*) *TaWRKY1* and *TaWRKY33* in *Arabidopsis* resulted in heat and drought stress tolerance (He et al., 2016). Other important regulators of transcription in plants are NAC transcription factors. They play an important role in biotic and abiotic stress signaling in plants (Nuruzzaman et al., 2013). The expression of NAC transcription factors is induced under drought conditions in vital crop plants like rice, soybean, maize, and wheat (Hao et al., 2011; Hong et al., 2016; Huang et al., 2015; Lu et al., 2012; Rabbani et al., 2003). Several NAC transcription factors have been demonstrated in improving drought stress tolerance through ABA signaling pathway in various plants like rice, wheat, tobacco, and *Arabidopsis* (Christiansen et al., 2011; Jeong et al., 2010; Ramegowda et al., 2012; Xue et al., 2011). Recently, expression of *OsNAC022* was increased fourfold after ABA treatment and *OsNAC022* overexpressing plants showed tolerance to drought and salt stress in rice (Hong et al., 2016). These findings prove that the transcription factors have multiplex roles in controlling ABA-responsive gene expressions in plants.

22.9 THE ROLE OF ABA IN ABIOTIC STRESS SIGNALING

Plants are sessile and are often exposed to various stresses in their natural habitat. Adverse environmental conditions disrupt the normal cellular functioning, thus decreasing the plants' productivity significantly. However, plants have devised adaptive mechanisms to combat adverse growth conditions. Triggering an array

of signaling networks is one of the major adaptive responses. ABA signaling has been established as an important signaling network triggered in response to abiotic stresses (Cutler et al., 2010; Singh and Pandey, 2012). ABA mediates drought, salinity, and cold stress responses and tolerance in plants (Huang et al., 2012; Qin et al., 2011). One of the crucial functions of ABA is to regulate stomatal movement under osmotic/drought stress. Higher levels of ABA promote stomata closure and inhibit the transport of positively charged ions across the guard cell membrane to regulate the rate of transpiration under water deficit conditions (Albert et al., 2017; Haworth et al., 2018). The onset of stress leads to elevated ABA concentration, which instigates an increase in cytosolic calcium concentration. An increase in cytosolic calcium concentrations results in activation of S type (slow activating channel) and R type (rapid transient) channels (Schroeder et al., 2001). SLAC1 anion channel allows release of anionic organic acid from vacuole to the cytoplasm (Sirichandra et al., 2009). Also, an increase in the guard cell ABA level activates the K^+ outward rectifying channel and inhibits the K^+ inward rectifying channel (KAT1, KAT2). Regulation of these channels leads to anion efflux from the guard cell, causing membrane depolarization, which further leads to the efflux of K^+ from the guard cell (Kim et al., 2010). This reduces the guard cell turgor leading to stomata closure. OST1 is an important protein kinase involved in regulating stomata aperture through ABA signaling (Sirichandra et al., 2009). OST1 is known to be induced under osmotic stress and targets inward rectifying potassium channel KAT1 to control its channel activity through phosphorylation (Sato et al., 2009). Thus, OST1 positively regulates stomata closure by negative regulation of KAT1 activity in an ABA-dependent manner (Umezawa et al., 2010). During water deficit conditions, ABA inhibits shoot growth and promotes root growth, thereby enhancing the root:shoot ratio (Li et al., 2017). Increasing the root growth enables the plant to efficiently absorb available water and hence prevents the plant from dehydrating. ABA is known to influence the activity of various aquaporins by regulating their gene expression or via posttranslational modification of aquaporins (Sharipova et al., 2016). During water scarcity, dephosphorylation of various aquaporins occur at the N-terminus in an ABA dependent manner. The posttranslational modification of aquaporins helps to reduce the water loss (Kline et al., 2010; Shatil-Cohen et al., 2011). Several studies of overexpression transgenic plants have unearthed the important roles of ABA in drought and salinity stress tolerance in various plants species. *OsbZIP* overexpression in rice plants conferred drought tolerance through ABA signaling pathway (Tang et al., 2012). The overexpression of ABA responsive transcription factor *AREB/ABF* or *BIS/AtDPBF* led to induction of stress-responsive gene like LEA (late embryogenesis abundant protein) (Bartels and Sunkar, 2005). LEA proteins act as molecular chaperones and protect the cell membrane integrity during stress. During osmotic stress, these proteins protect mRNA, lipid, and various enzymes from dehydration (Yamaguchi-Shinozaki and Shinozaki, 2006). Interestingly, exogenous application of ABA conferred drought tolerance in various flowering plants like *Salvia divinorum*, *Impatiens walleriana*, *Tagetes erecta*, and *Viola tricolor* (Harris et al., 2011). Recently, dehydrin genes are found to confer osmotic stress tolerance through ABA dependent pathway, in various plant species, including sorghum, maize, and *Prunus mume* (Bao et al., 2017; Halder et al., 2016; Zamora-Briseño and de Jiménez, 2016). Salt stress is detrimental to the plants as it hampers their photosynthetic efficiency and metabolic processes (Fernando and Schroeder, 2016). ABA is also known to mediate salinity stress response and tolerance in plants. Salinity stress has an inhibitory effect on the primary and lateral root growth. During salinity stress, lateral roots undergo a quiescent stage during which they develop thick casparian strips in the endodermis, a process supported by high ABA concentration. The thick casparian strips act as a barrier to salt diffusion (Duan et al., 2013). The HISTONE DEACETYLASE6 (HDAC6) played a crucial role in salt stress tolerance and seed germination in an ABA-dependent manner (Chen et al., 2010). Also, an important role of cuticle in stress signaling and ABA biosynthesis was elucidated. CED1 (9-CIS EPOXYCAROTENOID DIOXYGENASE DEFECTIVE 1) is an essential protein in the cuticle formation and *ced1* mutants are defective in ABA production, thereby showing sensitivity towards the osmotic stress (Wang et al., 2011). Recently, *Reaumuria trigyna RtWRKY* gene was induced by ABA and NaCl treatments and overexpression of *RtWRKY* in *Arabidopsis* plants showed enhanced salt tolerance (Du et al., 2017). The *AtDIF1* (*Arabidopsis thaliana* Drought induced F Box1) is induced upon ABA, drought, and salinity treatment. The *AtDIF1* overexpression contributes to salt tolerance by reducing Na^+ content and promoting seedling growth in transgenic plants through ABA signaling. However AtDIF1 had antagonistic effect under drought stress as transgenic plants showed reduced seedling growth (Gao et al., 2017). These results showed that AtDIF1 positively regulates salinity stress tolerance and negative regulates drought stress response.

In the recent past, various researchers have shown the role of ABA in cold stress tolerance as well. Cold-inducible dehydrin (WCS120) and ABA accumulation was reported to confer cold stress tolerance in wheat

(Kosová et al., 2012). Moreover, ABA and dehydrin accumulation results in increased cold acclimation induced frost tolerance in *Triticum monococcum* (Vanková et al., 2014). In prior studies, overexpression of wheat *WCOR410* (wheat cold regulated gene) in cucumber and potato, and *DHN24* (dehydrin 24) in strawberry, resulted in enhanced frost tolerance in transgenic plants (Yin et al., 2006). Overall, these findings advocate that ABA is an important stress hormone and enables the plants to stand against various stress conditions.

22.10 CONCLUSION

ABA is an important hormone involved in diverse plant processes and signaling networks. The recent breakthrough discovery of ABA receptors in plants has enabled researchers to understand the ABA signaling pathway comprehensively. Before understanding the role of ABA in different conditions, it is important to understand the biosynthesis of ABA in plants. ABA biosynthesis mainly occurs in the plastid and partly in the cytosol, where a number of intermediate products are formed with the help of several enzymes. Genetic manipulation of these enzymes, especially like that of NCED and AAO, would be vital to regulate the ABA level. Thus, a balance of ABA concentration could be achieved in stressed and unstressed conditions. Several groups of transcription factors, such as bZIP, AP2, WRKY, MYB and others, have been the major effectors of ABA signaling under stress and developmental events. These transcription factors receive the signal from upstream and perform transcriptional reprogramming at the downstream for adaptive response. Targeting transcription factors that are involved in ABA response for genetic manipulation has helped researchers to generate plants that show better tolerance to various abiotic stress. Several transgenic based studies have established the crucial role of ABA in osmotic, drought, salinity and cold in a spectrum of plant species. Therefore, ABA is a vital phytohormone required for plant adaptation to different stresses in the environment. In future, analysis of interactions of ABA signaling with other phytohormones such as cytokinin and ethylene, and crosstalk of signaling pathways, may provide the new paradigm in plant abiotic stress signaling.

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CHAPTER

23

Salicylic Acid–Mediated Defense Mechanisms to Abiotic Stress Tolerance

Farhan Ahmad[1], *Ananya Singh*[2] *and Aisha Kamal*[1]

[1]Department of Bioengineering, Integral University, Lucknow, Uttar Pradesh, India [2]Department of Biosciences, Integral University, Lucknow, Uttar Pradesh, India

OUTLINE

23.1 INTRODUCTION

Plant overall growth and development metabolism is well-coordinated and regulated by low molecular weight compounds that work as chemical messengers to perform a precise role in plants known as phytohormones (Voß et al., 2014). The active site of phytohormones may be close to the place of synthesis or may be transported to different location wherever prerequisite for the regulation of biological functioning either in normal or stressful environment (Khan and Khan, 2014; Per et al., 2018). The beneficial effect of

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phytohormones directly linked with plant growth and development and involving engineering for premeditated stress resistance has been well-established (Wani et al., 2016). The positive growth regulators of plants can be listed as auxins (AUX), gibberellic acid (GA), and cytokinins (CK). The growth regulator primarily involved in dormant and stress conditions, for example, salicylic acid (SA), abscisic acid (ABA), ethylene (ET), and recently identified compounds such as, jasmonates (JA) derived compound, brassinosteroids (BRs), and nitric oxide (NO), achieve much consideration to improve plant growth through stress tolerance. Among all, SA, chemically known as *2-hydroxybenzoic acid*, is a phenolic watchdog, involved directly or indirectly in growth and development, thermogenesis, and enhanced protection against disease (Khan and Khan, 2013; Khan et al., 2012, 2015a,b; Klessig, 2017). SA is fully dedicated to alleviating life-threatening consequences of abiotic stresses such as salinity tolerance (Cao et al., 2009; Ahmad et al., 2017), heat stress (Wang and Li, 2006; Khan et al., 2013a,b), metal stress (Nazar et al., 2015), etc. It was reported that exogenous spraying of SA enhanced photosynthetic capability; production of secondary metabolites such as proline, flavonoid, and saponin content; and promoted LOX inhibitory action, thus improving the antioxidant defense system of the plant (Khan et al., 2013a,b; Ibrahim et al., 2017). SA, either in the developmental stage or environmentally adverse conditions prompted the expression of stress-specific genes and proteins, the activation of molecular chaperone, mitogen activated protein kinases (MAPKs), and maintains balance between ROS formation and detoxification, to enhance tolerance against abiotic stress (Xu and Brosché, 2014). Further, the beneficial interactions of SA with other phytohormones in both biotic as well as abiotic response have also been well known with jasmonic acid (Khan et al., 2013a,b; Per et al., 2018), ethylene (Tirani et al., 2013; Nguyen et al., 2016), and brassinosteroids (Saini et al., 2015; Khatoon et al., 2017). However, the detailed mechanism of SA crosstalk with other phytohormones under stress is not well known. In this review, the crosstalk of SA with other phytohormones in boosting plant strength under ideal and adverse environments is deliberated with emphasis on the most suitable and justifiable mechanism, and other unresolved, unfamiliar perspectives have also been explored.

23.2 BIOSYNTHETIC PATHWAY AND MODIFICATIONS OF SALICYLIC ACID

The biosynthetic pathway for synthesis of SA adopted by plants encompasses two enzymatic pathways, that is, ICS (isochorismate synthase) and IPL (isochorismate pyruvate lyase), from a common precursor, chorismic acid (Strawn et al., 2007). The conversion of shikimic acid to SA is a multistep, enzyme catalyzed biochemical reaction via intermediates such as phenylalanine and cinnamic acid. The conversion of phenylalanine to SA accomplished by two routes in which transformation of orthocoumaric acid or benzoic acid by enzymatic action of BA 2-hydroxylase (BA2H) in PAL biosynthetic. The conversion of phenylalanine to cinnamic acid required essential phenylalanine ammonialyase enzyme and hence known as PAL biosynthetic pathway (Verberne et al., 1999; Hara et al., 2012). The IC pathway fundamentally is based on the assumption that conversion of chorismate to isochorismate is taking place through the action of IPL and is finally converted to SA. In another pathway, SA biosynthesis in plants has been reported from shikimic acid via chorismic acid and coumaric acid but the detailed mechanism is under question (An and Mou, 2011). The PAL and ICS pathways act independently, and it is well known that the PAL pathway is mainly responsible for the synthesis of SA. Thus the question arises whether the existence of PAL pathway is solely restricted to pathogen-prompted SA production (Chen et al., 2009). Further, the importance of the PAL pathway lends credibility that SA production is governed by intermediates from both the ISC and PAL pathways (Chen et al., 2009).

Once synthesized in the chloroplast, plant MATE proteins facilitate the transportation of SA from chloroplast to cytosol through EDS5 channel (Dempsey et al., 2011). EDS5 is localized to the envelope membranes of chloroplasts and is essential for SA biosynthesis and transportation (Feys and Parker, 2000). However, detailed transporter actions and the mechanism involved in the transportation of SA from chloroplasts is unknown. After transportation, SA undergoes various biochemical modifications such as glucosylation, methylation (MET), and amino acid (AA) conjugation that activates SA into an active growth regulator (Dempsey et al., 2011; Gao et al., 2015). Reversely, glucosylation and methylation also inactivates SA and facilitates the accumulation in vacuoles by making tonoplast more permeable to cross (Dempsey et al., 2011). The attachment of glucose molecules at the hydroxyl group resulted in synthesis of SA 2-*O*-β-D-glucoside (SAG) while at the carboxyl group salicylate glucose ester (SGE) was produced (Klessig, 2017). Whenever required, the SAG release free SA through hydroxylation reaction catalyzed by β-D-glucan glucohydrolases but the underlying mechanism of conversion is still not clear. Moreover attachment of methyl group to SA transformed it into methyl SA (MeSA), which respond in SAR response originating from an infected leaf to systemic tissues and stimulate conversion of MeSA to SA again (Park et al., 2007). Salicyloyl-aspartate has also been identified

in the plant, synthesized by WES1 belong to acyl adenylase family that increases the accumulation of SA in the cytosol by suppressing SA-Asp synthesis (Chen et al., 2013). The role and mechanism of amino acid conjugation is also not well-studied but may be associated with catabolic pathway of SA. The schematic representations of possible modes of SA biosynthesis, regulation, and modification have been shown in Fig. 23.1. Before discussing the significant role of SA in various abiotic stresses, an attempt to establish the importance of SA in plant growth and development has been made in the next section of the chapter.

23.3 ROLE OF SALICYLIC ACID IN PLANT GROWTH AND DEVELOPMENT

Plants usually secrete a wide range of secondary metabolites; among them, SA is a well-known phenolic compound that greatly influences germination of seeds, adventitious root initiation, transpiration, photosynthesis, flowering, thermogenesis, and membrane permeability (Vlot et al., 2009; Boatwright and Pajerowska-Mukhtar, 2013; Khan et al., 2015a,b; Verma and Agrawal, 2017). In recent years, extensive research on the ameliorative effect of SA in plants exposed to various abiotic stresses has been well documented, such as extreme temperature (high, low, chilling) (He and Liu, 2002; Wang et al., 2010; Khan et al., 2013a,b). Reports are available on the importance of SA and related compounds in plants facing extreme drought conditions (Kareem et al., 2017), salinity (Munns and Tester, 2008; Khan and Khan, 2014), and metals/metalloids (Chen et al., 2007; Zhang et al., 2015). Both endogenous production and exogenous application of SA have been recounted as determining factors in plant metabolism and have authoritarian rheostat over cellular ROS (Herrera-Vásquez et al., 2015). Seed germination is a basic agronomic trait that has impact on

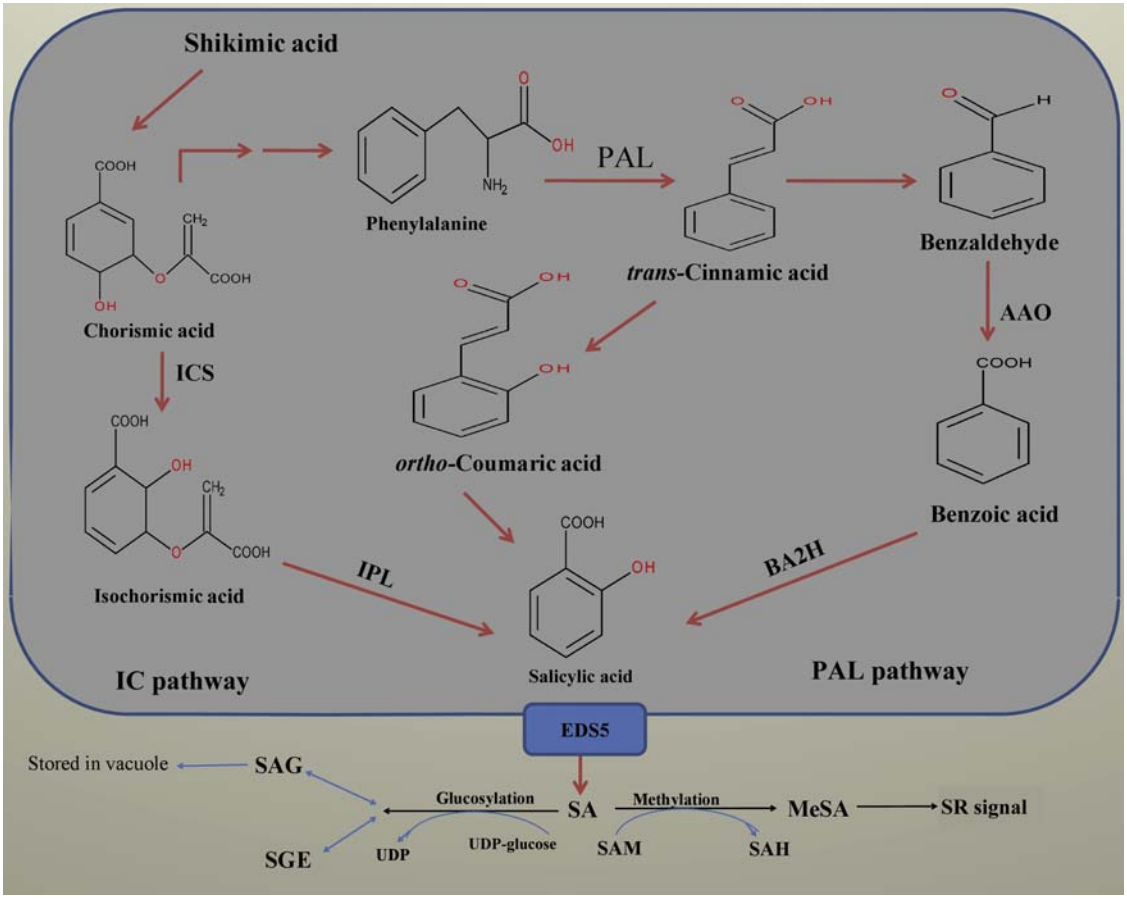

FIGURE 23.1 Steps involved in the biosynthesis and modification of salicylic acid from different pathways. Glucosylation process involved UDP-glucosyltransferases that glucosylate SA whereas methylation process was catalyzed by BA/SA carboxyl methyltransferase. SA has been shown to be sulfonated in vitro by members of the SOT family of sulfotransferases. Conversion back to SA is shown where evidence exists. MeSA is converted to SA by the methyl esterase activity of NtSABP2; the resultant increase in SA triggers systemic defenses. *SAH*, S-adenosyl homocysteine; *SAM*, S-adenosyl methionine; *UDP*, uridine diphosphate.

plant development and growth. It was observed that presoaking of seeds with SA at 1 mM concentration increased germination percentage, index, and mean germination time (MGT) in *Pisum sativum* exposed to high salinity (150 mM) through retaining relative water content and increased accumulation of proline (Ahmad et al., 2017). In another study, seed priming before sowing with low dose of SA (0.1 mM) showed better germination percentage in *Vicia faba* under salinity (Anaya et al., 2015). The dose-dependent role of SA in the germination process is still contradictory. For example, in *Arabidopsis* higher concentration of (1 mM) SA reduced the germination percentage under 150 mM NaCl while low concentration (0.1 mM) SA facilitated germination percentage (Lee et al., 2010). Besides soaking with SA, foliar applications to stressed plants are a promising approach in making plants tolerant. For instance, foliar application of SA (0.1% and 0.2%) improved plant height, anatomical leaf structure, stem length and dry weight, phenols and proline content, and nitrogen uptake in wheat plants exposed to salinity (El-Sayed and Merwad, 2015). In addition to this, foliar-applied SA improved growth, gas-exchange characteristics, and chlorophyll fluorescence in citrus under saline conditions (Khoshbakht and Asgharei, 2015). The foliar application of 0.5 mM SA on wheat plat plant alleviated the adverse effect of heat stress by increasing proline and ethylene formation (Khan et al., 2013a,b). Further evidence of foliar application of SA in combination of L-tryptophan enhanced drought tolerance by improving higher relative water content, leaf membrane stability index, chlorophyll, and potassium content (Rao et al., 2002). Reports are also available to foliar application in mitigating other abiotic stress such as metal toxicity, soil acidity, gaseous pollutants, extreme levels of light (Sulmon et al., 2015; Pereira, 2016), etc.

The SA biosynthetic pathway helps in maintaining the architectural structure of roots and associated meristem activity in *Oryza sativa* (Xu et al., 2017). In addition to this, SA has also been involved in regulating photosynthetic pigments and the antioxidant system, reducing superoxide formation, and ensuring better activity of photosynthetic enzymes such as Rubisco and carbonic anhydrase (Hayat et al., 2012; Khan et al., 2013a,b; Ma et al., 2017). Leaf senescence is a gradual process of cell death taking into account excess production of reactive oxygen species, decreased photosynthetic efficiency, the disintegration of cellular components, and degradation of biomolecules and nucleic acid (Khan and Khan, 2017; Thomas, 2013; Zhao et al., 2016). Advanced proteomic studies confirmed that SA-mediated expression of *PAD4*, *PR1*, and *SID2* induce leaf senescence in *PAT14 mutants* (Zhao et al., 2016). The progression from vegetative phase to reproductive phase has been influenced by natural conditions in coordination with hormonal regulations (Denay et al., 2017). The promising role of SA to induce flowering has been extensively explored for a couple of decades. Lee and Skoog (1965) observed that application of 4 μM SA to tobacco callus initiates flowering at very early stage. The mechanistic approach of SA to facilitate flowering comprises regulatory action of PAL (phenylalanine ammonia-lyase) enzyme, an important precursor of SA biosynthesis (discussed above) in *Pharbitis nil* (Wada et al., 2014). Controversially, negative regulation of SA by repressing floral gene such as FLC and flowering pathway cycles have also been reported. Plant senescence is the process of aging, either stress-induced or developmental aging, morphophysiologically described by discoloration of leaves, degradation of chlorophyll pigments, and initiation of necrosis (Vogelmann et al., 2012). The impact of SA to the senescence process has been long established through expression of several senescence-associated genes (SAGs), such as *SAG12* αVPE, γVPE (vacuolar processing enzymes), WRKY6, WRKY53 (transcription factors), and SEN1 (protease), which are undetectable in *Arabidopsis* plants (Morris et al., 2000; Rivas-San Vicente and Plasencia, 2011). However, SA's role in senescence still needs extensive scientific research at the molecular and physiological levels.

Modern agronomy practices face numerous abiotic stresses, for example, high salt accumulation, drought stress, chilling and high temperature stress, and metal stress, which have adverse impact on crops yield (Saud et al., 2013). Approximately, 25% of the worldwide agricultural land is plagued by drought and about 5%–7% land is polluted with salt accumulation (Ismail and Horie, 2017). According to the US Environmental Action Group, heavy metals toxicity has the severe impact on about 10 million people around the world and also affect the food quality of human and animals (ENS, 2006; Jaishankar et al., 2014). It has also been estimated that emission of greenhouse gases (CH_4, CO_2, and CFC) by 500–1000 ppm will cause an irregular high temperature by 3°C by the late twenty-first century. Therefore, the back-to-back deleterious effects of salinity, metal toxicity, drought, water stress, nutritional inadequacy, and frequent climate change have become more challenging.

23.4 SALICYLIC ACID-MEDIATED DEFENSE MECHANISMS TO ABIOTIC STRESS

23.4.1 Salicylic Acid Involvement in Modulation of Redox Homeostasis

Usually, generation and detoxification of ROS take place in plants in normal physiological conditions.

Plants thus use ROS as signaling molecules to develop steadfast pathways to protect themselves from ROS toxicity (Foyer and Noctor, 2013). Under stress conditions, disturbance in the equilibrium of ROS production and scavenging activity results in oxidative damages to membrane protein and degradation of nucleic acid (Anjum et al., 2012; Choudhury et al., 2017). Plants have innovative and multifaceted metabolic controls to ensure survival (Fahad and Bano, 2012). Tolerance stimulation in stress conditions demands the stimulation of the antioxidative defense system and scavenging systems under abiotic stress conditions (Khan and Khan, 2017; Khan et al., 2012, 2015a,b; Pasala et al., 2016; El-Mashad and Mohamed, 2012). The involvement of SA in regulating the antioxidant system to enhance tolerance under stress conditions has been extensively reviewed (Khan et al., 2015a,b; Sharma et al., 2017). Ma et al. (2017) showed that pretreatment of SA diminished injurious effects of salinity on growth and improved net photosynthesis rate by enhancing the antioxidant enzymes SOD, CAT, and POD activity in *Dianthus superbus*. Pretreatment of SA (0.05 mM) improved tolerance in *Brassica juncea* exposed to salinity stress due to enhanced activity of ascorbate glutathione (AsA−GSH) pathway (Nazar et al., 2015). It was also demonstrated that the hydrogen peroxide processing enzymes (SOD, CAT, and POD) were modulated by SA application on exposure to cold and drought stress (Mutlu et al., 2013; Saruhan et al., 2012). Heavy metal stress, for example, aluminum toxicity, increased the endogenous level of SA by regulating PAL and BA2H enzymatic pathway and increased expression of *GmNPR1* gene in soybean plants (Liu et al., 2013). The amelioration of oxidative damage caused by *Al* toxicity was directly linked to SA application by inducing H_2O_2 level, which acts as signaling molecule to activate the antioxidant system (Liu et al., 2017). In another study, *Triticum aestivum* exposed to Cd stress revealed SA biosynthetic pathway correlation with glutathione cycle and attained a degree of Cd tolerance (Kovács et al., 2014). Alleviating effects of SA (0.01 and 0.1 mM) by improving ASA (ascorbate), GSH (reduced glutathione), and redox ratios ASC/DHA (dihydroxyascorbate) and (GSH/GSSG) under drought stress caused by polyethylene glycol (PEG)in *Artemisia aucheri* was also observed (Abbaspour and Ehsanpour, 2016).

23.4.2 Salicylic Acid Interaction With Heat Shock Proteins, Reactive Oxygen Species and Mitogen-Activated Protein Kinase

The leading role of heat shock proteins (HSP) is to drive molecular chaperones and have a central role in stress tolerance. In response to stress, activation of HSP implicates a multistep pathway of homotrimer formation associated with transcriptional factor for stimulation of stress gene (Liu et al., 2013). Major plant reactions to stress encompasses activation of HSFs/HSPs associated with ROS shifting capabilities. The interaction between ROS and HSP is accompanied by mitogen-activated protein kinases (MAPK) and SA/ROS signaling reaction in MAK cascade activation (Cutler et al., 2010). In *Arabidopsis*, a model has been proposed by researchers to activate different mitogen-activated protein kinase (MPK3, MPK4, and MPK6) in response to a number of abiotic stresses (Ichimura et al., 2000; Teige et al., 2004). Thus, a model has been developed to demonstrate that MAP kinase kinase kinases (MEKKs) are stimulated as a consequences of phosphorylation of MAP kinase kinases (MKKs) that ultimately terminate in activation of MPKs (Jalmi and Sinha, 2015). In *Arabidopsis* roots, Mockaitis and Howell (2000), showed that phosphorylation of MPK was induced by SA. Profoundly, these cascades linked with SA-mediated downregulation signaling either lack the functionality of MEKK1 or MPK4 that induces the accumulation of SA (Suarez-Rodriguez et al., 2007). It is well understood that ROS generation is a basic factor for activating MAP kinase cascades and ANP1 (*Arabidopsis* NPK1-like protein kinase1) also activated by H_2O_2 and terminated by phosphorylation of MPK3/MPK6 in *Arabidopsis* plants (Kovtun et al., 2000). MEKK1-MPK4 cascade also has a commanding feature during ROS metabolism (Nakagami et al., 2006). However, these MAPK cascades are critically controlled by both SA and ROS levels. Since oxidative stress plays a central role in response to biotic and abiotic stresses, the redox homeostasis may be concluded to be an indicator for monitoring stress in plants. The interactions of SA and ROS with other important factors involved in various abiotic stresses have been demonstrated in Fig. 23.2.

23.4.3 Interaction of Salicylic Acid With Mineral Nutrients and Osmoprotectant

To perform normal physiological and biochemical functions, plants fundamentally need an adequate supply of macro- and microelements. It was estimated that about 60% of fertile soils have problems related with nutrient deficits thus reduced crop productivity (Bagheri et al., 2017). Nazar et al. (2015) found that SA increased sulfur absorption and nitrogen absorption, which is associated with antioxidant coordination in plants, and offered tolerance to salinity by provoking various physiological reactions. Foliar application of SA (200 ppm) on *Gossypium barbadense* with potassium

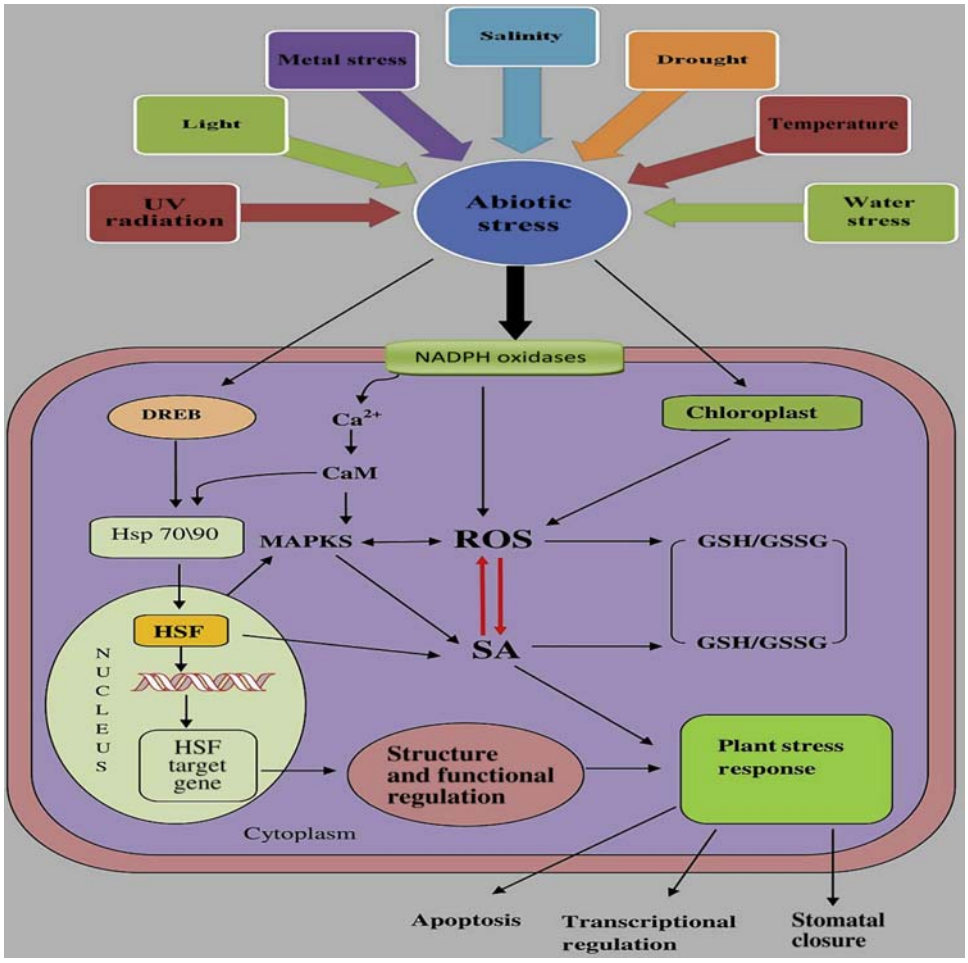

FIGURE 23.2 Interaction of salicylic acid and reactive oxygen species (ROS) with transcriptional factor, heat shock protein under abiotic stress. Reactive oxygen species (ROS) and Ca^{++} are known among others to play a prominent role as transducers (messengers) and mitogen-activated protein kinase (MAPK) cascades have been shown to be used by both types of stresses. Upregulation of transcription factors (TFs), pathogenesis related (PR) and defense genes, heat shock protein (HSP) genes, and further genes involved in protection against stress.

citrate (2.5 g/L) enhanced growth and yield and leaf chemical composition under salinity stress (El-Beltagi et al., 2017). Recent outcomes delivered that SA and Ca^{2+} improved the adverse effect of oxidative trauma by activating the antioxidant system and enhancing photosynthetic efficiency in *Triticum aestivum* (Yücel and Heybet, 2016). The mutual application of SA and S caused noteworthy diminution in Na/K proportions by modulating enzymatic and nonenzymatic antioxidant coordination in *Glycine max* exposed to salinity stress (Razmi et al., 2017).

Under extreme osmotic stress, there is accumulation of organic compounds such as glycine betaine (GB), proline, soluble sugars, and amines, which act as osmolytes to ensure survival of plants (Wani et al., 2016). These osmolytes are neutral and nontoxic molecules that maintain osmotic equilibrium between the cell's surroundings and the cytosol without affecting the metabolic process (Shabala et al., 2006). GB, chemically known as N,N,N-trimethylglycine, is an effective osmolyte ubiquitously found in plants for osmotic balance. GB at physiological pH is a dipolar but electrically neutral molecule that can hypothetically play a decisive role in operative guard against salt, drought, and extreme temperature stress (Wani et al., 2016; Masood et al., 2016). Plants achieved systematic acquired resistance (SAR) through SA-induced GB accumulation under drought and salinity stress (Jagendorf and Takabe, 2001). Further, 0.5 mM SA application to *Vigna radiate* overpowered ethylene formation and increased amino acid content, confirmed GA accumulation, and increased tolerance of plant under salinity stress (Khan and Khan, 2014). Another important osmolyte, proline, also gets accumulated in plants and activates ROS scavenging activity, regulates osmotic equilibrium, and maintains membrane stability under stress conditions (Iqbal et al., 2014). SA (1 mM) significantly increased proline content under

high level of NaCl in *Pisum sativum* plant; this might be due to enhanced activity of pyrroline-5-carboxylate reductase and γ-glutamyl kinase enzyme involved in proline biosynthesis (Misra and Misra, 2012; Ahmad et al., 2017). Pretreatment of SA (1 mM) was also reported to improve the level of total soluble sugars and protein content in pepper leaves during germination phase (Qados, 2015). Authors conclude from these findings that these molecules in association with SA help in reducing and prevent oxidative stress and maintain structural conformation of proteins in water scarcity.

23.5 CROSSTALK OF SALICYLIC ACID WITH OTHER PHYTOHORMONES UNDER ABIOTIC STRESS

Phytohormones showed multitude impact on plant health performance to numerous ecological cues (Griesser et al., 2015). To uphold a harmony amongst development and defense programming in plant systems, crosstalk between various phytohormones has been a striking assessment for plant researchers. The interactive multifarious signaling offered them ideal contenders for assigning defense reactions. Phytohormones act either self-sufficiently or through pathways interconnected with other growth regulators mutually or antagonistically (Kazan, 2015). Notably, hormonal crosstalk involved positive or destructive influence on synthesis, transport, and signaling of other stress hormones (Hu et al., 2017). The positive and negative crosstalk of SA with other growth regulators has been summarized in Table 23.1.

23.5.1 Salicylic Acid and Auxins

Auxins act as a key player in growth and development of plants by regulating plant metabolic processes as well as root differentiation and also in abiotic stress tolerance (Agtuca et al., 2014; Fahad and Bano, 2012). The antagonistic interaction of indole acetic acid (IAA) and SA signaling pathways hinder functionality of each other in maize plants, where exogenous application of IAA enhanced lateral root formation rather than primary roots development, and SA improved total root biomass (Agtuca et al., 2014). Overexpression of PR1 gene via SA application in auxin *TIR1*, *AFB2* mutants provides better understanding of SA-induced auxin signaling in salinity stressed plants (Iglesias et al., 2011). One of the common approaches of plants to deal with stress conditions is SA-mediated suppression of auxins studied in *Arabidopsis* (Atkinson et al., 2013). MicroRNA (miR393), the main regulator of TIR1, AFB2, and AFB (auxin receptors) was also stimulated by cold and salinity stress (Sunkar et al., 2007). Exogenous supplementation of SA caused repression of auxin-related genes and resulted in modulation of Aux/IAA proteins and inhibition of auxin responses in *Arabidopsis* (Wang et al., 2017). SA elicited the accumulation of abscisic acid (ABA) under both favorable and stress conditions, which thus helped in the osmotic adjustment, enhancement of photosynthetic pigments, and development traits in *Solanum lycopersicum* (Szepesi et al., 2009).

23.5.2 Salicylic Acid and Abscisic Acid

Abscisic acid (ABA) is usually known as a stress hormone mainly associated with abiotic stresses mitigation (Cramer et al., 2011; Vishwakarma et al., 2017). The ABA-dependent signaling pathway countersigns defense genes removing stress protection via transcriptional reprogramming of plant cell metabolism (Bali et al., 2017; Vishwakarma et al., 2017). The regulatory element of ABA receptor (PYR/PYL/RCAR) and protein phosphatase (PP2C) stress-protein subfamily was a major breakthrough in explaining ABA signaling pathway under stress (Fujii and Zhu, 2009; Soon et al., 2012). SA and ABA act provocatively to regulate cell cycle−related genes in rice plants (Meguro and Sato, 2014; Manohar et al., 2017). In *Agrostis stolonifera*, coapplication of abscisic acid, γ-aminobutyric acid (GABA), and SA enhanced drought resistance by protecting membrane strength and water retention (Li et al., 2017). Metabolic profiling studies proved that ABA, GABA, and SA have inclined metabolic route and also caused the differential alteration in metabolite accumulation under drought (Li et al., 2017).

The antagonistic performance of ABA and SA revealed that ABA kept up the SA-mediated protective mechanism by accumulation of SA, though this defense was perceived to be hindered when ABA was applied exogenously (Yasuda et al., 2008). Further, the endogenous SA levels in the Cd-stressed *Zea mays* were altered by ABA treatment (Szalai et al, 2013).

23.5.3 Salicylic Acid and Gibberellic Acid

Gibberellins (GAs) are naturally occurring tetracyclic diterpenoid carboxylic acids responsible for important process, that is, seed germination, shoot elongation, flowering and fruit development, etc. (Yamaguchi, 2008). They empower development and advance stage changes and are effectively involved in abiotic stress tolerances and adjustment (Colebrook et al., 2014). It has been reported that GAs improved seed germination under salinity (Kaya et al., 2009), as well as increased

TABLE 23.1 Interaction of SA With Other Plant Growth Regulator Under Optimal/Stress Condition

Phytohormones	Plant species	Interaction with SA	Stress/ optimal condition	Responses	Effect	References
Auxin	*A. esculentus*	Syn	Salinity	DPPH (2,2-diphenyl radical scavenging capacity), antioxidant activities	+	Esan et al. (2017)
	H. vulgare	Syn	Heavy metals	Cd-induced IAA mediated ROS	-	Tamás et al. (2015)
				Lipoxygenase or glutathione peroxidases along with stimulated root growth inhibition	+	
	Zea mays	Syn	Salinity	Micronutrients CO3 + , Mn2 + , Cu^{3+}, Fe^{2+} in roots, chl a, chl b, carotenoids, sugars, proline, and protein content	+	Fahad and Bano (2012)
Gibberellins	*Pisum sativum*	Syn	Salinity	Seed germination, protein and sugar content, antioxidant activity	+	Ahmad et al. (*In press*)
	Sorghum bicolor	Syn	Drought	Catalase, APX activities, germination percentage, and germination index	+	Sheykhbaglou et al. (2014)
	A. thaliana	Syn	Salinity	Modulation of biochemical and molecular GA-SA signaling mechanism, seed germination	+	Lee et al. (2010)
	Glycine max	Syn/Ant	Salinity	Upregulation of SA biosynthesis/decreased SA content, under increased GA3 and elevated SA under NaCl stress	+/-	Hamayun et al. (2010)
ABA	*A. thaliana*	Syn	Water stress	Promote stomatal closure, induce leaf senescence	+	Qi et al. (2015)
	S. lycopersicum	Syn	Salinity/ optimal	Osmotic adaptation, photosynthetic pigments, and growth attributes	+	Szepesi et al. (2009).
	T. aestivum	Syn	Drought	Soluble protein and soluble sugar content, photosynthetic efficiency	+	Sami et al. (2016)
	O. sativa	Ant	Optimal	Cell cycle progression	+	Meguro and Sato (2014)
				DNA replication, thymidine incorporation in the shoot apical meristem	-	
Jasmonic acid	*G. lemaneiformis*	Syn	Heat	Hsp70, Hsp60, signaling transduction	+	Wang et al. (2017)
	Citrus limon	Syn	Chilling	Total phenolics and phenylalanine ammonia lyase (PAL)	+	Siboza et al. (2014)
	O. sativa	Ant	Drought/ salinity	Transcripts and RSOs PR10 proteins	+	Clarke et al. (2009)
	A. thaliana	Syn	Heat stress	Electrolyte leakage	+	Schenk et al. (2000)
	Lycopersicon esculentum	Ant	Optimal	Pathogen related (PR) protein genes	+	Thaler et al. (1999)
Brassinosteroids	*B. juncea*	Syn	Metal stress (Pb)	Photosynthetic efficiency, gene expression	+	Kohli et al (2017)
	A. thaliana	Syn	Salinity	Transcription factor WRKY70 and WAK1, NPR1 defense gene expression	+	Divi et al. (2010)
	Arachis hypogaea	Syn	Metal stress (Cd)	Growth and metabolism	+	Khatoon et al. (2017)
NO	*Glycine max*	Syn	Salinity	CAT, APX, GPX, accumulation of proline	+	Simaei et al. (2012)
	T. aestivum	Syn	Chilling	Antioxidant activities SOD, POD, CAT	+	Esim and Atici (2015)

Syn, synergistic; *Ant*, antagonistic; + (positive response), – (negative response).

pigments, hill activity, and water holding capacity (Shah, 2007). GA signaling enables plants to adapt to adverse environmental conditions, and this can be mediated through SA regulation. GA and SA act in an organized manner to modulate pathogenesis associated protein expression (Miura and Tada, 2014) and activate defense system against abiotic stresses (Fayez and Bazaid, 2014). Inhibition of seed germination by SA occurs due to GA suppressible *WRKY* genes that block GA-induced α-amylase expression (Xie et al., 2007). The antagonistic association between SA and GA was also observed in *Arabidopsis* plant in trichome development under drought stress. Exogenous application of SA suppresses GA-initiated trichome development through downregulation of *Npr1*/*Nim2* gene (Traw and Bergelson, 2003).

23.6 INTERACTION AND INVOLVEMENT OF DELLA WITH SA-GA CROSSTALK UNDER STRESS

DELLA proteins stimulate ROS-detoxifying enzymes by altering ROS strength in abiotic/biotic stress (Achard et al., 2008). GAs activated in both salt and cold stress by cunning DELLA proteins and established noteworthy crosstalk with SA-ET signaling (Verma et al., 2016). Furthermore, upregulation of GA biosynthetic gene expressed itself after SA treatment followed by DELLA protein destruction degradation (Ding et al., 2015). SA and/or GA enhanced prominent CBF1 expression in tomato under cold stress (Zhao et al., 2016). It was reported that in *Arabidopsis thaliana*, GA stimulate *isocitratelyase* (*ICL*), an enzyme essential in lipid assimilation during seed germination also stimulated by SA (Bali et al., 2017).

23.6.1 Salicylic Acid and Ethylene

Ethylene (ET) is a self-effacing gaseous hormone, having manifold regulation in growth and development and acts as modulator between plant response to environmental stresses to attain maximum performance (Khan and Khan, 2014). It was shown that SA improved tolerance in *Arabidopsis* by inducing the expression of NPR1 gene (Jayakannan et al., 2015). A well-coordinated upregulation of ethylene TF genes such as *GmERF3*, *AtERF6*, and *CarERF116* (Sewelam et al., 2013; Deokar et al., 2015; Liu et al., 2017), while downexpression of *SodERF3* (Trujillo et al., 2008) was noticed upon SA application. During high temperature stress, exogenous spraying of SA improves plant growth by limiting ethylene production and enhancing proline metabolism to such a point that modulatory action of ACC synthase (1-aminocyclopropanel-carboxylic acid synthase) enzyme is held back (Khan et al., 2013a,b). According to Poór et al. (2013), ethylene is accountable for cell death under high salt concentration in *Lycopersicon esculentum* due to the excessive generation of ROS, whereas SA-induced reactive oxygen species production was not reported to be straightforwardly linked with ethylene. The chlorophyll as well as anthocyanin content was reduced noticeably by ethylene application, but SA ameliorates negative effects of salinity (Tirani et al., 2013). Further, SA facilitated the ethylene-dependent hypersensitive reaction in *Madia sativa* through reducing formation of lipid peroxidation and polyamines (Palma et al., 2013).

23.6.2 Salicylic Acid and Jasmonic Acid

Jasmonic acid is a naturally occurring lipid that performs as a regulator in germination and seedling growth and root development, induces tuber formation and gravitropism, embryo formation, determination and development of flowers, fruit ripening, and leaf senescence (Zheng et al.,2017). Additionally, JA plays a vital role in abiotic stress and is regarded as a stress modulator, and enhances stress tolerance by improving photosynthetic efficiency and antioxidant metabolism via modulating proteome profiling (Rincón-Pérez et al., 2016). Proteomic and transcriptomics analysis explored the upregulation of specific stress proteins modulated by JA and/or SA under abiotic stress (Van der Does et al., 2013). Usually, SA and JA signaling pathways interact antagonistically at the level of mitogen activated protein kinases (MAPK) under normal or stress conditions (Khan et al., 2013a,b; Bali et al., 2017).

Caarls et al. (2015) explained that SA inhibits JA action by downregulating the complex COI1-JAZ by altering GCC-box motifs in JA-responsive promoters. The downregulation of the JA pathway comprises MAPK, redox balances, and transcription factors such as *WRKY* regulated by SA (Pieterse et al., 2012). The inhibitory action of SA in *NPR1* suppressing the expression of *LOX2* and *VSP* gene, which are upregulated by JA (Spoel et al., 2003). It is interesting to note that SA-induced *NPR1* can activate GRX480, which in turn, can form a complex with TGA factors, and suppress the expression of JA-responsive genes (Backer et al., 2015).

Synergistic interaction between JA and SA are also evidenced; however, the reports are scanty in this context. In Citrus, coapplication of MeJA and SA enhanced synthesis and accumulation of total phenols and activity of phenylalanine ammonia lyase (PAL) and simultaneously deterring the action of polyphenol

oxidase (PPO) and peroxidase (POD) to chilling stress (Siboza et al., 2014). Moreover, the involvement of the transcription factor and ROS mechanism engaged by both SA and JA suggests feasible crosstalk connecting the JA and SA signal transduction pathway to cold stress (Sharma et al., 2017).

23.6.3 Salicylic Acid and Brassinosteroids

Brassinosteroids (BRs) are plant steroidal hormones that have gained much attention recently for their beneficial role to induce tolerance to plants exposed to a wide range of abiotic stress. BRs protect plants from a range of ecological distress, including extreme temperatures, drought, salinity, and pathogen attack, apart from playing a developmental role (Vardhini and Anjum, 2015; Ahmad et al., 2018). The collaborations of BR with SA reported to enhance tolerance to plants have been extensively studied under abiotic stress (Divi et al., 2010; Handa et al., 2017). BR is involved in upregulation of nonexpression of pathogenesis-related genes (NPR1) coupled with SA-mediated tolerance under salinity stress. In addition to this, expression of SA-induced PR genes has been upregulated by BR by modulating transcription factor *WRKY70* (Divi et al., 2010). Coapplication of SA and BR was found efficient to adjust under severe condition of salinity (Ding et al., 2012). However, BR mediated resistance does not require involvement of SA and further exploration by quantifying SA accumulation and expression analysis of *NahG* transgenic tobacco confirmed the independent mode of defense against pathogen attack (Nakashita et al., 2003; Saini et al., 2015). Further, crosstalk of SA and BR pathways negatively suppress immune response provoked by BR. For example, Brassinazole (Bz) reduced the susceptibility of *Pythium graminicola* observed in rice plants by downregulating of *NPR1* and *OsWRKY45* that directly linked with SA defense regulators pathway. Moreover, bioformulation of SA and 24-EBL and 24-epicastasterone enhanced the growth of millet plantlet by reducing lipid peroxidation under heat and salt stress (Litvinovskaya et al., 2016).

23.6.4 Salicylic Acid and Nitric Oxide

Nitric oxide (NO), a signaling molecule, alters gene expression thus regulating numerous physiological and metabolic pathways in a dose-dependent manner (Fancy et al., 2017). During the last two decades there has been a tremendous outburst in deciphering the role of NO in the germination process, photosynthesis, leaf senescence, and pollen growth (Esim and Atici, 2015). The stomata closure during stress begins with inactivation of Kin^+ pumps associated with NO and ROS interaction in guard cells induced by SA (Khokon et al., 2011). The reduction in oxidative stress and retaining equilibrium of ion-homeostasis thus protects the plant and promotes growth and photosynthetic efficiency when treated with sodium nitroprusside (SNP) as NO source (Zhang et al., 2015; Fatma et al., 2016). Under salinity stress, NO has been stimulated downstream of SA signaling in diminishing oxidative injury in osmotic stresses in *Triticum aestivum* seedlings (Naser Alavi et al., 2014). The combined application of SA (1 mM) and NO (0.1 mM) reduced the proportions of malondialdehyde (MDA), H_2O_2 and O_2^- amplified the activity of SOD, CAT, and POX, which subsidized to ROS generation in wheat under chilling stress (Esim and Atici, 2015). Exposure of Ni stress, pretreatment of SA plus SNP mitigates the detrimental effect of heavy metal by dropping root-to-shoot translocation of Ni, enhancing the chlorophyll content, and decreasing lipid peroxidation, H_2O_2, and proline accumulation in leaves of *Brassica napus* (Kazemi et al., 2010). The mutual accomplishment of SA and NO enriched Fe uptake and a smaller amount of leaf interveinal chlorosis in *Arachis* seedlings rather than individual application of SA or SNP under Fe-deficiency (Kong et al., 2014). Furthermore, synergistic consequence of SA + SNP enhanced activity of SOD, POD, APX, and GR exposed to UV-B stress. Under salinity, SA inhibited NO biosynthesis, demonstrating a conflicting relationship between NO and SA and showing dual response (Gémes et al., 2011).

23.7 CONCLUSION AND FUTURE PROSPECTS

SA is a multidimensional hormone used in physiological metabolic processes and defense mechanism tolerance to abiotic stresses. Studies on the physiological and molecular levels of plant tolerance to numerous stresses will be critical for improved management with prospective ecological alteration. It will be fascinating to investigate the additional roles of SA in the clash between plants and ecological stresses. However, some conflicting consequences are obtained in exploring exogenous SA application and endogenous SA levels depending upon the concentration applied. It is ambiguous if exogenous SA application directly or indirectly enhances endogenous SA levels and whether the effect of SA is coupled with ROS generation. In-depth study must be used to expose an advanced system of upregulation of SA regulated genes under the influence of abiotic stress, and the unambiguous network coordination with other phytohormones to search out precise information regarding plant reaction to abiotic stress.

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CHAPTER

24

Role of Methyl Jasmonates in Salt Stress Tolerance in Crop Plants

Bilal Ahmad[1], *Aamir Raina*[2,3], *Mohd Irfan Naikoo*[4] *and Samiullah Khan*[2]

[1]Plant Physiology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India
[2]Department of Botany, Mutation Breeding Laboratory, Aligarh Muslim University, Aligarh, Uttar Pradesh, India
[3]Botany Section, Women's College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India [4]Plant Ecology and Environment Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

OUTLINE

24.1 INTRODUCTION

Globally salinity is considered as one of the most serious problems of agriculture and allied sectors, especially in arid and semiarid regions. Increased soil salinity may be the main factor in making arable land unfavorable for a wide range of crop species (Epstein et al., 1980). The factors that lead to increased salinity are the usage of saline water for irrigation and application of fertilizer (Epstein et al., 1980). Saline soils are characterized with high levels of sodium and chloride content and thus exert severe abiotic stress on the growth and development of plants by inducing changes in most physiological and biochemical processes. Multiple factors are involved in the alleviation of salinity stress but phytohormones are thought to be among the most essential endogenous substances involved in the underlying mechanisms of tolerance or susceptibility of various plant species (Velitchkova and Fedina, 1998). Plants act in response to abiotic stresses by inducing the expression of different sets of genes whose products play a pivotal role in alleviating a wide range of stresses (Martinez et al., 2018). Phytohormones play an essential role in the plant's ability to acclimatize to environmental flux such as abscisic acid (ABA), a stress hormone known to be

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involved in abiotic stress response and tolerance (Peleg and Blumwald, 2011). Different plant hormones, such as jasmonic acid (JA) and its derivatives, are implicated in regulating plant defense mechanisms in response to environmental stresses including drought, low temperature, and salinity by inducing changes in gene expression (Wasternack and Parthier, 1997; Khan et al., 2012). Acting as stress modulator JAs can repress or augment plant stress responses (Agrawal et al., 2003). JAs have been well documented for their vital roles in plant responses to a wide range of abiotic stresses including drought (Brossa et al., 2011), salt (Dong et al., 2013; Qiu et al., 2014; Zhao et al., 2014), heavy metals (Maksymiec et al., 2005), and heat stress (Clarke et al., 2009). In the plant kingdom jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are cosmopolitan in distribution (Meyer et al., 1984; Schaller, 2011; Pirbalouti et al., 2014). The initial isolation of JA was reported from the culture filtrate of the fungus *Lasiodiplodia theobromae* (Aldridge et al., 1971). MeJA, one of the conjugates of JA, is an aromatic volatile compound originally known in flowers of *Jasminum grandiflorum* and is also known as a primary source to isolate MeJA (Demole et al., 1962; Avanci et al., 2010). MeJA can also considerably direct photosynthesis and antioxidant metabolism by modulating set of proteins. Apart from JA and MeJA, other JAs such as jasmonoyl ACC (JA-ACC), *cis*-jasmone, and jasmonoyl isoleucine (JA-Ile) with numerous biological functions have been documented (Rohwer and Erwin, 2008; Avanci et al., 2010; Wasternack and Kombrink, 2010). However, literature is very meager on JA crosstalk with other major phytohormones in plants under salt stress. Keeping in view the recent literature, this chapter dissects the role and underlying mechanisms of JA-regulated plant growth and development in response to salt stress, and presents crosstalk on and interaction-outcomes of JA.

24.2 JASMONATE BIOSYNTHESIS

Jasmonates, fatty acid derived cyclopentanones ubiquitously found in the kingdom *Plantae*, are vital regulators found in plants predominantly during various abiotic and biotic stress responses (Reinbothe et al., 2009). An array of metabolic events involving multiple signal transduction cascades regulates the Jasmonate biosynthesis and altered levels have been observed in stressed as well as unstressed plant tissues (Browse, 2009; Reinbothe et al., 2009). Characterized by a pentacyclic ring structure, jasmonates are the products of the oxidative metabolism of polyunsaturated fatty acids and belong to class of compounds called oxylipins, a family of oxygenated fatty acid derivatives. The biosynthesis of jasmonates takes place through the octadecanoid pathway analogous to the biogenesis of animal antiinflammatory prostaglandins (Wasternack and Hause, 2002), involving translocation of chloroplast membrane lipid intermediates to the peroxisomes through cytoplasm (León, 2013). Phospholipases belonging to the class D (PLDs), after activation through certain stimuli, act on the chloroplast membrane lipids releasing an 18 carbon phospholipid α-linolenic acid (C18:3) through oxidation (Fig. 24.1). This α-linolenic acid leads to the

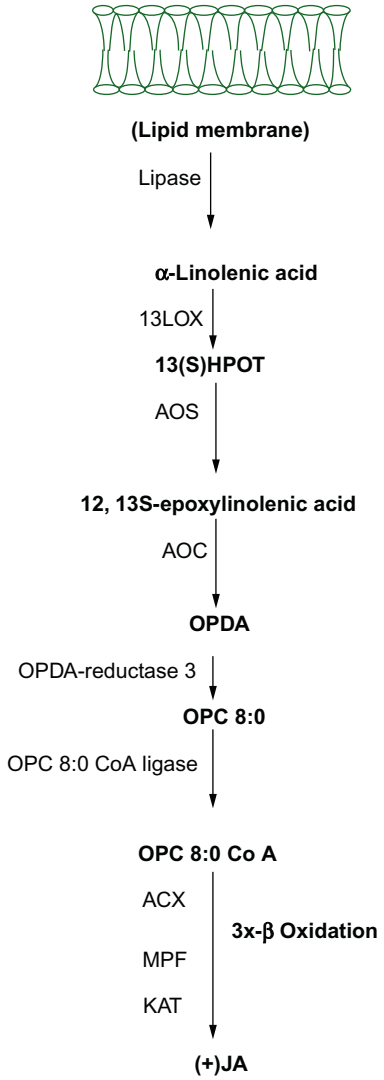

FIGURE 24.1 Pathway intermediates are abbreviated as 13-HPOT for (9Z11E15Z13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid (i.e., 13(S)-hydroperoxy linolenic acid), allene oxide for (12,13 (S)-epoxy-9(Z)), 11,15(Z)-octadecatrienoic acid, cis-(+)-OPDA for cis-(+)- 12-oxophytodienoic acid, and OPC8:0 for 3-oxo-2(2_(Z)- pentenyl)-cyclopentane-1-octanoic acid. The enzymes are indicated as 13-LOX for 13-lipoxygenase, AOS for allene oxide synthase, AOC for allene oxide cyclase, and OPR3 for 12-oxophytodienoate reductase 3. Source: *Adapted from Wasternack, C., Hause, B., 2002. Jasmonates and octadecanoids: signals in plant stress. responses and development. Prog. Nucl. Acid Res. Mol. Biol. 72, 165–221.*

production of 12-oxophytodienoic acid (OPDA) involving three steps. First a chloroplastic lipoxygenase (LOX) oxygenates α-linolenic acid to generate 13(S)-hydroperoxy linolenic acid derivatives (13-HPOT) (namely 13S (13S-HPODE) and 9S-hydroperoxy derivative of LA hydroperoxy octadecadienoic acids (9S-HPODE) (Vick and Zimmerman, 1984). This oxygenation is followed by cyclization leading to the generation of 12-oxophytodienoic acid (OPDA) (Bannenberg et al., 2009) by the help of allene oxide synthase (AOS; EC 4.2.1.92) and allene oxide cyclase (AOC) enzymes. AOS dehydrates the 13S-HPODE producing 12-oxophytodienoic acid (12-OPDA), which is recognized as allene oxide. Further biosynthesis of jasmonic acid from OPDA involves reduction and β-oxidation after which JA carboxyl methyltransferase (JMT) enzyme comes into play leading to the catabolization of β-oxidation product into its volatile equivalent methyl jasmonate (MeJA) (Song et al., 2000; Seo et al., 2011). OPDA is exported to the peroxisome where it is oxidized to 3-oxo-2-2′(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) by the help of the enzyme OPDA reductase (OPR; EC 1.3.1.4). Finally, shortening of the precursor molecule side chains brought about through three cycles of peroxisomal β-oxidation (Bannenberg et al., 2009; Lyons et al., 2013). The parent compound (octadecatrienoic acid, an 18:3 compound), from which jasmonic acid is derived through a series of enzyme catalyzed reactions, gives the pathway its name as the octadecanoid pathway. Occurrence of three of LOXs (LOX2, LOX3, and LOX4) has been observed in the genome of the *Arabidopsis thaliana* (Caldelari et al., 2011). AOS and AOC are reported to be chloroplastic enzymes (Ziegler et al., 2000; Hause et al., 2003). Moreover, OPDA is exported by the help of a carrier namely COMATOSE1/PEROXIMAL 1/PEROXISOME ABC TRANSPORTER (ABC CTS1/PXA1/PED3) from chloroplast to peroxisome (Zolman et al., 2000; Theodoulou et al., 2005; Footitt et al., 2007). Furthermore, the existence of a novel C16 cyclopentenone namely *dn*-OPDA has been revealed from the analysis of the oxylipin content of *Arabidopsis* leaves (Weber, 2002). Besides, an array of products are derived from JA after its biosynthesis, noteworthy among which are methyl ester of JA (MeJA) and certain conjugates like conjugate with hydroxylated didehydro derivatives and certain derivatives of sugars as well as amide conjugates with certain amino acids (JA-Ile) (Staswick, 2008). Certain proteins and enzymes necessary for the biosynthesis and perception of JA have been reproduced by the help of different plant species and a handful of like AOC, 13-AOS, ACYL-CoA-OXIDASE1, 13-LOX, JAR1, OPR3, and the SCFCOI1−JAZ-coreceptor complex have also been crystallized (Kombrink, 2012; Wasternack and Hause, 2013).

24.3 JASMONATE SIGNALING

Jasmonates, lipid-derived signals derived from the lipid constituents of some particular membranes by the help of some oxygenases, are perceived by protein receptors resulting in the activation of certain specific signal transduction cascades in plants (Wasternack and Hause, 2013). JAs mediate the plant responses to various types of biotic and abiotic stresses and in plant development. Quite a few molecules that operate as activators, repressors, and corepressors have been recognized in plants that polish up the gene expression regulated by JA itself and its various conjugates, and insights into the vital components of JA signaling and perception have been gained in addition to the elucidation of each and every step in their biosynthesis in the recent past due to the exhaustive efforts of researchers, which has increased our understanding regarding the functioning of the plant hormone (Wasternack and Strnad, 2016). JA actively participates in numerous metabolic reactions, notably conjugation with various amino acids, carboxylation, glucosylation, hydroxylation, methylation, and sulfation leading to several compounds with varied biological activities. The activity of these metabolites varies from being highly active, partly active, or inactive in specific processes. Carboxylation, hydroxylation, and sulfation lead to inactivation of JA signaling. 12-oxophytodienoic acid (OPDA), the JA-biosynthesis precursor, has been recognized as a JA-independent signaling compound (Wasternack and Strnad, 2016).

Among the chief regulators of JA signaling; JASMONATE ZIM DOMAIN (JAZ) proteins (ZIM-domain protein), Skp/Cullin/F-box complex (SCF^{COI1}), a type of E3 ubiquitin ligase, and also the 26S proteasome and F-box protein CORONATINE INSENSITIVE 1 (COI1), are the most noteworthy (Deshaies, 1999; Turner et al., 2002; Pauwels and Goossens, 2011). The discovery of the ubiquitin-proteasome system in plant signaling was an important breakthrough and it was observed to act as a central regulator in the sensing and signaling of plant hormone including JA. It is comprised of a protein complex consisting of three types of proteins (Skp1/Cullin/F-box proteins) called the SCF complex, which serves as an E3 ubiquitin ligase. At this complex, a target protein to be ubiquitinated is recognized by the F-box protein and consequently proteasomal degradation is carried out. COI1 serves as an F-box protein during perception and signaling of JA (Xie et al., 1998). The JAZ proteins play a pivotal role in the signaling cascade of JA (Thines et al., 2007). These proteins, which were discovered by chance and named as JASMONATE ZIM DOMAIN (JAZ) proteins (Yan et al., 2007), were found to be upregulated early by JA treatment or wounding.

These operate as repressors of JA signaling and consequently act as negative regulators of the gene expression. During the JA-induced gene expression, degradation of these repressors is brought about through the SCF^{COI1}-dependent 26S proteasome pathway. Pauwels et al. (2010) elucidated the molecular mechanisms through which JAZ proteins repress the expression of genes. It was shown that in *Arabidopsis*, the JAZ proteins conscript a type of corepressor called as Groucho/Tup1-type TOPLESS (TPL) and TPL-related proteins (TPRs) through a formerly uncharacterized adaptor protein, named as Novel Interactor of JAZ (NINJA). JAZ degradation allows the liberation of positively acting TFs like MYC2, which are then able to bind to the promoter sequences in the responsive elements of JA-responsive genes, leading to the initiation of transcription. During the resting state JA-Ile levels being very low, JAZ protein represses the attachment of the transcription factor MYC2 to the G-box. Enhanced endogenous levels of JA-Ile induced by any environmental or developmental signal results in the formation of SCF^{COI1}−JAZ coreceptor complex, which requires JA-Ile, one of the most bioactive compounds acting as the ligand during the formation of SCF^{COI1}-JAZ coreceptor complex. JA-Ile is active as an epimer (+)-7-iso-JA-Ile, synthesized by the enzyme jasmonoyl-isoleucine synthetase, which is encoded by *JAR1*, a member of the GH3 gene family (Fonseca et al., 2009; Staswick and Tiryaki, 2004; Westfall et al., 2012) JA-Ile level is very important as it plays a regulatory role and its level is primarily determined by the activity and hydroxylation of *JAR1* as well as its cleavage by certain amidohydrolases (Koo et al., 2011; Heitz et al., 2012; Aubert et al., 2015). During the scarcity of other plant proteins, the JA-Ile conjugate promotes interaction between COI1 and JAZ1 proteins specifically (Thines et al., 2007). Besides, JA-Ile conjugate-induced destruction of JAZ transcriptional repressors is responsible for the stimulation of downstream response genes (Staswick, 2008). Degradation of JAZ protein repressors can also be facilitated by their binding with F-box protein COI1, resulting in the release of transcription factors which are otherwise repressed (Thines et al., 2007; Pauwels and Goossens, 2011).

MYC2, a very essential member of the MYC-TF-family, is a master regulator of the signal transduction pathway of JA and this TF belongs to the basic helix−loop−helix (bHLH)-type (Kazan and Manners, 2013). Its chief role has already been established in a number of signal transduction pathways noteworthy among which are synthesis of auxin, ethylene, glucosinolates, tryptophan, and JA in addition to responses to wounding/insects, pathogens, oxidative stress, and ABA-dependent drought stress (Dombrecht et al., 2007; Kazan and Manners, 2008). MYC2 is also known to play a diverse part in various signaling pathways involved in different aspects of defense responses and development in *Arabidopsis* mediated through JA (Kazan and Manners, 2013). Worth noting is the MYC2-mediated regulation of crosstalk between different plant hormones like ABA, GAs, SA, and auxin, and also the establishment of a link between different signaling pathways such as phytochromes, light, circadian clock, and JA. Investigations at the molecular level have helped to gain insights into the regulation of transcription of JA-responsive elements through MYC2, which has improved our current understanding about the signal transduction pathway of JA in plants a great deal (Chen et al., 2011; Zhai et al., 2013a, b). MYC2 has been reportedly observed to involve distinct molecular mechanisms to differentially regulate the transcription of distinct JA-responsive genes thereby causing the transcriptional activation of various downstream target genes (Katsir et al., 2008; Chen et al., 2011; Zhai et al., 2013a,b). Reports regarding the occurrence of JAZ repressors with high affinity for various related TFs that belong to the bHLH family including MYC2, MYC3 and MYC4, GLABRA3 (GL3), ENHANCER OF GLABRA3 1 (EGL1), and TRANSPARENT TESTA 8 (TT8) are well documented, which actively participate in specific as well as overlapping JA signaling aspects (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu and Figueroa, 2011; Qi et al., 2011). MYC2 activity has been reported to be blocked due to the interaction of the JAZ repressors and the adaptor protein NINJA with the MYC2 in the absence of functional JAs and also due to the recruitment of the corepressors TOPLESS (TPL) and TPL-related proteins (reviewed by Pauwels and Goossens, 2011). However, reports regarding the mechanism of differential regulation of various JA-responsive genes at the protein level mediated through MYC2 are scanty. Furthermore, regulation of the target gene transcription can be regulated by MYC2-proteolysis. This has been confirmed by the discovery of a peptide stretch, 12 amino acids long, in the MYC2 transcription activation domain that has been assigned for both transcriptional activity of MYC2 as well as its proteolysis. In addition, phosphorylation of a threonine (Thr-328) residue has also been observed to be an important event in the MYC2-mediated regulation of transcription (Zhai et al., 2013a,b). One more breakthrough in the JA signaling was the discovery of a new regulating element called as JASMONOYL-L-ISOLEUCINE HYDROLASE 1 (JIH1), which has been observed to encode a novel homeostatic step in the signaling of the hormone along with additional JA-Ile inactivation steps like hydroxylation and carboxylation (Woldemariam et al., 2012). JIH1 has been shown to contribute in the defense responses of plants and to reduce the JA-Ile burst (Woldemariam et al., 2012). A number of new

endogenous bioactive conjugates mimicking the amino acid conjugates of JA have been revealed, which are designed by the conjugation of coronafacic acid (CFA) conjugates with the other amino acids, for example, (+)-7-iso-JA-Ala, (+)-7-iso-JA-Leu, (+)-7-iso-JAMet, and (+)-7-iso-JA-Val (Yan et al., 2007).

Recent studies have revealed the regulatory role of JAs in salt tolerance (Dong, et al., 2013; Qiu, et al., 2014; Zhao et al., 2014). Expression of *Triticum aestivumTaAOC1* gene, encoding allene oxide cyclase (AOC) enzyme, in *Arabidopsis* raises JA levels and increases the tolerance against salt stress, signifying the positive regulatory effect of JAs against salt tolerance. This salt tolerance imparted by the expression of *TaAOC1* occurs independent of the ABA as the transgenic expression of *TaAOC1* in aba2 (ABA-deficient mutant) did not compromise the salt tolerance. Contrary to this, *TaAOC1* expression in the myc2 mutant background impedes salt tolerance. It is therefore apparent that the branch of the JA biosynthetic pathway catalyzed by AOC imparts salt tolerance, which is MYC2-dependent but ABA-independent (Zhao et al., 2014). Likewise, the wheat gene *OPR1*, which encodes 12-oxophytodienoate reductase 1 enzyme, has been observed to mediate salt tolerance in *Arabidopsis* when overexpressed (Dong et al., 2013). But, in comparison with the ABA-independent TaAOC1-conferred salt tolerance, TaOPR1-induced salt tolerance is ABA-dependent. The possible mechanism underlying the TaOPR1-mediated salt tolerance appears to be the increased ability of TaOPR1 overexpressing plants to detoxify ROS in an ABA-dependent manner (Dong et al., 2013). Certainly, JAs are known to increase the salt tolerance in wheat as well as the antioxidant enzyme activities (Qiu et al., 2014); however, further study needs to be conducted regarding the JAs-induced TaOPR1-mediated salt tolerance as no instantaneous effect of TaOPR1 expression on the signaling of JA was observed (Dong et al., 2013).

JA-mediated salt tolerance is not limited to *Arabidopsis* only. In rice, a nuclear protein, namely RICE SALT SENSITIVE3 (RSS3), expressed in the root tip and known to promote cell elongation, is essential for root growth under salt stress. The promotion of root growth is caused by the suppression of root JA responses. Consequently, inhibition of root growth under salinity stress due to the loss of RSS3 function causes is endorsed to the stimulation of JA-responsive genes. RSS3 is known to interact with the components of JA signaling pathway, OsbHLH089 and OsbHLH094, as well as with OsJAZ9 and OsJAZ11, leading to the formation of a stable complex with OsbHLH089 and OsJAZ9, which is required for the repression of the OsbHLH094 transcriptional activity (Toda et al., 2013). Moreover, OsJAZ9, which is a negative regulator of salt tolerance, interacts with OsbHLH062 and suppresses it (Ye et al., 2009). OsbHLH062 is known to regulate the expression of important genes involved in ion homeostasis like *OsSKC1*, *OsHAK21*, and *OsHAK27* (Wu et al., 2015). A tomato mutant *res* (*restored cell structure by salinity*) was observed to accumulate JAs in its roots. This mutant revealed certain developmental alterations in the absence of salinity stress. Fascinatingly, however, on exposure to the salinity stress, the mutant was observed to restore the developmental alterations (Garcia-Abellan et al., 2015). Lastly, systemin, a recently discovered plant hormone that promotes JA production in tomato, is observed to promote salinity tolerance in an ABA-dependent manner. Transgenic tomato in which prosystemin is constitutively expressed have been observed to produce higher biomass enhanced stomatal conductance when exposed to salt stress in comparison with wild-type plants (Orsini et al., 2010). Jointly, all the above findings support that the positive role of JA in the regulation of salt tolerance in plants.

24.4 METHYL JASMONATES: MULTIFUNCTIONAL ROLES IN ABIOTIC STRESS TOLERANCE

Jasmonate and MeJA are supposed to play a dynamic role in alleviating biotic and abiotic stress tolerance especially salt stress. Apart from their role in stress tolerance, methyl jasmonates are involved in diverse developmental, growth, and physiological processes such as plant fertility, sex determination, reproductive processes, storage organ formation, root elongation, fruit ripening and senescence, oxidative resistance, and interaction with other phytohormones (Browse, 2009; Moreno et al., 2009; Avanci et al., 2010; Cipollini, 2010; Nafie et al., 2011; Khan et al., 2012; Khan and Khan, 2013; Per et al., 2018). Other physiological functions related to JA are stimulation of seed germination (Creelman and Mullet, 1997), chlorosis (Creelman and Mullet, 1997), buildup of storage proteins (Pelacho and Mingo-Castel, 1991), upregulation of antioxidant enzymes (Soares et al., 2010), seed and flower development (Wasternack et al., 2012), systemic resistance (Pieterse et al., 2002; 2012), elicitors of plant secondary metabolism (De Geyter et al., 2012), herbivory and wounding (Chung et al., 2008; Howe and Jander, 2008; Ballare, 2011; Erb et al., 2012), allelopathy (Baldwin, 2010), and senescence (Seltmann et al., 2010), etc. In addition, methyl jasmonates trigger plant defense mechanisms in response to insect-driven wounding, pathogens, and environmental stresses, such as low temperature, drought, and salinity (Cheong and Do Choi, 2003). Many researchers have reported the role of

methyl jasmonates in regulating gene expression; this has been documented in a wide range of crops such as *Arabidopsis* (Sasaki et al., 2001), grapevines (Marchive et al., 2013), rice (Liu et al., 2012), tomato (Boter et al., 2004) etc., and eventually leads to defense against abiotic stresses (Gfeller et al., 2010; Ballare, 2011; Su et al., 2011).

24.5 EFFECT OF SALT STRESS ON PLANTS

As per the reports of FAO (2011) soil salinity incurs loss of more than US$11,000 million annually and can affect more than 10% of the world's arable land, which greatly influences agricultural productivity (Tanji, 2002). Salt stress is one of the main abiotic stresses known to affect plant growth and development by inducing ionic, osmotic, and oxidative stress, with detrimental effects on plant yield (Golldack et al., 2014). The stress posed by high salt content in the soil is twofold. Firstly the high concentration of salt ions is toxic to plants and secondly, high salt content leads to water deficiency by lowering the water potential in the soil (Dar et al., 2015). Salinity is known to reduce the growth and development of glycophytes. Soil with electrical conductivity more than 4 dS/m and high content of salts such as sodium chloride and sodium sulfate imposes osmotic stress and ion-induced injury in a wide range of crops (Munns, 2002; Zhang and Mu, 2009). The extent of this injury is correlated to the concentration of NaCl, genotype, and the environment. Salt stress influences mineral distribution, carbon and nitrogen metabolism (Kim et al., 2004; Hakeem et al., 2012), membrane permeability (Gupta et al., 2002), chlorophyll biosynthesis (Khan and Abdullah, 2003), and leads to augmented ion toxicity and enhanced respiration rate (Sudhir and Murthy, 2004). High soil salinity leads to the generation of free radicals such as reactive oxygen species (ROS), which plays a vital role in nucleic acid damage, enzyme inactivation, and lipid peroxidation (Smirnoff, 1993; Khan and Khan, 2017). Salt stress also results in the increased ABA biosynthesis, which reduces stomatal aperture and eventually affects photosynthesis rate (Dar et al., 2015). Salinity induced drop in photosynthetic rate may be attributed to the decrease in the carboxylation rate at which RuBPC fixes CO_2. This may be caused by decline in either CO_2 concentration or in the activity of the enzymes that produce ATP and other reducing equivalents. Many researchers have advocated that the reduction in photosynthetic rate may be credited to two main factors: (1) an indirect effect mediated by stomatal closure causing a drop in CO_2 supply, or (2) a direct effect on the photosynthetic machinery independent of altered diffusion limitations. Essa (2002) have documented in soybean that salinity stress induces seed germination inhibition and reduces nodulation and seedling height, and decreases biomass accumulation and total plant yield. In a wide range of crop species salt stress alters diverse developmental processes, downregulates plant growth, and upregulates senescence and apoptosis during prolonged exposure. It is postulated that amongst diverse sources, irrigation and poor drainage are the main sources of soil salinity.

24.5.1 Jasmonates Counteract Salinity Stress

Phytohormone application is one of the strategies to counter the salinity stress in plants and the main ones involved are ABA, SA, BRs, and JAs. There is growing evidence that jasmonates alleviate the salt stress in plants (Table 24.1). Exogenous applications of MeJA can significantly alleviate salinity stress symptoms in soybean seedlings (Yoon et al., 2009). Moons et al. (1997a,b) in rice have documented that jasmonates induce salt stress-responsive protein in roots. Exogenous spray of JAs enhances dry mass production in the rice cultivars and the use of JA partially recovered the salt stress in them (Dar et al., 2015). Similarly Tsonev et al. (1998) and Velitchkova and Fedina (1998) have reported that MeJA pretreatment alleviates the salt stress and recovered the rate of CO_2 fixation in *Pisum sativum*. It has been pointed out that the treatment of barley seedlings with the jasmonates improved salt stress tolerance (Tsonev et al., 1998). In response to salt stress, the JA level increases in the leaves of *Iris hexagona* and roots of rice (Wang et al., 2001).

Increasing evidence supports the idea that JA can play a significant role in alleviating abiotic stress response (Kazan, 2015; Riemann et al., 2015). The JA-mediated responses are dependent on COI1, an F-box protein member of the SCFCOI1 ubiquitin-ligase complex (Xie et al., 1998). Chini et al. (2007) and Fonseca et al. (2009) have documented that the presence of the biologically active JA-Ile facilitate degradation of JAZ proteins by SCFCOI1 complex in an ubiquitin-dependent manner. JAZ proteins play a vital role in the repression of JA induced genes by binding to *bHLH* transcription factors such as MYC2, MYC3, MYC4, and MYC5 that are activators of JA responses (Chini et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011; Figueroa and Browse, 2012, 2015; Qi et al., 2015; Zhang et al., 2015). This inhibition of JAZ proteins is released by JA-lle mediated destabilization of JAZ by action of MYC2, MYC3, and MYC4 thereby activating the JA responses (Fernández-Calvo et al., 2011; Niu et al., 2011; Zhang et al., 2015). The JA responses include an inhibitory effect on primary root growth. Xie et al. (1998), Chen et al. (2011),

TABLE 24.1 Role of Methyl Jasmonates in Alleviating Salt Stress in Different Crop Species

Crop	Salt stress concentration	Studied parameters	Consequences on plants	Reference
Glycine max	60 mM NaCl	Plant growth, endogenous bioactive gibberellin (GA4), photosynthesis and transpiration rate	MeJA counteracted the negative effects of NaCl stress on plant growth, chlorophyll content, leaf photosynthetic rate, leaf transpiration rate, and proline content	Yoon et al. (2009)
Oryza sativa	50 and 75 mM NaCl	Molecular and physiological effects of jasmonic acid (JA) (< or = 10 μM), ABA, and salt stress in roots of rice	JA markedly induced a cationic peroxidase, two novel 32- and 28-kD proteins, acidic PR-1 and PR-10 pathogenesis-related proteins, and the salt stress-responsive SalT protein in roots	Moons et al. (1997a,b)
Iris hexagona	0, 100, 200, and 400 mM NaCl	Effects of salinity on abscisic acid (ABA), indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA) in leaves, stalks, fruits, and seeds of *Iris hexagona*	ABA and JA generally increased and IAA and SA declined in response to salinity; salinity significantly increased JA content in the apical parts of flower stalks	Wang et al. (2001)
Pisum sativum	30 mM NaCl	CO2 fixation and relative water content, and proline content	Pretreatment with JA-Me for 3 days before salt treatment diminished the inhibitory effect of NaCl on the rate of 14CO2 fixation, protein content, and activity and content of ribulose-1,5-bisophosphate carboxylase/oxygenase; the Na + and Cl- contents in leaves decreased in JA-Me pretreated plants	Velitchkova and Fedina (1998)
Hordeum vulgare	100 mM NaCl	Growth and photosynthesis	Both 100 mM NaCl and 25 μM JA treatment led to a noticeable decrease in net photosynthetic rate vs. intercellular CO2 concentration and the maximal rate of photosynthesis	Tsonev et al. (1998)
Oryza sativa	200 mM NaCl	Plant growth, root, and shoot length	Suppression of OsJAZ9, member of the JAZ subfamily, resulted in reduced salt tolerance, which was mainly due to the changes in K + homeostasis OsJAZ9 acts as a transcriptional regulator by forming a transcriptional regulation complex with OsNINJA and OsbHLH to fine tune the expression of JA-responsive genes involved in salt stress tolerance in rice	Wu et al. (2015)
Tamarix hispida	0.4 M NaCl	Adaptation to salty environments in roots of *Tamarix hispida*	Ninety redundant unique transcripts responsive to NaCl treatment were identified. Of them, 21 genes were novel or of unknown function while others were involved in the functional activities, such as ROS scavenging, lipid metabolism, osmolyte biosynthesis, signal transduction, transport, lignin synthesis, and homeostasis	Li et al. (2009)
Scenedesmus incrassatulus	175 mM NaCl	14CO2 fixation, free proline and malondialdehyde	Salt stress resulted in a reduction of growth and 14CO2 fixation and in an increase of accumulation of free proline and malondialdehyde; exogenously supplied methyl jasmonate did not considerably change the 14CO2 fixation, but increased proline and MDA accumulation in the cells	Fedina and Benderliev (2000)

(*Continued*)

TABLE 24.1 (Continued)

Crop	Salt stress concentration	Studied parameters	Consequences on plants	Reference
Mesembryanthemum crystallinum	200−500 mM	Plant growth, photosynthesis, and chlorophyll content	When plants are grown at high salt concentrations, salt is accumulated in the leaves and leaf osmotic pressure increases drastically; in the presence of high salt, the mode of primary carbon fixation switches from C3 to CAM	Schmitt et al. (1996)
Lycopersicon esculentum	100 mM NaCl	Lipoxygenase protein accumulation, transcripts of allene oxide synthase and proteinase inhibitor II, and activities of diacylglycerol kinase and phosphatidate kinase (enzymes involved in the hosphatidic acid and diacylglycerol pyrophosphate metabolism)	Steady-state levels of JA and related compounds were higher in the salt-tolerant cv. Pera than in cv. Hellfrucht Fruhstamm (HF) and JA levels in both cultivars changed in response to salt stress	Pedranzani et al. (2003)
Glycine max	100 mM NaCl	Shoot length, plant fresh weight and dry weight	It was noted that JA content increased in plants treated with PEG and NaCl, as maximum JA contents were found in plants treated with PEG followed by NaCl	Hamayun et al. (2010)
Triticum aestivum	150 mM NaCl	Plant height, root length, shoot dry weight, root dry weight	Jasmonic acid aids in regulating stress responses, plant growth, and development; JA could effectively protect wheat seedlings from salt stress damage Treatments with exogenous JA for 3 days significantly enhanced salt stress tolerance in wheat seedlings by decreasing the concentration of MDA and H2O2, the production rate of and increasing the transcript levels and activities of SOD, POD, CAT, and APX and the contents of GSH, Chl b and Car, which, in turn, enhanced the growth of salt-stressed seedlings	Qiu et al. (2014)
Iris hexagona	100, 200, 300 and 400 mM	Effects of salinity on abscisic acid (ABA), indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA) in leaves, stalks, fruits, and seeds	Leaves of plants exposed to 400 mM salt wilted and eventually died but lower salinities were tolerated; in young leaves, JA content increased in response to salinity concentration	Wang et al. (2001)
Hordeum vulgare	150 mM NaCl	Physiological parameters	Transcripts that showed significant upregulation under salinity stress are exemplified by jasmonate-responsive, metallothionein-like, late embryogenesis-abundant (LEA) and ABA-responsive proteins; downregulation in a category was observed for photosynthesis-related functions	Ozturk et al. (2002)
Hordeum vulgare	18 dS/m	Net photosynthetic rate per unit area, stomatal conductance to CO_2 (g_c), and transpiration rate	Photosynthetic and sodium ion accumulation responses were compared after salinity stress, JA treatment, and JA pretreatment followed by salinity stress; the JA-pretreated salt-stressed plants accumulated strikingly low levels of $Na+$ in the shoot tissue compared with untreated salt-stressed plants after several days of exposure to stress	Walia et al. (2007)

(*Continued*)

TABLE 24.1 (Continued)

Crop	Salt stress concentration	Studied parameters	Consequences on plants	Reference
Vitis vinifera	10, 20, 30, 40, 50, 85, 120, 155, 200, and 300 mM	Using two grapevine cell lines differing in salt tolerance, the response of jasmonate proteins a marker for salt adaptation, and markers for biotic defense were analyzed	JA in the absence of salt stress reduced growth by ~20% in both cell lines as compared with the control; salt stress signaling shares several events with biotic defense including activity of a gadolinium-sensitive calcium influx channel and transient induction of JAZ/TIFY transcripts; exogenous jasmonate can rescue growth in the salt-sensitive cell line	Ismail et al. (2012)
Oryza sativa	20, 40, and 80 mm NaCl	Plant growth, net photosynthetic rate, leaf water potential, and dry mass production	The decrease of JA concentrations in salt-tolerant cultivar was lesser than in the salt-sensitive cultivar plants in the shoot Postapplication in the stressed plants with 30 μm JA at 24 and 48 h after NaCl treatment recovered salt inhibition on dry mass production more effectively than application of JA at 48 and 24 h before salt stress, and during salt stress simultaneously	Kang et al. (2005)

and Fernández-Calvo et al. (2011) have reported that *Arabidopsis* null mutants that do not respond to JA signaling possess longer roots in the presence of JA. This was also supported by the results of Galvan-Ampudia and Testerink (2011) that plants develop shorter primary roots and alter the root system architecture in high soil with high salt concentration. The inhibition of root growth has been attributed to the cell division and elongation arrest (West et al., 2004; Geng et al., 2013). Hence, salt stress along with the activation of the JA pathway may have an inhibitory effect on primary root growth. As reported in recent literature, the activation of JA signaling pathway is correlated to salt stress response (Kazan, 2015; Riemann et al., 2015). According to Dong et al. (2013) attenuation of salt stress induced root growth inhibition in *Arabidopsis* by the overexpression of the wheat JA-biosynthesis gene OPR1. In contrast Toda et al. (2013) in rice (*Oryza sativa*) have documented that the interaction of OsbHLH089 and OsbHLH094 transcription factors with the salt-sensitive 3, a nuclear localized JAZ-interacting protein lacking a DNA binding domain, results in the formation of a ternary complex that regulates salt induced root cell elongation. However, Kurotani et al. (2015) in rice have reported that salt tolerance may be attributed to the conversion of JA-Ile to an inactive form as a result of overexpression of the *OsCYP94C2b* gene.

All these findings reveal that JA signaling may act as either a positive or negative regulator of the salt stress response in a conditional manner (Riemann et al., 2015). However, as per the reports of early workers such as Jiang and Deyholos (2006), Ma et al. (2006), Kilian et al. (2007), and Geng et al. (2013) have advocated salinity induces upregulation of JA-biosynthesis genes like *AOC1*, *AOC2*, *AOS*, *LOX3*, and *OPR3* in root cells. These results provide the basis the notion of salt stress mediated activation of the JA signaling pathway with wide impact on diverse developmental and physiological processes in plants. Additionally, in jai3-1, a JA-resistant mutant allele that encodes stabilized JAZ3 in *Arabidopsis*, showed an enhanced in root growth rate when compared with wild-type plants under salt stress on a temporal basis (Geng et al., 2013). This finding has reconfirmed that salt stress triggers activation of the JA signaling pathway in the roots leading to growth. Valenzuela et al. (2016) have shown that in the meristematic region and stele of the differentiation region of the *Arabidopsis* root, salinity stimulated activation of the JA signaling pathway in a JAR1-, COI1-, and proteasome-dependent manner. This activation is likely to occur with the participation of core components of the JA signaling pathway, such as COI1, JAZ3 and MYC2, MYC3 and MYC4, leading to arrest of cell elongation in the primary root. All these findings reveal that the salt stress response involves activation of the JA signaling pathway, resulting in inhibition of root growth in *Arabidopsis*.

24.5.2 Salt Stress Response Mediated by JA Signaling

Earlier expression studies showed that some genes implicated in JA biosynthesis were induced in the

roots by salt stress, signifying that the biosynthesis of this hormone is induced in roots by salt stress (Jiang and Deyholos, 2006; Ma et al., 2006; Kilian et al., 2007). Additionally Valenzuela et al. (2016) while studying the early JA-responsive gene activation in the roots during the salt stress response quantified JAZ expression profile, reported that eight out of nine analyzed *JAZ* genes are upregulated by salt stress. Together, these gene expression results strongly suggest that JA signaling is activated in the roots at early stages of the salt stress response. Chini et al. (2007), Thines et al. (2007), and Chung et al. (2008) have reviewed that wounding or exogenous spray of JA causes upregulation of JAZ that eventually turns off hormone signaling once the signal has been transmitted by employing negative-feedback mechanism. This JAZ upregulation has also been observed in the roots after 3 h of salt treatment (Valenzuela et al., 2016) thereby suggesting that JAZ proteins play a crucial role in the downregulation of the JA pathway. Xiao et al. (2004) have reported that coi1 mutants reflected reduced JAZ expression levels when compared with wild-type plants under salt stress. This result confirms that COI1, a core component of the JA-Ile coreceptor (Sheard et al., 2010), is imperative for JAZ transcript upregulation in the roots during the response to salt stress.

The salt stress mediated upregulation of *JAZ* genes in a COI1-dependent manner observed in the roots is likely to follow the canonical JA signaling pathway (Wasternack and Hause, 2013), with proteasome-dependent degradation of JAZ proteins. In *Arabidopsis*, Valenzuela et al. (2016) have reported that considerable activation of JA signaling after salt or JA treatment, mainly in the stele and lower part of the merismatic zone, involves the proteasome-mediated destabilization of JAZ1. The underlying mechanism of signal transduction induced by salt stress and integration from several upstream signals into upregulation of JA-Ile biosynthesis or downregulation of hormone catabolism is not fully understood yet and is under speculation. Detailed studies are required to reveal early events of JA-Ile homeostasis.

24.6 CONCLUSION AND FUTURE PERSPECTIVES

JAs have attained much attention in recent years owing to their considerable involvement in plant growth and development and in alleviating wide range of stresses. It is now clear that JAs are involved in a diversity of functions. JA induced responses are in harmony with diverse complex signals. There exists a complex interplay between JAs with other hormones' signaling pathways for regulating plant responses under salt stress conditions. JAs avert the deteriorating impact of salt stress on diverse developmental and physiological processes. The numerous jasmonate compounds and their different modes of action allow plants to respond specifically and flexibly to environmental flux. However, JA signaling and its roles in signaling crosstalk at the organ, tissue, or cell levels remains under speculation. As research on the biosynthesis and interaction of JA with other phytohormonal activity progresses, a large diversity of metabolites related to different phytohormones would play a role in plant defense and plant–environment interactions. Much work is to be done in the near future to find out the proper answers of the questions like action of JA metabolites, and identification of universal JA receptors, etc. Complete signaling pathways involving MAPKs, CDPK, TGA, SIPK, WIPK, and WRKY TFs are yet to be studied to understand the complete mechanism of action of JA.

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CHAPTER

25

Insights Into the Nitric Oxide Mediated Stress Tolerance in Plants

Parankusam Santisree[1], *Srivani S. Adimulam*[1], *Kapil Sharma*[2], *Pooja Bhatnagar-Mathur*[1] *and Kiran K. Sharma*[1]

[1]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India [2]Department of Plant Sciences, Repository of Tomato Genomics Resources, University of Hyderabad, Hyderabad, Telangana, India

OUTLINE

25.1 INTRODUCTION

Nitric oxide (NO) was first discovered by Joseph Priestley in 1772 as a colorless and odorless gas named "nitrous air"; just a couple of years later came his discovery of oxygen (O_2). NO was considered as a toxic gas for next two centuries, until it was shown to emit by air purging of herbicide-treated *Glycine max* leaves (Klepper, 1979). Thenceforward, NO has long been of major interest in both plant and animal research (Santisree et al., 2015), and in 1992, *Science* magazine crowned NO as "Molecule of the Year". A joint

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discovery that NO is produced by the mammalian species as a signaling molecule by Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad secured the Nobel Prize in Physiology or Medicine for 1998. During the last two decades, several other discoveries have revealed the biological significance of NO in both animals and plants. Despite rapid research in animals, NO research in plants has been gradual with increasing excitement.

25.1.1 Nitric Oxide Function in Plants

Although initial discoveries in plants recognized NO as an atmospheric toxic pollutant for plant foliage, it was eventually considered as a modulator of plant defense during pathogen attacks. The increasing number of reports demonstrated the role of NO in a plethora of plant development processes including seed germination (Arc et al., 2013), root formation, different stages of the seed development, gravitropism, stomatal movements, photosynthesis, mitochondrial functionality, senescence, plant maturation (Sun et al., 2017; Patel et al., 2017; Hasanuzzaman et al., 2018; Locato et al., 2016; Mostofa et al., 2015; Asgher et al., 2017), multiple abiotic (Fancy et al., 2017; Santisree et al., 2015; Parankusam et al., 2017; Adimulam et al., 2017; Tossi et al., 2012; Sehrawat et al., 2013; Ziogas et al., 2013) and biotic stress responses in plants (Vaishnav et al., 2018). In addition, a number of studies focused on describing the crucial role of NO in moderating various plant hormone-mediated development and stress responses (Asgher et al., 2017). Further, accumulation of NO has been shown to induce gene expression of defense proteins during stress conditions and recovery (Romero-Puertas et al., 2013; Fancy et al., 2017). Mounting evidence suggests the role of NO in maintaining cellular homeostasis by acting as an antioxidant and negating the intensity of oxidative damage caused by various stress treatments (Hasanuzzaman et al., 2012; Groß et al., 2013). Despite the emergent knowledge about NO-mediated plant stress responses such as decreasing reactive oxygen species (ROS) levels, protecting membranes from damage, osmolyte accumulation, and regulation of various hormone-mediated signaling events, its functional status has been far from clear. However, the short life and complex chemistry of this diffusible free radical makes NO research in living systems extremely challenging.

25.1.2 NO Synthesis in Plants

25.1.2.1 Nitric Oxide Synthase (NOS)

In animal tissues, NO generation occurs through at least three different nitric oxide synthase (NOS) genes namely neuronal NOS (nNOS), an endothelial enzyme (eNOS), and an inducible enzyme (iNOS). Though NO is known to exist functionally in plants, the origin and signaling of NO is inconclusive to date. The isolation of NOS enzymes in animals is really rapid compared with the decades of research in plants. It is speculated that NO is derived from four major sources in plants: NO synthase pathway, which is similar to that of animals (Negi et al., 2010), nitrate reductase (NR) pathway, and other enzymatic and nonenzymatic pathways (Sahay and Gupta, 2017). The generation of NO has also been suggested through reductive pathways including the mitochondrial electron transport system and from xanthine dehydrogenase/oxidase (Sahay and Gupta, 2017). Despite several possible NO generating pathways proposed in plants (Sahay and Gupta, 2017), identification of a definitive enzymatic pathway generating NO in plants is still awaiting. The first molecular evidence for gene encoding NOS like activity potentially involved in NO synthesis in plants came from *Arabidopsis thaliana* T-DNA insertion mutant (*atnos1*) based on the homology to a snail protein (Guo et al., 2014). However, later studies failed to detect any NOS activity in purified recombinant *AtNOS1* protein or in similar recombinant proteins encoded by orthologous genes from *Zea mays* and *Oryza sativa*. Moreover, this mutant also failed to induce NO synthesis in guard cells in response to abscisic acid (ABA) and other stimuli. These studies concluded AtNOS1 as a regulator of NO levels rather than the molecule of synthesis. Eventually it was renamed as *Arabidopsis thaliana nitric oxide associated* (*atnos1*) mutant (Parankusam et al., 2017). Even though a few other recent pharmacological studies in various plant species also suggested the existence of NOS-like enzyme in plants, the attempts of purifying the gene or protein are still underway (Negi et al., 2010; Fröhlich and Durner, 2011). Nonetheless, two genes with NOS like activity and approximately 40% homology to animal *NOS* genes have been reported in green algae *Ostreococcus tauri* and *Ostreococcus lucimarinus* (Foresi et al., 2015). Though we now have the first *NOS* genes identified in a green alga, identification of *NO synthase* gene in higher plants is still awaited. The divergence of this gene to a new nonanimal homologous during the course of evolution might have posed a challenge to isolate *NO synthase* genes from higher plants.

25.1.2.2 Nitrate Reductase (NR)

Apart from NOS, the other well-known NO-generating enzyme in plants is nitrate reductase (NR). It converts nitrite to NO in a NAD(P)H dependent manner. In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2*. The double mutant of *NIA* genes accumulate much less NO in guard cells in response to ABA. Eventually the NO generation by NR has been

confirmed in many other species, like *Helianthus annus*, *Spinacea oleracea*, *Zea mays*, *Cucumis sativus*, *Chlamydomonas reinhardtii*, *Triticum aestivum*, *Malaxis monophyllos*, and *Aloe vera* (Sakihama et al., 2002, Xu and Zhao, 2003). Additionally, NO can be generated nonenzymatically under low pH by the interaction of two nitrous acid (HNO_2) molecules derived from protonated nitrite, by reduction of NO_2 to NO by carotenoids under light or by oxidation of hydroxylamine, salicylhydroxamate in plant cell cultures, and also during electron transport processes in mitochondria and chloroplasts (Jasid et al., 2006). NO besides being endogenous in origin is also taken up by plants from the external environment. Endogenous NO is synthesized in parallel to ROS accumulation in various organelles like mitochondria, chloroplast, peroxisomes, and apoplast. Recently Gibbs et al. (2014) identified a unifying mechanism for NO sensing in plants based on targeted proteolysis of plant-specific group VII ERF transcription factors. They proposed N-end rule pathway proteolysis as an essential NO sensing mechanism throughout the plant life cycle. Though the research on NO is gaining popularity in recent years, the following controversial questions about NO are around the corner: (1) the major source of NO generation and biosynthesis mechanism, (2) the sensing mechanism of NO by different plant tissues, and (3) the degradation mechanism.

25.1.3 NO Signaling in Plants

As the sources of NO generation have begun to establish, the mechanisms of NO signaling are also increasingly being resolved in parallel. Being lipophilic and highly diffusible in nature, NO is involved in several signaling processes in plants (Baudouin, 2011). Invariably, it has been shown that NO modifies the activity of enzymes and some key signaling components, possibly through posttranslational protein modifications (PTMs). The major PTMs that are integral to NO signaling include protein *S*-nitrosylation and tyrosine nitration (Fancy et al., 2017). A number of S-nitrosylated proteins were identified in *Arabidopsis thaliana*, *Brassica juncea*, *Citrus aurantium*, and *Zea mays* after stress treatment (Mengel et al., 2013; Fan et al., 2014; Fancy et al., 2017). NO-mediated PTMs including nitrosylation, nitration, and carbonylation have been shown to influence ascorbate peroxidase activity under drought (Santisree et al., 2015). NO mediated S-nitrosylation prevented the inactivation of the antioxidant enzymes in *Antiaris toxicaria* thereby providing a way to mitigate the H_2O_2 concentration in plant cells and increasing desiccation tolerance of seeds (Fan et al., 2014; Santisree et al., 2015). Many target signaling components such as redox-associated proteins, the K^+ channel at the guard cell plasma membrane, AtRhobD, salicylic acid-binding protein 3, NPR1, TGA1, and auxin signaling TIR1 (Romero-Puertas et al., 2013; Lindermayr et al., 2010; Terrile et al., 2012) are known to be engaged in NO-mediated PTMs. Additionally, NO acts as a signaling molecule at the transcription level by enhancing the expression of stress related kinases and by its interaction with other signaling molecules and phytohormones (Lozano-Juste and León, 2011). NO stimulates myosin activated protein kinase (MAPK), which in turn causes the activation of stress related genes (Li and Xue, 2010). Another well-established method by which NO exerts its effect is by influencing the redox status of the cell. Modulation of guanylate cyclase represents an NO-specific mechanism of signaling and there are many reports showing that cyclic guanosine monophosphate (cGMP) is a facet of NO effects in plants (Mulaudzi et al., 2011). Recently, a novel guanylate cyclase that generates cGMP and binds NO has been described in *Arabidopsis thaliana* (Mulaudzi et al., 2011). Certain other molecules like Ca^{2+} and cyclic ADP-ribose (cADPR) are found to be involved in the downstream signaling of NO in plant stress responses (Mioto and Mercier, 2013). Moreover, the emerging high throughput omics platforms have been very useful in identifying various candidate genes associated with NO and NO-mediated PTMs (nitrosylation, nitration, and carbonylation) influencing plant stress conditions (Astier and Lindermayr, 2012; Shi et al., 2013; Begara-Morales et al., 2014). It is now imperative to integrate all these molecules and events into our existing knowledge of NO networks.

25.1.4 Modulation of Endogenous Nitric Oxide Levels in Plants

Given that NO is an important signal in plant stress responses, there is increasing interest in understanding the impact of altered NO levels in plants. Notwithstanding, the in vivo level of NO appears to be regulated by mutations in diverse genes. Previous studies have reported few mutants of *Arabidopsis thaliana* including *nia1nia2*, *noa1*, and *nox1*, which failed to exhibit certain developmental and stress responses due to defective NO accumulation, while *noe1* mutation in *Oryza sativa* resulted in higher NO accumulation (Desikan et al., 2002). The mutation in *CUE1* gene encoding a chloroplast phosphoenolpyruvate/phosphate translocator led to an increase in NO content and delayed flowering in the mutant (He et al., 2004). Meanwhile, the null alleles of *Arabidopsis thaliana HOT5* locus encoding S-nitrosoglutathione reductase (GSNOR), showed decreased tolerance to temperature stress due to an increase in levels of nitrate, NO, and

nitroso species (Santisree et al., 2017). Similarly, enhanced lateral root formation in arginase-negative mutant is associated with an increased NO levels (Santisree et al., 2015). Similarly mutation in *Arabidopsis* prohibitin (*PHB3*) gene leads to reduction in absicic acid (ABA)−mediated NO accumulation and auxin-induced lateral root formation (Wang et al., 2010a,b).

Although there is no NOS sequence identified in higher plants, few transgenic efforts have constitutively expressed rat and mammalian neural nitric oxide synthase (nNOS) genes in plants (Shi et al., 2014; Santisree et al., 2015). *35S::nNOS* transgenic lines of *Arabidopsis thaliana* displayed improved abiotic and biotic tolerance (Shi et al., 2011). Similarly, overexpression of rat *nNOS* increased NOS activity and endogenous NO level in transgenic *Oryza sativa* lines, that led to higher tolerance under both drought and salt stresses (Cai et al., 2015). Further, transgenic plants expressing *OtNOS* also displayed increased stomatal development and enhanced abiotic stress tolerance due to higher accumulation of NO (Santisree et al., 2015). The fragmentary molecular identities related to NO synthesis and signaling in plants makes the transgenic and genetic studies challenging.

Due to the inadequate molecular information, most of the current NO research in plants bank on exogenous application of NO-donors and inhibitors/scavengers (Table 25.1). So far this pharmacological approach has been used either to mimic an endogenous NO-related response or as a substitute for an endogenous NO deficiency. Exogenous NO donors or inhibitors have been combined with high throughput technologies to study the ability of NO in modulating plant stress responses at the genomic, proteomic, and postproteomic levels. A pioneering proteomic study in *Gossypium hirsutum* leaves treated with sodium nitroprusside (SNP) identified 166 differentially expressed proteins belonging to diverse pathways, followed by the identification of 167 phosphoproteins to be differentially phosphorylated in response to SNP (Meng et al., 2011). Similarly, proteome profiling revealed 172 downregulated and 76 upregulated proteins in *Cicer aritinum* leaves (Santisree et al., 2017). Few other studies attempted to understand the proteomic basis of NO mediated stress tolerance (Bai et al., 2011; Sehrawat et al., 2013; Yang et al., 2013; Fan et al., 2014). Nevertheless, high-throughput genomic and proteomic signatures of NO still need to be unfolded to further explore the complexity involved in its signaling under plant stress.

25.2 NO IN PLANT STRESS RESPONSES

High temperature and drought are perhaps the two major environmental factors limiting crop growth and yield worldwide (Prasad et al., 2011; Vile et al., 2012). Plants respond at the molecular, cellular, and physiological level by perception and transmission of stress signals followed by a series of responses (Fancy et al., 2017). Longer and severe stress episodes result in production of redox active molecules including reactive oxygen and reactive nitrogen species (RNS), respectively (Astier et al., 2016), which leads to abnormalities at the cellular level due to oxidation of proteins, lipids, and nucleic acids (Hayat et al., 2012). On the other hand, it was demonstrated that abiotic stress often induced NO generation that led to the activation of cellular processes for protection against oxidative stress. NO protects the plants from oxidative damage by enhancing the H_2O_2-scavenging enzymes activities thereby maintaining cellular redox homeostasis (Shi et al., 2014; Zheng et al., 2009). Moreover, exogenous NO donors have often been deployed successfully as priming agents to ward off abiotic stress induced losses in plants (Uchida et al., 2002; Hasanuzzaman et al., 2012; Santisree et al., 2015; Savvides et al., 2016). Although accumulation of NO during various stress conditions appears to be a general response in diverse plant species and tissues, its specificity has been established by using various inhibitors/scavengers such as 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) or L-N^G-Nitroarginine methyl ester; N(G)-Nitro-L-arginine methyl ester (L-NAME), which reversed these NO-mediated effects in many such studies (Santisree et al., 2015). NO plays a critical role in mitigating oxidative stress caused by unfavorable climate (Siddiqui et al., 2017; Table 25.2). Most of the studies reinforce the role of NO in detoxifying ROS either directly interacting with O^{-2} or indirectly by enhancing function of the antioxidant system (Tewari et al., 2008). Interaction of NO with O^{-2} forms peroxynitrite, which serves as a nitrating agent in regulating protein activity under stress conditions (Baudouin, 2011). Further, NO upregulated the activity and transcription of ascorbate peroxidase (APX) and glutathione reductase (GR), the two key enzymes in the ascorbic acid−glutathione (AsA-GSH) cycle in *Nicotiana tabacum* and *Cucumis sativus* leaves (Cui et al., 2011) and conferred resistance to abiotic stress. NO also rapidly reacts with oxygen species, hemes, thiols, and proteins to produce biochemical signals that directly and indirectly regulate enzymatic activity. However, the information available is sometimes contradictory, depending on the plant species, severity, and duration of the stress treatments (Begara-Morales et al., 2014). Despite an increasing number of reports on the role of NO as an endogenous signaling molecule in plants, there is still a large knowledge gap about underlying molecular mechanisms of its action that can sense and transduce NO signals.

TABLE 25.1 Various Forms of Nitric Oxide Modulators Used in Plants to Demonstrate the Role of NO Under Plant Abiotic Stress

	Plant species	Stress imposed	References
NO donors	*Medicago sativa;*	High temperature stress	Yu et al. (2014)
Sodium nitroprusside (SNP)	*Triticum aestivum; Zea mays; Vicia faba; Salpichora organifolia; Tagetes erecta; Lycopersicon esculentum*	Drought stress	Tian and Lei (2007); Hao et al. (2008); Gracia-Mata and Lamattina (2001); Gupta et al. (2013)
	Triticum aestivum	Chilling stress	Esim et al. (2014)
	Chrysanthemum morifolium; zea mays; Phaseolus vulgaris; phragmitis communis; Oryza sativa; Phaseolus radiates; Phragmitis communis; Gingiber officinale; Festuca anundinacea; Solanum lycopersicon	High temperature stress	Yang et al. (2011a,b); Li et al. (2013b); Song et al. (2008); Li et al. (2013); Chen et al. (2013); Siddiqui et al. (2017)
	Citrus grandis; Hibiscus moscheutos; Artemisia annua; Lolium perene; Triticum aestivum; Vigna unguiculata	Al[a] stress	Yang et al. (2016); Aftab et al. (2012); Bai et al. (2011); Sun et al. (2014); Sadeghipour (2016)
	Brassica junica; Arachis hypogeae; Trifolium repens; Typha angustifolia	Cd[a] stress	Dong et al. (2014); Zhao et al. (2016)
	Triticum aestivum; Pistia stratiotes	As[a] stress	Hasanuzzaman et al. (2013)
	Phaseolus vulgaris; Helianthus annuus; Zea mays; Glycene max	UV-B stress	Shi et al. (2005); Tossi et al. (2012)
S-nitroso-N_acetyl-D-penicillamine (SNAP)	*Cicer aritinum*	Salinity stress	Ahmad et al. (2016)
Diethylamine NONOate sodium	*Glycene max*	Salinity stress	Egbichi et al. (2014)
	Medicago sativa		
Inhibitors	*Salpichora organifolia; Tradescantia* sp; *Medicago sativa;*	Drought stress	Garcia-Mata and Lamattina (2001); Tian and Lei (2007); Hao et al. (2008)
Carboxy-2-phenyl-4,4,5,5, -tetramethylimidazoline-1-oxyl 3-oxide (cPTIO)	*Lactuca sativa; Zea mays*	High temperature stress	Deng and Song (2012); Li et al. (2013b)
	Nicotiana tabacam; Arabidopsis thaliana; Betula pendula	Ozone stress	Ederli et al. (2008); Ahlfors et al. (2009a)
Mutants	*Atnoa1, nia1nia2, gsnor1-3, respiratory burst oxidase homolog* mutants of *Arabidopsis thaliana*	Drought stress	Lozano-Juste and León (2011); She et al. (2004)
	nia1nia2 mutants of *Arabidopsis thaliana*	Chilling stress	Zhao et al. (2009); Liu et al. (2016)
	atgsnor1-3/hot5-2 mutants of *Arabidopsis thaliana*	Heat stress	Santisree et al. (2017)
	Homozygous mutants deficient of *atips1* of *Arabidopsis thaliana*	UV-B stress	Lytvyn et al. (2016)
Transgenics	*Arabidopsis thaliana* and *Oryza sativa* transgenic plants overexpressing *oxide synthase* gene (*nNOS*), *OtNOS* transgenic lines; *35S::nNOS* transgenic lines of *Arabidopsis thaliana*	Drought stress Salt stress	Cai et al. (2015); Shi et al. (2014)
	*Arabidopsis thaliana*tGLB3	Heat stress	Hossain et al. (2011)
	H7 plants overexpressing *Arabidopsis* hemoglobin 1 (*AHb1*), transgenic cucumber plants overexpressing *CsNOA1*	Chilling stress	Zhao et al. (2009); Cantrel et al. (2011); Bai et al. (2011); Xu et al. (2012a,b); Liu et al. (2016)

[a]*Al, aluminum; Cd, cadmium; As, arsenic.*

25.2.1 NO in Drought Stress Tolerance

Prevailing water deficit conditions limit crop yields worldwide (Simontacchi et al., 2015). NO, which is known to involve in various physiological processes of the plants, also plays a vital role in mitigating drought stress conditions in plants (Santisree et al., 2015). Many studies have reported an increased production of NO in drought-stressed plants depending on the duration and severity of the given drought stress (Santisree et al., 2015). Roots of *Cucumis sativus* when subjected to a mild water deficit for less than 10 h had slightly enhanced NO synthesis, while it increased to a greater extent with prolonged drought imposition for 17 h (Arasimowicz-Jelonek et al., 2011). Moreover, accumulation of NO as a result of application of exogenous donors in many reported studies also correlated well with the amelioration of drought stress, while the use of NO scavengers/inhibitors reversed this effect (Arasimowicz-Jelonek et al., 2011; Planchet et al., 2014).

The increase in NO production under drought stress has been correlated significantly to the decrease in stomatal conductance in *Vitis vinifera* (Patakas et al., 2010). Given the ability of NO to induce dark-induced stomatal closure in *Vicia faba* epidermal strips (She et al., 2004), a good number of studies confirmed the generation of NO in guard cells in response to drought and ABA by using a NO-sensitive fluorescent dye 4,5-Diaminofluorescein diacetate (DAF-2DA) (Planchet et al., 2014; Zimmer-Prados et al., 2014). Exogenous NO induces ABA synthesis by upregulating the expression of a key genes including *9-cis-epoxycarotenoid dioxygenase* and also negatively regulates the ABA sensitivity thereby enhancing plant tolerance to drought stress (Santisree et al., 2015). While, ABA failed to induce stomatal closure in *Atnoa1*and *nia1nia2* mutants of *Arabidopsis thaliana* having reduced endogenous NO levels, NO-deficient triple mutant *nia1nia2noa1-2* plants were hypersensitive to dehydration and ABA treatment in stomatal closure providing genetic evidence for the involvement of NO in ABA-mediated stomatal closure (Planchet et al., 2014; Lozano-Juste and León, 2011). Furthermore, the overaccumulation of NO in guard cells of *Arabidopsis thaliana* GSNO reductase mutant (*gsnor1-3*) has led to the defective ABA-induced stomatal closure due to the constitutive S-nitrosylation of open stomata 1 (OST1) reinforcing NO as a key intermediate in ABA-induced stomatal closure (Adimulam et al., 2017). Moreover, reduced NO accumulation and stomatal closure were observed in *respiratory burst oxidase homolog* mutant of *Arabidopsis thaliana* having a mutation in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase suggesting a link between H_2O_2 and NO accumulation (She et al., 2004). Broadly, NO enhances antioxidant enzyme activity and induce stomatal closure through ambiguous signaling pathways that require MAPKs, cGMP, and Ca^{2+}. In addition to MAPK, NO also activates other protein kinases such as osmotic stress-activated kinase, NtOSAK in *Nicociana tabacum* (Baudouin and Hancock, 2014). NO alters protein phosphorylation through the regulation of these kinases and also alters calcium flux to regain normal NO responses to occur in the guard cells under drought. NO increases the cGMP level, which acts as the secondary messenger in promoting the expression of stress related genes and secondary metabolites during water deficit conditions (Santisree et al., 2015).

It was reported that exogenous NO decreased drought-induced reduction in photochemical quenching in *Tagetes erecta* (Liao et al., 2012), while enhancing CO_2 assimilation and photosynthetic rate in *Rumex* leaves under osmotic stress (Li et al., 2013). In *Lycopersicon esculentum*, SNP treatment promoted the activity of *carbonic anhydrase* that catalyzes the interconversion of CO_2 and HCO_3, and thus indirectly maintain constant supply of CO_2 to *ribulose-1,5-bisphosphate carboxylase/oxygenase* (RuBisCo) in a concentration dependent manner. Additionally, NO ameliorates the stress effects on chloroplasts and combats drought-induced leaf senescence by antagonizing the effects of ethylene (Manjunatha et al., 2012). NO application has been shown to negate the drought-induced decrease in transcription of *psbA* gene encoding for D1 protein of PSII complex, thus protecting PSII functionality during grain filling (Wang et al., 2011; Procházková et al., 2013). Similarly, photochemical efficiency of photosystem II (PSII) increased by exogenous NO treatment in drought stressed *Populus przewalskii*, but decreased under prolonged drought stress conditions (Simontacchi et al., 2013). Conversely, thylakoids isolated from NO-treated *Spinacia oleracea* exhibited less photosynthetic activity indicating that NO can bind reversibly to PSII and inhibit electron transport (Misra et al., 2014).

Exogenous NO treatment under drought stress often results in reduced H_2O_2 content and lipid peroxidation in plants while high NO causes nitrosative stress (Farooq et al., 2009; Liao et al., 2012; Li et al., 2013). For instance, SNP treatment maintained higher relative water content and reduced ion leakage during drought stress in two turf grass species (Hatamzadeh et al., 2015). Similarly, NO-treated plants maintained high levels of antioxidant enzyme activities and less lipid peroxidation under drought stress in *Dendrobium huoshanense* and *Oryza sativa* (Farooq et al., 2009; Fan et al., 2014). Despite reducing the level of oxidative stress, NO also help in maintaining high concentrations of osmotically active solutes and amino acids. NO promoted drought-induced free proline accumulation in many plants (Farooq et al., 2009; Wang et al., 2011). NO also mediates the accumulation of glycine

betaine by stimulating the activity of *betaine aldehyde dehydrogenase* in the leaves of drought stressed *Zea Mays* (Hao et al., 2008). Conversely, neither depleting endogenous NO by its scavenger nor inducing by NO donor had significant effect on the accumulation of proline in *Medicago* seedlings. Meanwhile, drought stress decreased the DNA methylation levels in *Dendrobium huoshanense*, while NO increased the methylated sites clearly suggesting the ability of NO to alter gene expression under drought (Fan et al., 2014). Transgenic plants overexpressing the rat *neural nitric oxide synthase* gene (*nNOS*) in *Arabidopsis thaliana* and *Oryza sativa* exhibit enhanced drought tolerance than their untransformed controls (Cai et al., 2015; Shi et al., 2014). Moreover, *OtNOS* transgenic lines also exhibited better stomatal development compared with control plants. Although our knowledge on the role of NO in drought stress is still emerging, we find enormous potential of NO in mitigating drought induced adversaries in plants.

25.2.2 NO in Plant Salt Stress Tolerance

Soil salinity is one of the major abiotic stress factors for crop production impacting more than 45 million hectares of cultivated land (Slinger and Tenison, 2009; Fatma et al., 2016). With continuous increase in the demand for food, farmlands are being artificially irrigated in greater amounts, leading to increased salt accumulation in the soil. Excess accumulation of NaCl in soil limits the plant water and mineral uptake (Khan et al., 2012). In addition, the excess salt intake into the cytosol leads to osmotic imbalance and imposes toxic effects on cell membranes (Abeer et al., 2014). Higher salt concentrations cause oxidative stress due to excess production of ROS and thus hinder several metabolic processes (Fatma et al., 2016; Naser Alavi et al., 2014). In the past decade, function of NO in salt stress tolerance has gained a lot of attention among plant researchers (Yang et al., 2011a,b; Mostofa et al., 2015). It was reported that endogenous NO generation has increased in *Nicotiana tabacum* plants in response to salinity stress. External application of NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), to salinized plants enhanced the growth parameters, leaf relative water content, photosynthetic pigment production, levels of osmolytes, as well as the antioxidant enzyme activities and gene expression in *Cicer aritinum* (Ahmad et al., 2016). Moreover, exogenous NO enhanced salt tolerance by mitigating the oxidative damage, stimulating proton-pump and Na^+/H^+ antiport activity in the tonoplast thus promoting K^+/Na^+ ratio (Santisree et al., 2015). NO influences salinity tolerance by regulating plasma membrane H^+-ATPase and Na^+/K^+ ratio thereby generating a H^+ gradient that offers the force for Na^+/H^+ exchange (Zhang et al., 2006). For instance, NO has been implicated in enhancing K^+, Ca^{2+}, and Mg^{2+} content in salt-treated *Gossypium hirsutum* plants (Dong et al., 2014). Moreover, NO reacts with lipid radicals thus preventing salt stress induced lipid oxidation and decreasing membrane permeability (Kopyra, 2004; Fatma and Khan, 2014; Xu et al., 2010b). NO enhances the antioxidant defense systems in plants subjected to salinity by inducing the expression of redox-related genes (Zheng et al., 2009). The application of Diethylenetriamine-NONOate (DETA/NO) ameliorated long term salinity effects in *Glycene max* via the induction of antioxidant enzymes (Egbichi et al., 2014). Studies have provided evidence that NO mediated detoxification is partly by its ability to regulate ascorbate–glutathione cycle through S-nitrosylation (Camejo et al., 2013; Ziogas et al., 2013). It is evident from the studies that salt stress induces an increase in total S-nitrosylation especially S-nitrosylation of the glycine dehydrogenase P subunit, F1 ATPase β subunit, and isocitrate dehydrogenase (ICDH) implying the role of NO-mediated post-translational modifications in controlling respiratory/photorespiratory pathways (Fares et al., 2011; Camejo et al., 2013; Abat and Deswal, 2009; Begara-Morales et al., 2015). Conversely, salt stress induced O_2 might also reduce the S-nitrosylation level by interacting with S-nitrosoglutathione (GSNO/RSNO) (Fancy et al., 2017). However, the enhanced antioxidant enzyme activity due to foliar spray of NO in various crop plants has also resulted in increased plant growth under salt stress (Wu et al., 2011; Fatma and Khan, 2014). Similarly, exogenous application of NO was also proved beneficial in enhancing plant dry weight, shoot, and root length of salt-stressed wheat plants (Kausar and Shahbaz, 2013). Not only that, NO participates in enhancement of photosynthesis by inducing the photosynthetic pigments, adenosine triphosphate (ATP) synthesis, and two respiratory electron transport pathways in mitochondria under salt stress (Ruan et al., 2002). This was further ascertained by an improved photosynthesis by quenching excess energy and increasing in quantum yield of PSII by using exogenous NO in *Solanum melongena* seedlings (Wu et al., 2013). The exogenous application of NO has been reported to control the activity of phosphoenolpyruvate carboxylase kinase in *Sorghum bicolor* under salt stress (Monreal et al., 2013). NO application enhanced the photosynthetic potential of *Brassica juncea* under salt stress (Fatma and Khan, 2014). The sustained photosynthesis after application of NO under salt stress in *Lycopersicon esculentum* has been attributed to improved gas exchange parameters and chlorophyll fluorescence (Wu et al., 2011). A recent study in

TABLE 25.2 Various Studies Describing the Involvement of Nitric Oxide (NO) in Plant Abiotic Stress Tolerance

Stress imposed	Plant species	Response	References
Salinity stress	*Brassica nigra; Brassica juncea; Arabidopsis thaliana; Triticum aestivum; Lycopersicon esculentum; Sorghum bicoclor; Hordium vulgare; Cicer aritinum; Glycene max; Zea mays; Linum ustiatissimum; morua alba; Gossypium hirsutum*	Lipid peroxidation, ROS[a] scavenging Activation of antioxidant enzymes Altered gene transcription, enhanced photosynthesis	Khan et al. (2012); Fatma et al. (2016); Fatma and Khan (2014); Monreal et al. (2013); Ahmad et al. (2016); Egbichi et al. (2014); Dong et al. (2014)
Drought stress	*Triticum aestivum; Zea mays; Hordeum vulgare; Oryza sativa; Vicia faba; Cucumis sativus; Salpicora organifolia; Tagetes erecta; Malus hupensis; Spinacea oleracia; Populus przewalskii; Lycopersecon esculentum*	Stomatal closure, enhanced antioxidant defense, increased adventitious root length, reduced lipid peroxidation, mediating ABA[a] signaling	Santisree et al. (2015); Garcıa-Mata and Lamattina (2002); Tian and Lei (2007); Hao et al. (2008); Cheong et al. (2002); Simontacchi et al. (2013); Gupta et al. (2013)
Chilling stress	*Helianthus annus; Capsicum annum; Arabidopsis thaliana; Chirospora bugeana; Baccaurea ramiflora; Brassica juncea; Camallia sinensis; Lycopersicon esculentum; Triticum aestivum*	Synthesis of osmolytes, reprogramming of lipid signaling, negatively regulates sphingolipid phosphorylation, increases spermidine and spermine levels, increases antioxidant enzyme activity	Cantrel et al. (2011); Ashraf and Foolad (2007); Zhao et al. (2009); Chaki et al. (2011); Airaki et al. (2012); Zhao et al. (2009); Liu et al. (2016); Zhao et al. (2009); Bai et al. (2011); Xu et al. (2012a,b); Abat and Deswal (2009); Wang et al. (2012); Diao et al. (2016); Esim et al. (2014); Baudouin and Jeandroz (2015)
High temperature stress	*Medicago sativa; Pisum sativum; Chrysanthemeum morifolium; Nicotiana gluca; Helianthus annus; Triticum aestivum; zea mays; Phaseolus vulgaris; phragmitis communis; Oryza sativa; Phaseolus radiates; Gingiber officinale; Festuca anundinacea; Solanum lycopersicon; Cicer aritinum*	ROS scavenging enzymes, alleviated the expression of HSPs,[a] NO acts as signal molecule for the stress response, protects the plant from heat stress induced oxidative stress, plays an important role in H_2O_2[a] metabolism	Yu et al. (2014); Chaki et al. (2011); Hasanuzzaman et al. (2012); Li et al. (2013a); Song et al. (2008); Li et al. (2013); Chen et al. (2013); Siddiqui et al. (2017); Ziogas et al. (2013)
Heavy metal stress	*Oryza sativa; Arabidopsis thaliana; Nicotiana tabacum; Solanum lycopersicon; Panax ginseng; Brassica junica; Pisum sativum; Triticum aestivum; Citrus grandis; Hibiscus moscheutos; Artemisia annua; Phaseolus vulgaris; Secale cereal; Lolium perene; Vigna unguiculata; Antiaris toxicaria; Glycene max; Lupinus luteus; Arachis hypogeae; Pistia stratiotes*	NO helps challenge heavy metal stress by chelating the heavy metal at the root zone and preventing their accumulation in plant, regulating gene transcription level of APX,[a] GR,[a] and DHA,[a] increases the plasma membrane transport activity, and GSNOR[a] activity	Wang et al. (2011); Tewari et al. (2008); Mostofa et al. (2015); Xu et al. (2010a,b); Sun et al. (2014); He et al. (2012a,b); Aftab et al. (2012); Wang et al. (2010a,b); Bai et al. (2011, 2015); Sadeghipour (2016); Yang et al. (2016); Xiong et al. (2009); Arasimowicz-Jeloneck et al. (2012); Dong et al. (2014); Hasanuzzaman et al. (2013)
Ozone stress	*Arabidopsis thaliana; Nicotiana tabacam; Ginkgo biloba*	Exogenous application or endogenous synthesis of NO reduces the damaging effects of ozone by activating active oxygen scavenging enzymes	Ahlfors et al. (2009); Ederli et al. (2008); Xua et al. (2012)
UV-B	*Zea mays; Betula pendula; Pisum sativum; Solanum tuberosum; Helianthus annuus; Glycene max*	NO functions as a secondary messenger under UV-B stress, reduces UV induced photomorphogenic responses	Zhang et al. (2011); Tossi et al. (2012)
Wounding stress	*Arabidopsis thaliana; Pisum sativum; Nicotiana tabacum; Helianthus annus; Vicia faba; Triticum aestivum*	Ca^{2+} influx and ROS[a] production in NO[a] dependent pathway, acts as downstream signal molecule in wounding signal transduction	Huang et al. (2004); Chaki et al. (2011); Si et al. (2017)
Flooding stress	*Arabidopsis thaliana; Hordeum vulgare; Brassica japonicum*	Induces ethylene biosynthesis, maintains ATP[a] levels to prevent cell death	Wang et al. (2000)

[a]*ABA, abscisic acid; APX, ascorbate peroxidase; ATP, adenosine triphosphate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSNOR, S-Nitrasoglutathione reductase; H_2O_2, hydrogen peroxide; HSPs, heat shock proteins; NO, nitric oxide; ROS, reactive oxygen species.*

mustard has suggested that NO improves photosynthetic performance under salt stress more effectively by enhancing sulfur assimilation (Fatma et al., 2016). Besides, exogenous NO induced the accumulation of protecting molecules including proline, GB, total soluble proteins and total soluble sugars in *Cicer arietinum*, *Linum ustiatissimum*, and *Morua alba*, thus confirming its role in mitigating the adverse effects of salinity stress (Khan et al., 2012).

25.2.3 NO and Chilling Stress Tolerance in Plants

Exposure of plants to chilling stress severely limits the crop yields due to its deleterious effects including poor seed germination, stunted growth, delayed flowering, and altered gene expression (Minami et al., 2005). Previous studies have documented that an increase in NO synthesis was associated with cold acclimation in a number of plant species including *Arabidopsis thaliana*, *Helianthus annus*, and *Capsicum annum* (Singh et al., 2009; Zhao et al., 2009; Chaki et al., 2011; Airaki et al., 2012). Although, the temperature range for NO generation has not been clearly defined, its formation has been observed at 8ºC in pea leaves, at 4ºC in *Arabidopsis thaliana*, and at 0ºC in *Chorispora bugeana* (Zhao et al., 2009; Liu et al., 2011; Cantrel et al., 2011). Moreover, both NO-sensitive fluorescent probe and chemiluminescence confirmed the accumulation of NO after 1–4 h of chilling treatment in *Arabidopsis thaliana* (Cantrel et al., 2011). This chilling induced NO production was impaired in the *nia1nia2* nitrate reductase mutant and H7 plants overexpressing *Arabidopsis* hemoglobin 1 (*AHb1*) further supporting its role in chilling tolerance. While NR has been implicated in NO synthesis in *Arabidopsis thaliana*, loquat fruit, and *Baccaurea ramiflora* embryos under chilling stress (Zhao et al., 2009; Cantrel et al., 2011; Bai et al., 2011; Xu et al., 2012a,b), NO synthase inhibitors blocked NO accumulation in *Chorispora bungeana* suggesting the involvement of both NR and NOS like enzymes for NO synthesis during chilling stress (Baudouin and Jeandroz, 2015). Eventually, a number of other studies also confirmed the role of NO in alleviating cold injury (Zhao et al., 2009; Liu et al., 2011; Yang et al., 2011a,b; Tan et al., 2013; Diao et al., 2016). Recently, transgenic cucumber plants overexpressing *CsNOA1* constitutively had greater accumulation of soluble sugars, starch, and a lower chilling damage index, while suppression of *CsNOA1* expression resulted in opposite effects. Furthermore, ectopic expression of cucumber *CsNOA1* in the *A. thaliana noa1* mutant enhanced chilling tolerance and rescued the mutant phenotype due to an increase in endogenous NO levels (Liu et al., 2016). Consistent with this, exogenous NO donor has been shown to induce cold acclimation through the synthesis of osmolytes such as glycine betaine and proline (Ashraf and Foolad, 2007; Zhao et al., 2009; Wang et al., 2012) and reprogramming of lipid signaling and composition. Moreover, NO negated the chilling induced transient synthesis of phytosphingosine phosphate and ceramide phosphate in *Arabidopsis thaliana* (Cantrel et al., 2011; Lynch and Steponkus, 1987; Kawaguchi et al., 2000). Not only that, SNP treatment alleviated chilling injury in *Cynodon dactylon* by decreasing malondialdehyde (MDA) content and electrolyte leakage due to the stimulation of superoxide dismutase (SOD) and peroxidase (POD) activities (Fan et al., 2015). Similarly, application of exogenous SNP to cold stressed *Triticum aestivum* seedlings increased the tolerance by enhancing the synthesis of antioxidant enzymes (Esim et al., 2014).

It was documented that cold stress resulted in highest S-nitrosothiol formation followed by drought, high temperature, and salinity in *Brassica juncia*. Besides, 20 proteins predominantly associated with plant defense, photosynthesis, glycolysis, and signaling were found to be potentially S-nitrosylated after cold stress treatment in *Brassica juncea* (Abat and Deswal, 2009; Zhao et al., 2009). Importantly, Rubisco carboxylase is one among those enzymes shown to be inactivated by cold stress induced S-nitrosylation (Abat and Deswal, 2009). Collectively, the emerging picture suggests that NO regulates cold stress signaling by cell wall remodeling and promoting ROS detoxification in plants.

25.2.4 NO and High Temperature Stress Tolerance in Plants

Heat stress is defined as temperature above the optimal temperature that causes an irreversible damage to the growth and metabolism of plants (Johkan et al., 2011; Yamori et al., 2014; Awasthi et al., 2016; Santisree et al., 2017). Heat stress promoted NOS and GSNOR activities thereby increasing the accumulation of NO and S-nitrosothiols in plants suggesting a role for NO in heat stress mitigation (Yu et al., 2014). While exogenous application of NO donors has been able to reduce heat-induced cellular damage, depleting endogenous NO levels by cPTIO reversed these effects, establishing the functional specificity of NO in plant heat stress amelioration (Hasanuzzaman et al., 2013). The importance of NO homeostasis in heat stress tolerance has been highlighted by a null mutation in *atgsnor1-3/hot5-2* locus or RNAi line of *Arabidopsis thaliana*, where overaccumulation of NO correlated with high heat sensitivity (Parankusam et al., 2017). Further, NO scavenger could able to rescue heat sensitivity of these mutant lines of *Arabidopsis thaliana* (Parankusam et al., 2017).

Another major challenge under heat stress is the maintenance of membrane integrity. Exogenous application of SNP reduced electrolyte leakage and MDA content that substantially enhanced the survival percentage of *Zea mays* seedlings (Li et al., 2013b). In another study, SNP treatment recovered RWC, chlorophyll content, electrolyte leakage in heat stressed *Gingiber officinale* leaves (Li et al., 2013a). Heat stress reduced chlorophyll (chl) biosynthesis and caused great damage to photosynthetic apparatus followed by reduced yield (Parankusam et al., 2017). NO has been shown to negate this heat induced chlorophyll loss and also maintain the activity of photosystem II thereby sustaining photosynthesis in plants (Pospíšil, 2016). For example, pretreatment of SNP resulted in enhanced photosynthetic electron transport in heat stressed *Festuca arundinacea* (Chen et al., 2013). Similarly, SNP application reduced the rate of nonphotochemical quenching in heat shocked *Triticum aestivum* leaf discs and diverted more energy to PSII (Hossain et al., 2011). However, excess NO has been shown to inhibit electron transport by reversibly binding to thylakoid membrane complexes of *Pisum sativum* (Ziogas et al., 2013). Furthermore, heat-induced structural and functional changes in the thylakoid membrane often result in ROS formation (Pospíšil, 2016). Several studies evident the ability of NO in maintaining the cellular redox homeostasis by neutralizing harmful ROS produced by heat stress (Ziogas et al., 2013). Pretreatment with SNP enhanced ascorbate and glutathione contents and activities of antioxidant enzymes including monodehydroascorbate reductase, dehydroascorbate reductase, and glyoxalase I and II in heat stressed *Triticum aestivum* seedlings (Hasanuzzaman et al., 2012). Additionally, foliar application of SNP enhanced carotenoid levels thereby protecting against photooxidative damage caused by heat stress in *Chrysanthemum morifolium* (Yang et al., 2011a,b). However, prolonged heat stress was found to induce nitrosative stress in *Pisum sativum* due to increased S-nitrosylation (SNO) content (Parankusam et al., 2017). SNP pretreatment also helped in osmotic adjustment under heat stress by upregulating the *P5CS* gene in *Oryza sativa* seedlings and reducing putricine (PUT)/polyamine (PAs) ratio in *Gingiber officinale* (Uchida et al., 2002). It was shown that nearly 13 tyrosine-nitrated proteins including enzymes like ferredoxin−NADP oxidoreductase and carbonic anhydrase got induced by heat stress in *Chrysanthemum morifolium* seedlings (Chaki et al., 2011). However, detailed global molecular profiling by omics approaches are needed to increase our knowledge on the effect of NO and NO-mediated PTMs at subcellular level to mitigate nitrosative stress impacts induced by high heat.

25.2.5 NO and Heavy Metal Stress Tolerance in Plants

Heavy metals are a group of metals and metalloids that can alter the normal metabolic processes and become toxic to the growth of the plant beyond permissible concentrations (Sahay and Gupta, 2017; Oz et al., 2015). Copper (Cu), cadmium (Cd), aluminum (Al), and arsenic (Ar) are the most commonly reported heavy metals toxic to plants (Sahay and Gupta, 2017). They accumulate in the soil due to the application of fertilizers, mining, agricultural wastes, application of sewage sludge, and industrial activities. Once they enter into the plant cells through plasma membrane transporters, they affect the plants by direct interaction with DNA, proteins, and binding to atoms such as oxygen, sulfur, etc. (Choudhury et al., 2013; Chmielowska-Bąk et al., 2014). NO mitigate heavy metal stresses in plants mainly by two strategies. One, NO helps plants to challenge metal stress conditions by upregulation of antioxidant enzyme activity or by upregulation of defensive gene expression (Ortega-Galisteo et al., 2012). Two, NO regulates cellular free metal concentration either by excluding the heavy metal entry into the root or by preventing their cellular accumulation to a toxic level (Oz et al., 2015). However, the results are sometimes contradictory, where on one hand several reports evidence the protective role of NO in alleviating heavy metal stress adversaries, but on the other hand, NO together with heavy metals has been shown to contribute to the toxicity and growth inhibition of plants (Wang et al., 2014; Yun et al., 2016). Hence, more detailed studies will facilitate understanding of the exact roles of NO in regulating cellular responses to heavy metals (Xiong et al., 2010).

Studies suggested an increased NO production in the Cu-treated plants mainly in the root tissues (Tewari et al., 2008). Moreover, application of SNP effectively reduced Cu-induced toxicity and NH_4^+ accumulation in the leaves of *Oryza sativa* as well as in the adventitious roots of *Panax ginseng* (Tewari et al., 2008). Experiments showed the ability of NO in alleviating the growth inhibition induced by $CuCl_2$ due to the induction of H^+-ATPase activity in plasma membrane and also by enhancing the antioxidant system through regulating GSH/GSSG ratio (Yu et al., 2014; Wang et al., 2015; Xu et al., 2010a,b). In addition to regulation of antioxidant defense, NO is also shown to increase the RuBisCo activity and mineral uptake in Cu-stressed *Nicotiana tabacum* and *Loleumk perenne* respectively (Pető et al., 2011; Khairy et al., 2016).

Al affects crop growth and yield in acidic soils mainly by inhibiting uptake of minerals and nutrients. Exogenous application of SNP alleviated the effects of Al on root elongation in *Hibiscus moscheutos* (He et al.,

2012a,b). Moreover, SNP treatment promoted Al immobilization in roots by improving the root secretion of malate and citrate there by preventing Al accumulation inside the cells in *Artemisia annua* (He et al., 2012a,b; Aftab et al., 2012). Not only that, NO also enhances Al tolerance by regulating hormonal equilibrium in root apices of *Secale cereale* and *Triticum aestivum* (Sun et al., 2014). Besides, SNP treatment improved photosynthetic efficiency, gene transcription, and activity of major antioxidant enzymes of Al-stressed plants (Aftab et al., 2012; Sun et al., 2014; Bai et al., 2011; Sadeghipour, 2016). While increase in endogenous NO level has been observed by Cd-stress in soybean cell suspension cultures (Kopyra, 2004) and in roots of *Lupinus luteus* seedlings (Arasimowicz-Jelonek et al., 2011), a severe decline in the NOS-dependent NO production was observed under Cd stress in *Pisum sativum* leaves (Rodríguez-Serrano et al., 2009). Studies indicate that NO protects chlorophyll against cadmium stress in *Helianthus annuus* (Laspina et al., 2005; Groppa et al., 2008) and Cu stress in *Lolium perenne* (Dong et al., 2014). In another study, NO-induced Cd tolerance in *Oryza sativa* has been attributed to the ability of NO to restrict Cd entry by increasing pectin and hemicellulose content in the cell wall of roots, promoting phosphotic acid accumulation and phospholipase D activity in the plasma membrane (Xiong et al., 2009; Jhanji et al., 2012; Yang et al., 2016). Moreover, SNP treatment significantly decreased the level of ROS and lipid peroxidation due to the increased activities of antioxidant enzymes in Cd exposed *Lolium perenne* seedlings (Arasimowicz-Jelonek et al., 2011; Bai et al., 2011). Interestingly, SNP decreased the translocation of Cd metal in roots and stems of *Arachis hypogeae* followed by an increase in photosynthetic activity (Dong et al., 2014). Similarly, SNP treatment increased antioxidant enzyme activities in As stressed *Triticum aestivum* (Hasanuzzaman et al., 2013). Exogenous application of SNP also decreased As accumulation, which resulted in improved seed germination in mung bean (Ismail, 2012; Leterrier et al., 2012).

Zinc (Zn) is another essential micronutrient that becomes toxic to plants at higher concentration (Sahay and Gupta, 2017). Roots of *Triticum aestivum* display enhanced NOS activity followed by NO generation when exposed to Zn (Duan et al., 2007). Besides, exogenous NO also impacts Zn translocation from root to shoot, increase ascorbate content in *Triticum aestivum* plants (Buet et al., 2014). Meanwhile, NO treated *Triticum aestivum* plants show a decrease in H_2O_2 and nonprotein thiol content under Fe deficient conditions (Buet et al., 2014). Reports suggest that NO increased uptake of Fe and magnesium that subsequently resulted in improved chlorophyll synthesis, photosynthesis, and transpiration (Kong et al., 2016). Similarly, Nickel (Ni) induced oxidative stress was alleviated by exogenous application of NO in *Brassica junica, Solanum lycopersicum*, and *Triticum aestivum* (Mihailovic and Drazic, 2011; Kazemi, 2012; Wang et al., 2010a,b). Another study points to the reduced manganese (Mn) toxicity due to NO donor treatment in excised rice leaves (Srivastava and Dubey, 2012). Collectively, the available data on the involvement of NO in metal stress suggests that NO alleviates heavy metal toxicity by attenuating oxidative stress and enhancing the activity of antioxidant enzymes.

25.2.6 NO in Ozone Stress Tolerance

Ozone (O_3) is an oxidant gaseous molecule present in the stratosphere region protecting Earth from harmful UV-B radiation. The rising surface O_3 levels due to urbanization and the industrial revolution has become a serious concern for both for human health and vegetation (Monks et al., 2015). Increased accumulation of NO was observed in *Nicotiana tabacam* and *Arabidopsis thaliana* upon ozone exposure, which disappeared following the addition of 100 μM cPTIO (Ederli et al., 2008; Ahlfors et al., 2009; Xua et al., 2012). Moreover, *Atnoa1/rif1* mutant with decreased NO accumulation was found to be O_3 sensitive. Conversely, the O_3-sensitive *rcd1* mutant was found to be an NO overproducer suggesting the significance of NO generation during O_3 stress response. NO is required to modify hormone biosynthesis and signaling, and associated gene expression in plants during O_3 exposure (Ahlfors et al., 2009). Although both NO and ethylene dependent pathways are required for the O_3-induced upregulation of alternative oxidase *AOX1a*, only NO is indispensable for the activation of *AOX1a* gene expression. Activating alternative oxidase pathway is essential to reducing the formation of reactive oxygen species and thus allowing increased carbon flux through the tricarboxylic acid cycle to provide carbon skeletons for other cellular processes under limited ADP supply (Ederli et al., 2008). Interestingly, acute ozone fumigation of poplar has changed the total nitrite and nitrosothiol contents of leaves and altered the homeostasis of 32 S-nitrosylated proteins (Vanzo et al., 2014). Together, a total of 172 proteins have been shown to be S-nitrosylated in poplar callus and leaf due to ozone fumigation. Besides, ozone fumigation enhanced the activity of phenylalanine ammonia-lyase (PAL) by denitrosylation in poplar (Vanzo et al., 2014).

25.2.7 NO in UV-B Tolerance of Plants

In recent years the depleting ozone layer is allowing more UV-B radiation (280–320 nm) to reach Earth's

surface. Exposure to high doses of UV-B radiation leads to numerous harmful consequences in plant cells including dimers formation in DNA, genomic instability, overproduction of ROS, lipid peroxidation, followed by damage to the photosynthetic apparatus and membranes, and ultimately to PCD (Lytvyn et al., 2016; Krasylenko et al., 2013). Plants initiate various defense reactions including the activation of ROS scavenging systems, synthesis of UV-absorbing phenolic compounds, activation of DNA repair mechanisms, and upregulation of UV-B sensitive oxidative defense genes (Hideg et al., 2013). Studies indicate that upregulation of chalcone synthase gene responsible for flavonoid production by UV-B requires NO in *Arabidopsis thaliana* (Mackerness et al., 2001), while NO scavengers counteract this upregulation further asserting NO's role in flavonoid accumulation. A recent study suggests that treatment of *Arabidopsis thaliana* with SNP donor recovered the plant from UV-B inhibited root growth and altered morphology as well as had damaging effects on microtubular organization (Krasylenko et al., 2013). Similarly, a role of NO in UV-B induced stem elongation has been suggested for *Pisum sativum*. Although NOS-like source for NO generation during UV-B stress has been suggested in *Ginkgo biloba* (Tossi et al., 2012), the mammalian NOS inhibitors had no effect on UV-B-induced NO generation (Zhang et al., 2011). Conversely, a direct correlation between NR activity, NO emission, and nitrite (NO_2^-) has been shown to be essential for flavonoid accumulation under UV-B radiation, while cPTIO abolished this response in *Betula pendula* (Zhang et al., 2011).

Homozygous mutants of *Arabidopsis thaliana* deficient for inositol-3-phosphate synthase 1 (*atips1*), a key enzyme for biosynthesis of *myo*-inositol and its derivatives, displayed greater tissue-specific resistance to the action of UV-B. Moreover, pretreatment with low doses of SNP before UV-B exposure resulted in a tissue-specific protective effect in wild type, which was further enhanced in *atips1* (Lytvyn et al., 2016). Exogenous NO partially alleviated the UV-B effect by a decrease in chlorophyll content and oxidative damage to the thylakoid membrane in bean seedlings. In addition, the enhanced NO levels also help in maintaining cellular redox homeostasis under UV-B stress by activating antioxidants and secondary metabolite production. Consistent with this, SNP pretreatment prevented the oxidative stress progression in UV-B exposed *Phaseolus vulgaris* seedlings by decreasing H_2O_2 content, increasing the thiol group content and upregulation of active oxygen scavenging genes. Moreover, NO scavenger or inhibitor or cGMP inhibitor partially prevented the negative impact of UV-B on pollen germination and pollen tube growth suggesting the operation of cGMP mediated NO pathway during UV-B responses of *Paulownia tomentosa*. However, cataloguing of NO responsive genes, proteins, and other signaling partners under UV-B stress would decipher NO-dependent mechanisms of UV-B stress tolerance in plants.

25.2.8 NO in Wounding Stress Tolerance

Wounding stress in plants represents a range of mechanical injuries caused by both biotic (herbivory and infect feeding) and abiotic stresses (raining, wind, touching, hailing, etc.) (Gilroy et al., 2016). In cultivated crops, wounding may also occur during manual manipulation such as harvesting, handling, and storage. In *Pisum sativum* leaves, NO accumulated 4 h after wounding followed by an increase in the content of SNOs due to the induction of NOS and GSNOR activities (Gilroy et al., 2016). Interestingly, wounding triggered accumulation of GSNO and some other SNOs due to the downregulation of GSNOR activity, but without affecting the NO content in *Helianthus annus* hypocotyls (Chaki et al., 2011). The NO released under wounding in turn acts as a signal to cause a transient increase in the cytoplasmic Ca^{2+} concentration at the site of injury in *Vicia faba* and *Nicotiana tabacum* cell suspensions (Gilroy et al., 2016). More recently, extracellular ATP leaking from the injured cells was also suggested to act as a signal to carry stress response to surrounding healthy cells in plants as well as in animals (Casalongué et al., 2015). Intriguingly, extracellular ATP induced activation of Ca^{2+} influx, ROS production, and altered gene expression was also mediated through NO-dependent pathway (Chivasa et al., 2005; Song et al., 2008). Another study revealed that systemic accumulation of H_2O_2 and NO at local wound site enhanced cross tolerance to freezing in untreated systemic leaves in *Triticum aestivum* (Si et al., 2017). The mechanical stress induced NO burst has also been implicated in DNA fragmentation in *Arabidopsis thaliana* (Garcês et al., 2001). Although these results strongly support the critical role played by NO in wounding stress in plants, additional work is required to understand the detailed mechanism of its signaling in plants.

25.2.9 NO in Flooding Stress Tolerance

Flooding is a major limiting factor for crops growing on as much as 12% of the world's soils that suffer excess water (Bailey-Serres et al., 2012). In general, plants respond to flooding by forming root aerenchyma, initiating a hyponastic response and by initiating a quiescence phase to conserve bioenergy. The decline in mitochondrial respiration under hypoxia

leads to an increase in NADH and a drop in ATP levels. Flooding causes anaerobic conditions leading to intracellular NO_2 accumulation as an alternative source of NAD^+ to maintain a high glycolytic rate, which is often converted to NO by cytosolic NR in *Hordeum vulgare* roots (Dongen and Licausi, 2015). Besides NR, hypoxic stress-induced hemoglobin (Hb) also regulates NO levels in *Zea mays* cell cultures and *Medicago sativa* root cultures (Igamberdiev et al., 2005). The regulation of NO levels was also shown to be critical by maintaining high nitrate (NO^{3-}) concentrations and stress-induced Hb in *Arabidopsis thaliana* (Wang et al., 2000). Both increased Hb gene expression and activation of NR enzyme induce the generation of NO in order to maintain ATP levels and to prevent cell death under flooding (Igamberdiev et al., 2005). Detoxification of NO and nitrite produced due to denitrification in soybean nodules after flooding requires a functional plant oxygen carrier leghemoglobin (Sánchez et al., 2010). Hence, the reduction of nitrate by *Brassica japonicum* bacteroids leads to legheamoglobin-NO production within *Glycene max* nodules in response to hypoxia (Meakin et al., 2007). Moreover, flooding caused a decrease in *nifH* expression and nitrogenase activity in wild-type soybean nodules (Sánchez et al., 2010). Nevertheless, uncertainties not only on the source of NO, but also the complete NO-mediated mechanism under flooding, need to be thoroughly addressed.

25.3 NO AND PHYTOHORMONES CROSSTALK IN ABIOTIC STRESS TOLERANCE

Owing to its rich chemistry, NO involves interactions with a number of signaling molecules and phytohormones during abiotic stress responses in plants (Sami et al., 2017). A series of experimental reports established that NO modulates the biosynthesis, distribution, and degradation of phytohormones. The past decade has witnessed the interaction of NO with almost all the plant hormones mostly as a second messenger in the signaling cascades of various plant developmental and stress responses. For example, NO has been intensively involved with hormones such as GA, JA, ET, CK, and AUX during the regulation of stomata under environmental stress conditions (Sami et al., 2017; Nawaz et al., 2017). A majority of the research publications highlighted the interaction between ABA and NO under drought stress. Interestingly, both synergistic and antagonistic crosstalks have been described between NO and ABA depending on the physiological response and tissue during stress (Santisree et al., 2015). For example, NO is involved in the ABA induced stomatal closure by selectively activating intracellular Ca^{2+} channels through a cGMP/cADPR-dependent signaling pathway in *Vicia faba* guard cells (Mioto and Mercier, 2013). ABA induced NOS activity and NO levels have been shown to improve the thermotolerance of *Phragmites communis* calluses (Song et al., 2008). Guo et al. (2014) found that coordinated action between NO and ABA upregulated cold-induced MfSAMS1 expression, resulting in enhanced acclimation against cold stress. Similarly, the study using vp14 maize mutant defective in ABA synthesis suggests that the requirement of ABA accumulation after UV-B perception is critical to trigger the elevation of cytosolic Ca^{2+} concentration resulting in enhanced NOS-mediated NO production (Tossi et al., 2012). NO and Aux display an extensive signaling crosstalk during the development and remodeling of root architecture for the extraction of more water under drought stress Simontacchi et al., 2013. Additionally, several developmental studies indicate the involvement of NO in auxin mediated lateral and adventitious roots formation (Simontacchi et al., 2013). In agreement with this, interplay of NO-AUX was also evident by significant reduction in root meristem size in salt treated *Arabidopsis thaliana*. Interestingly, supplementation of NO reduces AUX degradation by downregulating IAA oxidase activity in *Medicago truncatula* under Cd stress as well as under Al toxicity in *Triticum aestivum* and *Secale cereale* (Xu et al., 2010a,b; He et al., 2012a,b). In another study, Cd induced NO accumulation promoted the stabilization of AUX repressor protein IAA17 in *Arabidopsis* through suppression of AUX carriers PIN1/3/7 (Kovacs and Lindermayr, 2013). Further, a positive correlation between AUX and NO has been suggested in enhancing ferric-chelate reductase activity in a Fe-deficient *Arabidopsis thaliana* plants (Chen et al., 2010). Similar to auxin, a positive interaction between NO and CK under drought was reported, wherein the treatment with CK regulated photosynthetic machinery by promoting NO signaling in *Zea mays* (Shao et al., 2010). Conversely, CK reduced NO levels to trigger stomatal opening in dark grown *Vicia faba* seedlings (Santisree et al., 2015). Foliar application of SNP delayed salt-induced leaf senescence by upregulating the expression of isopentenyl transferase (*IPT*) in *Gossypium hirsutum* seedlings (Kong et al., 2016). However, more studies are required to provide strong evidence for NO and CK interaction under abiotic stress. Previous studies have reported the participation of NO in SA-induced stomatal closure in *Arabidopsis thaliana* (Sun et al., 2010). A coordinated action of NO and SA was found to mitigate the damaging effects of osmotic stress in *Triticum aestivum* seedlings (Naser alavi et al., 2014). In another study, combined application of NO and SA improved Ca2 + / Mg2 + absorption, increased proline accumulation while mitigating the salt stress adversaries in *Glycene max*

seedlings. Similarly, combination of NO and SA has been shown to alleviate the toxic effects of Ni in *Brassica napus* and Cd in *Arachis hypogaea* (Kazemi et al., 2010; Xu et al., 2015).

The critical balance between NO and ethylene seems to be essential to prevent cold-induced injury during postharvest fruit ripening and seed conservation (Bai et al., 2011; Xu et al., 2012a,b). Akin to SA, ET induced by Cd stress reduced NO levels in *Pisum sativum* and *Glycine max* seedlings. Besides, both NO and ET are required to upregulate the plasma membrane H^+ -ATPase and alternative respiratory pathway to modulate ion homeostasis for improved salt tolerance (Wang et al., 2010a,b). A few studies indicate that NO treatment induces production of ethylene to regulate a few O_3 induced genes (Ahlfors et al., 2009). Additionally, NO plays a key role in programmed cell death (PCD) and the hyponastic responses by inducing ethylene biosynthesis during flooding stress (Pasqualini et al., 2012). It is well known from the literature that coordinated action between NO and plant hormones including ABA, JA, GA, and CK induce thermotolerance by activating the antioxidant machinery and upregulating the heat shock protein expression in plants (Zandalinas et al., 2016). Further, pretreatment of *Zea mays* seedlings with H_2O_2 rapidly induced endogenous H_2O_2, NO, and H_2S accumulation under heat stress, which was reversed by H_2O_2 scavenger dimethylthiourea and NO scavenger cPTIO, indicating that H_2O_2 induced heat stress tolerance was involved in the crosstalk between downstream components NO and H_2S (Li et al., 2013b). An interlink between NO and GA has also observed in promoting apical root growth in Al-stressed *Triticum aestivum* roots (He et al., 2012a,b). Additionally, NO induced reduction in total free polyamines, free put, spermidine (Spd), and polyamine oxidase activity was reported in salt stressed cucumber seedlings (Fan et al., 2013). Despite this evidence, the complete understanding of mechanisms underlying the intersection of NO signaling with other signaling molecules requires further study to explain how NO concomitantly interacts with hormone-related proteins at the posttranscriptional or translational level.

25.4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As climate change continues, our appreciation of how plants respond to stress is becoming increasingly significant. Accumulating evidence demonstrated a reversible accumulation of NO under a range of abiotic stresses (Fig. 25.1). Several studies indicate that the NO accumulation potentially enhances plant survival under stress conditions mostly by increasing cellular antioxidant defense. However, the functionality of NO accumulation depends on its concentration, location, duration, plant growth, and developmental stage and many internal and external factors. Although NO is recognized as a multitasking molecule with innumerable functions in plant stress responses, many questions remain unanswered. Most importantly, how does the stress-specific NO accumulation translate into a biological function that helps in stress amelioration? Another pertinent question is how the small NO molecule can influence modification of a massive number of molecules that enhance plants tolerance to stress. Since the sensitivity to stress varies from species to species, the stress ameliorating effects of NO in the given species under given stress may not be extrapolated to other species with different sensitivity. In some of the experiments, in vitro stress treatments used may not be comparable with the natural stress conditions that occur at

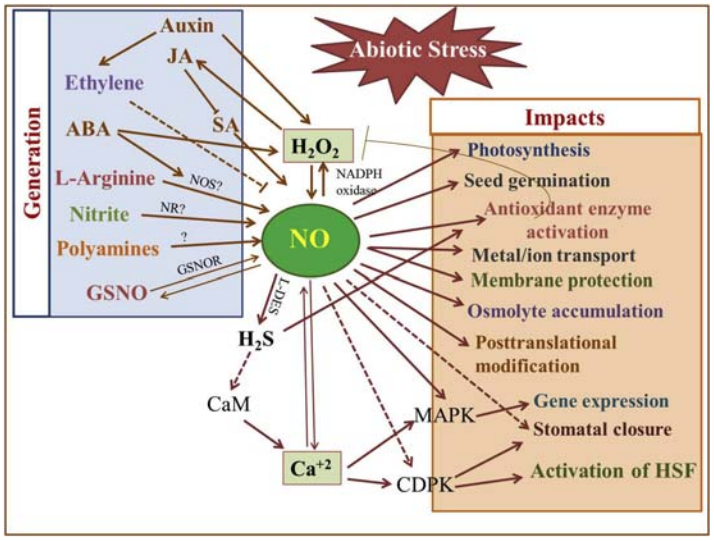

FIGURE 25.1 Schematic illustration for synthesis and functional roles of nitric oxide (NO) in plant responses to abiotic stress.Abiotic stress factors in plants led to the generation of reactive oxygen species (ROS) and nitric oxide (NO), which can effect gene expression. NO participates actively in signal transduction by altering the expression of a number of genes, such as protein kinases and transcription factors (TFs), triggering various physiobiochemical responses, including changes in general metabolism and ion/metabolite transport, stress responses, as well as protein degradation. NO either stimulates (normal end arrow) or inhibits (blunt end arrow) plant processes in coordination with various other signaling molecules under abiotic stress. The dotted line denotes the pathways not studied clearly. The double-sided arrow indicates the mutual regulation of molecules. *ABA*, abscisic acid; *JA*, jasmonic acid; *MAPK*, mitogen activated protein kinase; *NOS*, nitric oxide synthase; *NR*, nitrite reductase; *ROS*, reactive oxygen species; *SA*, salicylic acid; *TF*, transcription factor.

the field level. Therefore, how the NO signaling pathways function in an open environment can only be addressed by field level studies. Further, detailed studies that are more directly linked to yield, such as the role of NO in increasing protection to photosynthesis and osmolyte accumulation under stress, will add more value to the established antioxidant role of NO.

Despite growing knowledge about NO-mediated plant functions, detailed information on its origin and signaling under individual stress conditions has so far been elusive. Hence, the genetic screens used to explore the consequences of NO deficiency may not be conclusive unless multiple NO generating sources are disabled simultaneously. Clearly, blocking the NO generating sources without complete elucidation of the responsible molecular identities presents a big challenge for genetic and transgenic researchers. Although the use of exogenous donors/inhibitors offers an alternative until the molecular identities of NO generation in plants are better deciphered, it is critical to consider the stress treatment and the tissue type used to critically analyze the results to avoid ambiguous conclusions. Moreover, the method of NO application needs to be validated considering the cost, impact, and ease of use under large scale applications. In this context, encapsulation of NO donors in nanomaterials looks promising and has several advantages such as improved efficiency and controlled release over direct exogenous applications (Savvides et al., 2016).

Although there is sufficient evidence on the potential involvement of NO in various hormone-mediated plant growth processes, the crosstalk studies remain fragmental under individual stress conditions. Moreover, the high throughput -omics analysis of NO-mediated stress responses remain largely untapped, but more research in this direction will facilitate research aimed at the identification of NO targets in stress conditions. Together, exploration of NO metabolism and its interacting partners in plants and their physiological relevance under stress will be helpful to enrich our knowledge on NO functions.

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CHAPTER

26

Brassinosteroid Signaling and Complex Interplay of ROS, NADPH Oxidase, and MAPK Mediated Biotic and Abiotic Stress Acclimation in Plants

Deepesh Bhatt[1,*], *Mayank Sharma*[2,*], *Manoj Nath*[3], *Megha D. Bhatt*[4] *and Saurabh Badoni*[5]

[1]Department of Biotechnology, Shree Ramkrishna Institute of Computer Education and Applied Sciences, Veer Narmad South Gujarat University, Surat, Gujarat, India [2]Martin Luther University of Halle-Wittenberg, Halle, Germany [3]Indian Council of Agricultural Research, New Delhi, Uttar Pradesh, India [4]GSFC AgroTech Ltd. Gujarat State Fertilizers & Chemicals Ltd., Vadodara, India [5]Plant Breeding Division, International Rice Research Institute, Metro Manila, Philippines

OUTLINE

26.1 INTRODUCTION

Plants are constantly exposed to multiple biotic and abiotic stresses during their entire life, which include pathogen attacks and insect herbivory, high or low temperatures stress, drought and salinity stress, etc. Therefore, to survive, plants have evolved a range of intricate signaling mechanisms thus adapting towards these fluctuating environments. Plant growth regulators, also termed as phytohormones, are increasingly

*The authors (Deepesh Bhatt and Mayank Sharma) contributed equally to this work.

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recognized to play vital roles in plant stress adaptations. Several studies indicated that biological processes in plants are influenced by stressful conditions, which are regulated through different hormonal signaling pathways in plants (Teale et al., 2008). Phytohormones, like salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA), are primary signals that regulate responses to mitigate biotic and abiotic stresses in plants (Lorenzo and Solano, 2005; Mauch-Mani and Mauch, 2005). These stresses, if not properly regulated, may further lead to the generation of a key intermediate termed as reactive oxygen species (ROS). ROS are generated in response to stress as well as to regulate normal metabolic processes as a signaling agent (Mittler et al., 2011; Sewelam et al., 2016). Additionally, respiratory burst oxidase homologs (RBOHs) and NADPH oxidases are also known to be major components of ROS production system in plants (Suzuki et al., 2011; Kadota et al., 2015). Furthermore several other studies also propose that ROS plays a critical role in enhancing stress tolerance by activating mitogen-activated protein kinases (MAPKs), antioxidant enzymes, and other related transcription factors (Gechev et al., 2006). The generation of ROS has been shown to be a key process that is shared among many transcriptional pathways related to stress signaling. Thus at the molecular level, the perception of external stimuli results in the subsequent activation of stress signaling through some intermediate signal molecules. Brassinosteroids are a group of steroidal hormones and play a major role in various developmental pathways and are involved in stress response (Choudhary et al., 2012). Here in this chapter we will mainly emphasize how brassinosteroids are able to influence and regulate concomitant generation of ROS, NADPH oxidase, and MAPK, which act as intermediate components in the signaling cascade to regulate biotic and abiotic stress acclimation in plants.

26.2 BRASSINOSTEROIDS

Mitchell and coworkers explored a sixth class of plant hormones named brassinosteroids (BRs) in 1970 (Mitchell et al., 1970). BR was first isolated from the pollen of *Brassica napus*. BRs are polyhydroxysteroids similar to steroid hormones from animals (Clouse, 2011). Furthermore they play an important role in regulating various aspects of plant growth and development (Wei and Li, 2016). This includes cell division and elongation, tissue morphogenesis, plant defense, seed germination, and reproduction (Krishna, 2003; Ashraf et al., 2010). They also play a direct or indirect role in stress signaling in plants (Bajguz and Hayat, 2009). Approximately 70 different kinds of analogue of BRs have been identified to date from various plant species and tissue samples. Brassinolide has been reported as a most active BR identifies hitherto (Grove et al., 1979, Tang et al., 2016) (Fig. 26.1). BRs are continuously biosynthesized, mostly in pollens, immature seeds, roots, and flower (Takatsuto, 1994; Kutschera and Wang, 2012). Excess of BRs can be metabolized by plants via different chemical reactions to render them into inactive form. However, they can be converted into the active form whenever needed to maintain the BR homeostasis (Bishop and Yokota, 2001; Bajguz, 2007). Plant cell consists of various receptors for BRs, which recognize them and stimulate the downstream signaling processes. Characterization of BR-insensitive (*bri1*) mutants led to the discovery of the first BR receptor in *Arabidopsis thaliana* (Clouse et al., 1996). *BRI1* encodes a leucine-rich repeat-receptor like kinase (LRR-RLK) composing of an intracellular kinase domain and extracellular LRR domain (Li and Chory, 1997). The *bri1* mutants were shown to be male sterile with several developmental and tissue deformities. Three BRI1 homologs namely BRL1, BRL2, and BRL3 (BRI1 like 1, 2, and 3) were also reported to recognize BRs in *Arabidopsis* (Cano-Delgado et al., 2004), though they display a weak phenotype in respective knockout plants when compared with BRI1. A further molecular dissection, by two different scientific groups, contributed in identification of interacting partners of BRI1 named BRI1 associated kinase 1 (BAK1) or somatic embryogenesis receptor-like kinase (SERK3) (Hecht et al., 2001; Nam and Li, 2002). BAK1 interacts with BRI1 to persuade the BR signaling. The SERK family of proteins with five members seems to play an important role in BR signaling. SERK1 and SERK4 have a similar role as of BAK1/SERK3 in BR signaling. Just to avoid confusion it should be noted that BAK1 and SERK3 were discovered and named separately, however later on it came out that BAK1 and SERK3 are the same protein.

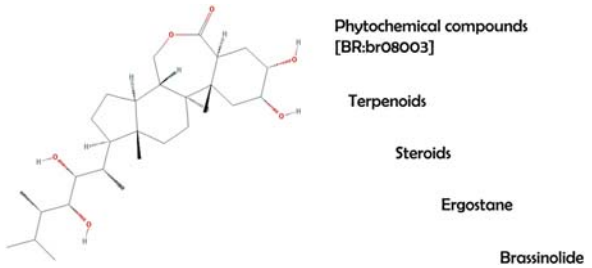

FIGURE 26.1 Chemical structure of the most active brassinosteroid 'brassinolide' $C_{28}H_{48}O_6$ (Image from PubChem) and its phytochemical compounds tree.

26.3 BRASSINOSTEROID SIGNALING IN PLANTS

BR signaling is a phosphorylation and dephosphorylation relay system among BR receptors, coreceptors,

and downstream transcription factors. In absence of BR, a BRI1-KINASE INHIBITOR 1 (BKI1) remains bound to BRI1 (Wang et al., 2005; Wang and Chory, 2006). The binding of BR to BRI1 and coreceptor BAK1 releases BKI1 and makes it available for 14-3-3 proteins that phosphorylate it and thus inhibit its binding to BRI1 (Gampala et al., 2007). Furthermore the mutual phosphorylation of BRI1 and BAK1 leads to activation of downstream signaling. The activation of BRI1 and BAK1 also requires the TWISTED DWARF 1 (TWD1/FKBP42) protein (Zhao et al., 2016). An activated BRI1 phosphorylates the downstream signaling proteins namely BR-SIGNALING KINASE (BSK1) and CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) kinase (Tang et al., 2008; Kim et al., 2009, 2011; Sreeramulu et al., 2013). After phosphorylation, this in turn leads to the activation of BRI1-SUPPRESSOR 1 (BSU1). BSU1 is a PP1-type phosphatase (Kim et al., 2011). The activation of BRI1 and BAK1 also requires the TWISTED DWARF 1 (TWD1/FKBP42) protein. The BSU1 further dephosphorylates and inactivates BRASSINOSTEROID INSENSITIVE 2 (BIN2). BIN2 is a G11SK3-like kinase and proposed to be degraded via its ubiquitinylation by a ubiquitin ligase KINK SUPPRESSED IN BZR1-1D (KIB1) (Zhu et al., 2017). Another protein, BES1/BZR1 (BRI1 EMS SUPPRESSOR 1/BRASSINAZOLE RESISTANT 1), a BR responsive transcription factor, plays a key role in activation of BR-induced genes. In absence of BR, the BIN2 phosphorylates BES1/BZR1 leading to its inactivation. But in the presence of BR, BIN2 is degraded and PROTEIN PHOSPHATASE 2A (PP2A) dephosphorylates BES1/BZR1 (He et al., 2001). The BES1/BZR1 later enters in the nucleus, binds to DNA and activates the BR-induced genes with the help of some additional transcription factors (Ryu et al., 2007). For a schematic view, refer to Fig. 26.2A–B.

26.4 TRANSCRIPTION FACTORS INVOLVED IN BR SIGNALING

Genome-wide analysis of genes regulated by BES1/BZR1 revealed that the expression of several thousand genes is influenced in BR signaling (Yu et al., 2011; Sun et al., 2010). The downstream BR signaling genes are either directly or indirectly regulated by BES1/BZR1. Several BES1/BZR1 interacting proteins and transcription factors have been identified so far. The complex interplay with these transcription factors in turn regulates the expression of BR signaling genes (Li, 2010). This include *PHYTOCHROME-INTERACTING FACTORS*

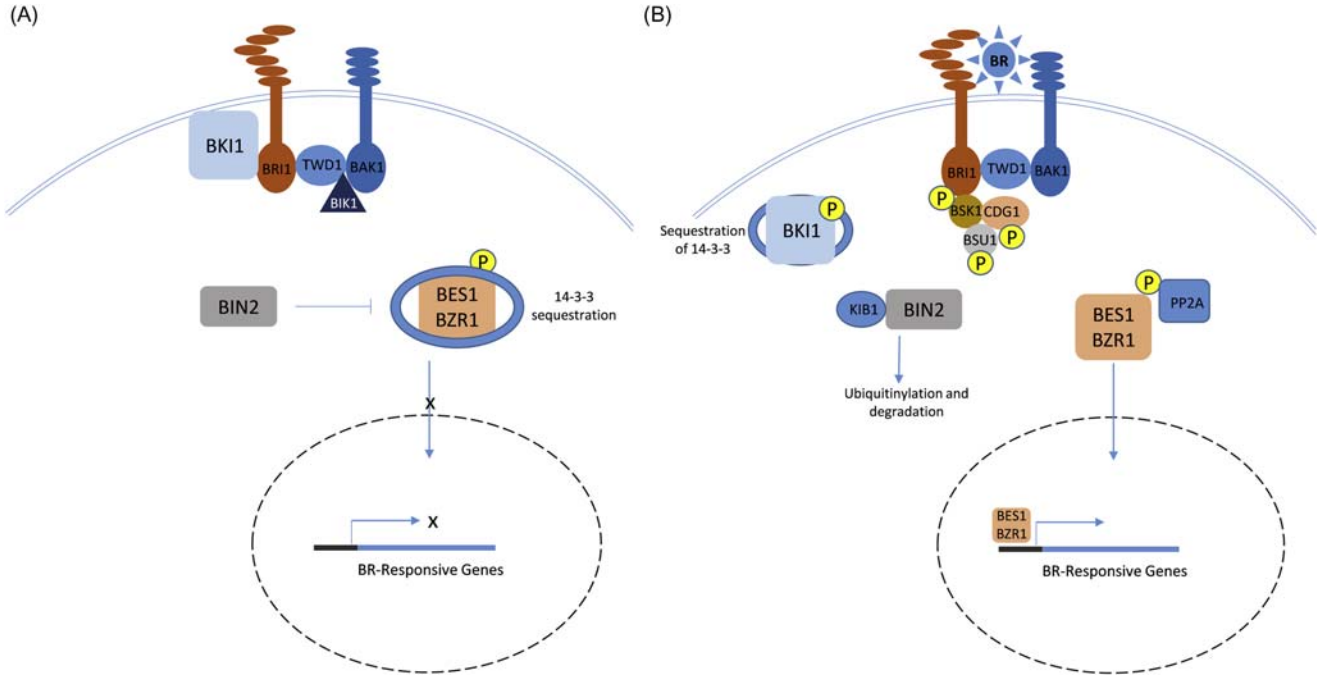

FIGURE 26.2 (A) A schematic view of BR-signaling in plant cell. A BR-regulated transcription factor BES1/BZR1 is the main player in BR-signaling. In absence of BR, it remains phosphorylated and cannot bind to BR responsive gene in the nucleus. 14-3-3 protein sequesters BES1/BZR1 and keeps it phosphorylated. (See Section 26.3 for details.) (B) A schematic view of BR-signaling in plant cell. When BR is available and binds to BR receptors and coreceptors, this phosphorylates several interacting proteins. A protein, BKI1, when phosphorylated, sequesters 14-3-3 protein thus keeping BES1/BZR1 free. PP2A dephosphorylates BES1/BZR1 and later it enters in the nucleus to activates the BR responsive genes. (See Section 26.3 for details.) *Source*: Model adapted from Nolan, T.M., Brennan, B., Yang, M., Chen, J., Zhang, M., Li, Z., et al., 2017. Selective autophagy of BES1 mediated by DSK2 balances plant growth and survival. Dev. Cell 41, 33–46.

TABLE 26.1 List of Proteins That Directly Bind to BES1 and or BZR1 and Regulate BR Responsive Gene Expression or Protein Stability

Gene name	Molecular activity	Binding partner	References
PIFs (PHYTOCHROME-INTERACTING FACTORS)	Regulation of BR responsive genes	BES1/BZR1	De Lucas and Prat (2014)
ARF6/8 (AUXIN RESPONSIVE TRANSCRIPTION FACTORS)	Regulation of growth and defense related genes	PIF4 and BZR1	Oh et al. (2012)
MYB30	Regulation of BR responsive gene induction and ABA response	BES1	Li et al. (2009)
HAT1 (HOMEODOMAIN-LEUCINE ZIPPER PROTEIN 1)	Control of BR responsive genes	BES1	Li et al. (2009)
BRAVO(BRs AT VASCULAR AND ORGANIZING CENTER)	Regulation of cell division in quiescent center	BES1	Vilarrasa-Blasi et al. (2014)
BIM1 (BES1-INTERACTING MYC-LIKE1)	Control of BR responsive genes	BES1	Yin et al. (2005)
TOPLESS and TOPLESS Related (TPR)	Antagonist of ABA signaling	BES1	Espinosa-Ruiz et al. (2017)
DSK2(DOMINANT SUPPRESSOR OF KAR2)	BES1 degradation during stress condition	BES1	Nolan et al. (2017)

(*PIFs*), *BES1-INTERACTING MYC-LIKE1* (*BIM1*), the auxin responsive transcription factors (*ARFs*), *ATBS1/PRE* (*ACTIVATION-TAGGED bri1 SUPPRESSOR1/PACLOBUTRAZOL-RESISTANCE*), *AIFs/IBH1* (*ATBS1-INTERACTING FACTORS/INCREASED LAMINA INCLINATION INTERACTING bHLH1*) *bHLH* transcription factors, etc. (Wang et al., 2009; Zhang et al., 2009; de Lucas and Prat, 2014; Oh et al., 2012; Wang et al., 2012). PIFs are the transcription factors regulated by light and circadian clock. *PIF4* has been validated to interact with BZR1 and forms a heterodimer, which together binds to the G-BOX (CACGTG) element present in the upstream promoter region of BR responsive genes. Similarly *BIM1* interacts with *BES1* and binds together with E-Box (CANNTG) element. The *ARF6* and *ARF8* also shown to interact with *BZR1* and later on bind to ARF motif (TGTCTC) present in the promoter region of BR signaling genes. BZR1, PIFs, and ARFs together activate the downstream basic HELIX LOOP HELIX (bHLH) transcription factors including ATBS1/PRE family protein, which works as antagonist to AIFs/IBH1 proteins and activate proteins, which promotes the cell elongation. Similarly, a number of other transcription factors are involved in BR signaling that are directly regulated by BES1/BZR1. A list of such proteins is included in Table 26.1.

26.5 ROLE OF RD26 IN BR SIGNALING

BR plays an important role in stress acclimation. Plants, being sessile in nature, try to reduce its growth and development to expense the resources in stress management (Claeys and Inze, 2013). BES1 is known to be involved in reduction of plant growth under stress conditions especially when the drought stress is encountered. *RESPONSIVE TO DESICCATION 26 (RD26)* is a NAC transcription factor, which has been shown to play a crucial role in abiotic stress signaling in plants. The expression of *RD26* gene is regulated by *BES1*. *BES1* binds to the BRRE-binding site present in the promoter region of *RD26* gene (Tran et al., 2004). BR response was found to be aberrated in plants overexpressing *RD26* gene. The plants showed a strong phenotype of reduced growth and development. *RD26* is induced under stress conditions thus providing a hint for *RD26* as a connecting link between stress signaling and BR responses (Fujita et al., 2004). Genome-wide analysis of these RD26OX plants revealed the antagonist expression of *RD26* and BR-responsive genes. The output provided a hint that *RD26* might inhibit BR responsive signaling under stress conditions (Chung et al., 2014). BES1 and *RD26* share their target genes, however one is an inducer and another is a repressor or vice versa. These two transcription factors are known to bind to E-Box element and BR-response element (BRRE) respectively. Both *BES1* and *RD26* were experimentally validated to be the interacting partner and together bind on to the DNA elements in an antagonistic fashion, thus neutralizing the gene regulation under normal plant growth conditions (Ye et al., 2017). While under stress conditions the expression of *RD26* increases this result in increased expression of BR-repressed genes and reduced expression of

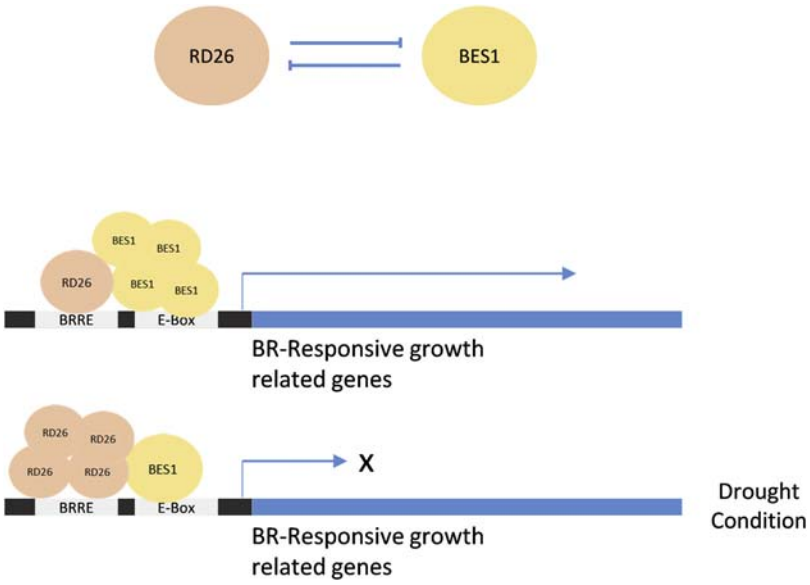

FIGURE 26.3 Antagonistic regulation of RD26 and BES1 during drought stress.RD26 and BES1 are known to compete with each other for binding to BR-responsive gene promoters. When plants experience drought stress, the expression of RD26 is elevated and this inhibits binding of BES1 to BR-responsive genes thus inhibiting their expression. (See Section 26.5 for details.)

BES1 targeted or BR-induced growth related genes. Extensive gene expression analysis of BR-responsive genes and *RD26* target gene under drought stress conditions (with external application of ABA) indicated that *RD26*, *BES1* and their target genes fluctuate in terms of expression to mitigate changing environmental conditions and sustain the plant growth (Fujita et al., 2004) (Fig. 26.3).

26.6 BR MEDIATED DEFENSE SIGNALING

The relationship of BR signaling and plant defense is similar to that of the BR signaling- drought crosstalk (Lozano-Durán and Zipfel, 2015). Plants need to utilize their resources carefully under stress conditions and this sometimes leads to a temporary inhibition of the growth and development while making the plant ready to tolerate the stress. As many BR responsive genes are involved in regulating plant growth and development, they are the first target to be suppressed to reduce the resource utilization and hence the crosstalk between BR-signaling genes and defense related genes needs to be established. Studies on the influence of BR signaling and defense indicated that PAMP triggered immunity or PTI in plants is somehow influenced by BR-receptors or BR-responsive genes. PTI is a defense mechanism in plants, which recognizes the pathogen associated molecular patterns (PAMPs) and activates the downstream defense signaling cascade. PAMPs are the chemical signature moiety derived from the pathogens and recognized by specific receptors present on the plasma membrane. Two main components of the BR signaling cascade, namely BAK1 and BRI1, have been shown to play an important role in PTI (Albrecht et al., 2012; Goddard et al., 2014). BAK1, a coreceptor for BR, also serves as coreceptor for bacterial PAMPs. It has been proved that bacterial flagellin (a PAMP) binds to its receptor FLAGELLIN SENSING 2 (FLS2) together with coreceptor BAK1 and hence it is proposed that bacterial PAMPs and BR compete with each other for BAK1 (Chinchilla et al., 2007). So in the case when the PTI is activated, the BR pathway is inhibited up to a certain extent and vice versa. Although several studies proposed different hypothesis for the mechanism of BAK1 action in PTI, the conclusion remains the same (Albrecht et al., 2012; Belkhadir et al., 2014). PTI in plants activates the ROS signaling, which leads to an oxidative burst in the cell, resulting in several defense related phenotypes like stomatal movement and callose deposition on the cell wall (Ingle et al., 2006; Nürnberger et al., 2004). An elevated BR signaling has been shown to reduce the oxidative burst in the plant cell and made plants more susceptible to the pathogen. For example, the overexpression of a BR biosynthetic gene has been shown to limit the plant's response against a bacterial PAMP, flg22 (Belkhadir et al., 2012). Moreover, the plants overexpressing BRI1 (BR receptor) resulted in similar phenotype indicating the antagonistic relationship between PTI and BR-signaling. A list of different experiments carried out to understand the effect of manipulation of BR-signaling genes on plants' pathogen defense has been summarized in Table 26.2 (also reviewed by Nolan et al., 2017). Another protein, BSK1, involved in BR-signaling, also plays a direct role in PTI via its interaction with FLS2 and positively regulates ROS production in the cell (Shi et al., 2013a,b). Two other members of BR-signaling related genes,

TABLE 26.2 Effect of Genetic Manipulation of BR-Signaling Genes on Disease Susceptibility

Host	Pathogen/PAMP	Genetic manipulation	Phenotype	References
Arabidopsis thaliana	flg22, elf19	BRI1 Overexprssion	Suppression of PTI, reduced ROS production	Belkhadir et al. (2012)
Arabidopsis thaliana	*Pseudomonas syringae* pv. tomatoDC3000	bik1 mutant	Enhanced Susceptibility	Lu et al. (2010)
Arabidopsis thaliana	flg22	bin2	Suppression of PTI, reduced ROS production	Lozano-Durán et al. (2013)
Arabidopsis thaliana	*Thrips tabaci*	bzr1-D mutant	Enhanced tolerance	Miyaji et al. (2014)
Arabidopsis thaliana	*Pseudomonas syringae* pv. tomatoDC3000	JUB1 Overexpression	Enhanced susceptibility	Shahnejat-Bushehri et al. (2016)
Arabidopsis thaliana	*Pseudomonas syringae* pv. tomatoDC3000	bes1 mutant	Enhanced susceptibility	Kang et al. (2015)
Hordeum vulgare	*Fusarium culmorum*	BRI1 mutation in kinase domain	Enhanced tolerance	Ali et al. (2014)
Brassica napus	*Sclerotinia sclerotiorum, Leptosphaeria maculans*	AtDWF4 Overexpression	Enhanced tolerance	Sahni et al. (2016)
Brachypodium distachyon	Necrotrophic fungus	bri1 mutation	Enhanced tolerance	Goddard et al. (2014)

namely *BIN2* and *BZR1*, were also reported to enhance tolerance against pathogen attack when inhibited or mutated (Lozano-Durán et al., 2013; Miyaji et al., 2014). Hence, providing supporting evidence for the hypothesis stating that the suppression of BR signaling decreases the susceptibility against pathogen attack. However this does not hold true for every experiment carried out and for different kinds of pathogen, indicating that the mechanism and interplay of BR-signaling is complex and the response might vary according to experimental setup. A study on *BES1* is one such example where the mutants were shown to behave differently for a fungal and a bacterial pathogen. BES1 was shown to be a potential candidate connecting the MAPK (mitogen-activated protein kinase) signaling pathway of plant defense to BR-signaling (Kang et al., 2015). Another example comes from *Brassica napus*, where the overexpression of a BR biosynthetic gene, *AtDWF4*, leads to enhance disease tolerance against fungal pathogens. While plants from Belkhadir et al. (2012), where bacterial PAMP were used, displayed compromised tolerance.

26.7 BR MEDIATED ROS SIGNALING AND ITS ROLE IN PLANT DEFENSE

ROS are often considered as major determinant of stress and serve as a key player in various signaling pathways in plant cells, entailing in photosynthetic regulation, perception of abiotic or pathogen response and hormonal action, programmed cell death (PCD), and other important growth and developmental pathways (Dat et al., 2000; Mittler, 2002; Mullineaux and Karpinski, 2002; Apel and Hirt, 2004; Khan and Khan, 2017). ROS consist of free radicals corresponding to superoxide ($\cdot O_2^-$) and hydroxyl radicals ($\cdot OH$), and nonfree radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In plants, ROS are mainly generated during the process of respiration, photosynthesis, and N_2 fixation in the chloroplast, mitochondria, peroxisome, cytosol, plasma membrane, and the apoplastic space. Furthermore, generation of ROS in cell wall bound peroxidases takes place in the apoplastic space. While, in the plasma membrane, an NADPH oxidase complex functions as the ROS-producing system (Bolwell, 1999). Suzuki and Mittler (2006) interpreted that ROS, like superoxide (.O_2-), are produced by NADPH oxidases during stress and trigger the downstream stress-response pathways and thereby induce several underlying defense pathways. Besides this, ROS lead to oxidative burst by initiating membrane depolarization and influx of calcium ions inside the cell, which evoke several pathways required for combating both biotic and abiotic stresses. This includes defense related pathways, production of antioxidant enzymes, dehydrins and heat shock proteins, synthesis of pathogenesis-related proteins (Gechev et al., 2006), MAPKs signaling (Apel and Hirt, 2004), etc.

H_2O_2 is one of the most stable ROS having the ability to behave both as an oxidant and as a reductant (Salin, 1991). Being a strong oxidant it may trigger localized oxidative damage leading to disruption of vital metabolic function and can also diffuse. Conversely, to mediate ROS signaling, H_2O_2 plays multiple roles by behaving as a signal, a mediator, and an effector molecule (Levine et al., 1994) and was found to accumulate in response to both biotic and abiotic stresses. Additionally, to reinforce the cell wall, H_2O_2 is utilized by apoplastic peroxidases that catalyze the cross-linking between and polysaccharides and cell wall extension, hampering the pathogen penetration (Passardi et al., 2004). Moreover, being as a secondary messenger in intracellular signaling pathways, it induces expression of genes involved in pathogen response and chilling acclimation (Chen et al., 1993; Prasad et al., 1994). It is also identified as one of the earliest factors involved in the transcriptional activation of defense related genes in Birch (Pellinen et al., 2002). Owing to stresses, sudden and transient increase of H_2O_2 is considered as a general alarm signal for subsequent eliciting the durable form of defenses that are helpful in facilitating protection against all these stresses (Mittler and Berkowitz, 2001). Additionally, H_2O_2 may also contribute in induction of systemic acquired acclimation (SAA), which is raised due to exposure of one part of the plant to high light that renders the unexposed parts resilient to high light (Karpinski et al., 1999). BR-induced H_2O_2 accumulation is caused by increased activity of NADPH oxidase as confirmed by measuring the activity of plasma membrane NADPH oxidase in plants treated with 24-epibrassinolide (EBR), which is a bioactive brassinosteroid derivative (Xia et al., 2009). Additionally, EBR was also shown to be effective in restoring the NADPH oxidase activity of brassinazole (BRZ, a specific inhibitor of BR biosynthesis) treated plants. Similarly exogenously applied H_2O_2 showed a similar pattern to that of EBR (Deng et al., 2016). Studies suggest that apart from BAK1 (for BRI1-associated receptor kinase 1), BIN2 (a GSK3/SHAGGY-like kinase), BSU1 (for BRI1 suppressor 1, a phosphatase) and, three other regulatory genes, RBOH (respiratory burst oxidase homolog), MAPK1 (mitogen-activated protein kinases), and MAPK3, were upregulated upon treatment with EBR but downregulated after BRZ treatment emphasizing their vital role (Xia et al., 2009).

Exogenous application of BR on the leaves of *Niotiana benthamiana* were shown to enhance the plant tolerance against several different pathogens (Nakashita et al., 2003). This is accompanied by accumulation of BR-induced MAPK and RBOHB (an NADPH oxidase B) genes and subsequent ROS burst. Eventually, the end products of the target genes directly take part into the cellular protection. Nevertheless, further studies are warranted to provide genetic evidence for NADPH oxidase's role in BR-induced ROS generation (Deng et al., 2016). This would pave the way to unveil the critical signaling components in BR signal perception and downstream stress responses, and to elucidate the molecular mechanisms involved in the crosstalk between BR and other hormones.

26.8 CONCLUSION

Elucidation of BR signaling pathways is still a challenging task for scientists worldwide. Its multifaceted nature and crosstalk with different growth and developmental pathways in plants make it a complex system to study. As far as stress signaling and BR crosstalk is concerned, a clear role of BR has been established. However, the availability of different model systems and different experimental approaches make it more complicated to untangle the exact pathway. The role of BR signaling in both biotic and abiotic stresses seems to be connected with ROS signaling and potential of an extensive crosstalk between two pathways can be visualized but the BR signaling research is still in its juvenile phase and many more factors are needed to be uncovered to have a clear view of this complex machinery in the plant cell.

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CHAPTER

27

Role and Regulation of Osmolytes and ABA Interaction in Salt and Drought Stress Tolerance

Guddimalli Rajasheker[1], *Gandra Jawahar*[1], *Naravula Jalaja*[2], *Somanaboina Anil Kumar*[1], *Palavalasa Hima Kumari*[1], *Devineni Lakshmi Punita*[1], *Appa Rao Karumanchi*[3], *Palakolanu Sudhakar Reddy*[4], *Polavarapu Rathnagiri*[5], *Nese Sreenivasulu*[6] *and Polavarapu Bilhan Kavi Kishor*[1]

[1]Department of Genetics, Osmania University, Hyderabad, Telangana, India [2]Department of Biotechnology, Vignan University, Vadlamudi, Guntur, India [3]Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, India [4]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India [5]Genomix CARL Pvt. Ltd., Kadapa, Andhra Pradesh, India [6]Grain Quality and Nutrition Center, Plant Breeding Division, International Rice Research Institute, Metro Manila, Philippines

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27.1 INTRODUCTION

Plants are constantly exposed to diverse abiotic stresses like salinity, drought, cold, high temperature, and high light intensity (photooxidative stress) during their life cycle. Such stresses initiate oxidative stress releasing reactive oxygen species (ROS) that comprise free radicals ($O_2^{\bullet -}$, $^1O_2$, $^{\bullet}OH$, $HO_2^{\bullet}$) and nonradical forms (H_2O_2). Production of ROS damages cellular fabric, disrupts metabolism, and causes functional loss of cell organelles. This leads to death of plants in the majority of the cases (Blokhina et al., 2003; Khan and Khan, 2017). To minimize the deleterious effect of ROS, plants develop mechanisms such as scavenging them with antioxidative enzyme systems like ascorbate peroxidase (APX), catalase (CAT), glutathione *S*-transferase, superoxide dismutase (SOD), etc., or quenching them with the help of nonenzymatic molecules such as ascorbic acid, reduced glutathione, α-tocopherol, carotenoids, flavonoids, and osmolytes such proline, glycine betaine (GB), mannitol, and trehalose (Khan and Khan, 2014; Khan et al., 2014, 2015; Per et al., 2018). Several compatible solutes or osmolytes are synthesized in bacteria as well as in plant systems when exposed to abiotic stresses. They protect protein structure stability and also scavenge the ROS as mentioned. Biosynthesis of certain osmolytes like proline and signaling contribute to the redox balance of cells under abiotic stress as well devoid of stress as has been pointed out by Per et al. (2017). However, multiple pathways exist for osmolyte biosynthesis in bacteria as well as in plants and the gene regulation is complex. The synthesis and accumulation of osmolytes is certainly controlled by phytohormones besides many other factors like mineral nutrients (Per et al., 2017). By interacting synergistically with proline and GB metabolism, phytohormones bring about stress tolerance in plants (Iqbal et al., 2014). But, the comprehensive role of hormones in modulating many osmolyte biosynthesis leading to salt and drought stress tolerance is not totally explored. Further, several signaling molecules like nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) play a pivotal role in the regulation of osmolyte biosynthesis (He and He, 2017). Accordingly, it is vital to understand the role of these molecules on osmolyte accumulation and metabolism to resolve the adaptive roles played by plants to avoid abiotic stresses. This review focuses on two such osmolyte molecules, proline and GB, which are well studied and modulated by ABA and other phytohormones and the associated signaling pathways. The regulation of other osmolytes by hormones is not well known, and hence not included here. Myriad functions that are performed by osmolytes during stress, plant growth, and development are also emphasized. This review may provide new insights and opportunities in modulating osmolyte metabolism to impart salt and drought stress tolerance to crop plants, thereby contributing to sustainable agricultural yields in future.

27.2 ABSCISIC ACID-SENSING MECHANISM OF PLANTS AND DOWNSTREAM EVENTS

Abscisic acid (ABA) plays an important role in seed development, dormancy, and acclimation of plants to abiotic stresses. When plants are exposed to salt or drought stresses, rapid de novo synthesis of ABA was observed in leaves. This leads to the closure of stomata, which helps in protecting the plants against evaporation of water (Schwartz and Zeevaart, 2010). Henson (1984) and Mohapatra et al. (1988) reported enhanced levels of ABA in tissues exposed to abiotic stresses. Further, ABA plays a pivotal role during drought stress by modulating many physiological responses that lead to plant adaptations to unfavorable conditions. Exogenous application of ABA can also induce several genes in plants that are not subjected to stress (Mundy and Chua, 1988). This indicates that ABA is associated with functions other than abiotic stress. ABA signaling pathway perceives and transmits the hormone stimulus to activate several of the downstream events in the plants (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). This comprises three protein classes, namely pyrabactin resistance (PYR)/pyrabactin resistance-like (PYL)/regulatory component of ABA receptor (RCAR), which regulate protein phosphatase 2C (PP2C) negatively (Fig. 27.1A), and the positive

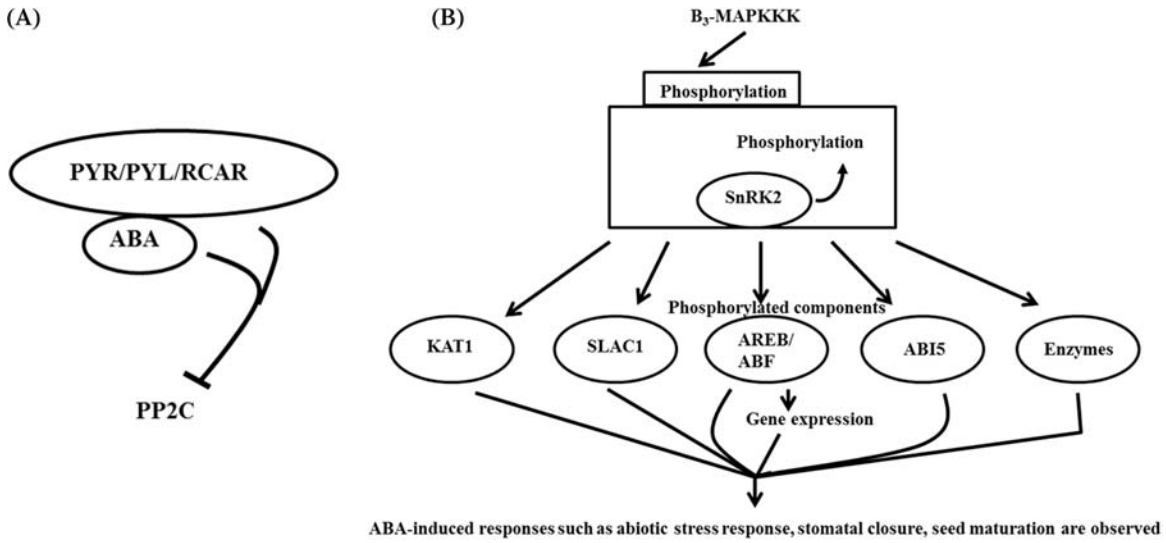

FIGURE 27.1 Abscisic acid signaling module. (A) An abscisic acid signaling module involves the presence of ABA, ABA receptors PYR/PYL/RCAR which bind to ABA and inhibit protein phosphatase 2C (PP2C). (B) PP2C inhibition activates SnRK2s through autophosphorylation. SnRK2 then phosphorylates the downstream targets initiating abiotic stress responses.

regulators sucrose nonfermenting 1–related protein kinase type 2 (SnRK2s) (Fig. 27.1B). When enough concentrations of ABA are not present in the plant systems, SnRK2s are inactivated by PP2Cs (Umezawa et al., 2010). Once ABA binds to PYR/PYL/RCAR receptors, they undergo conformational change that allows the binding of PP2C (Cutler et al., 2010; Seiler et al., 2014). Therefore, ABA-induced inhibition of PP2Cs leads to phosphorylation of SnRK2 and its activation (Boudsocq et al., 2007). It has been found that SnRK2 activity is sensitive to staurosporine, but not to hyperosmolarity or ABA. This infers that SnRK2 activation by phosphorylation is mediated by staurosporine-insensitive kinase. SnRK2s then can phosphorylate downstream proteins like ion channels, NADPH oxidases, and others (Sah et al., 2016). While a Raf-like kinase (B_3-MAPKKK) activates SnRK2, a casein kinase 2 phosphorylates SnRK2's carboxyl-terminal serine residues. This enhances SnRK2-PP2C interaction and thus, ABA can bring about activation of several of the downstream events.

27.3 ROLE OF ABSCISIC ACID IN OSMOLYTE BIOSYNTHESIS

27.3.1 Abscisic Acid–Dependent and –Independent Signaling Pathways and Proline Biosynthesis

Proline accumulation is a primary response to stress and is dependent on de novo synthesis (Verbruggen et al., 1993; Per et al., 2017). Therefore, the upstream signaling cascade must be in place for controlling proline biosynthesis as has been pointed out earlier (Hare et al., 1999). But, the signal transduction events controlling *P5CS1* are not completely known. However, it has been shown that different signaling pathways regulate *P5CS1* during cold and osmotic stress (Yoshiba et al., 1995; Igarashi et al., 2000; Hare et al., 1999). While *AtP5CS1* is strongly ABA-responsive, *AtP5CS2* is moderately responsive (Strizhov et al., 1997; Abrahám et al., 2003). Induction of *AtP5CS* transcript in salt-treated seedlings is associated with the early transcriptional response regulated by ABA signaling, but not observed by the deficiency of ABA biosynthesis in the *aba1 Arabidopsis* mutant (Strizhov et al., 1997). On the other hand, *AtP5CS2* mRNA level is inhibited by cycloheximide. Mutations *abi1* and *axr2* affecting ABA-perception in *Arabidopsis* decrease the accumulation of both *AtP5CS* mRNAs during salt stress. At the same time, ABA signaling functions defined by the *abi2* and *abi3* mutations have no effect on salt induction of the *P5CS* genes (Strizhov et al., 1997). Verslues and Bray (2006) pointed out that low water potential–induced proline accumulation requires ABA levels like those of wild-type levels. Estimations of ABA and proline levels in ABA-insensitive mutants, *abi1-1*, *abi2-1*, *abi3*, *abi4*, and *abi5* revealed that *abi4* had higher accumulation of proline at low water potential, but a reduced response to exogenous ABA. They found out that these responses could be modified by sucrose treatment. Also, while *abi1* had reduced accumulation of proline in response to low water potential and ABA application, *abi1-1* and *abi2-1* had enhanced ABA accumulation. ABA-insensitive mutants are impaired in their response to sugars also

(Gibson, 2005). It is known that sucrose inhibits ABA-induced proline accumulation (Verslues and Bray, 2006). It appears therefore, that ABI4 is connected to ABA and sugar-signaling in the regulation of proline accumulation (Verslues and Bray, 2006). Also, ABA-dependent induction of *P5CS1* expression is prevented by pretreatment with the hormone brassinosteroid (Abrahám et al., 2003). Thus, their analysis suggests the existence of both ABA-dependent and ABA-independent signaling pathways. Further, analysis of the *P5CS1* and *P5CS2* promoters showed the presence of *cis*-acting ABA-responsive elements only in *P5CS1*, but not *P5CS2*. Also, no dehydration-responsive element could be identified in the promoter sequences (Hare et al., 1999). In contrast, *P5CS2* is weakly induced by ABA. It has been discovered that MYB-type of transcription factors PHOSPHATE STARVATION RESPONSE1 (PHR1) and PHR1-LIKE1 (PHL1) bind to P5CS1 regulatory sequences in the first intron, which contains a conserved PHR1-binding site motif (Aleksza et al., 2017). Phosphate starvation of *Arabidopsis* seedlings led to the activation of P5CS1, proline dehydrogenase 2 (ProDH2), as well as an increase in proline content. Such an accumulation was not noticed in the ABA-deficient aba1-3 and ABA-insensitive abi4-1 mutants. They also noticed that ABA is implicated in growth retardation in such nutritional stress. These results point out that proline biosynthesis is modulated by a crosstalk between ABA signaling and regulation of phosphate homeostasis through PHR1- and PHL1-mediated transcriptional activation of the *P5CS1* gene (Aleksza et al., 2017). Factors affecting P5CS and proline dehydrogenase (ProDH), enzymes associated in proline metabolism, are shown in the Table 27.1.

27.3.2 Role of Hormones in the Regulation of P5CS and Proline Synthesis

The results of You et al. (2012) indicated that ornithine δ-aminotransferase (*OAT*) is strongly induced by ABA and indole-3-acetic acid, and is slightly induced by brassinosteroids and jasmonic acid (JA) indicating that *OAT* is responsive to multiple stresses. You et al. (2012) found out that the drought-induced expression of *OsOAT* is contributed by both ABA-dependent and ABA-independent pathways. Auxin upregulates the expression levels of both *P5CS1* and *P5CS2* genes, while cytokinin downregulates *P5CS1*, but enhances the expression of *P5CS2* (Yoshiba et al., 1995; Strizhov et al., 1997; Hare et al., 1999; Abrahám et al., 2003). Induction of *P5CS1* is light-dependent. Also, both ABA and salt stress strongly activate *P5CS1* gene in *Arabidopsis*. At the same time, ABA and salt stress weakly stimulate *P5CS2* gene and downregulate PDH gene expression in light-grown *Arabidopsis* plants (Abrahám et al., 2003). Thus, their experiments proved that proline accumulation is strongly dependent on light, salt stress, and ABA, which is due to the activation of *P5CS1* gene. At the same time, ABA and light-dependent activation of *P5CS1* gene is inhibited in dark-grown plants. Proline accumulation in response to ABA and salt stress is mostly controlled by light-dependent activation of *P5CS1* gene, but is inhibited by brassinosteroid signaling in *Arabidopsis thaliana* (Abrahám et al., 2003). In *Arabidopsis*, dark conditions downregulated *P5CS2*. Induction of *P5CS1* is increased in the ABA-hypersensitive pleiotropic regulatory locus1 (*prl1*) and brassinosteroid-deficient deetiolated 2 (*det2*) mutants. On the other hand, both ABA and salt stress increased the *P5CS2* gene induction only in *det2* mutants. Thus, proline accumulation is certainly controlled by ABA as well as salt stress via *P5CS1* gene. In the *SbP5CS* promoter region, a methyl jasmonate (MeJA)-responsive motif (TGACG-motif) was predicted, inferring that MeJA activates *SbP5CS* expression (Su et al., 2011). Salicylic acid (SA) positively regulated proline metabolism and helped in proline accumulation in several plants (Kanade, 2008; Misra and Saxena, 2009). SA induced proline accumulation might be due to increased P5CR activity and the stress protective effect of SA is perhaps controlled due to proline accumulation. SA treatment enhanced the accumulation of proline in barley shoots but not in roots (El-Tayeb, 2005). SA signaling is also associated with the expression of *P5CS2* after infection with avirulent *Pseudomonas* (Fabro et al., 2004). Indeed, SA-responsive element was noticed in the promoter region of *SbP5CS* (Su et al., 2011). Likewise, a gibberellin (GA)-responsive element, GARE, was predicted in the upstream region of *SbP5CS* gene. But, no experiment was conducted to show that GA activates *P5CS* expression. Brassinosteroids inhibited the expression of both *P5CS1* and *P5CS2*, and they could not stimulate *ProDH* expression (Abrahám et al., 2003). Thus, regulation of *P5CS1* appears to be rather complex. During stress conditions, other secondary messengers or hormones like NO and ROS such as hydrogen peroxide (H_2O_2) are known to mediate ABA signals and affect proline metabolism as has been shown by Desikan et al. (2002), Neill et al. (2008), and Yang et al. (2009). NO has been shown to be involved in the ABA-induced proline accumulation in wheat seedlings (Hai-Hua et al., 2004). While NO treatment enhanced the copper-induced proline accumulation (Zhang et al., 2008), the same was not noticed in *Brassica rapa* under salt stress conditions (Lopez-Carrion et al., 2008). It increased the activity of P5CS1, but downregulated the activity of ProDH in wheat (Hai-Hua et al., 2004). NO stimulated *P5CS1* expression in *Arabidopsis*, but inhibited the expression of *ProDH1* (Zhao et al., 2009). They also noticed that nitrate reductase rather than NO synthase

TABLE 27.1 Factors Affecting P5CS1, ProDH, and Proline Accumulation in Plants

Factor	Gene/expression	References
Phosphate starvation	*ProDH2 upregulation in A. thaliana*	Aleksza et al. (2017)
Light	*P5CS1 upregulation in A. thaliana*	Feng et al. (2016)
Heat/temperature	*P5CS1 upregulation in A. thaliana and Prunus persica*	Shin et al. (2016), Wei-Tao et al. (2011)
Hydrogen peroxide	*P5CS1 upregulation in A. thaliana*	Ben Rejeb et al. (2015)
Diphenylene iodonium	*P5CS1 downregulation in A. thaliana*	Ben Rejeb et al. (2015)
Menadione sodium bisulfite	*P5CS1 upregulation in A. thaliana*	Jiménez-Arias et al. (2015)
Salt stress	*ProT upregulation in Kosteletzkya virginica*	Wang et al. (2015)
Salt stress	*OAT upregulation in Kosteletzkya virginica*	Wang et al. (2015)
Phosphatidylinositol 3-kinase	*ProDH1 upregulation in A. thaliana*	Leprince et al. (2015)
H2S	*Downregulation of ProDH1 in Musa paradisica*	Luo et al. (2015)
Salt stress	*ProDH1 downregulation Saccharomyces cerevisiae and Helianthus tuberosus*	Huang et al. (2013)
Methyl jasmonate	*P5CS2 upregulation in Sorghum bicolor*	Su et al. (2011)
Nitric oxide	*P5CS1 upregulation in A. thaliana*	Zhao et al. (2009)
Carbon monoxide (CO)	*P5CS1 upregulation in Triticum aestivum*	Yuan et al. (2009)
Phospholipase C	*P5CS1 upregulation in A. thaliana*	Parre et al. (2007)
Phospholipase D	*P5CS1 downregulation under nonstress conditions in A. thaliana*	Thiery et al. (2004)
Salicylic acid	*P5CS2 upregulation in A. thaliana*	Fabro et al. (2004)
Pathogens	*P5CS2 upregulation in A. thaliana*	Fabro et al. (2004)
Rehydration	*ProDH1 upregulation in A. thaliana*	Satoh et al. (2002)
Brassinosteroids	*P5CS2 downregulation in A. thaliana*	Abrahám et al. (2003)
Metal	*P5CS1 upregulation/overexpression in Chlamydomonas reinhardtii*	Siripornadulsil et al. (2002)
Light	*P5CS1 upregulation in A. thaliana*	Hayashi et al. (2000)
Dark conditions	*ProDH1 upregulation in A. thaliana*	Hayashi et al. (2000)
Cold/Low temperature	*P5CS1 upregulation in Oryza sativa*	Igarashi et al. (1997)
Abscisic acid	*P5CS1 upregulation in A. thaliana*	Strizhov et al. (1997)
Indole-3-acetic acid	*P5CS1 upregulation in A. thaliana*	Strizhov et al. (1997)
Salt stress	*ProDH1 downregulation Saccharomyces cerevisiae and Helianthus tuberosus*	Peng et al. (1996)
Water stress	*ProDH1 downregulation in A. thaliana*	Kiyosue et al. (1996)
Salt stress	*P5CS1 upregulation in A. thaliana*	Yoshiba et al. (1995)
Drought	*P5CS1 upregulation in A. thaliana*	Savouré et al. (1995)
Low nitrogen	*P5CS1 upregulation in Vigna aconitifolia*	Delauney et al. (1993)

is responsible for NO-mediated regulation of proline accumulation and also freezing tolerance.

Experiments to unravel the upstream signaling pathway of *P5CS* gene resulted in the identification of phospholipase D, involved in water stress response and ABA signal transduction (Hallouin et al., 2002). But, phospholipase D downregulated *P5CS1* activity under normal and also abiotic stress conditions (Thiery et al., 2004). It is known that calcium plays a pivotal role in proline accumulation under salt stress conditions. But, $CaCl_2$ and phospholipase D treatment resulted in the upregulation of *P5CS1* gene. They suggested that calcium regulates phospholipase D as a downstream signal messenger. However, there are many gaps in our understanding of the activation of some of the enzymes involved in proline metabolism

and signaling pathways. Also, how some of these hormones regulated*P5CS* and *ProDH* expressions at the molecular level and controlled the fine tuning of proline accumulation or degradation during stress, plant growth, and development is still to be discovered and perhaps pivotal for a comprehensive understanding of this osmolyte. Nevertheless, these experiments revealed a link between proline, hormonal signal, and downstream stress responses in plants.

27.4 REGULATION OF PROLINE DEHYDROGENASE

Dark conditions upregulated *ProDH* gene in shoots. *ProDH* was inhibited by both ABA and salt stress in shoots and roots of light-grown plants unlike that of *P5CS1*. In *Arabidopsis*, while *prl1* mutation reduced the basal level of *PDH* gene expression, the *det2* mutation enhanced the inhibition of *PDH* by ABA (Abrahám et al., 2003). Thus, it appears that *PDH* expression is also regulated by ABA. In plants, the receptors histidine kinases (AHKs) and elements of the two-component system have been proposed to function in water stress responses by regulating various stress-responsive genes. But, not much information is available concerning AHK phosphorelay-mediated downstream signaling (Veerabagu et al., 2014). ProDH is associated with the catabolic process and converts proline to pyrroline-5-carboxylate (P5C). It is known that ProDH1 expression undergoes extensive regulation by exogenous and endogenous signals, but the mechanism of its transcriptional and posttranscriptional regulation is not known completely. Accumulation of ProDH1 is controlled by the ACTCAT *cis*-acting element (ACT-box) in the ProDH1 promoter (Satoh et al., 2002) via basic region leucine zipper (bZIP) transcriptional activators from the S1-group (Satoh et al., 2004). Analysis of ProDH1 regulation revealed that the S1-group members of TFs like bZIP1 and bZIP53 bind to the promoter of ProDH (Dietrich et al., 2011). But, not much is known about the functioning of C-group bZIP factors except perhaps AtbZIP63, which is a sensitive integrator of transient ABA and glucose signals under water stress (Matiolli et al., 2011). Veerabagu et al. (2014) have shown that the *Arabidopsis* type-B response regulator 18 (ARR18) acts as an osmotic stress response regulator in the seeds of *Arabidopsis*. This regulator affects the activity of the *ProDH1* promoter, controlled by C-group bZIP transcription factors. They showed that ARR18 interaction negatively interferes with the bZIP63 on the *ProDH1* promoter. Thus, regulation of ProDH via response regulators appears to be crucial for osmotic stress tolerance. However, such response regulators have not yet been discovered for other genes involved in the biosynthesis of other osmolytes.

27.4.1 Glycine Betaine Biosynthesis and Its Modulation by Abscisic Acid

GB interacts with plant hormones including ABA. Drought stress induced ABA accumulation was noticed first in corn, followed by GB accumulation (Zhang et al., 2012a,b). Thus, ABA and GB are positively associated with stress tolerance in plants. Both in wheat and pears, water stress has increased GB accumulation (Nayyar and Walia, 2004; Gao et al., 2004b). Zhang et al. (2012a,b) noticed enhanced GB accumulation with the application of ABA and also leaf relative water content and shoot dry matter production in two maize cultivars during water stress. They concluded that endogenous ABA is involved in modulating GB accumulation. Kurepin et al. (2015) reported upregulation of the genes associated with the GB biosynthetic pathway by increases in ABA and SA contents. An interplay between these hormones and GB appeared necessary for protection of photosynthesis under abiotic stress conditions. Synergistic effects of ABA and GB have been shown to protect photosynthetic apparatus in the cold acclimation process in higher plants (Kurepin et al., 2015). Transgenic plants overexpressing GB biosynthetic pathway genes produced better biomass in comparison with untransformed plants under stress (Hayashi et al., 1998). But, many researchers (Kurepin et al., 2013; Hüner et al., 2014) are of the opinion that GB in nonstressed plants can modify the production of endogenous phytohormones (ABA, ethylene, SA) associated with plant stress responses. It is not only drought stress conditions that increased the betaine aldehyde dehydrogenase (BADH) mRNA levels, even exogenously supplied ABA upregulated the BADH mRNA in leaves and roots of barley (Ishitani et al., 1995; Jagendorf and Takabe, 2001). Besides ABA and SA, JA also enhanced the GB accumulation in higher plants (Jagendorf and Takabe, 2001; Gao et al., 2004a). This implied that GB biosynthesis is under the hormonal control like that of proline. Taken together, it is suggested that a close interaction and synergistic effect of ABA and GB are necessary for effective acclimation of freezing and abiotic stress tolerance in plants. The above results also indicated that the hormone ABA transduces the signal for the biosynthesis of GB. But, how the signal is transduced and what are the different components associated in the pathway are not known. While proline biosynthesis is mediated by both ABA-dependent and ABA-independent signaling pathways (Hare et al., 1999), in contrast, GB biosynthesis appeared upregulated directly by ABA.

But, not much is known about the regulation of the genes associated with the biosynthesis of other osmolytes like proline derivatives, GB derivatives, trehalose, and sugar alcohols by ABA and other hormones.

27.5 SIGNALING MOLECULES AND OSMOLYTE SYNTHESIS

Environmental stresses result in the production of signaling molecules like NO, CO, and H_2S in plants (He and He, 2017). However, the molecular mechanisms associated with the induction of osmolyte synthesis are not totally known. Evidence exists that NO induces one of the important osmolytes, proline, in wheat (Hai-Hua et al., 2004), Chinese cabbage (Lopez-Carrion et al., 2008), and rye grass (Liu et al., 2010) by upregulating *P5CS* and downregulating *ProDH* genes. Ke et al. (2014) reported that NO is associated with salt tolerance by regulating proline metabolism in tobacco, thus indicating the importance of NO in proline synthesis during abiotic stress. Yuan et al. (2009), Zhang et al. (2012a,b) reported CO stimulated P5CS and suppression of ProDH in wheat and *Cassia obtusifolia* seedlings as well as salt stress alleviation. Luo et al. (2015) and Chen et al. (2016) reported chilling injury and drought stress alleviation respectively with H_2S treatment. Further, Tian et al. (2016) reported H_2S and proline ameliorated metal (Cd) stress in foxtail millet inferring a complex regulatory mechanism for proline synthesis and its relation to abiotic stress alleviation. However, the molecular mechanisms underlying activation of the biosynthetic pathway genes of proline metabolism and also the effects of these signal molecules on the biosynthesis of other osmolytes have not been understood so far.

27.6 FUNCTIONS OF OSMOLYTES DURING ABIOTIC STRESS

27.6.1 Osmolyte Accumulation and Osmotic Adjustment During Stress

Compatible solute accumulation, known for its osmotic adjustment, has long been recognized (Brown and Simpson, 1972; Borowitzka and Brown, 1974). Further, a correlation has also been found between the quantity of compatible solute and stress tolerance levels in plants (Storey and Jones, 1977; Flowers and Hall, 1978; Kishor et al., 1995). Amino acids play a vital role in protein biosynthesis. However, certain amino acids like proline accumulate under stressful environment and impart stress tolerance by maintaining cell turgor or osmotic balance, stabilizing membranes thereby preventing electrolyte leakage (Szabados and Savoure, 2010). When proline and GB were supplied exogenously, plants displayed salt stress tolerance. Both of them mediate upregulation of genes associated with antioxidant defense and glyoxalase systems and thus protect seedlings of rice from salt-induced oxidative damage (Hasanuzzaman et al., 2014). A large number of plants were genetically modified with the biosynthetic pathway genes associated with proline (reviewed in Kumar et al., 2014) and GB (reviewed in Khan et al., 2009), which displayed higher tolerance to salt stress. Higher accumulation of proline and GB in leaves of transgenic lines has been noticed in comparison with untransformed plants. This indicated that introduced genes are properly integrated and expressed in the host genome. It appeared that accumulation in leaves of stressed plants is regulated, at least in part, via the changes in the expression of biosynthetic pathway genes. But, the signals that provoke these changes in gene expressions have not been clearly identified in higher plants. It is not clear how osmolytes like proline and GB affect the cell turgor in plants and what signals are associated with them. It seems that in yeast, Synthetic Lethal of *N*-end rule 1 (Sln1) osmosensor histidine kinase monitors the changes in turgor pressure as demonstrated by Saito and Tatebayashi (2004). It has been shown that reduction in turgor pressure caused by hyperosmotic stress activates the mitogen activated protein kinase high osmolarity glycerol 1 (HOG1) through SLN1 branch of the glycerol pathway (Reiser et al., 2003). In higher plants, activity of the plant histidine kinase Cre1 (cytokinin response 1) is regulated by changes in turgor pressure like that of Sln1 in yeast. It is known that Cre1 complemented the deficient Hog1 response in Sln1 mutant yeast cells (Reiser et al., 2003). These authors proposed that Cre1 has dual functions in plants acting both as a cytokinin receptor and also as an osmosensor. In addition to the possible role of osmolytes in osmotic adjustment and in stabilizing membranes upon salt/water stress, they play several other important regulatory functions in stressed plants (Lokhande and Suprasanna, 2012). Sugars not only sustained the growth of sink tissues, but also affected sugar-sensing systems that regulated the expression, either positively or negatively, of a variety of genes involved in photosynthesis, respiration, and the synthesis and degradation of starch and sucrose (Hare et al., 1998). Accumulation of sugar alcohols such as mannitol, sorbitol, pinitol, and others might serve dual functions: facilitating osmotic adjustment and supporting redox control (Tarczynski et al., 1993; Shen et al., 1999). Similarly, other osmolytes exhibited multiple functions in plants (Bohnert and Shen, 1998; Shen et al., 1999; Elbein et al., 2003; Livingston et al., 2009).

27.6.2 Osmolytes and Protection of Photosynthetic Machinery During Abiotic Stress

Light including ultraviolet light-B (UV-B) radiation stress affects plant productivity drastically by inhibiting photosynthetic activity. Therefore, plants accumulate proline besides several other antioxidative and flavonoid molecules (Saradhi et al., 1995). As shown by Arora and Saradhi (2002), proline might protect the plants by scavenging the singlet oxygen or free radicals generated during light or UV-B radiation stress. A combination of NaCl and UV-B radiation showed an additive effect on most of the parameters studied in barley. UV-B treatment decreased the chlorophyll/carotenoid ratio in barley seedlings and also photochemical efficiency of PSII (Fedina et al., 2003). Fedina et al. (2003) pointed out that NaCl preexposure decreased H_2O_2 generation and alleviated the inhibitory effect of UV-B on PSII. Proline accumulated during NaCl preexposure might be one of the reasons for the observed tolerance of barley seedlings to UV-B radiation. Pretreatment of *Senedesmus* (algal member) with proline decreased lipid peroxidation and UV-B induced malondialdehyde (MDA) generation (Tripathi and Gaur, 2004). Thus far, the exact mechanism of light-dependent stimulation of proline biosynthetic pathway genes and proline accumulation are not known. It was shown by Uchida et al. (2002) that H_2O_2 pretreatment induces increased ROS scavenging enzyme activities and enhanced expression of P5CS, sucrose phosphate synthase, and the small heat shock protein 26 in rice. These experiments indicated that NO and H_2O_2 act as signaling molecules that modulate both salt and heat stress tolerance by regulating the gene expression associated with it. Likewise, transgenic *Arabidopsis* leaves that expressed choline oxidase (*COD*) gene for the accumulation of GB displayed enhanced levels of H_2O_2 in comparison with untransformed plants (Alia et al., 1999). Further, activities of the enzymes such as APX and CAT were higher in transgenics than in the wild-type plants. These results indicated that the H_2O_2 generated by overexpression of *COD* gene might have stimulated the expression of scavenging enzymes (Sakamoto and Murata, 2002). Thus, H_2O_2 generated during stress might play as a signal transducer in stimulating proline biosynthetic pathway gene *P5CS* as well as ROS scavenging enzymes under the influence of *COD*.

Photosynthesis is a major target of high-temperature as well as other abiotic stresses in plants and PSII is the most temperature-sensitive component. It is known that GB enhances the tolerance of photosynthetic machinery to photoinhibition (Sakamoto and Murata, 2002). Gorham (1995) indicated that GB protects enzymes and protein complexes against heat-induced inactivation. Photoinhibition involves photoinduced damage to PSII and the light-dependent repair of PSII complex (Aro et al., 1993). Abiotic stresses especially high temperature impair the activity of Rubisco enzyme also in several species (Haldimann and Feller, 2005). While D1 protein, one of the constituents of PSII, is damaged, steps are taken immediately by the plant to ensure the removal and replacement of the damaged D1 protein. GB plays a dual function of repairing the PSII complex during photoinhibition as well as protecting the complex proteins (Allakhverdiev et al., 2007). Alia et al. (1998) reported protection of transgenic *Arabidopsis* lines overexpressing GB biosynthetic pathway genes. This could be because of the protection of Rubisco activase by GB, which was later supported by Yang et al. (2005) in transgenic tobacco. Alia et al. (1999) found out that *codA* overexpression in *Arabidopsis* resulted in light stress tolerance. They also found that GB had no effect on photodamage, but participated in repair of the PSII complex. Holmström et al. (2000) revealed that overexpression of choline dehydrogenase (CDH) in tobacco resulted in the removal of photodamaged D1 protein and reconstitution of the functional PSII complex. It is believed that increased CO_2 assimilation rate in transgenic lines is associated with the Rubisco activase-mediated activation of Rubisco by GB (Yang et al., 2005). Proline has been found to reduce the inhibitory effects of NaCl on the activity of enzymes like Rubisco in vitro in *Tamarix jordanis* (Solomon et al., 1994). Papageorgiou and Murata (1995) showed that osmolytes prevented dissociation of the oxygen-evolving complex of photosystems II. Yang et al. (2005) also reported improved thermostability of the oxygen-evolving complex and the reaction center of PSII (Yang et al., 2007) when GB biosynthetic pathway genes are overexpressed. However, the mechanistic explanation of how exactly osmolytes, including GB, protect PSII under stress is not clear to date.

27.6.3 Osmolyte Accumulation and Oxidative Stress

Chloroplasts and mitochondria are the two powerhouses of plant systems. The redox state of these two cell organelles is maintained by a delicate balance between energy production and consumption. These organelles need to avoid always the excess production of ROS, especially under abiotic stress conditions. While optimal levels of ROS are useful for signal transduction and several developmental activities, excess amounts cause damage to the nucleic acids, oxidation of proteins and lipids, and degradation of chlorophyll molecules (Davies, 1987; Imlay and Linn, 1988). Plants need to utilize the redox cues that are generated both in chloroplasts and mitochondria not only for maintaining metabolic fluxes, but also for coping with

environmental changes *via* a complex network (Suzuki et al., 2012). The degradation pathway of proline is downregulated during osmotic stress, allowing free proline to accumulate. Miller et al. (2009) showed that overexpression of *MsProDH* in tobacco and *Arabidopsis* or impairment of P5C oxidation in the *Arabidopsis p5cdh* mutant did not change the cellular proline to P5C ratio under ambient and osmotic stress conditions. This reveals that excess P5C is reduced to proline in a mitochondrial-cytosolic cycle. This cycle involves conversion of proline by ProDH to P5C and back to proline by P5CR enzyme and is known to exist in animal cells (P5C-proline cycle). Miller et al. (2009) demonstrated that when an excess of exogenous L-proline is provided, it generates mitochondrial ROS by delivering electrons to O_2. This was demonstrated by them using mitochondria specific MitoSox staining of superoxide ions. When there is a lack of P5CDH enzyme activity, it has led to higher ROS production in the presence of excess proline (Miller et al., 2009). It appears therefore, balancing not only chloroplastic but also mitochondrial ROS production during enhanced proline oxidation is critical for avoiding proline related toxic effects. To avoid the generation of ROS production by P5C-proline cycling, plants must oxidize P5C back to glutamate by P5CDH.

Regulation of ROS is coordinated by both enzymatic and nonenzymatic mechanisms. Further, exogenous application of proline or genetic manipulation of its synthesis or degradation has amply demonstrated its role in plant responses to abiotic stresses in different species like tobacco, sugarcane, grapevine, and sorghum (Smirnoff and Cumbes, 1989; Okuma et al., 2004; Molinari et al., 2007; Ozden et al., 2009; Reddy et al., 2015). Proline has enhanced the primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photoinhibitory damage (Alia and Saradhi, 1991). They suggested that proline protects the components involved in water oxidation capacity by reducing the production of free radicals and/or scavenging the free radicals thereby reducing thylakoid lipid peroxidation. Alia et al. (1997) also proposed that proline produced a considerable reduction in the lipid peroxidation-linked formation of both conjugated dienes and MDA in the thylakoids during exposure to strong light. They demonstrated that proline is involved in reducing the photodamage in the thylakoid membranes by scavenging and/or reducing the production of singlet oxygen. Alia et al. (2001) utilized spin trapping electron paramagnetic resonance (EPR) spectroscopy for analyzing the singlet quenching action of proline. Their results show that proline is very effective in reducing the production of singlet oxygen ($^1O_2$). Quenching of $^1O_2$ by proline seems to be based on its capability to form a charge-transfer complex due to low ionization potential. Proline is also a scavenger of hydroxyl radicals ($OH^{\cdot}$) as shown by Smirnoff and Cumbes (1989). But, proline does not interact with superoxide radicals. Kaul et al. (2008) showed the free radical scavenging potential of L-proline using in vitro assay system. However, it is difficult to explain the exact mechanism of quenching of $^1O_2$ or $OH^{\cdot}$ by proline. Overall, it appears that proline can stabilize proteins, DNA, as well as membranes under stress conditions as has also been pointed out by Matysik et al. (2002). High proline producing genotypes of niger (*Guizotia abyssinica*) exhibited higher antioxidative enzymes compared with low proline producing lines (Sarvesh et al., 1996). Exogenous supply of proline alleviated the oxidative stress and increased the vase life of *Rosa hybrida* flowers (Kumar et al., 2010). This increase in vase life coincided with higher levels of endogenous proline, lower levels of superoxide radicals, and higher activity of PDH in proline treated flowers. Exogenous application of proline has also been shown to associate with antioxidative enzyme activities (Hoque et al., 2007a,b). Proline ameliorated the enzymatic inactivation of APX and peroxidase, while SOD and CAT activities were reduced in grapevine (Ozden et al., 2009). In transgenic sugarcane overexpressing *P5CS*, a negative correlation between proline and lipid peroxidation were observed. This suggested that proline might protect against osmotic stress by increasing antioxidant systems. De Campos et al. (2011) demonstrated that transgenic citrumelo plants were able to cope with water deficit better than untransformed controls. Since these transgenics expressing *VignaP5CSF129A* are able produce high endogenous proline levels, proline must have contributed to gas exchange parameters and elevated levels of antioxidative enzymes (APX, SOD) but not CAT, and thereby ameliorated the deleterious effects of drought-induced oxidative stress. Transgenic *Sorghum bicolor* plants overexpressing *P5CSF129A* also displayed higher proline and higher antioxidative enzyme activities under salt stress (Reddy et al., 2015). Kaushal et al. (2011) demonstrated that proline has induced heat tolerance in *Cicer arietinum* plants by protecting vital enzymes of carbon and antioxidative metabolism. Posmyk and Janas (2007) noticed a positive correlation between endogenous levels of proline content in seeds of *Vigna radiata* and germination upon exposure to chilling stress. When seeds of *V. radiata* were pretreated with proline, it had a stimulatory effect on germination. This increase in seed germination by exogenously supplied proline under chilling temperatures is attributed to its potential to stabilize cell membrane by quenching both $^1O_2$ and $OH^{\cdot}$. Heat stress induced H_2O_2 in *Saccharum* species, but pretreatment with proline and GB has substantially reduced the H_2O_2 production, improved the accumulation of soluble sugars, and protected the developing tissues from heat stress effects in sprouting sugarcane buds (Rasheed

et al., 2011). Ben Rejeb et al. (2015) investigated the role of NADPH oxidases, respiratory burst oxidase homologues (Rboh) in the induction of proline accumulation under NaCl and mannitol stress conditions. Both salt and mannitol stresses have increased H_2O_2 accompanied by accumulation of proline. They also found out that dimethylthiourea (a scavenger of H_2O_2) and diphenylene iodonium (an inhibitor of H_2O_2 production by NADPH oxidase) inhibited P5CS activity and proline accumulation under these stresses. Supporting this phenomenon, evidence was also presented in *Arabidopsis thaliana* knockout mutants lacking either AtRboHD or AtRbohF. Wild-type plants accumulated more proline than these mutants (Ben Rejeb et al., 2015). These results suggest that Rbohs contribute to H_2O_2 production in response to salt and mannitol stresses and help in proline accumulation in *Arabidopsis*.

Some of the sugars like trehalose (a nonreducing disaccharide) also protect the plants against oxygen radicals. Cell lines defective in trehalose synthesis were more sensitive to oxygen radicals than the wild-type indicating that trehalose protected the plants against oxidative stress. Oxygen radicals damage the amino acids in cellular proteins, but trehalose in the cells prevented this damage indicating that trehalose acts as a free radical scavenger. Hincha et al. (2002) also pointed out that when trehalose is present in high concentrations in cells, plants show resistance to heat, dehydration, and oxygen stress. Galactose as well as mannitol protected the cells, but not sucrose since sucrose does not have the ability to quench oxygen radicals (Benaroudj et al., 2001). Depending upon the species, many plants display high plasticity to accumulate various kinds of sugars (levels may vary in cells depending on the stage of the growth, nutritional status, and environmental conditions prevailing at that time) like raffinose series (Gala-1-6-sucrose and higher), stachyose, and other sucrose oligosaccharides that give protection against different stress conditions. While mannitol may function to shield susceptible thiol-regulated enzymes (such as phosphoribulokinase) from inactivation by hydroxyl radicals in plants, GB is not effective as a hydroxy radical scavenger (Shen et al., 1997; Smirnoff and Cumbes, 1989). Thus, several lines of research clearly indicate the ROS scavenging functions of osmolytes, but the exact molecular mechanisms are not yet completely known.

27.6.4 Osmolytes and Amelioration of NaCl- and Metal-Induced K^+ Efflux Under Stress

Potassium (K^+) homeostasis plays a central role during salt stress tolerance in the plant systems (Tester and Davenport, 2003). When plants are exposed to NaCl stress, a massive efflux of K^+ from plant cells is observed (Shabala et al., 2003; Chen et al., 2005). Prevention or mitigation of K^+ efflux is well correlated to salt stress tolerance in barley (Carden et al., 2003; Chen et al., 2005). It was not known until recently that compatible solutes are implicated with the K^+ transport under salt stress. Cuin and Shabala (2005) hypothesized that osmoprotectants may maintain cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the barley cells. They showed that either proline or GB at a concentration of 0.5–5 mM, when supplied exogenously, instantaneously reduced the NaCl-induced K^+ efflux from barley roots in a dose dependent manner. Proline at 5-mM concentrations reduced the hydroxyl-radical induced K^+ efflux in barley (Cuin and Shabala, 2007a). They also measured membrane potentials in addition to K^+ and Na^+ concentrations, which are consistent with the concept that cytosolic K^+ homeostasis is maintained by proline by preventing NaCl-induced leakage of K^+ from the cells. Proline may possibly control this through the increased activity of H^+-ATPase, controlling voltage-dependent outward-rectifying K^+ channels and creating the electrochemical gradient that is essential for ion transport processes (Cuin and Shabala, 2005). Thus, evidence has been provided for the first time for the regulation of ion fluxes across the plasma membrane by addition of proline/osmolytes. Cuin and Shabala (2007b) further showed that 21 out of 26 amino acids tested caused a significant mitigation of the NaCl-induced K^+ efflux. Surprisingly, both valine and ornithine prevented the NaCl-induced K^+ efflux significantly along with proline and maintained K^+ homeostasis. They pointed out that physiologically relevant concentrations of amino acids might contribute to salt stress adaptation by regulating K^+ transport across the plasma membrane. This might perhaps help the plants to maintain optimal K^+/Na^+ ratio, which is vital during salt stress. But, the exact mechanism underlying proline prevented K^+ efflux under stress is not known. Still though it is thought that free radicals can mediate this. Heavy metals such as Cu^{2+} when added to lower plants (algal members) also cause leakage of K^+ from the cells. Whether proline or other osmolytes are associated with Cu^{2+}/metal-induced K^+ leakage prevention in metal tolerant plants is not known.

27.6.5 Osmolytes and Their Metal Chelation Properties During Metal Stress

Several crop plants (*Cajanus cajan*, *Vigna mungo*, *Triticum aestivum*) accumulate proline in response to heavy metal stress (Alia and Saradhi, 1991; Bassi and Sharma, 1993). In seedlings of *Oryza sativa*, exogenous

supply of proline reduced the copper uptake (Chen et al., 2004). They noticed that proline supplement accompanied by Cu^{2+} exposure induce a barrier of Cu^{2+} influx and efflux in rice roots. It appeared that excess Cu^{2+} leads to inadequate proline in rice roots and results in the malfunction of copper transport barrier. An increase up to >20-fold in the proline content was noticed in leaves of metal nontolerant *Silene vulgaris* (Schat et al., 1997). The shoot proline content is higher than that in roots in *Silene*. On the other hand, root proline levels increased in *Lactuca sativa* with an increase in cadmium concentration (Costa and Morel, 1994). Not only nonmetal-tolerant plants, but also several metal-tolerant species like *Armeria maritima*, *Deschampsia cespitosa*, and *Silene vulgaris* have been reported to contain substantially higher constitutive proline levels when compared with nontolerant relatives (Farago and Mullen, 1979; Smirnoff and Stewart, 1987; Schat et al., 1997). But *A. maritima* plants, when grown in a noncopper site did not exhibit higher proline content (Farago and Mullen, 1979). Proline also accumulated in lower plants like algal members (*Anacystis*, *Chlorella*, *Scendesmus*, etc.) when exposed to heavy metal stress (Wu et al., 1995, 1998; Tripathi and Gaur, 2004). Thus, a large body of information existed with regard to proline accumulation under metal stress. Costa and Morel (1994) suggested that inhibition of proline oxidation is the reason for higher root proline levels under metal stress. Chen et al. (2001) pointed out that increased P5CR or OAT activities are responsible for higher proline accumulation in rice. Since water balance is disturbed under heavy metal stress, it is reasonable to speculate an increased proline synthesis and accumulation (Barceló and Poschenrieder, 1990). Chen et al. (2001) also suggested ABA mediated Cu-induced proline accumulation in rice leaves. Later, it has been reported that copper-induced proline synthesis in the green algal member *Chlamydomonas reinhardtii* is associated with NO generation. They further investigated the effect of Cu^{2+} and NO on the activity and transcript amount of *P5CS*, and observed that application of sodium nitroprusside (NO specific donor) is able to stimulate the P5CS activity in the Cu-treated algae (Zhang et al., 2008). Their results indicated that Cu-responsive proline synthesis is related to NO generation in *C. reinhardtii*.

Based on the existing information, the following speculations can be drawn for the possible metal stress mitigation by proline/osmolytes in plants. Proline may be acting as a metal chelator as demonstrated by Farago and Mullen (1979). They showed that Cu^{2+} in the roots of *A. maritima* existed as Cu-proline complex. Proline protected glucose-6-phosphate dehydrogenase and nitrate reductase activities in vitro against zinc and copper-induced inhibition due to the formation of a metal–proline complex (Sharma et al., 1998). Experiments conducted by Siripornadulsil et al. (2002) contradict these results. Their experiments revealed that transgenic *Chlamydomonas reinhardtii* expressing the mothbean *P5CS* gene exhibits tolerance to 100 μM cadmium and has 80% higher proline levels than the wild-type cells. They observed that cadmium does not bind to proline in transgenic algae but is coordinated tetrahedrally by sulfur of phytochelatin. In contrast to *P5CS*-expressing cells, in wild-type cells, cadmium is coordinated tetrahedrally by two oxygen and two sulfur atoms. These results suggested that free proline acts as an antioxidant in cadmium-stressed cells with higher reduced glutathione (GSH) levels. Enhanced GSH levels in turn facilitate phytochelatin synthesis and sequestration of cadmium, since GSH-heavy metal adducts are the substrates for phytochelatin synthase, as pointed out by Siripornadulsil et al. (2002). The above fact that cadmium is coordinated by oxygen and sulfur atoms supported the findings of Adhiya et al. (2002). Proline chelation of cadmium does not seem to be important since cadmium induces phytochelatins that can chelate the metal (de Knecht et al., 1994). Therefore, it is of interest to find out the role of proline or other osmolytes in binding metal ions that do not form complexes with phytochelatins as has been also pointed out by Sharma and Dietz (2006). The second possibility is that proline may be acting as an antioxidant during metal stress. Free radicals are generated under heavy metal stress, which could lead to oxidative stress. As mentioned in the earlier sections, both $^1O_2$ and $OH^{\cdot}$ can be scavenged by proline. Heavy metal exposure causes lipid peroxidation as well as K^+ efflux in algal members. Wu et al. (1995) observed that when *Anacystis nidulans* (*Cyanobacteria*) was exposed to Cu^{2+}, K^+ is effluxed out, but exogenously supplied proline reduced the leakage. Wu et al. (1998) also noticed that when proline was supplied exogenously to *Chlorella* species prior to copper treatment, it resulted in desorption of the adsorbed Cu^{2+} immediately after the addition of proline. These results indicate that one function of accumulated proline is to reduce the uptake of metal ions. Mehta and Gaur (1999) reported that *Chlorella vulgaris* accumulates proline within few hours of exposure to a wide range of heavy metals. Their experiments demonstrate that pretreatment of *C. vulgaris* with proline counteract metal-induced lipid peroxidation as well as K^+ efflux. Even in lichens *Trebouxia erici* (Bačkor et al., 2004), proline content is positively correlated to Cu^{2+} tolerance. Taken together, it is unlikely that proline binds to metals and chelates them during metal stress, but preferentially acts as an antioxidant molecule and detoxifies the ROS.

27.6.6 Role of Osmolytes in Membrane and Native Protein Structure Stabilizations

Galinski (1993) noticed thermal stability of enzymes in the presence of osmolytes. Several other lines of evidence show that osmolytes effectively protect plant enzymes against stress induced denaturation (Solomon et al., 1994; Yang et al., 2007). Arguments and counter-arguments were raised against and in favor of the effective compatible solute concentrations that are needed for protection of enzymes in vitro. While 500 mM has been suggested as an effective concentration for membrane stability, such high concentrations are often not detected in vivo (Bohnert and Shen, 1998). It appears that the osmolyte concentration may not be important since Zhao et al. (1992) have shown protection of thylakoid and plasma membranes against freezing damage under high as well as low concentrations. Experiments conducted to date indicate that the local concentration of membrane or protein surfaces is vital rather than the absolute concentrations for membrane stabilization or enzyme protection in plants undergoing stress.

Fructans are a class of polysaccharides known for their protective effects on liposomes during conditions of drying. Both bacteria (levan produced from *Bacillus subtilis*) and plants (inulin synthesized from chicory roots) have been found to protect liposomes from leakage during freeze-drying or air-drying (Hincha et al., 2000; Vereyken et al., 2003). Besides chicory, many grass species also accumulate osmolytes like fructans (Livingston et al., 2009). Differences exist between fructan molecules in their size, structure, and also tissue localization, which is vital for the survival of the whole plants under cold stress conditions (Livingston et al., 2005, 2006). Hincha et al. (2000) showed that inulin is a mixture of polysaccharides with a degree of polymerization (DP) between 10 and 30 (Hincha et al., 2000) and molecular masses 1600 and 5000. These researchers pointed out that during freeze-drying, inulin in phosphatidylcholine liposome preparations reduces the degree of leakage after rehydration by establishing H-bonds to the lipid P = O. But, high-DP fructans from oat and rye are not able to prevent leakage or fusion in liposomes during drying (Hincha et al., 2007). On the other hand, inulins and fructans from the same species with 7−10 degrees of polymerization (more soluble) do not precipitate during air-drying and provide protection to liposomes (Hincha et al., 2002, 2007; Vereyken et al., 2003). It appears that fructans are transported in the phloem of *Agave deserti* leaf tissues (Wang and Nobel, 1998). Similarly, it has been found that fructan DP3 is transported via the apoplast (phloem) in transgenic potato inferring that fructans in the apoplast protect the tissues from freezing/dehydration injury besides serving as a hexose reserve (Zuther et al., 2004). Levan from *Bacillus subtilis* has a DP of about 125, found to have higher solubility, but protects liposomes from leakage and fusion (Vereyken et al., 2003). Taken together, it appears that specific structural features of oligosaccharides determine their efficacy as membrane stabilizers during drying/freezing stress.

Some plants, especially anhydrobiotic organisms produce very high concentrations of trehalose, and other di- and oligosaccharides under abiotic stress conditions (Zentella et al., 1999; Elbein et al., 2003). In plants, sucrose also plays a similar role to that of trehalose in yeast (Anandarajah and McKersie, 1990). A large body of evidence suggests that trehalose, due to its structure and stereochemistry, depresses the phase transition temperature of the dry lipids, which maintains them in the liquid crystalline phase in the absence of water (Crowe and Crowe, 1988). Trehalose appears to preserve labile proteins during drying probably by interacting directly with the dry protein by hydrogen bonding between its hydroxyl groups and polar residues in the protein (Carpenter and Crowe, 1989). Two contrasting models that have been proposed to explain protective or stabilizing effects of compatible solutes on membrane/protein structures are (1) the preferential exclusion model (Arakawa and Timasheff, 1985) and (2) the preferential interaction model. In the first model, compatible solutes are excluded from the hydration shell of proteins that stabilize protein structure or promote protein/protein interaction under stress. But, Schobert (1977) is of the opinion that interactions between the compatible solutes and proteins are necessary, and protein's hydration shell is crucial for its structural stability. It appears that osmolytes interact with the hydrophobic domains of proteins and prevent their destabilization. The molecular mechanism for osmolyte-induced protein stability has been elucidated by Street et al. (2006). They pointed out that in the equilibrium protein folding reaction, unfolded (U) ↔ native (N), high concentrations of protecting osmolytes push the equilibrium of protein folding towards N, while denaturing osmolytes push it toward the unfolded form (U). It appears that the configuration of the protein backbone is the most important determinant of stabilization or denaturation. As yet, there is no universal molecular theory that explains the mechanism by which osmolytes interact with the protein to alter its stability in higher plants under abiotic stress conditions. However, more experiments will be necessary to gain a better insight into the membrane/protein stabilizing effects of osmolytes.

27.6.7 Osmolytes as Sources of Energy and Carbon Reserve During and After the Release of Stress

It is interesting to note that drought, salt, and flooding stresses increase generally soluble sugar concentrations in plants, but high light intensity, heavy metals, and ozone decrease sugar accumulation depending on the genotype (Gill et al., 2001; Morsy et al., 2007). It is possible that not all soluble sugars play identical roles during stress (Almodares et al., 2008). If sugars are accumulated in high concentrations, that can lead to downregulation of further energy synthesis (Koch, 2004; Chen, 2007). While low sugar content in the tissues increases photosynthesis, high sugar level promotes carbohydrate storage. Thus, changes in CO_2 assimilation are possible by sugar accumulation under stress. This is one of the ways perhaps to maintain energy homeostasis in plants during abiotic stress conditions as has been pointed out by Rosa et al. (2009a). Gupta and Kaur (2005) described that both glucose and sucrose act as sources of carbon and energy, but also as osmolytes (but not fructose) to maintain cell homeostasis. Oxidation of sugars via glycolytic and other pathways leads to the production of ATP, NADPH, and erythrose-4-phosphate, which can be utilized once the stress is released. Osmolytes like proline are involved in the alleviation of cytoplasmic acidosis and sustaining $NADP^+$/NADPH ratios at the levels required for metabolism (Hare and Cress, 1997). The functions of proline are dependent on spatial and temporal control of its synthesis as well as its catabolism. In turn, this helps the plants either to take up or release reductant and energy at a site/tissue location where it is necessary for metabolic functions (Sharma et al., 2011). Plants prefer NADPH over NADH as an electron donor for the biosynthesis of proline (Murahama et al., 2001). This helps the plants to regenerate $NADP^+$ in the chloroplast and thus prevents ROS production and photoinhibition as has been pointed out by Szabados and Savoure (2010). The work of Sharma et al. (2011) demonstrated that both *p5cs1* (involved in proline synthesis) and *pdh1* mutants (blocked in proline catabolism) are required for optimal growth at low water potential. Many lines of evidence suggest that the generation of $NADP^+$ and NADPH during proline synthesis and degradation respectively and maintaining a favorable ratio of $NADP^+$/NADPH are also critical for the survival of plants under stress. Once the stress is relieved, accumulated proline is oxidized in mitochondria and energy is released. Both proline and trehalose (Becker et al., 1996) are the major sugars and are consumed during flight in insects. Thornburg (2007) analyzed the content of proline in ornamental tobacco (LxS8 line) flowers, which was 2020 μM, while the concentration of other amino acids are in the range of only 114–547 μM. Bertazzini et al. (2010) found that artificial nectar containing proline is preferred by forager honeybees. Trehalose is stored in fungal spores and its hydrolysis helps in spore germination and is a source of carbon for synthesis of glucose (Thevelein, 1984). Thus, osmolytes act as both as a source of carbon and energy during and after release from the stress conditions.

27.7 OSMOLYTES AND SIGNALING PROCESSES

27.7.1 Proline and Signaling Processes

Proline propels two major signaling events like cellular survival as well as apoptosis. During proline oxidation, ROS are formed in the mitochondria, which have been implicated in the hypersensitive response in plants. Further, ROS leads to induction of intrinsic and extrinsic apoptotic cell death pathways in animals (Liu et al., 2006; Hu et al., 2007). Thus, ROS appear to be the main signal transducers for downstream responses during proline oxidation. However, several critical issues remain elusive in our understanding of these events. First, is there any threshold level of proline that is required for metabolic switchover from inducing survival pathways to cellular apoptosis? Second, it is not known if there are any other mediators or components that are associated with signaling phenomena during proline metabolism. Existing evidence suggests that proline biosynthetic pathway enzymes interact with redox proteins like thioredoxin (Liang et al., 2013). It would be interesting to find out if there are any other interacting partners of proline metabolic enzymes that play a role in cellular signaling networks leading to the triggering of downstream events.

27.7.2 Proline Metabolism and Signaling Pathways in Plant Senescence

Proline is also associated with plant senescence. Nearly 14-fold increase in proline content was recorded in petals of cut roses during the process of senescence. Enhanced activities of P5CS and ProDH were also noticed during the course of senescence (Kumar et al., 2009). While the expression of *ProDH2* in the vascular tissue and abscission zone of petals is regulated by the transcription factor bZIP11 (Hanson et al., 2008), *ProDH1* expression is modulated by bZIP1 and bZIP53 (Dietrich et al., 2011) in *Arabidopsis thaliana*. This infers that proline is catabolized rapidly

whenever sucrose levels are low in plants (Funck et al., 2010; Llorca et al., 2014). During senescence, proline metabolism also influences ROS signaling pathways that delay the process of senescence (Zhang and Becker, 2015). But, more studies are needed on the regulation of proline metabolic shifts that occur during senescence. Such studies may provide novel insights that rescue crop plants undergoing abiotic stress and also preserve postharvest agricultural products.

27.7.3 Osmolytes as Sensing Compounds and/or Growth Regulators

Sugars such as glucose and sucrose may act as sources of carbon and energy. But, fructose plays a different role from that of glucose and sucrose. Hilal et al. (2004) demonstrated that fructose acts as a precursor for the synthesis of lignin and several phenolic compounds, thus inferring that sugar accumulation under stress performs diverse roles. Sugars can act as primary messengers and regulate signals that control the expressions of genes (Gupta and Kaur, 2005; Gibson, 2005; Chen, 2007). Since sugars are rapidly metabolized and also interconverted depending upon the environmental stresses (Rosa et al., 2009b), it is difficult to pinpoint if sensing of soluble sugars depends upon their metabolism. But, it is known that sugar levels modulate differential expression of genes (Koch et al., 1992). Rook et al. (1998) demonstrated that sucrose-specific signaling pathways to be responsible for repression of *ATB2bZIP* transcription factor. Many genes are negatively regulated by sugars (sucrose, glucose, and fructose) at the transcription level (Yamaguchi-Shinozaki and Shinozaki, 2006). When 30 mM proline was applied exogenously, it ameliorated the salt stress effects in rice, but 40–50 mM levels resulted in poor growth (Roy et al., 1993). Overexpression of microbial genes for trehalose biosynthesis caused dwarfism and aberrant root development (Vogel et al., 1998). Müller et al. (1999) found such growth defects in transgenic rice producing trehalose. These findings have led to postulate that osmolytes might function as plant growth regulators. The plausible explanation that has been given is that small amounts of trehalose or trehalose-phosphate might be toxic to the plants. Else, trehalose metabolism may act as a signal in sugar sensing and partitioning of assimilates like other sugars (Müller et al., 1999).

27.8 CONCLUSIONS AND FUTURE PROSPECTS

ABA is central to the signal perception and subsequent transduction events during abiotic stress. The core signaling module regulates several downstream events including osmolyte biosynthesis and subsequently abiotic stress tolerance. Diverse osmolytes accumulated during abiotic stress conditions are regulated by many phytohormones. Osmolytes perform many vital functions such as osmotic adjustment, scavenging ROS, controlling the redox state, and cell survival and apoptosis during stress. However, the precise molecular mechanisms underlying the triggering of genes associated with several of the osmolyte biosyntheses and catabolisms (barring a few) are not completely known. Therefore, it is of prime importance to unravel the intricate networks, molecular mechanisms, and the signaling events leading to the better survival of crop plants exposed to different abiotic stress conditions. Such a comprehensive knowledge about molecular mechanisms and signaling events leading to the regulation of osmolyte biosynthesis and effective scavenging of ROS will enable us to develop strategies to genetically modify crop plants and use them for sustainable agricultural yields.

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CHAPTER

28

Regulatory Role of Proline in Heat Stress Tolerance: Modulation by Salicylic Acid

Noushina Iqbal[1], *Meher Fatma*[2], *Nafees A. Khan*[3] *and Shahid Umar*[1]

[1]Department of Botany, Hamdard University, New Delhi, India [2]Plant Molecular Biology, School of life Sciences, Jawaharlal Nehru University, New Delhi, India [3]Plant Physiology and Biochemistry Division, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

OUTLINE

28.1 INTRODUCTION

Heat stress is the continual rise in temperature for a longer period of time affecting plant growth and productivity (Fahad et al., 2017; Hatfield and Prueger, 2011; Khan et al., 2013a,b). With continual rise in temperature and global warming there is a need to develop heat-tolerant plants to protect food production and ensure crop safety. The complexity of global warming is of concern as it affects food security and risks extending famine if suitable tolerance strategies for plants are not well worked out. Plants, being immobile, cannot migrate and escape the extremities of temperature change and therefore have to develop avoidance or tolerance mechanisms. Avoidance or tolerance might decrease their production potential. According to the FAO Land and Nutrition Management Service (2009), the global agricultural aim for increasing the production of food by 70% by the year 2050 for approximately 2.3 billion additional people is facing dire obstruction in the context of poverty, hunger, and minor change with the environmental modification and exploitation of rare natural resources more economically. It is predicated that temperature increases of 1.5°C–5.8°C by 2100 will cause heat stress and create threats to agricultural production (Rosenzweig et al., 2001). Atmospheric CO_2 will rise with an increase in global temperature ranging from 1.1°C to 6.4°C depending on the basis of global emissions scenarios.

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High temperature affects plant physiology, biochemistry, and genetics, and protein structure and functions are affected in the whole development of the plant. The adverse effect of heat stress has been discussed in many studies explaining the famine and malnutrition conditions due to decreased crop nutritional values besides the physiological and biochemical changes (Bita and Gerats, 2013; Kazemi-Shahandashti et al., 2014; Larkindale and Knight, 2002; Siboza et al., 2014). Development of plants that can tolerate high temperature requires understanding the mechanism plants employ under heat stress.

Plants either employ antioxidants, proteins, ion transporters or osmoprotectants to cope with heat stress. Upon exposure to heat stress plants activate their signaling cascades and transcriptional machinery to reduce physiological and biochemical alterations (Hasanuzzaman et al., 2013). The reactive oxygen species (ROS) generated during stress are scavenged through the antioxidants or osmoprotectants (Khan and Khan, 2017). The heat shock proteins (HSPs) are just another heat tolerance strategy (Wang et al., 2014). Bita and Gerats (2013) reported that high temperature is sensed by sensors in different cellular compartments. These sensors send signals that modifications have to be done to incorporate changing temperature with increased membrane fluidity. As a result of temperature change the lipid based signaling cascade in the membrane sends a signal for increase in Ca^{2+} influx and cytoskeletal reorganization. These signal for the production of osmolytes and antioxidants. Phytohormone levels are also regulated during heat stress (Abeles, 1986; Khan et al., 2013,a,b; Lv et al., 2011). To produce heat-tolerant plants one strategy could be the regulation of osmolytes like proline by salicylic acid (SA). In the present chapter we will focus on the role of proline in heat tolerance together with its regulation by SA. Further, since hormonal regulation cannot be governed by a single hormone, the interaction between them should instead be dealt with to get better insight. We will also consider the relationship of SA with abscisic acid (ABA) and ethylene in regulating each other and proline for heat tolerance.

28.2 AFTERMATHS OF HEAT STRESS

Heat stress influences nearly every aspect of plants' growth and development at the physiological and molecular level and there have been major limitations to crop productivity (Bita and Gerats, 2013; Pasala et al., 2016; Wahid et al., 2007). It causes oxidative stress, membrane injury, protein degradation, enzyme inactivation, and DNA damage. Negative impact of heat stress on major world food crop yields is increasingly robust including reducing leaf photosynthesis and enhancing leaf senescence rate (Mathur and Jajoo, 2014; Sharma and Sharma, 2017; Sita et al., 2017). Moreover, extreme heat stress affects plant photosynthetic and transpiration efficiencies and negatively impacts on plant development, collectively affecting plant yield. The decreases in photosynthetic rate under heat stress attributes to lower internal plant CO_2, inhibition of photosynthetic enzymes, and synthesis of ATP, which produces chemical energy that is required for plant biochemical reactions. Heat stress also inhibited photosynthesis (Salvucci, 2008; Sage and Kubien, 2007). The inhibition of net photosynthesis by heat stress had been featured to an inability of rubisco activase to maintain rubisco in an active form. Hemantaranjan et al. (2014) reported that nonphotochemical quenching of chlorophyll fluorescence, the effective quantum yield of photochemical energy conversion, and the maximum yield of PSII were sensitive to temperature in Antarctic hairgrass and spinach. Moreover, Tripathy and Rebeiz (1986) and Porra (1997) elucidated in detail the biosynthesis of porphyrins and particularly chlorophyll during early greening stages of seedlings. In cucumber seedlings, biosynthesis of chlorophyll under chill and heat stress was affected by 90% and 60%, respectively. Inhibition of chlorophyll biosynthesis was partly due to impairment of 5-aminolevulinic acid biosynthesis both in chill and heat stress states. Also, protochlorophyllide synthesis was inhibited by 90% and 70% in chill and heat stress, respectively. Heat stress increases the rate of reproductive development, which shortens the time for photosynthesis to contribute to fruit or seed production and reduces total fruit or grain yield (Hall, 2001). Lobell et al. (2012) and Pradhan et al. (2012) noted that heat stress increased the supply of assimilate during grain filling in wheat plant, which was unable to fully compensate for the shorter duration of the grain filling period. Heat stress altered the regulation of senescence processes, such as reduced photosynthesis and leaf chlorophyll content, resulting in accelerated senescence (Harding et al., 1990; Yang et al., 2002; Zhao et al., 2007). Pradhan et al. (2012) reported decrease in leaf chlorophyll, individual grain weight, and grain yield in an increasing magnitude of drought or high temperature and with combined stress. Hays et al. (2007) reported grain abortion by 25% in one wheat cultivar and no response in another in response to a heat shock event 10 days after pollination. Those differences were attributed to genetic variation in heat tolerance. Tashiro and Wardlaw (1990) reported that heat shock also produced wheat kernels that are small, notched, and split that also significantly affect crop yield and quality. High temperatures had also been shown to reduce the grain filling period by 45%–60% by Shah and Paulsen (2003).

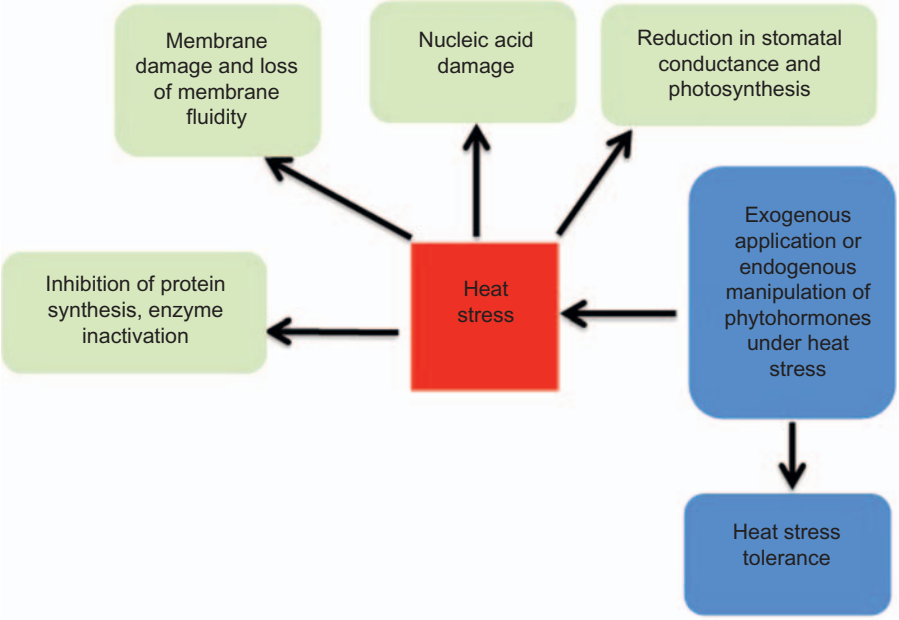

FIGURE 28.1 Major impacts of heat stress on plants and phytohormone coping mechanisms.

Each plant species has a specific temperature range characterized by a minimum, maximum, and optimum that affects the rate of plant growth and development. Those values were reviewed by Hatfield et al. (2008, 2011) for different species typical of grain and fruit production. Barlow et al. (2015) reported that extreme temperature, frost, and heat caused sterility and abortion of formed grains during frost and reduction in grain number and reduced duration of the grain filling period during heat stress in wheat plant. The report of Meehl et al. (2007) exposed that daily minimum temperatures would increase more rapidly than daily maximum temperatures leading to the increase in the daily mean temperatures and those changes had negative effects on grain yield. The effect of heat stress is increased under a high vapor pressure deficit because pollen viability depends on pollen moisture content which strongly depends on vapor pressure deficit (Fonseca and Westgate, 2005).

Under heat stress, Uchida et al. (2002) reported that pretreatment of H_2O_2 or NO induced not only ROS scavenging enzyme activities but also expression of oxidative stress–related genes in rice seedlings. Likewise, Larkindale and Knight (2002) reported that heat produced H_2O_2 and induced oxidative stress in plant cells and established that the production of H_2O_2 contributes to the transduction of the heat signal into the expression of HSPs and their downstream proteins (Königshofer et al., 2008). Likewise, Sanmiya et al. (2004) in tobacco plants and Xin et al. (2011) in wheat plant reported significant modifications in protein pattern. Sanmiya et al. (2004) and Reddy et al. (2016) noted that high temperature stress induced the synthesis of polypeptides and accumulated the different members of HSP families (96, 80, 70, 35, and 24 kDa). Heat stress or direct high temperature injuries caused protein denaturation and aggregation and increased fluidity of membrane lipids whereas slower heat injuries or indirect effect caused inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation, and loss of membrane integrity (Howarth et al., 2005). Heat stress also affected the organization of microtubules by splitting and/or elongation of spindles, microtubule asters formation in mitotic cells, and elongation of phragmoplast microtubules (Smertenkon et al., 1997). Fig. 28.1 shows the major impact of heat stress on plants.

28.3 PROLINE IN HEAT TOLERANCE

Proline accumulates under heat stress and helps in osmotic adjustment and plays a protective role (Szabados and Savouré, 2010; Kaur and Asthir, 2015; Per et al., 2017). Its synthesis mainly occurs from glutamate and proline metabolizing enzyme, pyrroline-5-carboxylate synthetase (P5CS), which reduces glutamate to glutamate-semialdehyde (GSA) and spontaneously to pyrroline-5-carboxylate (P5C). Finally proline formation occurs from the reduction of P5C by P5C reductase (P5CR). Proline catabolism occurs by the sequential action of proline dehydrogenase or proline oxidase (PDH or POX) resulting in P5C from proline, and P5C dehydrogenase (P5CDH), which regenerates glutamate from 5C. Alternatively, ornithine is transaminated by ornithine-delta-aminotransferase (OAT) producing GSA and P5C and then converted to proline. Besides glutamate this is another method of proline synthesis in

plants (Iqbal et al., 2014). Thus, proline level should increase with increase in P5CS, P5C, and OAT. However, increase in proline under heat stress is not ubiquitous since the increase under heat stress varies depending on different conditions. While proline was reported to increase in the leaves of tomato (Rivero et al., 2004) and tobacco (Cvikrová et al., 2012), it decreased in the germinating wheat seeds (Song et al., 2005) and remain unaltered in *Arabidopsis* plants (Hua et al., 2001; Rizhsky et al., 2004). This differential proline accumulation was due to altered expression of proline biosynthetic genes. Manipulation of proline could therefore be one potential heat tolerance mechanism. In *Arabidopsis* overexpression of pyrroline-5-carboxylate reductase gene (AtP5CR) in soybean improved heat tolerance (de Ronde et al., 2004). In heat stressed exposed leaves of sugarcane proline was responsible for increased pressure potential (Wahid et al., 2007). Proline helps in osmotic adjustment, increases the concentration of cell protoplasm to maintain normal membrane function under heat stress, and increases plants adaptability. Its capability to act as an osmoprotectant helps in stabilizing the antioxidant system and thus the deleterious effect of ROS (Reddy et al., 2015; Szabados and Savouré, 2010). Increased proline content was observed in leaf of lettuce seedlings under heat stress but the increase in heat-tolerant varieties was significantly higher than the nonheat-tolerant varieties (Han et al., 2013). Dobra et al. (2010), through the use of transgenic plant constitutively overexpressing a gene for the proline biosynthetic enzyme Δ2-pyrroline-5-carboxylate synthetase (P5CSF129A; EC 2.7.2.11/1.2.1.41) in tobacco, showed that the transformant with enhanced proline production had a more negative leaf osmotic potential and enhanced xanthophyll cycle pigments that provide protection to plants. In transgenic sugarcane plants with heterologous *P5CS* gene under control of stress inducible promoter, ABA-inducible promoter complex, Molinari et al. (2007) found that under stress, proline production increased more in the transgenic sugarcane leading to higher protection of chlorophyll and the PSII together with high Malondialdehyde (MDA) content responsible for enhanced antioxidant system. However, osmotic potential in the transgenic sugarcane with higher proline accumulation decreased, not supporting its role in osmotic adjustment. Thus, the role of proline depends upon species; however, we cannot ignore its role in heat tolerance. Rivero et al. (2004) suggested using NH_4^+ as N source rather than NO_3^- for heat tolerance. The tolerance was related to higher accumulation of proline with NH_4^+ than NO_3^-. Kocsy et al. (2005) reported that the genetic manipulation of proline in *Glycine max* caused enhanced activity of antioxidative enzymes and glutathione content under simultaneous drought and heat stress treatment. The role of proline in the protection of plants against simultaneous drought and heat stress was confirmed by de Ronde et al. (2000). Plants with higher proline content were less damaged compared with wild-type plants with lower proline. However, the effect of proline is not independent but relies on many factors, one among them being phytohormones. In the next section we are going to discuss the role of phytohormones in stress tolerance.

28.4 PHYTOHORMONES IN HEAT TOLERANCE

Hormones control a variety of functions in plants and their level determines plants' fate under stress situations. Together with proline, phytohormones also play a key role under heat stress. Per et al. (2017) reviewed that phytohormones play a role in modulating proline metabolism. *Arabidopsis* plants ectopically expressing the *Δ(1)-pyrroline-5-carboxylate synthetase 1* gene (*AtP5CS1*) under the control of the *HSP17.6II* gene promoter had enhanced proline biosynthesis but a decreased thermotolerance, most likely due to higher ROS production and inhibition of ABA and ethylene biosynthesis (Lv et al., 2011). Increased level of ABA, SA, and ethylene were observed under heat stress whereas, cytokinin (CK), auxin (AUX), and gibberellic acids (GAs) decreased causing premature plant senescence (Talanova et al., 2003; Larkindale and Huang, 2004; Larkindale et al., 2005). Binder and Patterson (2009) reported that the abscission of reproductive organs was caused by increased ABA and ethylene levels and reduced levels and transport of AUX. Hormones' association with heat stress tolerance may be fast to immediately provide adaptability or slow to enhance their defense pathway. Dobra et al. (2010) reported that under stress conditions the increased level of ABA is associated both with providing immediate protection through stomatal closure and slower metabolic changes coinciding with activation of defense pathways. Hays et al. (2007), in studying wheat genotypes, reported the role of ethylene in abortion of kernel and reduction in the weight of kernel in the heat susceptible "Karl 92" plants under heat stress. Inhibition of ethylene action in "Karl 92" by methylcyclopropane (1-MCP) before applying heat stress resulted in inhibition of both kernel abortion and reduction in kernel weight confirming the role of ethylene in heat stress induced damages. Heat stress evolved ethylene in such cases served as a signal for developmental arrest and senescence. Phytohormones work by reducing the heat induced oxidative damage as reported by Larkindale and Knight (2002). They applied SA, 1-aminocyclopropane-1-carboxylic acid (a precursor to ethylene), and ABA to plants and

observed not only protection from oxidative damage but also the insensitive mutants of ethylene; *etr-1*, the *ABA; abi-1*, and a transgenic line expressing *nahG* (consequently inhibited in SA production) showed increased susceptibility to heat and thus the role of these hormones in heat tolerance. In lettuce seeds exposed to high temperature, ethylene regulated thermoinhibition and the seeds' ability to germinate at high temperature was positively correlated to seed ethylene (Abeles, 1986).

Phytohormones are signal molecules that regulate plant growth and interact with proline metabolism to increase stress tolerance in plants. Reports are available for the synergistic interaction of phytohormones with proline metabolism for stress tolerance (Khan et al., 2013a,b; Iqbal et al., 2014; Iqbal et al., 2015; Per et al., 2017). However, not much is known about the interaction of proline with ethylene, ABA, and SA in respect to heat tolerance. Although, the relationship of nitrogen (N) with proline and regulation of N by phytohormones has been discussed (Iqbal et al., 2015) and the regulation of proline by different phytohormones under salt stress (Iqbal et al., 2014). However, not much is reported about regulation of proline by SA through interaction between the three hormones under heat stress.

Kumar et al. (2012) found that ABA induces tolerance to heat in seedlings of *Cicer arietinum* L. by accumulating osmoprotectants like proline, glycine betaine, and trehalose. ABA induces heat shock transcription factor 3, which acts synergistically with chimeric genes with a small HSP promoter (Rojas et al., 1999). Its role in induced thermotolerance has been supported (Jiang and Huang, 2001; Larkindale and Knight, 2002; Larkindale and Huang, 2004). Larkindale and Knight (2002) reported the induced thermotolerance in ethylene and ABA treated plants was through increased activity of antioxidative enzymes that reduced heat stress induced oxidative stress. They further showed that those mutants and transgenic plants that do not signal ABA and ethylene are more heat sensitive than wild-type plants. Lv et al. (2011) however, provided a completely different picture to what we usually see. He reported that proline overproducing mutants inhibit ethylene and ABA synthesis and increased oxidative stress via the proline/P5C cycle, decreasing thermotolerance in *Arabidopsis* seedlings.

SA treatment was reported to increase proline content in chickpea (*Cicer arietinum*) and increased heat tolerance compared with the control plants not receiving SA (Chakraborty and Tongden, 2005). Khan et al. (2013a,b) reported the role of SA in heat tolerance, showing that SA at 0.5 mM resulted in tolerating heat induced stress through increased proline production, which helped in maintaining osmotic and water potential necessary for photosynthesis. The increase in proline content was followed by the increase in proline metabolizing enzyme γ-glutamyl kinase (GK) and decrease in POX activity. Ethylene increased proline accumulation under salt stress to provide tolerance in mustard through the increase in proline metabolizing enzymes (Iqbal et al., 2015).

28.5 ROLE OF SALICYCLIC ACID IN HEAT TOLERANCE

Recently, SA has received great attention in the regulation of numerous plant developmental processes under abiotic stresses including heat stress (Khan et al., 2012; Khan and Khan, 2013; Khan et al., 2015). The mechanism of thermotolerance in plants by SA interaction gives an insight into some scientific approaches to modulate plants' responses for heat stress. The SA mechanism was reported by Oata (1975) and Pieterse and Muller (1977). They reported that SA-induced flowering by acting as a chelating agent. Their view was supported by Raskin et al. (1987), who confirmed that SA functioned as endogenous growth regulators of flowering and florigenic effects. It induces flowering, increases flower life, retards senescence, and increases cell metabolic rate. Furthermore, SA is a natural phenolic compound that acts as an endogenous signal molecule that plays a role in the regulation of plant growth, development, and responses to environmental stress. The positive effect of SA offered protection against a number of abiotic stresses, mainly heat stress. Exogenous application of SA is an important way to promote economic utilization by providing thermotolerance in many major crops, for example, in potato (Dat et al., 1998a), tomato (Shaheen et al., 2017; Senaratna et al., 2000), wheat (Sadak and Orabi, 2015), and *Arabidopsis* (Clarke et al., 2004; Larkindale and Knight, 2002) respectively. SA-mediated improved plant tolerance to heat stress has also been reported by He et al. (2002a); Wang et al. (2010); Khan et al. (2013a,b).

SA has been attributed to an increased CO_2 assimilation and photosynthetic rate and increased mineral uptake by the stressed plant (Vazirimehr and Rigi, 2014). Analysis of SA-mediated change in physiological, biochemical, molecular alternations under high temperature stress was proven by the application of SA in improving the phenology, photosynthesis, remobilization of carbohydrates, and yield under normal and late sown high temperature conditions. Larkindale and Knight (2002) reported that the transgenic *Arabidopsis* seedlings showing a bacterial SA-decomposing salicylate hydroxylase were less tolerant to heat stress. Cold/heat treated grape plant had shown cold or heat tolerance through the supplementation of SA (Wang and

Li, 2006). After both heat stress and recovery, SA declined electrolyte leakage and oxidative stress and improved maximum yield of PSII, Fv/Fm, and the quantum yield of the PSII electron transport in cucumber seedlings (Shi et al., 2006). Khan et al. (2013a,b) reported that SA treatment alleviated heat stress in wheat by increasing the production of proline and restriction of the stress ethylene formation. SA derivative acetyl SA application enhanced thermotolerance in potato microplants that simplified the production of virus free potato plants through tissue culture (Lopez-Delgado et al., 1998). Several other studies reported that exogenous application of SA or acetylsalicylate had been shown to enhance thermotolerance in *Arabidopsis* (Dat et al., 1998b; Clarke et al., 2004). SA marked differential antioxidant response by upregulating the Halliwell–Asada pathway in roots and attaining high peroxidase activity in both seedlings of maize under high temperature stress (Khanna et al., 2016).

SA pretreatment alleviated the heat stress through inducing decrease in net photosynthesis through maintaining higher Rubisco activation state and it accelerated the net photosynthesis recovery principally through effects on PSII function (Wang et al., 2010). Wang and Li (2007) reported that SA treatment maintained at higher net photosynthesis in grape leaves under heat stress. Wang and Li (2006) reported that spraying with a 0.1 mM solution of SA decreased thiobarbituric acid-reactive substances and relative electrolyte leakage in young grape leaves under heat stress, signifying that it induced intrinsic heat tolerance in grapevines by upregulating the antioxidant system. Wang et al. (2010) noted the effect of SA on photosynthesis of grape leaves before, during, and after heat stress. Heat stress led to decline in maximum yield of PSII, FPSII, and photochemical quenching and increased of Non-photochemical quenching (NPQ) in relation to the treatment of SA. Maximum quantum yield of PSII, FPSII, and photochemical quenching gradually rise and SA-treated leaves were always greater than control leaves during recovery. SA-treated and control leaves had much lower FPSII under heat stress and had greater thermal dissipation of excitation energy as measured by increased NPQ and with the recovery from heat stress, FPSII of SA-treated and control plants increased, and this was accompanied by increases in maximum yield of PSII and photochemical quenching and a rapid decline of nonphotochemical quenching. However, heat stress significantly reduced the crop productivity whereas in supplementation of SA played substantial role in combating the negative effect of it.

SA caused changes in net photosynthesis and changes in proline under heat stress suggesting that its application protects photosynthesis through alleviating the negative effects of heat stress on proline accumulation and ethylene formation (Khan et al., 2013a,b). Chakraborty and Tongden (2005) reported that SA induces heat tolerance via increase in antioxidative enzymes, protein, and proline in *Cicer arietinum* L. Sakhabutdinova et al. (2003) found that SA treatment to water stressed plants resulted in increase in ABA and development of antistress reactions like proline accumulation for stress tolerance. In tall fescue seedlings, SA increased the antioxidative enzymes to bring about salt tolerance under abiotic stress (He et al., 2002b). Krantev et al. (2008) reported cadmium tolerance in *Zea mays* through the increase in proline content.

In lentil, SA alleviated the negativity of salt stress by increasing P5CS, GK, and proline and thus maintaining turgor under salt stress (Misra and Saxena, 2006). SA treatment increased the proline content under stress (Szepsi et al., 2005**).** Similar proline increase after SA treatment was also reported in *Triticum aestivum*, *Avena sativa*, *Phaseolus vulgaris*, and *Lycopersicum* plants under oxidative stress (Tasgin et al., 2006). As SA increases proline so was proline associated with induction of SA signaling in tobacco (Chen et al., 2011). It was found that SA increases proline formation through regulating N-assimilation and was dependent on N source and application method (Iqbal et al., 2014; Tarighaleslami et al., 2012). Application of N and sulfur individually or in combination increased nitrate reductase activity (NRA), N content, proline accumulation in *Brassica juncea* to reduce salt stress (Rais et al., 2013). SA was reported to increase NR activity and activity of glutamine synthase in *Cucumis sativa* under drought stress (Jing-hong et al., 2012). Under salt stress, SA was responsible for increase in activity of NR and NiR in *Vigna radiata* (Akhtar et al., 2013). Application of SA increased proline accumulation through increased NRA in water stressed seedlings of *Amaranthus hybridus* (Umebese et al., 2009). However, this increase was found to be concentration dependent in *Vigna mungo* where low SA-induced NRA while high concentration of SA decreased it (Ramanujam et al., 1998). Thus SA might be responsible for increasing proline for heat tolerance via regulating N metabolism. Table 28.1 shows the effect of SA on proline content under different stresses.

28.6 INTERACTION BETWEEN SALICYCLIC ACID, ETHYLENE, AND ABSCISIC ACID FOR HEAT TOLERANCE

Now let us focus on the interaction between the three hormones to find out how they can regulate proline under heat stress to induce tolerance. The interaction between SA and ethylene was reported by

TABLE 28.1 Effect of Salicylic Acid on Proline Content Under Different Stresses

Plant species	Effect on proline	Stresses	References
Triticum aestivum	GK increases PROX decreases	Heat	Khan et al. (2013a,b)
Lentil	P5CS, GK, proline increases	Salt	Misra and Saxena (2006)
Hordeum vulgare cv Gerbel	Proline increases	Salt	El-Tayeb (2005)
Triticum aestivum	Proline increases	Salt	Shakirova et al. (2003)
Zea mays	Proline increases	Salt	Hussein et al. (2007)
Glycine max	SbPRP increase	Salt Drought Salicylic acid	He et al. (2002b)
Cicer arietinum L.	Proline increases	Heat	Chakraborty and Tongden (2005)
Triticum aestivum	Proline increases	Salicylic acid/heat	Azooz and Youssef (2010)
Zea mays	Proline decreases	Cadmium	Krantev et al. (2008)
Lycopersicum esculentum	Proline level decreases at higher salt level	Salinity	Zahra et al. (2010)

Khan et al. (2013a,b). Under heat stress SA worked as an inhibitor of ethylene synthesis inhibiting activity of ethylene synthesis enzyme 1-aminocyclopropane carboxylic acid (ACC) synthase (ACS) and brought ethylene to an optimum level from stress ethylene level. Optimal ethylene resulted in increased proline metabolism, N-assimilation and photosynthesis. Rao et al. (2002) found that SA is required for stress ethylene production and under ozone stress SA treatment increased ethylene production and ozone sensitivity to induce cell death. In canola plants ethylene treatment reduced chlorophyll and carotenoid content and increased lipid peroxidation while SA treatment after ethylene treatment reversed the ethylene induced oxidative damage and increased chlorophyll and carotenoid contents (Tirani et al., 2013). Lee et al. (1999) found inhibitory effect of SA on AUX-induced ethylene through inhibiting 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) enzyme activity probably through reversible interaction with Fe^{2+}, an essential cofactor of the enzyme. Nazar et al. (2015) reported restricted ethylene formation in drought stressed *Brassica juncea* upon SA treatment. SA inhibited the ACS activity to influence proline metabolism and photosynthesis.

Similar interaction between ABA and SA is also recorded. Both ABA and SA production increases in stress situations. Genes involved in ABA synthesis (*AtNCED* genes encoding 9-cisepoxycarotenoid dioxygenases) are expressed when *Arabidopsis* seeds are imbibed at high temperature. Li et al. (2017) reported that the actions of ABA and SA are different under stress. While ABA promotes the accumulation of organic acid, SA promoted the accumulation of amino acids, one among them being proline. ABA was found to suppress the SA signaling pathway in rice by repressing the transcriptional upregulation of WRKY45 and OsNPR1, the two key components of the SA signaling pathway in rice. ABA biosynthesis mutants (*aba1*, *aba2*, and *aba3*), and SA deficient mutant (*NahG* transgenic lines) showed weaker defects. The role of ABA in thermoinhibition was confirmed by Tamura et al. (2006) when they showed that *Arabidopsis* seeds both ABA-deficient and ABA-insensitive (*aba1 and abi3*) germinated at supraoptimal temperatures. The first biochemical step of ABA biosynthesis is catalyzed by AtNCED9 and it plays a major role in thermoinhibition (Toh et al., 2008). Both SA and ABA decreased oxidative stress and increased antioxidative enzymes activity and thereby increased chlorophyll and carotenoid contents, relative water content, membrane stability index, leaf area, and total biomass over control plants in water stressed wheat genotypes.

Xie et al. (2007) reported that for the suppression of GA-induced seed germination by SA and ABA, *HvWRKY38* gene in barley serves as a converging node of signal pathways for SA and ABA interaction. Audenaert et al. (2002) found that endogenous ABA level in wild-type plants increased plants' susceptibility to *B. cinerea* infection and they suggested ABA negatively regulates SA-dependent defense signaling, which in turn appears to be an effective plant defense mechanism against *B. cinerea*. The antagonistic interaction between ABA and SA was found in *Lycopersicon esculentum* where ABA was found to increase the susceptibility of tomato to necrotrophic fungus *Botrytis cinerea*. However, SA was found to provide protection against the fungus. Mutants with reduced ABA level were more resistant to the fungus and showed increase

in phenylalanine ammonia lyase activity (pathway for SA synthesis) and thus suggest the role of ABA in SA-dependent defense pathway in tomato.

Szepesi et al. (2009) found positive interaction between SA and ABA and SA was reported to increase ABA accumulation under salt stress; however, such an increase was dose dependent. At 10^{-4} M concentration SA led to ABA accumulation and enhanced activity of aldehyde oxidase responsible for conversion of ABA-aldehyde to ABA. Shakirova et al. (2003) reported high ABA level in SA-treated wheat seedlings that was responsible for proline accumulation under stress, which resulted in preadaptation of plants to stress. ABA is known to have a key role in the induction of the synthesis of a range of stress proteins (Rock, 2000) and its increased level under stress suggests a significant role of ABA in protection through changes in proline, in SA-induced preadaptation of wheat plants to stress. SA increases ABA content and proline accumulation under salt stress in wheat (Shakirova et al., 2003). Liu et al. (2006) reported that 100 μM ABA biosynthesis inhibitor inhibited not only increase in ABA content but also in SA formation during heat stress. This implies that increase in ABA content under heat stress is also responsible for increasing free SA level focusing on the relationship between the two hormones under heat stress. Both ethylene and ABA are stress hormones involved in germination, leaf abscission, flower senescence, fruit ripening, and in biotic and abiotic stress responses. Some functions of ethylene overlap with ABA throwing light on the interaction between ABA and ethylene pathway under normal or stress conditions. The signaling pathway of ABA and ethylene are generally antagonistic (Beaudoin et al., 2000; Anderson et al., 2004). They studied that ethylene insensitive mutant (*etr1*, *ein2*, *ein3*) and ABA-insensitive mutant (*aba1*, *aba2*, *abi1*, *abi2*) antagonistically affect the expression of stress-related genes and therefore differently modulate plants' abiotic stress responses. Both ethylene and ABA limit the formation of each other. In *Arabidopsis* higher level of endogenous ABA was found in ethylene insensitive mutants *ein2*/*era3* and *etr1* in comparison with higher ethylene level in ABA-deficient mutant aba2 (LeNoble et al., 2004; Chiwocha et al., 2005). This clearly shows that both ethylene and ABA action are opposite to each other restricting the formation of one another. However, we cannot say that such an antagonistic interaction is ubiquitously present in all plants and in all species. Generally plants' responses are species specific or depend on growth processes and conditions. Therefore, the synergistic interaction between ethylene and ABA cannot be ignored in regulating plants' development and stress responses. Ma et al. (2014) studied synergistic interaction between ethylene and ABA in inhibiting root growth. Ethylene-deficient mutants showed resistance to both ethylene and ABA while in ABA-deficient mutant only ABA resistance was present. In such case ethylene signaling was responsible for ABA-inhibited root growth (Beaudoin et al., 2000; Ghassemian et al., 2000). Thus ABA and ethylene signaling pathways have a close interplay in plant growth, development, and stress response. Hormone signals act either antagonistically or synergistically. The synergistic interaction was found when mutation in ethylene signaling

TABLE 28.2 Effect of Salicylic Acid on Ethylene and Abscisic Acid Under Different Abiotic Stresses

	Type of stress/condition	Plant species	Response	References
Ethylene	Heat stress	*Triticum aestivum*	Decreases stress ethylene	Khan et al. (2013a,b)
	Wounding	*Lycopersicon esculentum*	Ethylene decreased by preventing accumulation of ACS transcript	Li et al. (1992)
	Salt stress	*Vigna radiata*	Ethylene decreases	Khan et al. (2014)
	Cold stress	*Zea mays*	ACC decrease	Szalai et al. (2000)
	Ozone stress	*Arabidopsis mutants, eto1 and eto3*	Ethylene increases	Rao et al. (2002)
	Hypocotyl	*Vigna mungo*	Inhibits ACO enzyme activity	Lee et al. (1999)
	Drought stress	*Brassica juncea*	Restrict ethylene formation by restricting ACS enzyme activity	Nazar et al. (2015)
Abscisic acid	Salinity	*Solanum lycopersicum*	Increase	Szepesi et al. (2009)
	Salinity	*Triticum aestivum*	Increase	Shakirova et al. (2003)
	Shoot growth and cell cycle	*Rice*	Antagonizes ABA action	Meguro and Sato (2014)
	Seed germination	*Arabidopsis*	increased synthesis of ABA-regulated proteins	Rajjou et al. (2006)

enhanced seed dormancy by altering the sensitivities of germinating seeds to exogenous ABA (Beaudoin et al., 2000; Matilla and Matilla-Vazquez, 2008).

Larkindale et al. (2005) reported that root growth and seedling survival in ABA signaling mutants (*abi1 and abi2*) were more defective in acquired thermotolerance than basal thermotolerance while ethylene signaling mutants (*ein2 and etr1*) were more defective in basal than acquired thermotolerance, especially under high light. Table 28.2 shows the effect of SA on ABA and ethylene production under different conditions and the responses thereof.

28.7 CONCLUSION AND FUTURE PROSPECTS

Thus we can see that SA interacts with both ABA and ethylene and ethylene and ABA also interact with each other. Individually all of them have a role in heat tolerance and affect proline. Although it may be assumed that SA decreases ethylene content and decreased ethylene might increase ABA signaling to increase proline content for heat tolerance, however, this is not always true in all circumstances. Incidence of positive ABA−ethylene and ABA−SA interactions are also available. If we can figure out how these three hormones are interacting with each other under stress conditions to regulate proline metabolism, then mutations in hormones or mechanisms for enhancing proline accumulation through regulating the hormones to breed stress tolerant crop will be a promising strategy to combat heat stress in future and develop heat resistant plants. However, one thing is clear—that proline is normally involved in heat tolerance and SA, ABA, and ethylene affect proline under abiotic stresses. So, to develop heat resistant varieties manipulation of proline is an important technique.

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CHAPTER

29

Osmolyte Diversity, Distribution, and Their Biosynthetic Pathways

Gandra Jawahar[1], *Guddimalli Rajasheker*[1], *Parveda Maheshwari*[1], *Devineni Lakshmi Punita*[1], *Naravula Jalaja*[2], *Palavalasa Hima Kumari*[1], *Somanaboina Anil Kumar*[1], *Ruhi Afreen*[1], *Appa Rao Karumanchi*[1], *Polavarapu Rathnagiri*[3], *Nese Sreenivasulu*[4] and *Polavarapu Bilhan Kavi Kishor*[1]

[1]Department of Genetics, Osmania University, Hyderabad, Telangana, India [2]Department of Biotechnology, Vignan University, Vadlamudi, Guntur, India [3]Genomix CARL Pvt. Ltd., Kadapa, Andhra Pradesh, India [4]Grain Quality and Nutrition Center, Plant Breeding Division, International Rice Research Institute, Metro Manila, Philippines

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29.1 INTRODUCTION

Salt and water stresses, nutrient/mineral deficiencies, and extreme temperatures are the major environmental perturbations that adversely affect both growth and development and generate a threat to sustainable agriculture. Since abiotic stress is a complex phenomenon, it is necessary to understand the anatomical, physiological, biochemical, and molecular mechanisms associated with it. Development of crop plants that resist adverse environmental factors is an important approach since they can mitigate the problem of global food shortage in future. Environmental perturbations evoke multiple responses in the plant systems (Cheeseman, 2013; Khan et al., 2013a,b, 2017). While water deficit leads to osmotic stress, salt causes both ionic and osmotic stresses. Osmotic stress also leads to buildup of salt levels in and around the roots and inhibition of water uptake, cell expansion, and lateral root development (Munns and Tester, 2008). On the other hand, when Na^+ and Cl^- accumulate in plants under salt stress, it leads to a decline in photosynthesis with a simultaneous increase in chlorosis and cell death (Glenn et al., 1999). But, plants adapt a number of mechanisms that can help them to cope with such adverse conditions. Accumulation of both energetically cheap inorganic ions like Na^+ and Cl^- and low-molecular weight organic solutes such as proline and glycine betaine (*N*,*N*′,*N*′′-trimethylglycine) helps the plants in osmotic adjustment (Glenn et al., 1999). While the inorganic ions (Na^+ and Cl^-) accumulate in the vacuoles through complex biochemical mechanisms, osmolytes (amino acids like proline, tertiary sulfonium compounds (e.g., 3-dimethylsulfoniopropionate (DMSP)), quaternary ammonium compounds (e.g., glycine betaine, proline betaine, β-alanine betaine, and choline-*O*-sulfate), sugars (e.g., trehalose and others), and sugar alcohols or polyhydric alcohols) accumulation takes place primarily in the cytoplasm and to some extent in chloroplasts (proline and glycine betaine). The quaternary ammonium compounds possess a fully methyl substituted nitrogen atom and a negatively charged carboxyl group (in the case of betaines) or sulfate group (in the case of choline-*O*-sulfate) (Rhodes and Hanson, 1993; Gorham, 1995). While glycine betaine, β-alanine betaine, and proline betaine act as osmotic agents (based on growth assays), choline, an alcohol, without a negatively charged group is not an osmoprotectant per se (Kishor, 1988; Hanson et al., 1994). This review discusses the diversity, distribution, and biosynthesis of osmolytes that occur in different taxa, and the factors that regulate their accumulation. Multiple biosynthetic pathways; the interaction of hormones, especially abscisic acid in osmolyte regulation; and the wide array of osmolytes functions during salt and drought stress tolerance are discussed.

29.2 DIVERSITY AND DISTRIBUTION OF OSMOLYTES IN DIFFERENT PLANT SPECIES

29.2.1 What Are Osmolytes or Osmoprotectants

In plants, biochemical reactions require specific inorganic ions. But under stress, such as high NaCl conditions, the concentrations of the inorganic ions increase above the threshold levels that are typically found in plant cells. Such high concentrations generally perturb the protein functions. Contrarily, the effect of organic osmolytes on proteins is much less, in other words, compatible. Firstly, NaCl stress causes osmotic flux of water out of cells, and then elevates the concentration of all cellular constituents and inorganic ions (Burg et al., 2007). The perturbing inorganic ions are slowly replaced by organic osmolytes. Under different abiotic stress conditions, plants accumulate in high concentrations, a wide array of organic compounds, also known as compatible solutes or osmolytes. These compounds are highly soluble in water and do not interfere with normal physiological or metabolic activities in plants (Yancey, 2005; Kishor et al., 2005). They are nontoxic at high cellular concentrations and protect cellular components from dehydration. Hence, osmolytes are also commonly referred as osmoprotectants.

29.2.2 Why Are Osmolytes Compatible?

It has long been known that cells use osmolytes for osmotic adjustment under stress conditions. Osmolytes are not charged (or zwitterionic such as glycine betaine) at a neutral pH and are highly soluble in water (Bellantyne and Chamberlin, 1994). They modify the solvent properties of water and stabilize the internal osmotic potential (Yancey et al., 1982; Yancey, 2005). Many cells respond to hyperosmotic stress by accumulating nontoxic organic compatible solutes. Many different types of compatible solutes accumulate and have been classified as (1) water structure-makers, (2) promoters of protein association, (3) protein stabilizers, and (4) salting-out solutes and enzyme activators (Bellantyne and Chamberlin, 1994). Nonspecific binding of solutes to proteins (other than catalytic site) denatures proteins. On the other hand, solutes that are excluded from the surface of the proteins will stabilize folded protein structures (due to entropically unfavorable situation). Thus, osmolytes are excluded from the hydration sphere of proteins and protect the stability of folded proteins, and cellular components from dehydration injury (Low, 1985). Precisely for these reasons, osmolytes are also named as osmoprotectants.

29.2.3 Diversity and Distribution of Osmolytes

Organic osmolytes fall into few major chemical categories in all: small carbohydrates including mono-, di-, oligo-, and polysaccharides (e.g., glucose, fructose, sucrose, trehalose, raffinose, and fructans), sugar alcohols or polyols (e.g., glycerol, inositol, sorbitol, mannitol etc.), and inositol derivatives or methylated inositol like *o*-methyl-inositol, amino acids (alanine, arginine, glycine, proline, methylated proline-related compounds such as methyl-proline, proline betaine, and hydroxyproline betaine, other betaines such as glycine betaine, β-alanine betaine, choline-*O*-sulfate, taurine (observed among marine animals, but not in plants), etc.), amides such as glutamine, asparagine, nonprotein amino acids like γ-aminobutyric acid (GABA), pipecolic acid, citrulline, ornithine and amino acid derivatives (e.g., ectoine), methylammonium (*N*-trimethylamine oxide), methylsulfonium, tertiary sulfonium compounds and DMSP (Rhodes et al., 2002; Ashraf and Foolad, 2007). Ectoine is not discussed in this chapter since it is of bacterial origin. The above common osmolytes are synthesized from primary metabolic pathways and accumulated under conditions of abiotic stress in a wide range of plant families (Ahmad et al., 1981; Mansour, 2000; Slama et al., 2015). Being a proteogenic amino acid, proline occurs in all plant species. Glycine betaine is also the most common quaternary ammonium compound among plants exposed to a range of abiotic stresses (Guo et al., 2009).

Lower as well as higher plants synthesize and accumulate either one type or many types of osmolytes when exposed to one or multiple stresses simultaneously. But, all plants do not accumulate all kinds of osmolytes at the same level in the same tissues within the same or different families. Depending on the species and the type of environmental stress, the type and quantity or the level of accumulation (some species synthesize and accumulate low or high or may not accumulate) also varies to a large extent (Ashraf and Foolad, 2007; Szabados et al., 2011). Osmolyte synthesis in the cellular organelles is also important for low or high accumulation of glycine betaine as shown by Nuccio et al. (2000). Both glycophytes (e.g., *Citrus*) and halophytes accumulate several types of osmolytes at the same time (Gagneul et al., 2007). This indicates their synergistic mode of action when plants are exposed to abiotic stress conditions. Further, seasonal pattern, developmental stage of the plant, age of plant, type of tissue or organ and environmental parameters like temperature, moisture, nutritional status (including nitrogen) of the soil, pH of the medium greatly influence the accumulation (Hare et al., 1998; Murakeözy et al., 2003). Subcellular partitioning of several osmolytes is also reported depending on the growing conditions (Aubert et al., 1999; Kishor et al., 2005). While proline accumulates and also degrades rapidly, glycine betaine accumulates for a longer period (Gagneul et al., 2007) in comparison with proline. Accumulation of proline and glycine betaine can be cultivar or genotype specific. In *Guizotia abyssinica* (niger), both high and low proline accumulating cultivars have been found (Sarvesh et al., 1996). Similarly, some genotypes of *Sorghum bicolor* and corn accumulate glycine betaine, while others do not (Grote et al., 1994; Saneoka et al., 1995) inferring the occurrence of stress-tolerant and stress-susceptible genotypes within the species. Generally, accumulations of osmolytes are higher under stress due to increased biosynthesis or decreased degradation or both (Kishor et al., 2005; Khan et al., 2013, 2014; Per et al., 2017) indicating regulation of genes associated with such metabolic fluctuations and also homeostasis of biological reductants (Szabados and Savoure, 2010).

29.2.3.1 Quaternary Ammonium Compounds

Glycine betaine, β-alanine betaine, proline betaine, hydroxyproline betaine, choline-*O*-sulfate, and pipecolate betaine are the quaternary ammonium compounds found mostly in halophytic plant species (Ashraf and Harris, 2004). They are common among the members of the families Amaranthaceae (former Chenopodiaceae) and Plumbaginaceae (Slama et al., 2015). Among the many quaternary ammonium compounds, glycine betaine is the most commonly accumulated osmolyte when exposed to diverse abiotic stresses in plants (Hanson et al., 1991; Guo et al., 2009; Türkan and Demiral, 2009). Glycine betaine is accumulated in many halophytes with the exception of *Chenopodium quinoa*, *Noaea mucronata*, and others (Ruffino et al., 2010; Tipirdamaz et al., 2006). Nuccio et al. (1998) reported that endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase (CMO). Proline betaine and pipecolate betaine accumulate together in species like *Medicago sativa* and *Achillea* (Wood et al., 1991; Bonham et al., 1995). Stewart and Larher (1980) and Rosenthal (1982) reported that in Fabaceae, pipecolic acid is accumulated in high concentrations, which are derived from lysine. Biosynthesis of pipecolate betaine and hydroxypipecolate betaine is discussed under the biosynthesis part of this chapter.

29.2.3.2 Tertiary Sulfonium Compounds

Tertiary sulfonium compounds like DMSP are distributed in algae, in grasses like *Spartina alterniflora*, and in *Wollastonia biflora* (Otte et al., 2004). Methionine is the precursor for the synthesis of DMSP in both algae and higher plants (Kocsis and Hanson, 2000). While methionine is transaminated to form

4-methylthio-2-oxobutyrate in algae, it is methylated first to form *S*-methyl methionine and then converted to dimethylsulfoniopropionaldehyde (DMSP-ald) by some mechanism. It is believed that DMSP is a sulfur detoxifier and an osmoregulator and accumulates both in cytoplasm and vacuoles (Otte et al., 2004). Nakajima et al. (2014) isolated DMSP from a green sea alga and found that it suppresses Ehrlich ascites carcinoma.

29.2.3.3 Sugars and Sugar Alcohols

Several plants accumulate simple sugars such as glucose, fructose, sucrose, trehalose, and fructans under salt and drought stress conditions (Briens and Larher, 1982; Yuanyuan et al., 2009) in families like Amaranthaceae, Brassicaceae, Cyperaceae, Juncaceae, Plumbaginaceae, Poaceae, etc. (Slama et al., 2015). Both sugars and polyols are the dominant solutes in many plants, highly soluble in water and hence act as compatible solutes. The nonreducing disaccharide trehalose was first discovered in resurrection plants, but not widely distributed in higher plants (Lunn et al., 2014). Several cyclic polyols (e.g., pinitol) and noncyclic (e.g., mannitol, sorbitol) sugar alcohols accumulate both in halophytic and nonhalophytic species. While pinitol is common in the members of the families Aizoaceae and Fabaceae, mannitol occurs mostly in Combretaceae. Pinitol is widely reported in halophytic species and is derived from the methylation of myo-inositol. Epimerization of ononitol also leads to the production of pinitol (Sengupta et al., 2008). But, the biosynthesis of these osmolytes is an energy consuming process, requiring many molecules of ATP (Flowers and Colmer, 2008). Then, what makes plants use organic osmotica or osmolytes under stress in place of cheaper inorganic osmotica such as Na^+ and Cl^- is not known. Probably, glycophytes do not have the ability for tissue tolerance of Na^+/Cl^-, and hence depend on the accumulation of compatible organic solutes for osmotic adjustment under stress.

29.3 BIOSYNTHETIC PATHWAYS OF PROLINE AND ITS DERIVATIVES

29.3.1 Biosynthesis of Proline

Different routes of proline biosynthesis from glutamate and ornithine are shown in the Fig. 29.1. Also, proline derivatives like proline betaine are shown in Fig. 29.1. The differences that exist in the proline anabolic and catabolic pathways and the genes that encode the enzymes involved both in bacteria and plants, their localizations in different cellular compartments have been reviewed earlier (Kishor et al., 2005; Szabados and Savoure, 2010; Per et al., 2017). But, proline accumulation under stress conditions is not a universal phenomenon.

29.3.2 Biosynthesis of Proline Derivatives

Proline is converted to hydroxyproline by hydroxylation. Proline betaine is seen in members of Plumbaginaceae, Capparidaceae, Rutaceae, Labiatae, Compositae, and Leguminosae (Wyn Jones and Storey, 1981; Hanson et al., 1994). It has been pointed out that proline betaine catabolism contributes to rhizobial colonization of seedling roots (Phillips et al., 1998). Both proline and hydroxyproline can be converted to L-proline betaine (also called *N,N*-dimethyl-L-proline or stachydrine) and hydroxyproline betaine (known as *trans*-4-hydroxy-L-proline betaine or betonicine) respectively. Proline is first converted to *N*-methylproline by a methyl transferase and later to proline betaine by methylation again (Fig. 29.1). Likewise, hydroxyproline is converted first to *N*-methyl hydroxyproline by a methyl transferase enzyme. *N*-Methyl hydroxyproline is then converted to hydroxyproline betaine by methylation again (Fig. 29.1). Isotope-labeling studies by Trinchant et al. (2004) in alfalfa indicate that

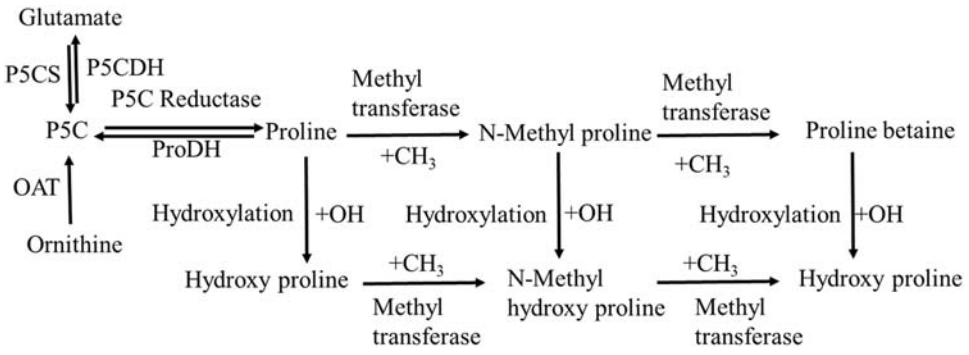

FIGURE 29.1 Biosynthesis of proline, proline betaine (stachydrine) and hydroxyproline betaine (betonicine) in bacteria and plants. *P5CS*, Δ^1-pyrroline-5-carboxylate synthetase; *P5C*, pyrroline-5-carboxylate; *P5CR*, P5C reductase; *ProDH*, proline dehydrogenase; *P5CDH*, P5C dehydrogenase; *OAT*, ornithine δ-aminotransferase; *CH_3*, methyl group; *OH*, hydroxyl group.

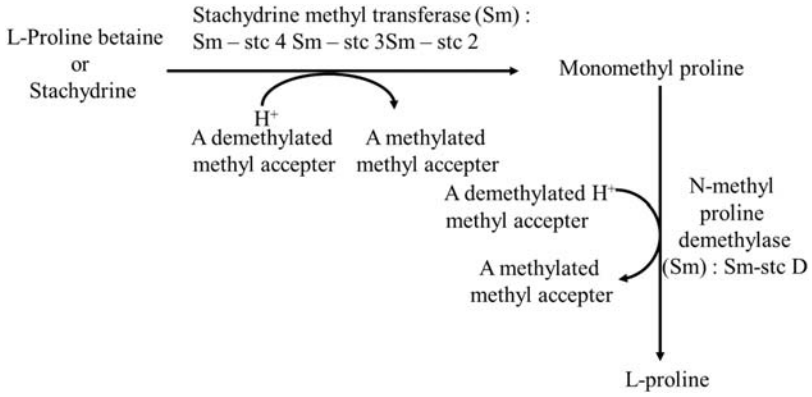

FIGURE 29.2 Catabolism of L-proline betaine to L-proline in *Sinorhizobium meliloti*.

[^{14}C] proline betaine is synthesized from L-[^{14}C] proline. Nolte et al. (1997) and Trinchant et al. (2004) reported biosynthesis of proline betaine (Fig. 29.1) as a long-term response to salinity in *Citrus* and *Medicago* species respectively. Both proline betaine and hydroxyproline betaine (albeit to a lesser extent) are simultaneously produced in species like *Citrus* that grow in chronically dry areas (Nolte et al., 1997). Proline betaine and hydroxyproline betaine protected bacteria against salt stress, but not *N*-methyl-L-proline and *trans*-4-L-hydroxyproline (Bashir et al., 2014). Hydroxyproline betaine is synthesized from hydroxyproline by a methyl transferase enzyme (Kishor et al., 2005). In species like *Citrus*, methylated derivatives of proline such as *N*-methylproline (hygric acid), 4-hydroxy-L-proline betaine, and *N*,*N*-dimethyl-L-proline accumulate to ameliorate abiotic stress (Servillo et al., 2011a). Both proline betaine and hydroxyproline betaine have been believed to be metabolically inert as cell protectants against salt, drought, and temperature stresses. But, Kumar et al. (2014) reported that several of the hydroxyproline betaine degradation pathway enzymes also function in proline betaine degradation in bacteria such as *Paracoccus denitrificans* and *Rhodobacter sphaeroides*. Proline betaine is ultimately converted back to glutamate in bacteria. Proline betaine catabolic pathway was repressed by both osmotic and cold stresses in these species. However, it is not known if such a catabolic pathway operates in plants also. Trinchant et al. (2004) showed that proline betaine is catabolized through sequential demethylations via *N*-methylproline and proline (Fig. 29.2). L-proline betaine is used by *Sinorhizobium meliloti* as a carbon and nitrogen source and as an osmoprotectant (Goldmann et al., 1991). Devoid of any stress, this organism degrades proline betaine. But, in osmotically stressed cells, it is strongly reduced resulting in significant amounts of accumulation (Gloux and Le Rudulier, 1989). Boscari et al. (2002) identified a DNA segment encoding a protein named as BetS in *Sinorhizobium meliloti*. This gene displays significant sequence identities to the choline transporter BetT of *Escherichia coli* and to the glycine betaine transporter OpuD of *Bacillus subtilis*. Thus, BetS appears as a major glycine betaine/proline betaine transporter needed for early osmotic adjustment in *Sinorhizobium meliloti*. L-proline betaine is degraded like glycine betaine that involves two sequential demethylations as shown in Fig. 29.2 and produces monomethyl glycine in bacteria (Smith et al., 1988). While the first demethylation reaction is catalyzed by a Rieske type iron-sulfur monooxygenase enzyme (Burnet et al., 2000), the second is carried out by oxidoreductase flavoproteins (Phillips et al., 1998). Using radioisotope [^{14}C], Trinchant et al. (2004) reported that proline betaine is catabolized through sequential demethylations via *N*-methylproline and proline in alfalfa. The compartmentalization of proline betaine and proline within root nodules revealed that salt stress induced an increase in cytosol and bacteroids (Trinchant et al., 2004). But, the existence of proline betaine degrading enzymes in higher plants (other than nodules) has not been reported yet. Also, Kim et al. (2017) discovered that *trans*-4-L-hydroxyproline also acts as a compatible solute in *Halobacillus halophilus*. This species accumulates glycine betaine, glutamine, proline, and ectoine to cope with salt stress conditions. It appears that *trans*-4-hydroxy-L-proline (Hyp) is accumulated in *H. halophilus* in response to salinity. This suggests that Hyp may be a vital compatible solute in this species. They have also identified the candidate genes associated with the biosynthetic pathway of Hyp.

29.4 BIOSYNTHETIC PATHWAYS OF GLYCINE BETAINE AND ITS DERIVATIVES

29.4.1 Biosynthesis of Glycine Betaine

Multiple pathways exist for the biosynthesis of glycine betaine (Fig. 29.3A–C). It is synthesized from choline (through a series of reactions, L-serine is converted

to choline in plants), which is converted to betaine aldehyde by CMO in higher plants (Fig. 29.3A). Ethanolamine is the precursor of choline, which is converted to monomethyl ethanolamine by the addition of a methyl group from *S*-adenosylmethionine (SAM). While choline is formed in cytosol, it is transported to the chloroplasts where glycine betaine is synthesized (Weretilnyk et al., 1989). Once it is synthesized, it is transported to different plant parts via phloem (Hattori et al., 2009) through proline porters in plants (common for both proline and glycine betaine). However, it is not known if proline porters also transport proline betaine and hydroxyproline betaine in higher plants. Like proline, younger leaves accumulated more glycine betaine than the older ones under stress (Chen and Murata, 2011; Khan et al., 2012; Masood et al., 2016). In barley, glycine betaine mostly accumulated in vascular tissues of leaves and the pericycle of roots. In most of the glycine betaine accumulating species, it is synthesized from betaine aldehyde by the action of the enzyme betaine aldehyde dehydrogenase (BADH) (Ashraf and Foolad, 2007; Fitzgerald et al., 2009). Nuccio et al. (1998) inserted a chloroplastic glycine betaine synthesizing pathway gene into tobacco and the transformants produced very little amounts. Contrary to this, a cytosolic glycine betaine synthesis pathway accumulated more glycine betaine (Nuccio et al., 2000). This suggested that subcellular localization influences the glycine betaine pathway flux. But in bacteria, three pathways exist. In soil bacterium *Arthrobacter globiformis*, choline is converted to glycine betaine by a single-enzyme choline oxidase (Fig. 29.3B), an H_2O_2-generating oxidase (Ikuta et al., 1977). In *E. coli*, choline is catalyzed by choline dehydrogenase (Fig. 29.3C) into betaine aldehyde. In extreme halophiles like *Ectothiorhodospira halochloris* and *Actinopolyspora halophila* glycine betaine is formed by a novel pathway from glycine through three *N*-methylations with SAM as a methyl donor, sarcosine and dimethylglycine as intermediates. The reactions are catalyzed by glycine sarcosine methyltransferase (GSMT) and sarcosine dimethylglycine methyltransferase (SDMT). GSMT first converts glycine to sarcosine and also sarcosine to dimethylglycine (with overlapping functions). Again SDMT catalyzes the methylation of sarcosine or dimethylglycine to dimethylglycine or glycine betaine respectively (Nyyssölä et al., 2000; Kimura et al., 2010). Similar pathway also exists in *Myxococcus xanthus* (Kimura et al., 2010). The enzyme BADH is common for both plants and animals that can convert betaine aldehyde (formed from choline) to glycine betaine. Thus, differences in glycine betaine pathway exist between bacteria and higher plants.

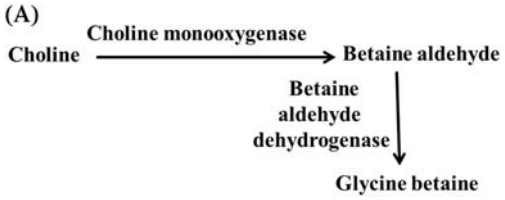

FIGURE 29.3A Biosynthesis of glycine betaine in *E. coli*.

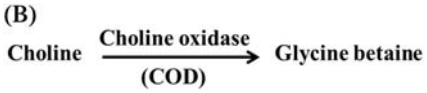

FIGURE 29.3B Biosynthesis of glycine betaine in higher plants.

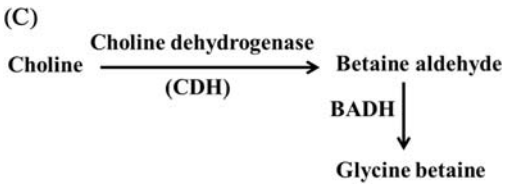

FIGURE 29.3C Biosynthesis of glycine betaine in *Arthrobacter globiformis*. *BADH* = Betaine aldehyde dehydrogenase.

29.4.2 Biosynthesis of Glycine Betaine Derivatives

Rathinasabapathi et al. (2001) reported biosynthesis of β-alaline betaine by the catalysis of SAM-dependent N-methylation of β-alanine via *N*-methyl β-alanine and *N*, *N*-dimethyl β-alanine. Glycine betaine requires oxygen for its biosynthesis, but not β-alanine betaine. β-Alanine betaine may be a more suitable osmoprotectant in halophytic species (e.g., *Limonium latifolium* and others) than glycine betaine under saline hypoxic conditions since it can avoid the requirement of oxygen for its biosynthesis (Hanson et al., 1991, 1994). The genus *Citrus* contains osmolytes like trigonelline (nicotinic acid betaine) and choline, but not GABA betaine (Servillo et al., 2011b). Nicotinic acid is converted to nicotinic acid betaine by nicotinic acid N-methyltransferase. Interestingly, *Citrus bergamia* and *Medicago sativa* produce pipecolic acid betaine or pipecolate betaine, also known as homostachydrine (Servillo et al., 2012). Accumulation of pipecolic acid betaine is reported under stress conditions in vegetables, *Citrus* species, *Medicago*, and *Achillea* (Wood et al., 1991; Moulin et al., 2006; Servillo et al., 2012). Pipecolate betaine is synthesized from pipecolic acid via N-methylpipecolate with the addition of methyl groups in each step (Fig. 29.4). Pipecolic acid is also converted to hydroxypipecolate by the addition of OH group. Hydroxypipecolate is first converted to N-methylhydroxypipecolate and then to hydroxypipecolate betaine with the addition of methyl groups

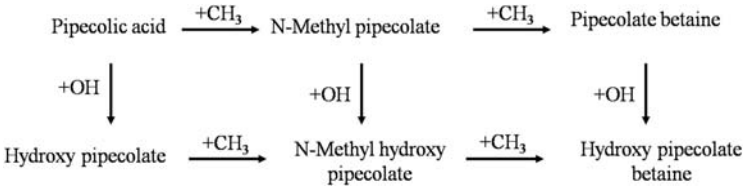

FIGURE 29.4 Biosynthetic pathway of pipecolate betaine and hydroxyl pipecolate betaine.

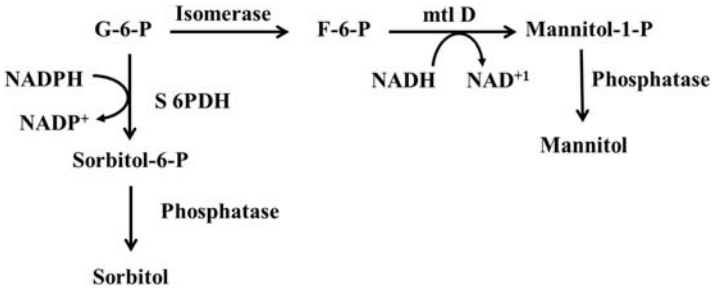

FIGURE 29.5 Biosynthesis of mannitol and sorbitol in plants. *G-6-P*, glucose-6-phospahate; *mtlD*, mannitol-1-phospahate; *S6PDH*, sorbitol-6-phosphate dehydrogenase; *mannitol-1-P*, mannitol-1-phosphate.

(Fig. 29.4). However, the enzymes that catalyze the pipecolic acid to the betaines are not exactly known. Presence of pipecolic acid in *Citrus* and *Medicago* species led to the postulation that it serves as a homostachydrine precursor through direct methylation. Thus, the pathways for the synthesis of proline and glycine betaine vary depending upon the species but all the pathways are not well characterized in higher plants.

29.5 BIOSYNTHETIC PATHWAYS OF TREHALOSE AND SUGAR ALCOHOLS

29.5.1 Biosynthesis of Trehalose (Sugar)

Biosynthesis of trehalose is complicated and three different pathways have been noticed to date. OtsA in *E. coli* and the enzyme trehalose phosphate synthase catalyze the transfer of glucose from UDP-glucose to glucose-6-phosphate (G-6-P) to produce trehalose-6-phosphate and uridine diphosphate (Cabib and Leloir, 1958; Roth and Sussman, 1966). Trehalose-6-phosphate is then converted to trehalose by dephosphorylation by phosphatase enzyme. Organisms that use OtsA pathway have a trehalose-P phosphatase or OtsB in *E. coli* to produce trehalose (Elbein et al., 2003). The second pathway for trehalose biosynthesis (e.g., *Pimelobacter* species) involves intramolecular rearrangement of maltose (glucosyl-a1-4-glucopyranoside) to convert the 1,4-linkage to the 1,1-bond of trehalose by the enzyme trehalose synthase (Nishimoto et al., 1996). Trehalose synthesis was also reported from some bacteria (e.g., *Sulfolobus acidocaldarius*) involving the conversion of maltooligosaccharides or starch to trehalose. Maltooligosyltrehalose synthase (TreY) catalyzes the conversion of maltodextrin to maltooligosyltrehalose and then maltooligosyltrehalose trehalohydrolase (TreZ) hydrolyzes this to trehalose (Maruta et al., 1996). In the third pathway (like in mushrooms), glucose is rearranged at the reducing end of a glycogen chain to convert the alpha 1,4-linkage to an a, a1,1-bond. A second enzyme then produces the disaccharide trehalose (Elbein et al., 2003).

29.5.2 Biosynthesis of Sugar Alcohols

Both mannitol and sorbitol accumulate in large quantities and are widespread in higher plants. Mannitol has been detected in more than 50 families. But, other sugar alcohols like dulcitol and ribitol are less represented in plants (Lewis and Smith, 1967). Glucose-6-phosphate is the precursor for the synthesis of sugar alcohols such as mannitol and sorbitol. G-6-P is first converted to fructose-6-phosphate (F-6-P) by an isomerase enzyme. F-6-P is then converted to mannitol and the reaction is catalyzed by mannitol-1-phosphate dehydrogenase (mtlD). Mannitol-1-phosphate is converted to mannitol by a phosphatase enzyme (Fig. 29.5). Mannitol not only acts a compatible solute but it also protects the plants against abiotic stress. Besides, it has a possible role in plant responses to pathogen attack (Stoop et al., 1996). Sorbitol-6-phosphate dehydrogenase (S6PDH) catalyzes the conversion of G-6-P to sorbitol-6-phosphate (S6P), releasing $NADP^+$. S6P produces sorbitol (Fig. 29.5) by dephosphorylation (Shen et al., 1999). *Mesembryanthemum crystallinum*, a facultative halophyte, produces rare sugar alcohols like pinitol (ID-3-*O*-methyl chiro-inositol) from inositol (a polyol and a 6-carbon compound). Pinitol is accumulated during salt and drought stress in this halophyte and also in many plants. In the pathway, a methylation step is catalyzed by a position specific SAM-dependent *O*-methyl transferase. Ononitol is then converted to pinitol by an

epimerase reaction that requires NAD^+ and/or NADPH (Loewus and Dickinson, 1982). But tracer studies with labeled inositols revealed that the biosynthesis of D-pinitol in several leguminous plants like *Medicago sativa*, *Trifolium incarnatum*, *Simmondsia chinensis* does not occur by epimerization of sequoyitol. It only occurs via D-ononitol (Dittrich and Brandl, 1987). Thus, rare sugar alcohols like pinitol and ononitol are produced not only in halophytic species, but also in glycophytes like in leguminous plants.

29.6 CONCLUSIONS

Diverse osmolytes have been identified in a wide range of microbial and plant species with multiple biosynthetic pathways. Unfortunately, the biosynthetic pathway enzymes and the corresponding genes have not yet been isolated in higher plants. However, it has been recognized that osmolytes perform multifarious functions during abiotic stress conditions though the exact molecular mechanisms are not completely known. Thus, several gaps exist in our understanding of the osmolyte biosynthesis and their precise regulation at the cellular level.

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CHAPTER

30

Role and Regulation of Osmolytes as Signaling Molecules to Abiotic Stress Tolerance

Titash Dutta[1], *Nageswara Rao Reddy Neelapu*[1], *Shabir H. Wani*[2,3] *and Challa Surekha*[1]

[1]Department of Biochemistry and Bioinformatics, Institute of Science, Gandhi Institute of Technology and Management (GITAM Deemed to be University), Visakhapatnam, Andhra Pradesh, India [2]Mountain Research Centre for Field Crops, Sher-e-Kashmir University of Agricultural Sciences and Technology, Srinagar, Jammu and Kashmir, India [3]Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, United States

OUTLINE

30.1 INTRODUCTION

Abiotic stresses, predominantly salinity, drought, flooding, metals, and extreme temperatures contribute to significant loss in global crop production. The scenario is further aggravated by the growing population, which is expected to cross 9.1 billion in 2050 (Sah et al., 2016). According to a survey, it was estimated that 60% increase in production of important crops such as cereals and legumes is necessary to meet the food supply of the growing population (FAO, 2010).

Abiotic stress factors are considered detrimental for crops as they minimize photosynthesis, photosynthetic pigments, mineral use efficiencies, pollen viability, seed quality, and yield (Khan et al., 2012, 2013, 2014, 2015, 2016; Nath et al., 2017; Sade et al., 2017).

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Acquaah (2007) reported that exposure to abiotic stresses lead to 70% reduction in crop yield annually. Plants are unable to avoid stress exposure due to their sessile nature. Abiotic stresses severely impair the cellular homeostasis and increases reactive oxygen species (ROS) accumulation, eventually leading to plant death (Khan and Khan, 2017; Zandalinas et al., 2018). Subsequently changes in global climate pattern and diminished rainfall have increased the severity and frequency of the abiotic stress conditions. Thus, the world food security is under constant threat as yield loss of agriculturally important crops (rice, maize, wheat, and barley) has been associated with climate change and abiotic stresses (Jaleel et al., 2009; Khan and Khan, 2013).

Plants at the molecular level trigger a sequence of biochemical events in response to the abiotic stress conditions. Stress tolerance is conferred by transcriptionally regulating specific gene families (Joshi et al., 2018). These genes families are classified into three categories based on their function. The first category contains genes involved in osmoprotection such as osmoprotectants, antioxidant enzymes, late embryogenesis abundant (LEA) proteins, and heat shock proteins. The second category comprises the genes coding ion transporters such as Na^+/H^+ channels and aquaporins (facilitates water uptake). The third category includes the genes responsible for signal perception and transcriptional regulation such as mitogen-activated protein kinases (MAPKs), salt overly sensitive kinases (Ji et al., 2013), ethylene-responsive element binding proteins, heat shock transcriptional factors, CBF/DREB (C-repeat-binding /dehydration-responsive element), bZIP (basic-domain leucine zipper), NAC (NAM, ATAF, and CUC), MYC (myelocytomatosis oncogene)/MYB (myeloblastosis oncogene), Cys2/His2 zinc-finger motifs, and WRKY protein domains (Umezawa et al., 2006). During the last decade, plant breeders focused on developing potential stress tolerant cultivars by incorporating transgenic approaches in conventional breeding techniques. The transgenic cultivars exhibit enhanced qualitative and quantitative traits and induce stress tolerance (Wani et al., 2016; Bhatnagar-Mathur et al., 2008).

Salt and drought stress are responsible for cellular dehydration. Loss of intracellular water to denaturation of cellular proteins alters the cellular homeostasis. To minimize the water loss, maintain cellular integrity, and protect the proteins, plants have evolved strategies to accumulate specific organic molecules classified as osmolytes or compatible solutes. The hallmark of these osmolytes includes low molecular weight and high solubility. Proline, glycine betaine (GB), polyols, sugar alcohols, and soluble sugars are the important osmolytes that accumulate in plants in response to abiotic stresses. These osmolytes are also involved in scavenging of ROS, maintaining cellular redox potential and pH, osmotic adjustments, and stabilizing proteins structures and membranes.

Thus, osmolyte biosynthesis and their accumulation in plants contribute towards osmoprotection thereby conferring abiotic as well as secondary (osmotic and ionic) stress conditions. Genome-wide screening and transcriptome analysis of major cereals, pulses, and in *A. thaliana* exposed to abiotic stresses led to identification of a set of genes involved in the biosynthetic pathway of these osmolytes (Suprasanna et al., 2016; Chakraborty and Sairam, 2017). Thus, the focus lies in developing transgenic cultivars by introducing the candidate genes of these osmolytes to enhance stress tolerance and crop productivity (Surekha et al., 2015). Several transgenic cultivars have been developed successfully by targeting osmoprotection. The transgenic plants exhibited improved grain yield, biomass, and abiotic stress tolerance. These include rice (Garg et al., 2002; Su and Wu, 2004), potato (Zhang et al., 2011b), wheat (Sawahel and Hassan, 2002), tomato (Park et al., 2007), maize (Quan et al., 2004), tobacco (Szabados and Savouré, 2010), soyabean (Qin et al., 2017) and pigeonpea (Surekha et al., 2014).

The present review article focuses on identifying the various osmolytes that plants accumulate, and their specific role and regulation as signaling molecules in response to abiotic stresses. Extensive research on their biosynthetic pathways highlighted the candidate genes essential for their synthesis. The review also features the transgenic plants developed bearing the candidate biosynthetic genes thereby enhancing osmoprotection and abiotic stress tolerance.

30.2 OSMOLYTE MEDIATED ABIOTIC STRESS RESPONSES

Exposure to abiotic stress initiates morphological, physiological, metabolic, and molecular changes that significantly hamper plant development and survival. Prolonged exposure to salinity inhibits plant growth as observed in the biphasic response model: initial phase (disruption of cellular homeostasis) and the final phase (disruption of ionic homeostasis) (Munns, 2002; Adem et al., 2014; Wani et al., 2017). To counter these stress conditions, plants have evolved three strategies, namely, ion exclusion, Na^+ compartmentalization into vacuoles, and osmoprotection (Munns, 2002; Wani et al., 2017).

In stressed plants, osmoprotection is achieved by increasing the synthesis and accumulation of specific osmolytes such as GB and proline (Ashfaque et al., 2014; Masood et al., 2016; Per et al., 2017). The osmolytes are generally characterized by their low

molecular weight, high polarity, solubility, and hydrophilic nature. These features allow the solutes to protect the membrane protein structures during stress conditions. The osmolytes accumulated in plants are grouped into three classes: (1) free amino acids (proline), (2) quaternary amines (GB and polyamines (PAs)), and (3) sugars and sugar alcohols (trehalose, fructans, mannitol, and sorbitol) (Yancey, 2005; Roychoudhury et al., 2015). GB and trehalose serve as osmoprotectants by stabilizing the membranes and the quaternary structures of proteins. Mannitol acts as a free-radical scavenger. Proline functions as a vital storage sink for carbon and nitrogen as well as a free-radical scavenger (Kaur and Asthir, 2015). Moreover it also lends stability to subcellular structures (membranes and proteins), and maintains the cellular redox potential under abiotic stress. Due to their role in osmoprotection, these organic osmolytes are termed as osmoprotectants (Chen and Murata, 2002; Surekha et al., 2014; Roychoudhury et al., 2015; Blum, 2017).

Osmolyte mediated abiotic stress response involves compartmentalization of osmolytes at the subcellular level, thereby reducing the water potential and enhancing osmoregulation. Niu et al. (1995) reported reduced water potential is associated with regulation of tissue water content in saline stressed soils. They also function as molecular chaperones by reducing protein misfolding, stabilizing integral protein structures and membrane. Osmolytes form strong hydrogen bonding with these protein structures, which prevents their denaturation and simultaneously enhances their stability and integrity (Kumar, 2009; Slama et al., 2015). Moreover they also scavenge hydroxyl radicals (Ozgur et al., 2013). Oxidative damage is the major detrimental effect of abiotic stress characterized by elevated levels of ROS affecting the organelles due to cellular toxicity. Osmolytes scavenge hydroxyl radicals, singlet oxygen species (Roychoudhury and Chakraborty, 2013) thus lowering the lipid peroxidation level, which serves as plant ROS status indicator. The importance of osmolytes has been thoroughly exploited by researchers towards development of transgenic cultivars able to express the candidate biosynthetic genes leading to enhanced osmolyte accumulation and abiotic stress tolerance (Singh et al., 2015; Wani et al., 2017).

Osmolyte accumulation is triggered by the onset of abiotic stresses (salt, drought and cold). The stress stimuli is first sensed by the primary sensors (histidine kinase) and is passed onto specific transcriptional factors (MYC/MYB, AREB/ABF, NAC/ZF-HD, DREB, etc.) that regulate the expression of specific genes involved in the biosynthesis of osmolytes (Zhou et al., 2016). The entire stress perception and signaling is mediated by the MAPK pathway (Fig. 30.1). Stress

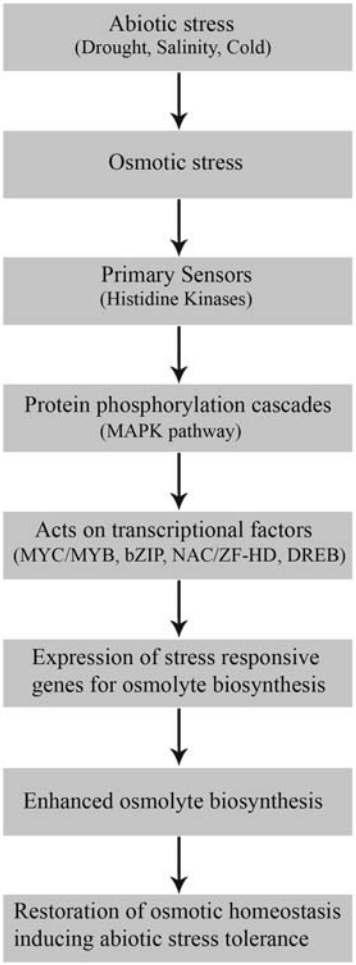

FIGURE 30.1 Abiotic stress signal perception, transduction, and response.

stimuli perception is carried out via a protein phosphorylation cascade. The signals of the plasma membrane receptors activate MAP kinase kinase kinases (MAPKKK), which subsequently activates MAP kinase kinases (MAPKK) and MAPK via reversible phosphorylation (Rodriguez et al., 2010). The MAPK regulates the transcription factors (TFs) that in turn regulate the osmolyte biosynthetic genes.

30.3 BIOSYNTHESIS, ACCUMULATION, AND REGULATION OF OSMOLYTES IN RESPONSE TO ABIOTIC STRESS

30.3.1 Proline

Proline is structurally an α-amino acid derivative, and plays a key role in regulating plant growth and development under stressed environment. The high solubility of proline in water (1.54 kg/L) makes it an automatic choice for an ideal solute (Huang et al., 2008).

Proline synthesis is common in innumerable plant species both under stressed and nonstressed environments. Under nonstressed circumstances proline is involved in regulation of seed formations and embryo development (Mattioli et al., 2009) and it functions as precursor for enzyme and protein synthesis (Nanjo et al., 1999; Vives-Peris et al., 2017). Moreover proline enhances the stability of protein secondary structures due to its superior conformational rigidity (Lehmann et al., 2010; Funck et al., 2012) and also serves as a reservoir for storing cellular carbon and nitrogen during the plant recovery phase (Kavi Kishor et al., 2005).

A high level of proline accumulation is frequently observed when plants are exposed to salt and drought stressed environments (Per et al., 2017; Mansour and Ali, 2017). Under such conditions, it serves as an osmoprotectant by restoring the adverse effects of the secondary stress factors (oxidative and osmotic). As plants are exposed to salinity over a prolonged period, high levels of proline get accumulated in the cytoplasm, which regulates the cellular homeostasis and redox potential (Heuer, 2003) and protein secondary structural stability (Suprasanna et al., 2014), and scavenges free radicals thereby suppressing oxidative stress (Kavi Kishor et al., 2014; Matysik et al., 2002).

Endogenous proline production in all plant species at an optimum level is essential for its use as an osmoprotectant or compatible solute. However, studies reveal that important cereals including wheat, rice, and maize are not able to synthesize adequate levels of proline necessary to negate the deleterious effects of salinity and drought and promote plant revival (Slama et al., 2015). Therefore, to enhance the endogenous levels of proline, the plants were treated with exogenous supply of proline (Ashraf and Foolad, 2007). Exogenous application of proline in alfalfa (Ehsanpour and Fatahian, 2003), ice plant (Shevyakova et al., 2009), *A. thaliana* (Hare et al., 2003), rice (Bhusan et al., 2016), maize (Alam et al., 2017), *Brassica juncea* (Arif et al., 2017), and soybean (Sabagh et al., 2017) improved the endogenous proline accumulation. However in some plants, such as spinach and rapeseed, exogenous application was associated with adverse side effects due to noncompatibility of the accumulated proline with intracellular environments (Sulpice et al., 1998). Such drawbacks persuaded scientists to elucidate the machinery of biosynthetic pathways of these solutes and identify enzymes that play a key role in their synthesis. These results have been incorporated to develop transgenic plants so as to upregulate the endogenous level of osmolytes thereby inducing abiotic stress tolerance.

In plants, cytosol is the site of proline synthesis and can be accomplished by any of the two pathways: glutamate or ornithine pathway (Kavi Kishor et al., 2005; Suprasanna et al., 2014; Zarattini and Forlani, 2017) (Fig. 30.2). The glutamate pathway is completed in two enzyme dependent steps and requires two NADPH molecules. The first step involves ATP dependent phosphorylation of glutamate thereby activating it. This is followed by reduction of the activated glutamate to glutamatic-γ-glutamyl kinase (GSA) and its subsequent cyclization to pyrroline 5-carboxylate (P5C). The cyclization of GSA to P5C is catalyzed by pyrroline 5-carboxylate synthetase (P5CS) enzyme. The final enzyme catalyzed step of the glutamate pathway involves reduction of the P5C to proline by pyrroline 5-carboxylate reductase (P5CR) enzyme. The alternative ornithine pathway uses ornithine as the precursor for proline biosynthesis instead of glutamate. The steps involve transamination of ornithine to P5C catalyzed by Orn-D-aminotransferase and reduction of P5C to proline by the enzyme P5CR.

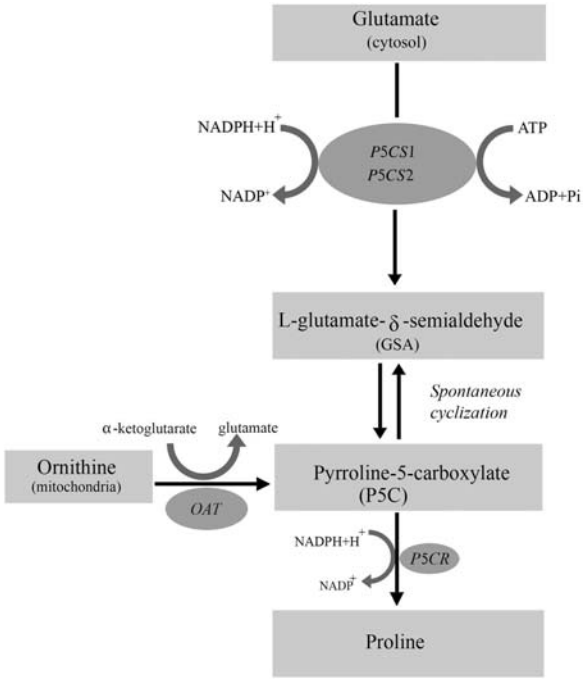

FIGURE 30.2 Proline biosynthesis pathway. *P5CS1/P5CS2*, pyrroline 5-carboxylate synthetase; *P5CR*, pyrroline 5-carboxylate reductase; *OAT*, ornithine D-aminotransferase.

The mechanism regulating proline biosynthesis has been studied in the model plant *A. thaliana* (Yoshiba et al., 1995), tobacco (Delauney and Verma, 1993), and rice (Lutts et al., 1999; Joseph et al., 2015). The findings identified the two enzymes P5CS and P5CR as potential candidate genes capable of regulating proline production under abiotic stressed environments. Two genes, *P5CS1* and *P5CS2*, code for the enzyme P5CS whereas a single gene codes for P5CR enzyme. These two genes have been incorporated in many economically important crops to develop their transgenic counterparts

capable of increased proline accumulation under stress thus negating the adverse effects of osmotic stress induced by salt and drought stress.

Kishor et al. (1995) developed transgenic cultivars overexpressing the *P5CS* gene from *Vigna aconitifolia* (moth bean). The transformed cultivars showed increased proline accumulation compared with the nontransformed (wild) plants by 10- to 18-fold. The enhanced levels of proline contributed to flower development and increased root and plant dry biomass. There was one major concern: the negative feedback inhibition of the P5CS enzyme due to increased proline accumulation. This glitch was taken care of when Hong et al. (2000) developed the *P5CS129A* gene, which was a mutated version of the *V. acontifolia* P5CS enzyme. They substituted the phenylalanine (Phe) residue at the 129th position with an alanine (Ala) residue. The mutated version of the enzyme did not exhibit negative feedback inhibition and increased proline accumulation by 36-fold in the transformed tobacco. Kumar et al. (2010) achieved similar results when they developed transgenic rice overexpressing the mutated *P5CS129A* gene. Moreover, the transgenic lines showed lower lipid peroxidation level when exposed to 150 mM NaCl stress.

The same *V. acontifolia P5CSF129A* gene increased fourfold more proline accumulation in first generation of transgenic *Cajanus cajan* lines under 200 mM NaCl stress thereby inducing salt tolerance (Surekha et al., 2014). The transgenic lines showed increased chlorophyll content and lower lipid peroxidation levels, which resulted in better growth and development. Similarly, Gleeson et al. (2005) overexpressed the same gene in *Larix leptoeuropaea* (forest tree), proline content increased significantly by 30-fold in the transgenic plants in comparison with its wild type counterparts. The transformed cultivars exhibited remarkable growth and recovery when subjected to salt (200 mM NaCl) and low temperature (4°C) stress.

The rice *OsP5CS1* and *OsP5CS2* genes were expressed together in tobacco by Zhang et al. (2014). The transgenic cultivars of the second generation (T_2) recorded 3.2-fold increased proline accumulation along with improved root length and mean fresh weight in comparison with its wild type counterparts treated with 200 mM NaCl. Similarly Chen et al. (2013) coexpressed the *Phaseolus vulgarisP5CS* (*PvP5CS1* and *PvP5CS2*) genes in the model plant, *A. thaliana*. The transgenic lines were subjected to different salt concentrations (0, 100, and 200 mM NaCl) and they accumulated 1.6 times and 1.9 times more proline at 100 and 200 mM NaCl concentration respectively.

The other enzymes involved in proline biosynthesis and degradation (P5CS, P5CR, ornithine aminotransferase (OAT) and P5CDH) have also been exploited to develop transgenic rice (Su and Wu, 2004; Karthikeyan et al., 2011), sugarcane (Guerzoni et al., 2014), wheat (Vendruscolo et al., 2007), olive (Behelgardy et al., 2012) carrot (Han and Hwang, 2003), Jerusalem artichoke (Huang et al., 2013), *Kosteletzkya virginica* (Wang et al., 2015), and sweet potato (Liu et al., 2014) with enhanced abiotic stress tolerance (Table 30.1). All these experiments aimed at improving proline accumulation in transgenic plants highlight the function of these enzymes in regulating the osmolyte levels depending on the environmental conditions. Moreover proline also restores cellular homeostasis by reducing oxidative damage scavenging free radicals.

30.3.2 Glycine Betaine

GB represents quaternary amines and is a derivative of *N*-trimethyl glycine. The properties of GB that make it a suitable osmolyte includes its low molar mass, water solubility, amphoteric nature, and nontoxicity even in high concentrations. Its amphoteric nature allows its interaction with hydrophilic and hydrophobic side chains of macromolecules (Gupta and Huang, 2014). In plants, GB serves as an effective compatible solute by regulating cellular osmotic homeostasis, shielding photosystem II and thylakoid membranes, maintaining protein stability and reducing oxidative stress (Khan et al., 2009; Allakhverdiev et al., 2003; Kurepin et al., 2017). But very few plants, namely sugar beet, maize, spinach, and barley are natural GB accumulators (Kishitani et al., 1994; Chen and Murata, 2008). However, the amount of GB accumulated is negligible and is triggered when plants are exposed to abiotic stress (Wani et al., 2013). The major agricultural plants such as potato, rice, eggplant, and tomato are naturally nonaccumulators of GB (De Zwart et al., 2003; Park et al., 2004). The cause can be attributed to loss of functional domains, premature stop codons, and shortened transcripts of the genes involved in the GB biosynthetic pathway (Rasheed et al., 2017). To overcome the shortcomings, researchers aimed at developing transgenic lines of these nonaccumulators harboring candidate GB biosynthetic genes from other species to increase the endogenous GB levels (Khan et al., 2009; Chen and Murata, 2011).

In plants GB biosynthetic pathway takes place in the stroma of the chloroplast and involves the precursor choline molecule (Sakamoto and Murata, 2002). The two steps involve oxidation of choline to betaine aldehyde catalyzed by choline monooxygenase (CMO) followed by its oxidation to GB. The final step is catalyzed by betaine aldehyde dehydrogenase (BADH) and involves the cofactor NAD^+ (Wani et al., 2013;

TABLE 30.1 List of Transgenic Plants Overexpressing Candidate Genes for Proline Accumulation

S. no.	Transgene	Host	Target crop plants/trees	Remarks	References
1	P5CS	*Vigna aconitifolia*	*Nicotiana tabacum*	Enhanced 10−18 fold proline accumulation and induced salt tolerance	Kavi Kishor et al. (1995)
2	P5CSF129A	*V. aconitifolia*	*O. sativa*	Enhanced proline accumulation and salt tolerance	Kumar et al. (2010)
3	*P5CS*	*V. aconitifolia*	*Larix leptoeuropaea*	Alleviates oxidative stress, high chlorophyll content and 30-fold enhanced proline accumulation	Gleeson et al. (2005)
4	*P5CS*	*V. aconitifolia*	*Saccharum officinarum*	Enhanced proline content, biomass production, low lipid peroxidation level, and oxidative stress protection;	Guerzoni et al. (2014)
5	*P5CSF129A*	*Vigna acontifolia*	*Cajanus cajan*	enhanced proline accumulation, seed germination rate, chlorophyll content, low lipid peroxidation level	Surekha et al., 2014
6	*P5CS*	*Vigna aconitifolia*	*Daucus carota*	Enhanced proline accumulation, biomass production, and low lipid peroxidation level	Han and Hwang (2003)
7	*P5CS*	*Vigna aconitifolia*	*T. aestivum*	Enhanced proline accumulation, biomass production, and low lipid peroxidation level	Sawahel and Hassan (2002)
9	*PvP5CS1, PvP5CS2*	*P. vulgaris*	*A. thaliana*	Increased 1.9 times proline content, flower and seed development	Chen et al. (2013)
10	*P5CR*	*A. thaliana*	*Glycine max* L.	Increased proline accumulation, RWC and WUE content, high spikelet fertility	De Ronde et al. (2000)
11	*P5CS*	*A. thaliana*	*Olea europaea*	Increased proline accumulation, ionic homeostasis	Behelgardy et al. (2012)
12	*P5CS*	*A. thaliana*	*S. tuberosum*	Enhanced proline accumulation, tuber yield, and biomass production	Hmida-Sayari et al. (2005)
13	OsP5CS1 and OsP5CS2	*O. sativa*	*N. tabacum*	Enhanced 3.2 times proline content, biomass production, oxidative stress protection	Zhang et al. (2014)
14	*OAT*	*O sativa*	*O sativa*	Proline accumulation, water retention, spikelet fertility, and increased biomass	You et al. (2012)
15	*HtP5CS*	*Helianthus tuberosus L.*	*Helianthus tuberosus L.*	Enhanced proline accumulation and salt tolerance	Huang et al. (2013)
16	*IbP5CR*	*Ipomoea batatas (L.)*	*Ipomoea batatas (L.)*	Enhanced proline accumulation and salt tolerance.	Liu et al. (2014)
17	*P5CS*	*Vigna aconitifolia*	*Medicago truncatula*	Enhanced proline accumulation and salt tolerance	Verdoy et al. (2006)
18	*KvP5CS1*	*Kosteletzkya virginica*	*Kosteletzkya virginica*	Enhanced 6.83 times proline content and salt tolerance	Wang et al. (2015)
19	*LrP5CS1, LrP5CS2*	*Lilium regale*	*A. thaliana*	Enhanced proline accumulation and tolerance to salt, drought, and osmotic stress	Wei et al. (2016)

Chen and Murata, 2002) (Fig. 30.3). Scientists exploited the genes involved in the GB biosynthetic pathway (bacterial *codA*, CMO, and BADH) to develop transgenic varieties of agriculturally important crops such as rice, potato, soybean, groundnut, and maize, among other plants (Sawahel,2003; Ranganayakulu et al., 2013). Significant increase in yield and growth parameters was observed in barley, soybean, wheat, maize, tobacco, beans, and sunflower exposed to abiotic stresses (Ashraf and Foolad, 2007). The *codA* gene of *Arthobacter* spp. has been introduced in many plant species such as *A. thaliana*, *Solanum tuberosum*, *Zea mays*, *S. lycopersicum*, and *Lycopersicon esculentum* (Giri,2011; Quan et al., 2004; Wei et al., 2017; Yu et al., 2017). The transgenic lines showed increased GB accumulation, which enhanced photosynthetic activity, plant development, and crop yield under abiotic stress conditions.

Sakamato and Murata (1998) overexpressed the *A. globiformis codA* gene in rice (non-GB accumulator) and observed that the transgenic varieties accumulated 5.3 mmol/g fresh weight of GB. Park et al. (2004)

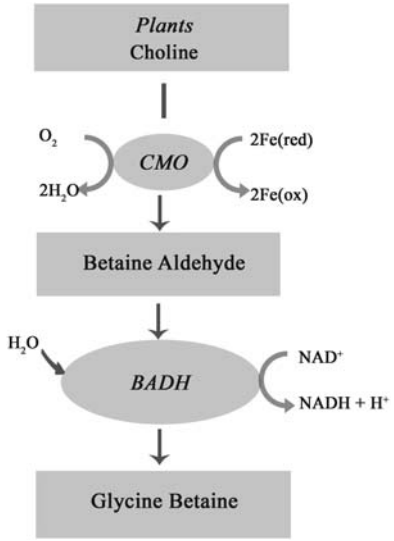

FIGURE 30.3 Glycine betaine biosynthesis pathway. *CMO*, choline monooxygenase; *CDH*, choline dehydrogenase; *COD*, choline oxidase; *BADH*, betaine aldehyde dehydrogenase.

developed transgenic tomato harboring the same *codA* gene and exposed it to salt (200 mM NaCl) and low temperature (4°C) stress. Under salt and low temperature stress, the transgenic tomato plants accumulated 3 times and 5 times more GB respectively than the nontransformed plants and exhibited 10%−30% increase in fruit formation. Enhanced GB accumulation in the transgenic varieties contributed towards improving the net photosynthetic rate, activity of antioxidant enzyme, and lowering the lipid peroxidation levels in the transgenic lines.

Similarly, Ahmad et al. (2014) overexpressed the *A. globiformis codA* gene in potato and exposed the plants to salt (100 mM NaCl) and low temperature (4°C) stress. The transformed varieties were characterized by normal growth, improved plant biomass, and high tuber yield both under salt and low temperature stress. The transgenic potato tuber yield was reported to be 44 g/plant while it was only 33.6 g/plant in the wild type (WT) plants. Wei et al. (2017) also transformed tomato with the same *A. globiformis codA* gene. The transgenic varieties exhibited increased GB accumulation in comparison with the nonaccumulator wild types. Moreover there was significant improvement in photosynthetic rates due to restoration of chlorophyll content, and activity of antioxidant enzymes in lowering ROS content.

The *BADH* gene coding for BADH enzyme that regulates the oxidation of betaine aldehyde to GB has also been exploited to enhance GB accumulation in sweet potato. Fan et al. (2012) developed transgenic sweet potato harboring the *SoBADH* gene isolated from *Spinacia oleracea*. The amount of GB accumulation in the transgenic plants doubled conferring tolerance to salt, cold, and oxidative stress. Enhanced GB accumulation resulted in improved integrity of cellular membranes, net photosynthetic activity, and ROS scavenging. Transgenic tomato developed by Li et al. (2014) carrying the *SoBADH* gene exhibited high photosynthetic capacity even at 42°C. Enhanced GB accumulation increased the D_1 protein content thereby preventing photosystem II from denaturation. Moreover, there was significant decrease in H_2O_2 content and superoxide radical in the transgenic tomato plants. Wang et al. (2010) reported 170.7 μmol/g dry weight GB accumulation in chloroplast of transgenic wheat lines harboring the BADH gene isolated from cyanobacteria under salt and drought stress. These experiments highlight the regulatory role of GB in improving plant growth and development under abiotic stress conditions. Similar transgenic lines have been developed with improved GB accumulation potential and have been listed in Table 30.2.

30.3.3 Polyamines

PAs belong to the class of aliphatic amines and are present in bacteria, animals, and plants (Hussain et al., 2011; Wimalasekera et al., 2011; Zhang et al., 2011a,b). The PAs found in plants include putrescine (Put), spermidine (Spd), and spermine (Spm) (Tiburcio et al., 2014; Sharma et al., 2017). PAs have been found to significantly regulate plant stress and development physiology during the stages of elongation stage, floral development, fruit ripening, and cellular apoptosis (Alcázar et al., 2010; Feng et al., 2011; Alet et al., 2012).

In plants, arginine (Arg) serves as the precursor for diamine putrescine (Put) synthesis and involves the sequential action of three enzymes: arginine decarboxylase (ADC), agmatine iminohydrolase (AIH), and *N*-carbamoyl Put amidohydrolase. Putrescine biosynthesis can be alternatively carried out in a single step by converting mitochondrial ornithine to Put catalyzed by the enzyme ornithine decarboxylase (ODC). The second PA, spermidine, is synthesized from Put by the action of spermidine synthase (SPDS) while spermine (tetramine) is formed from spermidine. This reaction is catalyzed by spermine synthase (SPMS) and decarboxylated *S*-adenosylmethionine (dcSAM) provides the aminopropyl group essential for the synthesis of spermidine and spermine (Fig. 30.4). The aminopropyl donor group is obtained from methionine via two enzyme catalyzed reactions by *S*-adenosylmethionine (SAM) synthase and SAM decarboxylase (SAMDC) (Takahashi et al., 2010; Moschou et al., 2008).

Transcriptome analysis of PAs revealed their accumulation in plants subjected to salt, drought, low and high temperature, and oxidative stresses (Romero et al., 2018;

TABLE 30.2 List of Transgenic Plants Overexpressing Candidate Genes for Glycine Betaine Accumulation

S. no.	Transgene	Host	Target crop plants/trees	Remarks	References
1	*codA*	*Arthrobacter globiformis*	*Oryza sativa* L.	Enhanced 5.3-fold GB accumulation and induced salt tolerance	Sakamoto and Murata (1998)
2	*codA*	*Arthrobacter globiformis*	*Solanum lycopersicum*	Enhanced fivefold GB accumulation and induced salt tolerance	Park et al. (2004)
3	*codA*	*Arthrobacter globiformis*	*Solanum tuberosum*	Enhanced GB content and tolerance to salt and chilling stress	Ahmad et al. (2014)
4	*SoBADH*	*Spinacia oleracea*	*Ipomoea batatas (L.)*	Enhanced GB accumulation and tolerance to salinity, low temperature and oxidative stress	Fan et al. (2012)
5	*codA*	*Arthrobacter globiformis*	*Solanum lycopersicum*	Induced GB accumulation, antioxidant enzyme activity, and salt tolerance	Wei et al. (2017)
6	*SoBADH*	*Spinacia oleracea*	*Solanum lycopersicum*	Enhanced GB accumulation and heat stress	Li et al. (2014)
7	*BADH*	*Cyanobacteria*	*Triticum aestivum*	Induced 170.7 μM/g GB accumulation and salt tolerance	Wang et al. (2010)
9	*BvCMO*	*Beta vulgaris*	*Nicotiana tobacum*	Enhanced fivefold GB accumulation and salt tolerance	Zhang et al. (2008)
10	*GSMT and DMT a*	*Aphanothece halophytica*	*O. sativa*	Enhanced GB biosynthesis, salt and cold stress tolerance	Niu et al. (2014)
11	*BADH*	*E. coli*	*M. sativa*	Enhanced GB accumulation and salt tolerance	Yan et al. (2012)
12	*OsBADH1*	*O. sativa*	*N. tabacum*	Salinity stress tolerance	Hasthanasombut et al. (2010)
13	*SIBADH*	*Suaeda liaotungensis*	*Solanum lycopersicum*	Enhanced GB accumulation under salt stress	Wang et al. (2013)
14	*codA*	*Arthrobacter globiformis*	*Lycopersicon esculentum*	Enhanced seed germination rate, GB accumulation, and tolerance to salt and drought.	Goel et al. (2011)
15	*codA*	*Arthrobacter globiformis*	*N. tabacum*	Enhanced antioxidant enzyme activity by 50% and salt tolerance.	Jing et al. (2013)
16	*betA*	*Escherichia coli*	*Zea mays*	Enhanced GB, seed germination rate, and tolerance to drought	Quan et al. (2004)
17	*codA*	*Arthrobacter globiformis*	Transgenic *populus* spp.	Enhanced GB accumulation, photosystem II integrity, and multiple stress tolerance	Ke et al. (2016)

Ma et al., 2017; Tavladoraki et al., 2012). To gain more insights in their role in inducing stress tolerance, exogenous application of these PAs was carried out for plants growing in stressed environments.

Exogenous application of PAs under abiotic stress has been extensively studied in plants species such as tomato, rice, wheat, barley, cucumber, soybean, Welsh onion, etc. (Yiu et al., 2009; Li et al., 2013; Sagor et al., 2013; Sequera-Mutiozabal et al., 2017). Nahar et al. (2016) exogenously applied spermine (0.2 mM) on mung bean species growing under high temperature (40°C), salt (200 mm NaCl), and drought stress. The plants exhibited lowered ROS status depicted by less H_2O_2 content, lipoxygenase activity, and malondialdehyde activity (marker for membrane peroxidation) in comparison with the controls (nontreated). Moreover, activity of important antioxidant enzymes such as glutathione (GSH), superoxide dismutase (SOD), catalase, and glutathione peroxidase was significantly enhanced. Sánchez-Rodríguez et al. (2016) also reported similar findings when they treated the drought-tolerant cultivar (Zarina) and drought-sensitive cultivar (Josefina) and subjected them to water stress. However, in both studies, it was observed that the effect of the exogenously supplied Pas reduced drastically as the duration of stress was increased. This drawback prompted scientists to enhance the endogenous PAs levels by developing transgenic varieties harboring genes encoding the PA biosynthesis enzymes (ADC, ODC, SPDS, and SAM) (Gill and Tuteja, 2010; Gupta et al.,2013; Shukla and Mattoo, 2013).

Kasukabe et al. (2004) overexpressed the *Cucurbita ficifolia* spermidine synthase gene (*CsSPDS)* in the model plant *A. thaliana* under the regulation of a

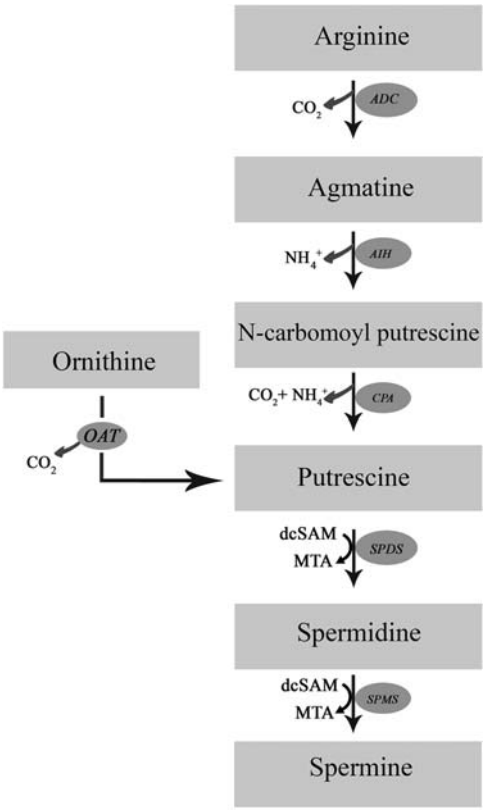

FIGURE 30.4 Polyamines biosynthesis pathway. *ADC*, arginine decarboxylase; *AIH*, agmatine iminohydrolase; *CPA*, *N*-carbamoylPut amidohydrolase; *SPDS*, spermidine synthase; *SPMS*, spermine synthase; *dcSAM*, decarboxylated S-adenosylmethionine; *MTA*, 5-methylthioasdenosine.

constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter. The transformed *A .thaliana* lines exhibited twofold more accumulation of spermidine under salt (200 mM NaCl), low temperature (4°C), and drought stress when compared with its wild types. Capell et al. (2004) engineered rice with the *Datura stramonium* ADC gene (*DsADC)* and expressed it under the inducible monocot Ubi-1 promoter. The transformed rice varieties showed threefold increase in endogenous putrescine levels and also facilitated spermidine and spermine synthesis under drought stress. In another study, Franceschetti et al. (2004) developed transgenic tobacco expressing the *D. stramonium* SPDS gene (*DsSPDS*) and reported high spermidine accumulation, restoration of tissue water, and chlorophyll content facilitating plant growth and development under 200 mM NaCl stress.

He et al. (2008) studied the enzymatic and nonenzymatic antioxidant capacity of *Pyrus communis* harboring the *SPDS* gene from apple exposed to 200 mM NaCl stress. The transformed pear varieties showed enhanced spermidine accumulation and antioxidant enzyme activity, and low levels of malondialdehyde and hydrogen peroxide levels (markers of ROS status). The transgenic lines exhibited high ROS scavenging that helped in shielding membrane and protein structures from denaturation. Similar studies led to the development of many transgenic plants (Table 30.3) expressing the different PA biosynthetic genes and were associated with enhanced endogenous PA accumulation inducing abiotic stress tolerance.

TABLE 30.3 List of Transgenic Plants Overexpressing Candidate Genes for Polyamines Accumulation

S. no.	Transgene	Host	Target crop plants/ trees	Remarks	References
1	*CsSPDS*	*Cucurbita ficifolia*	*A. thaliana*	Enhanced twofold accumulation of spermidine, inducing abiotic stress tolerance	Kasukabe et al. (2004)
2	*DsADC*	*Daturastramonium*	*O. sativa* L.	Enhanced threefold putrescine accumulation inducing drought tolerance	Capell et al. (2004)
3	*DsSPDS*	*D. stramonium*	*N. tobacum*	Enhanced spermidine accumulation, chlorophyll content, and plant growth	Franceschetti et al. (2004)
4	*SPDS*	*Maluspumila*	*Pyruscommunis*	Enhanced spermidine content and ROS scavenging, inducing salinity tolerance	He et al. (2008)
5	*SAMDC*	*Saccharomyces cerevisiae*	*Lycopersicon esculentum*	1.7- to 2.4-fold higher levels of spermidine and spermine, heat stress tolerance, CO_2 assimilation	Cheng et al. (2009)
6	*AsADC*	*Avena sativa* L.	*O. sativa L.*	Enhanced polyamine accumulation inducing abiotic stress tolerance	Roy and Wu (2001)
7	*SAMDC*	*Tritordeum*	*O. sativa L.*	three and fourfold increase of spermidine and spermine, enhanced seed germination, salt tolerance	Roy and Wu (2002)
8	*MdSPDS1*	*Malusdomestica*	*Pyrus communis* L.	Enhanced spermidine accumulation leading to salt and osmotic stress tolerance	Wen et al. (2008)
9	*LcSAMDC1*	*Leymuschinensis*	*A. thaliana*	Enhanced spermine, proline, and chlorophyll content under salt and cold stress	Liu et al. (2017)
10	*SAMDC*	*Homo sapiens*	*Solanumlycopersicum*	Enhanced polyamine accumulation, delayed ripening, and improved postharvest storage	Madhulatha et al. (2014)
12	*AvADC*	*Avena sativa* L	*Medicago truncatula*	Enhanced polyamine content, seed yield, and desiccation stress tolerance	Duque et al. (2016)

30.3.4 Sugars and Sugar Alcohols

Prolonged exposure to abiotic stress interferes with the cellular metabolism of carbon and alters the cellular levels of certain sugars and polyols (alcohol derivatives of sugars). Sugars (trehalose and fructose) and polyols (mannitol, sorbitol, and ononitol) have been studied in relation to abiotic stress and it has been observed that plants accumulate high levels of these osmolytes (Wani et al., 2016; O'Hara et al., 2013). These low molecular weight molecules regulate cellular osmolarity, ROS scavenging, and stability of protein and membrane structures. Moreover they also serve as molecular chaperones and carbon reservoir (Gupta and Huang, 2014; Parvaiz and Satyawati, 2008). This section highlights the biosynthetic pathways and the regulatory function of the various sugars and their alcohol derivatives in connection to abiotic stress tolerance. Moreover, the successfully engineered transgenic plants harboring potential biosynthetic genes of these osmolytes have also been enlisted in Table 30.4.

TABLE 30.4 List of Transgenic Plants Overexpressing Candidate Genes for Sugar and Sugar Alcohols

S. no.	Transgene	Host	Target crop plants/trees	Remarks	References
1	*ScTPS1*	*Saccharomyces cervisiae*	*N. tabacum*	0.17 mg/g FW in leaves trehalose content, shunted growth, drought tolerance	Romero et al. (1997)
2	*ScTPS1*	*Saccharomyces cervisiae*	*Solanum tuberosum*	Twofold trehalose accumulation, salt and drought tolerance	Yeo et al. (2000)
3	*ScTPS1*	*Saccharomyces cervisiae*	*Solanum lycopersicum*	2.5-fold trehalose accumulation, salt, and drought tolerance	Cortina and Culiáñez-Macià (2005)
4	*otsA and otsB*	*E. coli*	*Oryza sativa* L.	3–10 fold trehalose accumulation, salt, drought, and low temperature tolerance	Garg et al. (2002)
5	PyTPS	*Porphyrayezoensis*	*Oryza sativa* L TP309	Enhanced trehalose accumulation, seed germination, and yield	Guo et al. (2014)
6	*GfTPS*	*Grifolafrondosa*	*N. tabacum*	12-fold more trehalose content, abiotic stress tolerance	Zhang et al. (2005)
7	*CvTPS1 + CvTPS2*	*Saccharomyces cervisiae*	*Arabidopsis thaliana*	Enhanced plant growth and abiotic stress tolerance, twofold trehalose content	Miranda et al. (2007)
9	*CvTPS1 + CvTPS2*	*Saccharomyces cervisiae*	*M. sativa*	Enhanced plant growth and abiotic stress tolerance, enhanced trehalose content	Suárez et al. (2009)
10	*SacB*	*Bacillus subtilis*	*N. tabacum*	0.35 mg/g fructans accumulation, 55% enhanced plant growth under drought stress	Pilon-Smits et al. (1995)
11	*SacB*	*Bacillus subtilis*	*Beta vulgaris* L	Accumulated 0.5% more fructans, inducing drought stress tolerance	Pilon-Smits et al. (1999)
12	*1-SST*	*Lactuca sativa*	*N. tabacum*	High soluble carbohydrate, fructose, fructan content inducing cold stress tolerance	Li et al. (2007)
13	*mtlD*	*E. coli*	*Arabidopsis thaliana*	Enhanced mannitol (3 μmol/g FW), germination rate, optimum shoot and root growth under salt stress	Thomas et al. (1995)
14	*mtlD*	*E. coli*	*N. tabacum*	6 μmol/g FW of mannitol inducing salt tolerance	Tarczynski et al. (1992)
15	*mtlD*	*E. coli*	*Triticum aestivum*	Enhanced mannitol content, plant height, dry and fresh weight under salt stress.	Abebe et al. (2003)
16	*mtlD*	*E. coli*	*Solanum tuberosum*	Enhanced mannitol level leading to 65% plant survival under salt stress.	Rahnama et al. (2011)
17	*HVA1 + mtlD*	*Hordeumvulgare and E. coli*	*Zea mays*	Improved rate of plant survival, shoot and root biomass under multiple abiotic stress conditions	Nguyen et al. (2013)
18	*MpS6PDH*	*Maluspumila*	*N. tabacum*	0.2–130 μmol/g FW sorbitol content in response to salt stress	Sheveleva et al. (1998)
19	*MpS6PDH*	*Maluspumila*	*Diospyros kaki*	14.5–61.5 μmol/g FW sorbitol content and high chlorophyll content under salt stress	Gao et al. (2001)

30.3.4.1 Trehalose

Trehalose, a nonreducing disaccharide is composed of two glucose units and has been found in bacteria, fungi, and higher plants (Djilianov et al., 2005; Elbein et al., 2003). Trehalose biosynthesis in plants is accomplished in two enzyme catalyzed reactions. In the first step, uridine diphosphate glucose (UDP-glucose) and glucose-6-phosphate are catalyzed by the enzyme trehalose-6-phosphate synthase (TPS) forming trehalose-6-phosphate (T6P) intermediate. In the final step, the intermediate T6P is converted to trehalose via a dephosphorylation reaction catalyzed by trehalose-6-phosphate phosphatase (TPP) (Iordachescu and Imai, 2011; Paul et al., 2008; John et al., 2017) (Fig. 30.5A).

Trehalose has been associated with protection of protein and membrane structures of plants exposed to abiotic stress (Garg et al., 2002; Wingler, 2002). It shields the structures against denaturation and is also involved in regulation of cell proliferation, cell differentiation, and cellular homeostasis (Wani et al., 2016). Transcriptome analysis of *Arabidopsis* revealed the presence of 11 TPS and 10 TPP enzymes respectively while nine TPS and nine TPP enzymes have been found in the case of rice. To suppress the deleterious effects of abiotic stress, many scientists focused on exploiting the role of trehalose either by treating plants with trehalose supplements or by developing transgenic plants overexpressing the candidate biosynthetic genes.

Sadak (2016) studied the effect of exogenous effect of 500 mM trehalose on fenugreek plants under drought stress. They reported that the plants exhibited increased chlorophyll content, protein, and flavonoid content when compared with their WT counterparts. In radish a very small trehalose supplement (25 mM) increased root dry biomass, antioxidant enzyme activity (SOD and peroxidase), chlorophyll a content, total soluble sugar, and free proline content after 45 days exposure to drought stress (Akram et al., 2016).

The rapid progresses in the field of genetic engineering has enabled researchers to harness the potential trehalose biosynthetic genes (TPP and TPS) from plants as well as prokaryotes and develop transgenic varieties. These transgenic lines are designed to enhance endogenous trehalose accumulation, which in turn protects the plants from the deleterious effects of abiotic stress (Iordachescu and Imai, 2008; Sah et al., 2016). The ScTPS1 gene was isolated from yeast and used to develop the first transgenic tobacco plants expressing the TPS enzyme. The trehalose accumulation in the transgenic cultivars was recorded as 0.17 mg/g fresh weight in leaves. Though the plants exhibited stunted growth and lancet-shaped leaves they were tolerant to prolonged drought exposure (Romero et al., 1997). The yeast (*ScTPS1*) gene was overexpressed in transgenic potato (Yeo et al., 2000) and tomato (Cortina and Culiáñez-Macià, 2005) respectively under the regulation of a 35S CaMV promoter and exposed to salt, drought, and cold stress. Both the transgenic plants showed enhanced trehalose accumulation leading to abiotic stress tolerance.

Garg et al. (2002) developed transgenic rice varieties expressing the *E. coli* trehalose biosynthetic genes (*otsA*

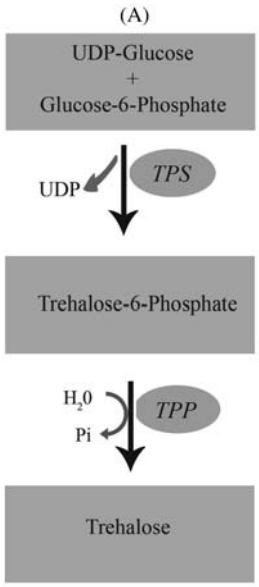

FIGURE 30.5A Trehalose biosynthetic pathway.
TPS: trehalose-6-phosphate synthase; *TPP*: trehalose-6-phosphate phosphatase; UDP: uridine diphosphate.

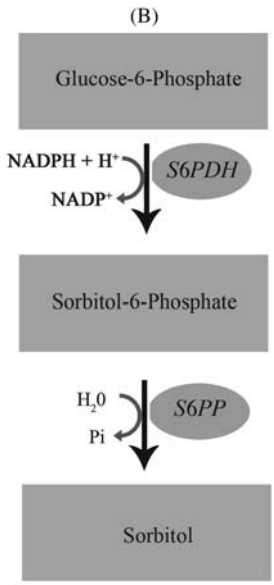

FIGURE 30.5B Sorbitol biosynthesis pathway.
S6PDH: sorbitol-6-phosphate dehydrogenase; *S6PP*: sorbitol-6-pyrophosphatase.

and *otsB*) under the control of a stress responsive promoter. The transformed plants accumulated 3- to 10-fold more trehalose resulting in improved growth, less photooxidative damage, and maintenance of mineral homeostasis under salt, drought, and low temperature. Leaf rolling and wilting were common in nontransformed plants whereas the transformed plants exhibited significant shoot development. Guo et al. (2014) expressed *Porphyra yezoensis* (*PyTPS*) gene in a particular rice variety (TP309). The transgenic plants were characterized with improved seed germination rate and yield parameters due to increased trehalose accumulation.

Zhang et al. (2005) reported significant trehalose (2.1−2.5 mg/g fresh weight) accumulation in transgenic tobacco plants expressing the *Grifola frondosa* trehalose synthase gene. The transgenic plants accumulated trehalose up to 2.1−2.5 mg/g fresh weight while the level of trehalose remained untraced in the controls (nontransformed). High levels of trehalose accumulation were reported in transgenic lines harboring the *A. thaliana* (*AtTPS1*) gene, which increased net photosynthesis, improved antioxidant enzyme activity, and lowered peroxidation levels (Vinocur and Altman, 2005). Miranda et al. (2007) coexpressed the yeast TPS1 and TPS2 gene in *Arabidopsis* and the same gene construct was later introduced in *Alfalfa* (Suárez et al., 2009). In both the experiments, the transformed varieties were able to thrive in drought, heat, freezing, and salt stress conditions and showed normal growth and development.

30.3.4.2 Fructans

Fructose polymers are referred as fructans and are synthesized from sucrose molecule characterized by transfer of a fructosyl group in two steps catalyzed by sucrose:sucrose 1-fructosyltransferase (1-SST) and sucrose:fructan 6-fructosyltransferase(6-SFT) respectively (Fig. 30.5C). Under abiotic stress condition, plants accumulate fructan in vacuoles and they serve as carbohydrate sinks that are utilized during the nutrient recovery phase (Vijn and Smeekens, 1999; Konstantinova et al., 2002)

The genes involved in fructan biosynthesis have been used to engineer transgenic tobacco, potato (Van Der Meer et al., 1994), rice (Kawakami et al., 2008), and sugar beet varieties tolerant to abiotic stresses. Pilon-Smits et al. (1995) developed transgenic tobacco expressing the *Bacillus subtilis* gene (*SacB*) under the constitutive (CaMV 35S) promoter. The transgenic plants accumulated higher fructan levels (0.35 mg/g fresh weight) under drought stress and growth rate was enhanced by 55%. Moreover the transgenic lines recorded 33% and 59% increase in fresh and dry weight when compared with their wild type. The same gene when expressed in sugar beet accumulated sevenfold more trehalose predominantly in roots

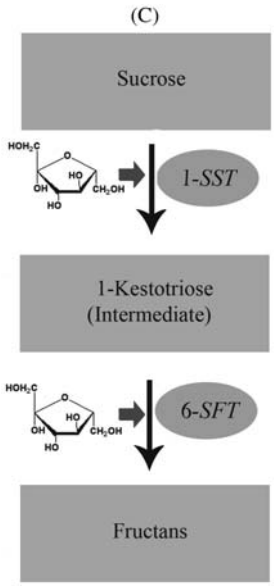

FIGURE 30.5C Fructan biosynthesis pathway.
1-SST: sucrose:sucrose 1-fructosyltransferase; *6-SFT*: sucrose:fructan 6-fructosyltransferase.

under drought stress in comparison to its WT (Pilon-Smits et al., 1999).

Li et al. (2007) also focused on transgenic tobacco expressing the sucrose 1-fructosyltransferase (*1-SST*) gene isolated from *Lactuca sativa*. The transgenic lines exhibited increase in soluble carbohydrate content, fructan, and fructose content and net photosynthesis but similar antioxidant enzyme activity (SOD) under low temperature stress (4°C) in comparison with wild types.

30.3.4.3 Mannitol

Mannitol belongs to the class of sugar alcohols and is a 6-carbon molecule. Three enzymes, namely, mannose-6-phosphate isomerase, mannose-6-phosphate reductase, and mannose-1-phosphate phosphatase sequentially catalyze the synthesis of mannitol from fructose-6-phosphate (Loescher et al., 1992) (Fig. 30.5D). In plants, enhanced mannitol accumulation is triggered when exposed to abiotic stress and regulates stability of macromolecules and ROS scavenging (Llanes et al., 2013). Thomas et al. (1995) introduced the *E. colimtlD* gene into *Arabidopsis* (nonmannitol accumulator). Mannitol dehydrogenase is the major rate limiting enzyme involved in conversion of fructose-6-phosphate to mannitol-1-phosphate. The transgenic plants were shown to accumulate increased mannitol levels (3 μmol/g fresh weight), which resulted in improved biomass and germination rate, and normal shoot and root development even when exposed under 400 mM NaCl stress. The same *E. colimtlD* gene was inserted in

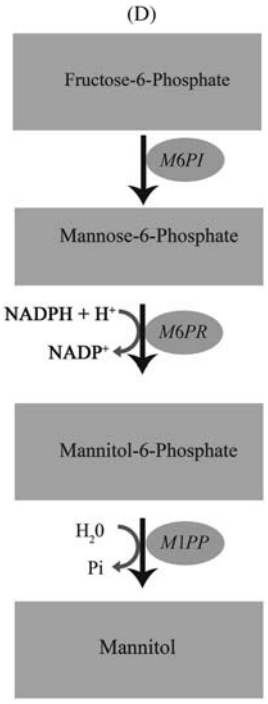

FIGURE 30.5D Mannitol biosynthesis pathway.
M6PI: mannose-6-phosphate isomerase; *M6PR*: mannose-6-phosphate reductase; *M1PP*: mannose-1-phosphate phosphatase.

tobacco to develop its transgenic lines (Tarczynski et al., 1992). The transgenic tobacco lines accumulated mannitol (6 μmol/g fresh weight) unlike the wild types. Moreover the transgenic lines thrived in 150 mM NaCl stress and did not show reduction in dry weight as in the case of wild types, which showed 44% reduction in dry weight.

Rahnama et al. (2011) introduced the *E. coli*(*mtlD*) gene in potato and exposed both the transformed and the control varieties to100 mM NaCl stress. The transformed plants only observed 17.3% reduction in shoot fresh weight while the reduction was 76.5% weight in the nontransformed potato lines. In another study, Nguyen et al. (2013) designed a gene construct of *Hordeum vulgare* HVA1 (a group 3 LEA protein) and *E. coli*(*mtlD*) genes under the control of rice actine promoter (Act1). This construct was expressed in maize exposed to high salinity (300 mM NaCl) and drought stress. The successfully transformed varieties expressing the *HVA1 + mtlD* construct exhibited better shoot and root biomass and sustained throughout the stress period while their wild type counterparts failed to survive.

30.3.4.4 Sorbitol

Sorbitol also belongs to the category of sugar alcohol and is produced from glucose-6-phosphate by the action of sorbitol-6-phosphate dehydrogenase (S6PDH) and sorbitol-6-pyrophosphatase (S6PP) enzymes (Fig. 30.5B). The genes regulating the sorbitol biosynthetic pathway have been exploited to engineer some transgenic plants aimed for developing abiotic stress tolerance. Sheveleva et al. (1998) expressed the sorbitol-6-phosphate dehydrogenase (*S6PDH*) gene from apple in tobacco. The transformed varieties accumulated sorbitol (2−130 μmol/g fresh weight) when subjected to 200 mM salt stress and were more stress tolerant in comparison with its WT plants. Gao et al. (2001) also expressed the same *S6PDH* gene in *Japanese persimmon* and exposed them to 200 mM NaCl stress. The transgenic lines accumulated sorbitol (14.5−61.5 μmol/g fresh weight) in comparison with its wild types (naturally nonaccumulator). The accumulation of sorbitol was attributed to enhanced photosynthetic activity and chlorophyll content increased in the transgenic lines thereby conferring salt stress tolerance.

30.4 CONCLUSION AND FUTURE PROSPECTS

Abiotic stresses are responsible for significant damage to crop productivity annually and the situation is further aggravated due to changes in world climatic pattern and global warming. The major research conducted these days focuses on evolving new techniques aimed at enhancing plant stress physiology. The major concern remains in ensuring sustainable crop productivity for the ever-growing population. Under such a scenario, the diverse role of osmolytes in conferring abiotic stress tolerance can be harnessed to develop potential transgenic varieties of agriculturally important plants. Extensive studies have been conducted to elucidate the biosynthetic pathways of these osmolytes and the valuable inputs have translated to the identification of many candidate genes regulating the biosynthetic pathways of the above discussed osmolytes. The transgenic cultivars expressing the candidate genes in plants improved osmolyte accumulation significantly and contributed to specific stress tolerance. However all the developed transgenic plants discussed so far have not been subjected to field trials and all responses are only true to laboratory conditions. Hence, special focus should be made to develop transgenic plants that can successfully thrive in field conditions. Moreover plants being sessile are exposed to multiple abiotic stresses simultaneously, hence it is of utmost importance in developing transgenic plants capable of expressing multigenic traits (simultaneous expression of osmolyte biosynthesis genes and other genes involved in stress tolerance like ion transporters, aquaporins, transcriptional factors, LEA proteins, etc.).

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CHAPTER

31

Proteomics Insights Into Salt Stress Signaling in Plants

Arajmand Frukh[1], *Altaf Ahmad*[2] *and Tariq Omar Siddiqi*[1]

[1]Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India
[2]Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

OUTLINE

31.1 INTRODUCTION

Plants are continuously being subjected to a number of environmental stresses that diminish the productivity of various economically important crops (Sewelam et al., 2016; Khan and Khan, 2017). Environmental stress conditions include biotic stress, such as pathogen infection and herbivore attack, and abiotic stress, such as excess salt, drought, heat, cold, etc. (Zhu, 2016). Among all, salt stress is a major environmental constraint to agricultural productivity (Ji et al., 2013). It is estimated that more than 20% of the total irrigated land on Earth has been affected by salinity, which is expected to continue to increase over time

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(Ji et al., 2016). This increase in salinity on the other hand is the major cause of decrease in crop productivity throughout the world. The increase in the salt concentration in soil occurs either naturally by means of rock weathering, salt deposited from saline wind, rain near the coast, and soil evaporation or by anthropogenic activities, which include intense irrigation, land clearing, poor agricultural practices, etc. (Hafiz Che-Othman et al., 2017). Salinity imposes both osmotic as well as ionic stress in plants (Munns and Tester, 2008). In the early phase, inhibition of water uptake, cell elongation, root development, formation of new leaves occurs, while in the later phase, salt ions accumulate and cause premature senescence, disruption in enzyme functionality, and inhibition of photosynthesis (Khan et al., 2012, 2014; Roy et al., 2014; Shelke et al., 2017). It affects most of the developmental stages of plants from seed germination to reproductive stage. Plants survive against these different environmental challenges by a complicated protection mechanism. One such mechanism is the ability to sense complex stress factors to activate complex signaling pathways to respond appropriately (Sewelam et al., 2016). To improve the adaptability of plants, a better understanding of plant signaling pathways is the need of the hour.

Plants have evolved a complex signaling system to enable cell-to-cell communication during growth and development. This communication is highly responsible for plants to adjust their metabolism, growth, and development to a highly dynamic environment especially during stress conditions. Plants are unable to relocate during unfavorable environmental conditions and instead rely on intricate signaling pathways that work at the local and systemic level to withstand stress (Carella et al., 2016). The crucial part of the biological research is to study the nature and mechanism of signaling events. Stress signaling pathways in plants are complex and they don't involve only the components that transfer a "signal" from receptors to TFs or other intracellular effector proteins (Kaufmann et al., 2011). The signaling mechanism also relies on intricate networks, which encompass feedback loops, crosstalk with other signaling components, and the integration of information related to the internal state of the cell (Choudhary and Mann, 2010). Although genomics and transcriptomics have conferred the role of various genes that activate multiple signal transduction pathways they have been far less successful in identifying the signaling components involved. This may be because of the fact that there is redundancy in pathways and in components of pathways, or lethality by mutated genes encoding the proteins (Peck et al., 2001).

Genomics can offer clues regarding signaling molecules. However, the genome of an organism is static and does not necessarily reflect the pool of genes that are actually transcribed or translated into proteins in a given individual in a given environment (Song and Lin, 2017). The transcriptome analyses of gene expression at the mRNA level have contributed greatly to understand the complex signaling mechanism. However, the level of mRNA does not always correlate well with the level of protein. This is mainly due to posttranscriptional modifications. Therefore, it is insufficient to predict protein expression level from quantitative mRNA data (Yan et al., 2006). Thus, studies via alternative methods are required to complement existing genetic studies to elucidate the complex pattern of signaling. Proteome analysis aims at the complete set of proteins encoded by the genome and thus complements the transcriptome studies.

31.2 PROTEOMICS: EDGE OVER OTHER TECHNOLOGIES

Proteomics is evolving phenomenally as one of the major tools of "omics" to identify proteins. Proteomics has many applications. Among them signaling is one of the components. The term *proteomics* was coined in 1997 in analogy with genomics, the study of the genome. The word *proteome* is actually a combination of protein and genome and was coined by Mark Wilkins in 1994. The proteome is the entire set of proteins produced by a living organism and proteomics deals with the study of proteome. Proteomics has advanced over time to enrich the understanding of salt stress mechanism in plants. While the other two fields of "omics," that is, genomics and transcriptomics, deal with the analysis of genes, regulatory elements, and their transcripts, the field of proteomics deals with the analysis of proteins (Ashwin et al., 2017). Proteomic based technologies have been widely used in several crop species to understand the changes in the cellular activities at the protein level under salt stress.

Proteins are the important macromolecules exhibiting diverse functions in plant stress tolerance. They act as enzymes, exhibit protective functions, interact with other proteins and other biomolecules, and scavenge ROS (reactive oxygen species) either directly via chemical reactions or indirectly via metal cofactors. It is therefore highly important to study changes in proteome composition under stress to uncover key proteins involved in mechanisms underlying plant acclimation to stress. However, it is quite evident that the diverse functionality of one protein depends on its subcellular localization, PTMs, and interacting partners. Therefore, studies of particulate protein, PTMs, as well as protein–protein interactions are extremely important. The major focus of proteomics studies in the future would probably shift from a mere identification of

differentially expressed proteins to a proper characterization of protein function in plant stress response (Kosová et al., 2013). 2-DE (2-dimensional electrophoresis) coupled with mass spectrometry are the widely used quantitative proteomics methods. Nonetheless, the defects of low rate of protein detection, low reproducibility, and difficult isolation of hydrophobic proteins restricted the full potential of 2-DE in systematic analysis of proteomic changes (Sun et al., 2017). This technical disadvantage of 2-DE gave rise to the gel-free based protein quantitative approach, which is also regarded as shotgun proteomics.

31.3 TECHNICAL ADVANCES IN PROTEOMICS

There are ideally three critical stages in proteomic approaches, that is, sample preparation, gel/column-based protein/peptide separation, and identification of proteins using MS (mass spectrometry) (Ashwin et al., 2017). In case of model systems including human, yeast, and bacterial proteomes advanced proteomic technologies are being developed but they may not be directly applied to plant tissues (Timperio et al., 2008). In the case of plant tissue the main critical step for proteome analysis is sample preparation because of rigid cell wall, presence of secondary metabolites like phenolic compounds, polysaccharides, etc. These secondary metabolites usually cause protein precipitation during the disruption of tissues. Therefore, it is difficult to obtain high quality protein in case of plants. Moreover, roots and fruits have got the least amount of protein content and high amount of interfering substances, which makes it challenging to extract protein for proteome analysis. There are many techniques of extracting proteins from plant tissues for proteomic studies but phenol and trichloroacetic acid (TCA)-based extraction methods have been found to give superior protein yield and good quality 2-DE gels for certain plant tissues (Wang et al., 2016). Further, the sample preparation methodology should be compatible with the downstream proteomic strategies for separation, identification/quantification, and analysis (Agrawal et al., 2011).

MS has emerged as an indispensable tool for proteomics over the years as the technology has advanced in its robustness, accuracy, sensitivity, and selectiveness in identification and quantification of proteins. Besides this, increased speed and accuracy in matching proteins/peptides from vast database searches in in silico tools for MS data analysis have been rapidly refined. Also de novo assisted database search for highly reliable protein/peptide predictions and quantifications of labeled or label-free proteins/peptides, the algorithms for de novo sequencing for organisms that do not have reference genome/proteome database, are evolving continuously. Many proteomic approaches have emerged owing to these technological advances, which can be broadly classified into global and targeted profiling of proteomes (Liebler and Zimmerman, 2013).

31.3.1 Global Proteome Analysis

Global proteome analysis is the most common approach for proteome analysis. This approach is generally preferred whenever there is comparative analysis of two or more proteomes or to establish a reference proteome map. The global proteome profiling approach can be further classified into two broad categories, that is, gel-based and gel-free/shotgun approaches as represented in Fig. 31.1. Gel-based approaches include 1DGE (one-dimensional gel electrophoresis), 2DGE, 2DE−DIGE (two-dimensional−differential in gel electrophoresis), and 3DGE. These are the preferred techniques used in amalgamation with MS (Görg et al., 2009). On the other hand, gel-free or shotgun approaches include isotope-coded affinity tags (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), stable isotope labeling by amino acids in cell culture (SILAC), multidimensional protein identification technology (MudPIT), and deep proteome analysis approaches. In the gel-free proteomics approach, the extracted protein mixture is directly subjected to trypsin digestion and the digested peptides are chromatographically separated and analyzed by MS. Shotgun or gel-free proteomics approach can raise the number of different proteins that can be recognized from complex samples, contrasted to more traditional gel-based approaches (Hakeem et al., 2013).

31.3.1.1 Two-Dimensional Gel Electrophoresis (2DGE)

In plant proteomics, 2DE remains one of the main methods of choice for the reproducible separation of proteins and their isoforms in complex extracts (Ngara and Ndimba, 2014). The standard approach for 2DE in the analysis of plant proteins uses immobilized pH gradient (IPG) gels in the first dimension for charge separation and then an orthogonal separation, in the presence of SDS (sodium dodecyl sulfate), to resolve the proteins according to their molecular mass (Cash and Argo, 2009). Using this technique, different biological samples are compared and the proteins that show differential expression are revealed in the form of spots individual gels following silver or Coomassie Blue staining. Those proteins that are of our interest are then identified by MS. The limitation of 2DE is the lack of reproducibility and quantitation.

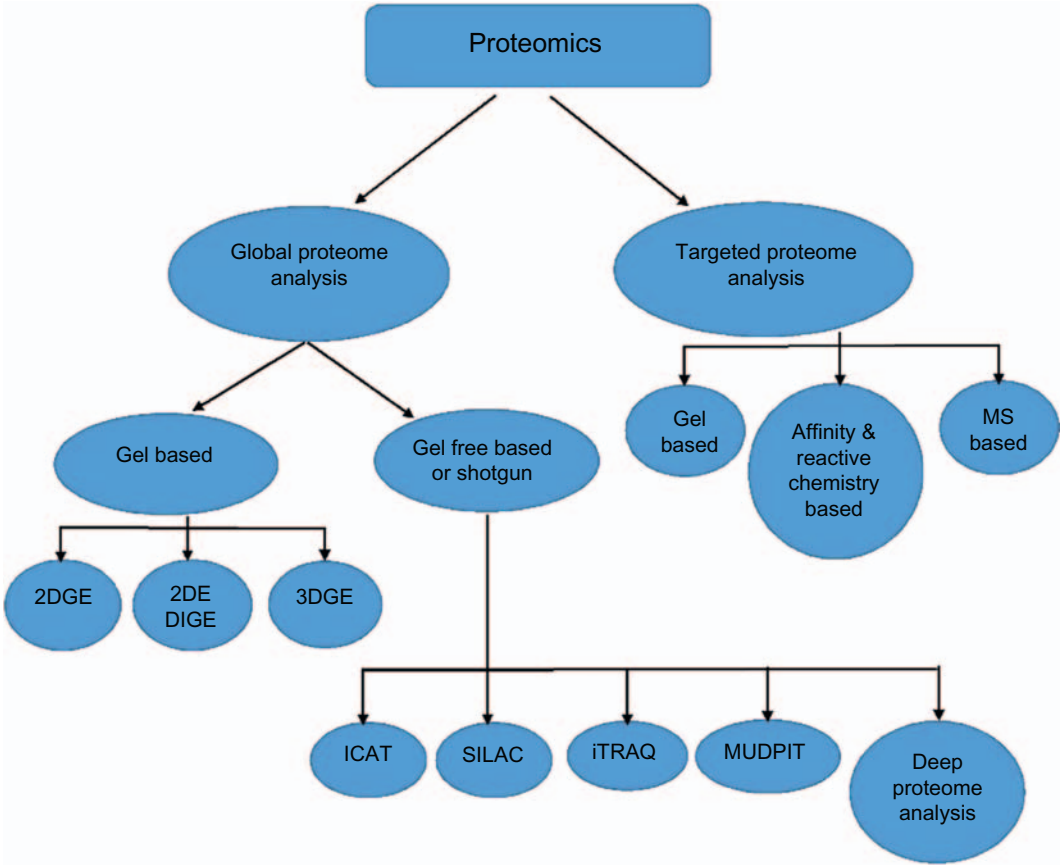

FIGURE 31.1 Schematic representation of types of proteomic techniques used for the analysis, identification, and quantification of proteins. 2DGE two-dimensional gel electrophoresis, 2DE−DIGE two-dimensional−differential in gel electrophoresis, 3DGE three-dimensional gel electrophoresis, ICAT isotope coded affinity tags, SILAC stable isotope labeling with amino acids in cell culture, iTRAQ isobaric tag for relative and absolute quantification, MudPIT multidimensional protein identification technology.

31.3.1.2 Three-Dimensional Gel Electrophoresis (3DGE)

Three-dimensional (3D)-gel electrophoresis has advancement over 2DE because the protein analysis is carried out in a separation medium that extends substantially in all three spatial dimensions. 3DGE was developed to overcome some of the limitations of 2DGE such as lack of accuracy in protein identification and variance in relative quantification of spots that occur mainly due to comigration of proteins. In this method the analysis of the proteins occurs according to three independent separation parameters, that is, native size, pI, and molecular mass (MM) (Ventzki and Stegemann, 2010). Another advantage of this method is that it aids in unambiguous identification of posttranslational modification (PTM) of proteins (Rabilloud, 2013). Despite its high accuracy than 2DE this principle is yet to be employed in unraveling the signaling pathway related proteome.

31.3.1.3 Isotope-Coded Affinity Tag (ICAT)

ICAT is the first in vitro method that permits tagging of proteins and peptides of all types of biological samples using stable isotopes. It was developed by Gygi and his associates in 1999 (Gygi et al., 1999). This technique employs ICAT reagent, which comprises of mainly three structures, that is, iodo-acetamide group or N-ethymaleimide, a spacer or linker arm, and biotin. Iodo-acetamide group or N-ethymaleimide is highly reactive group that binds with the thiol groups (cysteines) of proteins and leads to its alkylation. A spacer or linker arm is meant for introduction of mass shift by incorporation of different isotopes in different samples. Isotopes introduce mass difference of in the labeled or unlabeled amino acid residues producing light and heavy tags. Biotin is an affinity tag that captures all the cysteine containing peptides from the mixture and in this way assists purification of labeled peptides (Zargar et al., 2016). This technique adopts the strategy of chromatographic fractionation of ICAT labeled tryptic peptides, followed by identification and quantification of proteins using tandem MS (Shiio and Aebersold, 2006). No doubt, this technique offers accuracy as samples are similarly treated by protease and in this way experimental variations are prevented.

Moreover, sample complexity is also reduced due to tagging of only cysteine residues but it is also associated with loss of information leading to lower proteome coverage. Also the expensive tags have made the ICAT approach inapt for use.

31.3.1.4 Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

SILAC is a simple in vivo technique that was first developed in 2002 (Ong et al., 2002). Isotopic lysine ($C_6H_{14}N_2O_2$) and arginine ($C_6H_{14}N_4O_2$) amino acid tags are mainly used in this strategy. This technique works on the principle of incorporation of nonradioactive heavy isotopes in either amino acids or metabolites in the culture medium, which can be easily identified by tandem MS (Geiger et al., 2011). However, in the case of plant cells only partial labeling of plant proteins with selected stable isotope-coded amino acids appears to be feasible due to the autotrophic nature of plant cells allowing the synthesis of amino acids from inorganic nitrogen (Oeljeklaus et al., 2009). This is the main reason that the strengths of the SILAC strategy could not be fully exploited for the analysis of plant proteomes. Moreover, the approach is relatively more expensive and cumbersome compared with other shotgun proteomic approaches.

31.3.1.5 Isobaric Tag for Relative and Absolute Quantification (iTRAQ)

The strategy of iTRAQ was introduced by Ross and his associates in 2004, and involves isobaric labeling of peptides (Ross et al., 2004). This strategy has been utilized efficiently to explore the diverse molecular mechanisms occurring in plants. Among the shotgun approaches iTRAQ is the most preferred and relatively less sophisticated method for proteomic studies. The iTRAQ reagents usually consist of an N-methyl piperazine reporter group that introduces mass shifts, a balance group that is required to maintain the overall mass of isobaric tag, and an N-hydroxy succinimide ester group that is reactive with the primary amines of peptides. The balance groups, which are mainly carbonyl group, function to make the labeled peptides from each sample isobaric and the quantification is facilitated through analysis of reporter groups that are generated upon fragmentation in the mass spectrometer. Reporter groups have a mass range varying from 114 to 117 Da and 113 to 121 Da, which is compensated by balance group having a range from 28 to 31 Da and 184 to 192 Da (Rauniyar and Yates, 2014). There are currently two mainly used reagents: 4-plex (mass tag = 145 Da) and 8-plex (mass tag = 305 Da), which can be used to label all peptides from different samples/treatments. These samples are then pooled and usually fractionated by nano liquid chromatography and analyzed by tandem mass spectrometry (MS/MS). The sensitivity of detecting peptides is higher in iTRAQ than other shotgun approaches because the labels are isobaric and uniform to all peptides (Evans et al., 2012).

31.3.1.6 Multidimensional Protein Identification Technology (MudPIT)

MudPIT is the first gel-free approach developed for complex proteome analysis (Washburn et al., 2001). In case of multidimensional protein identification technology (MudPIT) biphasic or triphasic microcapillary columns are coupled to high-performance liquid chromatography, tandem mass spectrometry, and database searching. This multidimensional separation results in the increase in the sensitivity and identification of the low abundant peptides increases due to which an exhaustive list of proteins present in a sample is generated (Florens and Washburn, 2006). MudPIT has been proven to be an excellent tool for both qualitative and quantitative proteomic analysis. Although this technique is less complex and cheaper than iTRAQ, quantitation accuracy is relatively less and largely dependent on sample preparation.

31.3.1.7 Deep Proteome Analysis

Deep proteome analysis, also known as deep proteomics, is one of the approaches used to get the maximum coverage of proteins present in a complex protein mixture. The basic workflow of this technique relies on three protein separation techniques, that is, 1D PAGE, in-gel IEF (iso-electric focusing) and reverse phase LC–MS (liquid chromatography–mass spectrometry). The enhancement of the resolving power is achieved by gel fractionation and trypsin digestion steps after 1D-PAGE (Atanassov and Urlaub, 2013). To obtain the maximum and deeper coverage of peptides sometimes MudPIT approach is followed after trypsin digestion of fractionated gel slices. All these combined approaches coupled with Orbitrap MS enhance the sensitivity and peptide coverage by many folds, which in turn improves the identification of LAPs (linear azole-containing peptides) and posttranslationally modified proteins. Due to these advantages this is the most often used approach in recent years, often used for nonmodel plants.

31.3.2 Targeted Proteome Analysis

Targeted proteome analysis, as the name indicates, is the selective analysis in which only a few selective or targeted peptides are quantified. Earlier the targeted protein analysis was carried out by ELISA (enzyme linked immunosorbent assay) but it requires specific

antibodies and complete information of the proteins to be analyzed and thus it is not suitable for analysis of novel proteins. Targeted proteome analysis is mainly used for identification of interacting proteins or proteins of interest (immunoprecipitation) and identification of posttranslationally modified proteins. The profiling or identification of specific protein or selective group of proteins in this case is carried out by the use of motif or PTM-specific stains, antibodies (immunoassay) or targeted MS assays. Among these methods MS-based approaches are gaining more attention and utility over others because of the advantage of multiplexing many target peptides with quantitative information in a single experiment. Moreover, it has got the ability to systematically configure any protein or protein modifications of interest (Liebler and Zimmerman, 2013).

31.3.2.1 *Gel-Based Targeted Proteomics*

In case of gel based targeted proteomics protein-specific or PTM-specific staining procedures are followed after global profiling of proteins using 2DGE. PTM specific stains like ProQ-Diamond used for the detection of phosphoproteins, Lissamine Rhodamine B sulfonyl hydrazine (LRSH) used for the detection of glycoproteins are used for visualizing the proteins of interest. Another way of detection of phosphoproteins is by comparative analysis between phosphatase treated proteome profiles and untreated profiles, which would aid in mapping differential migration of proteins (Yamagata et al., 2002). Some web-based tools are also there that aid in putative identification of modified proteins in in silico gels. The limitation of this approach is that it can only detect certain PTMs like phosphorylation, glycosylation, acetylation, and ubiquitination after MS analysis (Mann and Jensen, 2003). These limitations are the main reason that the gel based targeted proteome analysis is not followed nowadays.

31.3.2.2 *Affinity and Reactive Chemistry-Based Proteomics*

For the large scale study of proteins or peptides of interest in a complex sample enrichment or purification is required. Immobilized metal affinity chromatography (IMAC) using transition metals such as Fe^{3+}or Ga^{3+} (Ficarro et al., 2002) as well as titanium dioxide (TiO_2) (Macek et al., 2009) or zirconium dioxide (ZrO_2) (Kweon and Håkansson, 2006) chromatography, often used in combination with strong cation exchange (SCX) chromatography, are the most common enrichment strategies used to date. Immunoprecipitation using phosphotyrosine-specific antibodies is another enrichment method frequently employed. This technique is usually employed for those low abundant peptides/proteins that tend to elude MS detection following other enrichment strategies (Kaboord and Perr, 2008).

31.3.2.3 *Mass Spectrometry–Based Targeted Proteomics*

There are a number of technologies that can be used to study proteomes, but arguably none is more powerful than mass spectrometry. The goal of a targeted proteomics experiment is to monitor or select a few proteins of interest with high sensitivity, reproducibility, and quantitative accuracy. Although various mass spectrometric platforms have been used for targeted proteomics analysis only a few tools like triple quadrupole, quadrupole trap (Q-Trap), and linear trap quadrupole-orbitrap (LTQ-Orbitrap) are widely employed. The triple quadrupole mass spectrometer operates as a dual mass filter that allows molecular ions of predetermined masses to be selected for fragmentation in the instrument. Peptide ions travel into the first mass filter, which is programmed to select specific "precursor" ions on the basis of their m/z ratio for fragmentation. The second mass filter then selects the target "product" ions, which are guided to the detector for quantification, resulting in a trace of signal intensity versus retention time for each precursor ion. The process of detecting specific signals or modifications in triple quadrupole is called selected reaction monitoring (SRM). Similarly, the process of detecting multiple modifications is called multiple reaction monitoring (MRM) (Liebler and Zimmerman, 2013). SRM is the most mature mass spectrometry–based technology but as it is laborious new methodologies are on the horizon. Sequential window acquisition of all theoretical spectra (SWATH) is one such approach in which complex mass spectra generated by data-independent acquisition are queried for the presence of specific peptides using libraries of qualified peptide fragment spectra. Another new approach called parallel reaction monitoring (PRM) provides increased speed, sensitivity, and selectivity because all transitions are monitored in parallel in a single analysis (Gallien et al., 2014). Despite many advances in detection and processing algorithms, MS-based label-free quantification methods suffer from light precision errors between samples. For this reason the strategy of isotope dilution is implemented with SRM/MRM/PRM techniques. After isotope labeling, the true and precise abundance of each protein or peptide is determined by standards such as absolute quantification (AQUA), quantification concatamers (QconCAT), and protein standard absolute quantification (PSAQ). QconCAT, PSAQ, and AQUA methods are highly specific and sophisticated, and find a better scope in clinical applications.

31.4 SALT STRESS IN PLANTS

Salinity imposes two kinds of stresses to plants; osmotic stress arising due to increased osmotic pressure, and ionic stress due to the increase in the levels of toxic ions like Na^+ and Cl^- leading to ionic imbalance (Flowers and Colmer, 2008). Osmotic stress causes cell membrane disruption in plants. Plants respond to osmotic stress, with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings. These signals activate responses to deal with extreme conditions. Ionic stress on the other hand disrupts the mineral relations of plants. Na^+ induce K^+ deficiency and Cl^- inhibit NO^{3-} absorption, which eventually generates imbalances and nutritional disorders and finally may confront the structure and composition of plant cells, injure macromolecules, and consequently lead to senescence of the leaves (Hakeem et al., 2013). Although the general effects of salinity stresses on plant growth and development have been studied, their influence at the physiological and biochemical levels is not well understood (Manuchehri and Salehi, 2014).

31.5 RESPONSE OF PLANTS TOWARDS SALT STRESS

31.5.1 Osmotic Stress

Osmotic stress occurs due to the lower osmotic potential of the soil because of the higher concentration of soluble salts in saline soils. Whenever plants are exposed to osmotic stress, they exhibit a wide range of responses at the molecular, cellular, and whole-plant levels. These include morphological and developmental changes, adjustment in ion transport, and metabolic changes (Xiong and Zhu, 2002). Plants tend to lose water to the external environment during osmotic stress, which in turn causes decrease in the turgor pressure. As a result of loss of turgor pressure the main processes of cell division and cell expansion are highly affected. This indirectly inhibits the plant growth. The decreased osmotic potential induces specific signaling events in plants, which include MAPK signaling, calcium signaling, phospholipid signaling, and ABA signaling (Kosová et al., 2013).

31.5.2 Ionic Stress

Ionic effect is considered as a continuous, long-term effect of an accumulative nature since it usually depends on the intracellular salt ion levels, which increase with the duration of salinity stress and also with aging processes of a given plant tissue. Salinity stress leads to an increase in the activity of salt ions in soil water surrounding plant root cells. This results in the entry of Na^+ ions into the cytoplasm passively via nonselective cation channels because of the negative electrical potential of plant cell cytoplasm. The excessive Na^+ ions harm plant growth and metabolism. To diminish these harmful effects sequestration of excessive Na^+ ions is required for which the SOS pathway paves the way.

31.6 SALT STRESS SIGNALING PATHWAYS

31.6.1 Salt Overly Sensitive (SOS) Signaling Pathway

Salt stress leads to ionic stress, which is crucial for normal plant growth. Hence, ion homeostasis by compartmentalization is an essential process for growth of plants under stress conditions. Glycophytes and halophytes both cannot tolerate high NaCl concentration. Therefore, salt is either transported or sequestered in older tissues to protect plants from salt stress. Although a number of possible salt signaling pathways for ionic homeostasis have been proposed, none is established in terms of signaling proteins and inputs and outputs. One exception is the SOS pathway, which emerged recently as a result of combinatorial approaches of genetic, molecular, and biochemical analysis. The SOS signaling transduction pathway is a major regulatory mechanism for ion homeostasis.

The Na^+ ions that enter the cytoplasm are transported to vacuoles via Na^+/H^+ antiporter. There are usually two types of H^+ pumps present in the vacuolar membrane: vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase (V-PPase) (Chinnusamy et al., 2005). Between the two pumps V-ATPase is the most dominant one and plays an important role in ion homeostasis. The SOS signaling pathway consists of three major proteins, SOS1, SOS2, and SOS3. SOS1 encodes plasma membrane Na^+/H^+ antiporter, which is one of the possible Na sensors and is essential in regulating Na^+ efflux at cellular level. Na^+/H^+ antiporter also plays a critical role in controlling long distance Na^+ transport from root to shoot. SOS1 protein has 10–12 transmembrane domains and a long tail (of more than 700 amino acids) to sense Na^+ and is supposed to exist in the cytoplasm. Another protein, that is, SOS5 containing two putative AGP-like (arabinogalactanproteins-like) domains and two alternatively organized fasciclin-like domains residing in the outer surface of the plasma membrane also seems as a candidate for being a Na^+ sensor, although limited data is available about the function of this protein

(Türkan and Demiral, 2009). SOS2 gene encodes serine/threonine kinase, which is activated by Ca^+ signals elicited by salt stress. The serine/threonine kinase protein has got N-terminal catalytic domain and C-terminal regulatory domain (Liu et al., 2000). The regulatory domain contains 21 amino acid long FISL motif (also called NAF domain), which serves as a site of interaction for Ca^+ binding SOS3 binding protein. SOS3 encodes EF-hand Ca^{2+} binding protein, also called myristolylated Ca^+ binding protein. This protein has an N-terminus myristolylation site, which plays an important role in conferring salt stress (Ishitani et al., 2000).

Whenever there is increase in the Na^+ level, intracellular Ca^+ levels get elicited. The cytosolic Ca^+ perturbations are decoded by Ca^{2+} sensing proteins such as calcineurin B-like proteins (CBLs) and their cooperative partners CBL interacting protein kinases (CIPKs) (Knight and Knight, 2001). This sharp increase in the Ca^+ levels facilitates the binding of SOS3 protein with SOS2. The SOS3 protein activates SOS2 protein by releasing its self-inhibition. The SOS3-SOS2 complex is then loaded onto the plasma membrane and their interaction results in the activation of kinases (Guo et al., 2004). Activated kinase phosphorylates SOS1 protein thereby increasing its transport activity. The phosphorylation of SOS1 results in increased Na^+ efflux reducing the Na^+ toxicity (Gupta and Huang, 2014). *SOS1* transcript present in *Arabidopsis* plants was found to be upregulated by NaCl treatment (Zhu, 2001). Moreover, molecular studies have also shown that *SOS1* gene expression is regulated by SOS2 and SOS3 (Huang et al., 2012). SOS3-SOS2 protein complex also downregulates the activity of low affinity Na^+ transporter HKT1 under salt stress so as to regulate the entry of Na^+ ions into cells (Mahajan et al., 2008). SOS2 also influences vacuolar Na^+/H^+ exchanger activity to sequestrate Na^+ ions into vacuolar compartment and thus facilitates the process of ion homeostasis (Fig. 31.2). SOS2 also regulates vacuolar H^+/Ca^+ antiporter CAX1, which is independent of SOS3 and thus maintains the Ca^+ homeostasis. It also interacts with AB12, which restores the homeostasis after stress condition by dephosphorylating the proteins that are phosphorylated by SOS2 (Ohta et al., 2003). Phosphorylation of SOS1 by the SOS2-SOS3 kinase complex activates SOS1 and results in increased tolerance to NaCl and enhanced Na exclusion in yeast (Quintero et al., 2002).

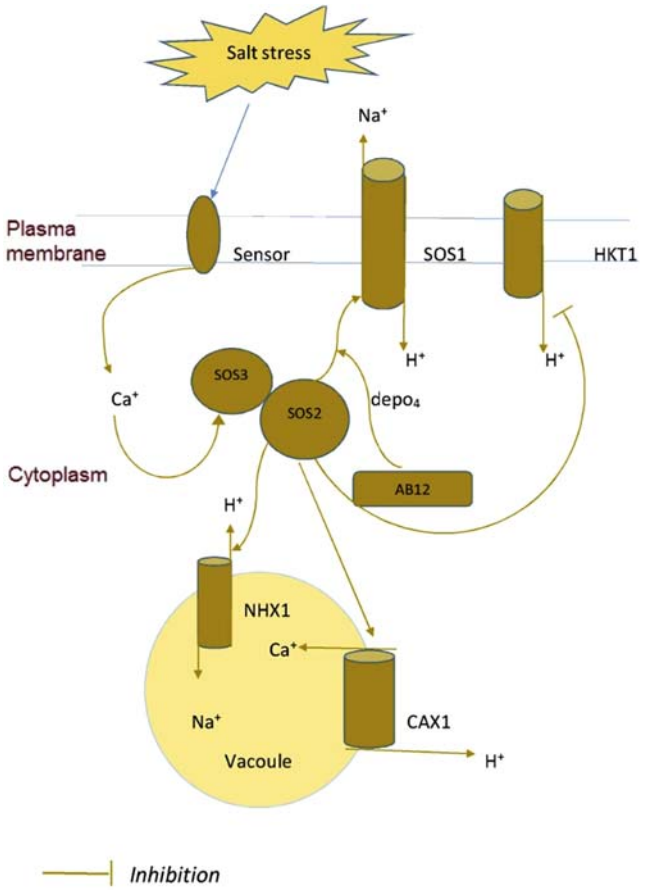

FIGURE 31.2 SOS (salt overly sensitive) pathway for the regulation of ion homeostasis during salt stress. Excessive Na^+ ions are sensed by unknown sensors at the plasma membrane, which leads to elicitation of cytosolic Ca^{2+} ions. Ca^{2+} signals are perceived by SOS3, which activates the SOS2 kinase. The SOS3-SOS2 complex phosphorylates plasma membrane Na^+/H^+ antiporter SOS1 to efflux Na^+ out of the cytoplasm. Further, it inhibits HKT1 activity so as to restrict Na^+ ions into the cytoplasm thereby regulating ion homeostasis. SOS2, independent of SOS3, regulates NHX1 activity to transport Na^+ from cytosol to vacuole so as to regulate cellular Na^+ levels. SOS2 also regulates vacuolar H^+/Ca^+ antiporter CAX1 to maintain the Ca^+ homeostasis. It also interacts with AB12, which restores the homeostasis after stress conditions by dephosphorylating the proteins that are phosphorylated by SOS2.

31.6.2 Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

The MAPK kinase pathways are intracellular signal modules that mediate signal transduction from the cell surface to nucleus. These are usually osmolarity signaling modules as they respond to the osmotic stress caused by various abiotic stress components (Kaur and Gupta, 2005). The MAP kinase family belongs to the CMGC group of the eukaryotic protein kinase superfamily. All MAPKs are characterized by the unique signature motif that distinguishes them from other eukaryotic protein kinases. This signature motif consists of part of the phosphorylation lip and the "P + 1" substrate-binding pocket, both of which are located on loop 12 of the three-dimensional structure of the MAP kinase and are critical for MAPK activity (Kültz and Burg, 1998). MAPK is one of the most studied

signaling mechanisms in plants. MAPK cascade is composed of 3 protein kinases: MAP3K, MAP2K, and MAPK. MAPK cascade has been mainly studied in *Arabidopsis*, which contains more than 80 MAPKs, 10 MAP2Ks, and 20 MAP3Ks. It has been observed that under salt stress MAPKs respond to the stress stimuli. The salt stress activated MAPKs are activated by secondary signals rather than primary osmotic stress signals. The rapid activation of multiple MAPKs, including MAPK3, 4, and 6, has long been observed in plants in response to biotic as well as abiotic stimuli (deZelicourt et al., 2016). These MAPKs are activated by MAP2Ks, which in turn are activated by MAP3Ks in a sequential manner via phosphorylation (Colcombet and Hirt, 2008) (Fig. 31.3). An activated MAPKKK (MAP3K) first phosphorylates two serine and/or threonine residues (S/T-X_{3-5}-S/T) located in the activation loop of MAPKK (MAP2K), which in turn phosphorylates the T-X-Y motif present in the activation loop of MAPK and in this way triggers the activation of MAPK (Hamel et al., 2012; Danquah et al., 2014). Activated MAPK is imported into the nucleus, where it phosphorylates and activates specific downstream signaling components. The sequential activation of MAPK cascade eventually results in the activation of transcription factors, phospholipases, cytoskeletal, and microtubule associated proteins and also results in the expression of number of genes that respond to the stress stimuli (Taj et al., 2010). In case of *Arabidopsis* it was observed that MKK2 acts as a key signal transducer for salt stress (Teige et al., 2004). The activity of GMK1 (*Glycine max* MAP kinase 1) was found to be induced and regulated by posttranslational modification with increasing salt concentrations (Im et al., 2012). Ectopic expression of ZmSIMK1 (*Zea mays* salt-induced mitogen-activated protein kinase 1) in *Arabidopsis* resulted in increased resistance against salt stress suggesting that ZmSIMK1 plays an important role in salt stress (Gu et al., 2010). In *Arabidopsis* it was found that MKK5 is involved in NaCl-induced salt stress via regulating the expression of iron superoxide dismutase gene (Xing et al., 2015).

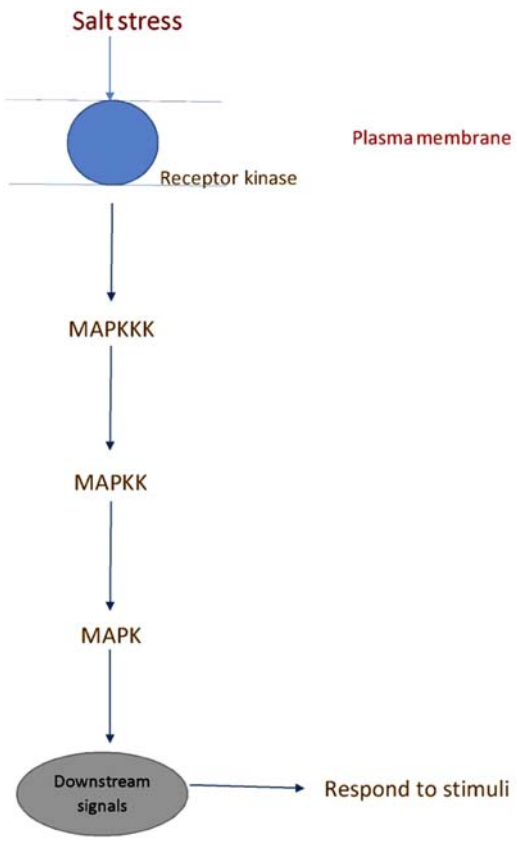

FIGURE 31.3 MAPK (mitogen activated protein kinase) cascade. In this pathway the salt stress induces receptor kinases present in the plasma membrane, which in turn causes the phosphorylation of MAPKKK. The MAPKKK causes the downstream signals by the activation of MAPKK and MAPK via sequential phosphorylation, ultimately leading to response to salt stress stimuli.

31.6.3 ABA-Signaling Pathway

ABA belongs to class of isoprenoids (terpenoids) and also belongs to the most important phytohormones involved in the plant growth, development, and adaptation to various stress conditions. ABA plays a vital role in controlling downstreaming response essential for adaptation to stress (Raghavendra et al., 2010). ABA signaling cascades, synchronized with the plant growth stage and other hormone levels regulate important abiotic stress responses (Park et al., 2016). One of the most important advances in the past few decades has been the identification of ABA receptors and the elucidation of the core ABA-signaling pathway. Stress induced biosynthesis of ABA primarily occurs in vascular tissue but it exerts its response in various cells, including distant guard cells (Kuromori et al., 2010). ABA signaling transduction occurs via central signaling module made up of proteins belonging to three classes: pyracbactin resistance/pyracbactin resistance like/regulatory component of ABA receptors (PYR/PYL/RCARs), which act as ABA receptors; protein phosphatase 2Cs (PP2Cs), which act as negative regulators; and SNF1-related protein kinase 2s (SnRKs), which act as positive regulators (Danquah et al., 2014). PYR/PYL/RCAR, a family of soluble START domain proteins, plays a central role in ABA perception (Melcher et al., 2010). Members of this family like PYR1, PYL1, and PYL2 were biochemically shown to directly bind to ABA. The entire family of PYR/PYL/RCAR is capable of activating ABA signaling response, which indicates that nearly all members can function as ABA receptors (Fujii and Zhu, 2009).

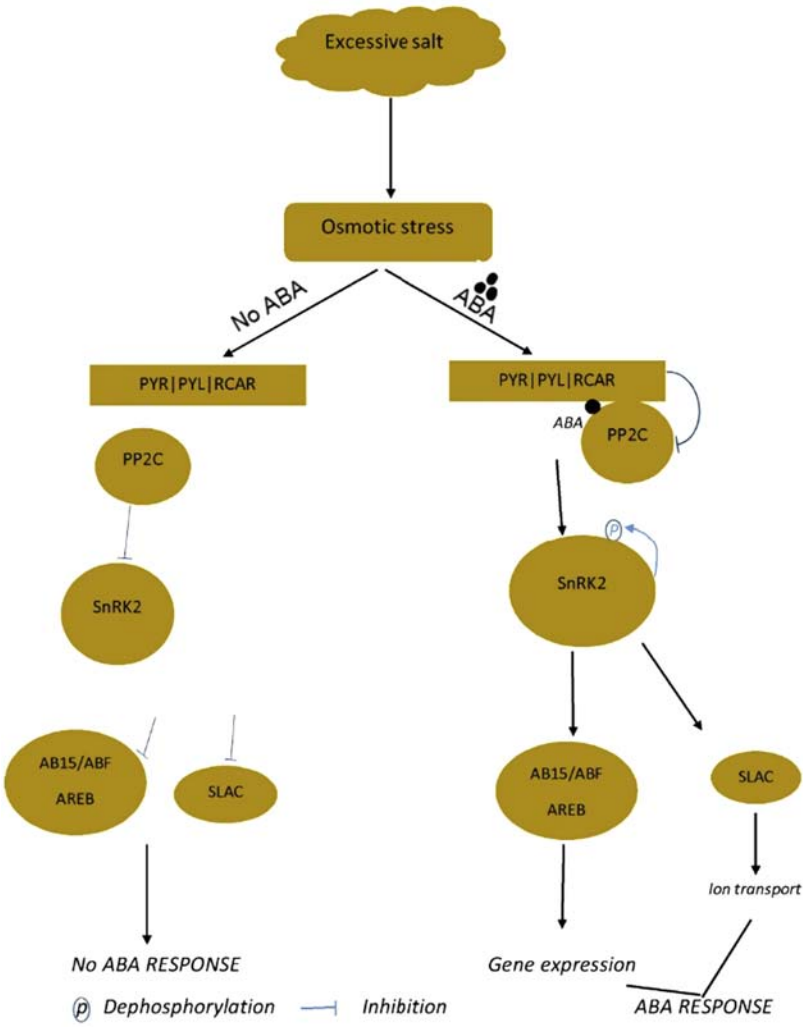

FIGURE 31.4 ABA signaling events during salt stress. Salt stress causes osmotic stress in plants, which is responded to by ABA. Whenever there is presence of ABA, the ABA receptors PYR/PIL/RCAR proteins bind to ABA and in turn inhibit PP2C activity, allowing the activation of SnRK2 through autophosphorylation. Active SnRK2 kinases phosphorylate downstream target proteins to induce ABA response. In absence of ABA, PP2C is active because of the inhibition of ABA receptor proteins and hence it inhibits the activity of SnRK2. Due to inhibition of SnRK2 there is no downstream signals and hence no ABA response.

PYLs bind ABA in presence of clade A PP2Cs, such as ABI1 (ABA-INSENSITIVE 1), ABI2, HAB1 (Homology to ABI1), and PP2CA. This is the main reason these PP2Cs are considered as coreceptors. In the absence of ABA, PP2Cs dephosphorylate SnRK2 kinases including SnRK2.1, 2.3, and SnRK2.6 in a serine residue in the kinase activation loop. This phosphorylation is necessary for kinase activity (Soon et al., 2012). There is docking of kinase activation loop of SnRK2 into the active site of PP2Cs, while the insertion of the conserved ABA-sensing tryptophan of PP2Cs into the kinase catalytic cleft occurs. In this way the kinases are kept inactive by blocking their catalytic cleft and by dephosphorylating the activation loop ultimately leading to the inhibition of enzyme activity (Miyazono et al., 2009; Melcher et al., 2010). In the presence of ABA, the ABA receptors PYR/PYL/RCAR proteins bind to ABA and in turn inhibit PP2C activity, allowing the activation of SnRK2s through autophosphorylation. This allows the SnRK2s to rely upon the ABA signal to downstream effectors (Boudsocq et al., 2007) (Fig 31.4). 10 SnRK2 were activated by hyperosmolarity induced by NaCl in *Arabidopsis*, indicating an important role of the SnRK2 family in osmotic signaling (Boudsocq et al., 2004). In rice it was found that overexpression of *OsPP1* enhanced tolerance to high salt treatment (Liao et al., 2016).

31.6.4 Ca^{2+}/Calmodulin (CaM) Signaling Pathway

Calcium was first described as an essential macronutrient element in plants, which is taken by roots and then delivered to shoot via xylem. Ca^{2+} ions represent an important signaling molecule and a convergence point of many disparate signaling pathways. The cytosolic Ca^{2+} in plant cells increases in response to various abiotic stresses. Salt stress-induced Ca^{2+}-dependent signaling network has been reported to mediate Na^{+} homeostasis and salt resistance (Mahajan et al., 2008). An elevation in cytosolic free calcium due

to opening of stretch-dependent or voltage-gated calcium channels is the secondary signal caused by many different environmental stresses. Cytosolic calcium waves are produced within the cytoplasm by successive recruitment of particular Ca^{2+} channels to coordinate cellular response. The local elevation of cytosolic Ca^{2+} generates secondary messengers such as IP3 (inositol trisphosphate) or cADPR that activate a relay of spatially separate Ca^{2+} channels (Drøbak and Watkins, 2000). The information encoded in transient Ca^{2+} changes is decoded by an array of Ca^{2+}-binding proteins, which fall into two main classes, referred to as sensor relays and sensor responders. Sensor relays, which include calmodulin (CaM), CaM-related proteins and calcineurin B-like proteins (CBLs), function through biomolecular interaction. Sensor responders such as the Ca^{2+}-dependent protein kinases (CDPK) function at first through intramolecular interactions and undergo a Ca^{2+}-induced conformational change that alters the protein's own activity or structure (Lecourieux et al., 2006). Calmodulin (CaM) is a small (17 KDa), highly conserved, acidic protein. It has two globular domains each containing two EF hands connected by a flexible α-helical linker (Luan et al., 2002). CaM appears to be regulatory protein and induces large changes in interhelical angles as Ca^{2+} is bound. There is influence of particular target proteins for affinity of CaM for Ca^{2+}. CaM-like proteins differ from the CaM in containing more than 148 amino acid residues and have between one and six EF hand motifs. CDPKs are serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF-hand motifs that can directly bind Ca^{2+}. Some CDPKs have an N-terminal myristoylation motif suggesting potential association with membranes. Calcineurin B-like proteins (CBLs) are relatively a new class of calcium sensors that are essential in the SOS signaling pathway to reinstate cellular ion homeostasis. There are several other proteins that bind Ca^{2+} but do not contain EF hand motifs. These include the phospholipase D (PLD), annexins, calreticulin, and Pistil-expressed Ca^{2+} binding protein (PCP) (Tuteja and Mahajan, 2007). The information that is decoded by calcium binding proteins during stress gives rise to a cascade of downstream effects, including altered protein phosphorylation and gene expression patterns (Sanders et al., 2002). Studies on *Arabidopsis* revealed that expression of OsMSR2 (*Oryza sativa* L. Multistress-Responsive gene 2), a novel calmodulin-like protein gene conferred enhanced tolerance in it under high salinity (Xu et al., 2011). Similarly, in another study of *Arabidopsis*, overexpression of a salt-induced CaM gene from soybean, *GmCaM4*, conferred salt stress tolerance through the upregulation of DNA-binding activity of a MYB transcription factor MYB2 (Zeng et al., 2015). Studies also provided the information that CaM methylation plays a regulatory role in *Arabidopsis* under salt stress (Banerjee et al., 2013). In another study the mRNA of CaM-4 was significantly induced in response to salt stress in soybean (Park et al., 2004).

31.6.5 Phospholipid Signaling Pathway

Membrane lipids give rise to various signaling messengers, such as phosphatidic acid (PA), diacylglycerol (DAG), DAG-pyrophosphate (DAG-PP), lysophospholipids, free fatty acids (FFAs), oxylipins, phosphoinositides, and inositol polyphosphates (Wang et al., 2007). Phosphatidic acid acts as a messenger in osmotic stress. Signaling PA is generated by two principal routes in plants. One is the direct PA production by phospholipase D (PLD)-catalyzed hydrolysis of common membrane phospholipids. Another is phospholipase C (PLC) hydrolysis of phosphatidylinositol (4, 5) bisphosphate PI(4, 5)P2 followed by phosphorylation of DAG by DAG kinase (DGK). PLD and PA have been suggested to affect osmotic stress−induced production of proline (Thiery et al., 2004). Moreover, it has been found that 1-butanol, which is an inhibitor of PA production by PLD, reduces NaCl-induced H^+-ATPase activation, whereas applied PA stimulated H^+-ATPase activity (Zhang et al., 2006). These results suggest that PLD and PA play an important role in salt stress. Under salt and hyperosmotic conditions, PLC-produced DAG is phosphorylated to PA by DGK and it has been found that the PLD and PLC/DGK reactions are activated differentially in response to different stimuli (Zonia and Munnik, 2004). PA regulates ABI1 function by inhibiting its phosphatase activity and by sequestering it in the plasma membrane thereby leading to the closure of stomata. Phosphoinositides (PIs) are phosphorylated phosphatidylinositols and they include three monophosphorylated PI3P, PI4P, and PI5P; three bisphosphorylated PI(4, 5)P2, PI(3, 4) P2, and PI(3, 5)P2; and one trisphosphorylated PI(3, 4, 5)P3. PIs are important signaling molecules that regulate actin organization, membrane trafficking, endo/exocytosis, and ion channels. Increase in PA and PIP_2 with respect to time and dose was observed in rice under the effect of salinity (Darwish et al., 2009). In another study rapidly increased PI(4, 5)P2 synthesis in response to NaCl treatment was observed in *Arabidopsis* suggesting its important role in salt tolerance (DeWald et al., 2001).

31.6.6 ROS Signaling Pathway

ROS are produced during abiotic stresses and it has been hypothesized that ROS production can be the primary symptom of phytotoxicity. ROS are capable of

inducing cellular damage by degradation of proteins, inactivation of enzymes, alterations in the gene, and interfering in various pathways of metabolic importance. ROS includes hydrogen peroxide (H_2O_2), superoxide radical ($O2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) etc. (Khan and Khan, 2013, 2017). In plants, NADPH oxidases, respiratory burst oxidase homologs (RBOHs), play a key role in the network of ROS production. In recent years, several studies have revealed that plant RBOHs are involved in a multitude of different signaling pathways including stomatal closure, plant defense, and acclimation to different abiotic stresses (Suzuki et al., 2011). Plant RBOHs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, and six conserved transmembrane-spanning domains. RBOHs have a cytosolic N-terminal extension comprised of two Ca^{2+}-binding EF-hand motifs and phosphorylation target sites that are important for their activity (Kimura et al., 2012). Salt stress induces elevation in cytosolic Ca^{2+} ions leading to the activation of Rboh (Lecourieux et al., 2006). Once activated, superoxide ($O_2^{\bullet-}$) is produced at the apoplast via the function of RBOH proteins, which is then dismutated to H_2O_2 by SOD. $O2^{\bullet-}$ and H_2O_2 act as secondary messengers in plants by regulating diverse function of growth and development. It is quite evident that ROS act as intracellular signaling molecules, but the controversy lies in the fact that how they can set specific signaling duties. This controversy arises because the specificity in signaling pathways is generally mediated via the noncovalent binding of a ligand to its cognate receptor through a shape-complementary fit between macromolecules but ROS deliver signaling events via chemical reactions that lead to protein modifications (Nathan, 2003). The chemical characteristics and the biological activities of each ROS reveal an answer to this question. $O_2^{\bullet-}$ is unstable and is not able to diffuse through membranes because of its negative charge, which makes this ROS a relatively poor signaling molecule. On the other hand H_2O_2 is relatively stable and can diffuse through biological membranes because it is not charged, which makes it fit for signaling. The intracellular signal transduction is mediated by H_2O_2 through chemoselective oxidation of Cys residues in signaling proteins, such as glutathione, thioredoxins, and peroxiredoxins (Paulsen and Carroll, 2009). High indiscriminate reactivity of $OH^{\bullet}$ limits its diffusion to sites of production. $^1O_2$ could deliver specific signaling events mainly through spatial aspects of ROS production because the half-life time of $^1O_2$ is very short and as a result it can react directly only with molecules in close proximity to its production location. In recent years rapid progress has been made in defining ROS as a major signal in diverse biological processes in plants. These are considered as highly controlled signaling molecules, which are able to transfer the environmental signals, with other signaling intermediates, to the genetic machinery (Polidoros et al., 2005).

In plant cells, the ROS production is strictly regulated by ROS scavenging pathways involving enzymatic and nonenzymatic antioxidants. The major antioxidants that play crucial role in ROS detoxification include ascorbic acid (AA), α-tocopherol, glutathione, catalase (CAT), peroxidases (POX), superoxide dismutase (SOD), glutathione reductase (GR), etc. SOD is considered as a first-line defense against ROS (Khan and Khan, 2014; Khan et al., 2015, 2017). It converts superoxide to H_2O_2 while APX, GPX, and CAT detoxify H_2O_2. AA is used as a substrate by ascorbate peroxidase (APX) to reduce H_2O_2 to H_2O in the ascorbate−glutathione cycle and generate monodehydroascorbate, which further dissociates to AA and dehydroascorbate (Choudhury et al., 2013). The ROS signaling pathway can regulate additional pathways that are physically nonadjacent to the pathway in which it was formed (Nathan, 2003). One such example is the activation of a secondary messenger such as a MAPK or a plant hormone by ROS, which in turn activate remote signaling pathways.

31.6.7 Jasmonic Acid (JA), Ethylene (ET), and Salicylic Acid (SA) Signaling Pathways

JA is one of the important signaling phytohormones that play an essential role in plant development as well as respond to biotic and abiotic stresses (Khan et al., 2012; Per et al., 2018). JA synthesis usually occurs from the α-linolenic acid precursor through the octadecanoid pathway. JA is activated by enzymatic coupling to isoleucine amino acid. The resulting JA-Ile functions as a ligand promoting assembly of a coreceptor complex between the F-box protein COI1 and JA ZIM-domain (JAZ) proteins (Thines et al., 2007). JAZ proteins bind to bHLH (basic helix−loop−helix) transcription factors (e.g., MYC2, MYC3, MYC4, and MYC5) that are activators of JA responses repressing their transcriptional activity and turning off the expression of the early JA-responsive genes (Qi et al., 2015; Zhang et al., 2015). JAR1 (jasmonate resistant 1) is a JA-amidosynthetase that catalyzes the conversion of JA to JA-Ile, which is in turn recognized by the COI1-JAZ coreceptor (Sheard et al., 2010). After destabilizing JAZ by action of JA-lle, transcription factors are released from repression activating early JA responses. This switching on of JA responses leads to the attenuation of hormone signaling by induction of the JA-responsive JAZ genes to avoid the inhibitory effect that overactivation of JA responses has on plant

growth (Zhang and Turner, 2008). JAZ proteins are transcriptional repressors that prevent the transcription of target genes under low JA-Ile levels, and are specifically ubiquitinated when JA-Ile accumulates under biotic stress. JA-Ile is therefore a master switch controlling various aspects of plant immunity/adaptation. Exogenous supply of JA confirmed that it acts as a positive regulator of salt tolerance in wheat (Qiu et al., 2014). In *Arabidopsis* elevated JA levels promoted salt tolerance, suggesting that JAs positively regulate salt tolerance (Zhao et al., 2014). Although role for jasmonates for the adaptation to salt stress has been suggested (Fujita et al., 2006), molecular mechanisms of the role of jasmonates for salt stress-signaling are still mostly unclear.

Ethylene is a simple gaseous hormone synthesized through three enzymatic reaction steps: in the first reaction methionine is converted to *S*-adenosyl-methionine (*S*-AdoMet) by *S*-adometsynthetase, after this the direct precursor of ethylene ACC(1-aminocyclopropane-1-carboxylic) is synthesized from *S*-AdoMet by ACS (aminocyclopropane-1-carboxylic acid synthase), and finally ethylene is produced through the oxidation of ACC by ACO (ACC oxidase) (Lin et al., 2009; Khan et al., 2017). The main components of ethylene signal transduction model include five ethylene receptors, a negative regulator CTR1 (CONSTITUTIVE TRIPLE RESPONSE1), a key positive regulator EIN2 (ETHYLENE INSENSITIVE2), primary transcription factors EIN3/EILs (ETHYLENE-INSENSITIVE-LIKE), and many downstream ethylene-response factors. The main ethylene signaling components that pave the way to downstream the signal of EIN3 are ERFs (ethylene-responsive element binding factors). During normal conditions, ethylene receptors interact with CTR1 and activate it, which in turn phosphorylates EIN2 and prevents its translocation into nucleus, therefore inhibits ethylene signal transduction. But whenever ethylene production is induced by environmental signals, it binds with receptors to inhibit their interaction with CTR1. This leads to the inactivation of CTR1, causing the dephosphorylation and cleavage of EIN2 (Tao et al., 2015). There is translocation of C-terminus of EIN2 into nucleus to stabilize downstream transcription factors EIN3/EILs (Wen et al., 2012). The whole ethylene signaling pathway possibly mediates the stress signal transduction as the whole pathway is involved in salinity stress response in plants. Ethylene receptors have been found to have contrasting roles in seed germination in *Arabidopsis* during salt stress. ETR1 and EIN4 stimulate ABA biosynthesis to inhibit germination. On the other hand ETR2 suppresses ETR1 and EIN4, reduces ET levels, and improves germination under salt stress (Wilson et al., 2014). Also the lack of ETO1 (*ETHYLENE OVERPRODUCER1*) function in *Arabidopsis* conferred soil-salinity tolerance through improved shoot Na/K homeostasis, effected via the ETHYLENE RESISTANT1−CONSTITUTIVE TRIPLE RESPONSE1 ethylene signaling pathway (Jiang et al., 2013).

Salicylic acid (SA) is a phenolic compound and it is involved in the regulation of some of the important plant physiological processes such as photosynthesis, nitrogen metabolism, proline (Pro) metabolism, production of glycinebetaine (GB), antioxidant defense system, and plant−water relations under stress conditions and thereby provides protection in plants against abiotic stresses (Nazar et al., 2011; Miura and Tada, 2014; Khan et al., 2015, 2012). Salicylic acid is synthesized via SID2-dependent and SID2-independent biosynthesis. To induce defense signaling, SA binds to some specific receptors including SA methyl transferase 1 (SAMT1) and SA-binding protein 2 (SABP2). Transcriptomic-profiling study on tomato revealed that SABP2 was induced by salinity suggesting involvement of SABP2 in the salt tolerance mechanisms (Sun et al., 2010). Another SA receptor, NPR1 (nonexpresser of PR proteins 1) emerged as a master regulatory protein of SA-dependent defense responses (Wu et al., 2012). *Arabidopsis* npr1 mutant showed enhanced growth during salt stress (Hao et al., 2012). On the other hand NPR1-hyper accumulating *Arabidopsis* double mutant (npr3npr4) failed to undergo programmed cell death (Fu et al., 2012). This controversial role of NPR1 during salt stress suggests that salt tolerance in plants can be controlled by both NPR1-independent and NPR1-dependent mechanisms (Jayakannan et al., 2013). One of the major actions of SA is the inhibition of catalase, a major H_2O_2 enzyme scavenging, thereby increasing cellular concentrations of H_2O_2, which acts as a second messenger and activates defense-related genes (Ananieva et al., 2002).

31.7 PROTEOMICS APPROACH IN UNDERSTANDING SALT STRESS SIGNALING PATHWAYS

Plants' response to salinity at the proteomic level has been investigated in both halophytes as well as glycophytes but there are only few reports on differential protein abundance of some salinity signaling involved protein. The main reason for this is that the signaling involved proteins are mostly low abundant proteins, which are hardly detectable on the 2DE gels and for proteome analysis 2DE is the most preferred technique. However, the gel free based techniques have more or less overcome this problem.

Proteomic studies of rice (Li et al., 2010) and *S. salsa* (Li et al., 2011) revealed that the calcium binding

proteins are regulated by salinity while studying the salt stress induced plasma membrane–associated proteome of rice shoots. This observation was further confirmed through the study of salt stress induced differential proteome response in the shoots of *Aeluropus lagopoides* (Sobhanian et al., 2010) and proteomic changes in maize roots under saline conditions (Zörb et al., 2010). Among all calcium binding proteins the most studied ones are calreticulin and calmodulin. During the study of root proteome of barley under salt stress, annexin, a calcium dependent membrane protein that mediates osmotic stress and ABA signaling transduction, was found to be upregulated in salt tolerant barley line. Annexins sense Ca^{+} signals induced by ABA signaling and transmit them to downstream signaling pathways. This study also confirmed the role of JA during salt stress because the most important components involved in the biosynthesis of oxylipins (JA), that is, linolate 9S-lipoxygenase 1 and lipoxygenase 2, were found to be increased under salt stress (Mostek et al., 2015). Some other researchers also found that the proteins involved in JA biosynthesis are regulated by salinity. Among these proteins two 12-oxophytodienoate reductase proteins involved in JA biosynthesis were found to be reduced by salt stress in sensitive cucumber plants (Fan et al., 2015); and the enzymes such as allene oxide synthase, 4-coumarate-CoA ligase-like 4, lipoxygenase 2.2 were found to be upregulated under higher salt stress level in durum wheat (Capriotti et al., 2014). One of the proteins, that is, 14-3-3, which is ubiquitous and multifunctional, interacts with calcium dependent protein kinases

TABLE 31.1 List of Proteins Involved in Salt Stress Signal Transduction Identified by Proteome Analysis

S. NO	Signaling proteins identified	Proteomic technique used	Plant species
1	**I.** Upregulation of serine/threonine protein phosphatase, PP1 catalytic subunit (PPP1C), and serine/threonine protein phosphatase 2B regulatory subunit (PPP3R) **II.** Downregulation of COI-1 protein, which is involved in JA response **III.** CTR-1 protein, involved in ethylene signaling, was found to be downregulated	iTRAQ coupled with LCMS/MS	*Carex rigescens*[b]
2	**I.** Calmodulin-1/11/16, a Ca^{2+} binding protein was found to be downregulated in salt tolerant variety **II.** 14-3-3 like protein E was found to be regulated by salt stress	iTRAQ coupled with SCX and LCMS/MS	Cotton[a]
3	**I.** LysM domain receptor like kinase-4 involved in signal transduction was found to be differentially regulated	iTRAQ coupled with LCMS/MS	Maize[c]
4	**I.** Calmodulin 490 proteins (F2E7M2 and A0MMD0) showed significant decrease under salt stress	iTRAQ coupled with nano-HPLC-MS/MS	Barley[d]
5	**I.** Probable mannitol dehydrogenase involved in signaling was differentially expressed **II.** Histone deactylase signaling protein was regulated under salt stress	2D-PAGE coupled with MALDI-TOF/TOF-MS	*Azolla microphylla*[f]
6	**I.** Type 1 MADS box transcription factor involved in stress transduction was found to be upregulated under salt stress **II.** SNF-1 related kinase was upregulated under salt stress **III.** SOS2 was found to be upregulated under salt stress **IV.** Remonin family protein and zinc finger protein, putative were also upregulated	2D-PAGE coupled with MALDI-TOF/TOF-MS	Mustard[g]
7	**I.** Serine/threonine protein kinase was found to undergo posttranslational modifications **II.** PI3-kinase was also found to be modified **III.** Acid phosphatase was also found to be modified under salt stress	1D-PAGE coupled with LC-MALDI-TOF/TOF	*Citrus aurantium*[e]

[a]*Gong, W., Xu, F., Sun, J., Peng, Z., He, S., Pan, Z. and Du, X., 2017. iTRAQ-based comparative proteomic analysis of seedling leaves of two upland cotton genotypes differing in salt tolerance. Frontiers in plant science, 8, 2113.*

[b]*Li, M., Zhang, K., Long, R., Sun, Y., Kang, J., Zhang, T. and Cao, S., 2017. iTRAQ-based comparative proteomic analysis reveals tissue-specific and novel early-stage molecular mechanisms of salt stress response in Carex rigescens. Environ. Exp. Bot., 143, 99–114.*

[c]*Luo, M., Zhao, Y., Wang, Y., Shi, Z., Zhang, P., Zhang, Y., Song, W. and Zhao, J., 2017. Comparative proteomics of contrasting maize genotypes provides insights into salt-stress tolerance mechanisms. J. Proteome Res., 17(1), 141–153.*

[d]*Shen, Q., Yu, J., Fu, L., Wu, L., Dai, F., Jiang, L., Wu, D. and Zhang, G., 2017. Ionomic, metabolomic and proteomic analyses reveal molecular mechanisms of root adaption to salt stress in Tibetan wild barley. Plant Physiol. Biochem.*

[e]*Tanou, G., Filippou, P., Belghazi, M., Job, D., Diamantidis, G., Fotopoulos, V. and Molassiotis, A., 2012. Oxidative and nitrosative-based signaling and associated post-translational modifications orchestrate the acclimation of citrus plants to salinity stress. Plant J., 72(4), 585–599.*

[f]*Thagela, P., Yadav, R.K., Mishra, V., Dahuja, A., Ahmad, A., Singh, P.K., Tiwari, B.S. and Abraham, G., 2017. Salinity-induced inhibition of growth in the aquatic pteridophyte Azolla microphylla primarily involves inhibition of photosynthetic components and signaling molecules as revealed by proteome analysis. Protoplasma, 254(1), 303–313.*

[g]*Yousuf, P.Y., Ahmad, A., Ganie, A.H., Sareer, O., Krishnapriya, V., Aref, I.M. and Iqbal, M., 2017. Antioxidant response and proteomic modulations in Indian mustard grown under salt stress. Plant Growth Regul., 81(1), 31–50.*

during stress conditions and is hence involved in many cellular signaling pathways. This protein has been found to be involved in salt signaling transduction through proteomic studies of rice (Malakshah et al., 2007), *P. patens* (Wang et al., 2008), and mangrove (Wang et al., 2014). Proteomic study of bread wheat revealed that the ethylene receptor (ETR) and three isoforms of receptor protein kinase (RPK) were stimulated by salinity, which suggests the possible role of ethylene and ABA signaling pathways in salt response (Peng et al., 2009). Another protein involved in the synthesis of ethylene, that is, ACC oxidase, was found to be downregulated under salt stress in *Tangut nitraria*, which validates the earlier reports of the role of ethylene signaling in response to salt stress (Cheng et al., 2015). The role of ABA signaling was also confirmed while studying *Brachypodium distachyon* in which protein PP2C 70 and serine/threonine protein kinase SAPK8 were found to be phosphorylated under salt stress, which indicates that these proteins function in the phosphorylation status in response to salt stress (Lv et al., 2014). The number of signal transduction proteins was found to be regulated while studying proteomic changes in salt stressed radish. These proteins include annexin 1 and annexin E1, calcium dependent protein kinase 2 and calcium dependent protein kinase 21, calmodulin 5, calmodulin like protein 13 and calmodulin like protein 20, mitogen-activated protein kinase 3, phospholipase C and phospholipase D (Sun et al., 2017). Reportedly, one of the members of mitogen-activated protein kinase pathway, that is, mitogen-activated protein kinase 4, was found to be modulated under saline conditions, which further signifies the implication of this pathway in salt stress signaling (Cheng et al., 2015). Phosphorylation of polyphosphoinositide phosphatase 1016 under salt stress implies that the phospholipid signaling pathway has its role to play during salt stress (Lv et al., 2014). Moreover, the increased level of iniostol1;3,4-triphosphate 5/6- kinase in *A. thaliana* (Ndimba et al., 2005) and *S europaea* (Wang et al., 2009) in the earlier reports also provides insights of phospholipid signaling pathway under salt stress. Some of the salt stress induced proteins related to signaling are enlisted in Table 31.1.

31.8 CONCLUSION AND FUTURE PERSPECTIVES

2-DE is considered as the most preferred technique for comparative studies of dynamic protein profiling during developmental or stress responses. However, the poor resolution of the low abundant proteins has led to introduction of shotgun proteomics as a complementary approach to address these technical limitations. Besides this the major challenge in the field of abiotic stress proteomics research is to unravel the process of PTMs and interactomics (protein−protein interactions) so that the major components involved in signaling pathways can be explored. Also the crosstalk between signaling pathways needs to be understood to find out how plants interact with and adapt to the stress conditions. Hopefully, in the coming years the integration of plant proteomics will deliver the raw information to predict which protein forms, PTMs, and protein complexes are present at a specific moment in a given tissue upon stress so that the information can be used to reveal the mechanism of salt tolerance and further implemented to generate tolerance in those crops that fail to survive under saline conditions.

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CHAPTER

32

Heat Shock Proteins (Hsps) Mediated Signalling Pathways During Abiotic Stress Conditions

Kummari Divya, Pooja Bhatnagar-Mathur, Kiran K. Sharma and Palakolanu Sudhakar Reddy

Cell, Molecular Biology & Genetic Engineering Group, Research Program—Genetic Gains, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India

OUTLINE

32.1 INTRODUCTION

During their entire life span plants encounter several abiotic stresses and also experience various complex environmental interactions comprising multiple factors. Plants have evolved with specific adaptive mechanisms to cope with stress conditions (Meena et al., 2017; Khan and Khan, 2017). When plants are exposed to various stresses plant metabolism is disturbed (Massad et al., 2012; Khan et al., 2012, 2013; Per et al., 2017, 2018), which eventually affects the plants' growth and productivity up to >50% (Wang et al., 2003; Shao et al., 2008). A crucial step in plants' stress tolerance mechanism to abiotic stress is the timely recognition of the stress signals and activation of the composite signaling cascades of defense in a specific and efficient manner (Chinnusamy et al., 2004; AbouQamar et al., 2009; Andreasson and Ellis, 2010; Khan et al., 2016). To protect against different stresses, plants activate several specific stress-responsive mechanisms by perceiving

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the stress signals, then producing molecules through signal transduction by up/down regulation of the many sets of mechanisms and their genes (Wang et al., 2003; Per et al., 2017, 2018). Products of stress-induced genes are classified into two major groups: (1) proteins, which directly protect against stresses include chaperones, Lea proteins, antifreeze proteins, and detoxification enzymes; and (2) some of which regulate gene expression and signal transduction pathways include transcription factors (TFs), protein kinases, and enzymes (Basu and Roychoudhury, 2014). Among them, Hsps come under the umbrella of chaperones, which show important stress-related chaperone functions in plants under abiotic stress conditions (Hendrick and Hartl, 1993; Bartels et al., 2007; Reddy et al., 2010, 2011, 2014a,b, 2016). Chaperones include specific stress-related proteins and are involved in protein synthesis, targeting, maturation, degradation, membrane stabilization, and protein renaturation (Reddy et al., 2014, 2016; Díaz-Villanueva et al., 2015). In the presence of abiotic stresses, there is an assembly of some chemical messengers that positively affects a plant's stimulus to the stresses and hence protects it from different aggressors (Pastori and Foyer, 2002; Rasmussen et al., 2013; Khan et al., 2015, 2016). Plants' reflex in adverse stress conditions is by altering the expression of a complex array of genes and elucidation of the biochemical and molecular pathways (Fig. 32.1). Genes associated with these pathways have been the foremost focus of research over the last two to three decades and the mechanisms by which these genes and their products interact remain relatively less focused.

Commonly, Hsps shield cells from injury and assist in revival and endurance after a return to normal growth conditions (Al-Whaibi, 2011). Under stress, by default Hsps play as molecular chaperones whereas under nonthermal stress their function can be changed (Timperio et al., 2008). It is implied that Hsps play a consistent role as molecular chaperones by regulating the enfolding, aggregation, transport, and degradation of the proteins in the plants (Hu et al., 2009; Tripp et al., 2009; Gupta et al., 2010; Al-Whaibi, 2011; Reddy et al., 2010, 2011, 2016). Based on their protein size, Hsps can be divided into five families that have been shown to have important stress-related chaperone functions in the plants (Török et al., 2001; Reddy et al., 2016). Each Hsp family has a distinctive mechanism and the role of each family is summarized in the subsequent sections (Table 32.1). Hsps' function in signal transduction has been established from experimental results that Hsp90 and Hsp70 proteins are associated with a number of signaling molecules, including v-Src, Raf1, Akt, and steroid receptor protein kinases like MAPK and Ca^{+2} (Sato et al., 2000; Nollen and Morimoto, 2002; Wang and Huang, 2017). The components or signaling molecules and cochaperones of both ABA-dependent and independent signaling pathways that activate the TFs needs to be defined genetically for assessing whether a signaling pathway is specific to a particular stress or is involved in crosstalk with

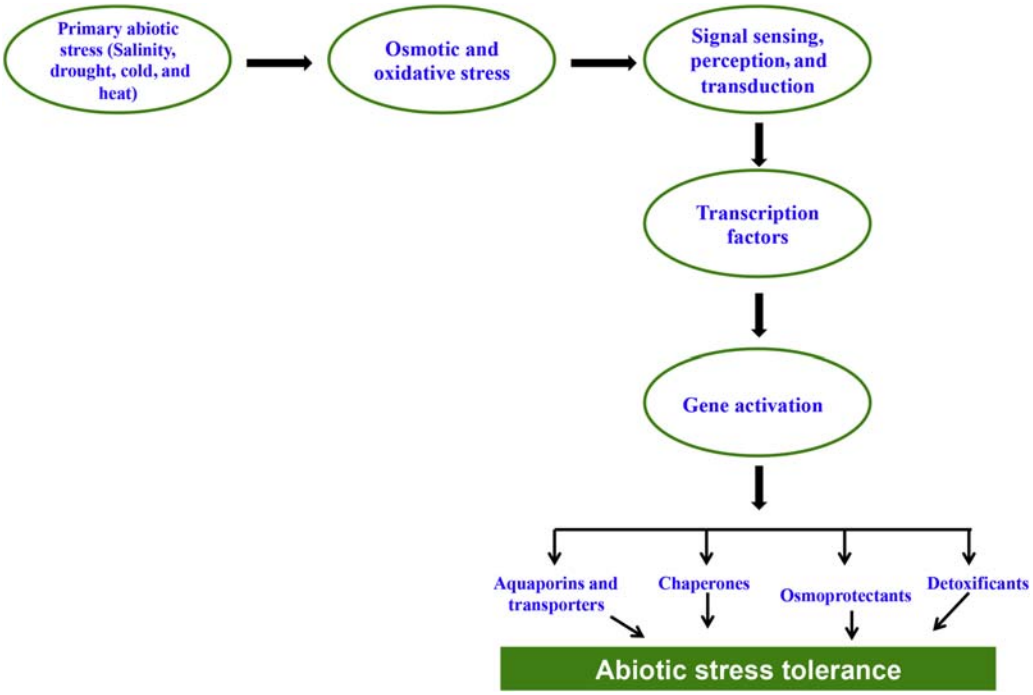

FIGURE 32.1 Complex signaling network of the plant response against different abiotic stress.

TABLE 32.1 Different Classes of Heat Shock Protein Families, Their Location and Functions

Classes	Representative members	Location	Major functions
Hsp100/Clp	Hsp100	Cytosol, mitochondria	Disaggregation, unfolding
Subfamily:		Chloroplast	
Class I	ClpB, ClpA/C	Chloroplast	
	ClpD		
Class II	ClpM, ClpN		
	ClpX, ClpY		
Hsp90	Hsp90	Cytosol	Facilitating maturation of signaling molecules, genetic buffering
		Chloroplast	
		Mitochondria	
		Endoplasmic reticulum	
Hsp70	Hsp/Hsc70	Cytosol	Preventing aggregation, assisting refolding, protein import and translocation, signal transduction, and transcriptional activation
Subfamily:	Hsp70	Chloroplast, mitochondria	
DnaK	Bip	Endoplasmic reticulum	
	Hsp91	Cytosol	
Hsp110/SSE			
Chaperonin/ Hsp60	Cpn60	Chloroplast, mitochondria	Folding and assisting refolding
	CCT	Cytosol	
Subfamily:			
Group I			
Group II			
sHsp		Cytosol	Preventing aggregation, stabilizing nonnative proteins
Subfamily:		Cytosol	
I	Hsp17.6	Chloroplast	
II	Hsp17.9	Endoplasmic reticulum	
III	Hsp21	Mitochondria	
	Hsp26.2	Membrane	
IV	Hsp22		
V	Hsp23		
VI	Hsp22.3		

other pathways (Yoshida et al., 2014). Signal transduction in plants under various environmental stresses have been divided into three major types, that is, (1) osmotic/oxidative stress signaling through MAP kinases, (2) Ca^{+2}-dependent signaling, and (3) reactive oxygen species (ROS) signaling (Xiong et al., 2002). Resolving the mechanisms that control Ca^{+2}, MAPK, and ROS signaling in cells during abiotic stress can provide an efficient way to increase plant tolerance to the environmental stresses. These signaling mechanisms are emphasized more in the following sections pertaining to Hsps as well as the functionality of different Hsps and their crosstalk in different signaling mechanisms.

32.2 A GENERAL ACCOUNT ON HEAT SHOCK PROTEINS

Abiotic stresses induce a set of special class of proteins called heat shock proteins (Hsps) or stress-induced proteins (Reddy et al., 2016). Based on their protein size, Hsps were classified into five subfamilies including small Hsp (sHsp), Chaperonins, Hsp70, Hsp90, and Hsp100 (Wang et al., 2004; Kotak et al., 2007; Gupta et al., 2010; Reddy et al., 2016). Hsps are involved in maintaining the cellular equilibrium, protein conformation, preventing aggregation, and hence preserving the nonnative protein in a competent state for further remodeling, which is attained by other Hsps/chaperones (Lindquist, 1986; Wang et al., 2004). The denatured or incorrectly folded proteins make aggregates, and they can be resolubilized by Hsp100/Clp followed by refolding, or degraded by proteases (Mishra et al., 2016). Some Hsps/chaperones (e.g., Hsp70, Hsp90) accompany the signal transduction and transcription switch on the synthesis of Hsps (Wang et al., 2004; Al-Whaibi, 2011). The detailed role of each Hsp family is emphasized in the subsequent sections of this chapter.

32.2.1 Small Heat Shock Proteins

Small heat shock proteins (sHsps) have a unique alpha-crystallin domain (ACD) containing 80–100 amino acid residues located in the C-terminal region (Seo et al., 2006). sHsps have several characteristic features including the degradation of the proteins that cannot fold back and ATP independent chaperonic function (Miernyk, 1999; Reddy et al., 2014, 2015). The sHsps cannot refold nonnative proteins, but they can bind to partly folded or denatured substrate proteins and prevent permanent unfolding or incorrect protein aggregation (Sun et al., 2002). Neoteric study revealed that under in vitro conditions, sHsp18.1 from *Pisum sativum* as well as the sHsp16.6 from *Synechocystis* sp. PCC6803 binds to unfolded proteins and allows further refolding by Hsp70/Hsp100 complexes (Mogk et al., 2003). Studies in apple indicated that the

mitochondrial sHsp protects the NADDH:ubiquinone oxidoreductase (complex I) during heat stress (Downs and Heckathorn, 1998). AsHsp17 modulates the expression of photosynthetic genes through ABA-dependent and independent signaling pathways (Sun et al., 2016). In addition to abiotic stress, sHsps also play a role in biotic stress by associating with Hsp70/Hsp40 chaperone complex (Rousch et al., 2004). Another sHsp20 is known to specifically interact with I-2, which confers resistance to *Fusarium oxysporum* (Simons et al., 1998).

32.2.1.1 Chaperonins

Hsp60 comes under chaperonins and is involved in facilitating the vast range of newly synthesized proteins, and translocates to organelles such as chloroplasts and mitochondria (Bukau and Horwich, 1998; Wang et al., 2004). Plant chaperonins commonly play a major role in assisting the plastid proteins such as Rubisco (Tompa and Kovacs, 2010). Hsp60 family proteins bind to different types of proteins after their transcription and prior to folding to avoid their aggregation (Parsell and Lindquist, 1993). *Arabidopsis* chlCpn60a mutated species was shown to exhibit defects in chloroplast development and subsequently in the plant development (Apuya et al., 2001). Antisense Cpn60b in transgenic tobacco plants showed drastic phenotypic alterations, including slow growth, delayed flowering, stunting, and leaf chlorosis (Zabaleta et al., 1994). Hsp10 is a cochaperone that functions together with Hsp60 in an ATP-dependent manner and helps in folding, assembly, and sorting of proteins (Bukau and Horwich, 1998). The Hsp60-Hsp10 complex enhances the osmotic as well as salt stress tolerance in transformed *E. coli* and yeast cells (Wang et al., 2004).

32.2.1.2 Heat Shock Protein 70

The abundant Hsps present in eukaryotic cell is Hsp70 proteins. Hsp70 proteins play a role as chaperones for newly synthesized proteins to stop their accumulations as aggregates, and assist in accurate folding and help in translocation (Su and Li, 2008; Wang et al., 2014). Hsp70 protein binds in an ATP-dependent manner to hydrophobic patches of moderate unfolded proteins and prevent protein aggregation (Mayer and Bukau, 2005; Reddy et al., 2010). Hsp70 along with other Hsps behave as molecular chaperones and play an important role in protecting plant cells from the detrimental effects of abiotic stresses including heat stress (Rousch et al., 2004). Hsp70-sHsp17.6 chaperone complex may play a role in cross adaptation to temperature stress induced by heat or cold pretreatment in grape plants (Zhang et al., 2008). Under heat stress, Hsc70 cognates are essential for general cellular functions due to their involvement in the control of protein homeostasis, categorizing of proteins by interaction with mitochondrial and chloroplast protein import complexes, and making a connection to the proteasomal degradation pathway through ubiquitin (Hartl and Hayer-Hartl, 2002; Mirus and Schleiff, 2009). Chaperone complex (SGT1b-Hsp70-Hsp90) with other client protein (COI1) helps in hormone signaling by stabilizing the client protein. The Came/AtBAG5/Hsc70 signaling complex plays a pivotal role in modulating the plant senescence (Li et al., 2016). Salicylic acid (SA) is a crucial molecule in signaling, which activates several plant defense mechanisms, and enhances the expression of Hsp70, which further helps in modulation of the heat shock response (HSR) in tomato seedlings (Cronjé et al., 2004), tobacco protoplasts (Cronjé et al., 2004), and seedlings of *Arabidopsis*. Another receptor that could straight away bind to mycobacterial Hsp70 is the chemokine receptor CCRS, which has a considerable significance for signaling cascades induced by Hsps (Floto et al., 2006). Hsp70 protein forms guidance complex with other proteins and directs import bound to the protein precursor to be transferred through the membranes into the organelles such as chloroplast and mitochondria (Jackson-Constan et al., 2001). Other studies indicate that Hsp70B found in the stroma of chloroplasts takes part in photoprotection and the repairing of photosystem II during and after the photoinhibition (Schroda et al., 1999). Study on *A. thaliana* also indicated the necessity of Hsp70 found in the stroma of chloroplast for the differentiation of germinating seeds and its tolerance of heat stress (Su and Li, 2008).

32.2.1.3 Heat Shock Protein 90

Hsp90 class proteins are the most abundant class of Hsps with ~1% of total proteins in prokaryotic and eukaryotic cells. Hsp90 increases up to 4%–6% of total protein under elevated heat stress conditions (Wegele et al., 2004). Hsp90 proteins majorly reside in the cytoplasm, and are rarely seen in the other organelles like endoplasmic reticulum (ER), mitochondria, and chloroplasts (Hao et al., 2010; Reddy et al., 2011). Hsp90 proteins are seen to interact and bind with Hsp70 in many chaperone complexes in signaling and trafficking (Pratt and Toft, 2003). Hsp90 proteins are involved indirectly along with other proteins in the process of signal transduction. Hence, losing the activity may lead to different disturbances in cells. Hsp90 interacting proteins are categorized into three types, which include auxiliary proteins (cochaperones), regulatory factors (regulators), and substrate proteins (substrates) (Xu et al., 2012). Accessory proteins are involved in the modulation of ATP enzymatic function of Hsp90s in cytoplasm and help in interactions between Hsp90s and

the substrates (Johnson and Brown, 2009; Zuehlke and Johnson, 2010). All Hsp90 proteins have the TPR-binding site in their structure and help in interaction of the TPR domain-containing proteins (Aviezer-Hagai et al., 2007). Under control conditions, *Arabidopsis* ROF1 interacts with Hsp90 through the TPR domain and targets into the cytoplasm (Aviezer-Hagai et al., 2007) but under heat stress, ROF1-Hsp90 makes a complex with HsfA2 and migrates to the nucleus and regulates sHsp transcription to increase heat tolerance (Meiri and Breiman, 2009). In control state, *A. thaliana* Hsp90 negatively inhibited Hsf, but under heat stress modulated positively (Yamada et al., 2007). The Hsp90-TIR1 module integrates the temperature and auxin signaling to regulate plant development (Wang et al., 2016). Hsp90s chaperonic activity mainly depends on phosphorylation and stimulates heme to modulate heme-regulated inhibitor (HRI) through casein kinase II or secondary phosphorylation (Szyszka Kramer and Hardesty, 1989). Hsp90s affect the folding and activation of a wide variety of substrate proteins, most of which are kinases and TFs involved in signal transduction and regulatory processes (Pratt et al., 2004; Krukenberg et al., 2011). Hsp90 has also been seen to function in brassinosteroid (BR) signaling (Samakovli et al., 2014). This highlights the importance of the Hsp90s' role in signaling of hormones in plants and animals. Gene silencing of RPS4 results in cell death, which is dependent on the three plant signaling components, EDS1, SGT1, and Hsp90 (Zhang et al., 2004). These studies substantiate that Hsp90 is an important signaling component under abiotic stress conditions.

32.2.1.4 Heat Shock Protein 100

The Hsp100 family belongs to the AAA ATPase super family with a wide range of functional properties (Agarwal et al., 2001). Besides playing a role in protein aggregation and misfolding, the Hsp100 families are seen to be crucial for disaggregation and/or degradation of proteins (Singh and Grover, 2010). Removal of degraded polypeptides, which are harmful due to misfolding, accumulation, or denaturation is crucial for maintaining equilibrium of the cells. A characteristic activity of Hsp100 is a reactivation of accumulated proteins by resolubilization of nonfunctional protein aggregates as well as in assisting to degrade damaged and irreparable polypeptides (Parsell and Lindquist, 1993; Bösl et al., 2006; Kim et al., 2007). Hsp101 is also seen to be playing a crucial role in high temperature stress tolerance, which can be used in genetic engineering of plants. This may improve sustenance during situations of acute environmental stress (Queitsch et al., 2000).

32.2.1.5 Heat Shock Transcription Factors

In the plant genome, ~7% of the coding sequences constitute TFs and among them, the majority are of large gene families in comparison with their counterparts in animals and yeasts, such as Hsfs (Udvardi et al., 2007). To survive under adverse conditions, plants have greater number of Hsfs in comparison with other systems like mammalian cells. Hsfs belong to a multigenic family with more than 21 in *Arabidopsis* (Scharf et al., 2012), 24 in tomato (Fragkostefanakis et al., 2015), 52 in soybean (Scharf et al., 2012), and at least 56 in wheat (Xue et al., 2014) are anticipated and may be involved in stress responsiveness. Hsfs are seen to be involved in plant response to various abiotic stress conditions. Hsfs specifically bind to the heat stress elements (HSEs) present in the promoters of target genes, which further activate the genes (Baniwal et al., 2004; Sakurai and Enoki, 2010; Scharf et al., 2012). In tomato, only two Hsfs, HsfA2 and HsfB1, are induced by heat (Scharf et al., 1990), whose expression is modulated by HsfA1 (Mishra et al., 2002). In fact, the interaction of HsfA2 with HsfA1 is required for the colocalization of HsfA2 into the nucleus (Scharf et al., 1998). HsfA2 is the most predominant Hsf during heat stress conditions (Mishra et al., 2002). Under heat stress, AtHsfA1d and AtHsfA1e together bind to the HSE *cis*-element of the *AtHsfA2* gene and activate the AtHsfA2 expression (Nishizawa-Yokoi et al., 2011). Hsfs playing a role in drought and salinity stresses are modulated by ABA-dependent and ABA-independent signaling pathways (Yoshida et al., 2008). At high temperature, the expression of the Hsps is modulated by Hsfs (Saidi et al., 2011; Scharf et al., 2012). Further, Hsfs translocate to the nucleus in heat stress where they bind to HSEs of respective promoter regions of Hsp genes (Voellmy and Boellmann, 2007; Scharf et al., 2012). The alternative regulatory systems for the activation of different Hsp expression are seen to be operated in plants by modulating WRKY, DREB2A, and DREB2C TFs (Scharf et al., 2012). Besides WRKY, there are more than 90 TFs in *Arabidopsis* that have the binding sites for CAM that comprise the Calmodulin-binding transcription activator (CAMTA). Expression of the genes coding to these proteins increases in response to heat conditions (Reddy et al., 2011), but it is unknown if this increase is important for Hsp expression or not. HsfA2, HsfA3, and HsfA7a of *Arabidopsis* are considered as the strongest activators of HS transcription during recovery and are responsible for the heat-acclimation phenotype (Charng et al., 2007; Nishizawa et al., 2006; Schramm et al., 2008). Some studies have also concluded that Hsfs play a role in the sensing of ROS. Mittler and Zilinskas (1992) and Storozhenko et al. (1998) have revealed the presence of

a HSF-binding sequence in the 5' region of the gene that codes for H_2O_2-scavenging enzyme Apx1. In addition, Hsf and HSE (PgApx1) specificity to each other and their expression profile shows a critical interlink in heat and oxidative stress signaling pathways, which are known to play an important part in understanding the mechanisms involved in plant abiotic stress tolerance (Reddy et al., 2009). Furthermore, HsfA4A carries out the function of *Arabidopsis* H_2O_2 under oxidative stress (Miller and Mittler, 2006). HvHsfB2c is coexpressed in the main hub of sHsps and hence it may be modulating the expression levels of different sHsps in barley (Reddy et al., 2014). DREB2A signaling is mediated by HsfA3, which again induces the expression of Hsfs (Schramm et al., 2008; Yoshida et al., 2008). It has been shown that calmodulin is an essential component in *Arabidopsis* signal transduction, and with the help of a yeast two-hybrid assay, it has been demonstrated that calmodulin-binding protein kinase3 interacts with heat shock factor (Hsf) AtHsfA1a (Liu et al., 2008). Wang and Huang (2017) also demonstrated that transcriptional regulation of HsfA2c-mediated heat tolerance involving lipid and calcium signaling pathways in tall fescue.

32.3 HEAT SHOCK PROTEIN INDUCTION PHENOMENA IN PLANTS

Hsp synthesis in plants, including rice, *Medicago* and tomato was qualitatively and quantitatively dependent on the type of condition and nature of tissue (Hernandez and Vierling, 1993; Pareek et al., 1998; Hu et al., 2009; Fragkostefanakis et al., 2015; Zhou et al., 2016). A further study on expression of cytoplasmic class of proteins in leaves, flowers, and developing seed pods in *Medicago sativa* was carried out. Results indicated the recurrent formation of these proteins in flowers and buds, but no expression in the leaves (Hernandez and Vierling, 1993). Heat stress is an increase in temperature for a short term at 37–38°C, which has no negative impact, but enhances the ability of plants to sustain the subsequent damaging heat treatment, termed heat shock; this phenomenon is known as induced or acquired thermotolerance (Saidi et al., 2011). The induced thermotolerance of plants is seen to be dependent on the expression of Hsps. Plant Hsp101 is seen to play the main role in induced thermotolerance (Queitsch et al., 2000). Expression of Hsp90 in rice plant indicated that the Hsp90 was present after 2 h of heat stress (from 28°C to 45°C), and its quantity was high and stable even after heat stress (4 h) and after return to normal conditions. It was also found that Hsp90 (Hsp85 and Hsp87) could be induced by other than heat stress, such as salinity, drought, and cold (Pareek et al., 1998). Hsp100/ClpB proteins tend to interact tightly with sHsps that are incorporated into the protein aggregates under heat shock, thus promoting the disaggregation activity of Hsp100/ClpB (Rikhvanov et al., 2007). Hu et al. (2009) examined a global expression profiling with heat stressed rice seedling, and then compared the results with the previous rice data under cold, drought, and salt stresses. They found that Hsps and Hsfs might be important molecules in crosstalk of different stress signal transduction networks. The expression of Hsps and its factors' Hsfs was induced largely by heat, cold, salinity, and osmotic stresses. The response to other stress factors depended on protein class and tissue. For example, under stress, high expression response for class Hsp20 was recorded with high similarity of their information. Wounding the roots of the plant stimulated (after 12 h) the expression of several genes, including Hsp20, Hsp70, and Hsp100. High expression of Hsps and Hsfs was observed under UV-B stress in aerial tissues (shoot), but in nonaerial tissues (root system) there was no expression (Swindell et al., 2007).

32.4 ABIOTIC STRESSES INDUCE THE HEAT SHOCK PROTEIN–MEDIATED SIGNALING PATHWAYS

Abiotic stress response of plants is governed through the signaling pathways knitted at the cellular as well as molecular levels (Knight and Knight, 2001). Perception of abiotic stress initiates the signals that activate downstream signaling cascades and transcriptional controls and simultaneous pathways (Wang et al., 2003; Vij and Tyagi, 2007). The first step in the signal transduction pathway is the perception of the signal, which is carried out by receptors/sensors as phytochromes, histidine kinases and receptor-like kinases, G-protein-coupled receptors, and hormones. And then, secondary signaling molecules such as inositol phosphatase, ROS, and ABA are produced. Eventually, the secondary molecule-mediated modulation of intracellular Ca^{2+} level activates protein phosphorylation cascades (i.e., MAP kinases, CDP kinases, protein phosphatase, SOS3/protein kinase, etc.), TFs, and stress-responsive genes (Boguszewska and Zagdańska, 2012; Gong et al., 2013). For genetically engineering stress tolerance traits in plants, proper insights of both endpoints and the precession are required for signaling pathways (Chinnusamy et al., 2004; Akpinar et al., 2012). Taking into account the first principle concepts and the developments of late, succeeding sections briefly give an insight into the significance of ROS, calcium and calcium-regulated proteins, and MAPK cascades in signaling pathways in plants under different abiotic stresses. The different signaling molecules and varied Hsps and Hsfs activated in response to elevated temperatures are given in Table 32.2.

TABLE 32.2 Summary of the Key Signaling Mechanisms Modulating the Expression of Heat Shock Proteins in Plants

Signaling component	Plant	Treatment (°C)	Remarks	References
Membrane and Ca^{2+}	*Arabidopsis*	22	Induction of AtHsp18.2 triggered by Ca^{2+} and inhibited by Ca channel blockers and EGTA	Liu et al. (2005)
		37		
	Tobacco	25	Hsp70 induced by membrane fluidizers and prevented by membrane rigidifiers	Suri and Dhindsa (2008)
		37		
		>38	Acquired thermotolerance diminished by EGTA and enhanced by Ca^{2+}	
	Alfalfa	37	Ca^{2+}require for MAPK induction	Sangwan et al. (2002)
	Maize	27	DNA-binding activity of Hsfs induced by Ca^{2+}	Li et al. (2004)
	Physcomitrella	22	Membrane fluidizers induced Ca^{2+} influx, triggered HSR and enhanced thermotolerance	Saidi et al. (2009)
		>27	Elicited Ca^{2+} influx. Blocking Ca^{2+} entry abolished the expression of Hsps and reduced thermotolerance	
Calmodulin and kinase	*Arabidopsis*	37	AtCaM3 induced by elevated temperature. DNA-binding activity of Hsfs reduced in cam3 mutant	Zhang et al. (2009)
		37	At HsfA1 a specifically phosphorylated by At CBK3in the presence of CaM and Ca^{2+}; binding activity of Hsfs to HSEs impaired in AtCBK3 mutants and improved in the overexpressors; accumulation of Hsps reduced in AtCBK3 mutants and enhanced in the overexpressors	Liu et al. (2008)
	Tobacco	37	Hsp70 accumulation repressed by MAPKK inhibitors	
	Maize	27	DNA-binding activity of Hsfs induced by CaM and reduced by CaM antagonist	Li et al. (2004)
		44		
	Wheat	37	CaM antagonists decreased Hsp26 and Hsp70 expression	Liu et al. (2003)
	Physcomitrella	36	Pretreatment with kinase inhibitor reduced HSR and negatively affected thermotolerance	Saidi et al. (2009)
H_2O_2 and NO	*Arabidopsis*	20	DNA-binding activity of Hsfs occurred in protein extracts from H_2O_2-treated cells	Volkov et al. (2006)
		23	HsfA2 and Hsp25 were induced by treatment with H_2O_2	Banti et al. (2010)
		20	Exposure to H_2O_2 induced expression of hsp17.6 and hsp18.2	
		37	Heat increased endogenous H_2O_2 levels.	Volkov et al. (2006)
			Hsp expression is reduced by peroxide scavenger; peroxide scavenger blocked Hsf DNA-binding activity	
		37	DNA-binding activity of Hsfs and Hsp18.2 expression were reduced in noa1 mutant and rescued by the addition of an NO donor	Xuan et al. (2010)
	Tobacco	45	Endogenous NO levels increased during heat shock	Gould et al. (2003)
		36	Small Hsp induction reduced by inhibitor of H_2O_2 generation	Königshofer et al. (2008)
Cytoskeletonand protein denaturation	*Arabidopsis*	20	Expression of HsfA2, Hsp70A, and small Hsps induced by chemical generation of misfolded AZC-mediated Hsp70A expression reduced in hsfA2 mutant	Sugio et al. (2009)
	Tobacco	25	Microfilament and microtubule destabilizers induced Hsp70 accumulations	Suri and Dhindsa (2008)
		37	Hsp70 accumulation repressed by microfilament and microtubule stabilizers	

(*Continued*)

TABLE 32.2 (Continued)

Signaling component	Plant	Treatment (°C)	Remarks	References
	Rice	28	Activation of Oshsp17.3 promoter induced by AZC	Guan et al. (2010)
	Physcomitrella	40	Heat-denatured luciferase was not sufficient to induce Hsps when extracellular Ca^{2+} was immobilized	Saidi et al. (2009)
Hsp90 inhibition	*Arabidopsis*	22	Hsp90 inhibitors activated the transcription of Hsps and increased thermotolerance	Yamada et al. (2007)
		37	Hsp90.2 binds HsfA1d in the absence of heat; heat treatment inhibited Hsp90 activity	
Hsp90 inhibition	*Physcomitrella*	37	ROF1-Hsp90 complex translocates to the nucleus by heat via interaction with HsfA2	Meiri and Breiman (2009)
		22	Hsp90 inhibitors induced a Ca^{2+}-dependent HSR	Saidi et al. (2009)

32.4.1 Reactive Oxygen Species

ROS and its reaction products are the most significant second messengers that actively participate in stress signal transduction (Gong et al., 2013; Chakradhar et al., 2017; Khan and Khan, 2017). ROS have been credibly evidenced to play a significant role in signaling, redox-sensing mechanisms, and plant survival under abiotic stresses (Mittler, 2002; Mittler et al., 2004, 2008, 2010; Gill and Tuteja, 2010). A number of researchers have considered H_2O_2 as an active signaling molecule in plants, where a variety of cellular responses are accomplished due to redox-sensing-mediated H_2O_2 accumulation (Bhattacharjee, 2005; Del Rio and Velez-Pardo, 2006; Halliwell, 2006; Kovalchuk, 2010; Ashfaque et al., 2014; Khan et al., 2016). Though little information is available on ROS-mediated induction of the redox-sensing mechanisms and the associated signaling pathways, the contribution of ROS-induced signaling in the activation of defense genes and subsequent stress tolerance/specific acclamatory responses has been widely accepted (Åslund et al., 1999; Goyer et al., 2002; Locato et al., 2009). Researchers provided the clues in support of H_2O_2 as a central metabolite and diffusible signal that has the capacity to induce a number of defense related genes (Chen et al., 1993; Prasad et al., 1994). Chloroplastic ROS contributing to the signaling cascade of the *Hsp* genes were not affected by apoplastic H_2O_2 or H_2O_2 produced by the plasma membrane (Scarpeci et al., 2008). Increase in temperature would enhance the mitochondrial production of ROS in plant cells (Pucciariello et al., 2012; Suzuki et al., 2012; Kreslavski et al., 2012). An increase in mt$\Delta\psi$ (potential difference across the inner mitochondrial membrane) (Rikhvanov et al., 2007; Pyatrikas et al., 2014; Pavlova et al., 2009; Pulyaevskaya et al., 2011) and ROS production (Saidi et al., 2011;Volkov et al., 2006; Miller and Mittler, 2006) is essential for the activation of Hsp expression under heat stress. Inhibitors and uncouplers of mitochondria suppress the increase in mt$\Delta\psi$ and inhibit the activation of the Hsp expression at elevated temperatures, even though these agents sometimes activate Hsp expression in the absence of heat stress (Rikhvanov et al., 2007; Pyatrikas et al., 2014). Likely, the addition of antioxidants inhibits Hsp expression under heat stress (Volkov et al., 2006; Saidi et al., 2011; Mittler et al., 2012). Production of ROS under heat stress is probably associated with changes in $[Ca^{2+}]$ cyt and with hyperpolarization of the mitochondrial membrane. On the other hand, the rate of ROS generation in mammalian mitochondria is known to enhance with an increase in mt$\Delta\psi$ (Korshunov et al., 1997). Similar concepts can be applied to plant cells. The increase in mt$\Delta\psi$ induced by extracellular ATP (Sun et al., 2012) and camptothecin (Weir et al., 2003) stimulated ROS production, whereas the protonophore CCCP known to diminish mt$\Delta\psi$ inhibited this process. Hence, mt$\Delta\psi$ level can be among the factors determining ROS formation under heat stress. Apparently, plant cell mitochondria participate in the regulation of Hsp expression by controlling not only the $[Ca^{2+}]$ cyt level but also ROS generation. This also shows the crosstalk between different signaling pathways in the regulation of Hsps.

32.4.2 Mitogen Activated Protein Kinases

Mitogen activated protein kinases (MAPKs) are the best-studied plant protein kinases that connect different receptors/sensors to cellular and nuclear responses (Tena et al., 2001; Sinha et al., 2011). The MAP kinase pathways facilitate signal transduction from the surface of the cell to the nucleus and are extensively used as osmolarity signaling modules. The environmental

signals are initially perceived by specific receptors, which on being activated initiate a cascade to transmit the signal intracellularly and activate nuclear TFs to induce expression of specific target genes. Evidence shows that plants quickly activate MAPK when exposed to multiple abiotic stress stimuli (Kiegerl et al., 2000; Ligterink and Hirt, 2001). MAPKs are dual function kinases that are stimulated through phosphorylation of their two amino acids in sequential order, starting from the MAPK kinase kinase (MAPKKK) to MAPK kinase (MAPKK) to MAPK (Fig. 32.2). A series of subfamilies (i.e., MAP4K, MAP3K, MAP2K, MAPK) are chronologically activated in this MAPK cascade as a result of various environmental stimuli that in turn activate TFs like Hsf, phospholipases, or microtubule-associated proteins, and the expression of Hsps (Wang et al., 2003; Sasabe et al., 2006) (Fig. 32.2). MAPK modules in plant tolerance to abiotic stress is illustrated, in which a tobacco MAPKKK ANP orthologue, NPK1, was expressed in an active form in *Arabidopsis thaliana*. NPK1 mediates H_2O_2 regulated gene expression in plants (Kovtun et al., 2000). In *Arabidopsis*, MAPK6 targets AtHsfA2, phosphorylates it on T249, and changes its intracellular localization under HS conditions (Evrard et al., 2013). AtHsfA4A interacts with the MAP kinases MPK3 and MPK6 and is phosphorylated in vitro on three distinct sites, with Ser-309 being the major phosphorylation site (Pérez-Salamó et al., 2014).

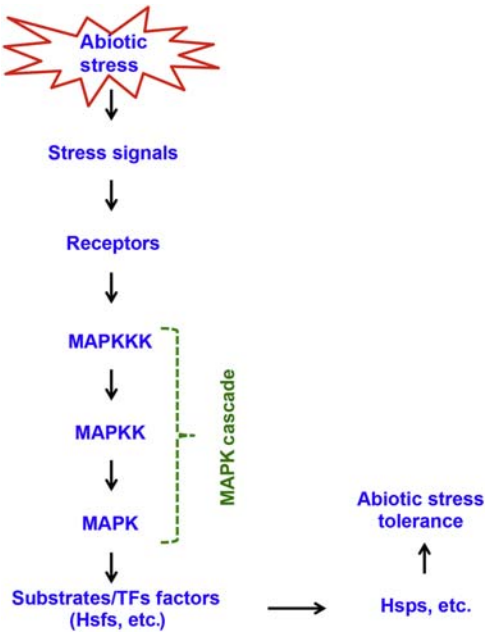

FIGURE 32.2 Predicted model of MAP kinase cascade pathway represents the cascade mechanism of MAPK phosphorylation system that serves as a link between receptors, stress signals, and downstream signaling components such as TFs to activate genes and induce abiotic stress tolerance.

Nishizawa-Yokoi et al. (2010) described that AtHsfA2 was regulated by the accumulation of polyubiquitinated proteins generated by the inhibition of 26S proteasome and AtHsp90.

32.4.3 Calcium and Calcium-Regulated Proteins

In plants, Ca^{2+} serves as an important, ubiquitous second messenger and regulates many physiological processes (Tuteja and Sopory, 2008; Tuteja, 2009; Boudsocq et al., 2010). Ca^{2+} channels, pumps, and exchangers (carriers) control the plant Ca^{2+} homeostasis maintenance under a variety of stimuli through the regulation of diverse Ca^{2+} transport systems (Gong et al., 2013; Boudsocq et al., 2010; Kudla et al., 2010). In addition, reduction in Ca^{2+} mobility, localization, and spatial concentration elevations are facilitated by the abundance of buffering Ca^{2+} sensors (Dodd et al., 2010). Ca^{2+} sensor groups, namely, sensor relays [calmodulin (CaMs), calcineurin B-like (CBL)], and sensor responders, that is, sensor protein kinases [CDPKs, calcium and calmodulin-dependent protein kinases (CCaMKs)] lack any intrinsic enzymatic activity, directly activated upon Ca^{2+} binding, decode cellular Ca^{2+} signals, and transmit the Ca^{2+} induced modification to target proteins (Fig. 32.3).

Activation and inactivation of Hsf are determined by the degree of its phosphorylation and dephosphorylation, because of which it should be clear that Ca^{2+}activates Hsf indirectly, by modulating the activity of protein kinases and phosphatases (Voellmy and Boellmann, 2007). Serine residue phosphorylation at position 230 leads to transcriptional activity of a human Hsf1. This process is carried out by Ca^{2+}/CaM dependent kinase II (CaMK II) (Holmberg et al., 2001). It is evident that Ca^{2+}/CaM binding protein kinases (CBK) and Ca^{2+}dependent protein kinases (CPK) in plants perform a similar function (Fig. 32.3). It is seen that CBK3 phosphorylated HsfA1a (Liu et al., 2008), while CPK3 and CPK13 phosphorylated HsfB2a (Kanchiswamy et al., 2010a; Kanchiswamy et al., 2010b), which activated Hsp expression in heat stressed *A. thaliana*. MAP kinase (HAMK), which is activated by heat, may also be involved in Hsf phosphorylation. HAMK was shown to be a Ca^{2+} dependent kinase and activated under heat stress in cultured tobacco cells, which is necessary for the expression of Hsp70 (Suri and Dhindsa, 2008). Dephosphorylation of certain serine residues in Hsf also leads to its activation. In *A. thaliana* dephosphorylation is performed by serine/threonine phosphatase PP7. It was shown that PP7 interacts with both CaM and Hsf. A mutation in *PP7* gene inhibited the expression of Hsps under heat stress (Liu et al., 2007). It is not excluded that the ability of Hsp70 and Hsp90 to regulate Hsf activity

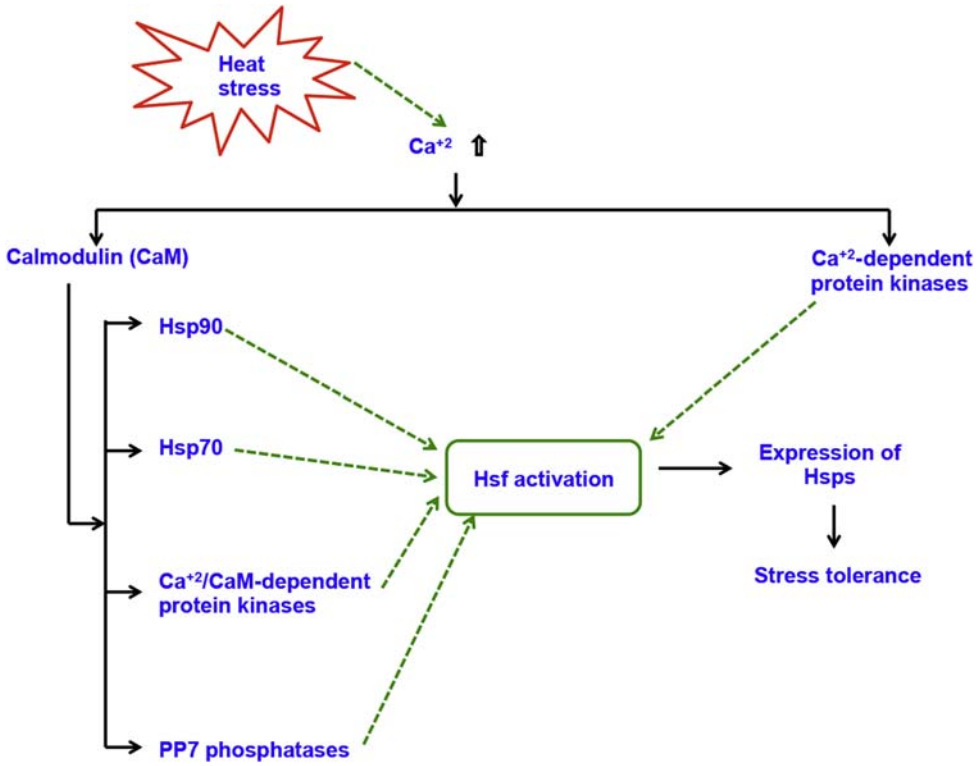

FIGURE 32.3 Mechanism depicting Ca-dependent activation of Hsf and Hsps in heat stressed plants.

depends on [Ca^{2+}] cyt. As seen, Hsp70 contains the CaM binding site (Sun et al., 2000). Although Hsp90 is devoid of such site, its activity can be modulated by adapter proteins possessing the CaM binding site (Meiri and Breiman, 2009; Saidi et al., 2011; Mittler et al., 2012). Activation of Hsps through calcium ion in heat stress conditions sustains the plants in heat stress conditions (Fig. 32.3).

32.5 CROSSTALK BETWEEN REACTIVE OXYGEN SPECIES, MITOGEN ACTIVATED PROTEIN KINASES CASCADES, CA^{+2} AND HEAT SHOCK FACTOR/HEAT SHOCK PROTEINS

Abiotic stresses are perceived by different signaling mechanisms, among which few are specific, and some may have crosstalk at different stages. Intricate teamwork and interaction of signaling mechanism is adopted by abiotic-stressed plants to perceive transducer stress signals and finally to transduce stress responses. ROS, TFs, Ca^{2+} and Ca^{2+}-regulated proteins, and MAPK cascades are credibly being evidenced to sport a significant role in plant abiotic stress signaling cascades. Induction of changes in Ca^{2+} in the cytosol is observed with hormonal signals (Poovaiah et al., 1993). The initiation of MAPK in plants has also been reported to be evoked by H_2O_2 (Desikan et al., 2001; Grant et al., 2000; Neill et al., 2002; Song et al., 2008; Palavan-Unsal and Arisan, 2009). Involvement of Ca^{2+}-dependent MAPK pathways in signaling of abiotic stress in plant cells is also known (Wurzinger et al., 2011). However, although much has been attained in the area of plant abiotic stress signaling pathways, efforts should be made with the assistance of powerful molecular tools, including transcriptome and proteome analysis, to understand more about molecular mechanism(s) underlying ROS and Ca^{2+} sensing and signal transduction.

Hsp as well as Hsfs are the most important factors in crosstalk between various stress signaling transduction networks as understood through the comparative study of global expression profiling in heat, salt, and drought stresses in rice seedlings (Hu et al., 2009). A number of recent studies have shown proofs in context to interrelations among Hsfs, Hsps, ROS, and ROS scavengers under heat stress conditions. To activate Hsfs directly, elevated temperatures enhance the accumulation of ROS, which again activates Hsfs, either straight away or indirectly through triggering the MAPK pathway. The Hsfs tend to bind to HSE in the 5' upstream region of different genes like Hsf, Hsp, miRNA398, and ROS scavenging genes. The miRNA398-dependent repression of SOD scavengers may have a role in the quick ROS accumulation upon exposure to heat.

This supports the triggering of Hsfs, which then boosts induction of the heat stress response in a lower duration. sHsp protein plays a crucial part in safeguarding PSII and PSI during chilling stress in low illumination in tobacco (Guo et al., 2007). These findings suggest that Hsps are not active only in heat stress, but are also active in other stresses by activating a cascade of signaling reactions. In the longer term, the induction and stabilization of other scavengers would start to suppress ROS levels to avoid excessive cellular damage.

32.6 GENETIC ENGINEERING OF HEAT SHOCK PROTEINS SIGNALING MOLECULES

Different approaches have been made to produce stress resistant plants for tackling global warming. The molecular mechanism of abiotic stress tolerance depends on activation and modulation of genes that are related to specific stress conditions. Abiotic stress sustenance in plants can be achieved by the combination of molecular and traditional plant breeding (Wang et al., 2003; Vinocur and Altman, 2005) and alternatively through forward and reverse genetic approaches (Lavania et al., 2015; Driedonks et al., 2015; Usman et al., 2014; Reddy et al., 2016). There is much evidence that shows that a broad conservation of the Hsp/chaperone web as a multiple stress defense mechanism admits all land plants. Indeed, plant alteration was shown to be strongly dependent on Hsps as shown from the diversification of the Hsp families. Proteomic and genomic studies have also revealed a positive correlation between the expression of Hsps and tolerance to different abiotic stress conditions. Transgenic plants that are overexpressing either of Hsps or Hsfs possess enhanced antioxidant activities, higher osmolyte levels, and higher expression of the stress inducible genes, which is indicative of the activation of different stress-responsive mechanisms by Hsps and their role in the cross stress tolerance of the plants (Li et al., 2012; Lee et al., 2012; Zhou et al., 2012b; Zou et al., 2012; Mu et al., 2013; Song et al., 2014; Wang et al., 2015; Masand and Yadav, 2016; Wan et al., 2016). Overexpression and knockout studies showed Hsp70 plays a protective role during dehydration stress in tobacco, soybean, and citrus (Yu et al., 2015). *OsAhl1* of rice was shown to directly induce *Hsp101* and *Hsp90* expression leading to drought resistance (Zhou et al., 2016). *OsHsp90-2* and *OsHsp90-4* were also seen to be upregulated under drought, cold, heat, and salt stresses (Zhang et al., 2016). *OsHsp90-2*, when introduced in *E. coli*, was sufficient to induce resistance to heat, huge salinity conditions, and drought (Reddy et al., 2011; Zhang et al., 2016). Similar regulations of these Hsfs and Hsps were seen in tomato in stimuli to heat, drought, and salinity (Fragkostefanakis et al., 2015). In wheat, overexpression of *Triticum HsfA6f* was demonstrated to direct the expression of several Hsps, leading to thermotolerance (Xue et al., 2014). Increased expression of soybean GmHsp90s decreases the destruction of abiotic stresses in *Arabidopsis thaliana* (Xu et al., 2013). The expression of *ZmHsf06* (*Zea mays*) is sufficient to confer heat and drought stress resistance in *Arabidopsis*. Identification of Hsf signaling from monocots to dicots definitely shows an intense preservation of Hsp-based multiple stress responses in crops.

Hsps repair the structure of the protein and the target at incorrectly aggregated and nonnative proteins for removing it out of the cells (Cho and Hong, 2006). It was found that both the overexpression of Hsc70 and the use of a dominant negative (DN) form of Hsp90 disrupted ABA-mediated stomata closure, thereby negatively affecting water loss in stress conditions. The targets of Hsp90 and Hsc70 are not known yet, but must be downstream of *SnRK2* as it was fully activated after ABA treatment, despite the use of an Hsp90 inhibitor (Clément et al., 2011). *NtHsp70-1* is one among such proteins, which is constitutively overexpressed in tobacco to find out its role in plant drought response and tolerance. The tolerance of transgenic seedlings to drought was enhanced and their optimum water content was sustained after drought stress (Cho and Hong, 2006). *Hsp24* from *Trichederma* was seen to develop significantly higher resistance to salt, drought, and heat stress in yeast (Liming et al., 2008). Expression of the *CaHsp26* gene in transgenic tobacco showed that the mRNA accumulation of *CaHsp26* was triggered by heat stress (Guo et al., 2007). Overexpression of soybean *GmHsfA1* can increase the thermotolerance of transgenic soybeans because of the activation under HS of downstream genes, such as *GmHsp70*, *GmHsp22*, and other *GmHsps* (Zhu et al., 2006). *HsfA2* has been identified to be the dominant Hsf in tomato and *Arabidopsis* based on its high activator potential for transcription of *Hsp* genes and the strong accumulation under conditions of long-term HS or repeated cycles of HS and recovery (Mishra et al., 2002; von Koskull-Döring et al., 2007). Studies on tomato HsfB1 with a variant of lysine residue that was replaced by its *Arabidopsis* counterpart showed that *HsfB1* and *HsfB2b* may promote the activity of *HsfA1* under HS conditions by repressing *Hsps* that interfere with the nuclear migration of HsfA1s, an activator of the early HS response (Ikeda et al., 2011). In *Arabidopsis*, overexpression of *RcHsp17.8* enhanced the SOD activity (Jiang et al., 2009) whereas overexpression of *ZmHSP16.9* in tobacco enhanced POD, CAT, and SOD activity (Sun et al., 2012). Overexpression of the LeCDJ1 DnaJ protein-coding gene also known as

J-protein or Hsp40 (Qiu et al., 2006) in tomato resulted in improved thermotolerance, accompanied by increased Apx and SOD activity after heat stress and reduced accumulation of O_2^- and H_2O_2. Also, tomato plants overexpressing the DnaJ/Hsp40 *LeCDJ1* showed both higher heat and chilling tolerance (Kong et al., 2013, 2014a) and overexpression of BRZ-INSENSITIVE-LONG HYPOCOTYLS 2 (BIL2), a mitochondrial-localized DnaJ/Hsp40 family member, enhanced resistance against salinity and high light stress (Bekh-Ochir et al., 2013). Zhang et al. (2009) could demonstrate that AtCaM3 knockout mutant plants in *Arabidopsis* were more sensitive to heat stress, whereas high expression of CaM3 showed enhanced thermotolerance. Moreover, CaM3 is supposed to be involved in the activation of Hsfs shown by electrophoretic mobility-shift assays, real-time quantitative reverse transcription–polymerase chain reaction, and Western-blot analyses (Zhang et al., 2009). *HsfA3* helps in DREB2A signaling inducing the expression of Hsfs (Schramm et al., 2008; Yoshida et al., 2008). The multiprotein bridging factor1c (MBF1c) modulates several genes, including *DREB2A* and two classes of B Hsfs. *MBF1c* overexpression in plants revealed increased tolerance to heat, osmotic, and biotic stress (Bechtold et al., 2010; Suzuki et al., 2005, 2011).

Overexpression of *Hsf3* in *Arabidopsis* has shown enhanced functionality of *Apx* at the time of postheat-stress recovery and expressed solid induction of *Apx2* in comparison with the wild-type *Arabidopsis* (Panchuk et al., 2002). In a knockout Apx1, crops accumulated H_2O_2 in mild light stress in which *Hsf21* was seen to be increased in light stress initial stages (Davletova et al., 2005a, Pnueli et al., 2003). When wild-type cells are put on with H_2O_2, accumulation of transcripts that encode for *Hsf21* are seen to be aggregated (Davletova et al., 2005b). A variant of *Hsf21* showed reduced expression of *Zat12*, which is a H_2O_2-responsive zinc finger protein wanted for expression of *Apx1* in transgenic plants (Davletova et al., 2005a). From these studies, the critical function of Hsfs in initial sensing of H_2O_2 and *Apx1*, *Apx2*, and *Zat12* expression is revealed.

32.7 CONCLUSIONS AND FUTURE PERSPECTIVES

The ubiquitous role of Hsps is seen in stabilization of proteins and maintaining the homeostasis in the cell. Heat stress muddles cellular homeostasis, which leads to developmental aberrations, growth retardation, and eventually plant death in some cases. Heat stress acts initially upon the quaternary structure of the folding protein, activity of which is lost due to exposure to elevated temperatures, which have deleterious effects on plant metabolism. This results in the removal of connections between the signaling pathways. In this context, further elucidation is required to understand how plants better respond to heat and other major abiotic stresses from the physiological and molecular perspectives. Still, we need to understand precisely how Hsps/chaperones participate in sensing, translocation of immune receptors, signal transduction, and transcriptional activation of several stress genes. Hence, first what needs to be focused upon is understanding the way plants respond to heat stress and utilizing this knowledge in the development of thermotolerant crops.

Many abiotic stress signaling components have been identified from mutants or functional genomic studies. Still, there is only a fragmentary view of understanding the abiotic stress signaling pathways. Functional genomics approaches by forward and reverse genetics will continue to be imperative to break through these complex pathways. Conventional genetic screens have yielded immensely important insight into stress signal transduction; this approach may ultimately be limited due to functional redundancy of components within the signaling pathways. Molecular screens based on reporter genes are a better way to identify upstream signaling components that control subsets of responses that may not manifest as visible tolerance phenotypes. Detailed characterization of mutant phenotypes will provide an indication if a signaling component function is in a specific pathway or is involved in multiple pathways. Analysis of spatial and temporal expression patterns, in combination with biochemical analysis, will be required to firmly establish specificity or crosstalk of the signaling mechanisms and their pathways.

More elucidation is required to explore opportunities to better understand how plants respond to major abiotic stresses and their cross connectivity. Multiple genes affected under abiotic stresses imply that there could not be a single marker for protection against stress. There is a lot of crosstalk taking place between various hormonal pathways, and the exact nature of this crosstalk during simultaneous biotic and abiotic stress is yet to be investigated. Researchers should look forward for defined set of markers to predict tolerance towards a particular stress with a definite degree of affirmation. Though much has been achieved in the context of plant abiotic stress signaling pathways, efforts should be made with the aid of powerful modern molecular tools, including transcriptomic and proteomics technologies, to get more insights into molecular mechanism(s) underlying various signaling cascades, that is, ROS and Ca^{2+} sensing signal transduction pathways. Mutations or edits enhancing Hsf/Hsps expression or activity undoubtedly are valuable targets to engineer multi-stress resistant crops through

recently discovered CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat-CRISPR associated 9) system and TILLING (targeted induced local lesion in genomes), which would allow a rapid technology transfer in crops.

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CHAPTER

33

C_4/CAM Facultative Photosynthesis as a Means to Improve Plant Sustainable Productivity Under Abiotic-Stressed Conditions: Regulatory Mechanisms and Biotechnological Implications

Renata Callegari Ferrari and Luciano Freschi

Department of Botany, Institute of Biosciences, University of São Paulo, São Paulo, Brazil

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33.1 INITIAL CONSIDERATIONS

When referring to carbon concentrating mechanisms (CCMs), it is important take first a brief overview of the selective pressures that lead to their evolution. Under low CO_2 availability, the oxygenase activity of ribulose-1,5-biphosphate-carboxylase oxygenase (Rubisco) is favored, resulting in the production of 3-phosphoglyceric acid (PGA) and phosphoglycolate (PG), a toxic molecule capable of inhibiting photosynthesis, instead of PGA alone (Erb and Zarzycki, 2018; Miziorko and Lorimer, 1983). As a result, all plant lineages evolved the photorespiratory pathway, a complex mechanism that involves coordinated chemical reactions in chloroplasts, peroxisomes, and mitochondria. Photorespiration leads to the conversion of PG into less toxic products and yields back part of the CO_2 that would have been lost due to the formation of PG (Sage et al., 2012). For this reason, although energetically expensive, photorespiration is essential to photosynthetic organisms (Eisenhut et al., 2008).

Different methods for reconstructing atmospheric history converge in estimating that, for the last 40 million years until present date, atmospheric CO_2 concentration has decreased from over 800 ppm to the current 390 ppm (Beerling and Royer, 2011; Zhang et al., 2013). In this scenario, photorespiration limits carbon fixation in plants performing C_3 photosynthesis, being intensified by high temperatures or stress conditions that promote stomatal closure and consequently lead to a decline in intercellular CO_2 levels (Keeley and Rundel, 2003). As atmospheric CO_2 levels dropped significantly, some photosynthetic organisms evolved CCMs, which are mechanisms capable of increasing inorganic carbon availability inside cells (Moroney et al., 2013; Raven et al., 2008). CCMs are responsible for a significant portion of Earth's inorganic carbon assimilation since they occur in a vast diversity of phylogenetic groups (Raven et al., 2008). These pathways have diversified among cyanobacteria (Badger et al., 1980, 2002), eukaryotic algae (Giordano et al., 2005; Meyer and Griffiths, 2013), basal embryophyte lineages (Raven et al., 2008), and flowering plants, either terrestrial or aquatic (Keeley and Rundel, 2003; Maberly and Madsen, 2002; Raven et al., 2008; Sage, 2004; Silvera et al., 2010). Among the distinct CCMs currently known, the C_4 photosynthesis and the Crassulacean acid metabolism (CAM) are particularly relevant for flowering plants; therefore, they will be the focus of this chapter. We do not intend to exhaustively review the biochemical, anatomical, regulatory, and evolutionary similarities and differences between the C_4 and CAM systems, but instead we aim to discuss the compatibility between these two photosynthetic adaptations, the environmental regulation and signaling events controlling both syndromes, and the potential biotechnological implications of engineering C_4 and CAM together into one crop species.

33.2 C_4 AND CRASSULACEAN ACID METABOLISM: SIMILARITIES AND DIFFERENCES

In the low CO_2 availability scenario, when increasing CO_2 assimilation involved higher transpiration losses, C_4 and CAM lineages evolved independently and irradiated, resulting in increased fitness under specific environmental conditions (Edwards and Ogburn, 2012; Ehleringer and Monson, 1993). These two pathways recruited existing genes, formerly involved in anaplerotic functions, to perform photosynthetic-related roles (Edwards and Ogburn, 2012). On their evolutionary trajectories, C_4 and CAM evolved from C_3 ancestors, and probably the first few characteristics opted lead to their final divergent phenotypes (Sage, 2002). CAM is considered to have arisen first, since it is present in basal lineages such as lycophytes, ferns, isoetids, and cycads, whereas C_4 probably evolved more recently (Edwards and Ogburn, 2012; Ehleringer and Monson, 1993; Keeley and Rundel, 2003; Silvera et al., 2010). C_4 is present in 19 angiosperm families, mostly grasses, representing roughly 3% of known species (Sage, 2017), while CAM is more widespread, occurring in 35 families (about 6% of total species number) (Silvera et al., 2010). C_4 and CAM can be considered as photosynthetic syndromes, since both comprise a whole set of morphoanatomical and biochemical attributes, and may be associated with specific ecological niches.

33.2.1 Defining C_4 and Crassulacean Acid Metabolism

Both pathways utilize organic four carbon (4-C) acids as temporary reservoirs of CO_2. C_4 photosynthesis, or dual-cell C_4, represents a spatial specialization, where CO_2 is prefixed into 4-C acids in mesophyll cells (MC) and transported to bundle sheath cells (BSC), the latter being the only cells containing Rubisco and performing the photosynthetic carbon reductive (PCR) cycle (Monson, 1999). On the other hand, CAM is a temporal specialization, with carbon prefixation into 4-C acids occurring at night, followed by daytime remobilization of acids to release CO_2 when the PCR cycle is active (Winter and Smith, 1996).

The steps involved in each mechanism can be described in a comparative manner, as similar biochemical modules are required in both these photosynthetic adaptations. As highlighted in recent next-generation sequencing and biodesign studies (Borland et al., 2014;

Schluter et al., 2016; Yang et al., 2015), both C_4 and CAM pathways require a coordinated series of reactions comprising distinct modules (e.g., carboxylation, decarboxylation, CO_2 acceptor regeneration, transfer acid generation, anatomy, stomatal control), for which the participating enzymes and regulatory proteins have been identified over the years (Table 33.1). Despite efforts in dissecting the components of these modules, uncertainties remain, particularly regarding the role played by many transporters in both syndromes (Hibberd and Covshoff, 2010; Schluter et al., 2016).

33.2.2 Modules Common to Both C_4 and Crassulacean Acid Metabolism Plants

33.2.2.1 Carboxylation and Decarboxylation Modules

A common set of enzymes and regulatory proteins are responsible for the carboxylation and decarboxylation modules in both syndromes (Fig. 33.1, Table 33.1). In C_4, the carboxylation module is restricted to MC whereas the decarboxylation module is confined to BSC, and both take place during the daytime (Monson, 1999). In CAM, the carboxylation and decarboxylation modules occur in the same cells (Fig. 33.1), but during four distinct phases: carboxylation occurs at night when stomata are open (phase I); decarboxylation occurs during the day when stomata are closed (phase III); dawn and dusk are transition moments (phases II and IV, respectively), when phosphoenolpyruvate carboxylase (PEPC) activity declines and Rubisco initiates CO_2 assimilation (phase II), or vice versa (phase IV) (Winter and Smith, 1996).

The first carboxylation by PEPC is one of the key steps for both the C_4 and CAM photosynthesis (Fig. 33.1, Table 33.1). Because of its fundamental role, PEPC regulation has been widely studied and extensively reviewed (e.g., Chollet et al., 1996; Izui et al., 2004; Lepiniec et al., 1994; Nimmo, 2000). Allosteric regulation of PEPC involves effector molecules, being glucose-6-P (G-6-P) and triose-P positive effectors, and malate and aspartate negative regulators (Chollet et al., 1996). Different isoforms of PEPC are encountered in higher plants, involved both in photosynthetic and anaplerotic reactions. According to Christin et al. (2014), there are two major groups of PEPC genes in plants: *PPC-1* and *PPC-2*. The *PPC-1* group comprises all CAM- and C_4-related genes and was duplicated many types in different plant lineages, while *PPC-2* genes are related to C_3 housekeeping functions and are present in a single copy in plant genomes. The *PPC-1* lineage is divided in different groups: *PPC-1E2* and *PPC-1E1*, the latter containing the CAM and C_4 specific genes (Christin et al., 2014).

The most significant difference between PEPC isoforms is the substitution of an alanine for a serine residue near the N-terminal domain (Chollet et al., 1996). This serine can be phosphorylated, which is a critical step in the regulation of PEPC activity. A PEPC kinase is responsible for phosphorylating this serine residue, thus activating PEPC, whereas a typical mammalian-type protein phosphatase 2A (PP2A) converts PEPC back to its inactive form by dephosphorylating this residue (Nimmo, 2000). The activation of PEPC occurs in opposite moments of the diel cycle for C_4 and CAM (daytime and nighttime, respectively) and results in lower sensitivity to malate inhibition and higher sensitivity to G-6-P activation in both cases (Chollet et al., 1996).

Compared with the conserved role played by PEPC in all C_4 and CAM plants, distinct decarboxylation enzymes can be found among representatives of both syndromes. In fact, three types of C_4 photosynthesis can be defined according to the decarboxylation enzyme employed (i.e., NAD-ME, NADP-ME, and PEPCK types), which are accompanied by ultrastructural differences involving chloroplast position in the BSC (Kanai and Edwards, 1999). In the NADP-ME type, chloroplasts are arranged centrifugally and have reduced grana stackings; most oxaloacetate (OAA) is converted to malate, which is metabolized in MC and BSC chloroplasts. In NAD-ME type, chloroplasts and mitochondria are arranged centripetally and show well-developed grana stackings; most OAA is converted to aspartate in the cytoplasm, which is transferred to BSC mitochondria to be decarboxylated. While grana-rich chloroplasts are related to NADP and ATP production due to higher photosystem II activities and linear electron flow, lower grana content is related to ATP production and photosystem I–mediated cyclic electron flow (Edwards et al., 2004). In the PEPCK-type, chloroplasts also have developed grana stackings, and there may be a 30% contribution or more from NAD-ME decarboxylation (Bräutigam et al., 2014; Kanai and Edwards, 1999). Recently, this type has been questioned due to metabolite and energetic specific requirements that may be hard to maintain in BSC (Wang et al., 2014). Overall, it is more likely that NAD- or NADP-ME act as the main decarboxylating enzyme and PEPCK acts as a second decarboxylating enzyme instead of an exclusive PEPCK type (Bräutigam et al., 2014; Muhaidat et al., 2007; Wang et al., 2014).

The decarboxylation enzyme employed may also vary in CAM according to the species, and options are the same as employed by C_4. In addition, the transitory carbohydrate pool converted into phosphoenolpyruvate for CO_2 assimilation can be starch or soluble sugars (Borland et al., 2016; Holtum et al., 2005), resulting in an array of combinations of transitory carbohydrate pool and decarboxylation system.

TABLE 33.1 Core Candidate Genes for C_4 and CAM Syndromes Based on Recent Literature

Module	Gene	Type	Protein name	Reaction
Carboxylation		C_4/CAM		
	CA		Beta carbonic anhydrase	$H_2CO_3 <=> CO_2 + H_2O$
	PEPC		Phosphoenolpyruvate carboxylase	H_2O + phosphoenolpyruvate + $HCO_3^- <=>$ phosphate + oxaloacetate
	V-ATPases		V-type proton ATPase	ATP + H_2O + H^+ (in) = ADP + phosphate + H^+ (out)
	ALMT		Aluminum-activated malate transporter	Presumably controlling malate influx and efflux in and out of the tonoplast
	VPPases		Pyrophosphate-energized vacuolar membrane proton pump	PP => 2 P
Decarboxylation		C_4/CAM		
	NADP-ME		NADP-dependent malic enzyme	Malate + $NADP^+ <=>$ pyruvate + CO_2 + NADPH
	NAD-ME		NAD-dependent malic enzyme	Malate + $NAD^+ <=>$ pyruvate + CO_2 + NADH
	PEPCK		Phosphoenolpyruvate carboxykinase	ATP + oxaloacetate <=> ADP + phosphoenolpyruvate + CO_2
	DiT2/DCT		Dicarboxylate transporter	Malate (out) + X => malate (in) + X
	DIC		Dicarboxylate carrier	Malate (out) + P or SO_4^- (in) => malate (in) + P or SO_4 (out)
	Pyruvate exporter		Unknown pyruvate exporter in mitochondria and chloroplasts	Pyruvate (in) + X => Pyruvate (out) + X
CO_2 acceptor regeneration[a]		C_4/CAM		
	PPDK		Pyruvate, phosphate dikinase	ATP + pyruvate + phosphate <=> AMP + phosphoenolpyruvate + diphosphate
	AMK		AMP kinase	AMP + ATP => 2 ADP
	PPase		Pyrophosphorylase	PP => 2 P
	BASS2		Sodium/pyruvate cotransporter BASS2	Pyruvate (out) + Na^+ (out) => pyruvate (in) + Na^+ (in)
	NHD		Na/H antiporter	H^+ (out) + Na^+ (in) => H^+ (in) + Na^+ (out)
	PPT		Phosphoenolpyruvate transporter	PEP (in) + H^+ (in) + P (out) => PEP (out) + H^+ (out) + P (in)
	Proton pyruvate transporter		Unknown chloroplast pyruvate importer	Pyruvate (out) + H^+ (out) => pyruvate (in) + H^+ (in)
Transfer acid generation[b]		C_4		
	MDH		Malate dehydrogenase	Malate + $NAD(P)^+ <=>$ oxaloacetate + NAD(P)H
	AlaAT		Alanine transaminase	Alanine + 2-oxoglutarate <=> pyruvate + glutamate
	AspAT		Aspartate transaminase	Aspartate + 2-oxoglutarate <=> oxaloacetate + glutamate
	DiT1/OMT		Dicarboxylate transporter 1	OAA (out) + malate (in) => malate (out) + OAA (in)
	DiT2/DCT		Dicarboxylate transporter 2	OAA (out) + aspartate (in) => aspartate (out) + OAA (in)

[a] *Although a PEP regeneration module has only been named separately for C_4, the reactions involved also occur in CAM and, for this work, it was considered here as part of the CO_2 acceptor regeneration module.*

[b] *The role played by many transporters is still unclear for both syndromes; therefore, we summarize the most probable candidate reported in the literature (Schluter et al., 2016).*

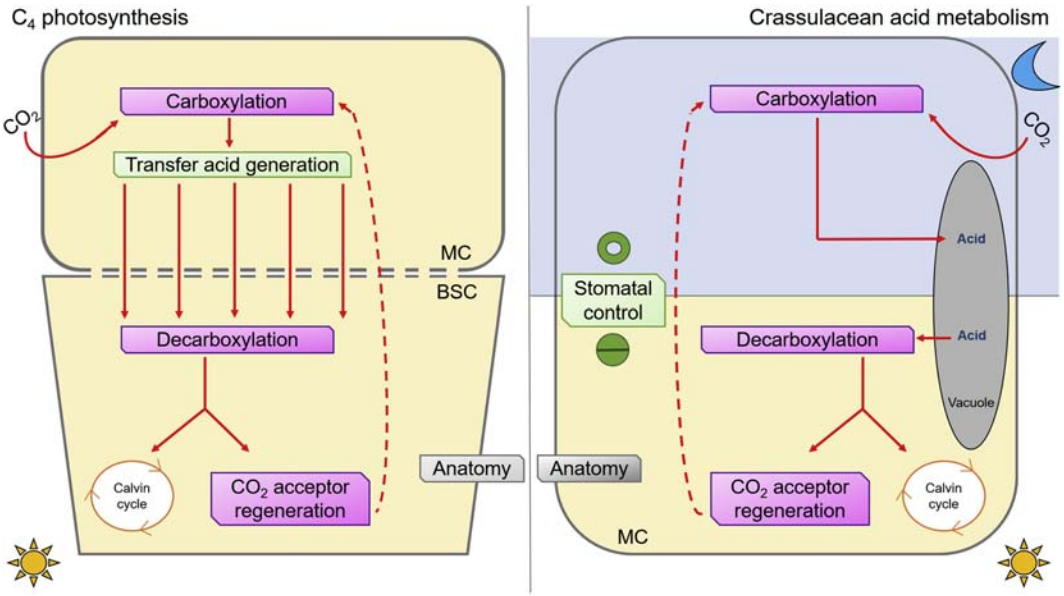

FIGURE 33.1 Comparative scheme between C_4 photosynthesis and Crassulacean acid metabolism. Boxes indicate modules composed of a set of genes. Exclusive modules are indicated in green boxes. Common modules are indicated in purple boxes. Gray boxes indicate modules that are different in composition but share a common theme. Red arrows indicate the carbon pathway inside the cells. *BSC*, Bundle sheath cell; *MC*, mesophyll cell.

33.2.2.2 *Anatomy Module*

Anatomical traits are encompassed in an anatomy module, but although the theme is common, different attributes are involved in each syndrome (Fig. 33.1, Table 33.1). Most C_4 plants present Kranz anatomy, named after the well-developed layer of BSC surrounding vascular bundles and containing all Rubisco and glycine decarboxylase activity of the leaf (Hibberd and Covshoff, 2010; Sage, 2004). However, there are also a few examples of single-cell terrestrial C_4 photosynthesis, indicating that the presence of Kranz anatomy is not mandatory (Voznesenskaya et al., 2001). In these cases, dimorphic chloroplasts can be found inside a single MC, where there is asymmetrical distribution of C_4-related enzymes and organelles between the distal and proximal regions of the cytoplasm, as in *Borszczowia aralocaspica*, or between internal and external regions, as in *Bienertia cycloptera* (Edwards et al., 2004; Voznesenskaya et al., 2001, 2002).

Different types of Kranz anatomy can be found among dual-cell C_4 representatives based on the position of MC, BSC, vascular bundles, and other tissues, also varying between eudicots and monocots (Dengler and Nelson, 1999; Edwards et al., 2004; Muhaidat et al., 2007). However, there is no well-specified correlation between specific anatomical and biochemical types (Muhaidat et al., 2007). Other important traits associated to Kranz anatomy, all aiming to provide short distances for metabolite diffusion but at the same time avoid CO_2 leakage, are (1) low M:BSC ratio with numerous plasmodesmata connecting these two cell types, (2) increased vein density, (3) thin leaves, and (4) a diffusion barrier in or around BSC, with either suberization of cell walls or high concentration of organelles at peripheric regions (Dengler and Nelson, 1999; Edwards et al., 2004; Sage, 2004). Specific mesophyll and bundle sheath *trans*- and *cis*-acting elements for core C_4 enzymes have been identified and some have already been tested successfully (Wang et al., 2017). Moreover, tissue specificity in C_4 leaves is guaranteed via epigenetic, transcriptional and posttranscriptional, translational and posttranslational regulation (Fig. 33.2; Reeves et al., 2016).

In line with the need of accumulating large quantities of 4-C acids in vacuoles of MC overnight, the most frequent anatomical features related to CAM are (1) increased MC size, (2) reduced intercellular air space (IAS), (3) reduced mesophyll surface exposed to IAS (L_{mes}) per unit area, and (4) increased leaf thickness compared with C_3 and C_4 species (Nelson et al., 2005). This results in more succulent leaves and prevents CO_2 efflux during the day, although an increase in succulence itself is not related to higher CAM expression (Nelson and Sage, 2008).

33.2.3 Exclusive Modules: Transfer Acid Generation and Stomatal Control

Lastly, there are two exclusive modules, one related to each syndrome alone. The transfer acid generation

module (Table 33.1) is very relevant for dual-cell C_4 representatives since metabolites must flow between two different cell types (i.e., MC and BSC) (Schluter et al., 2016). As the type of transfer acid formed in MC and the place of decarboxylation in BSC may vary, this module involves a variable number of genes depending on the species. Essentially, it comprises proteins responsible for transporting or generating metabolites in different cell compartments such as mitochondria, chloroplasts, and the cytosol (Kanai and Edwards, 1999; Schluter et al., 2016).

On the other hand, the so-called stomatal control module is particularly relevant in CAM research. In C_3 and C_4, stomata remain open during the day and closed during the night. In contrast, in most CAM plants stomata remain closed during the day (phase III) probably due to the increase in partial CO_2 pressure (pCO_2) from daytime acid decarboxylation, followed by nocturnal stomata opening (Borland and Taybi, 2004; Hartwell, 2005). Stomata may also remain open during transitional phase II in some species, promoting a burst in CO_2 assimilation at the beginning of the light period (Winter and Holtum, 2014). In the CAM species *Agave americana*, several protein kinase-encoding genes, including *HIGH LEAF TEMPERATURE 1* (*HT1*), *OPEN STOMATA 1* (*OST*), and *SnRK2.10*, presented rescheduled expression compared with their orthologs in *Arabidopsis thaliana* (Abraham et al., 2016). Furthermore, *PHOTOTROPIN 2* (*PHOT2*), which encodes a blue light receptor, showed an inverted pattern of expression in the CAM plant model *Kalanchöe fedtschenkoi* compared with that observed in C_3 species (Yang et al., 2017). Also, genes related to ion channels in guard cells, such as *POTASSIUM TRANSPORTER 2/3* (*AKT2/3*) and *CHLORIDE CHANNEL FAMILY* (*CLC-c*), showed reciprocal expression behavior in *A. americana* compared with that described in *Arabidopsis* (Abraham et al., 2016). Therefore, these genes exemplify some of the promising candidates associated with the inverted stomatal control typically found in CAM plants (Abraham et al., 2016; Yang et al., 2017).

33.3 ENVIRONMENTAL CUES CONTROLLING C_4 AND CRASSULACEAN ACID METABOLISM

As stated by Lüttge (2004), environmental cues are defined as an input act upon organisms, referred to as receivers, and this interacting network will result in an output at community and ecosystems level. The input affects genotypes and phenotypes of receivers, the latter including morphotype and physiotype. C_4 and CAM, both including genotype and phenotype components, represent two cases of parallel convergent evolution, where unrelated taxa from various phylogenetic backgrounds have evolved similar mechanisms in two independent ways (Christin et al., 2013, 2014; Muhaidat et al., 2007; Sage, 2017; Silvera et al., 2010). This makes the study of how representatives from both syndromes respond to the environment particularly interesting since a diverse array of strategies and different magnitudes of response has been observed.

In C_4 species such as the traditionally studied monocots maize (*Zea mays*) and sugarcane (*Saccharum officinarum*) or in CAM crops such as *Agave americana* or *Opuntia ficus-indica*, the CCM is constitutively expressed. Among CAM plants, there are also facultative species, whose CAM expression is highly influenced by environmental cues, thereby representing valuable tools for dissecting the regulatory mechanisms responsible for adjusting the CCM expression in response to the surrounding environmental conditions (Winter and Holtum, 2007; Winter and Holtum, 2014). In addition, increasing attention has been dedicated to the so-called C_3–C_4 intermediates, which may shed light on evolutionary paths leading to full C_4 expression (Christin et al., 2011).

An interesting strategy to understand how external cues affect organisms is to study these intermediate and facultative phenotypes, because the first may help to understand evolutionary paths, and in the latter, the timing and inducing factors can be controlled without developmental interference (Christin et al., 2011; Winter and Holtum, 2014; Winter and Holtum, 2007).

33.3.1 Modulation of C_4 by Environmental Cues

The relationship between dual-cell C_4 and environmental cues can be traced in an evolutionary panorama or considering phenotypically plastic responses. Regarding the latter, a great description of environmental variables—such as light, water, nitrogen, and temperature—and how they modulate photosynthetic aspects of C_4 plants was made by Long (1999). When referring to the biochemical types of C_4, NAD-type grasses were able to enhance water use efficiency (WUE) at leaf level in a higher proportion than NADP-ME type grasses, which was probably related to stomatal adjustments (Ghannoum et al., 2002).

Dual-cell C_4 requires more specialized structural arrangements when compared with single-cell C_4, C_3, and CAM, and presents more restricted geographic distribution than the latter two types. This can be correlated to a reduced potential for phenotypic plasticity when compared with C_3 plants, as stated by Sage and McKown (2006). These authors highlight that

acclimation processes must involve coordinated changes in the different tissues involved in C_4 to keep functional stoichiometries. Besides, when compared with C_3 plants, dual-cell C_4 representatives display restricted potential to modulate leaf thickness, due to the enlarged BSC, the costs associated with changes in vein density, and a limited capacity to modulate Rubisco content. However, there are exceptions to this assumption, since *Flaveria bidentis* and maize have been shown to exhibit plastic acclimation responses to low-light conditions (Pengelly et al., 2010; Bellasio and Griffiths, 2014), indicating that this subject needs further investigation. Plants exhibiting single-cell C_4 do not face such structural constraints since they can more easily modulate photosynthesis-related processes within a single photosynthetic cell (Edwards et al., 2004; Voznesenskaya et al., 2001). Perhaps, in this case, more plastic responses could be observed, but no experimental evidence has been reported yet.

Similarly, little is known about the phenotypic plasticity within C_3–C_4 intermediate species. This photosynthetic type is considered a transitional stage between ancestral C_3 and fully expressed C_4, as it presents biochemical and anatomical features of both photosynthetic types (Christin et al., 2011; Monson and Moore, 1989). In a different point of view, C_3–C_4 plants can also be treated as a successful adaptive strategy, not merely as a transitional stage, as is the case for *Mollugo verticillata* and *M. nudicaulis*. These two species are weeds that show a cosmopolitan distribution, suggesting that the intermediate photosynthetic type may have contributed to their wide geographical distribution (Christin et al., 2011).

Differently from most C_4 and C_3–C_4 intermediates, the aquatic species *Eleocharis vivipara* stands out as a remarkable example of C_4 expression controlled by environmental circumstances (Ueno et al., 1988). *E. vivipara* produces C_3 photosynthetically active culms when underwater, but emerged C_3 culms wither away and new C_4 culms develop after a few days (Ueno et al., 1988; Ueno, 2001). The C_4 culms have already been characterized biochemically and anatomically (Ueno, 2001), and C_4-related gene expression is not strictly dependent on Kranz anatomy (Uchino et al., 1998).

33.3.2 Modulation of Crassulacean Acid Metabolism by Environmental Cues

It is increasingly accepted that a higher plasticity can be found between and within CAM plants than in most C_4 species (Edwards and Ogburn, 2012). Compared with the rare examples of C_3–C_4 facultative plants (e.g., *E. vivipara*, *E. baldwinii*), a significantly higher number of C_3-CAM facultative species are described in the literature (Silvera et al., 2010; Winter et al., 2015). Moreover, even obligate CAM species display some plasticity in CAM expression during early developmental stages (Freschi et al., 2010; Winter and Holtum, 2007). In this sense, CAM has been considered a trait with continuous distribution oppositely to an all-or-nothing adaptive response (Silvera et al., 2010; Winter et al., 2015).

Besides the variation in plasticity (i.e., constitutive versus facultative CAM), differences in the magnitude of nighttime acid accumulation and the diel pattern of stomata opening also greatly vary among CAM, giving rise to the following CAM modes: (1) CAM cycling, in which gas exchange is limited to daytime, with nocturnal acid production due to respiratory CO_2 recycling; and (2) CAM-idling, which is an emergency process triggered during periods of prolonged drought and heat. The latter state is characterized by the complete closure of stomata over the diel cycle associated with a small nocturnal acid production from respiratory CO_2 recycling (Cushman, 2001). Both these modes are considered weak versions of CAM as the amount of acids accumulated overnight is usually lower than in plants performing classical CAM.

Facultative plants frequently perform weak CAM for variable portions of their life cycles; therefore, the adaptive advantages of facultative CAM have been questioned (Herrera, 2009). However, even if a low level of CAM is induced, this may improve plant survival by increasing WUE, promoting a positive carbon balance, intensifying the photoprotection of the photosynthetic machinery, and, consequently, improving reproductive success (Herrera, 2009; Winter and Holtum, 2014).

Due to the absence of clear anatomical traits characterizing CAM and the fact that $\delta^{13}C$ values in facultative CAM species tend to be closer to C_3 plants, detailed physiological studies are required to properly identify facultative CAM (Silvera et al., 2010; Winter et al., 2015). This may contribute to an underestimation of the total number of facultative C_3-CAM species (Silvera et al., 2010). Nevertheless, confirmed facultative CAM species have been described in Bromeliaceae, Crassulaceae, Montiaceae, Piperaceae, Portulacaceae, Talinaceae, and Clusiaceae families (Winter and Holtum, 2014). In most facultative species, CAM expression can be induced or intensified by drought either alone or in combination with other environmental stresses such as nutrient availability and changes in temperature, light intensity, and photoperiod (Silvera et al., 2010). In the halophyte *Mesembryanthemum crystallinum*, either drought or salt stress strongly induce CAM (Cushman et al., 1990; Herppich et al., 1992; Winter and Holtum, 2007), and although a great deal of physiological, signaling, and molecular studies have

been performed in this facultative model (Bohnert et al., 1988; Cushman et al., 1990, 2008; Herppich et al., 1992; Holtum and Winter, 1982; Winter and Holtum, 2007), much still remains to be elucidated.

Besides *M. crystallinum*, constitutive and facultative CAM species belonging the tropical genus *Clusia* have also been intensively studied over the years (Lüttge, 1996, 2006). CAM induction may be under developmental control as in *C. rosea*, *C. alata*, and *C. hilariana*; under environmental control in a fully reversible manner as in *C. pratensis*; or even exhibit intermediary influences as in *C. minor* (Winter and Holtum, 2007). Given the rapid, intense, and completely reversible induction of CAM observed in *C. pratensis* in response to drought, this species has emerged as an interesting model for future studies on the regulation of facultative CAM (Winter and Holtum, 2014).

33.4 STRESS SIGNALING NETWORKS CONTROLLING C_4 AND CRASSULACEAN ACID METABOLISM

As presented so far, C_4 and CAM are both complex syndromes, involving multiple genes, complicated metabolite dynamics, biochemical adjustments, and sometimes anatomical specializations. This implies the existence of equally complex signaling routes, as stated by Freschi and Mercier (2012) for CAM signaling, and here extended to C_4. Intricate transcriptional, translational, posttranslational, and metabolic regulatory changes are supposedly required to facilitate the establishment and functioning of either CCM in a given tissue (Fig. 33.2). This includes, but is not limited to, coarse changes in enzyme content/activity and the fine-tuned control of regulatory proteins (e.g., transcription factors, kinases, phosphatases) (Taybi et al., 2002).

Most of the studies regarding the establishment of the C_4 machinery has been based on the developmental gradient in leaves (e.g., Li et al., 2010; Pick et al., 2011), since C_4 induction upon environmental variables is rare, with a few exceptions in the *Eleocharis* genus (Ueno, 2001) as previously mentioned.

In *E. vivipara* submerged plants, abscisic acid (ABA) was shown to induce Kranz anatomy formation and C_4-like biochemical traits (Ueno, 1998) whereas in the C_3-C_4 intermediate *E. baldwinii*, full C_4 was induced after ABA treatment causing auxin signaling and changes in the transcriptional profile of genes involved in the glycolytic pathway, ion and metabolite transporters, citrate metabolism, among other processes (Chen et al., 2014). Recent evidence also indicates that PEPC content and activity is increased in light-exposed *Amaranthus hypochondriacus* leaf disks upon ABA treatment (Aloor et al., 2017), suggesting that it may be advantageous for C_4 plants to keep PEPC activated by ABA under drought stress conditions.

Although still fragmented, our current knowledge on the stress signaling cascades controlling CAM expression is relatively more detailed than for C_4

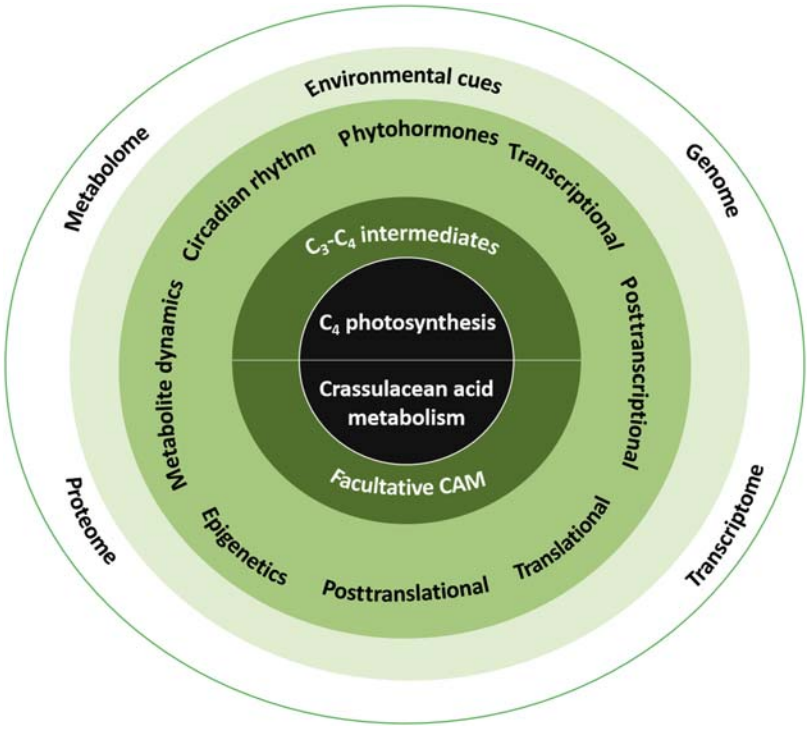

FIGURE 33.2 C_4 and CAM are both complex syndromes that are regulated in many different levels. The study of intermediary and facultative systems for C_4 and CAM, respectively, using omics technologies is very promising in understanding the regulatory processes. The two inner circles represent both photosynthetic metabolism types and their variations. The two outer circles represent the regulatory levels and omics fields that can be used to explore them.

photosynthesis (Taybi et al., 2002; Freschi and Mercier, 2012). Most studies have been performed in the C_3-CAM halophyte *M. crystallinum*, but data is also available for a few other CAM species including pineapple (*Ananas comosus*) and *Kalanchöe blossfeldiana*. The signaling cascades controlling CAM expression in these species have been extensively reviewed first by Taybi et al. (2002) and later by Freschi and Mercier (2012); therefore, they are briefly discussed here.

Data indicate that ABA and cytokinins (Cks) play opposite roles in controlling CAM expression (Chu et al., 1990; Schmitt and Piepenbrock, 1992; Thomas and Bohnert, 1993; Thomas et al., 1992), which agrees with the contrasting impact of water availability on the biosynthesis of these two hormonal classes (Hare et al., 1997; Pospisilova et al., 2000, 2005; Tanaka et al., 2006). Two major lines of evidence support a promotive role of ABA on CAM expression. First, exogenous ABA has been shown to increase the activity and/or mRNA levels of CAM-related enzymes both in detached leaves and intact plants (Chu et al., 1990; Dai et al., 1994; Forsthoefel et al., 1995a,b, 2010; Taybi et al., 1995, 2002; Tsiantis et al., 1996). Second, stress-induced CAM expression is normally accompanied by an increment in endogenous ABA content (Freschi et al., 2010; Taybi et al., 1995, 2002). However, studies indicate that a parallel, non-ABA-dependent, signaling route is also involved in controlling CAM expression in response to environmental stresses (Freschi et al., 2010; Taybi et al., 2002).

In contrast, accumulating evidence implicates Cks as negative regulators of CAM expression in facultative CAM species as the stress-induced increase in CAM expression in facultative plants is usually accompanied by a reduction in endogenous Ck levels (Freschi et al., 2010; Peters et al., 1997), and exogenous Cks generally represses CAM induction in response to stress conditions (Freschi et al., 2010; Peters et al., 1997; Schmitt and Piepenbrock, 1992). Some studies also indicate that exogenous methyl jasmonate (MJA) can also limit CAM expression in droughted *M. crystallinum* plants (Dai et al., 1994; Schmitt and Piepenbrock, 1992), though the roles of endogenous MJA still remain elusive. More recently, gibberellin levels have been reported to decrease and not recover in *Aptenia cordifolia* plants exposed to successive drought stress, being thus connected to a drought stress memory response in CAM expression (Fleta-Soriano et al., 2015).

So far, only few second messengers have been demonstrated to control CAM expression in facultative plants (Freschi et al., 2010; Taybi et al., 2002). Among them, the influx of extracellular calcium (Ca^{2+}) has been identified as a key downstream event in both ABA-dependent and -independent signaling pathways leading to CAM expression (Freschi et al., 2010; Taybi et al., 2002). Data also indicate that inositol-1,4,5-triphosphate (IP3) and nitric oxide participate in the ABA-dependent signaling cascade promoting CAM expression (Taybi et al., 2002).

In addition to studies focused on specific signaling events, the generation of genomic and transcriptomic databases for both facultative and obligate CAM species has also recently opened a window of opportunities for more detailed characterization of the stress signaling cascades controlling CAM expression (Abraham et al., 2016; Brilhaus et al., 2016; Wai et al., 2017). In *Talinum triangulare*, ABA-responsive transcription factors were among the most upregulated transcription factors during the drought-triggered CAM induction (Brilhaus et al., 2016). In addition, studies also indicate that several families of miRNAs presumably target key CAM-related enzymes, and many of these miRNAs display a circadian pattern of expression in *Ananas comosus* (Wai et al., 2017). Cluster analysis of gene expression patterns leading to the generation of coexpression modules have also been increasingly employed to identify less obvious candidate genes potentially involved in controlling CAM expression. For example, *CONSTANS*-like genes and *REVEILLE* transcription factor-encoding genes have been grouped with PEPC and NADP-ME genes, thereby suggesting their potential involvement in controlling the CAM machinery in *K. fedtschenkoi* (Yang et al., 2017).

Metabolite fluctuation is also reported as an important layer of control in CAM plants (Borland and Taybi, 2004). In *Agave americana*, besides malic and fumaric acid nocturnal fluctuation, ascorbic acid was also shown to accumulate during the night, presumably providing antiredox power against reactive oxygen species in this CAM plant (Abraham et al., 2016). Glycolysis and carbohydrate metabolism-related transcripts have been highlighted as a very important step for CAM regulation (Brilhaus et al., 2016; Yang et al., 2017). Moreover, the transcripts' abundance of core CAM and starch degradation-related enzymes was drastically altered in a starch and CAM-deficient *M. crystallinum* mutant, thereby reinforcing the role of carbohydrates in CAM regulation (Taybi et al., 2017).

33.5 C_4/CRASSULACEAN ACID METABOLISM COMPATIBILITY

C4 and CAM have been considered incompatible syndromes for various reasons: (1) they show differential regulation of enzymes involved in the carboxylation and decarboxylation steps; (2) the metabolite transport dynamics of each syndrome is strikingly

different; and (3) structural arrangements for each syndrome differ, favoring Kranz anatomy in C_4 and succulence in CAM (Sage, 2002). In addition to this list, Sage (2002) also highlights that the evolutionary pathways selecting for each of these photosynthetic behaviors appear to be exclusive. Thus, the simultaneous evolution of C_4 and CAM in a single organism would be unnecessary.

One of the most stunning cases, the order Caryophyllales, contains many C_4 and/or CAM origins including its intermediates variants (Kellogg, 1999), being considered a hotbed for these CCMs (Edwards and Ogburn, 2012). To illustrate the photosynthetic diversity of Caryophyllales, the following representatives of the order are here listed: *Borszczowia aralocaspica* and *Bienertia cycloptera* (Chenopodiaceae), the two species known to perform single-cell C_4 in a family of many dual-cell C_4 (Edwards et al., 2004); cacti (Cactaceae), traditionally known to be obligate CAM plants (Ocampo and Columbus, 2012); and the "Portullugo" clade, as referred to by Edwards and Ogburn (2012), comprising the sister-groups Molluginaceae and Portulacaceae, that each evolved C_4 independently (Christin et al., 2011, 2014). Both families contain C_3, C_4, and C_3–C_4 intermediates, and the latter, monogeneric, also includes C_4–CAM species (Edwards and Ogburn, 2012). Recently, Christin et al. (2015) studying C_4 evolution in Caryophyllales reinforced that genetic enablers were present in C_3 ancestors and genes were coopted into C_4 when already expressed in a C_4-manner. However, the identity of the coopted candidates varies in different lineages inside Caryophyllales, representing true independent evolution events of C_4 photosynthesis.

The genus *Portulaca* is an exception to the C_4–CAM incompatibility assumption. *Portulaca* comprises about 60 species of C_4 annual weeds, of which at least six can undergo CAM induction upon environmental stimuli: *P. oleracea*, *P. grandiflora*, *P. pilosa*, *P. australis*, *P. cyclophylla*, and *P. digyna* (Guralnick and Jackson, 2001; Guralnick et al., 2002; Holtum et al., 2017; Koch and Kennedy, 1980, 1982; Ku et al., 1981; Winter and Holtum, 2017). This metabolic plasticity has been better studied in *P. grandiflora* and *P. oleracea* (Christin et al., 2014; Guralnick and Jackson, 2001; Guralnick et al., 2002; Mazen, 1996, 2000), revealing that both leaves and stems display the induction of CAM-like features under stress conditions (Guralnick et al., 2002; Koch and Kennedy, 1980, 1982; Mazen, 1996; Voznesenskaya et al., 2010).

The first discovery was carried out in *P. oleracea*, when leaves were shown to increase nocturnal acidity and perform a low level of CO_2 assimilation in the dark after prolonged periods of water deprivation or under short-day conditions (Koch and Kennedy, 1982, 1980). Similar findings of a CAM-like behavior were made around the same time for *P. grandiflora* (Ku et al., 1981), and after a decade, the records of CAM in these two species plus *P. pilosa* (previously known as *P. mundula*) were confirmed (Kraybill and Martin, 1996). Moreover, leaf PEPC content was shown to increase in *P. oleracea* as the drought period was prolonged, associated with opposite diel changes in PEPC kinetic properties characterized by a peak in PEPC activity during the day and higher malate sensitivity at night in well-watered plants, and the exact opposite pattern in drought-stressed individuals (Mazen, 1996, 2000).

In line with these findings, the comparison of the global transcriptional profile of well-watered and droughted *P. oleracea* plants led to the identification of two PEPC-encoding genes differentially regulated under each condition: while *PoPPC1E1c* was highly expressed during the night in droughted plants, *PoPPC1E1a'* was predominantly expressed during the day and in well-watered samples (Christin et al., 2014). Therefore, *PoPPC1E1c* and *PoPPC1E1a'* were probably recruited by the CAM and C_4 pathways, respectively, which may have facilitated the evolution of regulatory mechanisms to allow the expression of both CCM in leaves of this plant species. According to the evolutionary history of PEPC genes and considering the existence of other CAM plants with close phylogenetic proximity, C_4 probably evolved on top of a CAM-performing organism (Christin et al., 2014), and the CAM pathway may have been lost in most species of the genus. In addition, the appearance of different types of C_4 in different *Portulaca* species (e.g., *P. oleracea* is NAD-ME type and *P. grandiflora* is NADP-type) reinforces this hypothesis, where C_4 would have evolved independently in each species (Voznesenskaya et al., 2010).

Based on immunoblot results, Lara et al. (2003, 2004) proposed a first hypothetical model of C_4–CAM compatibility in *P. oleracea*. Most *Portulaca* leaves have three types of cells: water storage cells (WSC), whose contribution to overall carbon gain may be minimal due to their small number of chloroplasts, and photosynthetically active MC and BSC (Lara et al., 2003, 2004; Voznesenskaya et al., 2010). In the hypothetical model, malate generated from CAM at night in WSC and MC would be shuttled to BSC for decarboxylation during the day (Lara et al., 2004).

The compatibility between C_4 and CAM observed in certain *Portulaca* species is much more than an evolutionary curiosity, as these species represent a particularly interesting model for exploring the molecular, biochemical, and signaling mechanisms responsible for allowing the occurrence of these distinct photosynthetic modes within a single individual (Yang et al., 2015). Among the six C_4–CAM facultative *Portulaca* species currently identified, *P. oleracea* display short

size, rapid growth, flat leaves, and abundant production of long-lived seed (Zimmerman, 1976), making this species a particularly attractive target for future research on C_4/CAM compatibility.

33.6 ENGINEERING C_4 AND CRASSULACEAN ACID METABOLISM: CHALLENGES AND POSSIBILITIES

In the context of climate change, drier and warmer conditions are expected to occur in many important agricultural regions of the world (IPCC, 2014). Given the significant adaptive advantages of C_4 and CAM pathways under hot and dry environments compared with C_3 photosynthesis, it is perhaps unsurprising that ambitious initiatives aiming to engineer either CCMs into C_3 crops have being initiated: the C_4 Rice project (von Caemmerer et al., 2012; von Caemmerer and Furbank, 2016; Furbank, 2016) and the CAM Biodesign project (Borland et al., 2014, 2015; Hartwell et al., 2016; Yang et al., 2015). Both initiatives are long-term endeavors (10 years or more) and agree on the intensive use of omics technologies to provide essential information on poorly understood aspects of both cycles. Moreover, since both syndromes involve hundreds or thousands of genes, considerable efforts have been done by both projects to develop new technologies to engineer plants for complex, multigene constructs (Czarnecki et al., 2016; Yang et al., 2015).

Though the engineering of functional C_4 into rice or any other species has not been achieved yet, many important accomplishments have been obtained in recent years, such as massive advances in understanding the components and regulatory processes responsible for the C_4 functioning (e.g., Wang et al., 2017), the successful increase of chloroplast, mitochondria, and plasmodesmata number in rice BSC (Wang et al., 2017), among others. Similarly, The CAM Biodesign initiative has also led to impressive advances in understanding CAM biology, including the development of an omics dataset for several key CAM model species (Abraham et al., 2016; Yang et al., 2017) and key functional genomics data (Boxall et al., 2017; Dever et al., 2015). In addition to engineering CAM in C_3 crops, the CAM Biodesign initiative also aims to explore the photosynthetic plasticity of CAM plants for high biomass production in marginal lands and habitat restoration (Borland et al., 2011).

33.6.1 Engineering Crassulacean Acid Metabolism Into C_4

In addition to the current efforts to increase drought tolerance in C_4 crops (Lopes et al., 2011), another elusive possibility would be to engineer inducible CAM into C_4 crops. C_4 and CAM have been considered incompatible to occur in the same cells (Sage, 2002), but as is the case for some *Portulaca* species, stress-induced CAM has been shown to occur in C_4-performing organisms (Guralnick and Jackson, 2001; Guralnick et al., 2002; Holtum et al., 2017; Koch and Kennedy, 1980, 1982; Ku et al., 1981; Winter and Holtum, 2017). Although an obligate CAM pathway would maximize WUE, a partial commitment to CAM, specifically when abiotic stress conditions challenge the functioning of C_4, may prove to be beneficial by increasing the survival of the C_4 crops until favorable environmental conditions are restored (Borland et al., 2014). Therefore, C_4 and CAM would not necessarily co-occur in a given tissue, providing that the stress-induced CAM is preceded by the downregulation of the C_4 pathway.

A strong CAM response would require, most of all, a compact mesophyll for storing large amounts of nocturnal organic acid, and inverted stomatal control. On the other hand, CAM cycling seems to be more plausible to engineer into a C_4 plant, as this mode of CAM does not require changes in the diel stomata pattern, nor much vacuolar space to accumulate the comparatively limited acids generated overnight (Borland et al., 2014). Although performing CAM cycling would only contribute with a small additional input in carbon supply, this could mean an increase in survival under dry seasons.

33.6.2 Parts List for Crassulacean Acid Metabolism Into C_4

Engineering CAM or C_4 in C_3 plants involves transferring genes directly involved in either machinery as many of the modules required for either syndrome are absent in C_3-performing species. In contrast, many genes and biochemical steps are common in both CCMs, so perhaps the most important and challenging task in engineering weak facultative CAM into C_4 plants would be to coordinate the timing of expression of components of each CCM depending on the environmental conditions. Therefore, instead of transferring genes involved in CAM or C_4 to C_3 plants, perhaps one of the major challenges in engineering facultative CAM in a C_4 species may rely in understanding the transcriptional regulation of genes involved in the C_4 machinery aiming to the future adjustments in their expression patterns via genome editing technologies.

For example, the main players of the carboxylation module required for CAM functioning are already present in the MC of C_4 plants, only functioning in a

different moment of the diel cycle. Therefore, an important challenge to engineer the carboxylation module of CAM in a C_4 species would be to invert the timing of PEPC expression and activation in the MC and guarantee available carbonic anhydrase (CA) activity. Adjustments to promote the nighttime accumulation of acids in the vacuoles and the subsequent transport of these molecules back to the cytosol during the day will also be necessary.

The decarboxylation module in CAM plants also take place in MC, whereas in dual-cell C_4 species this module is in the BSC. In *P. oleracea* plants under drought stress, it has been proposed that the acids accumulated overnight inside MC vacuoles are transferred to BSC during the day (Lara et al., 2004). Therefore, it seems tempting to propose that, with some adjustments, the acid transport mechanisms and decarboxylating enzymes already present in a C_4 plant would be enough to decarboxylate the acids accumulated overnight in a C_4–CAM engineered plant.

A hypothetical scheme of CAM cycling to be engineered into C_4 as means to improve drought stress tolerance in crops is presented in Fig. 33.3, which is based on current knowledge available in C_4–CAM facultative plants (Lara et al., 2004). Although accomplishing this idea would take place in a more distant future compared with the other bioengineering initiatives, technological progress achieved through other projects, such as the C_4 rice and the CAM Biodesign projects, may shed light on this C_4–CAM engineering endeavor and bring it closer to realization. Our discussion has not, by any means, exhausted such a rich and interesting research topic, which should be revisited as our current understanding on C_4 and CAM functioning and compatibility is increased.

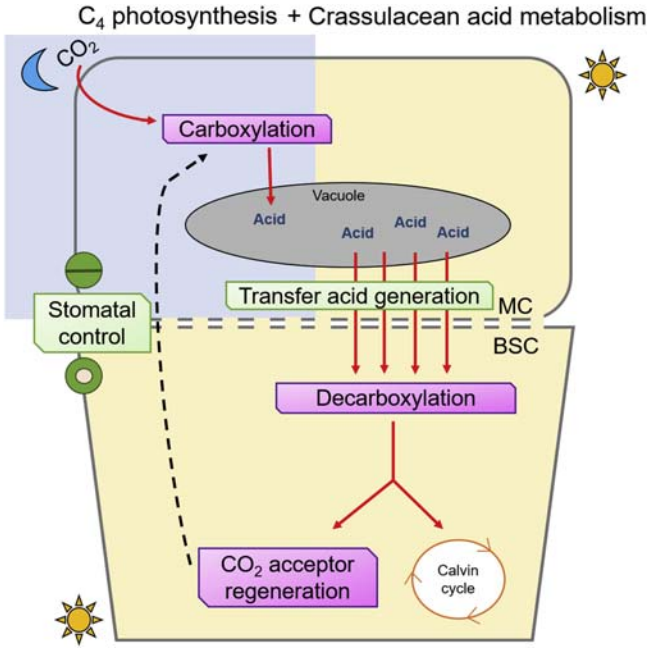

FIGURE 33.3 Hypothetical scheme of CAM cycling to be engineered into C_4 as means to improve drought stress tolerance in crops, based on current knowledge available in C_4–CAM facultative plants (Lara et al., 2004). Respiratory CO_2 would be used in nocturnal acid formation on mesophyll cells. Acids stored overnight would then be transported to the B to be decarboxylated during the day. Red arrows indicate the carbon pathway inside the cells. *BSC*, Bundle sheath cell; *MC*, Mesophyll cell.

33.7 CONCLUDING REMARKS

In summary, there is still much room for improving our current knowledge on the complexity involving C_4 and CAM functioning and regulation, especially regarding metabolite transport, stomatal control, and molecular and hormonal events regulating the expression of these CCMs. Given the vast array of biochemical and anatomical variation found among C_4 and CAM plants, it seems plausible to anticipate that equally diverse regulatory mechanisms have evolved to control the distinct C_4 and CAM genotypes and physiotypes currently known. Therefore, generating and comparing data for different candidate species seems a valid approach to provide a broad and more complete overview of the regulatory mechanisms controlling C_4 and CAM pathways. As both syndromes display higher WUE and lower crop water demand compared with C_3 plants, C_4 and CAM have been increasingly proposed as promising systems for bioengineering approaches in a context of climate change. A common initial step in these bioengineering initiatives is the use of omics approaches to unravel new candidate genes involved in both the core CCM pathway and the regulatory processes to produce a parts list for each syndrome. Besides exploring C_4 and CAM in obligate organisms, further attention should be devoted to the few known examples of C_4–CAM facultative plants, as they may help to explain how these syndromes interconnect, thereby holding a great value as a living blueprint for engineering CAM into C_4 crops.

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CHAPTER

34

Protein Kinases and Phosphatases in Stress Transduction: Role in Crop Improvement

Thammineni Chakradhar[1], *Ramesha A. Reddy*[2] *and Thummala Chandrasekhar*[3]

[1]Thakora Crop Sciences Pvt. Ltd., Anantapur, Andhra Pradesh, India
[2]Seri-Biotech Research Laboratory, Central Silk Board, Bangalore, Karnataka, India
[3]Department of Environmental Sciences, Yogi Vemana University, Kadapa, Andhra Pradesh, India

OUTLINE

34.1 INTRODUCTION

Due to their sessile nature, plants are exposed to various kinds of adverse environmental cues. This has makes the plant cell evolve with different signaling enzymes/molecules that can perceive environmental stimuli, transduce across membranes and cytoplasm, interact with regulatory elements in the genome, and finally elicit appropriate adaptive response. The majority of these signaling enzymes belong to kinase group. Plant protein kinases are a class of enzymes that catalyze phosphorylation of functional proteins through which signal cascades transduce. Protein kinases are mainly constituted of a larger group of phosphoryltransferases that transfer the terminal phosphate from ATP to the substrate protein. Protein phosphorylation

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induces the conformational changes in the protein kinase structure to regulate its activity. A network of such signals stimulates the production of second messenger molecules such as phorbol ester, phorbol myristate acetate (PMA) and Ca^{2+} (Melissa et al., 2012). The substrate proteins interact with Ca^{2+} in presence of phorbol ester, PMA, and lowers their Km value. This promotes the phosphorylation of serine, threonine residues in kinase enzymes using terminal phosphate of ATP. Ca^{2+} is an important secondary messenger that acts as a catalyst in signal transmission (Batistic and Kudla, 2004). Ca^{2+} binds to the protein kinases like CDPKs, CIPKs, and MAPKs and makes them transmit stress signals to the transcriptional cascade, through phosphorylation. Plant protein kinases contain a highly conserved domain of 250−300 amino acids, which are responsible for phosphorylation transferase activity. The activity of protein kinases depends on the concentrations of cytoplasmic Ca^{2+}, which in turn causes development and metabolic changes in plant cells. During experimenting on Ca^{2+} activated phosphorylation, Harmon et al. (2001) identified the calcium-dependent protein kinases in soybean, which are unique in nature. In short, difference in the concentrations of cytoplasmic Ca^{2+} is a cause for the promoting and inhibiting activity of protein kinases in plants. The changes of cytosolic Ca^{2+} level in response to various environmental stimuli such as drought, salinity, extreme temperature, light, pathogenic infection, and pH alter the protein phosphorylation in plant cells. CDPKs with N-terminal kinase catalytic domain contiguous with a terminal calmodulin-like Ca2 + -binding domain is the best example of calcium-dependent protein phosphorylation in plants (Ludwig et al., 2004). Moreover, another type of CDPK with the sequence of CB1 has also been recognized in plant that can change cytoplasm calcium levels and was found to be involved in protein phosphorylation (Watillon et al., 1993). Different environmental stress signals utilize common protein kinases to transduce the stimuli to nucleus. Apart from external signals, plant protein kinases are also involved in cellular morphogenesis and development such as cell division, self-incompatibility, initiation of mitosis, and elongation. Crosstalk of signaling molecules is a common phenomenon in plant signal transduction. Hormonal, reactive oxygen species (ROS), abiotic and biotic signaling pathways are interdependent through a complex network involving ROS molecules like H_2O_2, Ca^{2+}, ABA, inositol 1,4,5-triphosphate (IP3), etc. The continuous exposure to various environmental cues has led the plants to evolve with different kinds of kinases with special and functional specificity. Further evolutionary process has created duplications and divergence in basic kinase groups to various extent and led to the formation of different subfamilies. A detailed description of structural divergence of plant protein kinases has been elaborately reviewed by Lehti-Shiu et al. (2012). Protein kinases occupy a large group of functional genes in eukaryotes. Initial investigations proved that the plant genome contains Ser/Thr and His protein kinases whereas the animal genome contains Tyr protein kinase in addition to the abovementioned kinases. Based on phosphorylation activity between the conserved amino acids and substrates, protein kinases are broadly categorized into (1) serine-tyrosine kinases, (2) serine-threonine kinases, and (3) histidine kinases. Depending on functional diversity plant protein kinases have also been categorized into (1) RLKs, (2) MAPKs, (3) CDPKS, (4) CDKs, and other less significant kinases like SNF1/AMPK, PDK1, HKs, DGKs, etc. Each group of plant protein kinases is involved in specific environmental signal, or developmental response, forming a unique signal cascade. But often the signaling components in one cascade participate in other signaling pathways forming a complex network. Calmodulin-like domain protein kinases (CDPKs) and receptor-like kinases (RLKs) are unique to plants and are lacking in other eukaryotes. RLKs in plants perform as the receptor of Tyr kinase (RTKs) in an animal system. Histidine kinases (HKs) exist in bacteria and eukaryotes, with different modes of operation in both. Transgenic expression of different signaling kinase genes are described in Table 34.1.

Plants are equipped with many enzymatic and nonprotein components to cope with abiotic stress. As indicated above plant protein kinases are important signaling enzymes that perceive and transfer the external environmental stresses down to the nucleus to get the defense response. The role of kinases is more projected in the defense mechanism in biotic stress rather than in abiotic stress. Most of the abiotic stress responsive genes in plants are grouped as ABA dependent and ABA-independent. SnRK2 are plant specific kinases belonging to serine/threonine group and found to be expressed in all abiotic stress conditions (Kulik et al., 2011). In response to ABA hormone, SNF1 (sucrose nonfermenting 1) a SnRK2 kinase, binds to ABRE/ABF transcription factor and activates ABA responsive physiological process (Umezawa et al., 2009). Different classes of plant protein kinases, including MAPKs and RLKs, play vital role in cellular metabolic signaling and physiological process during abiotic stress (Wankhede et al., 2013; Jaggi et al., 2013). The salt overly sensitive (SOS) pathway is a signaling pathway that plays a crucial role in salt stress adaptation. SnRK3, a SNF like protein kinase, interacts with SOS3 protein to activate plasma membrane bound Na^+/H^+ antiporter. The phosphorylation of Na^+/H^+ antiporter,

TABLE 34.1 Plant Protein Kinase Gene Used in Genetic Engineering for Abiotic Stress Tolerance

Functional category	Gene	Transgenic crop/plant	Tolerance	Mechanism of action	References
RLK	(FLORAL ORGAN NUMBER1) FON1	Rice	Drought stress	Through action of ABA responsive genes	Feng et al. (2014)
	(*Leaf Panicle 2*) LP2	Rice	Drought stress	Through interaction of aquaporin and drought related TF (DST)	Wu et al. (2015)
	OsSIK1	*Arabidopsis*	Drought stress	ABA mediated signaling	Ouyang et al. (2010)
	MsSIK1	*Arabidopsis*	Drought stress	Improved water use efficiency and controlled stomatal movement	Guo et al. (2016)
	TaPRK2697	Wheat	Salt stress	Enhanced Na? efflux	Ma et al. (2015)
	(Phloem intercalated with xylem-like 1) AtPXL1	*Arabidopsis*	Cold stress	Auto phosphorylation other signaling components	Chang et al. (2015)
	GsLRPK	*Arabidopsis*	Cold stress	Activation of cold specific TFs like KIN1 and COR15b	Yang et al. (2014)
	LRR-RLK-VIII	*Arabidopsis*	Heavy metal stress	Ethylene-related pathways	Fu et al. (2014)
	WAK1	*Arabidopsis*	Heavy metal stress	Efficient functioning of vacuole membrane porters and long root morphology	Sivaguru et al. (2003)
	OsWAKL124	Rice	Heavy metal stress	Efficient sequestration to vacuoles	
	GsRLCK	*Arabidopsis*	Drought stress	Through ABA signaling molecules	Sun et al. (2016)
	PnRLK-1	*Arabidopsis*	Salt stress	Efficient ROS scavenging	Zhang et al. (2015)
	PsLecRLK	Tobacco	Salt stress	Efficient osmotic and ionic management	Vaid et al. (2015)
	OsSIK2	Rice	Drought and salt stress	Through activation of DREB transcription factor (TF)	Chen et al. (2013a,b)
	AtLRK10L1.2	*Arabidopsis*	Drought stress	Through ABA signaling	Lim et al. (2015)
MAPKKK	*Nt*NPK1		Drought stress	Through higher photosynthesis rates	Shou et al. (2004)
	OsMKK6		Abiotic and biotic	Efficient functioning of ROS scavenging genes, DREB & WRKY TFs	Kumar and Sinha (2014)
	OsDSM1		Dehydration stress	Through activation of DREB TFs	Ning et al. (2010)
MAPKK	GhMKK1		Drought and salt stress	Efficient ROS regulation	Lu et al. (2015)
	ZmMKK1		Chilling and defense responses	Enhancing antioxidant enzyme activity and increasing osmolytes accumulation	Cai et al. (2014)
	PtMAPKK4	Tobacco	Salt tolerance	Efficient free radical scavenging and long root morphology	Zhang et al. (2014)
MAPK	OsMPKK10.2		Drought and bacterial blight	Through activation of DREB TFs	Ma et al. (2018)
	ZmSIMK1		Drought and salt stress Also improves seed germination	Efficient ROS regulation	Wang et al. (2014)

(*Continued*)

TABLE 34.1 (Continued)

Functional category	Gene	Transgenic crop/plant	Tolerance	Mechanism of action	References
	ZmMPK5		Salt and defense tolerance	Improved ROS scavenging activity	Zhang et al. (2014)
	OsMAPK33		Salt stress	Efficient ion transportation	
	OsBWMK1				Cheong et al. (2003)
CDPK	OsCPK4		Salt and drought stress	Preventing membrane lipid peroxidation	Campo et al. (2014)
	OsCPK12		Salt and drought stress	ROS production and scavenging	Asano et al. (2012)
	OsCDPK2		Salt and drought stress	Inhibits seeds development	Morello et al. (2000)
CIPK	CaCIPK25	Tobacco	Dehydration tolerance, improved germination	Long root morphology	Meena et al. (2015)
	TaCIPK29	Tobacco	Salt stress	ROS scavenging	Deng et al. (2013)
	MdSOS2L1	Tomato	ROS scavenging; antioxidative metabolism	Drought and oxidative stress	Hu et al. (2016)
	ZmCIPK16	*Arabidopsis*	Efficient K^+/Na^+ homeostasis and ROS scavenging	Salt stress	Wang et al. (2012)

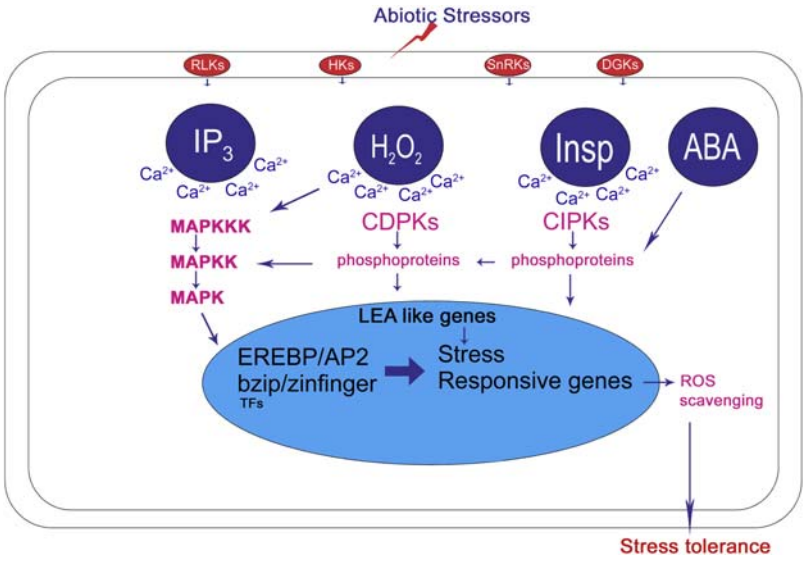

FIGURE 34.1 Schematic representation of abiotic stress signal transduction in plants. The external abiotic stress signals perceived by membrane bound receptor-like kinases (RLKs), histidine kinases (HKs), SNF1-related protein kinases (SnRKs), and diacylglycerol kinases (DGKs) are transduced to cytoplasm. The secondary messengers like inositol 1,4,5-triphosphate (IP_3), hydrogen peroxide (H_2O_2), and abscisic acid (ABA) sense these signals through calcium ions (Ca^{2+}) and make conformational changes in the mitogen activated protein kinase (MAPK) cascade, which in turn regulates the master controls, that is, transcription factors in the nucleus, to get the right response.

that is, SOS1 was mediated by MPK6, in response to NaCl during salt stress (Quintero et al., 2011). Another group of protein kinases that has a prominent role in abiotic stress adaptation are mitogen-activated protein kinases (MAPK). Many studies highlighted the MAPK role in different abiotic stress conditions (Kim et al., 2011). Another important study indicated the interplay of MAPK compounds with ROS signaling molecules (Kim et al., 2011). Wei et al. (2014a,b) have demonstrated that RLKs have a major share in abiotic stress signaling followed by CDPKs and MAP kinases. MAP kinases can interact with ABA mediated signaling components and elicit stress response (Ara and Sinha, 2014). The hierarchy of various components in signal transduction and their interacting partners is depicted in Fig. 34.1.

34.2 RECEPTORS LIKE KINASES

Cell surface RLKs are a major group of plant protein kinases. RLKs contain conserved serine/threonine catalytic domain that transduces external environmental signals through phosphorylation and dephosphorylation mechanisms. The processes controlled by RLKs include gas exchange (through stomatal control), disease reactions, and self-incompatibility. *Arabidopsis* is reported to have 610 RLK genes, whereas rice contains 1132 members of RLKs (Shiu et al., 2004). Plant RLKs are unique in nature, consisting of components resembling other eukaryotes. ZmPK1, a putative RLK, was the first cloned and characterized plant protein kinase and was reported to contain a transmembrane catalytic domain and a long highly conserved N-terminal region (Walker and Zhang, 1990). Moreover leucine-rich repeat RLKs (LRR-RLKs) were the most studied surface RLKs and found to be involved in drought resistance (Morillo and Tax, 2006). A leucine-rich RLK, GHR1 (guard cell hydrogen peroxide resistant 1) was found as the initial component in ABA, H_2O_2 mediated signaling pathway of stomatal movement in *Arabidopsis* (Hua et al., 2012). Another RLK from *Arabidopsis*, that is, RLK7 was found involved in seed germination and confers oxidative stress tolerance (Pitorre et al., 2010). Osakabe et al. (2013) have observed high water use efficiency (WUE) with drought tolerance in transgenic *Arabidopsis* plants overexpressing RPK1. The interaction of RLKs with aquaporins (plasma membrane intrinsic proteins) on the membrane could be a possible mechanism of ABA mediated drought stress tolerance in plants. FERONIA is also a group of RLKs identified from *Catharanthus roseus* (CrRLKs) that are found to activate transcription factors involved in mechanical and abiotic stress tolerance through ABA-independent signaling cascade (Chen et al., 2017). FON1, a LRR-RLK gene was initially isolated from rice, perceives drought signals and phosphorylates downstream ABA signaling molecules to confer drought tolerance (Feng et al., 2014). Several other RLKs were identified that are involved in ABA-independent mechanism and have been proposed to play an important role in the drought tolerance mechanism. OsSIKI1/OsSIKI2 are S-domain receptor kinases in plants and their overexpression leads to drought tolerance in many plant systems tested by the action of stomatal regulation and ROS detoxification (Ouyang et al., 2010; Chen et al., 2013a,b). MsSIKI1 is also a LRR-RLK gene that promotes drought tolerance through improved water use efficiency (Guo et al., 2016). Another group of RLKs that are well studied in plants are the membrane bound lectin RLKs (Lec RLKs). A LecRLK from *Pisum*, that is, PsLecRLK, was found to be expressed efficiently under salinity (Vaid et al., 2015). PnRLK-1 is a cytoplasmic type of RLK found involved in salt tolerance mechanism through efficient ROS scavenging (Zhang et al., 2014).

Calcium regulated RLKs (CRLKs) are activated by Ca^{2+} signaling and play a vital role in cold stress tolerance in plants. CRLK1 is a membrane bound kinase molecule that was found to accumulate during cold stress and phosphorylate MAP kinase cascade to transmit cold stress signals to candidate genes in the genome (Furuya et al., 2013). AtPXL1 (phloem intercalated with xylem-like 1) is a LRR-RLK gene from *Arabidopsis* and found highly expressed by cold stress (Chang et al., 2015). GsLRPK is another LRR-RLK gene that autophosphorylates by cold stress, leading to acclimation (Yang et al., 2014).

34.3 MITOGEN ACTIVATED PROTEIN KINASES

MAPKs are the most studied plant protein kinases that connect environmental signals to the TFs/genes in the nucleus and make them to express accordingly. Three classes of MAPKs are recognized in plants that are activated through phosphorylation in sequential order starting from MAPK kinase kinase (MAPKKK) to MAPK kinase (MAPKK) to MAPK. MAPKs contain a conserved dual function kinase domain that has threonine/tyrosine/serine phosphorylation residues in their active domain site (T-loop). The stress stimuli are perceived by membrane bound receptors and transmitted to MAP cascade through common secondary messenger molecules like inositol phosphate (IP_3) and ROS molecules like hydrogen peroxide (H_2O_2) as mentioned above. An interaction between H_2O_2 and MAPK molecule under stressful condition has been well explained in *Arabidopsis* (Tena et al., 2001). These secondary messenger molecules raise calcium concentrations in cytoplasm that trigger the initiation of protein phosphorylation in MAPK cascade to express the defense genes. Many attempts have been made to overexpress MAPKK derivative genes in different genetic backgrounds and the results are encouraging with superior performance compared with their nontransgenic counterparts. MAP kinase cascade is a circuit of various enzymes and each component has a prominent role and functions in a coordinate way with its interacting molecules. Transgenic overexpression of a single MAPK gene may not confer tolerance to complex traits to full extent as disproportionate concentrations of overexpressed enzymes could lead to limitation of interaction molecules or feedback inhibition. The concept of mutagen constructs with simultaneous expression of all MAPK genes involved in a signal cascade has been proposed by Moustafa et al. (2014). Multigene cloning and their transgenic

overexpression was successfully demonstrated and found to be a successful biotechnology method to mitigate complex traits like drought, salt, and heat (Roy et al., 2011). A brief description of MAPK genes and their heterologous expression is presented in Table 34.1.

34.3.1 MAPKKKs

The whole genome-wide analysis of *Arabidopsis* has revealed the occurrence of approximately 80 MAP3Ks (Hashimoto et al., 2012). Wang et al. (2017) has identified 155 MAPKKK genes in wheat, and studied their phylogenetic relations with other crops. They also demonstrated tissue specific expression profile of selected MAPKKKs, under different simulated conditions using RNAseq analysis. Considering the phylogenetic relation and similarity among the catalytic domain, MAPKKKs are broadly categorized into two different families, that is, the Raf family that contains a highly conserved kinase domain at C-terminal and MKEE family with less conserved amino groups (Ichimura et al., 2002). Several individual MAP3K genes were isolated, characterized, and could establish their function in the stress tolerance mechanism. Functional characterization of cDNA clones from *N. benthamiana* has revealed the role of MAP3Ks in pathogen induced programmed cell death (PCD). OMTK1 is another MAPKKK gene, isolated from *Medicago*, and was found to be activated by H_2O_2, during stress and cell death defense mechanism (Nakagami et al., 2004). Transgenic overexpression of NPK1, a kinase domain of MAPKKK from tobacco, was reported to increase abiotic stress tolerance in maize (Shou et al., 2004). Most of the studies focused on MAP3Ks indicated their role in hypersensitive reactions through PCD. But Ning et al. (2010) has identified a putative MAP3K gene, that is, OsDSM1, whose overexpression leads to drought tolerance in rice.

The high occurrence of MAPKKK genes in cytoplasm compared with MAP2Ks and MAPKs indicates the interaction of more than one MAPKKK with single MAPKK and MAPK in the MAP cascade. The results obtained by Hashimoto et al. (2012) have supported this thought. Hashimoto et al. (2012) identified three novel MAPKKK genes and demonstrated a parallel, linear signaling pathway involving 3 MAPKKKs in its signaling cascade leading to programmed cell death (PCD). MEK1, a novel MAP3K gene in *Arabidopsis*, is activated by both biotic and abiotic stimuli, but the downstream signal module is different in both cases. This is due to the scaffolding mechanism of MAKKK proteins (Jalmi and Sinha, 2015). This indicates the high functional diversity of MAPKKKs, being the upstream proteins in MAP cascade.

34.3.2 MAPKKs

Mitogen activated protein kinase kinase (MAPKKs) are the central component in the MAPK cascade and are the junction point where the convergence and divergence of other signaling pathways takes place. The whole genome sequence analysis of *Arabidopsis* has revealed the occurrence of 10 MAPKK genes, which have been classified into four groups based on the similarity of their protein sequence (Sinha et al., 2011). Eight novel MAPKK genes were identified in rice and one among them, that is, OsMKK6 has been characterized and demonstrated its interaction with OsMPK3 (Kumar and Sinha, 2014). Compared with MAPKs fewer MAPKKs are present in cytoplasm indicating more than one MAPK interaction to each MAPKK. MAPKKs are reported to express under cold and pathogen stress. Transcriptome analysis in paddy has revealed that *OsMKK4* and *OsMKK6* are strongly regulated by salt and cold stresses (Kumar et al., 2008). Another MAPKK enzyme, OsMKK4, was found to be involved in heavy metal stress tolerance in rice (Rao et al., 2011). A MAP2K, identified as *OsWNK1* (with no lysine kinase 1) in rice is involved in circadian rhythm and abiotic stress has been reported. Wankhede et al. (2013) have reported that OsMKK6EE is responsible for cellular protection under UV stress and also established MKK6EE's role in rice blast disease. In the same study, global gene expression analysis was performed for the overexpressed constitutive active *OsMKK6* lines. Constitutive expression of Nt MeK2 (NtMAPKK2) in tobacco has efficiently expressed defense related genes when compared with controls, on artificial pathogen inoculation. Kumar and Sinha (2014) has studied the genes that upregulate in OsMKK6 overexpressed transgenic rice plants. Xu et al. (2008) have explained the role of MAPKK9 in transducing signals in salt stress and proposed ABA mediated defense pathway in *Arabidopsis*. They further identified the interacting molecule for MAPK3 in salt stress signal cascade. In another example, a cDNA clone of MAPKK from populous (PtMAPKK4) was overexpressed in tobacco and results showed increased water stress tolerance and salt tolerance up to 150 mM NaCl (Wang et al., 2017). The same group further studied tissue specific expression pattern of PtMAPKK4 and found more expression of transcript in leaves followed by roots.

34.3.3 MAPKs

Based on the amino acid composition in the activation loop the MAPKs are broadly categorized into two major groups, that is, TEY motif kinases and TDY motif kinases. TEY motif is further divided in to subgroups A, B, and C and TDY motif consists of only

subgroup D (Opdenakker et al., 2012; Hamel et al., 2006). MAPKs are the last component in the MAP kinase cascade and are fewer in numbers, compared with MAP2Ks and MAP3Ks. Many genes encoding MAP kinase have been identified from different species and their role in gene expression related to biotic and abiotic stress has been studied. Twenty MAPKs are identified in *Arabidopsis* whereas 15 MAPKs are reported from rice. Since a lower number of MAPKs are available to their upstream counterparts, high functional diversity and crosstalk has been observed with MAPK genes. At MPK6 and At MAPK3 reported from *Arabidopsis* and their orthologous genes SIMK and SAMK from *Medicago* were found to share both biotic and abiotic stress signals and elicit the appropriate gene action (Cheong et al., 2003). BWMK1 was the first characterized MAPK isolated from indica rice and found involved in pathogen signaling and also in wound/mechanical signaling cascade (He et al. 1999). A cDNA clone of MAPK (OsMAPK33) isolated from rice was found to be involved in salt stress tolerance. Overexpression and gene silencing studies of OsMAP33 indicated its negative regulation action in confirming salt tolerance in transgenic plants through efficient functioning of ion transport genes (Lee et al., 2011). Several independent MAPKs are found to be involved in plant developmental process (Wang et al., 2007). Studies showed that drought stress resulted in the activation of OsMSRMK2 and OsMAPK5 in rice plants, indicating the role of MAP kinase genes in abiotic stress (Agrawal et al., 2002). Cheong et al. (2003) have demonstrated the interaction of MAP kinase with the promoter region of a transcription factor EREBP1, that in turn activates stress related genes in rice.

34.4 CALCIUM KINASES

Calcium plays a pivotal role in plant adaptation to different biotic and abiotic stresses by modulating the signaling network that induces stress responsive genes. Perturbations from normal growth conditions result in elevated cellular calcium levels in the cytoplasm through influx from calcium stores such as endoplasmic reticulum, vacuole, mitochondria, and cell wall and then rapidly returns to basal level by Ca^{2+} efflux to generate calcium spikes or signatures (Yang and Poovaiah, 2003), that further act as secondary messengers. Consequent to spike in cellular calcium levels, calcium sensors, and calcium binding proteins are activated and phosphorylate downstream regulatory proteins including transcription factors. The activated regulatory proteins alter the gene expression pattern resulting in altered metabolism and physiology followed by phenotypic response to stress conditions. The transient increase in cytosolic Ca^{2+} in response to stress signal is sensed by a plethora of calcium sensors or calcium binding proteins, which are estimated to be about 250 in *Arabidopsis* (Day et al., 2002). To date, the major classes of Ca^{2+} sensors identified and characterized include (1) calmodulin (CaMs) and calmodulin-like proteins, (2) calcium-dependent protein kinases (CDPKs/CPKs), and (3) calcineurin B like proteins (CBLs) (Kudla et al., 2010). Differential affinity of these families of proteins to Ca^{2+} coupled with their subcellular localization determines their activities. Calcium sensors and effectors transduce stress signals by changing their conformation upon binding to the Ca^{2+} ion, which triggers their association with target proteins and/or activation of kinase activity.

34.4.1 Calcium-Dependent Protein Kinases

Unlike CaM and CBLs, which acts as only Ca^{2+} sensors, CDPKs can sense, respond, and translate the Ca^{2+} signature generated by different environmental stresses into downstream effects. CDPKs have been identified in protozoans in addition to plants, but not in animals or the fungal genome so far (Valmonte et al., 2014). CDPKs represents a multigene family, estimated at about 34 CDPK genes in *Arabidopsis* (Cheng et al., 2002), 31 CDPK genes in rice (Ray et al., 2007), 20 CDPK genes in wheat (Li et al., 2008), and representing 29 CDPK genes in tomato (Wang et al., 2016a,b). CDPKs have three conserved domains, one at the N-terminus variable domain that is fused to the serine/threonine protein kinase domain and another at the C-terminus, and one more CDPK activation domain (CAD) exists near the C-terminus. The CAD domain possesses an autoinhibitory region and four EF hands for binding Ca^{2+} ion (Singh et al., 2018).

Stress specific Ca^{2+} signatures generated are decoded by CDPKs through sensing and phosphorylation of downstream regulatory proteins. Several studies of genome-wide expression revealed that CDPK genes show differential expression in response to different stress conditions and also during developmental stages (Singh et al., 2018). CDPKs are often considered as positive regulators of abiotic stress tolerance; consequently, overexpression of CDPKs in plants imparts enhanced stress tolerance. During water deficit conditions, closure of stomata is a crucial adaptation to prevent water loss through transpiration. It has been shown that CDPKs regulate stomatal movement through phosphorylation of ion channels, as cpk3cpk6 double mutant shows impaired ABA activation through slow anion channel (SLAC) protein (Mori et al., 2006) interactions. In addition to CPK3 and CPK6, CPK21 and CPK23 were also reported to activate SLAC proteins. In *Arabidopsis*,

CPK8 was found to regulate Catalase3 (CAT3) through phosphorylation by interaction and it is observed that absence of CPK8 activity leads to susceptibility to drought stress (Zou et al., 2015). In rice, CPK14 and CPK21 positively regulate drought, salinity, and ABA signaling through phosphorylation of Di19−4 transcription factor and 14−3-3 protein (Wang et al., 2016a,b; Chen et al., 2017). A few CDPKs were also enhanced by the accumulation of compatible solutes in response to drought stress through enhanced gene expression. Rice CPK9 was induced in response to drought, salinity, and ABA. Further, overexpression of CKP9 improves drought stress tolerance while mutant show increased sensitivity to stress (Wei et al., 2014a,b). Overexpression of AtCPK32 resulted in upregulation of ABA responsive genes such as RAB18, RD29A, and RD29B. This indicates that the AtCPK32 acts as a positive regulator of ABA-mediated stress signaling pathway. Further, overexpression of AtCPK4 and AtCPK11 also confers abiotic stress tolerance mediated by ABA-signaling transduction pathway (Zhu et al., 2007). The mutant of AtCPK23 results improved drought and salt tolerance but was not involved in ABA signaling pathway, which means AtCPK23 is the negative regulator for the ABA-mediated signaling pathway (Ma and Wu, 2007). Some of the CDPKs were also involved in ABA-independent signaling pathways under abiotic stress conditions. The AtCPK11 interacted with the zinc finger protein family of AtDi19-1 gene, and as a result induced drought tolerance (Rodriguez Milla et al., 2006). AtCPK1 also showed calcium sensing nature during stress conditions (Hwang et al., 2000). According to Wan et al. (2007), 17 OsCPK transcripts were identified in rice with response to cold, drought, salt, and heat stresses, which includes OsCPK1, 4, 6, 7, 8, 9, 10, 13, 14, 15, 16, 17, 19, 23, 24, 25, and 29. CDPKs in rice showed elevated expression when the crop is exposed to cold stress. Consequently, overexpressing of CDK13 along with a calreticulin interacting protein conferred cold tolerance in rice (Komatsu et al., 2007). *Arabidopsis* plants confer cold tolerance when *Populus* CPK10 and *Vitis amurensis* CPK20 were overexpressed (Chen et al., 2013a,b; Dubrovina et al., 2015). Tomato CPK2 showed higher expression when exposed to 42°C (Chang et al., 2009). Overexpression and silencing of CPK12 in rice conferred the role in salt stress tolerance through regulation of ROS scavenging enzymes (Asano et al., 2012).

34.4.2 Calcineurin B-Like Proteins and CBL-Interacting Protein Kinases:

CBLs are plant specific Ca^{2+} sensor proteins without kinase activity. CBLs were first identified in *Arabidopsis* and they are named after functional counterparts in yeast, calcineurin B. CBLs interact with CBL-interacting protein kinases (CIPKs), to relay perceived $Ca^{2}+$ signal to induce downstream gene expression and response. CBLs contain four EF hands, helix−loop−helix structure for Ca^{2+} binding, and are responsible for interaction with CIPKs. The number of amino acids present between EF hands is highly conserved in CBLs; there are 22 amino acids between EF1 and EF2, 25 amino acids between EF2 and EF3, and 32 amino acids between EF3 and EF4 (Xi et al., 2017). However, their first EF hand is unique and possesses distinguishing features in comparison with other Ca^{2+} sensor proteins, as Ca^{2+} binding loop as insertion of two amino acids in addition to the regular 12 amino acids (Nagae et al., 2003). CIPKs display a conserved domain architecture consisting of N-terminal, serine/threonine protein kinase domain, junction domain and at the C-terminal, CIPK specific NAF/FISL motif is required for interaction with CBLs present. The NAF/FISL domain, named for its highly conserved amino acids, aspargine (N), alanine (A), phenylalanine (F), isoleucine (I), serine (S), and leucine (L), is required and sufficient to mediate binding with CBL proteins (Mao et al., 2016). In a few CIPKs, domain at the C-terminus can specifically interact with phosphatase 2C (Ohta et al., 2003). There are about 10 CBLs and 26 CIPKs genes in *Arabidopsis*, while the rice genome encodes 11CBLs and 34 CIPKs. The CBLs specifically interact with a subset of CIPKs in Ca^{2+} dependent manner for efficient transmission of signaling (Kudla et al., 2018). There are about 8 CBLs and 20 CIPKs genes identified in the grapevine genome (Xi et al., 2017).

In studies of gene expression and functional characterization, CBL-CIPK has been reported to play an important role in plant abiotic stress response. The physiological role of the CBL-CIPK complex was first identified while studying salt tolerance mechanism in *Arabidopsis*. Interaction of CBLs with CIPKs not only activate the kinase domain but is also important for localization of the resulting functional complex to different subcellular compartments that is driven by lipid modification of CBLs. This process determines the complex regulation of signaling, as the same CIPK can function in a different cellular compartment membrane by interacting with different CBLs. For example, CBL4 (SOS3) interacts with CIPK24 (SOS2) and recruits to the plasma membrane to activate the Na^{+}/H^{+} antiporter (SOS1) at the plasma membrane, and the same CIPK24 is targeted to the vacuole when it interacts with the vacuole targeted CBL10, which leads to salinity tolerance (Qiu et al., 2002; Kudla et al., 2018). Several studies highlighted that overexpression of CIPKs conferred tolerance to different abiotic stresses. A few examples include an overexpression of CIPK6 in cotton (He et al., 2013) and overexpression of

CIPK24 to salt tolerance (Huertas et al., 2012). Over expression of CIPK3 in *Arabidopsis* increased tolerance to drought, salt, low temperature, and ABA (Kim et al., 2003). It has been shown that CIPK31 is involved in stress tolerance when rice plants are exposed to stress (Piao et al., 2010). Transgenic *Brassica* producing higher levels of CBL1-CIPK6 has enhanced tolerance to high NaCl, mannitol, and ABA compared with wild type (Chen et al., 2012). The loss-of-function cipk21 mutant was hypersensitive to salt and osmotic stress conditions and CBL2 and CBL3 were found to interact with CIPK21 and target to tonoplast under salt stress (Pandey et al., 2015). It was found that the CBL-CIPK complex is more responsive to environmental stress than the developmental process (Xi et al., 2017). The CBL1/9-CIPK23 complex plays an important role in ABA mediated closure of stomata in *Arabidopsis*, where this complex phosphorylates and activates slow anion channel associated1 (SLAC1) and slow anion channel1 homolog3 (SLAH3) proteins and that resulting closure of stomata. The CBL9-CIPK3 complex was involved in salt induced ABA signaling as indicated from changes in ABA regulated gene expression in mutants (Mao et al., 2016).

34.4.3 CDPK-Regulated Protein Kinases

In addition to CDPKs and CIPKs some other classes of calcium regulated protein kinases are identified such as CDPK-regulated protein kinase (CRK) and Ca2 + /CAM kinase (CCaMK), which are yet to be explored in detail but play a major role in the abiotic stress signaling mechanism. The CRKs has N-terminal myristoylation motif, which helps in plasma membrane associations. And also they have degenerated EF-hands, which are incapable of binding calcium ions (Harmon, 2003). A good potential substrate such as AtGLN1 (cytosolic glutamine synthetase involved in nitrogen assimilation) has been identified for AtCRK3 by using yeast two-hybrid approach that regulates mainly nitrogen mobilization during leaf senescence (Li et al., 2006).

Most of the CDPK-related protein kinase (CRKS) expression studies were reported in *Arabidopsis* and maize plants. All of the CRKs have kinase activity towards Ca2 + and CaM independently. The example is that AtCRK3 and ZmCRK exhibit autophosphorylation and substrate phosphorylation to Ca2 + and CaM independently (Furumoto et al., 1996; Du et al., 2004). With AtGLN1 (cytosolic glutamine synthetase take part in N assimilation) as a substrate for AtCRK3 gene using yeast two-hybrid approach, those interactions mainly induced nitrogen mobilization during leaf senescence (Li et al., 2006). Moreover, the AtCRK1 has affinity to bind with CaM-isoforms such as AtCaM2, 4, 7, and 8, which stimulates kinase activity of AtCRK1 (Wang et al., 2004).

34.5 SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASES

One of the well-characterized protein kinases involved in stress responses is the group of sucrose nonfermenting 1 (SNF1)-related protein kinases SnRKs (Halford and Hey, 2009). SnRKs are grouped into three subfamilies, that is, SnRK1, SnRK2, and SnRK3 (Halford and Hey, 2009). Recent studies have indicated the pivotal roles of plant-specific subgroups of SnRK2 and SnRK3 in the link between abiotic stress and abscisic acid (ABA) signaling to regulate metabolic pathways (Hrabak et al., 2003; Halford and Hey, 2009). Further evidence has shown that SnRK2 proteins function as positive regulators of ABA signaling for stress responses, as well as development, in plants (Umezawa et al., 2013). The SnRK2 family consists of 10 members, which includes SRK2A, SRK2J, and SnRK2.10 in *Arabidopsis* and in rice SAPK1−10 was reported (Hrabak et al., 2003; Kobayashi et al., 2004). They are further classified into three subclasses based on their domain structures (Kobayashi et al., 2004). Most SnRK2 proteins are activated by abiotic stresses, while the members of subclasses 2 and 3 are also activated by ABA (Boudsocq et al., 2004, 2007; Kobayashi et al., 2004). In the current model, ABA-induced activation is largely explained by the interaction between SnRK2s and protein phosphatase type 2C (PP2C) proteins in the ABA signaling pathway (Leung et al., 1994, 1997; Meyer et al., 1994; Saez et al., 2004; Nishimura et al., 2007; Umezawa et al., 2009; Cutler et al., 2010). In the absence of ABA, group A PP2Cs physically bind to SnRK2s to dephosphorylate SnRK2s, resulting in the inhibition of ABA signal transduction. But in the presence of ABA, SnRK2 will be released from such inhibitory regulation by PP2C, because the soluble ABA receptor PYR/PYL/RCAR inhibits PP2C activity (Umezawa et al., 2009). In *Arabidopsis*, detailed analyses of subclass 2 (SRK2F/SnRK2.7, SRK2C/SnRK2.8) and subclass 3 (SRK2D/SnRK2.2, SRK2I/SnRK2.3, SRK2E/SnRK2.6) have revealed their redundant functions in ABA signaling for abiotic stress responses and developmental controls (Yoshida et al., 2002; Fujii et al., 2007, 2009; Fujii and Zhu, 2009; Nakashima et al., 2009; Mizoguchi et al., 2010). Overexpression of SnRK2 (AtSRK2C/SnRK2.8, TaSnRK2.3, TaSnRK2.4, TaSnRK2.7, and TaSnRK2.8) genes resulted in enhanced abiotic stress tolerance in *Arabidopsis* (Umezawa et al., 2004; Mao et al., 2010; Zhang et al., 2010). Overexpression of *SnRK2* genes in *Arabidopsis* induced the upregulation of several

important stress responsive genes, including RD29A and DREB1A/CBF3, and ABA biosynthetic genes, such as ABA1, under normal conditions (Umezawa et al., 2004). Early and quick expression of such key genes was noticed under abiotic stress conditions in the same study, indicating their role in stress tolerance. The rice *SAPK4* overexpression exhibited enhanced salt tolerance and major aspects of its tolerance were explained by changes in the expression of genes related to ion homeostasis and oxidative stress responses (Diédhiou et al., 2008). In the above examples of SnRK2 overexpression studies, the results clearly indicated that SnRK2 can function in abiotic stress responses in plant cells, through the modulation of stress response related gene expression. Comparative genomics studies have demonstrated that the core components of ABA signaling, that is, PYR/PYL/RCAR, SnRK2, and PP2C, are well-conserved in land plant species suggesting the evolutionary conservation of key molecular system (Umezawa et al., 2010). Indeed, the conserved molecular characteristics of SnRK2, such as transcriptional induction by abiotic stresses and activation by stress and/or ABA, have been reported for SnRK2 genes not only in *Arabidopsis* and rice, but also in other crop plants like maize (Huai et al., 2008) and wheat (Holappa and Walker-Simmons, 1995; Gómez-Cadenas et al., 1999; Mao et al., 2010; Zhang et al., 2010).

34.6 DIACYLGLYCEROL KINASES

Diacylglycerol kinases (DGKs) are important signaling kinases that exist in all higher plants. Unlike other plant kinases, DGKs participate indirectly in abiotic stress tolerance. DGKs play a crucial role in synthesis of phosphotidic acid (PA), an important secondary messenger molecule that functions during abiotic stress in plants. DAG (diaceylglycerol) is a well-characterized membrane that binds secondary messenger molecules in higher plants and functions during abiotic stress conditions. DAG (diaceylglycerol) is a precursor of PA phosphorylated by DGK giving rise to PA. The primary stress signals such as drought, cold, and salinity or stress respondent hormones like ABA, ethylene initiates the synthesis of PA through DGKs. Apart from stress tolerant molecules, PA acts as a precursor in the synthesis of triacylglycerols and phosphoglycerolipids, important components of cell wall synthesis. After its synthesis PA may be further metabolized to give PPI (Escobar-Sepúlveda et al., 2017). Phospholipase C (PLC) acts on PPI yielding two important secondary messenger molecules, that is, DAG and IP3. DAG and IP3 activate many kinases in cytoplasm in response to Ca^{2+} concentrations. Plant DGKs exist in many isoforms with conserved catalytic domain, containing ATP binding domain. Stress specific expression profile of DGK genes indicated their role in drought and salt tolerance (Zimmermann et al., 2014).

34.7 HISTIDINE KINASES

Plant HKs are membrane bound proteins with a highly conserved two-component system of operation. Like yeast HKs, plant HKs contain a CHASE domain, transmembrane domain, and receiver domain. The N-terminal motifs are variable depending on the functional diversity of HKs, whereas the C-terminal domain is highly conserved with kinase activity. The transfer of phosphoryl group to aspartate domain takes place at the C-terminal residue. Since their discovery many HKs have been identified and characterized in plant species like rice, maize, *Arabidopsis*, soybean, poplar, etc. (Pareek et al., 2006). Plant HKs mostly perceive ethylene and cytokinin stimuli and are involved in their signaling pathways. Based on their functional diversity plant HKs could be categorized into ethylene receptors, cytokinin receptors, osmosensors, and other less significant HKs. *Arabidopsis* AHK5/CK12 and CK11 are osmosensing type HKs, whereas AHK4 (CRE1/WOL), AHK3, and AHK2 belong to ethylene receptor HKs. Plant HKs exhibit cold, drought, and salt stress tolerance and evidence also confirms their role in some biotic stress tolerance (Nongpiur et al., 2012). Gene expression profile of AtHKs indicates abundance of AtHK1 and AtHK5 transcripts in root tissue (Iwama et al., 2007). This data supports the results of root phenotype in transgenic *Arabidopsis* overexpressing AtHK1.

Constitutive expression of AtHKI in *Arabidopsis* has conferred in water stress tolerance and improved resistance to changes in osmolality (Tran et al., 2007). Heterologous expression of a corn HK (ZmHK9) showed improved water stress tolerance in *Arabidopsis*, through control of stomatal openings indicating the role of HKs in the drought stress mechanism (Wang et al.,2012). AHK5/CK12 is another HK involved in gourd cell control through H_20_2 regulation (Desikan et al., 2008).

34.8 GENOMICS EFFORTS IN PLANT PROTEIN KINASE TOWARDS CROP IMPROVEMENT

The innovations in sequencing chemistries have heavily reduced the cost per data point. The sequence information together with bioinformatics tools has created many genomic tools that help in improving crop productivity. Genomics based breeding tools have

become an integral part of breeding programs in many institutes and industry. Genomic selection (GS) and genome-wide association studies (GWAS) for dissecting complex traits are a few innovative genomic tools that help in identification of rare QTLs and predicting superior breeding lines that have great impact on crop improvement. The precision in selection has increased with identification of SNP markers for complex traits.

The increased number of genome sequencings and improvements in trait association methods has led to the discovery of gene families using genome-wide markers. This helps in accurate classification of protein kinases and to establish phylogenetic relations among different crop species. It also helps in designing accurate markers for various kinase genes that participate in abiotic stress tolerance and helps in improved selections. Recently Guo et al. (2013) identified 18 CDPK and 7CRK genes in watermelon using whole genome sequencing and found structural polymorphism in one gene that is responsible for abiotic stress tolerance in this species. Rudrabhatla et al. (2006) has applied genome-wide study to identify tyrosine kinase genes in *Arabidopsis* using animal tyrosine kinase motifs. Recently genome-wide expression analysis of the MAPK gene family was performed in *Cicer arietinum* using informatics tools and great functional diversity among MAPK genes in different tissues under abiotic stress conditions was found (Singh et al., 2018). Similarly a comprehensive expression profile of MAPK genes in *Brassica* was studied using sequence similarities in the *Brassica* database (BRAD) using qRT-PCR (Lu et al., 2015).

Genomic selection is an important tool in recent molecular breeding, where the performance of genotypes can be predicated without phenotyping. During a recent study on identifying maize lines for drought tolerance, genomic selection method was employed and among the top 10 significant SNPs found contributing to drought tolerance, SnRK2 (SnSNF1-related protein kinase 2) was identified as one of the genes, indicating the role of plant protein kinases in abiotic stress tolerance (Umezawa et al.,2013). During genome-wide association study for salt tolerance using a core set of rice genotypes, many functional genes including MAP kinase genes were recognized as candidates responsible for salt tolerance (Patishtan et al., 2017). A genome-wide association study for dissection of the genomic region for leaf angle trait in *Brassica* has revealed a CDPK related gene (CRK5) is closely associated to the trait (Sun et al.,2016). Genome-wide transcriptome profiling in maize crop has revealed the profound role of protein kinases in drought tolerance. RNA-seq and analysis is an excellent method for global gene expression analysis in plants. Sequence analysis of drought and cold stress cDNAs in cassava showed 12 CDPKs and 4 MAPKs (Lu et al., 2015). Furthermore, the study revealed crosstalk between protein kinase genes like SnRK2 indicating the common mechanism in abiotic stress tolerance.

34.9 CONCLUSIONS

Protein kinases are the largest group of molecules involved in various biotic and abiotic stress regulation in plants and hence have attracted researchers all over the globe. Most of the work on plant protein kinases was focused on identification and structural characterization of various groups of kinase genes belonging to different species. But functional validation of plant kinase genes and their specific role in metabolic pathways needs to be elucidated. Most of the research works are focused on overexpression of single kinase genes and the transgenic plants showed a certain degree of tolerance to abiotic stress under study. Plant kinase genes act in a circuit fashion and functionally get activated through phosphorylation by their neighboring component in the cascade. Hence overexpression of a single gene in a signal cascade may not address the problem as a whole. The technological revolution in molecular biology has made multigene cloning and expression of whole pathway genes in single transfer. The recent advancements in genomic technologies has allowed scanning the genomic regions at low cost and faster rate. Identification of functional polymorphisms between the extreme genotypes will facilitate parental line selections and crop improvement programs.

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CHAPTER

35

Nanoparticles and Abiotic Stress Tolerance in Plants: Synthesis, Action, and Signaling Mechanisms

Syed Uzma Jalil[1] *and Mohammad Israil Ansari*[2]

[1]Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow, Uttar Pradesh, India
[2]Department of Botany, University of Lucknow, Lucknow, Uttar Pradesh, India

OUTLINE

35.1 INTRODUCTION

Abiotic stresses are the main constraints that adversely affect the crop productivity and plant growth. Realizing the increased incidences of diverse abiotic stresses due to both natural as well as anthropogenic activities, the scientific community has a major concern to mitigate their effect to increase the yield potential of crops. According to FAO reports, there is a major challenge among the scientific community to increase world food crop production by 70% (FAO, 2009). Therefore, in such changing environmental scenario there is a constant need to identify a new area of research to overcome the technological challenges in addressing the yield barrier, resource use efficiency, and development of environmentally accepted technology. In recent years, nanobiotechnology is gaining momentum to occupy the promising position of mitigating the constraints associated with abiotic and biotic stresses to obtain a sustainable and secure future of agriculture worldwide. Plants being sessile organisms are constantly exposed to environmental variations and multiple stress factors alone or in combination throughout their life. However, plants develop various

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mechanisms to respond to adverse conditions but their responses may vary considerably even in the same plant species. Therefore, identification of tolerant plant material or augmentation of stress tolerance in plants is always the prime concern in terms of sustainable agriculture and crop production. Nanotechnology, a novel approach that allows innovative study in various extents, and nanotechnological findings could open up new way in the field of biotechnology and agriculture (Torney et al., 2007; Perez-de-Luque and Diego, 2009).

Nanoparticles are small molecules of 1−100 nm dimensions (Roco, 2003) including very small size, nanoparticles may also acquire some other physiochemical properties, that is, improved reactivity, large surface area, malleable pore size, as well as diverse morphology (Nel et al., 2006). Nanoparticles retain a high surface dynamism and large surface to volume ratio that improves their reactivity as well as biochemical activity; these characteristics of nanoparticles may show diverse activities and impact (Dubchak et al., 2010). In the present circumstance, nanoparticles have potential to boost plant growth and development, used as herbicides, nanopesticide, and nanofertilizers, etc. that can proficiently release their content in required amounts to target cellular organelles in plants. There is an extensive scope of nanotechnology in the agriculture sector and the potential uses of nanoparticles are still unknown, particularly their role and mechanism in plant growth and development (Manzer et al., 2015). Application of fertilizers in agriculture is a common practice to increase the productivity and maintain growing food demand. Since fertilizers play a vital role in crop growth, development, and production, they must be applied in bulk quantity and most remains unutilized by plants, because of several inherent aspects (Singh et al., 2015). Therefore, there is a constant need to develop novel approaches with the support of nanotechnology and nanoparticles that not only increase the crop production and yield, but also minimize the nutrient losses of fertilizers and augment their effective availability to plants. Development of nanofertilizers or nanoencapsulated nutrients could be an effective tool in this direction towards sustainable production substantially by effective release of nutrients and availability. Application of nanofertilizers may provide a suitable alternative to increase resource use efficiency and help to reduce soil toxicity created due to accumulation of chemical fertilizers and pesticides in the soil. Most of the chemical fertilizer applied in the field remains unutilized by plants and gets accumulated in the soil leading to increased soil toxicity; therefore application of nanofertilizers could help to reduce such problems (DeRosa et al., 2010; Nair et al., 2010).

Plants cannot move from their growing place so they cannot escape from environmental stress conditions, that is, salinity, drought, chilling, heat, heavy metals, waterlogging, UV radiation, etc. These stresses produce reactive oxygen species (ROS) in plants and cause oxidative burst. Extreme generation of ROS degrades macromolecules and membrane lipids (Foyer and Noctor, 2000), prompts toxicity in cells (Shen et al., 2010; Yadav et al., 2014), as well as conquers growth of plant (Begum and Fugetsu, 2012; Khan et al., 2012a,b, 2013, 2014a,b, 2015, 2016). Plants have an antioxidant system to mitigate oxidative stress by scavenging ROS (Khan and Khan, 2014, 2017). While, plants counteract osmotic stress by increasing the production of trehalose and polyols, that is, inositol, sorbitol, glycerol, etc., as well as amino acids (betaine, glycine, proline, and taurine) that sustain the required osmotic level in the plant's cells. During hypoxia, roots are not able to provide required oxygen to the plants, which results in depletion of energy as well as adapts the plants with low vigor, though, to retain vigor level plants modify their metabolic rate and shift over from starch metabolism to fermentation (Banti et al., 2013). On responding to heavy metal stress, plants accumulate polyphosphates, metal-chelates, and organic acids that results in limiting as well as requisitioning of toxic metals in the plasma membrane. Furthermore, nanoparticles play an important role in the growth and development of plants, and are also involved in the protection of plants against different abiotic stress conditions (Khan et al., 2017). The nanoparticles imitate the activities of antioxidative enzymes and scavenge these ROS (Rico et al., 2013a,b; Wei and Wang, 2013). Small size and large surface area of nanoparticles are available to toxic metals for binding, thus condensed the accessibility and toxicity of heavy metals (Worms et al., 2012). Photosynthesis is an important process of plants; however, during abiotic stress conditions, nanoparticles improve photosynthesis rate by conquering oxidative and osmotic stress and defending the photosynthetic system (Fig. 35.1) (Haghighi and Pessarakli, 2013; Qi et al., 2013; Siddiqui et al., 2014). Therefore, the response of plants to nanoparticles varies from plant species and type or concentration of nanoparticles (Lin and Xing, 2007). Apart from their beneficial effects several nanoparticles show toxicity symptoms (Slomberg and Schoenfisch, 2012; Begum and Fugetsu, 2012). Exposure of some nanoparticles prompts oxidative stress and causes decline in germination rate, root and shoot length, loss of photosystem and crop yields (Barhoumi et al., 2015; Da Costa and Sharma, 2016; Wang et al., 2016), and nutritive value of crop plants (Peralta-Videa et al., 2014). The nanoparticles also alter expression of genes involved cell biosynthesis, cell organization, electron transport, and energy pathways in biotic and abiotic stress responses (Landa et al., 2012; Kaveh et al., 2013;

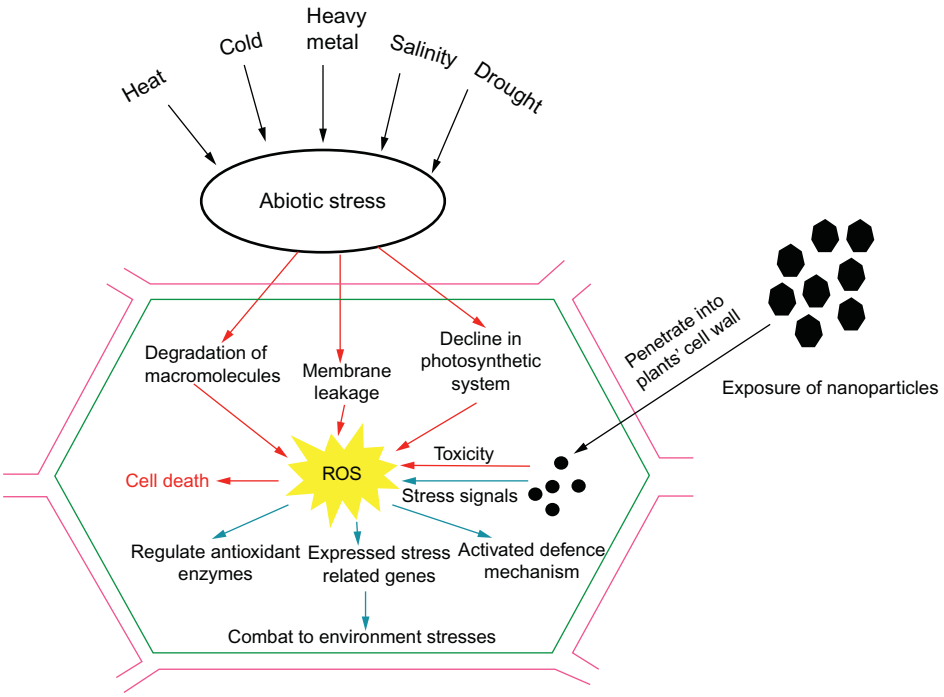

FIGURE 35.1 **Mechanism of nanoparticles under abiotic stress condition in plant cell.** The red arrows show the effect of abiotic stress and toxic effect of nanoparticles that leads to cell death. Blue arrows show the positive role of nanoparticles to act as stress signals that activate the defense mechanism in plants to mitigate abiotic stresses. *ROS*, reactive oxygen species.

Aken, 2015). Therefore, the present chapter is focused on the potential applications of nanoparticles in the agriculture sector, exploiting peculiar properties of different nanoparticles and their impact on crop plants especially toward growth and mitigation of abiotic stress tolerance in plants to achieve sustainable agriculture.

35.2 PLANT AND ABIOTIC STRESS

Plants are constantly affected by various adverse environmental conditions; among them abiotic stress is the prime cause of limiting crop production worldwide, reducing more than 50% average yields for most major crop plants (Bray et al., 2000; Wang et al., 2003). The effect of abiotic stresses on the plant is influenced by its extent or strength. The plant needs optimum abiotic environmental conditions for proper growth. Any alteration from such optimal environmental conditions, which is insufficient in the physical or chemical condition, is considered as abiotic stress, and also critically impacts the growth, development, as well as productivity of plant (Bray et al., 2000). Abiotic stresses includes temperature (heat, cold, and freezing), irradiation, drought, waterlogging, heavy metals, nutrients deficiency, and salinity.

Several abiotic conditions, that is, salinity, alkalinity of soils, toxicity by heavy metals, extremely high or low temperature, etc. (Bromham et al., 2013; Khan and Khan 2013; Pasala et al., 2016) have effects on the growth and development of plants that negatively impact on the production of agroeconomic crop plants (Emamverdian et al., 2015). Diverse acidic conditions in soil adversely influence nutrients in soil, which causes nutrient deficiency in plants causing them to lose their normal physiological ability of growth and development (Rorison, 1986). Primary and prolonged salinity treatment causes toxicity in the plant cell as well as disrupts the osmotic equilibrium. The effect of ionic followed with osmotic stresses leads to disturbing the growth and development of plants (Munns and Tester, 2008). Abiotic stress conditions are the main reason for the generation of ROS. Production and elimination of ROS is in balance under normal conditions; however during abiotic stress condition equilibrium of ROS is disturbed by increasing ROS production, which causes phytotoxicity by adversely impacting the structure and function of the proteins. The production of ROS occurs in the cell organelle, that is, mitochondria, peroxisomes, and chloroplasts in plants. Oxygen radicals and hydrogen peroxide are produced in mitochondria due to the overreduction of the electron transport chain. Chloroplasts are the main source of the production of O_2 and H_2O_2 (Davletova et al., 2005). These superoxides are transformed to hydrogen peroxide either impulsively or by superoxide dismutase enzyme. In the peroxisomal matrix, the oxidation of xanthine and hypoxanthine to uric acid in the presence of the enzyme xanthine oxidase generates O_2^- radicals (Halliwell and Gutteridge 2000). They damage the biomolecules, that is, proteins, lipids, carbohydrates, and DNA, which results in the death of the cells (Foyer and Noctor 2005).

Plants acclimatize to sudden variation and adverse abiotic conditions due to their inherent metabolism (Simontacchi et al., 2015). Fluctuation in the environmental conditions might disturb the metabolic equilibrium of the plant (Foyer and Noctor, 2005), and prompts the plant to harbor metabolic and genetic processes in the cell (Apel and Hirt, 2004; Gill and Tuteja, 2010). Plants retain defense mechanisms assimilated to combat the abiotic stress conditions (Yolcu et al., 2016). These mechanisms lead reprogramming of metabolic processes in the plant system (Heil and Bostock, 2002; Swarbrick et al., 2006; Shao et al., 2008; Bolton, 2009; Massad et al., 2012) to assist biophysicochemical progressions of the abiotic stress conditions (Mickelbart et al., 2015).

35.3 MODE OF ACTION OF NANOMATERIALS UNDER ABIOTIC STRESSES

Plants have ability to adapt or cope with adverse environment conditions, for example, chilling, salinity, drought, heat stress, etc. Cellular and molecular responses of plants to these abiotic stresses have been studied widely (Yoshioka and Shinozaki, 2009; Hirayama and Shinozaki, 2010; Duque et al., 2013). The preliminary response of plants against abiotic stresses comprises a transitory escalation of cytoplasmic Ca^{2+}, raised intracellular secondary messengers (inositol, polyphosphate), ROS, abscisic acid, and increase in mitogen-activated protein kinase (MAPK) pathways (Hirt, 1997; Nakagami et al., 2005; Bailey-Serres and Mittler, 2006; Alcázar-Román and Wente, 2008; Gill and Tuteja, 2010; Baxter et al., 2013). The advanced level of stress response includes regulation of proteins involved in protection from cellular damage, and regulation of the expression of stress-specific genes (Xiong et al., 2002; Mahalingam and Fedoroff, 2003). Secondary metabolites play a key role in plants to combat abiotic stress conditions by stabilizing cell structure, protection of photosystem from ROS, signal transduction, and biosynthesis of polyamines (Dixon and Paiva, 1995; Dixon et al., 2002; Edreva et al., 2008; Oh et al., 2009). During abiotic and biotic stress conditions, the plant cell wall acts as a physical barrier to stress perception and plays a vigorous role in plant adaptation (Degenhardt and Gimmler, 2000). Extracellular peroxidases are involved in the modification of the cell wall and accumulate ROS and oxidative stress when encountering stresses (Passardi et al., 2004; Daudi et al., 2012; Roet et al., 2006). Oxidative stress activates generation of ROS, accumulation of phenylpropanoid, biosynthesis of enzymes, and regulation of gene expression in plant defense response (Fig. 35.1) (Minibayeva et al., 2009; Daudi et al., 2012).

There are several studies that indicated that nanoparticles' mediated effect on plants growth and development is concentration dependent. Nanoparticles upregulate the antioxidant enzyme activities (Laware and Raskar, 2014). Laware and Raskar (2014) conducted a study to assess the impact on onion seedlings when exposed to TiO_2 nanoparticles; the results suggested that TiO_2 nanoparticles elevate the activity of superoxide dismutase enzyme and it further enhanced with increasing nanoparticle concentration. However, seed germination and seedling growth in onion were improved at low concentration of TiO_2 nanoparticles whereas the effect was reversed (suppressed) at higher concentrations. Beside superoxide dismutase, which showed concentration dependent increase, there was significant induction of hydrolytic enzyme (amylase) and catalase as well as peroxidase enzyme activities, although enzyme activity was higher at lower concentration (10−30 μg/mL) of TiO_2 and decreased at higher concentration (40 and 50 μg/mL) (Laware and Raskar, 2014). Some studies suggested that TiO_2 and SiO_2 nanoparticles have shown potential to enhance seed germination as well as growth of *Glycine max* seeds (Lu et al., 2002).

35.3.1 Drought Stress

Drought is among the most frequently occurring abiotic stresses, and has significantly contributed to limit crop production in arid regions (Martínez-Vilalta and Piñol, 2002). Studies indicated that application of different fractions of Si nanoparticles improves the plant tolerance toward drought stress, that is, hawthorns (*Crataegus* sp.) showed increased drought tolerance. The physiological and biochemical responses vary in seedlings of hawthorn to different doses of Si nanoparticles at different level of dehydration stress from temperate to severe stress. The results suggested the positive effect on photosynthesis parameters, malondialdehyde (MDA), water content, ion leakage of membrane, leaf pigments, proline, as well as carbohydrate contents by pretreatment of Si nanoparticles. Perhaps the involvement of Si nanoparticles in maintaining critical physiological and biochemical attributes induces drought tolerance in seedlings of hawthorn under drought stress (Ashkavand et al., 2015). Application of silicon on two sorghums (*Sorghum bicolor* (L.) Moench) cultivars having different drought susceptibility showed improved drought tolerance irrespective of their drought susceptibility by lowering shoot to root ratio, which perhaps suggested the improved root growth and the maintenance of the photosynthetic rate. These findings could be attributed to improve the drought tolerance of sorghum via the augmenting water uptake efficiency of plants

(Hattori et al., 2005). Silicon can be potentially used to mitigate effects of drought stress impact to some extent. A study conducted by Pei et al. (2010), suggested that exposure of low concentration (1.0 mM) of sodium silicate could moderately alleviate the harmful effects of drought stress in wheat. Although the exact mechanism is unclear silicon partially improves shoot growth, increases the leaf chlorophyll contents, and maintains leaf water potential in stressed plants. Moreover, it also reduces membrane lipid peroxidation in wheat (Pei et al., 2010). Application of Zn could increase the radicle growth in germinated seeds and high Zn content in grains can increase the seed viability and establishment especially in Zn-deficient areas (Cakmak et al., 1996; Degenhardt and Gimmler, 2000). Sedghi et al (2013) demonstrated that ZnO nanoparticles have the potential to increase seed germination percentage and germination rate in soybean as compared with those subjected to water stress. It was further suggested that ZnO nanoparticle application under drought stress reduces fresh and dry weight of seeds, which shows that ZnO nanoparticles were effective for using germination and growth of seedlings and improved resistance to drought stress (Sedghi et al., 2013). Iron is an important micronutrient and plays a crucial role in plant growth and development; its deficiency leads to significant changes in plant metabolism and causes chlorosis.

Therefore, iron in plants under drought stress may play a pivotal role in drought tolerance. Several studies indicated that the application of micronutrients can be used to ameliorate the effects of drought and salinity stresses. A study revealed the significant effect of iron nanoparticles under drought stress in plants on traits like number of bolls per branch, number of seeds per boll, the thousand-seed weight, and yield at probability level. Foliar application of iron nanoparticles exhibited drought stress mitigating effects on biomass and oil content of safflower cultivars. Application of Fe nanoparticles also enhances biomass at two stages of flowering and granulation, although it was better at the flowering stage than seed formation in contrast to drought stress conditions without Fe nanoparticle application (Davar et al., 2014). The mitigation of the adverse effect of drought stress using titanium nanoparticle foliar application on wheat has also shown promising results on certain agronomic traits such as starch and gluten. The results suggested that application of 0.02% TiO_2 nanoparticles exhibited enhancement in various agronomic traits, that is, plant height, ear weight, ear number, seed number, thousand-seed weight, final yield, biomass, harvest index including gluten, and starch content under drought stress (Jaberzadeh et al., 2013). Advances in silver nanoparticles (AgNPs) application were also appreciated to reduce negative effects of drought stress on lentil (*Lens culinaris* Medic). A study suggested the significant effect of different concentrations of PEG and silver nanoparticles on germination rate and germination percentage, root length, root fresh, and dry weight in lentil seeds. Moreover, application of AgNPs could be attributed to mitigating water stress, mediating loss of plant growth and yield (Hojjat, 2016).

35.3.2 Salinity Stress

Salinity is a major abiotic stress factor. It limits food production and deteriorates the growing demand of food crops. Salinity is a major concern in the scientific community to attain sustainable crop production. Since, the majority of major crop plant species belong to the lycophyte category, they are susceptible to salt stress; hence it is the most critical environmental stress that can cripple crop productivity (Flowers, 2004; Munns and Tester, 2008). Salinity stress causes the negative impact on various biochemical and physiological processes that are associated with plant growth and yield. Lowering of soil osmotic potential, creation of nutritional imbalance, enhancing specific ionic toxicity (salt stress), or one or more combinations of these factors are some of the common implications of salinity stress experienced by plants (Ashraf, 1994). Some other vital processes like photosynthesis, protein synthesis, lipid metabolisms, etc. are severely affected by salinity stress within a plant (Parida and Das, 2005).

Nanotechnology is recently gaining the attention of researchers because of their wide application in diverse sectors including agriculture. Application of nanofertilizers is among the most promising methods that can potentially enhance plant resource use efficiency and reduce environmental toxicity due to accumulation of unused chemical fertilizers and pesticides in the soil. Plants utilize a much lower amount of chemical fertilizers and pesticides than the amount applied to the soil, therefore the rest of the chemicals remain unused and accumulate in soil to increase soil toxicity. The application of nanofertilizers could be a potential approach to address such issues of soil toxicity and other associated stress problems. It has been reported that Si nanoparticles and silicon fertilizer exhibited promising effects on physiological and morphological traits on vegetative features of basil under salinity stress. This was evident from results that indicated significant increase in growth and development indices, chlorophyll content, and proline level in basil (*Ocimum basilicum*) under salinity stress, when treated with Si nanoparticles and silicon fertilizer. Results suggested this could be due to tolerance induction in plants thereby mitigating the effect of salinity stress in

basil (*Ocimum basilicum*) (Kalteh et al., 2014). Other studies also revealed the salinity stress mitigating capability of SiO_2 nanoparticles. Application of SiO_2 nanoparticles have shown potential increase in chlorophyll content, leaf fresh weight, leaf dry weight, proline accumulation, and upregulation of antioxidant enzyme activity under salinity stress. Such increases may be corroborated to enhancing the abiotic stress tolerance in plants (Haghighi et al., 2012; Kalteh et al., 2014). Application of Si nanoparticles on lentil (*Lens culinaris* Medik.) genotypes under salinity stress revealed significant increase in germination of seed and growth of seedling, whereas there was significant reduction in germination percent and seedling growth due to the salinity stress under without treatment of nanoparticles. Adding SiO_2 nanoparticles not only enhances seed germination and early seedling growth, but also increases other related traits in lentil genotypes under salinity stress. Therefore, SiO_2 nanoparticles ameliorate different defense mechanisms of plants against salt toxicity (Sabaghnia and Janmohammad, 2015). Stress mitigation effects of Si nanoparticle were also studied in tomato seeds and seedlings under salt stress. The results suggested the reduced salt toxicity impact on seed germination, and root length and plant dry weight in basil (*Ocimum basilicum*), exposed under salinity stress (Haghighi et al., 2012). Salinity stress reduces the crop growth and yield because of the Na^+ ion toxicity and nanoparticles (SiO_2) have been suggested to decrease the ionic toxicity leading to enhance crop growth and yield, thus helping crop improvement under adverse conditions (Savvasd et al., 2009). Other studies in maize suggested that fresh and dry weight of shoot and root increase under salinity stress after the application of SiO_2 nanoparticles (Gao et al., 2006). One strategy by which silica nanoparticles are used to mitigate salinity stress in plants is to reduce Na^+ ion concentration, perhaps by reducing Na^+ ion absorption by plant tissues. Since the primary impact of salinity stress on plant growth is due to reduction of osmotic potential and toxicity of Na^+ ion, Si nanoparticles may help to improve plant growth under salinity stress following Na^+ ion toxicity (Raven, 1982). Multiwalled carbon nanotubes exposed to broccoli under salinity stress reported to induce water uptake and transportation by increasing the net assimilation of CO_2 and aquaporin transduction, slightly changing the properties of salt stressed root plasma membrane to alleviate the stress and increasing growth (Martínez-Ballesta et al., 2016).

35.3.3 Chilling Stress

Chilling stress is produced by the very low temperatures that cause damage in the cells of the plant (Hasanuzzaman et al., 2013). Distortion of permeability as well as ion leakage from the membrane are the discrete impacts of chilling stress, which negatively affect the plant by reducing germination and growth (Welti et al., 2002; Suzuki et al., 2008). Conversely, vulnerability to chilling stress differs among the species; the plants with greater tolerance ability express low damage of membrane as compared with sensitive plants (Maali Amiri et al., 2010; Heidarvand et al., 2011). However, TiO_2 nanoparticles retain the ability to reduce the negative effect of chilling stress by decreasing the damage of plant membrane and ion leakage (Mohammadi et al., 2013). Photosynthesis is the integral mechanism of plant system that is much susceptible to chilling stress. Plants suffering from chilling stress express damage in the photosystem by reducing chlorophyll content, transpiration rate, CO_2 assimilation, as well as photosystem enzyme (Rubisco) degradation (Yordanova and Popova, 2007; Liu et al., 2012). Effect of nanoparticles on the photosystem have been concluded by increasing production of Rubisco enzyme (Gao et al., 2006), light immersion ability of chloroplast (Ze et al., 2011), as well as inhibiting the production of ROS (Giraldo et al., 2014). Exposure to TiO_2 nanoparticles increases the Rubisco and chlorophyll binding protein gene expression (Hasanpour et al., 2015), activities of antioxidant enzyme (Mohammadi et al., 2014), leaf pigments, and improves susceptibility to chilling stress. Plants that suffered with cold stress upregulated *MeCu/ZnSOD* and *MeAPX2* genes and increased the monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase activities that scavenge ROS, which results in repressed oxidative stress, that is, lipid peroxidation, chlorophyll degradation, and H_2O_2 generation and finally ensured stress tolerance (Xu et al., 2014). However, nanoparticle exposure along with chilling stress have shown increased growth and biochemical physiognomies of plants exposed to cold stress (Hawrylak-Nowak et al., 2010; Azimi et al., 2014; Haghighi et al., 2014; Kohan-Baghkheirati and Geisler-Lee, 2015).

35.3.4 Heat Stress

Heat stress involves high temperature at such severe level for a long enough time to result in irretrievable loss to development as well as growth of plants (Wahid, 2007). Heat stress increases the ROS generation and produces oxidative stress, which results in membrane lipid degeneration and membrane ion leakage, followed by degradation of protein (Moller et al., 2007; Savicka and Skute, 2010; Karuppanapandian et al., 2011) along with reduced rate of photosynthesis and chlorophyll content (Prasad et al., 2011). Application of

Se nanoparticle in low concentration reduced the effect of heat stress by increasing hydration ability, chlorophyll content, and plant development (Haghighi et al., 2014). Low concentration of Se nanoparticles shows antioxidative properties to plants whereas high concentration of Se nanoparticles induces oxidative stress (Hartikainen et al., 2000; Hasanuzzaman et al., 2014). During heat stress, plant synthesized some heat shock proteins and molecular chaperones (Schulze et al., 2005). Heat shock proteins assist other proteins in sustaining their constancy in stress conditions (Wahid, 2007) and are involved in heat stress resistance. It has been reported that multiwall carbon nanotubes are involved in upregulating gene expression of heat shock proteins, for example, HSP90 (Khodakovskaya et al., 2011). Furthermore, exposure to CeO_2 nanoparticle in maize causes excessive generation of H_2O_2 and upregulation of *HSP70* (Zhao et al., 2012). Additionally, treatment with TiO_2 nanoparticles reduced the effect of heat stress by stomatal opening regulation (Qi et al., 2013).

35.3.5 Heavy-Metal Stress

Heavy metal stress becomes a serious threat to crops worldwide by increasing toxicity and reducing growth of plants (Rahimi et al., 2012; Chibuike and Obiora, 2014). Under heavy metal stress, plants reduce their growth due to deficiency of essential nutrients by disturbance of uptake of vital supplements as well as suppression of enzyme activities (Capuana, 2011). Supplement of heavy metals increases extreme ROS production, which causes oxidative damage in the cell by altering cell structure, reduces membrane permeability, and degrades proteins (Rascio and Navari-Izzo, 2011; Sharma et al., 2012). Conversely, plants developed defense systems to combat heavy metal stress. Furthermore, plants produce metal-chelates, organic acids, and polyphosphates that limit the uptake of heavy metals and efflux of metal ions and activation of antioxidant enzymes that scavenge ROS production. However, the activation of these defense systems is important for resistance to heavy metal stress. It has been reported that synthesized nanoparticles are extremely active in lessening heavy metal−induced phytotoxicity (Gunjan et al., 2014; Tripathi et al., 2015). Nanoparticles are very small and have large surface area due to these properties; nanoparticles easily enter into plant cells and retain high affinity to the heavy metals. It has been studied that exposure of quantum dots reduces accessibility of Cu and Pb (Worms et al., 2012). However, if heavy metals cross the cell wall and the biophysical barriers and go into the plant cell, plants respond to the stress by accumulating biomolecules and nutrients, and by activating antioxidant enzymes to combat heavy metal stress. Although, exposure to TiO_2 nanoparticle limits cadmium toxicity and increases photosynthesis rate as well as plant growth (Singh and Lee, 2016). In addition, hydroxyapatite nanoparticle treatment in *Brassica juncea* lessens cadmium toxicity (Li and Huang 2014). It has been observed that the supplement of Si nanoparticle in growth media alleviates toxicity of chromium in pea (Tripathi et al., 2015). Furthermore, treatment of cowpea with gold ion stress incites the reduction of Au^{3+} to nontoxic gold nanoparticle by the phenolic compounds of germinating seeds (Shabnam et al., 2014).

35.4 SIGNALING MECHANISM OF NANOPARTICLES DURING ABIOTIC STRESS CONDITIONS

Plants have a network of defense systems; however, the exact recognition and transduction of stress to the defense system and precise sequence and stimulation of these defense systems in response to stress conditions before the stress-induced destruction are decisive for the fortification of the plant's cell. According to the existing data on nanoparticle−plant interactions during abiotic stress conditions, it was observed that production of ROS is a common response of plants to all stresses. ROS play a role as a stress signal to trigger the plant's defense system as well as aggravate cellular damage (Dat et al., 2000). Though, nanoparticles not only induce the ROS (Qi et al., 2013; Oukarroum et al., 2012; Van Hoecke et al., 2008; Ma et al., 2010; Simon et al., 2013), but also imitate antioxidant enzyme activities to scavenge ROS (Fig. 35.1) (Rico et al., 2013a,b; Wei and Wang, 2013). The dichotomy of actions ought to be deliberately broken down to the solution that nanoparticles give fortification against ROS whereas nanoparticles are also involved in the cause of oxidative stress (Fig. 35.1). This investigation might be handled by researching the part of nanoparticles in plant signaling. While, exhaustive mechanism of nanoparticles is not well known, conversely, via proteomic and genomic approaches it will be conceivable to understand the mechanism of nanoparticles in plants in abiotic stress conditions. It has been reported that treatment with Ag and $AgNO_3$ nanoparticles altered the proteins involved in the regulation of redox as well as metabolism of sulfur in the roots of *Eruca sativa* due to its physiochemical properties (Vannini et al., 2013). Exposer of Ag and Ag^+ nanoparticles coated with polyvinylpyrrolidone (PVP) on *Arabidopsis thaliana* regulated the gene expression of stress related genes (Kaveh et al., 2013). MiRNAs have been involved in the regulation of biological processes in plants and animals, and in addition play significant roles in plant

responses to abiotic and biotic stresses (Macovei et al., 2012; Frazier et al., 2014). Association of nanoparticles with miRNAs likewise reveals insight into the mechanism of nanoparticles under abiotic stress conditions. Exposure of TiO_2 and Al_2O_3 nanoparticles on tobacco plants is involved in the upregulation of miRNA expression as well as response to metal stress, though enhanced concentration of these nanoparticles displayed wilting, reduced biomass, leaf sizes, and leaf counts, as well as decrease root growth (Frazier et al., 2014; Burklew et al., 2012). It has been studied that the exposure of zero valent iron nanoparticle on *Arabidopsis* upregulated the gene expression of *AHA2* (involved in stomatal opening process), which enhanced drought tolerance (Kim et al., 2015). Furthermore, the treatment of TiO_2, multiwall carbon nanotubes, and Ag nanoparticles on *Arabidopsis thaliana* suppressed the expression of root-development as well as phosphate starvation genes under different stress conditions (García-Sanchez et al., 2015). In a recent study, it has been reported that exposure of NiO nanoparticles on barley (*Hordeum vulgare*) induces overproduction of ROS that favored oxidative stress as well as increased lipid peroxidation in the plant. Wherever, cotreatment with SiO_2 nanoparticle on nano-NiO treated plant provoked proficient antioxidant response, reduced the levels of lipid peroxidation, and stimulated the redox pathway that mitigates the phytotoxicity of the NiO nanoparticles. This study revealed that SiO_2 nanoparticles play a protective role in barley in response to NiO nanoparticle stress (Soares et al., 2018).

A signaling network activates the defense system in plants, which prompts the molecular mechanism in response to particular stress conditions. Calcium ions involved in signal transduction during various stress conditions act as a second messenger. Sensitivity of stress signals causes enlistment of calcium ions to the cytosol via calcium ion channels, which causes enhancement in calcium ion level in the cytosol, which is recognized through calcium ion-binding proteins that cause alterations in gene expression as well as plant adaptation to stress conditions (Tuteja and Mahajan, 2007; Khan et al., 2014a,b). It has been observed that nitric oxide (NO) prompts the enhancement of cytosolic calcium ions in plant cells during various biotic and abiotic stress conditions (Lamotte et al., 2006; Khan et al., 2012a,b) and therefore calcium ions persuade the synthesis of nitric oxide (Del Rio et al., 2004; Corpas et al., 2004). It has been reported that Ag nanoparticle treatment on *Oryza sativa* roots revealed the involvement of nanoparticle responsive proteins in regulation and signaling of calcium ions, protein degradation, cell wall synthesis, transcription, oxidative stress response pathway, cell division, and apoptosis (Mirzajani et al., 2014). In addition, it has been supposed that Ag nanoparticles bind with calcium ion channels or Ca^+/Na^+ ion pumps via calcium ion receptors that affect cell metabolism (Goyer, 1995; Mirzajani et al., 2014). It has been also studied that that interaction of C60 nanocrystals instigated functional modulation of the Ca^+/calmodulin-dependent protein kinase II (Miao et al., 2014). Furthermore, the application of cadmium sulfide QDs on *Arabidopsis thaliana* caused overexpression of calcium-binding protein CML45 as well as calcium-dependent protein kinase 23 (Marmiroli et al., 2015). These calcium-binding proteins have been revealed to control stress responses, and overexpression caused improved resistance in plants against several abiotic stress conditions (Delk et al., 2005; Xu et al., 2011; Boudsocq and Sheen, 2013).

Nanoparticles enhanced the activity of nitrate reductase enzyme in plants, which increased the concentration of nitric oxide to modulate immune response in plants (Carpenter et al., 2012; Shahrokh et al., 2014; Chandra et al., 2015). Conversely, it has been reported that nitric oxide causes nanoparticle-induced toxicity and activates the antioxidant genes expression as well as suppresses the lipid peroxidation and generation of ROS (Chen et al., 2015). Relating the mechanism of nanoparticles with calcium ions revealed that nanoparticles impersonate calcium ions and bind with calcium-binding proteins that stimulate the cascade of stress responsive genes (Mirzajani et al., 2014). Additionally, the treatment of nanoparticles enhances the expression of cell elongation, cell division, as well as stress responsive genes (Almutairi, 2016; Khodakovskaya et al., 2011, 2012). Multiwall carbon nanotubes penetrate the cell wall of the plant; considering this characteristics, carbon nanotubes can be sensed by plants as a stress stimulus like biotic stress. Therefore, the complete mechanism of signal transduction in plants induced by nanoparticles needs further study (Khodakovskaya et al., 2012). Additionally, studies on nanoparticle-induced phytotoxicity have shown enhanced generation of ROS that act as toxic compounds as well as signaling molecules in plant cells (Fig. 35.1). The different roles of ROS are precisely governed by their production as well as scavenging activity; disproportion in any of these processes will result in extreme production or diminished accessibility of ROS, which leads to oxidative stress or disruption of signaling respectively. Conversely, this symmetry is sustained by incessant production as well as scavenging of ROS. Moreover, it has been noticed that higher concentration of nanoparticles showed toxic effect, whereas its lower concentrations show beneficial or no influence on the plant's system. It concludes that lower concentration of nanoparticles sustains an active antioxidant defense system that regulates the generation of ROS

into a precise concentration adequate for signaling but incapable of causing damage (Syu et al., 2014).

35.5 CONCLUSION

Nanoparticles lessen abiotic stress-induced damage by stimulating the defense mechanism of plants. The very small size of nanoparticles enables them to easily penetrate as well as control ion channels, which supports germination of seed and plant growth; furthermore, their large surface area assists high absorption as well as targeted delivery of molecules. On the other hand, nanoparticles are involved in the production of ROS and cause phytotoxicity. The elevated concentration of ROS by exposure of nanoparticles could be related to the intensification of stress signals, which trigger defense mechanisms in plants at proficient mode. Concerning the mechanism of nanoparticles, the available reports are emerging and are still too inconsistent to illustrate the complete mechanism. However, reported data illuminate that nanoparticles imitate calcium ions or a signaling substance in cytosol as recognized by calcium-binding proteins or by nanoparticle-specific proteins. Thus, initiation of the signaling substance promotes gene expression, which results in improved resistance to stress. It is concluded that the function of nanoparticles in plant systems requires additional research at the molecular and cellular level and it is greatly necessary to confirm whether nanoparticles are involved in stress promoters or stress inhibitors.

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Further Reading

Corral-Diaz, B., Peralta-Videa, J.R., Alvarez-Parrilla, E., Rodrigo-García, J., Morales, M.I., Osuna-Avila, P., et al., 2014. Cerium oxide nanoparticles alter the antioxidant capacity but do not impact tuber ionome in *Raphanus sativus* (L). Plant Physiol. Biochem. 84, 277–285.
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Plant Signaling Molecules

Role and Regulation Under Stressful Environments

Edited by
M. Iqbal R. Khan
Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi, India

Palakolanu Sudhakar Reddy
Cell, Molecular Biology and Genetic Engineering Group, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Telangana, India

Antonio Ferrante
Department of Agricultural and Environmental Sciences, University of Milano, Milano, Italy

Nafees A. Khan
Department of Botany, Aligarh Muslim University, Aligarh, India

Plant Signaling Molecules explores stress tolerance mechanisms mediated by different "signaling molecules" in plants for achieving sustainability under changing environmental conditions through mechanistic strategies. This book includes a wide range of signaling molecules, exploring the present status and future prospects of the role and regulation of such molecules at physiological, biochemical, molecular, and structural levels under abiotic stresses. Understanding how these mechanisms direct plant responses provides an important contribution to anticipating and addressing underlying mechanisms. This book is designed to enhance the mechanistic understanding of signaling molecules and will be an important resource for graduate students, researchers, professors, and plant scientists. This book will also assist in developing stress tolerant crop plants to achieve sustainability under changing environmental conditions.

Key Features

- Provides a compendium of knowledge related to plant physiology, plant biochemistry, and plant molecular responses for adaptation of plants under stressful environments.
- Focuses on plant signaling molecules under stressful environments and identifies treatments able to enhance tolerance to abiotic stresses.
- Illustrates specific physiological and molecular pathways that are considered key points for plant adaptation or tolerance to abiotic stresses.

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