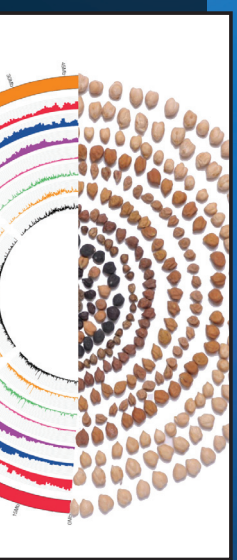
Translational Genomics for Crop Breeding

Volume I: Biotic Stress

Editors: **Rajeev K. Varshney**
Roberto Tuberosa

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Edited by

Rajeev K. Varshney
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Foreword

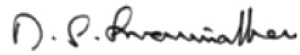
Drs. Rajeev K. Varshney and Roberto Tuberosa have done a great service by bringing out this important book, *Translational Genomics for Crop Breeding, Vol. 1: Biotic Stresses*. This volume deals with the application of genomics in crop breeding for biotic stress tolerance. It will be useful to refer briefly to the transformational role the new genetics based on genomic applications is playing today in improving agriculture, industry, medicine, and environment, following the elucidation of the double-helix structure of the DNA molecule 60 years ago by James Watson, Francis Crick, Maurice Wilkins, and Rosalind Franklin. Their discovery opened up uncommon opportunities for the advancement of science as related to all aspects of life. During recent decades, many Nobel Prizes in Physiology and Medicine have gone to molecular geneticists. At the same time, public concern about the proper measurement of risks and benefits has grown, particularly in the fields of agricultural and food biotechnology. Biotechnology provides an opportunity to convert bioresources into economic wealth. This has to be done in such a manner as to ensure no adverse impact either on the environment or on human and animal health. The bottom line of Indian national agricultural biotechnology policy should be the economic well-being of farm families, food security of the nation, health security of the consumer, protection of the environment, and the security of our national and international trade in farm commodities.

This volume is an epitome of advances in the area of translational genomics application for improving crops with resilience to important components of biotic stress. Integration of high-throughput genotyping with precise phenotyping is the key for dissecting mechanism of complex traits at the molecular level. There are a number of races and biotypes known for a particular disease and insect, and so it is necessary to have a complete knowledge of the causal organism so that race-specific or biotype-specific resistance can be attained. This encourages optimal and target approach to breeding for the trait of interest. Hence, a more holistic approach and, more importantly, a holistic perspective such as that of systems biology is the need of the hour. The chapters in this volume not only provide in-depth review of the problem at hand but also enlighten readers about the advances and possibility of integrating genomics approach in tackling a research problem. In addition, the successful example and success stories discussed are thought provoking to young plant scientists and make them prepare for the challenges ahead.

New approaches for identifying marker-trait association such as genome-wide and candidate gene association studies are gaining fast acceptance due to advantages such as amenability to phenotype at multi-location for multiple traits and genotyping only once, not at each generation. In addition, marker-trait association is validated simultaneously in order to allow the deployment of markers directly in the breeding

program. Another upcoming and promising approach termed as genomic selection is fast gaining importance among the crop specialists. It relies on the genomic-assisted breeding values, rather than phenotypic selection alone, in order to select the lines for crossing and advancing them to next generation. These approaches along with others are covered comprehensively in this book.

I hope this book will be widely read by scientists and scholars, since we must harness the best in the new genetics to overcome the serious threats to human well-being caused by malnutrition, hunger, and disease. The contents of the book show the ways to enhancing productivity in perpetuity without ecological harm. I congratulate and thank Drs. Varshney and Tuberosa for their labor of love in helping harness the best in modern science for enhancing the quality of human life.



Chennai
Date: June 15, 2013

MS Swaminathan
Founder Chairman
M S Swaminathan
Research Foundation

Preface

Recent years have witnessed significant progress in the area of crop genomics mainly due to advances in next-generation sequencing and high-throughput genotyping. Such advances are driving genomics-assisted breeding (GAB), a discipline that has grown tremendously during the past decade, particularly for its applications to improve crop productivity and quality. This quantum leap has been possible through the continuous effort and dedication of those engaged in the translation of the findings of genomics research into improved genotypes and populations. As we anticipate a further reduction in genotyping/sequencing cost, translational genomics is expected to become a more integral part of crop breeding.

Biotic stress is one of the major factors behind crop losses. While a number of reports have been available on genomics approaches such as deciphering marker-trait association either through linkage or association mapping, some success stories have also been reported in recent years on translational aspects of this genomics research in crop breeding. However, the ever-changing and dynamic world of causal organisms of diseases and pests pose serious challenges to crop specialists to identify new resistant alleles and to target disease and pest resistance as well as to accelerate development of superior lines with enhanced resistance to biotic stresses. Therefore, there was an urgent need for a book in which translational genomics activities for resistance to key pests and diseases, success stories completed and in progress, and useful take-home messages from GAB efforts in different crops would be compiled. Along these lines, the 16 chapters of *Translational Genomics for Crop Breeding, Volume 1: Biotic Stresses* include not only details on the aforementioned issues but also address perspectives and challenges in translational genomics for developing superior varieties and lines with enhanced resistance to biotic stresses.

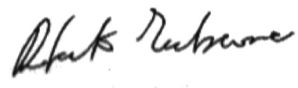
We thank the authors (Appendix I) of different chapters for their commendable effort in summarizing the published and unpublished research and putting all the pieces together in a well-knitted, up-to-date manner, for the benefit of the research challenge in hand. In addition, the cooperation they have extended in terms of timely completion and revision of chapters is greatly appreciated. While editing this book, the strong support received from many other colleagues (Appendix II) willing to review the chapters is equally appreciated. Their constructive comments and suggestions have been instrumental in further improving the contents.

The editors are also grateful to colleagues and staff from their respective laboratories who helped complete the editing of the two volumes in parallel with their demanding responsibilities. In particular, Manish Roorkiwal, B. Manjula, Pawan Khera, and Mahendar Thudi helped RKV with the editorial work. The editors also wish to thank their respective families, as the editorial work for this book took away precious moments they should have spent together with their families. RKV is thankful to his wife Monika for her constant encouragement and

support, and to Prakhar (son) and Preksha (daughter) for their love and cooperation. Similarly, RT is equally thankful to his wife Kay for her support and editorial help. RKV would also like to extend his sincerest thanks to Dr. William D. Dar, Director General, ICRISAT, for his guidance and support in completing this book. The cooperation and help received from Justin Jeffryes, Anna Ehlers, Kelvin Matthews, Erin Topp of Wiley Publishers, and Shikha Sharma of Aptara Corp. during various stages of development and completion of this book are gratefully acknoweldged. RKV would also like to mention that the book was edited during the tenure of RKV as Director, Center of Excellence in Genomics (CEG), ICRISAT, Hyderabad (India), Theme Leader – Comparative and Applied Genomics (CAG), Generation Challenge Programme (GCP) and Adjunct positions at the University of Western Australia, Crops Research Institute of Guangdong Academy of Agricultural Sciences (GAAS), China and BGI-Hongkong Research Institute, China.

We hope that this book will be helpful and useful as a ready guide to students, young researchers, crop specialists, GAB and translational genomics practitioners, and policy makers for developing crops more resilient to biotic stress.

Hyderabad, India (Rajeev K. Varshney)
June 10, 2013



Bologna, Italy (Roberto Tuberosa)
June 11, 2013

Chapter 1

Translational Genomics in Crop Breeding for Biotic Stress Resistance: An Introduction

Rajeev K. Varshney and Roberto Tuberosa

Abstract

Biotic stresses pose a major threat to crop productivity. Crops are challenged by a plethora of biotic stresses, but only a limited number of key pests and diseases cause the vast majority of economic losses in a particular crop. Plant protection measures such as application of pesticides and deployment of resistant gene(s)/quantitative trait loci (QTLs) into cultivars have so far been quite successful in curtailing the losses; however, these measures have also led to the constant evolution of new biotypes/pathotypes/strains/races of pest and disease organisms. Hence, there is a continuous need to identify genomic regions that can impart resistance against these variants. The availability of large-scale genomic resources in many crop species has enhanced our understanding on the path to developing host-plant resistance. As a result, numerous race-specific gene(s) and QTLs have now been identified and cloned with the help of molecular markers. It is quite exciting that these genomic regions are being introgressed into breeding programs of many crops. The objective of this book is to critically review the current availability and utilization of genomic tools for major biotic stresses in important cereals, legumes, vegetables, and tuber and oilseed crop. The book also summarizes the success stories achieved through application of genomics-assisted breeding (GAB), as well as the scope for deployment of modern breeding methods such as marker-assisted backcrossing (MABC) and genomic selection in the era of next-generation sequencing (NGS) technologies, which have the potential to advance the genetic gains for enhancing resilience against biotic stress. This chapter summarizes highlights of different chapters included in the book that is expected to be a resource for young researchers, GAB practitioners, and policy makers for employing better strategies toward achieving food security.

Introduction

Several biotic and abiotic stresses challenge crop productivity. Breeders try to develop superior lines by making crosses and selecting the best lines based on their agronomic performance, but the entire process is expensive and takes several years. During the past two decades, remarkable progress in the area of genomics and molecular genetics has greatly improved our basic

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understanding of resistance to biotic stresses and tolerance of abiotic stresses. Genomics approaches can enhance the precision and efficiency of breeding programs through a better prediction of phenotype from a given genotype – process generally referred to as genomics-assisted breeding (GAB) (Varshney et al. 2005).

Among different GAB approaches, the marker-assisted backcrossing (MABC) approach has been quite successful in transferring the target genomic regions in elite cultivars (Varshney et al. 2012). MABC for gene pyramiding coupled with selection for the genetic background of the recurrent parent and recombination at the target region(s) could lead to faster and better product delivery, thereby increasing productivity and improving livelihoods of the smallholder farmers (Collard et al. 2008).

Biotic stress caused by pests and diseases continues to pose a significant risk to crop productivity in spite of years of investments in research and development aimed at understanding host-plant interaction and finding more effective methods to control it (Lucas 2011). It has been estimated that even after the deployment of pesticides and improved cultivars in the target environment with resistance to biotic stresses, yield losses resulting from pests and diseases can still reach 20-30% (Oerke 2006). This loss may be attributed to the constant and rapid evolution of new virulent pathogens/pests such as Ug99 for wheat stem rust (Levine and D'Antonio 2003), as well as to their spread to new regions in response to climate change and the adoption of different agricultural practices (e.g., minimum tillage).

Abiotic stresses, such as drought, salinity, cold, submergence, mineral toxicity, and others, also hamper growth, yield, and yield quality of crop plants. In fact, these abiotic stresses represent the main cause of crop failure worldwide, reducing average yields for major crops by more than 50%. Overall, as compared to biotic stresses, abiotic stresses pose more serious constraints to crop production, particularly in view of rapidly deteriorating environmental conditions. Quality traits are the other important class of target traits that breeders select for in order to improve crop productivity as well as nutritional quality.

In recent years, large-scale genomic resources have been developed and are being utilized in breeding programs for several crop species (Varshney et al. 2009; Tuberosa et al. 2011). These advances in genomics research have greatly contributed to the conversion of so-called orphan crops to genomic resources-rich crops (Varshney et al. 2009, 2010) and to the enhanced precision and speed of breeding programs. In several cases, GAB has delivered superior lines that have been used for developing new varieties or hybrids (Simpson et al. 2003; Sundaram et al. 2008; Ceballos et al. 2012; Singh et al. 2012). However, introgression of QTLs has not always been successful in crop breeding, and even less so for the improvement of tolerance to abiotic stresses (Collins et al. 2008). Therefore, GAB practices have also offered some lessons to the molecular breeding practitioners.

In view of the above, the two volumes on *Translational Genomics for Crop Breeding* compile a number of manuscripts that report on success stories either completed or still in progress, as well as the lessons learned from GAB work on different crops. Volume I compiles 16 chapters that review the current status and recent advances in the application of GAB approaches for biotic stress resistance. Volume II is a compendium of 13 chapters on GAB for enhancing abiotic stress tolerance and improving crop quality.

This introductory chapter of Volume I provides key highlights of GAB applications to enhance biotic stress tolerance. Since the majority (estimated to be ca. 60-70%) of our major caloric intake is obtained directly or indirectly from cereals, the first five chapters summarize the progress on the improvement of biotic stress tolerance in five major cereals, namely rice, maize, wheat, barley, and sorghum. The contribution of legumes to enhancing nutrition in the daily diet has been largely recognized apart from their well-known ability for nitrogen-fixation. The next five chapters deal with GAB

applications for important biotic stresses in legumes, namely soybean, peanut, common bean, cowpea, and chickpea. Two additional chapters deal with GAB for enhancing the tolerance of potato and tomato to late blight, one of the most devastating diseases of these two important vegetable crops. The three final chapters highlight GAB efforts toward improving disease resistance in lettuce, cassava, and *Brassica* species.

Improving Disease Resistance in Cereals

Bacterial blight (BB), effected by *Xanthomonas oryzae* pv. o*ryzae* (*Xoo*), is a major constraint for rice production, with reported yield losses of up to 50% (Ou 1985). Recently several genes and QTLs have been identified for various virulent strains. Chapter 2 by Kou and Wang provides a comprehensive review of and valuable insights to understanding the interaction between rice and *Xoo* pathogen. This review provides strategies and prior knowledge for effective deployment of resistance genes in target environment against *Xoo* pathogen. Until now, more than 35 BB rice resistance genes have been identified and 7 of these have been isolated. MABC has been quite successful in the case of BB, and various genes such as *Xa4*, *xa5*, *Xa7*, *xa13*, *Xa21*, *Xa23* in single or in pyramided form have been introgressed in popular varieties/parental lines such as, Samba Mahsuri, Pusa Basmati 1, Minghui 63, and have been developed and released in India and China (Gopalakrishnan et al. 2008; Sundaram et al. 2008; Perumalsamy et al. 2010; Huang et al. 2012; Singh et al. 2012).

Chapter 3 by Jamann, Nelson, and Balint-Kurti provides a comprehensive survey of the genetic basis of disease resistance in maize, especially against fungal diseases. In the past, bi-parental linkage mapping was commonly adopted for mapping important genes and QTLs. However, in recent years, modern mapping approaches such as nested association mapping (NAM), which is an effective combination of linkage and linkage-disequilibrium approaches, are becoming increasingly popular (Yu et al. 2008). The chapter reports on the use of the NAM approach to identify genomic regions responsible for three important diseases in maize, namely southern leaf blight, northern leaf blight, and gray leaf spot (Benson et al. 2011; Kump et al. 2011; Poland et al. 2011). In addition, the authors outline the potential of genomic selection to accelerate the breeding efforts for disease resistance, especially in cases where small-effect and environment-sensitive QTLs are involved, as in *Aspergillus* ear rot and aflatoxin accumulation (Warburton et al. 2009). These genetic studies provide an insight into the disease resistance mechanism, thereby helping molecular breeders understand the genes to be used for their deployment in elite cultivars.

In the case of wheat, among several other diseases, Fusarium head blight (FHB) is an age-old and severe one (Leonard and Bushnell 2003). Importantly, contamination caused by fusarium secondary metabolites, known as mycotoxins, poses a major threat to animal and human health (Van Egmond 2004). Extensive QTL studies for FHB resistance have led to the identification of 19 meta-QTLs spread across wheat chromosomes (Buerstmayr et al. 2009; Liu et al. 2009; Löffler et al. 2009). These GAB efforts for FHB have been summarized in Chapter 4 by Hermann Buerstmayr, Maria Buerstmayr, and Schweiger and Steiner. A closely linked codominant marker is always a prerequisite for making any MABC program a success. In particular, *Umn10*, a PCR-based marker linked to a major gene (*Fhb1*) located on the long arm of chromosome 3B and explaining 40-50% of phenotypic variance (Rosyara et al. 2009), is being used routinely in breeding programs of both hexaploid and tetraploid wheat.

In barley, improving virus resistance is one of the top research priorities because it has a serious impact on its production, particularly in Western Europe. Much work has been done in the recent past toward identification of resistance genes for four major viruses affecting barley (Ordon et al.

2009). As a result, molecular markers are now available for fast introgression. In a recent study, improved DH-lines have been developed for Barley Yellow Dwarf Virus through markers (Riedel et al. 2011). Chapter 5 by Ordon and Perovic covers recent advances toward development of genomic tools for transferring virus resistance into elite cultivars via GAB. The authors also highlight the importance and use of allele mining and utilization of high-throughput SNP technologies for carrying out precision breeding activities in barley.

In sorghum, *Striga* is the most damaging obligate parasite pest that leads to yield loss of up to 90% (Ejeta 2007). It is particularly severe in East Africa and some regions in the United States and Asia. Although much progress has been made toward QTL analysis and Marker-assisted selection (MAS) for improving resistance to *Striga*, the molecular mechanisms behind the establishment of parasitism are still not well understood. In Chapter 6, Deshpande, Mohamed, and Hash describe several aspects for elucidating the molecular mechanisms of *Striga* resistance through development of bioassays, exploring the pathway, and identifying the stages as entry points for breeding resistance to *Striga*, as well as GAB approaches to developing sorghum lines with enhanced resistance to *Striga.* The authors also discuss the utility of next-generation sequencing (NGS) technologies for identifying the functional basis of *Striga* resistance.

Improving Disease Resistance in Legumes

Among different legumes, soybean, known for its edible oil and protein content, is an important industry crop. North America and South America are the major production areas, accounting for nearly 86% of total soybean production worldwide (http://www.soystats.com). Cyst, root-knot, and reniform nematode are the major pests of soybean, with annual losses of more than $1 billion (Koenning and Wrather 2010). Chapter 7 by Vuong, Jiao, Shannon, and Nguyen provides a comprehensive review of nematode resistance in soybean. This work highlights the different nematode problems, their biology and candidate genes for host plant response. Notably, the continuous effort toward the identification of genetic markers closely linked to soybean cyst nematode has led to the development and release of three varieties, namely JTN-5503, JTN-5303, and JTN-5109 in the United States, which are essentially gene pyramids of *Rhg1, Rhg4*, and *Rhg5* (Arelli et al. 2006, 2007; Arelli and Young 2009).

Grown in more than 100 countries, peanut is one of the most widespread legume crops in the world (Nwokolo 1996). Chapter 8 by Burow, Leal-Bertioli, Simpson, Ozias-Akins, Chu, Denwar, Chagoya, Starr, Moretzsohn, Pandey, Varshney, Holbrook, and Bertioli describes molecular mapping and MAS for several diseases and pest challenges faced by peanut. As to improving the resistance to root knot nematode, a serious problem in the United States caused by *Meloidogyne* species, the effectiveness of MAS has been demonstrated through the development and release of a nematode-resistant variety 'NemaTAM' in the United States (Simpson et al. 2003). With the availability of more than 6,000 SSR markers, extensive studies have also led to the identification of QTLs with high phenotypic variance for resistance to late leaf spot and rust (Sujay et al. 2012) and tomato spotted wilt virus (Qin et al. 2012). In addition, this chapter presents the prospects and progress of the International Peanut Genome Project toward sequencing the peanut genome, which should help in the identification of candidate genes for stress tolerance and to accelerate GAB in peanut (http://www.peanutbioscience.com/peanutgenomeproject.html).

In common bean, the fungal pathogen *Colletotrichum lindemuthianum* (Sacc. & Magnus) causes a devastating disease known as anthracnose. Several resistance genes against race-specific isolates for anthracnose have been reported in the past. Ferreira, Campa, and Kelly in Chapter 9 report on the inheritance pattern of

the pathogen and the related allelism tests, and discuss GAB approaches for anthracnose resistance. Furthermore, the authors propose a new system of naming anthracnose resistance gene(s) based on the location on the genetic map. Efforts toward marker-assisted introgression in common bean have led to the release of variety 'USPT-ANT-1' with gene *Co-4*^{2} conferring resistance to anthracnose in the United States (Miklas et al. 2003). Recently, line A3308 carrying genes *Co-2* and *Co-3/9* for anthracnose and bean common mosaic (BCM) resistance by genotype *I* + *bc-3* has also been developed (Ferreira et al. 2012).

Cowpea is an important leguminous crop in the tropical and subtropical areas, especially in Latin America, Asia, and Sub-Saharan Africa (Singh et al. 1997). Recent advances in the development of genomic tools in cowpea have enabled the identification of molecular markers for resistance to critical biotic stresses. This notwithstanding, application of modern breeding approaches is still in its infancy. In Chapter 10, Huynh, Ehlers, Close, Cissé, Drabo, Boukar, Lucas, Wanamaker, Pottorf, and Roberts review initial MABC work for various disease resistance and genomic resources available for carrying out GAB in cowpea. The transgenic approach has also been discussed as an option to increase resistance to pod borer and cowpea weevil, as the level of resistance to these pests in the available germplasm is negligible.

Chickpea is another important leguminous crop, mainly grown in Asia and the Mediterranean regions of the world, which is highly nutritious and rich in protein, carbohydrates, and vitamins (Abu-Salem and Abou-Arab 2011). India is the largest producer of chickpea in the world, accounting for more than 65% of global production (FAO 2011). Among important biotic stresses, *Fusarium* wilt and *Ascochyta* blight can cause yield losses of more than 90% (Singh and Reddy 1991, 1996). Efforts to develop genomic resources have led to the identification of molecular markers for agronomic as well as biotic stress, paving the way for GAB activities in this crop (Varshney et al. 2013a). In Chapter 11, Millan, Madrid, Imtiaz, Kharrat, and Chen extensively review disease resistance aspects in chickpea. Furthermore, as genome sequencing of 90 chickpea lines is now available, molecular breeding efforts can now be accelerated to develop tolerant lines for disease resistance (Varshney et al. 2013b).

Improving Disease Resistance in Vegetables

Potato is one of the major staple and vegetable crops, covering more than 100 countries, with an annual production of more than 300 million tons (FAO 2011). *Phytophthora infestans*, which causes late blight, is the main, devastating disease in potato, with an annual yield loss of more than $3 billion (Duncan 1999). Chapter 12 by Śliwka and Zimnoch-Guzowska discusses recent advances in discovering, identifying, mapping, and cloning the resistance genes in potato. This information could be quite useful for the deployment of race-specific resistance in improved lines for target environments.

Tomato is another major vegetable crop for which late blight is a major devastating disease causing vast yield loss. In Chapter 13, Nowicki, Kozik, and Foolad make a special emphasis on late blight resistance in tomato. The chapter provides comprehensive insight into the disease, its chemical control, and GAB aspects. Furthermore, the recently sequenced tomato genome (Tomato Genome Consortium 2012) and *Phytophthora* genome (Haas et al. 2009) provide much-needed understanding of *R-Avr* interaction for late blight. Molecular breeding activities have been quite successful in imparting resilience against late blight, and several varieties such as NC1 CELBR, NC2 CELBR, Mountain Magic, and Mountain Merit have been developed by stacking two genes (*Ph-2* + *Ph-3*) and released in the United States (Gardner and Panthee 2010; Panthee and Gardner 2010).

Lettuce, one of the most commercially important leafy vegetables, has an annual production of more than 23 million tons (FAO 2011).

The crop is grown for a variety of purposes such as salad, stem, and oilseed. The crop is challenged by many biotic stresses leading to huge economic losses. In Chapter 14, Simko reviews recent developments in MAS for resistance to downy mildew, corky root, lettuce mosaic, and lettuce dieback. To achieve these traits, both public and private sectors are routinely utilizing allele-specific assays in their breeding programs. Furthermore, details and current status regarding mapping efforts for other important traits are discussed. Important progress has been made in generating large-scale genomic resources/platforms in lettuce, such as an EST database that includes sequences of more than 700 candidate resistance genes (McHale et al. 2009), microarray chip with more than 6.5 million feature Affymetrix genechip (Stoffel et al. 2012), and complete genome sequencing of cultivated and wild lettuce (https://lgr.genomecenter.ucdavis.edu/; Lavelle et al. 2013), which promises to facilitate faster diagnostics, gene expression analysis, high-throughput genotyping, and cloning of genes.

Improving Disease Resistance in Cassava and *Brassica*

In addition to the aforementioned cereal, legume, and vegetable crops, Volume I includes GAB activities in cassava and *Brassica*, two other important crops for human diet. Cassava, a starchy root crop, is a major food source for more than 800 million people in Sub-Saharan Africa, Asia, and South America. It is cultivated on more than 20 million hectares, with an annual production of more than 240 million tons (FAO 2011). Cassava suffers from several biotic stresses and is highly vulnerable to viral diseases. Cassava mosaic disease (CMD), caused by cassava mosaic Gemini virus, is one of the major viral diseases of cassava, causing reported yield loss of up to 40% (Taylor et al. 2004). Much success has been achieved in identification of molecular markers for CMD, and MAS for this trait is currently being employed in several popular cultivars of Africa and India. The release in 2010 of cassava cultivar CR41-10 in Nigeria, made possible through the activities of the CGIAR Generation Challenge Program (GCP), is the first example of MAS-derived product in cassava (Ceballos et al. 2012). In Chapter 15, Okogbenin, Moreno, Tomkins, Fauquet, Mkamilo, and Fregene present an informative and critical review of GAB activities in cassava.

The agricultural and horticultural uses of the *Brassica* genus contribute an important part to the human diet and to the global economy. Like with all other crops, a plethora of pests and diseases curtail the yield in *Brassica*. In Chapter 16, Li and McVetty review the recent progress on the genetics and gene mapping for disease resistance in *Brassica* species. Tangible progress has been achieved toward GAB for resistance to blackleg and clubroot. However, the development of MAS of sclerotinia stem rot has seen slower progress, mostly because germplasm accessions with high levels of resistance have yet to be identified.

Summary and Outlook

In summary, this volume presents recent advances, useful insights, and comprehensive reviews for GAB approaches to improve biotic stress tolerance in a range of crops. Although the potential for utilization of GAB in crop improvement programs appears almost endless, its application varies greatly among different crop species, reflecting to a certain extent the state-of-the-art genomics of each single species and their economic importance. In crops such as rice, maize, wheat, and barley, MAS and MABC is already well integrated in breeding programs, whereas in many others, the deployment of molecular breeding activities is under way. Notably, GAB for several traits has recently been initiated in orphan crops.

Thanks to the advent of NGS, it has become possible to generate reference genome sequence data of the main crops and also to (re)sequence several varieties/lines. In parallel, modern genetic mapping approaches such as

genome-wide association studies (GWAS; Rafalski 2010; Hamblin et al. 2011) and nested association mapping (NAM; Yu et al. 2008; McMullen et al. 2009) for trait mapping and modern breeding methodologies like marker-assisted recurrent selection (MARS) (Charmet et al. 1999) and GS (Heffner et al. 2009; Jannink et al. 2010) are being increasingly adopted in several crop species. In addition, molecular breeding decision support tools such as an integrated system for marker-assisted breeding (ISMAB) (https://www.integrated breeding.net/ib-tools/breeding-decision/ismab), OptiMAS (http://moulon.inra.fr/optimas/index .html), GS modules (Pérez-Rodríguez et al. 2012; de Los Campos et al. 2013), and platforms like Integrated Breeding Platform (IBP) (https://www.integratedbreeding.net/) are being developed. These advances are expected to accelerate GAB for a range of traits, including biotic stress resistance in crop breeding.

As mentioned earlier, Volume II of this series documents the application of genomics for abiotic stress tolerance and quality traits in several crops. Therefore, together with Volume II, this volume provides an informative and critical update of genomics applications in crop breeding. We hope these chapters will allow young researchers, including graduate students and postdoctoral scholars, to better appreciate GAB and encourage them to devote their career to this exciting area of crop improvement. Additionally, we hope that GAB practitioners as well as policy makers will find these volumes useful for developing the road map toward a more effective improvement of target crops in their respective geographical areas.

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Chapter 2

Bacterial Blight Resistance in Rice

Yanjun Kou and Shiping Wang

Abstract

Rice is one of the most important cultivated food crops. Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the major constraints for sustainable production of rice. Researchers have made tremendous progress in trying to elucidate the interaction between rice and *Xoo*. The genomes of three *Xoo* strains have been sequenced. Some factors affecting pathogenicity of *Xoo*, such as type III secretion system, effectors translocated by type II and III secretion systems, have been identified. In rice, a number of genes contributing to qualitative and quantitative resistance against *Xoo* have been characterized. At least 37 major disease (*MR*) genes have been identified and named, and 7 (*Xa1*, *Xa3/Xa26*, *xa5*, *xa13*, *Xa21*, *xa25*, and *Xa27*) of them have been isolated. Importantly, some key components functioning in *Xa3/Xa26*- and *Xa21*-mediated defense signaling pathways have been characterized, which is helpful to understand molecular mechanisms of qualitative resistance to BB. At least 74 resistance QTLs against *Xoo* have been identified in different rice cultivars interacting with different *Xoo* strains. One major resistance QTL (*WRKY45*) and eight minor resistance QTLs (*NRR*, *WRKY13*, *OsDR8*, *MPK6*, *GH3-1*, *GH3-2*, *GH3-8*, and *C3H12*) have also been identified. The wealth of information about molecular components that function in rice defense response is now accessible for rice improvement in breeding programs.

Rice (*Oryza sativa* L.) is perhaps the most widely cultivated food crop worldwide; it is consumed by approximately 50% of the world's population, and its consumption has been dramatically increased in many parts of the world (White 1994). Various factors affect rice productivity, including diseases. Bacterial blight (BB) is the most devastating bacterial disease of rice. It occurs in epidemic areas of the world and can result in yield loss of up to 50% (Ou 1985). Traditional management methods, including cultivation strategies, chemical control, and biological control, are useful tools to combat BB. However, these methods can be labor intensive, expensive, and may cause environment pollution. The most economical and environmentally friendly way to control BB is to use resistant varieties carrying major disease resistance (*MR*) genes and/or resistance quantitative trait loci (QTLs) in combination with agricultural management practices. Resistance genes and QTLs have been identified and provide valuable resources for developing broad-spectrum and/or durable resistance against BB in rice breeding programs.

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The Disease and Pathogen

BB, also called "kersek" at early growth stage of the plant, is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and is one of the oldest known crop diseases. It was first reported by the farmers of Fukuoka (Japan) in 1884 (Yamanuki et al. 1962). Subsequently, it was found in various parts of Asian countries, Australia, African countries, and the United States. BB occurs in both temperate and tropical regions, but outbreaks are more frequent in irrigated and rainfed lowland areas. Severe epidemics often occur with strong winds and continuous heavy rains (Ou 1985). *Xoo* may be seed-borne and can be spread by irrigation water, but this is disputed (Mizukami 1961; Premalatha and Devadath 1983). The pathogen may survive on infected cultivated rice plants or other hosts (wild rice and gramineous weeds) over winter (Ou 1985). Under favorable conditions, *Xoo* invades rice leaves through hydathodes or wounds, multiplies in the intercellular space of the underlying epithem, and spreads into the plant through the xylem vessels, resulting in yellow lesions with wavy margins along the veins that may systemically extend to the sheath (Figures 2.1A, 2.1B). BB is observed on both seedlings and adult plants and peaks at the flowering stage.

Xoo is a gram-negative bacterium that is rod-shaped, round-ended, motile, and slime-producing with a polar flagellum. The length and width of individual cells are approximately 0.7 to 2.0 μm and 0.4 to 0.7 μm, respectively. Bacterial colonies on nutrient solid media are yellow, round, and convex (Webster and Gunnell 1992) (Figure 2.1C). *Xoo* is aerobic, catalase-positive, able to produce acids from carbohydrates, and unable to use nitrate. The optimal temperature range for *Xoo* growth is 25°C to 30°C (Bradbury 1984). Identification and classification of the bacterial pathotypes of *Xoo* are helpful for resistance breeding and disease control of BB. However, the morphological, physiological, and biochemical characters of different pathotypes are identical (Reddy and Reddy 1990). Based on the infection responses elicited in rice lines, Japanese *Xoo* strains have been classified into 6 virulence groups (I to VI), Philippines *Xoo* strains have been classified into 10 virulence groups (race 1 to 10), Chinese *Xoo* strains include 7 virulence groups (C1 to C7), and Indian *Xoo* strains can be classified into 13 clusters and 5 broad groups (Ezuka and Horino 1974; Vera Cruz 1984; Fang 1990; Nayak et al. 2008).

The genomes of three *Xoo* strains, including Japanese strain MAFF311018, Korean strain

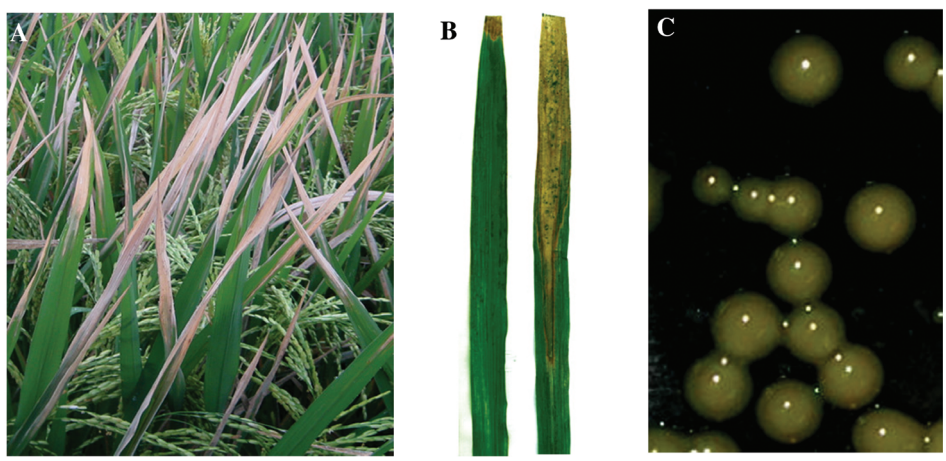

Fig. 2.1. Bacterial blight disease of rice. (A) Rice cultivar infected by *Xoo*. (B) Infected rice leaves after artificial inoculation of *Xoo*. (C) *Xoo* colonies. For a color version of this figure, please refer to the color plate.

KACC10331, and Philippine strain PXO99A, have been sequenced (Lee et al. 2005; Ochiai et al. 2005; Salzberg et al. 2008). The *Xoo* genome is a single circular chromosome of about 50 million bases (Mb), and it contains nearly 5,000 open reading frames (ORFs). It features remarkable plasticity and evolves rapidly. There are large numbers of major rearrangements and indels between the three strains, which contributes to the genomic variation in *Xoo*. This genomic variation explains the diversity of *Xoo* genotypes and pathotypes (Salzberg et al. 2008).

Factors Affecting Pathogenicity of *Xoo*

Key to *Xoo* pathogenicity is the type III secretion system that is encoded by hypersensitive response and pathogenicity (*Hrp*) genes (Boch and Bonas 2010). The *Hrp* gene cluster is necessary for pathogenicity in susceptible hosts and for a hypersensitive response in resistance plants and nonhost plants. In the *Xoo* genome, the *Hrp* gene cluster includes 26 genes that have a high sequence similarity (Ochiai et al. 2005). These genes are regulated by two crucial components, HrpG and HrpX, in the *Xanthomonas* genus. The expression of *HrpX* gene is upregulated by HrpG protein (Koebnik et al. 2006).

The type III secretion system translocates effector proteins into plant cells to support bacterial virulence, proliferation, and dissemination. The largest effector family of *Xoo* is the transcription activator-like (TAL) effector family (also called the avrBs3/pthA family) (Boch and Bonas 2010). A common feature shared by TAL effectors is the central repeat region that consists of 1.5 to 28.5 repeats, with each repeat containing 33–34 amino acids, and contributes to binding the *cis*-elements named UTP (upregulated by TAL effector) boxes of plant gene promoters, the amino-terminal translocation region, the carboxyl-terminal nuclear localization signal, and carboxyl-terminal acidic transcription activator-like domain (Boch et al. 2009; Kay and Bonas 2009; Yuan and Wang 2012). TAL effectors function as specific transcriptional activators in the plant cell nucleus. The specificity of DNA recognition by the TAL effector is determined by the variable amino acids at residues 12 and 13 of each repeat. However, some TAL effectors have been identified as avirulence (Avr) proteins in disease resistance (*R*) gene-mediated *Xoo* resistance (Boch and Bonas 2010).

In addition to the effectors translocated by the type III secretion system, the other important virulence factors of *Xoo* are extracellular enzymes and polysaccharide and a diffusible signal factor (Feng et al. 1996; Büttner and Bonas, 2010; He et al. 2010). The extracellular enzymes, such as endoglucanases, xylanase, cellobiosidase, and esterase, are secreted by the type II secretion system of *Xoo* to degrade the plant cell wall (Büttner and Bonas 2010). The extracellular polysaccharide protects bacteria against environmental stress. Null mutation of *rpfC* in *Xoo* strain T3000 substantially influences the synthesis of extracellular polysaccharide and virulence in rice (Feng et al. 1996). The diffusible signal factor is a cell-cell communication signal, and it can affect the expression of virulence genes (He et al. 2010). Repeats in the structural toxin (RTX toxin), which has functions in biofilm development, cellular adherence, and eukaryotic cell targeting, represent another type of important virulence factors among gram-negative bacteria (Coote 1992; Satchell 2011). Several RTX toxins, including phenylacetic acid, trans-3-methylthioacrylic acid, and 3-methylthio-propionic acid, have been identified in *Xoo* (Noda et al. 1989). Thus, RTX toxins may also be virulence factors of *Xoo*. In addition, the *rax* genes (such as *raxA*, *raxB*, *raxC*, and *raxST*) of *Xoo* are involved in secretion by the type I secretion system and sulfation of peptide Ax21 (activator of *Xa21*-mediated immunity), which elicit rice Xa21 protein-mediated resistance (Lee et al. 2009).

Xoo Resistance in Rice

Overview of Disease Resistance Mechanism in Plants

Physical and biochemical barriers provide a first line of defense against potential pathogen attack. These constitutive defenses include the presence of many preformed barriers such as waxy epidermal cuticles, cell wall, bark, antimicrobial enzymes, and secondary metabolites. However, pathogens have evolved strategies to breach these passive defense barriers. When *Xoo* enters a leaf apoplast through hydathodes or wounds, the plant relies on its innate immune system to detect the invading organisms and activate inducible defenses.

The current view of plant-pathogen interactions has revealed that the innate immune system consists of a two-branched defense response. The first branch is pathogen (microbe)-associated molecular patterns (PAMPs/MAMPs)-triggered immunity (PTI) or basal resistance, which is initiated by the direct recognition pathogen PAMPs through plant pattern-recognition receptors (PRRs) (Jones and Dangl 2006; Boller and Felix 2009). PRRs are plasma membrane proteins. PAMPs, which are essential for microbe fitness or survival, are relatively conserved molecules within a class of microbes during evolution, such as flagellin, peptidoglycan, and lipopolysaccharides. The other branch is effector-triggered immunity (ETI) or race-specific resistance that is activated on direct or indirect detection of pathogen effectors by plant proteins encoded by *R* genes (Jones and Dangl 2006; Thomma et al. 2011). R proteins are either intracellular, plasma membrane, or extracellular, and each of these R proteins recognizes one or a few specific effectors. Pathogen effectors are rapid evolving, which results in loss of function of R proteins.

After the presence of PAMPs or effectors activates PPRs or R protein, the plant receptors transfer the defense signal to downstream components encoded by defense-responsive or defense-related genes, which leads to defense responses. Defense-responsive genes are characterized by their response to a pathogen attack via changed expression levels or posttranslational modifications of their encoding proteins (Kou and Wang 2010). In general, PTI is a relative weak defense response and ETI is a high-level defense response. However, strong PTI and weak ETI have also been reported (Thomma et al. 2011). Furthermore, PAMPs and effectors as well as PRRs and R proteins cannot be strictly maintained, because there is a continuum between PTI and ETI (Thomma et al. 2011). For example, rice *Xa21*-mediated *Xoo* resistance is triggered by a narrowly conserved PAMP, Ax21, and Xa21 protein is considered to be both a PRR and an R protein (Lee et al. 2009). In addition, the defense signaling pathways initiated by PRRs and R proteins are partially overlapping (Kou and Wang 2010).

According to the speed and strength of the plant response to pathogen invasion, plant resistance can be divided into two major categories: qualitative or complete resistance and quantitative or partial resistance. Qualitative resistance is a rapid and high level of defense response mediated by *MR* genes, including *R* and *PRR* genes that confer a high level of resistance. More than 30 *MR* genes that mediate qualitative resistance and have different resistance spectra against *Xoo* have been named. Quantitative resistance is controlled by multiple genes or resistance QTLs and can be broad spectrum and/or durable (Kou and Wang 2010). A large number of resistance QTLs have been identified in the interactions of different rice varieties and *Xoo* strains (Kou and Wang 2012).

In addition to innate immunity, plants have different types of induced resistance, including systemic acquired resistance (SAR) and induced systemic resistance (ISR). Genetic studies in *Arabidopsis* revealed that NPR1 (non-expressor of pathogenesis-related genes 1) is important for SAR, and TGA transcription factors are repressors of SAR (Vlot et al. 2009). Some evidence supports rice having a similar SAR pathway for *Xoo* resistance. Overexpression of rice NH1,

which is a sequence and functional ortholog of *Arabidopsis* NPR1, results in enhanced resistance to *Xoo* (Chern et al. 2005). In rice, NH1 interacts with TGA2.1 transcription factor and negative regulator of resistance (NRR). TGA2.1 negatively regulates basal defense responses to *Xoo* (Fitzerald et al. 2005). Rice NRR negatively regulates SAR in *Arabidopsis* and basal and *Xa21*-mediated *Xoo* resistance in rice (Chern et al. 2005, 2008). It is also known that a rice mitogen-activated protein kinase, MPK6, negatively regulates SAR in rice-*Xoo* interaction (Shen et al. 2010). ISR of plants against pathogens is a widespread phenomenon that activates multiple defense mechanisms including increased activity of pathogenesis-related gene (PR) proteins. Attenuated UV-mutant *Xoo* strains have been documented to induce rice ISR against BB (Thein and Prathuangwong 2010).

Qualitative Resistance to *Xoo*

Asian-cultivated rice (AA genome) consists of two major subspecies, *indica* (*O. sativa* L. ssp. *indica*) and *japonica* (*O. sativa* L. ssp. *japonica)*. At least 37 *MR* genes against *Xoo* have been identified and designated in a series from *Xa1* to *Xa36*, with one symbol having been used for two different genes (Table 2.1). Most of these genes were identified in Asian-cultivated rice while only a few were identified from wild rice species, which were then introgressed into cultivated rice. It is generally accepted that R proteins encoded by dominant *R* genes recognize specific pathogen effectors and initiate defense signal transduction leading to rapid and race-specific disease resistance in most plant-pathogen systems, including rice *R* gene-mediated resistance to fungal pathogen *Magnaporthe oryzae* (Dangl and Jones 2001; Martin et al. 2003; Liu et al. 2010). However, more than one-third of identified *MR* genes against *Xoo* confer recessive resistance, namely *xa5*, *xa8*, *xa9*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa25/Xa25(t)*, *xa26(t)*, *xa28(t)*, *xa31(t)*, *xa33(t)*, and *xa34(t)* (Table 2.1). Only 7 (*Xa1*, *Xa3/Xa26*, *xa5*, *xa13*, *Xa21*, *xa25*, and *Xa27*) of the 37 identified *MR* genes against *Xoo* have been isolated. Most of the characterized *MR* genes encode proteins that are different from the most common R protein, such as nucleotide-binding site (NBS)-leucine-rich repeat (LRR) protein (Liu et al. 2010). This feature suggests that the molecular mechanisms of qualitative resistance in rice-*Xoo* system are more complicated than in other plant-pathogen systems.

Xa1

Xa1, localized on the long arm of chromosome 4, was used in Japanese rice breeding for BB resistance from 1967. It confers resistance to Japanese *Xoo* race I, which is the most dominant race in Japan. *Xa1*, which was cloned by a map-based cloning strategy from the *japonica* rice cultivar Kogyoku and *indica* rice line IRBB1, encodes a cytoplasmic NBS-LRR protein (Yoshimura et al. 1998) (Figure 2.2). The expression of *Xa1* can be induced by *Xoo* and wounding. The induction of expression is speculated to be involved in enhanced resistance to *Xoo* (Yoshimura et al. 1998).

Xa3/Xa26

Xa3/Xa26 gene, localized on the long arm of chromosome 11, was isolated as *Xa26* from an *indica* rice cultivar Minghui 63 (AA genome) with a map-based cloning strategy. It encodes a plasma membrane–localized LRR receptor kinase-type protein with an extracellular LRR domain, a transmembrane motif, and a cytoplasmic kinase domain (Sun et al. 2004). Further study revealed that *Xa3*, a previously named *MR* gene, and *Xa26* are actually the same gene, which was then renamed as *Xa3/Xa26* (Xiang et al. 2006) (Figure 2.2). *Xa3/Xa26* gene confers relatively broad-spectrum resistance to different *Xoo* races; rice cultivars carrying *Xa3/Xa26* gene have been widely used in rice production in China for a long period of time (Xu et al. 2004; Gao et al. 2010; Li et al. 2012). The *Xa3/Xa26* alleles, *Xa3/Xa26-2* from wild rice *Oryza officinalis* (CC genome) and *Xa3/Xa26-3* from the CC

Table 2.1. Summary of major disease resistance genes against *Xoo* in rice

Gene	Resistance to *Xoo* race	Donor cultivar[a]	Chromosome	Reference[a]
Xa1	Japanese race I	Kogyoku, IRBB1	4	Yoshimura et al. 1998
Xa2	Japanese races I and II	IRBB2	4	He et al. 2006
Xa3/Xa26	Chinese, Philippine, and Japanese races	Minghui 63, IRBB3	11	Sun et al. 2004, Xiang et al. 2006
Xa4	Philippine races	IRBB4	11	Sun et al. 2003
xa5	Philippine and Japanese races	IRBB5	5	Iyer and McCouch 2004
Xa6	Philippine race 1	Zenith	11	Sidhu and Noori 1978a
Xa7	Philippine races	IRBB7	6	Chen et al. 2008
xa8	Philippine races	PI231128	7	Sidhu and Noori 1978b
xa9	Philippine races	Sateng	11	Singh et al. 1983
Xa10	Philippine and Japanese races	IRBB10	11	Gu et al. 2008
Xa11	Japanese races	IR8		Goto et al. 2009
Xa12	Japanese and Indonesian races	Kogyoku, Java14	4	Ogawa et al. 1978
xa13	Philippine race 6	IRBB13	8	Chu et al. 2006
Xa14	Japanese races and Philippine races 3 and 5	CBB14	4	Tan et al. 2004
xa15	Japanese races	M41 Harebare mutant		Noda 1989
Xa16	Japanese races	Tetep		Noda 1989
Xa17	Japanese races	Asominori		Ogawa et al. 1989
Xa18	Burmese races	IR24, Miyang23, Toyonishiki		Ogawa and Yamamoto 1986
xa19	Japanese races	XM5 (mutant of IR24)		Taura et al. 1991
xa20	Japanese races	XM6 (mutant of IR24)		Taura et al. 1992
Xa21	Philippine and Japanese races	IRBB21	11	Song et al. 1995
Xa22(t)	Chinese races	Zhachanglong	11	Wang et al. 2003
Xa23	Indonesian races	*O. rufipogon* (CBB23)	11	Zhou et al. 2005
xa24(t)	Philippine race 6	DV86	2	Wu X. et al. 2008
	Philippine race 9	Minghui 63	12	Liu et al. 2011
xa25/Xa25(t)				
Xa25	Chinese and Philippine races	HX-3 (somaclonal mutant of Minghui 63)		Gao et al. 2005
xa26(t)	Philippine races	Nep Bha Bong		Lee et al. 2003
Xa27	Chinese strains and Philippine races 2 to 6	IRBB27	6	Gu et al. 2005
xa28(t)	Philippine race 2	Lota sail		Lee et al. 2003
Xa29(t)	Chinese races	*O. officinalis* (B5)	1	Tan et al. 2004
Xa30(t)	Indonesian races	Y238	11	Cheema et al. 2008
xa31(t)	Chinese races	Zhachanglong	4	Wang et al. 2009
Xa32(t)	Philippine races	C406	11	Zheng et al. 2009
xa33(t)	Thai races	Ba7	6	Korinsak et al. 2009
xa34(t)	Chinese race V	BG1222	1	Chen et al. 2011
Xa35(t)	Philippine races	*Oryza minuta* (Acc. No. 101133)	11	Guo et al. 2010
Xa36(t)	Philippine races	C4059	11	Miao et al. 2010

[a]Rice cultivars or rice lines and references are those reporting the characterization of the genes or fine-mapping the genes.

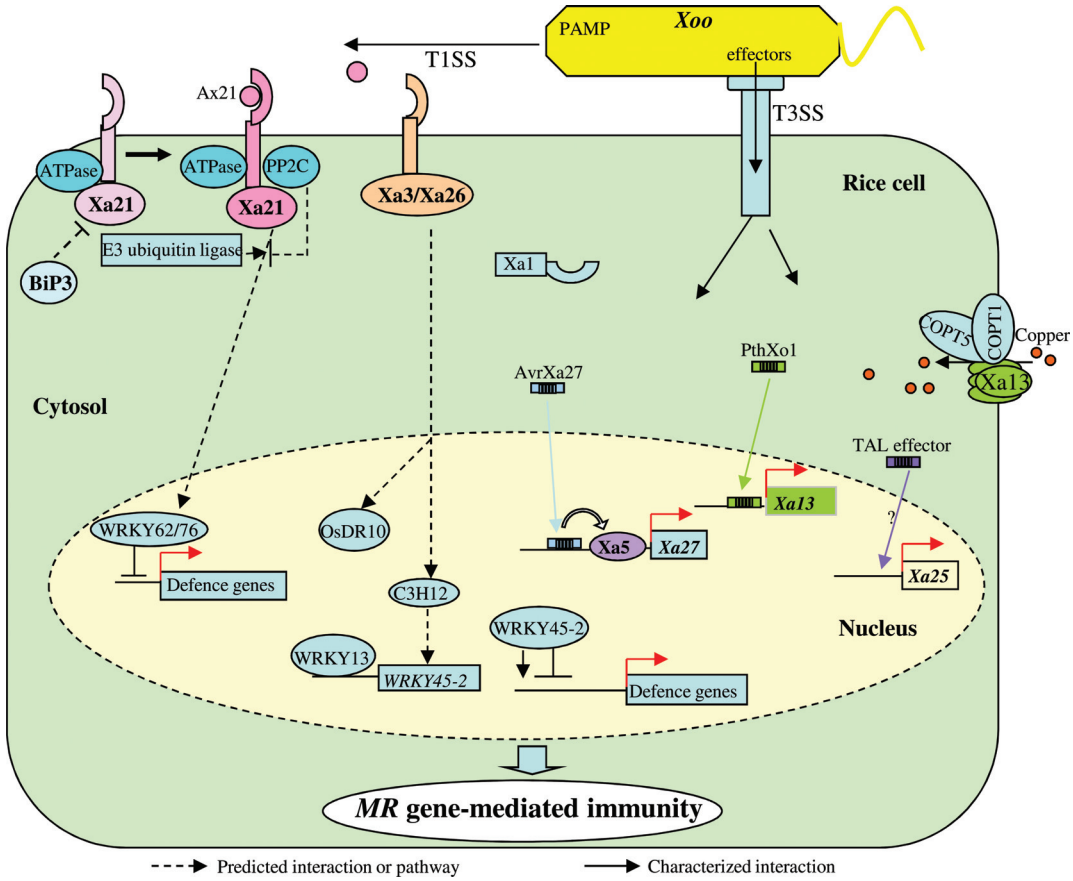

Fig. 2.2. Molecular mechanisms of characterized major disease resistance gene-mediated resistance to *Xoo*. For a color version of this figure, please refer to the color plate.

genome of wild rice *Oryza minuta* (BBCC genome), encode proteins with high sequence similarity to the Xa3/Xa26 protein and can mediate a similar spectrum of resistance against *Xoo* (Li et al. 2012). The speciation of the AA and CC genomes is approximately 7.5 million years ago. These characteristics suggest that the *Xa3/Xa26* locus may confer a durable resistance.

Xa3/Xa26-mediated resistance is influenced by the genetic background and the developmental stage of a plant. This gene confers higher level of resistance in a *japonica* background than in an *indica* background, and rice plants carrying *Xa3/Xa26* gene have full resistance to some *Xoo* strains at both seedling and adult stages, but have full resistance to other *Xoo* strains at adult stage (Yang et al. 2003; Sun et al. 2004; Cao et al. 2007a). Further study has demonstrated that the expression level of *Xa3/Xa26* gene is associated with genetic background- and development-controlled resistance (Cao et al. 2007a; Zhao et al. 2009). *Xa3/Xa26*-mediated resistance is dose dependent: as the expression of *Xa3/Xa26* gene increases, the plant's resistance increases. A *japonica* background facilitates the expression of *Xa3/Xa26* gene compared with an *indica* background. In addition, the expression of *Xa3/Xa26* gene gradually increases with development and reaches the highest level at the maximum tillering to booting (panicle development) stages. Rice plants constitutively overexpressing *Xa3/Xa26* have a high level and broad

spectrum of resistance to *Xoo* at both seedling and adult stages, without any effects on morphology and agronomic performance (Gao et al. 2010). Furthermore, other factors may also contribute to genetic background-controlled resistance conferred by *Xa3/Xa26* gene in addition of the one influencing *Xa3/Xa26* expression (Zhou et al. 2009).

Domain swap analyses suggest that the LRR domain of Xa3/Xa26 protein is an important determinant of race-specific recognition during rice-*Xoo* interaction; in addition, the juxtamembrane region of this protein also appears to contribute to resistance specificity (Zhao et al. 2009). Four components in Xa3/Xa26 protein-initiated defense-signaling pathway have been identified (Figure 2.2). Although they function downstream of Xa3/Xa26 protein in the defense signaling leading to *Xoo* resistance, these components can mediate a broad-spectrum resistance compared with Xa3/Xa26 protein. For example, WRKY45-2, a WRKY-type transcription factor, positively regulates rice resistance to *Xoo*, *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) causing bacterial streak, and *M. oryzae* causing fungal blast (Tao et al. 2009). WRKY13, which is also a transcription factor and functions upstream of WRKY45-2 in the rice-*Xoo* interaction, positively controls rice resistance to *Xoo* and *M. oryzae* (Qiu et al. 2007; Tao et al. 2009). C3H12, a nucleic acid–binding protein upstream of WRKY45-2 in the rice-*Xoo* interaction, promotes rice resistance against *Xoo* and *Xoc* (Deng et al. 2012; Deng H. and Wang S. unpublished data). *OsDR10*, a gene of *de novo* origin and encoding an unknown protein, negatively regulates rice resistance to *Xoo*, and transgenic plants with suppressed expression of *OsDR10* gene have been shown to have broad-spectrum resistance to *Xoo*, including the *Xoo* strain that is compatible with *Xa3/Xa26* gene (Xiao et al. 2009). OsDR10 protein appears to function upstream of WRKY13 in the rice-*Xoo* interaction.

Xa3/Xa26 gene belongs to a tandem clustered multiple gene family, and paralogs of this family have a similar tissue-specific expression pattern (Sun et al. 2006; Xu S et al. 2007; Xu L et al. 2008). One paralog of this family, *MRKa* gene, can mediate partial resistance to *Xoo* when it is overexpressed (Cao et al. 2007b). The kinase domain of MRKa protein can partially replace the function of the kinase domain of Xa3/Xa26 protein in *Xoo* resistance, suggesting that the functions of the paralogs in this family may be partially conserved. This hypothesis is also supported by a recent report that another paralog of this family, *NRKe* gene, regulates rice response to raised temperature (Zhang et al. 2011). The kinase domain of Xa3/Xa26 protein can replace the function of the kinase domain of NRKe protein in response to temperature change.

xa5

The recessive *xa5*, localized on the short arm of chromosome 5, was first identified in varieties of the DZ192 group in 1977 (Iyer and McCouch 2004). It mediates specific resistance to Japanese races and Philippine races 1, 2, 3, and 5 by restriction of bacterial movement, but not multiplication (Iyer and McCouch 2004; Iyer-Pascuzzi et al. 2008). This gene was cloned by a map-based cloning approach combined with allele sequence analysis (Iyer and McCouch 2004), and further complementation testing confirmed this gene (Jiang et al. 2006). The *xa5* encodes a typical gamma subunit of transcription factor IIA (TFIIAγ), which is one of general transcription factors required for transcription by RNA polymerase II (Iyer and McCouch 2004). There are two nucleotide substitutions in the recessive allele, which results in an amino acid substitution of dominant (susceptible) *Xa5* gene. It is speculated that *Xoo* TAL effectors usurp parts of plant basal transcription machinery to regulate rice gene expression; the missense mutation of *xa5* allele does not compromise its general function in transcription, but it may evade TAL virulence functions (Gu et al. 2009; Boch et al. 2010). Thus, *xa5* displays resistance to *Xoo*. *Xoo avrXa5* is an avirulence gene, which encodes a TAL-type protein, corresponding to *xa5* (Zou et al. 2010).

The *xa5* showed a constitutive expression pattern in different tissues, and the resistance of *xa5* is not dose dependent (Iyer and McCouch 2004; Jiang et al. 2006).

xa13

The *xa13* gene localized on the long arm of chromosome 8, originally identified in cultivar BJ1, recessively confers resistance to Philippine *Xoo* race 6 (PXO99) (Ogawa et al. 1987). The *indica* rice line IRBB13 carrying only *xa13* against *Xoo* is resistant to >50% of *Xoo* strains/isolates collected from major rice-growing areas of China and India (Shanti et al. 2001; Singh et al. 2003; Li et al. 2009). This gene was isolated from IRBB13 by a map-based cloning strategy (Chu et al. 2006). The *xa13* and its dominant allele *Xa13*, which is also named *Os8N3* and *OsSWEET11* (Yang B et al. 2006; Chen L et al. 2010), encode polytopic plasma membrane proteins of the MtN3/saliva family (Figure 2.2; Yuan et al. 2010). Promoter swap analysis confirmed that dominant *Xa13* is a susceptibility gene, and the cause of the functional difference of recessive *xa13* and dominant *Xa13* in rice-*Xoo* interaction is their promoter regions (Yuan et al. 2009). The expression of dominant *Xa13* is induced by the direct binding of the TAL effector PthXo1 of *Xoo* strain PXO99 to the *cis*-element, the UPT_{PthXol} box, on the *Xa13* promoter (Yang B. et al. 2006; Römer et al. 2010; Yuan et al. 2011). PXO99 is more sensitive to copper, an essential micronutrient of plants and an important element for a number of pesticides in agriculture, than other *Xoo* strains (Yuan et al. 2010). The Xa13 protein cooperates with two plasma membrane–localized copper transporter-type proteins, COPT1 and COPT5, to promote removal of copper from xylem vessels, where *Xoo* multiplies and spreads to cause disease (Figure 2.2). Thus, the redistribution of copper in rice plants facilitates *Xoo* spread in rice and results in disease. Promoter mutations in dominant *Xa13* result in recessive *xa13* whose promoter lacks the UPT_{PthXol} box. PXO99 cannot induce the expression of recessive *xa13* and the copper level in xylem vessels can suppress *Xoo* growth, which results in rice resistance to *Xoo* infection (Yuan et al. 2010).

Suppressing the expression of dominant *Xa13* can result in the same level of resistance to PXO99 as mediated by recessive *xa13* in rice; suppressing recessive *xa13* can generate plants that are immune to PXO99 (Chu et al. 2006). Since *xa13* recessively regulates rice resistance, suppressing *Xa13/xa13* is one choice to improve rice *Xoo* resistance in hybrid rice-breeding programs. However, both dominant *Xa13* and *xa13* are required for reproductive development. Pathogen-induced or tissue-specific promoters can be used to specifically suppress *Xa13/xa13* in the infection sites.

Xa21

Xa21, localized on the long arm of chromosome 11, was first identified in wild rice *Oryza longistaninata*. It confers resistance to diverse races of *Xoo* from eight different countries and has been used for breeding programs since the 1970s (Wang et al. 1996). This gene was cloned by using a map-based cloning strategy in *indica* rice line IRBB21 (Song et al. 1995). It encodes a plasma membrane–localized LRR receptor kinase protein (Figure 2.2). *Xa21*-mediated resistance is not expressed in the early developmental stages and gradually increases from the seedling stage to later stages, with 100% resistance at the adult stage (Century et al. 1999). The gradually increased expression of *Xa21* gene during rice development is associated with development-controlled *Xa21*-mediated resistance (Zhao et al. 2009). Ectopic expression of *Xa21* gene can generate rice plants with a high level of resistance to *Xoo* at both seedling and adult stages (Zhao et al. 2009; Park et al. 2010b).

Xa21 gene is a single polymorphic determinant that confers resistance to *Xoo* strains expressing *avirXa21* (Lee et al. 2008). Thus, *Xa21* is considered to be an *R* gene. The *avirXa21* gene was isolated and later renamed *Ax21*

(activator of *Xa21*-mediated immunity; Lee et al. 2009). Ax21 protein is conserved across a microbial genus, and a sulfated 17-amino acid synthetic peptide of the N-terminal region of Ax21, axY^{S}22, which is 100% conserved in some pathogens including *Xoo* and *Xoc*, is sufficient for Ax21 activity (Lee et al. 2009). Ax21 protein is consistent with the definition of PAMPs. Thus Xa21 protein is also considered to be a PRR (Lee et al. 2009). This is a typical example of PRR and R protein not being strictly separate (Lee et al. 2009; Thomma et al. 2011).

Several key components of Xa21 protein-initiated defense signaling pathway have been identified. Five Xa21 binding proteins – E3 ubiquitin ligase/XB3, WRKY62/XB10, protein phosphatase 2C (PP2C)/XB15, ATPase/XB24, and Bip3 (also known as glucose-regulated protein 78) – were identified (Fig. 2). The E3 ubiquitin ligase interacts with the kinase domain of Xa21 protein and acts as a substrate for the Xa21 serine and threonine kinase; it is necessary for full accumulation of the Xa21 protein and *Xa21*-mediated immunity (Wang et al. 2006). Xa21 protein binds to WRKY62 when its juxtamembrane motif and serine/threonine kinase catalytic activity are present. WRKY62 functions as a negative regulator in basal resistance and *Xa21*-mediated resistance (Peng et al. 2008). Another WRKY transcriptor, WRKY76, also functions as a negative regulator of *Xa21*-mediated defense (Seo et al. 2011). PP2C, which interacts with the juxtamembrane motif and kinase domain of Xa21 protein, can dephosphorylate autophosphorylated Xa21; it negatively regulates *Xa21*-mediated resistance (Park et al. 2008). ATPase is physically associated with the juxtamembrane motif and kinase domain of Xa21 protein *in vivo*, and it can enhance autophosphorylation of Xa21 protein by its enzymatic activity. Transgenic rice plants overexpressing ATPase are compromised for *Xa21*-meditated resistance because Xa21 protein is degraded via endoplasmic reticulum–associated degradation in the presence of Ax21 protein (Chen X et al. 2010). The endoplasmic reticulum chaperone Bip3 can interact with Xa21 protein *in vivo*. Rice plants overexpressing *Bip3* have decreased Xa21 protein accumulation and inhibited Xa21 protein processing, which results in compromised *Xa21*-mediated resistance (Park et al. 2010a).

Xa21 and Xa3/Xa26 are the same type of proteins and have 53% sequence similarity (Song et al. 1995; Sun et al. 2004). Domain swap analyses have revealed that the defense signaling pathways initiated by Xa21 and Xa3/Xa26 proteins may partially overlap; the LRR domains are important determinants of race-specific recognition of the Xa21 and Xa3/Xa26 proteins (Zhao et al. 2009). Furthermore, the juxtamembrane motifs of the two proteins may also influence the pathogen recognition specificity, in addition to being important for protein stability (Xu et al. 2006; Zhao et al. 2009).

xa25

The recessive gene, *xa25*, localized on the centromeric region of chromosome 12, confers resistance to Philippine *Xoo* race 9 (PXO339). It also encodes a plasma membrane protein of the MtN3/saliva family similar to *xa13* (Liu et al. 2011). The *xa25* gene was isolated from *indica* rice cultivar Minghui 63 by a map-based cloning strategy. The encoding proteins of recessive *xa25* and its dominant allele *Xa25* have eight amino acid differences. Furthermore, there are nucleotide differences in their promoter regions. The expression of dominant *Xa25*, but not recessive *xa25*, was rapidly induced by PXO339 but not other *Xoo* strains that are compatible with recessive *xa25*. The nature of the *xa25*-encoding protein and its expression pattern in comparison with its dominant allele *Xa25* in rice-PXO339 interaction suggest that the dominant *Xa25* may be a race-specific susceptible gene and the recessive *xa25* may be a *Xoo*-induced expressional non-reaction mutant similar to the recessive *xa13*. The rice MtN3/saliva family contains more than 20 paralogs. Some MtN3/saliva proteins from different species can mediate glucose transport (Chen L. et al. 2010, 2012). Further

study is needed to elucidate the biochemical function of Xa25 protein in rice-*Xoo* interaction.

Genetic studies have revealed that recessive *xa25* gene has the nature of dominance reversal; it mediates *Xoo* resistance recessively at seeding stage but dominantly at adult stage (thus it was named *Xa25(t)* in Chen et al. 2002; Liu et al. 2011). However, transgenic rice plants carrying both recessive *xa25* and its dominant allele *Xa25* as a transgene are susceptible to *Xoo* at both seedling and adult stages, confirming that *xa25* is a recessive gene. This dominance reversal of *xa25*-carrying plants is associated with reduction of *Xoo*-induced expression of dominant *Xa25* at the adult stage as compared to *Xa25* expression at seedling stage.

Xa27

Xa27, localized on the long arm of chromosome 6, mediates resistance to diverse strains of *Xoo*, including Chinese *Xoo* strains and Philippine *Xoo* races 2 to 6. It was isolated from *indica* rice line IRBB27 by map-based cloning (Gu et al. 2005). *Xa27* encodes an apoplast protein of 113 amino acids that has no distinguishable sequence similarity to proteins from organisms other than rice (Wu L. et al. 2008). The resistant and susceptible alleles of *Xa27* encode an identical protein, whereas the promoters of this pair of alleles have crucial sequence differences that determine the specific recognition of *Xoo* (Gu et al. 2005). The resistance of *Xa27* is dose dependent. The TAL effector AvrXa27 from *Xoo* induces *Xa27* expression by binding to the UPA box (upregulated by AvrBs3) of the *Xa27* promoter (Boch et al. 2009). However, the recessive *MR* gene *xa5* can attenuate the *Xa27*-mediated resistance in rice, which suggests that *Xoo* TAL effector could not use protein encoded by the recessive *xa5* as a transcription machinery to activation of *Xa27* (Gu et al. 2009).

Fine-Mapped MR *Genes*

In addition to the fine-mapping of the seven characterized *MR* genes, eight other *MR* genes, including *Xa2*, *Xa4*, *Xa7*, *Xa10*, *xa24*, *Xa30*, *Xa31(t)*, and *xa34(t)*, have been fine-mapped. The *Xa2* gene is mapped to an approximately 190-kb region on the long arm of chromosome 4 (He et al. 2006). The *Xa4* gene is defined by a 47-kb DNA fragment on the long arm of chromosome 11, and the *Xa4* locus is linked or tightly linked to the *Xa3/Xa26* locus (Sun et al. 2003, 2004). *Xa7* is located in a 118.3-kb region on the long arm of chromosome 6 (Chen et al. 2008). *Xa10* is mapped to a 74-kb region on the long arm of chromosome 11 (Gu et al. 2008). The *Xa22(t)* is localized to a 100-kb region of chromosome 11, and this locus is also tightly linked to *Xa3/Xa26* locus (Wang et al. 2003). The recessive *xa24* is mapped to a 71-kb DNA fragment on the long arm of chromosome 2 (Wu X. et al. 2008). The *Xa30* is mapped to a 38-kb region on the long arm of chromosome 4 (Cheema et al. 2008). The *Xa31(t)* is limited to a length of about 100 kb on the long arm of chromosome 11 (Wang et al. 2009). The recessive *xa34(t)* is defined to a 204-kb DNA fragment near the centromeric region of chromosome 1 (Chen et al. 2011). The fine-mapping information of these genes will facilitate breeding programs by marker-assisted selection (MAS). Furthermore, the isolation of these *MR* genes will deepen understanding of molecular mechanism underlying BB disease in general, and the opportunity to develop functional markers for more precise breeding.

Quantitative Resistance to *Xoo*

Researchers commonly study quantitative resistance by identifying disease resistance QTLs. At least 74 QTLs against *Xoo* have been identified in different rice cultivars interacting with different *Xoo* strains (Figure 2.3; Li et al. 1999; Luo et al. 1998; Yu et al. 2003; Wang et al. 2005; Li et al. 2006; Yang CD et al. 2006; Hu et al. 2008; Kou et al. 2010; Fu et al. 2011; Deng et al. 2012). These QTLs are distributed on all 12 chromosomes. Several resistance QTLs span a large segment of a chromosome, indicating the poor quality of the data. However, resistance QTLs

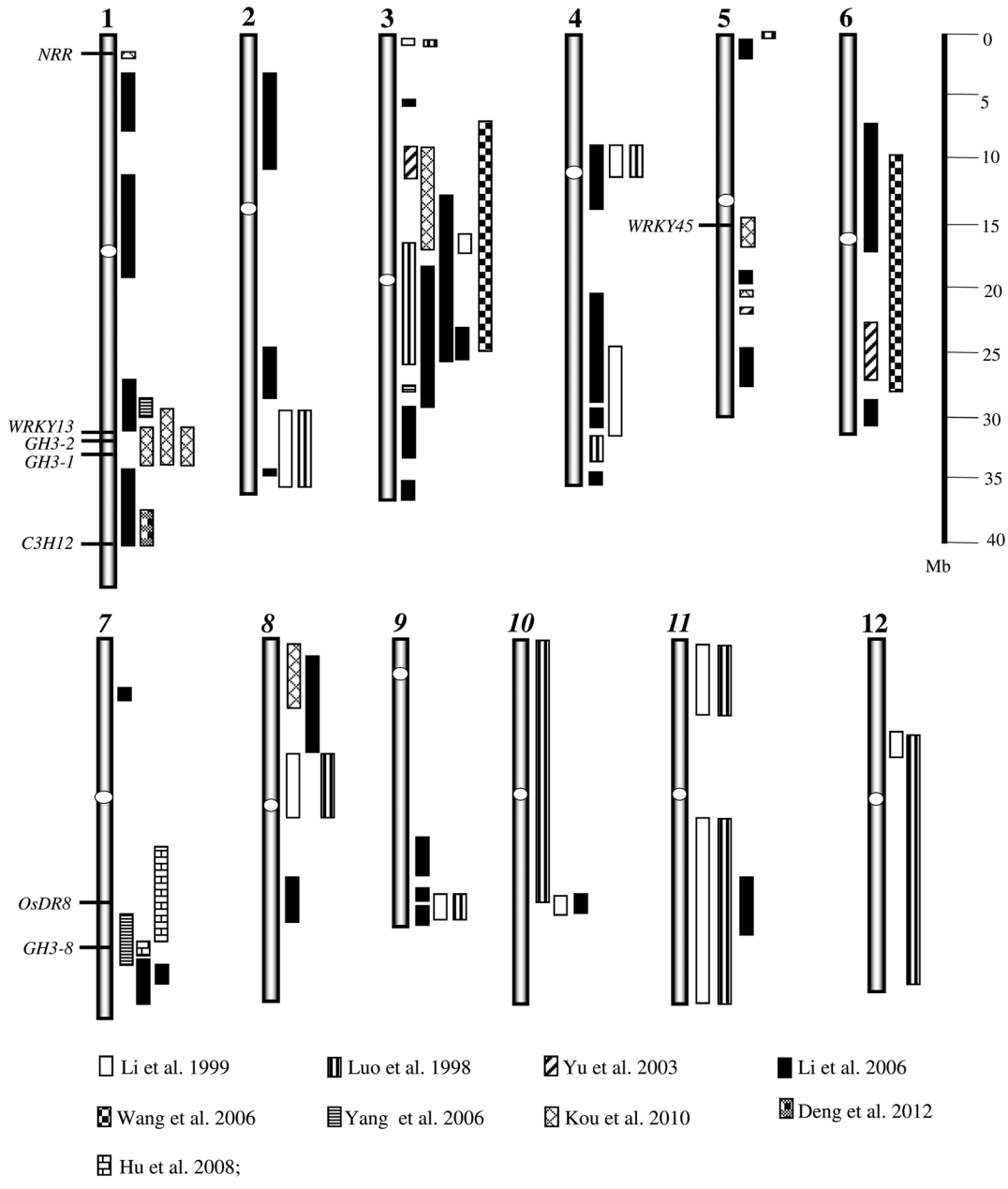

Fig. 2.3. Physical map of rice resistance QTLs to *Xoo*. Chromosome size (million bases) is shown by the scale on the left based on the Gramene database (http://www.gramene.org/markers). The positions of characterized genes contributing to resistance QTL are presented to the left of the chromosomes. The positions of QTLs are indicated with different patterns to the right of each chromosome.

identified by different research groups are frequently colocalized, which suggests the possibility of a real QTL and/or a QTL with a broad spectrum of resistance against *Xoo*.

Characterized Genes Contributing to Resistance QTLs

Recently, great progress has been made in characterizing the genes contributing to resistance QTLs against *Xoo* in rice. At least one major QTL (*WRKY45*) that explains more than 10% phenotypic variation and eight minor QTLs (*NRR*, *WRKY13*, *OsDR8*, *MPK6*, *GH3-1*, *GH3-2*, *GH3-8*, and *C3H12*) that explain less than 10% phenotypic variation against *Xoo* have been characterized (Hu et al. 2008; Kou et al. 2010; Fu et al. 2011; Deng et al. 2012). These genes provide the preliminary information for understanding the molecular basis of rice quantitative resistance to *Xoo*.

All the characterized genes contributing to resistance QTLs against *Xoo* belong to defense-responsive genes based on the features of their encoding proteins and biochemical functions or predicted functions in rice-*Xoo* interaction. The encoding proteins of these genes appear to function in the MR protein–mediated defense pathway or a basal defense pathway either as positive or negative regulators in rice resistance to *Xoo*. *WRKY45* locus has at least two alleles, *WRKY45-1* and *WRKY45-2*, which encode proteins with a 10-amino acid difference (Tao et al. 2009). *WRKY45-1* acts as a negative regulator, whereas *WRKY45-2* is a positive regulator in rice resistance to *Xoo*. As described previously, *WRKY45-2*, *WRKY13*, and *C3H12* all function in *Xa3/Xa26*-initiated defense signaling pathway and *NRR* functions in *Xa21*-initiated defense signaling pathway in rice-*Xoo* interactions. *OsDR8* encodes an enzyme-like protein involved in thiamine biosynthesis (Wang et al. 2006). *OsDR8*-suppressing plants showed compromised resistance to *Xoo* accompanied by reduced thiamine level; exogenous application of thiamine restored the resistance of the transgenic plants, suggesting that accumulation of thiamine regulated by *OsDR8* may be required for resistance to *Xoo*. Mitogen-activated protein kinase (MAPK) cascades have a pivotal role in PTI and ETI. Several rice MAPKs have been reported to function either as an activator or a suppresser in rice resistance to *Xoo*. The MPK6 is a two-faced player in rice-*Xoo* interactions; it functions as a positive regulator in local resistance to *Xoo*, whereas it is a negative regulator for SAR after *Xoo* infection (Shen et al. 2010). Auxin facilitates *Xoo* invasion of rice (Ding et al. 2008; Fu et al. 2011), and indole-3-acetic acid (IAA) is a major form of auxin in most plants. *GH3-2* and *GH3-8* encode IAA-amido synthetases that deactivate IAA by conjugating it to an amino acid, which prevents IAA-induced loosening of the cell wall, the natural protective barrier of plant cells against pathogens. Thus, *GH3-2* and *GH3-8* contribute to basal resistance. *GH3-1* may function in the same way as *GH3-2* and *GH3-8* in rice resistance against *Xoo* (Domingo et al. 2009; Kou et al. 2010).

More than one gene may contribute to a resistance QTL. *WRKY13* and *GH3-2*, which are tightly linked on rice chromosome 1, may collectively contribute to a minor resistance QTL conferring resistance to *Xoo* (Fu et al. 2011).

Other Genes Contributing to Quantitative Resistance

A number of defense-responsive genes have been reported to positively or negatively regulate partial resistance to *Xoo*, such as *NH1*, *XB3*, *TGA2.1*, *Spl11*, *WRKY*62, *WRKY71*, *WRKY76*, *MPK5*, *MPK12*, and *Rac1*. However, their association with resistance QTLs to *Xoo* has not been reported (Kou et al. 2010). As previously described, *NH1* and *TGA2.1* are involved in SAR, and *XB3*, *WRKY62*, and *WRKY76* function in a *Xa21*-mediated defense pathway. The mutant of *Spl11* confers broad-spectrum resistance to both *Xoo* and *M. oryzae* (Zeng et al. 2004). Overexpression of *WRKY71* in rice resulted in constitutive expression of *NH1* and *PR1b* and

enhanced resistance to *Xoo* (Liu et al. 2007). Two MAPK genes also regulate quantitative resistance. *MPK5*-suppressing rice plants display increased resistance to *Xoo* (Xiong and Yang 2003). MPK12 (BWMK1) positively regulates rice resistance to *Xoo* (Seo et al. 2011). Another key component of rice defense signaling, Rac1, which is a small GTPase of Rho type, functions in basal resistance to *Xoo* as a regulator of reactive oxygen species and programmed cell death (Ono et al. 2001).

In addition, *R*-type gene or defeated *R*-type gene also contribute to quantitative resistance. Activation of *MRKa*, a member of the *Xa3/Xa26* gene family, displayed partial resistance to *Xoo* (Cao et al. 2007b). A member of the *Xa21* gene family, *Xa21D* that encodes only the LRR domain of Xa21 protein, confers partial resistance to *Xoo*, and its resistance spectrum is identical to that of *Xa21* gene (Wang et al. 1998). The *MR* gene *Xa4*, which confers qualitative resistance to Philippines *Xoo* race 1 and 4, can act as a recessive QTL and mediate partial resistance against new virulent *Xoo* races (Niño-Liu et al. 2006). The recessive *xa5* mediates qualitative resistance to Philippine race 1, 2, 3, and 5, but it also has moderately resistance to Philippine race 4 (Wan and Zheng 2007).

Control of Bacterial Blight

Agronomic Practices for Disease Management

Different strategies including integrated disease management combining cultivation methods, chemical control, and biological control have been used to combat this disease. Weed hosts, volunteer seedlings, rice stubble and ratoons, and infected plants are important sources of *Xoo* inoculums. Thus, utilizing pathogen-free seed, removing contaminated sources to keep field clean, and allowing fallow fields to dry are the control options to suppress inoculums.

Chemical control of *Xoo* in rice fields began in the 1950s (Niño-Liu et al. 2006). An ideal agent for chemical control is a pesticide that effectively kills *Xoo* or inhibits its multiplication. Although pesticides are efficient in controlling BB, they can lead to environmental contamination and pesticide-resistant pathogens. Biological control is accomplished by using antagonistic organisms, such as *Bacillus* species, to protect rice plants. In contrast to chemical control, biological control is a more environmentally friendly and cost-effective method.

Breeding for Rice Resistance to Bacterial Blight

Although the agronomic practices are useful in controlling BB, most of these strategies are labor intensive. Utilization of resistant varieties with agricultural management practices is a more effective way to control BB. Conventional breeding is irreplaceable in resistance breeding. It is achieved by hybridization and phenotypic selection, in which the experience of breeders plays a major role. In the past, *MR* genes and resistance QTLs have been used in rice improvement by conventional breeding. However, conventional breeding is painstaking and time-consuming and may not be applicable for certain types of quantitative resistance (Kou and Wang 2010, 2012).

In the last decades, rice genomic research has generated a wealth of information about gene function. These advances are now accessible for rice improvement and have been applied in MAS and genetic engineering in breeding programs. MAS can be a “shortcut” in breeding programs because it reduces the number of generations that must be developed to have a viable product that can be released to the farmers; it also can make conventional breeding more efficient by using genetic markers. This technology has already proven to be a useful tool for rice breeding to control BB. Minghui 63(*Xa21*) carrying the *MR* gene *Xa21* is the first BB-resistant rice cultivar developed by MAS in China (Chen et al. 2000). MAS is also effective for pyramiding more than one *MR* gene in rice improvement. A MAS-developed rice cultivar, Tubigan 7, which has an IR64 background and was introgressed with three *MR* genes (*Xa4*, *xa5*, and

Xa21), was released in the Philippines (Toenniessen et al. 2003). "Angke" and "Cone," which carry *Xa4* + *xa5* and *Xa4* + *Xa7*, respectively, are derived from an existing popular variety by using MAS; both cultivars are well accepted by farmers and consumers in Indonesia (Jena and Mackill 2008). An aromatic resistance cultivar developed by MAS is Pusa 1460 (Improved Pusa Basmati 1), and this Indian aromatic germplasm harbors *xa13* and *Xa21* (Gopalakrishnan et al. 2008). Another successful application of MAS to improve resistance to BB is RP BIO 226 (Improved Samba Mahsuri). It was derived from introgression of *xa5*, *xa13*, and *Xa21* into a premium quality rice variety (Samba Mahsuri) in India (Sundaram et al. 2008). QTLs are valuable resources for durable and broad-spectrum resistance. MAS can provide an efficient approach to using major resistance QTLs for rice improvement. However, the success of this approach depends on the genetic backgrounds (Kou and Wang 2010) and the nature of the effect being introgressed. MAS also may not be applicable for employing minor resistance QTLs in breeding because it is costly; thus it might not be worth to pursue QTLs with small effects. In addition, pyramiding multiple QTLs may bring undesired traits into an improved cultivar due to linkage drag.

Genetic engineering provides another opportunity for rice breeding with capacity to break the reproductive isolation between species and make the best use of germplasm resources. Genetic engineering can also make the best use of a gene to improve BB resistance. For example, *Xa3/Xa26*-mediated resistance is influenced by the developmental stage and the genetic background. Expression of *Xa3/Xa26* using a constitutive promoter can enhance *Xa3/Xa26*-mediated resistance in an unfavorable genetic background and generates resistant rice plants at both seedling and adult stages but without influencing agronomic performance (Cao et al. 2007a; Gao et al. 2010). A transgenic approach may also be applied in improving BB resistance by using a single minor resistance QTL. For example, *WRKY13*, *GH3-2*, *GH3-8*, *OsDR8*, or *C3H12* can be used alone for the improvement of rice BB resistance by manipulating its expression with appropriate promoters (Hu et al. 2008; Kou and Wang 2010; Deng et al. 2012). The transgenic approach may especially enable the use of resistance QTLs whose functions depend on upstream signaling in an unfavorable genetic background.

Conclusion and Future Prospects

In the last decade, researchers have made substantial progress in trying to understand the interaction between rice and *Xoo*. A number of genes contributing to qualitative and quantitative resistance against *Xoo* have been characterized. These genes provide a basis for further exploring the defense signaling network and elucidating the molecular mechanisms of rice resistance. These genes also provide multiple choices for rice resistance improvement by different approaches.

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Chapter 3

The Genetic Basis of Disease Resistance in Maize

Tiffany Jamann, Rebecca Nelson, and Peter Balint-Kurti

Abstract

This chapter presents an overview of diseases important to global maize production, outlines the current understanding of the genetic underpinnings for resistance to these diseases, and explores how these findings can be used to improve maize. With a primary focus on fungal diseases, we review the current understanding of qualitative and quantitative resistance. In order to dissect the genetics of quantitative resistance to three important diseases, new datasets and resources have been utilized. A number of populations have been evaluated for various maize diseases, including bi-parental populations, association mapping panels, and the nested association mapping population. By generating lists of genes suspected to be involved in the interaction between plant and pathogen, both genome-wide association mapping and nested association mapping have provided hints about the biology of disease resistance. As part of the study of the architecture of disease resistance, both single-disease resistance and multiple-disease resistance have been explored. Multiple-disease resistance is rare, but some genes apparently confer resistance to multiple pathogens. As high-resolution mapping becomes available, the challenge remains to translate this knowledge into breeding outcomes. Marker-assisted selection can be used to utilize these results, but there is a disconnect between the wealth of mapping information and the application of this data. Genomic selection is emerging as a powerful tool for maize improvement. The challenge, however, remains to apply mapping studies and basic biology to plant breeding to decrease the amount of maize lost to pathogens.

Introduction

Biotic stresses constrain maize production worldwide, affecting food security and prices. Population growth and the use of grain for biofuels spurs demand, while climate variability, as well as rising costs of fertilizer and water, challenge supply. Crop losses caused by maize diseases worldwide, excluding viruses, have been estimated at 4–14% of global annual harvest (Oerke 2006). It is thus increasingly important to reduce losses due to diseases. Several pathogens cause grain yield losses, whereas others contaminate maize seeds with mycotoxins, a widespread hazard to human and animal health (Wild and Gong 2010).

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Because of maize's importance, its genetics and biology have been the focus of considerable research effort in the public and private sectors. A number of advanced breeding and genomic resources have been developed for understanding the genetics of resistance in maize, including populations derived from bi-parental crosses (e.g., Coe et al. 2002; Szalma et al. 2007; Belcher et al. 2012), several association mapping panels (Flint-Garcia et al. 2005; Yan et al. 2011), a nested association mapping population (McMullen et al. 2009; Yu et al. 2008), and large genomic and sequence datasets (Ganal et al. 2011; Gore et al. 2009). These resources have been utilized, at least to some extent, to better understand the genetic architecture of resistance for multiple diseases (e.g. Kump et al. 2011; Poland et al. 2011; Wisser et al. 2006; Wisser et al. 2011).

With its advanced genetic and genomic resources, maize can be used both as a model system for understanding plant-pathogen interactions and as a practical system in which these basic biological findings can be applied in breeding programs to address farmers' production constraints. The challenge is to produce more resistant varieties in the context of various scientific and resource constraints and within the organization of the global maize breeding infrastructure. The purpose of this chapter is to summarize the current understanding of the genetic basis of disease resistance in maize and to note some of the challenges and frontiers in its application.

Understanding the Intruders: Diseases of Maize

Historically, maize has suffered major losses due to disease, with perhaps the best-known epiphytotic being the southern leaf blight (SLB) epidemic caused by *Cochliobolus heterostrophus* in 1970-71 in the United States. At the time of the epidemic, the *T-urf13* gene conferring cytoplasmic male sterility was widely used in maize hybrid seed production. About 85% of the U.S. maize crop carried this gene in 1970 (Ullstrup 1972). As it turned out, *T-urf13* also conferred specific hyper-susceptibility to a toxin produced by *C. heterostrophus* race T (Wise et al. 1999). The ensuing SLB epidemic of 1970 was one of the most economically damaging plant disease epidemics of all time: yield loss throughout the United States for that season was estimated at 20-30%, with some areas suffering 50-100% losses (Ullstrup 1972). The amount of maize lost to the disease was much larger than, for instance, the amount of potato lost during the Irish late blight epidemic of the 1840s. Because susceptibility was under very simple genetic control, simply switching to germplasm lacking *T-urf13* was sufficient to rapidly control the disease in the following seasons. It should be noted that the cause of the epidemic was not an overall lack of genetic diversity, but rather the ubiquity of a single-disease susceptibility gene within elite germplasm.

Today, global maize diseases that pose threats to yield and human health include fungal diseases that attack the leaves, stem, and ear (Balint-Kurti and Johal 2009). Globally important foliar diseases include southern leaf blight (SLB) caused by *C. heterostrophus*, southern rust caused by *Puccinia polysora*, common rust caused by *Puccinia sorghi*, northern leaf blight (NLB) caused by *Setosphaeria turcica*, and gray leaf spot (GLS) caused by *Cercospora zeae-maydis* and *Cercospora zeina.* Diplodia and Fusarium stalk and ear rots and Fusarium and Aspergillus kernel and ear rots are also important in many regions. Diseases of regional importance include tar spot complex (caused by *Phyllachora maydis* and *Monographella maydis*) in Latin America and maize streak virus (MSV) in Africa (Shiferaw et al. 2011). In addition to established diseases, emerging diseases, such as banded leaf and sheath blight (BLSB) in Asia (Pingali 2001) caused by *Rhizoctonia solani*, also pose potential future constraints to maize production. Maize diseases have been reviewed elsewhere more extensively (Balint-Kurti and Johal 2009; Pratt and Gordon 2006; White 1999).

The emergence of some diseases is related to changes in farming practices and production. For example, the increase in BLSB is correlated with an increase in maize production near rice paddies (Pingali 2001). *R. solani*, causal agent of BLSB, has a broad host range, and isolates virulent on rice can also infect maize (Pascual et al. 2000). Reduced tillage, which allows inoculum to overwinter in stalk debris on the soil surface and to reinfect maize the following season, has increased the distribution and severity of diseases such as GLS (Latterell and Rossi 1983; Ward et al. 1999).

In some cases, agronomic techniques or biocides are used to manage diseases. Diversity at the population level can be used to suppress disease progress (e.g., Mundt 2002), so synthetic populations or other open-pollinated varieties may present opportunities for deployment of population-level genetic diversity for disease management. Varietal resistance is, however, the dominant and most convenient approach to disease management in crops in general, as well as in maize. For purposes of this chapter, we consider genetic resistance in the context of analyzing or breeding individual genotypes. The overall genetic diversity available within maize is high compared to most crop species (Goodman 1983; Tenaillon et al. 2001; Sachs et al. 2009), and the primary gene pool is a rich source of disease resistance alleles for crop improvement. While the genetic diversity among elite hybrids is relatively low (Smith et al. 1992), diverse exotic germplasm can be utilized to identify and introduce novel disease resistance genes and alleles into maize varieties (Goodman 1999).

Understanding the System: Genetic Architecture of Disease Resistance in Maize and Biological Insights

Plant disease resistance is often categorized as qualitative (complete) or quantitative (partial) based on the extent of disease in a "resistant" interaction (Vanderplank 1968). While these categories are often presented as quite distinct, there is a gray area between these types of resistance in practice (Poland et al. 2009; see discussion later in the chapter). A number of qualitative resistance genes (often referred to as major genes) have been cloned in a number of different plant systems (e.g. Ellis et al. 2000; Sanseverino et al. 2010). Most major genes function by detecting the presence or the activity of pathogen-derived proteins and then inducing a rapid, localized defense response (called a hypersensitive response) at the point of infection, which limits pathogen growth (Bent and Mackey 2007). This type of defense is often referred to as effector-triggered immunity or ETI (Jones and Dangl 2006), and major genes of this type have been referred to as R-genes. R-genes generally provide high levels of resistance and are easy to manipulate in breeding programs. However, they generally provide only race-specific resistance and are often easily overcome by the pathogen, such that they are typically not durable in an agricultural context (McDonald and Linde 2002).

At least 17 qualitative resistance genes have been identified and mapped for several diverse maize diseases, including maize streak virus, NLB, and southern and common rust (Wisser et al. 2006). Four of these genes (*Rp1*, *Rp3*, *Rxo1*, and *Hm1*) have been cloned. *Rp1*, *Rp3*, and *Rxo1* all carry the domain typical of R-genes, the nucleotide binding site-leucine rich repeat (NBS-LRR) (Collins et al. 1999; Webb et al. 2002; Zhao et al. 2005). *Rp1* and *Rp3* confer resistance to specific races of common rust while *Rxo1* confers resistance to bacterial stripe of maize. *Hm1*, which confers resistance to *Cochliobolus carbonum* race 1, encodes an NADPH-dependent HC-toxin reductase that detoxifies HC-toxin produced by the fungus (Johal and Briggs 1992). Interestingly, the *Hm1* gene is extremely widespread in maize and consequently only a few lines that lack the *Hm1* gene are susceptible to *C. carbonum* race 1. Furthermore, genes with high homology to *Hm1* are present in all grass genomes tested. Specific

silencing of the *Hm1* homolog in barley rendered the plant susceptible to *C. carbonum* race 1. *Hm1* appears to have evolved early in the grass lineage, possibly under selection for resistance to HC-toxin (Sindhu et al. 2008).

Quantitative, or partial, disease resistance is generally controlled by multiple loci, each with relatively small effects. In general, this form of resistance is more durable in the field than qualitative resistance and is therefore agronomically important (McDonald and Linde 2002). The underlying mechanisms associated with quantitative disease resistance in plants are not well understood. To date, the identity of five quantitative genes or gene clusters associated with disease resistance in plants have been determined (Broglie et al. 2006; Fu et al. 2009; Fukuoka et al. 2009; Krattinger et al. 2009; Manosalva et al. 2009). These genes appear to be unrelated and confer resistance by a variety of mechanisms, although these mechanisms are not entirely clear at this point. They include an NBS-LRR gene (Broglie et al. 2006), a START kinase (Fu et al. 2009), an ABC transporter (Krattinger et al. 2009), a proline-rich protein of unknown function (Fukuoka et al. 2009), and a family of germin-like proteins (Manosalva et al. 2009). This diversity of gene classes is consistent with the emerging consensus that variation in quantitative disease resistance in plants is likely based on variation in genes involved in a number of different mechanisms and pathways (Kliebenstein and Rowe 2009; Poland et al. 2009).

Generally, disease resistance quantitative trait loci (dQTL) are thought to be race nonspecific (Vanderplank 1968), but there are multiple examples of race-specific QTL (e.g., Kolmer and Leonard 1986; Leonards-Schippers et al. 1994; Marcel et al. 2008; Qi et al. 1999; Talukder et al. 2004). Therefore, to ensure the effective deployment of dQTL, it is important to assess the effectiveness of the resistance with respect to the pathogen populations against which the resistance is intended to perform. Preliminary assessments can be made by testing source germplasm and/or derived lines with pathogen isolates considered to represent the target population. Candidate germplasm should be tested over a number of different environments to ensure as much as possible that the resistance is broadly effective.

Numerous dQTL studies in maize have been carried out. Genotypic variation has been associated with variation in resistance to all classes of disease, including viral, bacterial, and fungal leaf blights, ear rots, and stalk rots (e.g. Ali et al. 2005; Brown et al. 2001; McMullen et al. 1994; Ming et al. 1997; Paul et al. 2003; Pernet et al. 1999; Robertson-Hoyt et al. 2006; Xia et al. 1999). A synthesis of 50 studies reporting the locations of 437 QTL associated with resistance to 19 maize diseases identified QTL on both arms of all 10 maize chromosomes (Wisser et al. 2006). The composite map showed 89% of the maize genome to be associated with dQTL intervals, reflecting the low resolution of the mapping procedures employed, as well as indicating that there are large numbers of dQTL in the maize genome.

In recent years, new resources and datasets have been generated to gain a more precise idea of the genetic architecture underlying quantitative disease resistance in maize. Maize is well suited to association mapping (Yan et al. 2011) due to the high genetic diversity among lines (Liu et al. 2003). The generally low levels of linkage disequilibrium found in maize (Remington et al. 2001) mean that, given an appropriate population and accurate genotypic and phenotypic data, association mapping has the potential to resolve QTL to their causal genes and potentially nucleotides. A number of maize association mapping populations have been developed in the public sector, including a 300-line panel (Flint-Garcia et al. 2005) mentioned below that has been evaluated for NLB, SLB, and GLS (Wisser et al. 2011).

Association mapping in maize was formerly limited to the analysis of candidate genes (i.e., genes already suspected of being important in controlling variation for the trait of interest) (Harjes et al. 2008; Krill et al. 2010; Wilson et al. 2004). The increasing quantity of genotypic

information now permits genome-wide association studies (GWAS), in which the entire genome is scanned for marker-trait associations in an unbiased way (Belo et al. 2008; Cook et al. 2012). One difficulty with GWAS is that the multiple test corrections associated with the very large number of tests conducted lead to very high significance thresholds, such that even for traits with high heritabilities, few significant 'hits' may be identified. In a GWAS of kernel starch, protein, and oil traits, with broad-sense heritabilities ranging from 83% to 91%, no significant associations were identified after multiple test corrections (Cook et al. 2012). Significant or otherwise intriguing GWAS "hits" need to be validated independently, using mutants, transgenics, and/or fine-mapping studies.

Another new breeding tool utilized to understand the genetic architecture of disease resistance in maize is the nested association mapping (NAM) population (McMullen et al. 2009; Yu et al. 2008). The NAM population consists of 25 linked recombinant inbred populations of ~200 lines each. Each of these populations is derived from a cross between B73 and one of a set of 25 diverse lines. Analyzed as a single population, the NAM population has unprecedented mapping power due to its large size (~5,000 lines) and the effective combination of linkage and linkage-disequilibrium approaches (Yu et al. 2008). This, in theory, allows resolution to the single-gene level (Cook et al. 2012; Poland et al. 2011; Tian et al. 2011). The NAM population has been evaluated for SLB, NLB, and GLS (Benson et al. 2011; Kump et al. 2011; Poland et al. 2011). The genetic architectures controlling variation in resistance to SLB and NLB were found to be broadly similar: 32 and 29 dQTL were identified for the two diseases respectively. These dQTL were of relatively small effect and no epistatic interactions were identified. In these respects, the genetic architectures controlling variation in SLB and NLB resistance were similar to those controlling other quantitative traits, including flowering time and various leaf and kernel composition traits that have been analyzed in this population (Buckler et al. 2009; Cook et al. 2012; Tian et al. 2011).

The results of NAM GWAS provide a preliminary look at the genes that may underlie the trait of quantitative disease resistance. GWAS revealed more than 200 associations with specific SNPs for SLB and NLB resistance traits in the NAM population. It is likely that in many cases, the SNPs identified are at or very near to the actual causal genes (Cook et al. 2012; Tian et al. 2011). For both NLB and SLB, many of the associated SNPs were within or adjacent to genes that have been previously implicated in disease resistance or the defense response. For NLB, genes implicated by GWAS included many defense candidates including those encoding serine-threonine protein kinases, receptor-like kinases, antifreeze proteins, a germin protein, and an ABC transporter, among others. Results were similar for SLB, with candidate genes including those encoding serine-threonine kinases, an ABC transporter, a GST, and an LRR receptor kinase, among others. The identification of receptor-like kinases as candidate genes for quantitative resistance loci for both diseases is consistent with the hypothesis that modest levels of resistance are associated with host recognition of conserved pathogen features. Recognition of "pathogen-associated molecular patterns" (PAMPs) has been linked to disease resistance in several cases and is associated with partial restriction of pathogen infection (Bent and Mackey 2007). As previously noted, genes implicated by GWAS need to be confirmed with complementary evidence.

There are several lines of evidence suggesting that some loci may condition resistance to more than one disease (reviewed by Poland et al., 2009; Kou and Wang 2010; Krattinger et al., 2009). Loci conditioning multiple disease resistance (MDR) would make breeding for disease resistance more efficient. In synthesizing the results of 50 mapping studies, Wisser et al. (2006) found that dQTL were non-randomly distributed in the maize genome. At several

loci, dQTL for different diseases were clustered, suggesting the presence of genes conferring multiple disease resistance. Other QTL mapping studies and analyses of introgression lines have provided additional evidence for the existence of MDR genes and loci in maize with respect to a variety of disease combinations (Balint-Kurti et al. 2010; Belcher et al. 2012; Chung et al. 2011; Kerns et al. 1999; Welz et al. 1999; Zwonitzer et al. 2010).

The questions remain as to whether the observed MDR is due to linkage or pleiotropy and whether MDR is the rule or the exception. To address these questions, we examined MDR to three foliar diseases of maize: GLS, SLB, and NLB. These three diseases are caused by fungi in the class Dothideomycetes and share somewhat similar modes of pathogenesis (Beckman and Payne 1982; Jennings 1957). It may be that genes conferring MDR target aspects of the pathogenesis process that are shared among these pathogens. Analyzing the disease ratings for an association panel of 300 diverse lines, Wisser et al. (2011) observed significant genotypic correlations between resistances to the three foliar diseases of maize, supporting the MDR hypothesis. Using an initial dataset of 858 SNPs, these authors reported the association of a glutathione-S-transferase gene with resistance to the three diseases.

Poland (2010) analyzed the correlations among disease ratings for these same diseases among the NAM founders (the 26 parents of the population), across the 5,000 lines of the population, and within the 25 individual families comprising the population. Correlations between the diseases were the highest within the diverse inbred founders, followed by the correlations among the 5,000 recombinant inbred lines (RILs), and correlations within RIL populations were the weakest. The modest disease correlations within RIL populations were not strongly supportive of the MDR hypothesis, suggesting instead that a large proportion of the strong correlations among parental lines could be due to the fact that some of the parental lines (those bred for disease-conducive environments) carry sets of resistance loci for multiple diseases.

A comparison of the QTL identified for SLB, NLB, and GLS resistance in the NAM population allows a fairly explicit examination of the MDR hypothesis. A comparison of QTL and SNPs provides evidence for some loci with pleiotropic effects. In the NAM population, 23 genetic positions were identified for which quantitative resistance loci for two or more diseases co-localized. At these loci, the estimated allele effects from each founder inbred were compared. At seven of these loci, allele effects were positively correlated, as would be expected for MDR genes or loci (Poland 2010). When GWAS results for NLB and SLB were compared, three genes (a predicted leucine zipper transcription factor and two unknown proteins) were identified as carrying SNP loci with significant associations with resistance to both diseases (Kump et al. 2011; Poland et al. 2011).

While there is evidence that some individual loci confer resistance to more than one disease, this phenomenon does not apparently explain the wider trends of pleiotropic QTL and correlated resistances. From our work to date, it seems clear that, at least with respect to SLB, GLS, and NLB, most of the genetic disease resistance and in particular most of the dQTL of larger effect that we observe are disease specific (Zwonitzer et al. 2010). While a number of lines of evidence suggest the presence of MDR genes conferring resistance to SLB, GLS, and NLB, it seems likely that many of these MDR loci individually have relatively small effects and may be below the detection threshold as individual loci (Balint-Kurti et al. 2010).

Both in terms of understanding the nature of resistance and in choosing loci with complementary functions, it would be desirable to know the ways in which different QTL influence the process of pathogenesis. Analysis of specific dQTL using near-isogenic lines (NILs) differing only for a specific locus permits a better understanding of their quantitative and qualitative phenotypes than can be achieved in segregating

families. NILs can be used to validate QTL, test association mapping hits and characterize QTL. Characterization of two NLB QTL on chromosome 1 revealed that they influenced the pathogenesis process in distinct ways. The QTL in bin 6 reduced the pathogen's success during the initial stages of infection, whereas the QTL in bin 2 reduced the extent of the pathogen's invasion of the leaf vasculature (Chung et al. 2010).

Another important question about the agronomic use of disease resistance concerns the trade-offs that may exist with other traits. There are several examples from other plant systems in which the presence of genes associated with both qualitative and quantitative disease resistance incur a yield cost (Heidel et al. 2004; Orgil et al. 2007; Tian et al. 2003; Todesco et al. 2010). The only study addressing this question in maize thus far has been on the yield costs associated with the large-effect dQTL *Rcg1* for anthracnose stalk rot resistance (Frey et al. 2011). In this case, NILs have been used to show that there are no fitness costs associated with *Rcg1* in non-diseased conditions and there is a yield benefit associated with *Rcg1* under inoculated conditions (Frey et al. 2011).

Translating Knowledge to Action: Breeding for Disease Resistance

As new resources and technologies permit the identification of dQTL with unprecedented precision, a key challenge will be translating the knowledge gained from mapping studies into breeding outcomes. This can be achieved through marker-assisted selection (MAS), including genomic selection (GS) and/or cisgenesis (direct transfer within species). Marker-assisted backcrossing and "forward crossing" are well suited for the manipulation of genes with large effects (Holland 2004), while genomic selection is proving particularly useful for improving on traits with low heritability and/or under polygenic control (Heffner et al. 2009).

In the past, the low resolution of mapping results meant that recombination could readily separate the marker being used for selection from the desired allele. The use of "perfect" markers (those targeting the polymorphism that causes the phenotype of interest) avoids this problem (Lande and Thompson 1990). As we know more about the genetic architecture of disease resistance in maize, we come closer to having perfect markers. Historically, there has been a trade-off between conventional breeding approaches and MAS in terms of cost and time, with MAS being faster but more costly and conventional breeding schemes being slower but cheaper (Morris et al. 2003). This led breeding efforts with constrained budgets, including many public breeding programs, to focus on conventional breeding schemes (Morris et al. 2003). However, this is changing as marker technologies improve and genotyping costs decline. In one recent study, MAS in maize was shown to be more cost-effective than phenotypic selection when selecting for resistance to multiple foliar pathogens (Asea et al. 2011) and for maize streak virus (Abalo et al. 2009). The advantages of MAS will become more compelling as genotyping costs continue to decline and more useful trait-marker associations become available for selection. Challenges associated with the effective deployment of MAS have been discussed in several recent reviews (Holland 2004; Hospital 2009; Johnson 2004).

When a trait is controlled by multiple QTL, or when multiple traits are being considered in a breeding program, a form of MAS known as genomic selection (GS) can be employed (Goddard and Hayes 2007; Meuwissen et al. 2001). In GS, specific loci associated with a trait are not identified and selected for; instead, the effect of every marker is fitted as an effect in a linear model and genome-wide marker information is used to make selections. The availability of low-cost, high-throughput genotyping methods has made GS a feasible and attractive form of MAS. Using simulations, GS was predicted to result in up to a 43% greater genetic gain over

marker-assisted recurrent selection, depending on levels of heritability and number of QTL (Bernando and Yu 2007). For polygenic traits with low heritability in maize, both GS and MARS outperformed phenotypic selection in terms of genetic gains (Bernardo and Yu 2007). GS has the potential to improve disease resistance traits in plants. Its use has been proposed for achieving durable stem rust in wheat (Rutkoski et al. 2011), and it has the potential to increase genetic gain for traits with low heritability (Heffner et al. 2009). Thus, GS could improve gains for resistance to maize diseases, including those such as *Aspergillus* ear rot and aflatoxin accumulation, for which it is notoriously difficult to achieve genetic gains and for which dQTL have small effects and are highly influenced by the environment (Brooks et al. 2005; Paul et al. 2003; Warburton et al. 2009).

GS is gaining favor in maize breeding as high-throughput genotyping and extensive phenotypic datasets are generated. Because GS does not require the identification and careful characterization of loci and genes associated with variation in traits of interests, it may seem that QTL identification and characterization (previously seen as the basis of MAS) is now unnecessary. However, basic science, plant pathology, and QTL mapping can still inform breeding programs (including GS programs) in several ways. These include understanding the mechanisms of resistance, improving phenotyping methods, and identifying sources of diverse alleles. An understanding of the mechanisms of resistance associated with specific dQTL can be used to predict complementary combinations of loci and alleles. For instance, knowing that one dQTL is associated with variation in susceptibility to penetration, while another is associated resistance to vascular invasion (Chung et al. 2010), can allow a breeder to target both stages of pathogenesis by selecting for both QTL. Analysis of phenotyping methods and development of new phenotypic assays can also enhance breeding efficiency. For example, a method developed by Mideros et al. (2009) to estimate *A. flavus* biomass by qPCR enables the separation of components of resistance such as fungal infection in multiple tissues and aflatoxin accumulation (Mideros 2012). Presumably, by breeding for components of resistance, difficult traits can be improved.

By identifying and characterizing new, diverse alleles at mapped loci and characterizing the effects they can have on disease, public sector research into the genetic architecture of disease resistance can prove useful. The NAM analysis allowed outstanding alleles to be identified across a relatively broad set of maize germplasm. These insights might contribute to the strategic selection of parents in a breeding program, as the incorporation of known sources of resistance is essential to the success of GS for disease resistance (Rutkoski et al. 2011). It is important to keep in mind that major genes can mask the effects of minor genes in GS scenarios, such that quantitative resistance is not selected (Rutkoski et al. 2011). When this occurs, breeders could select for resistance-associated loci based on prior knowledge. Basic research has and will continue to define major and minor gene loci, and these data can be incorporated into GS algorithms. Public mapping efforts to identify causative genes and polymorphisms can provide a basis for markers to include in genomic selection models. In addition, while GS populations may not be evaluated specifically for some diseases and traits, it is desirable to include resistance for these diseases.

Disease resistance has long been and remains an attractive target trait for genetically modified crops (Godfray et al. 2010). Despite significant resources devoted to this area, few commercially viable plants with transgenically conferred disease resistance traits are available; the exception are a few virus resistance traits (e.g., Gonsalves 1998). This is due to a combination of factors. Biological considerations include small allele effects, narrow spectra, and potentially short durability of certain transgenically conferred disease resistance traits, as well as their yield costs (see above; Hammond-Kosack and Parker 2003). Nonbiological considerations include the cost of

developing transgenic lines, the availability of specific intellectual property, and public opposition to the deployment of the technology. New insights concerning plant quantitative resistance, plant basal defense mechanisms (Lacombe et al. 2010), and mechanisms and applications of RNA interference (Nowara et al. 2010; Wulff et al. 2011; Yin et al. 2011), however, are likely to lead to some practical applications in the foreseeable future. As more disease resistance alleles are cloned, it may be increasingly feasible to use these alleles via direct gene transfer among maize lines. Transfer of genes within different members of the Poaceae can also be effective: the maize NBS-LRR gene *Rxo1* protects rice against *Xanthomonas oryzae* pv. *oryzae* (Zhao et al. 2005).

Another potential impact of transgenics in maize is the reduction of mycotoxins due to pest resistance provided by transgenic expression of toxins from *Bacillus thuringiensis* (Bt). Bt maize is thought to decrease mycotoxin accumulation because insect damage provides an entry point for the fungus and limiting insect damage in turn decreases mycotoxin accumulation (Dowd 2001). Lower levels of fumonisin have been associated with the use of Bt maize (Hammond et al. 2004). In 2004, the reduction of fumonisin and deoxynivalenol damage through the use of Bt maize was estimated to have an annual economic impact of $17 million (Wu et al. 2004). Bt maize has been shown to reduce aflatoxin accumulation when insect pressure is high (Williams et al. 2002; Williams et al. 2005; Windham et al. 1999; Wu 2006).

Conclusions

To meet increasing demand for maize, yield constraints must be overcome. Diseases pose an important constraint in many parts of the world, including Asia, Sub-Saharan Africa, and Latin America (Pingali 2001). A number of new genomics-based resources have been developed for public maize research in the past few years, and have been employed to better elucidate disease resistance in maize. As a result, the genetic architecture and biology of resistance is better understood, but the challenge remains to translate this knowledge into improved disease resistance in maize varieties.

Understanding the biology of disease resistance can inform the search for genes effective in conditioning resistance, and thus contribute to harnessing genetic diversity for crop improvement. Loci conferring resistance to multiple pathogens are of particular interest and have been identified, but are relatively rare in maize. The mechanisms underlying such resistance and whether there are associated pleiotropic effects affecting other important agronomic traits, such as yield, are thus of fundamental interest. A knowledge of the multiple functions of defense-related genes can thus inform breeding decisions. Understanding the genetic architecture and biochemical pathways that underlie disease resistance will provide a route by which to do so.

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Chapter 4

Genomics-Assisted Breeding for Fusarium Head Blight Resistance in Wheat

Hermann Buerstmayr, Maria Buerstmayr, Wolfgang Schweiger, and Barbara Steiner

Abstract

Fusarium head blight (FHB) of wheat and other small-grain cereals is a devastating plant disease in many parts of the world. The cultivation of resistant varieties plays a key role in integrated management of this disease. FHB resistance in wheat is a truly quantitative trait, governed by polygenes and modulated by the environment. Breeding productive cultivars with superior FHB resistance is therefore not trivial and requires significant investments. In the past decade, numerous studies have been performed to decipher the inheritance of FHB resistance in wheat. Quantitative trait loci (QTL) for FHB resistance have been discovered at all wheat chromosomes. Genomics-assisted breeding for improvement of FHB resistance has been implemented in several breeding programs. Marker-assisted selection for relatively large-effect QTL was an effective approach to introgress known QTL into regionally adapted germplasm. Recent advances in genome-wide selection offer great promise to select for even small-effect QTL in breeding populations. In this chapter we summarize and discuss results obtained in marker-assisted breeding experiments for improvement of FHB resistance in hexaploid and tetraploid wheat.

Introduction

Fusarium head blight (FHB), also known as Fusarium ear blight (FEB) of wheat, has been first described more than a century ago by W.G. Smith in England (cited by Arthur 1891). A complex of several Fusarium species have been associated with the disease. *Fusarium graminearum* (Schwabe) Group 2 [teleomorph, *Gibberella zeae* (Schw.) Petch], *F. culmorum* (Wm. G. Smith) Sacc. and *F. avenaceum* (Corda ex Fr.) Sacc. [teleomorph *G. avenacea* (Cook)] appear as the most relevant species, depending on cultural practices and climatic conditions (Parry et al. 1995). FHB can lead to yield losses due to reduced kernel set and kernel weight and quality losses due to reduced seed and baking qualities. The major problem associated with FHB is contamination of the harvest with fungal secondary metabolites knows as mycotoxins. In order to protect consumers from mycotoxicosis, numerous countries have established maximum allowed levels for the most prevalent Fusarium mycotoxins in cereals and cereal products

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(Van Egmond 2004). As an example, the EU regulation allows a maximum deoxynivalenol (DON) content in unprocessed bread wheat of 1.25 ppm, in bread and bakeries of 0.5 ppm, and in baby food of 0.2 ppm (Anonymous 2005).

In the past two decades, several review articles have been published on Fusarium diseases of cereals covering different aspects. Parry et al. (1995) reviewed the significance of the disease with an emphasis on phytopathological aspects. Goswami and Kistler (2005) provided a comprehensive survey of *Fusarium graminearum*. Reviews of conventional breeding for FHB resistance were published by Mesterhazy (1995), Miedaner (1997), and Mesterhazy et al. (1999). Placinta et al. (1999) documented the worldwide occurrence and significance of Fusarium mycotoxins. The first review on molecular markers for FHB resistance in wheat by Kolb et al. (2001) summarized the early findings in this field, and later Anderson (2007) listed some of the more stable FHB QTL and reviewed the advantages of marker-assisted FHB resistance breeding. A comprehensive monograph edited by Leonard and Bushnell (2003) described a range of aspects of Fusarium diseases of small-grain cereals, including the pathogen, the associated mycotoxins, resistance breeding, and other control options, as well as the social and economic impact of the disease. Bai and Shaner (2004) reviewed the management and resistance to FHB in wheat and barley, including the knowledge on FHB resistance QTL mainly from a North American perspective. Buerstmayr et al. (2012) provided an updated survey of Fusarium resistance breeding by conventional, genomics-assisted, and transgenic approaches.

The disease occurs regularly in many wheat-growing regions with moderate to low severity, depending on weather conditions and agricultural practices. Heavy epidemics with severe yield and quality losses have been reported quite a few times from various regions of the world. As an example, heavy epidemics of FHB have been reported in North America in 1919, 1928, 1932, and 1935 (Stack 2003). An extraordinary series of FHB epidemics struck different areas of North America during the 1990s, resulting in about $1.3 billion of total direct losses and $4.8 billion of losses for the accumulative economic impact of FHB (Johnson er al. 2003). In parts of China, such as the Yangtze valley, severe FHB epidemics have been reported 4 times and moderate epidemics 15 times during the 1957-1984 period (Liu 1985). Even in cases when disease severity is moderate to low and visual symptoms appear at modest incidence, the crop can be contaminated with Fusarium toxins way above the tolerable level.

The Fusarium species involved in FHB live a saprophytic life for the most part of the year. Fusarium is an opportunistic pathogen. Specifically, at anthesis the cereal head becomes vulnerable to Fusarium infection. Airborne or splash-dispersed spores germinate at the glumes and fungal hyphae enter through the open floret and infect living ear tissue. In order to reduce the damage due to Fusarium head blight integrated control measures are needed, aiming at either reducing the disease pressure or avoiding infection even if inoculum is abundant (Parry et al. 1995). Agronomic measures that aim at reducing the amount of inoculum are well known, for instance by removal of plant remains from the previous crop through careful ploughing. However, because of other reasons modern farms nowadays often apply minimum- or no-till practices, which in turn enhance the disease pressure. Chemical control is not straightforward and only moderately effective, because current fungicides slow down Fusarium growth to some extent only and the time window of the fungicide application is very narrow (Mesterhazy 2003), and fungicide applications add to farming costs. Therefore, the use of resistant cereal cultivars has to play a key role in integrated Fusarium control and prevention of mycotoxin contamination of cereals and cereal products, as stated by Placinta et al. (1999: 22): “It is clear that legislation for the control of these mycotoxins is now overdue and that further work is required to exploit cereal genotypes

that are resistant to diseases caused by toxigenic Fusarium phytopathogens."

Until the 1990s, FHB resistance breeding relied entirely on phenotypic selection. This approach is practical, but also resource demanding and time consuming (Spanakakis 2003). In nature the occurrence of Fusarium epidemics depend on the local agronomic practice and weather conditions. Therefore, in a breeding situation, phenotypic selection for resistance is usually done in separate screening nurseries. To make regular progress in selection, artificial inoculation is indispensable in most situations (Ruckenbauer et al. 2001; Dill-Macky 2003). FHB resistance is a complex trait and not one single, simple way of measuring FHB resistance is practiced. The concept of resistance to initial infection (type 1) and resistance to fungal spread from an infected floret along the rachis (type 2) first described by Schroeder and Christensen (1963) is still widely accepted. In addition, further types or components of resistance to FHB have been described (Mesterhazy 1995; Mesterhazy et al. 1999). Specific methods for testing genotypes for the different types of resistance have been proposed (Dill-Macky 2003). Generally an FHB epidemic is provoked by providing infectious material (fungal spores or mycelia) at the proper time of infection (anthesis) and environmental conditions (high humidity) which stimulate the disease. For a more detailed review on inoculation and evaluation methods, see Dill-Macky (2003).

Breeders who included selection for improved FHB resistance in their breeding programs successfully developed improved cultivars for this trait. A common observation was that short-straw cultivars were more FHB susceptible than tall cultivars, indicating a pleiotropic effect of stem-shortening alleles on reducing FHB resistance (Spanakais 2003). Later studies showed that particularly the semi-dwarfing allele *Rht-D1b* and, to a lesser extent, *Rht-B1b* appear associated with increased FHB susceptibility (Miedaner and Voss 2008; Voss et al. 2008; Srinivasachary et al. 2009). Plant height *per se* seems to play a considerable role in this context (Yan et al. 2011). FHB resistance was considered a truly quantitative trait, modulated by polygenes and environmental conditions, but the number and chromosomal location of FHB resistance quantitative trait loci (QTL) remained unknown before the 1990s.

Since the early 1990s, significant research investments have been undertaken to better understand the inheritance of Fusarium resistance in wheat in order to derive knowledge-based and focused breeding strategies. This research effort was inspired through the urgent need to incorporate FHB resistance in regionally adapted cultivars because of startling losses due to FHB epidemics in the 1990s (McMullen et al. 1997; McMullen 2003; Johnson et al. 2003), and at the same time the availability of novel molecular genotyping tools and statistical methods for genetic mapping of polygenes (Tanksley 1993).

A comprehensive synopsis of the knowledge on mapped QTL for FHB resistance was provided by Buerstmayr et al. (2009), who summarized the results from 52 QTL-mapping studies, 9 research articles on marker-assisted selection, and 7 articles on marker-assisted germplasm evaluation. They illustrated the position of published QTL in a consensus linkage map and provided extensive tables summarizing the essential information on FHB resistance QTL. In a QTL meta-analysis, Löffler et al. (2009) used the results from 30 mapping populations and highlighted 19 meta-QTL on 12 wheat chromosomes, which were, to a large extent, in agreement with the results from Buerstmayr et al. (2009). Liu et al. (2009) performed a QTL meta-analysis of FHB resistance in wheat. They grouped FHB resistance QTL into 43 clusters on 21 wheat chromosomes and identified 19 confirmed QTL on 8 chromosomes. A survey of FHB resistance QTL found in European winter wheat was published by Holzapfel et al. (2008). QTL for FHB resistance have been reported on all 21 wheat chromosomes (Buerstmayr et al. 2009; Löffler et al. 2009; Liu et al. 2009).

Genomics-assisted wheat breeding has been reviewed recently by Gupta et al. (2010), who also list a few cases where genomics-assisted breeding for FHB resistance improvement was performed. Gupta et al. (2010) stated that marker-assisted selection (MAS) is being practiced for improvement of a variety of traits in wheat around the world. Marker-trait associations have been discovered for a number of genetically relatively easy but difficult-to-score traits. Hence MAS has been found useful for the improvement of such traits. However, for improvement of highly complex polygenic traits, novel approaches using high throughput and dense genotyping and new selection strategies such as AB-QTL mapping, mapping-as-you-go, marker-assisted recurrent selection, association mapping, and genome-wide selection (GS) should be further developed (Gupta et al. 2010). This statement appears very appropriate for FHB resistance breeding. Assessing genetic differences in FHB resistance is not trivial and requires well-replicated disease resistance tests usually done in specific nurseries. Several reports showed that MAS was efficient for the fixation of a limited number of well-characterized QTL, as outlined in the following paragraphs.

In addition to detectable QTL, FHB resistance is also modulated by an unknown number of medium- to small-effect QTL that usually remain undiscovered in conventional QTL mapping. Selection for such minor resistance factors was only possible through sophisticated phenotypic selection until recently. In order to better exploit the potential of genomics-assisted breeding for polygenic traits, genome-wide selection (GS), first proposed by Meuwissen et al. (2001), offers great promise also for crop plant improvement (Heffner et al. 2009, 2010). So far no peer-reviewed research paper using GS for FHB resistance has been published, but initial results appear highly encouraging (Rutkoski et al. 2012; Hofstetter et al. 2011). Up to now, only a subset of the reported FHB resistance QTL has been independently validated and an even lower number of QTL have been applied in marker-assisted breeding (Buerstmayr et al. 2009).

Genomics-Assisted Breeding for FHB Resistance

Although a number of transgenic approaches for improving FHB resistance have been performed (see Buerstmayr et al. 2012 for review), this article does not cover transgenics. This chapter focuses entirely on genomics-assisted breeding for resistance improvement by utilizing the plant's native resistance genes. In order to apply marker-assisted breeding, it is necessary to know the position and effect of resistance QTL. Whereas mapping of QTL is still resource-intensive, because it requires extensive investments in genotyping and phenotyping, the application of markers indicative of resistance QTL in cultivar improvement is comparably quick and easy.

Table 4.1 illustrates a survey of published QTL validation and marker-assisted germplasm improvement studies for Fusarium head blight resistance in wheat. The earlier studies in the field of genomics-assisted breeding were not yet explicit MAS projects, but relied on QTL validation populations that were used to assess the resistance-improving effects of various QTL alleles in specific populations. In more recent studies, MAS has been performed by moving the desired QTL alleles into regionally adapted lines and evaluating the selection response associated with the specific QTL. Until now, no FHB resistance QTL has been cloned, therefore linked markers are the only option for MAS. Currently, an almost perfect marker is available only for *Fhb1*: the marker *Umn10* (Liu et al. 2008). Very recently, initial results on genomic selection for improving FHB resistance have been published (Rutkoski et al. 2012).

MAS for the Major FHB Resistance Gene *Fhb1*

The first, and to date best, validated FHB resistance QTL is derived from the Chinese spring wheat cultivar Sumai-3, and is mapped to chromosome arm 3BS. This QTL was independently discovered by Waldron et al. (1999), Bai

Table 4.1. QTL validation and marker assisted selection studies for Fusarium head blight resistance in wheat

Source of resistance allele	Chromosome	FHB reduction (%)[a]	Explained variation (%)	Markers	Main FHB trait (additional traits)	Association with	Plant material	Phenotyping[b]	Comment[c]	Reference
Sumai-3	3BS	59.9	30–31	*Xgwm533*	FHB spread		36 HRSW lines	*F. graminearum,* SFI: severeal exp.	QTL validation in breeding lines	Del Blanco et al. (2003)
Ning 7840	3BS	51.9	–	*Xgwm389, Xgwm533, Xbarc147*	FHB spread		Ning 7840/ Wheaton and Ning 7840/IL89–7978	*F. graminearum*, SFI: greenhouse exp.	MAS with 6 markers on 3B	Zhou et al. (2003)
DH181 (Sumai-3)	3BS	41.4	17	*Xgwm533 – Xgwm493*	FHB spread		DH181/AC Foremost, 174 DH	*F. graminearum*, SFI: 3 greenhouse exp.	MAS with 8 markers	Yang et al. (2003)
DH181 (Sumai-3)	6B	51.2	21	*Xgwm644*						
DH181 (Sumai-3)	3BS + 6B	68.5	36	*Xgwm533 & Xgwm644*						
93FHB21 (Ning8331)	3BS	42.4	48	*Xgwm389 – Xgwm493*	FHB spread		AC Foremost/ 93FHB21, 76 DH	*F. graminearum*, SFI: 1 greenhouse exp.	MAS with 8 markers	Yang et al. (2003)
93FHB21 (Ning8331)	5AL	14.1[ns]	5	*Xgwm291*						
93FHB21 (Ning8331)	6B	11.5[ns]	6	*Xgwm644*						
Sumai-3	3BS	32.0	–	*Xgwm493 – Xgwm533*	FHB spread		Sumai-3 x Australian wheat, four crosses	*F. graminearum*, SFI: 1 controlled exp.	MAS with 2 markers	Xie et al. (2007)
Wuhan 1	4B	28.1	–	*Xwmc238, Xgwm149*	FHB severity (kernel infection, DON content)	plant height	HC374/3*98B69-L47	*F. graminearum*, SPRAY: 2 field exp.	MAS in BC lines with 8 markers	McCartney et al. (2007)
Nyu-Bai	3BS	10.7	–	*Xgwm533, Xgwm493*		plant height				

(*continued*)

Table 4.1. (*Continued*)

Source of resistance allele	Chromosome	FHB reduction (%)[a]	Explained variation (%)	Markers	Main FHB trait (additional traits)	Association with	Plant material	Phenotyping[b]	Comment[c]	Reference
Nyu-Bai	3BSc	1.1[ns]	–	*Xgwm566, Xwmc231, Xwmc625, Xwmc693*		plant height				
Wuhan 1	2D	35.7	–	*Xwmc245, Xgwm608*	FHB severity (kernel infection, DON content)		BW301*3/HC374	*F. graminearum*, SPRAY: 2 field exp.	MAS in BC lines with 5 markers	McCartney et al. (2007)
Nyu-Bai	5AS	11.9[ns]	–	*Xwmc705*						
Nyu-Bai	3BS	–15.1[ns]	–	*Xgwm533, Xgwm493*						
Sumai-3	3BSc	21.3	–	*Xgwm566, Xwmc307, Xwmc418*	FHB severity (kernel infection, DON content)		98B08*A111/ 3*Kanata	*F. graminearum*, SPRAY: 2 field exp.	MAS in BC lines with 6 markers	McCartney et al. (2007)
Sumai-3	5AS	12.0[ns]	–	*Xgwm154, Xwmc705, Xgwm304*		plant height				
Sumai-3	3BS	25.0 (0–52)	–	*Xgwm493, Xbarc133, Xgwm533*	FHB spread (FHB severity, kernel infection)		19 QTL F4:5 NIL pairs from 13 crosses	*F. graminearum,* SPRAY: 2 field exp., grain spawn: 2 field exp., SFI: 3 controlled environment exp.	QTL validation in breeding lines	Pumphrey et al. (2007)
Sumai-3	3BS	–	46	*Xbarc133, Xgwm493*	FHB spread		793 breeding lines from 82 families	*F. graminearum*, SFI: 1 greenhouse exp.	Family-based and association-based QTL mapping, 13 markers	Rosyara et al. (2009)

CM-82036	3BS	33.0	–	*Xgwm389, Xgwm533, Xbarc133*	FHB severity	CM-82036/Remus// Nandu/3/Frontana/ Remus//Munk	*F. culmorum*, SPRAY: 4 field exp.	MAS and QTL validation with 6 markers	Miedaner et al. (2006), Wilde et al. (2007)
CM-82036	5A	32.1	–	*Xgwm156, Xgwm304a*					
Frontana	3A	15.6	–	*Xgwm720*					
CM-82036 & Frontana	3BS+5A+3A	54.9							
CM-82036	3BS	58.8	–	*Xgwm389, Xgwm533, Xbarc133*	DON content	CM-82036/Remus// Nandu/3/Frontana/ Remus//Munk	*F. culmorum*, SPRAY: 4 field exp.	MAS and QTL validation with 6 markers	Miedaner et al. (2006), Wilde et al. (2007)
CM-82036	5A	42.8	–	*Xgwm156, Xgwm304a*					
Frontana	3A	33.8	–	*Xgwm720*					
CM-82063 & Frontana	3BS+5A+3A	78.1							
Wangshuibai	4B	63.7	–	*Xbarc180-Xbarc117-Xgwm415-Xgwm304-Xmag3794*	FHB severity	Nanda2419/ Wangshuibai// 3*Mianyang 99–323	*F. graminearum*, SPRAY: 1-2field exps.; grain spawn: 2 field exp.	marker-assisted NIL development for individual QTL	Xue et al. (2010)
Wangshuibai	5A	70.0	–	*Xbarc20-Xgwm513-Xgwm192-Xgwm149-Xcdf22-Xwmc349*					
Wangshuibai	3BS	20.2 ^{ns}	–	*Xgwm389-Xgwm533-Xbarc147-Xgwm493*					
Nanda2419	2B	56.0	–	*Xwmc474-Xwmc499*					

(*continued*)

Table 4.1. (*Continued*)

Source of resistance allele	Chromosome	FHB reduction (%)[a]	Explained variation (%)	Markers	Main FHB trait (additional traits)	Association with	Plant material	Phenotyping[b]	Comment[c]	Reference
Wangshuibai	4B	26.0^{ns}	–	*Xbarc180-Xbarc117-Xgwm415-Xgwm304-Xmag3794*	FHB spread		Nanda2419/ Wangshuibai// 3*Mianyang 99–323	*F. graminearum*, SFI: 2 exp.	marker-assisted NIL development for individual QTL	Xue et al. (2010)
Wangshuibai	5A	25.5^{ns}	–	*Xbarc20-Xgwm513-Xgwm192-Xgwm149-Xcdf22-Xwmc349*						
Wangshuibai	3BS	84.0	–	*Xgwm389-Xgwm533-Xbarc147-Xgwm493*						
Nanda2419	2B	78.7	–	*Xwmc474-Xwmc499*						
CM-82036	3BS	15.9	–	*Xgwm389, Xgwm533, Xbarc133*	FHB severity		two BC_3 derived populations in two winter wheat recurrent paretns	*F. culmorum*, SPRAY: 10 field exp.	QTL validation in BC lines	Von der Ohe et al. (2010)
CM-82036	5A	24.4	–	*Xgwm304a and Xgwm156*						
CM-82036	3BS + 5A	32.4	–							
CM-82036	3BS	35.0	–	*Xgwm533, Xbarc133, Umn10, Xgwm493*	FHB severity		15 BC_2 derived families in 9 winter wheat recurrent parents	*F. graminearum*, SPRAY: 3 field exp. & *F. culmorum*, SPRAY: 3 field exp.	QTL validation in BC families	Salameh et al. (2011)

CM-82036	5A	14.0	–	*Xgwm156, Xgwm293, Xgwm1057*		plant height				
CM-82036	3BS + 5A	42.0	–							
G16-92	2BL	23.5[ns]	–	*Xgwm47*	FHB severity	plant height	Dream/Lynx// Brando /3/G16-92/Hussar//LP235.1	*F. culmorum:* SPRAY: 4 exp.	MAS with 3 markers	Wilde et al. (2008), Miedaner et al. (2009)
Dream	6AL	19.1[ns]	–	*Xgwm82*		plant height				
Dream	7BS	9.2[ns]	–	*Xgwm46*						
Dream & G16-92	2BL+6AL+7BS	36.2	–			plant height				
Dream	6AL	27.0	–	*Xgwm82*	FHB severity	plant height	Dream/4*Lynx, 127 BC_2F_4 lines	*F. culmorum,* SPRAY: 3 field exp.	QTL validation in BC lines	Häberle et al. (2007)
Dream	7BS	27.0	–	*Xgwm46*		plant height				
Dream	6AL + 7BS	36.0	–			plant height				
Cansas	1BL	42.0	–	*Xwmc728 - Xgwm259*	FHB severity	heading date	Cansas/Ritmo, F_4 derived sister lines	*F. culmorum,* SPRAY: 4 field exp.	QTL validation in breeding lines	Häberle et al. (2009)

[a]Relative reduction in FHB severity or other FHB-related trait compared to the group of lines with the susceptible allele(s) (=100%); ns = not significant.
[b]SPRAY = spray inoculation, SFI = single floret inoculation.
[c]MAS = marker-assisted selection, BC = back-cross, NIL = near isogenic line.

et al. (1999), and Buerstmayr et al. (2002, 2003) and later rediscovered in a number of studies using Sumai-3 or related Asian lines as resistance donors (see references in Buerstmayr et al. 2009; Löffler et al. 2009; Liu et al. 2009). This QTL was designated *Qfhs.ndsu-3BS* (Anderson et al. 2001). In high-resolution mapping populations segregating at *Qfhs.ndsu-3BS*, this locus was fine-mapped as a single Mendelian gene with high precision and therefore renamed *Fhb1*. Flanking markers bracketing *Fhb1* within a 1.2 cM interval are now available (Liu et al. 2006; Cuthbert et al. 2006), including the PCR marker *Umn10*, a nearly perfect PCR marker for this resistance gene (Liu et al. 2008). It was therefore logical that this QTL was the first candidate for marker-assisted selection of FHB resistance in wheat.

Del Blanco et al. (2003) evaluated 36 phenotypically selected hard red spring wheat breeding lines obtained through several cycles of crossing to North Dakota adapted bread wheat genotypes and deriving their FHB resistance from Sumai-3 using 152 microsatellites primer pairs. Among these, two SSR loci were associated with type 2 FHB resistance: *Xgwm533* and *Xgwm274*, both mapping to chromosome 3B. The marker *Xgwm533* explained 30% of the phenotypic variation for type 2 FHB resistance in this set of breeding lines. A second SSR marker on the long arm of chromosome 3B, *Xgwm274*, was also associated with FHB resistance, but at lower stringency.

Zhou et al. (2003) evaluated SSR markers linked to *Fhb1* in two Ning-7840 (Aurora/Anhui-11//Sumai-3) derived segregating populations. SSR markers in the *Fhb1* genomic region were strongly associated with type 2 FHB resistance: lines with the resistant allele in this region showed on average about half the number of Fusarium-damaged spikelets in single-floret inoculated trials compared to lines possessing the alternative allele. Selection using markers linked to *Fhb1* appeared highly efficient. Despite that, the variation in FHB severity among lines within each QTL class was large, indicating that further unknown QTL and environmental factors influenced FHB severity in these populations.

Yang et al. (2003) performed MAS in two doubled haploid populations descending from crosses of Sumai-3 derivatives and the elite Canadian spring wheat cultivar AC Foremost. Markers near *Fhb1* explained 12-36 % of the phenotypic variation for type 2 FHB resistance. Lines with the Sumai-3 allele at *Fhb1*-linked SSR markers had an average reduction in FHB severity of 40% compared to a group with the AC-Foremost alleles.

In several four-way crosses and backcrosses using Sumai-3 as resistance donor and Australian wheat lines as recurrent parents, lines possessing *Fhb1*, identified by SSR markers near this locus, had a significant average reduction of around 30% type 2 FHB severity compared to lines with the alternative alleles at this QTL (Xie et al. 2007).

Pumphrey et al. (2007) used $F_{3:4}$ head rows from the University of Minnesota wheat-breeding program to establish series of near-isogenic $F_{3:5}$ lines using markers in the *Fhb1* region. They generated 19 NIL pairs descending from 15 crosses. Lines possessing *Fhb1* showed average reductions of 23% for type 2 disease severity ratings and 27% for infected kernels in the harvested grain. The variation in the resistance-improving effect of *Fhb1* was large: NIL pairs with and without *Fhb1* differed in type 2 FHB severity from 0% to 52% (Pumphrey et al. 2007).

In breeding populations composed of 82 families and a total of 793 individuals, Rosyara et al. (2009) performed linkage and association mapping based QTL analysis in the extended family pedigrees for the *Fhb1* region. Markers linked to *Fhb1* explained 40-50% of the variation for type 2 FHB resistance, and *Fhb1* was mapped with high precision near the SSR marker *Xbarc133*, which is in full agreement with previous QTL-mapping results (Buerstmayr et al. 2009). Rosyara et al. (2008) showed that family-based QTL mapping in breeding populations was a very practical approach to combine-QTL

mapping, QTL validation, and the development of improved breeding lines with high prospective for cultivar release. This approach offers a big advantage over traditional QTL mapping, which is often based on experimental populations, usually not suitable to deliver adapted cultivars directly.

Many breeding programs use *Fhb1* successfully as an important resistance source. Reliance on a narrow genetic basis bears the potential risk of resistance breakdown. Fortunately, resistance for FHB in wheat appears to be neither race-specific nor species-specific (van Eeuwijk et al. 1995). No breakdown of an FHB resistance gene has been reported, and FHB resistance is therefore considered durable at the present time (Buerstmayr et al. 2012). The risk of pathogen adaptation is possibly low because Fusarium is an opportunistic, facultative pathogen that survives well as a saprophyte, other than, for instance, the cereal rusts and powdery mildew.

MAS for QTL Other than *Fhb1* and MAS for Multiple QTL Simultaneously

A repeatedly detected QTL for type 2 resistance has been mapped to chromosome 6BS and a QTL governing mainly type 1 resistance at chromosome 5A, both again descending from Asian resistance sources (Buerstmayr et al. 2009). The QTL on 6B has been fine-mapped 2 cM from *Xgwm644* by Cuthbert et al. (2007) and designated *Fhb2*. The QTL on 5A has been fine-mapped between *Xgwm304* and *Xgwm415* in Wangshiubai-derived populations by Xue et al. (2011) and designated *Fhb5*. In two QTL validation populations analysed by Yang et al (2003) in addition to *Fhb1* as described earlier, a significant effect was associated with the SSR marker *Xgwm644* at chromosome arm 6BS (most likely *Fhb2*) in one population.

Somers et al. (2005) created a multiway backcrossing scheme for assembling complex genotypes. This included two backcrosses and selection for a total of six FHB resistance QTL, orange blossom wheat midge resistance (*Sm1*), and leaf rust resistance (*Lr21*). In addition, the genetic background was monitored with markers to accelerate restoration of the elite genetic background at each backcross. This approach resembles a breeding-by-design scheme where one aims to pyramid desired alleles in an "optimal" genotype (Peleman and van der Voort 2003). Somers et al. (2005) were successful in establishing the desired lines within a relatively short period of only 25 months, but they did not report about a phenotypic evaluation of the new germplasm carrying the desired alleles. Therefore, the obtained selection gain with this approach remains unknown.

A fairly large QTL evaluation and marker-assisted breeding project was performed by McCartney et al. (2007), who aimed to transfer QTL from three exotic sources—Nyu-Bai, Sumai-3, and Wuhan-1—into elite Canadian spring wheat cultivars and measured the effects on three traits indicative for FHB resistance (visual scores of FHB index and severity, percent of Fusarium-damaged kernels, and DON content in the harvested grains) and several agronomic and grain quality traits. They reported that the 4B FHB resistance QTL from Wuhan-1 was the most efficient QTL improving FHB resistance, but at the same time it was associated with an increase in plant height. The Wuhan-1 2D, Nyu-Bai 3BSc, Sumai-3 3BSc, Nyu-Bai 5AS, and Sumai-3 5AS alleles were also effective FHB resistance alleles. There was a tendency that recombinant lines possessing two or three resistance alleles had higher resistance than the lines with single resistance alleles. The three measures for FHB resistance—visual scores, percent of Fusarium-damaged kernels, and DON content in the harvested grains—were highly correlated.

Miedaner et al. (2006), generated a four-parent recombinant population involving two regionally adapted German spring wheat cultivars (Munk, Nandu) and two experimental lines carrying either the FHB resistance QTL at 3BS and 5A derived from CM-82036 (Buerstmayr et al. 2002, 2003) or the QTL on 3A from

Frontana (Steiner et al. 2004). All three QTL led to a decrease in FHB severity and in DON content. The 3A QTL from Frontana was less effective compared to the 3B and 5A QTL from CM-82036. Lines with the 3BS and 5A QTL alleles from CM-82036 combined were the most resistant group, which showed reduced DON content by 78% and reduced FHB rating by 55% compared to the susceptible QTL class. The same population was used by Miedaner et al. (2008) to select resistant lines by phenotypic selection only and to genotype these with SSR markers linked to the three QTL on 3B, 5A, and 3A *a posteriori*. Among the 135 FHB resistant selections, 121 carried either two or three QTL combined, and only one selected FHB-resistant line did not possess any of the SSR marker alleles linked to the resistance QTL, indicating the expected QTL contributed to a large extent to resistance performance in this selection experiment. A study by Wilde et al. (2007) compared selection response between phenotypic selection and marker-based selection in the same segregating population used by Miedaner et al. (2006, 2008). A sophisticated three-step phenotypic selection approach, without any marker evaluation, resulted in the highest overall selection gain, but selection gain per unit time was highest in the marker-based selection approach, because selection cycles could be performed much faster. MAS was thus very efficient in fixing desired QTL resistance alleles quickly, but phenotypic selection obviously also gathered unknown or small-effect QTL. Within each QTL group, variation in FHB resistance was large. Therefore, even after fixing known QTL by marker-based selection, phenotypic selection for unknown or small-effect QTL should be done in order to select lines with excellent resistance performance.

Xue et al. (2010) developed and evaluated NILs for four FHB resistance QTL in the susceptible cultivar Mianyang 99-323 using SSR markers flanking these QTL. All four QTL significantly improved FHB resistance. The QTL on 3B improved type 2 resistance, the QTL on 4B and 5A from Wangshuibai improved type 1 resistance, and a QTL from a rather susceptible cultivar Nanda2419 on chromosome 2B enhanced both types of FHB resistance.

It is an ongoing discussion among breeders whether one should use non-adapted germplasm for crossing in an applied cultivar development program. Usually several rounds of backcrossing and rigorous selection are needed to restore lines with high-yield performance and specific environmental adaptation plus the desired alleles from the non-adapted donor. This is exactly the situation that faces, for instance, a winter wheat breeder who aims to incorporate FHB resistance from an Asian spring wheat donor in a high-yielding regionally adapted winter wheat cultivar. In such cases, marker-assisted backcross breeding has great potential. Two complementary projects have been performed recently by von der Ohe et al. (2010) and Salameh et al. (2011) to evaluate such an approach. Both projects were based on the FHB-resistant line CM-82036 as donor with the well-known QTL alleles on 3B and 5A. Von der Ohe et al. (2010) used two German winter wheat cultivars, one moderately resistant and one highly susceptible, as recurrent parents for backcrossing and developed BC_3-derived lines with both, one, or no QTL from CM-82036. These BC_3-derived lines were tested for FHB resistance in inoculated trials and for agronomic and yield traits in performance trials across four locations and two seasons. Salameh et al. (2011) used the same resistance donor and generated BC_2-derived lines in nine different European winter wheat cultivars. The donor cultivars were chosen to range from moderately resistant to highly susceptible. BC_2-derived sister lines were selected with both, one, or none of the QTL from CM-82036. The selected experimental lines were evaluated for FHB resistance in six inoculated experiments and a subset of lines for yield and agronomic performance in three non-inoculated field trails. These two companion studies yielded highly congruent results, with minor differences. Generally progeny lines possessing one or both QTL from CM-82036 showed improved FHB

resistance. Interestingly, in the results by von der Ohe (2010), the 5A QTL had a larger resistance-improving effect, whereas Samaleh et al. (2011) found a larger effect of the 3B QTL. The final resistance level of the derived lines depended to a large extent on the background resistance of the recurrent parent. For example, incorporating resistance QTL into a moderately resistant cultivar led to highly resistant progeny; incorporating the QTL into a highly susceptible cultivar improved this to a moderately susceptible line. There was no systematic negative effect of the spring-wheat-derived QTL on yield and other agronomic traits. Some resistant lines with spring wheat QTL alleles were equal to or even slightly better in yield than the recurrent cultivars. All in all, these results underscore the fact that utilization of exotic QTL is highly promising when a rigorous backcrossing and selection scheme is applied. Markers are a perfect tool to select the desired genotypes quickly and efficiently.

MAS for FHB Resistance QTL Available in European Winter Wheat

To date, only a few reports applying marker-assisted selection for FHB resistance QTL of non-Asian origin are available (Häberle et al. 2007, 2009; Wilde et al. 2008; Miedaner et al. 2009). The potential advantage in using so-called native resistance, as opposed to exotic resistance sources, is that regionally adapted breeding lines can be selected easier and faster, because fewer rounds of crossing and selection are needed in order to select agronomically acceptable varieties. Three reports are based on the FHB-resistant German winter wheat variety Dream, with previously mapped QTL at chromosome arms 6BL, 7BS, and 2BL (Schmolke et al. 2005). Häberle et al. (2007) selected BC_2F_4 lines for the QTL at 6BL and 7BS using the susceptible cultivar Lynx as recurrent parent. Lines with individual QTL expressed about 27% reduced FHB severity and lines with both QTL combined expressed 36% reduced FHB severity compared to lines with the Lynx alleles. At the same time, both QTL were associated with increased plant height. Wilde et al. (2008) explored marker-based selection, and Miedaner et al. (2009) compared marker-based selection to phenotypic selection. They used an experimental population entirely based in European winter wheat. A population of 600 progeny was derived from a double-cross combining three FHB resistance QTL alleles (6AL and 7BS from the cultivar Dream and one QTL on chromosome 2BL from the line G16-92) with two high-yielding, susceptible winter wheat varieties. Both marker-based selection and phenotypic selection led to a selection response toward increased resistance. Total gain by selection was larger in phenotypically selected variant, but resistance gain per unit time was larger in the marker-selected variant. The allele substitution effect of the QTL in this MAS project was lower compared to the estimated effects of the QTL alleles in the preceding mapping projects.

Häberle et al. (2009) validated an FHB resistance QTL on chromosome arm 1BL descending from the German cultivar Cansas in F_4-derived sister lines that increased FHB resistance by 42% compared to lines lacking the positive allele. Marker-assisted breeding using well-validated QTL detected in European winter wheat thus appeared as effective as marker-assisted breeding for exotic QTL from Asian spring wheat sources.

MAS for Improving FHB Resistance in Tetraploid Wheat

Because durum wheat is highly FHB susceptible, and only little genetic variation for this trait is available in durum wheat, it appeared logical to introgress QTL from bread wheat to durum wheat. It is easy to transfer A and B genome chromosome segments between these species by crossing and selection, but the progeny thus attained was not necessarily better in resistance. Yet, among about

100 lines selected from Sumai-3 x *T. durum* backcross lines about 5 lines were significantly improved in FHB resistance, indicating that this approach is feasible but not as simple as originally anticipated (own unpublished results). The phenotypic expression of FHB resistance genes in a *T. durum* background seems suppressed. A potential suppressor for FHB resistance has been located to chromosome 2A in wild emmer wheat (Stack et al. 2002, Garvin et al. 2009). In search of resistance sources for durum wheat improvement, accession of its close relatives such as cultivated emmer (*T. dicoccum*) or wild emmer (*T. dicoccoides*) have been evaluated and a number of QTL-mapping studies have been performed using such material (Buerstmayr et al. 2009). Recently, promising sources of FHB resistance have been discovered among Tunisian *T. durum* accessions (Huhn et al. 2012), and this germplasm has already entered durum wheat breeding. Ghavami et al. (2011) performed traditional and association mapping of breeding populations derived from crosses with Tunisian lines. This study revealed several QTL, with the largest effect mapping to chromosome arm 5BL. Association mapping in breeding populations had the big advantage that QTL discovery and development of regionally adapted breeding lines was done simultaneously. All in all, improvement of FHB resistance in durum wheat seems achievable but requires intensified research and breeding efforts.

Conclusions and Summary

Well-mapped and validated QTL are those on chromosomes 3BS (*Qfhs.ndsu-3BS*, *Fhb1*), 5AS (*Qfhs.ifa-5A, Fhb5*), 4B (*Fhb4*), and 6BS (*Fhb2*), all deriving from Asian spring wheat sources. In addition, a few QTL from European winter wheat were used in MAS projects. For the purposes of MAS, diagnostic markers are available for only *Fhb1*, and highly successful MAS for *Fhb1* has repeatedly been reported. Several more FHB QTL were used in MAS programs, especially in cases where breeders were familiar with marker allele types of the QTL donors and the recipient germplasm. The number of diagnostic markers should be increased for QTL to be easily adopted by breeders. Therefore, the emphasis of future research activities should be to detect more diagnostic markers for the most repeatable FHB QTL reported. Fine-mapping populations can be used both for QTL validation and finding diagnostic markers with close linkage to the FHB QTL. Alternatively, association mapping exploiting linkage disequilibrium in breeding populations may yield tightly linked markers for relevant resistance QTL. MAS was efficient in the fixation of a limited number of well-characterized FHB resistance QTL, as outlined in the previous paragraphs. In addition to detectable QTL, FHB resistance is certainly also modulated by an unknown number of medium- to small-effect QTL that usually remain undiscovered in conventional QTL mapping. High-throughput genotyping methods, using either dense SNP genotyping platforms or sequence-based genotyping, became available recently, which enable unprecedented marker density and at competitive cost. This novel development and the refinement of statistical tools for handling large genotype and phenotype data open the way to apply genome-wide selection (GS) also for small-effect QTL.

While in hexaploid wheat, both conventional and marker-assisted breeding for improving FHB resistance has made significant progress, in tetraploid durum wheat, good sources of resistance are still sparse and more work is needed to identify more resistant germplasm and to decipher its FHB resistance.

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Chapter 5

Virus Resistance in Barley

Frank Ordon and Dragan Perovic

Abstract

Barley (*Hordeum vulgare* L.), which is a diploid species with 2n=2x=14 chromosomes and a genome size of about 5.2 Gb, is one of the most important crop species worldwide. It is the host for more than 50 different viruses, of which the soil-borne *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) and the aphid-transmitted *Barley yellow dwarf virus* (BYDV) and *Cereal yellow dwarf virus* (CYDV) have a serious impact on barley production due to high yield losses frequently observed in susceptible barley crops. Breeding for resistance/tolerance is the only way to ensure barley production in the growing area of fields infected by soil-borne viruses and to prevent infections by aphid-transmitted viruses in an environmentally sound manner. In barley, genomic tools are available today that pave the way to (1) efficient marker-based selection procedures for virus resistance, (2) the enhanced isolation of resistance genes via a map-based cloning approach, and (3) efficient allele mining and allele-based breeding strategies for virus resistance. The present state and future perspectives of breeding for virus resistance in barley using these genomic tools are briefly reviewed.

Introduction

Barley (*Hordeum vulgare*) ranked fifth in acreage among major crops in 2010, covering about 47.5 million hectares worldwide, and it was number two in Europe next to wheat, with an acreage of about 23 million hectares. On the worldwide level, in 2010, barley yields averaged about 2.6 tons per hectare; in the European Union they were 4.3 tons per hectare. However, in the main barley-growing countries of Western Europe (France, Germany, Belgium, Netherlands, United Kingdom, and Denmark), average barley yields today are nearly 7.0 tons per hectare (all statistics are from http://faostat.fao.org). Barley is mainly used for animal feed, malting, and, to a lesser extent, directly for human nutrition.

Barley is a diploid species (2n=2x=14) that was cultivated in the Fertile Crescent some 10,500 years ago (Zohary and Hopf 2000), and it is regarded as one of the founder crops of Old World agriculture (Badr et al. 2000). Barley has a genome size of about 5.2 Gb (Arumaganathan and Earle 1991), and due to diploidy,

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inbreeding, and the small chromosome number, extensive genetic studies have been conducted in barley starting from morphological and isozyme markers (e.g., Benito et al. 1988) via restriction fragment length polymorphisms (RFLPs, Graner et al. 1991), PCR-based marker techniques (e.g., Varshney et al. 2007), and high-throughput SNP genotyping (Close et al. 2009) to genome sequencing (Mayer et al. 2011).

Aside from fungal pathogens, barley is a host to more than 50 different viruses (http://www.agls.uidaho.edu/ebi/vdie/famly064.htm# Horde um%20vulgare). However, on the worldwide level, only a few viruses cause economically important diseases. These are the soil-borne *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV), belonging to the genus *Bymovirus* within the family *Potyviridae*, as well as the aphid-transmitted *Barley yellow dwarf virus* (BYDV) and *Cereal yellow dwarf virus* (CYDV), which are members of the genera *Luteovirus* and *Polerovirus*, respectively. Besides this, *Wheat dwarf virus* (WDV) belonging to the genus *Mastrevirus* and being transmitted by the leaf hopper *Psammottetix alienus* causes severe yield losses in barley in restricted areas of Europe. But, although intensive screening programs for resistance to WDV have been conducted (Vacke and Cibulka 2001; Bukvayova et al. 2006), tolerance to WDV has been only observed in the barley cv. "Post" up to date, and nothing is known about the genetics of this tolerance (Habekuss et al. 2008).

First molecular maps for barley have been developed in the early 1990s (Graner et al. 1991), and since that time molecular markers have been developed for many resistance genes and quantitative trait loci (QTL) against the aforementioned viral pathogens (Ordon et al. 2009). Today, high-density maps (e.g., Sato et al. 2009; Close et al. 2009), physical maps (Schulte et al. 2011), and much sequence information (Mayer et al. 2011) are available, opening the way for genomics-based breeding for virus resistance in barley.

The present chapter focuses on resistance to the economically most important barley viruses, namely BaMMV/BaYMV and BYDV/CYDV, giving a brief overview on (1) biological properties and economic importance of BaMMV/BaYMV and BYDV/CYDV, (2) sources and genetics of resistance or tolerance, respectively, (3) molecular markers available, (4) strategies for molecular breeding, and (5) genomics-assisted breeding for virus resistance in barley.

Important Viral Pathogens of Barley

Barley Yellow Mosaic Virus/ Barley Mild Mosaic Virus

Barley yellow mosaic virus disease was first detected in Japan in 1940 (Ikata and Kawai 1940). Today it is known in East Asian countries and especially in Europe, where the disease is present in numerous countries (Kühne 2009). In Europe, barley yellow mosaic virus has become one of the most important diseases of winter barley since its first detection in Germany in 1978 (Huth and Lesemann 1978).

Both BaMMV and BaYMV have filamentous particles with two modal lengths of 500-600 nm and 250-300 nm, containing two species of ssRNA (Huth et al. 1984). Sequencing data revealed that BaMMV and BaYMV share only a limited sequence homology (e.g., Peerenboom et al. 1992), meaning that these two viruses are related but distinct members of the genus *Bymovirus.* In Japan, at least seven strains of BaYMV and two strains of BaMMV are known (Kashiwazaki et al. 1989), while in Europe, two strains of BaYMV and three of BaMMV have been described (Habekuss et al. 2008). Both viruses are transmitted by the soil-borne plasmodiophorid *Polymyxa graminis* (Adams et al. 1988), which is distributed worldwide (Anonymous 2011; Thompson et al. 2011). Therefore, chemical measures are not efficient to prevent yield losses, which can reach 50%. Therefore, the only way to ensure winter barley cultivation

in the growing area of infected field is breeding for resistance.

Barley Yellow Dwarf Virus / Cereal Yellow Dwarf Virus

Barley yellow dwarf is caused by a group of related viruses (see below) inducing leaf discoloration and dwarfing, resulting in high yield losses (e.g., Lister and Ranieri 1995). It was first detected in 1951 in California (Oswald and Houston 1951). The agents of BYD are single-stranded RNA viruses with a genome size of 5.3-5.7 kb and isometric particles of about 25 nm in diameter, which are transmitted in a persistent manner by aphids. In a first step, according to the different transmission efficiency of different aphid species, five different strains of BYDV were distinguished (Rochow 1969; Rochow and Muller 1971). Today, this group of viruses is subdivided into three species belonging to the genus *Luteovirus*, namely *Barley yellow dwarf virus* (BYDV)-PAV, BYDV-PAS, and BYDV-MAV, two species known as *Cereal yellow dwarf virus* (CYDV)-RPS and CYDV-RPV belonging to the genus *Polerovirus*, and another three species, namely BYDV-GPV, BYDV-RMV, and BYDV-SGV, also classified as members of the *Luteoviridae* family but not yet being assigned to any genus (http://ictvonline.org/virusTaxonomy.asp?version=2009). Due to global warming and longer periods of warm temperatures in autumn and winter, it is expected that these aphid-transmitted viruses will become even more important in the future in some parts of the world, for example, in northern Europe (Habekuss et al. 2009).

Breeding for Virus Resistance – Some Case History

Sources and Genetics of Resistance

The starting point for each breeding program for virus resistance in barley has been the extensive screening for resistance. With respect to BaMMV/BaYMV, genotypic differences concerning the reaction to the different members of the barley yellow mosaic virus complex were observed, but it turned out that resistance to BaMMV and BaYMV is quite frequent within the primary gene pool of barley (e.g., Kawada et al. 1991; Ordon and Friedt 1993; Habekuss et al. 2008). Genetic analysis has shown that different recessive resistance genes are present in barley (Goetz and Friedt 1993; Ordon and Friedt 1993). In addition, two dominant resistance genes have been identified in the secondary gene pool, namely in *Hordeum bulbosum* (Ruge et al. 2003; Ruge-Wehling et al. 2006), and very recently the first dominant resistance gene has been identified also in cultivated barley (Kai et al. 2012).

In contrast to BaMMV/BaYMV, no complete resistance against BYDV/CYDV is known in barley, but different genes conferring tolerance have been identified. The first was *ryd1*, detected in the spring barley cultivar "Rojo" (Suneson 1955). This gene was not used in barley breeding due to its low efficiency. Furthermore, *Ryd2* and *Ryd3*, with similar effects against BYDV-PAV and BYDV-MAV, were identified in Ethiopian landraces (Schaller et al. 1964; Niks et al. 2004). The effect of the semi-dominant *Ryd2* gene located on chromosome 3HL (Collins et al. 1996) depends on the genetic background, environmental conditions, and virus isolate (Schaller et al. 1964; Schaller 1984). Besides this, different alleles may be present at this locus (Catherall and Hayes 1970; Chalhoub et al. 1995). Recently, a gene called *Ryd4*^{Hb}, conferring complete resistance to BYDV-PAV, has been transferred from *Hordeum bulbosum* to cultivated barley (Scholz et al. 2009). Furthermore, additional QTL for tolerance against BYD have been identified in barley (Kraakman et al. 2006; Scheurer et al. 2001; Toojinda et al. 2000). Results of these screening programs and genetic analyses revealed that there is a broad genetic variation available concerning resistance to BaMMV/BaYMV and sufficient variation for tolerance to BYDV/CYDV, which can be employed in barley breeding.

Molecular Markers for Virus Resistance

With respect to the insect-transmitted BYDV/CYDV, viruliferous aphids have to be available for efficient breeding for resistance/tolerance on the phenotypic level, while effective selection procedures against BaMMV/BaYMV require uniformly infested fields or mechanical inoculation procedures in the greenhouse followed by DAS-ELISA (for details, cf. Palloix and Ordon 2011). Furthermore, it has to be taken into account that rearing of viruliferous aphids and artificial infection are difficult to integrate into applied barley-breeding schemes, and that with respect to BaMMV/BaYMV, symptom development is strongly influenced by the climatic conditions during winter and spring time, leading to the situation in which a reliable selection for virus resistance on the phenotypic level cannot be conducted every year.

Therefore, soon after the first molecular marker techniques, such as Restriction Fragment Length Polymorphisms (RFLPs), became available, followed by PCR-based techniques like Random Amplified Polymorphic DNA (RAPDs), Simple Sequence Repeats (SSRs), and Amplified Fragment Length Polymorphisms (AFLPs), attempts were made to develop molecular markers for virus resistance genes in barley, facilitating efficient marker-based selection procedures. An overview on markers available for major virus resistance genes in barley is given in Table 5.1.

Based on these markers, different marker-based selection procedures have been developed. Together with doubled haploid techniques, which are routinely used in barley breeding today, respective markers facilitate a reliable and fast selection for virus resistance; for example, doubled haploid populations may be screened directly *in vitro* and only those plantlets carrying the resistance encoding allele have to be transferred to the greenhouse (for overview on molecular breeding for virus resistance, cf. Friedt and Ordon 2007; Ordon et al. 2009; Palloix and Ordon 2011).

In general, virus resistance is identified in genetic resources that are rather un-adapted to productive growing systems (e.g., Ordon and Friedt 1994). Therefore, to combine virus resistance with superior agronomic performance, time-consuming backcrossing procedures are needed. This holds especially true if recessive resistance genes like in the case of BaMMV/BaYMV must be incorporated, as a selfing generation is needed after each backcross to identify homozygous recessive genotypes on the phenotypic level. In contrast to this, in marker-based procedures, the recessive resistance encoding allele can be directly followed by a co-dominant marker or a dominant one showing an additional fragment linked to the resistance-encoding allele (Ordon et al. 2003, 2009), thus saving one generation per backcrossing cycle. Furthermore, backcrossing schemes can be additionally enhanced if in parallel the genomic portion of the recurrent parent is determined, for example, by efficient high-throughput Single Nucleotide Polymorphism (SNP) genotyping (Close et al. 2009), as in many species (Uptmoor et al. 2006) a strong deviation from the theoretically expected portion of 75% in BC_1 was observed.

Furthermore, molecular markers facilitate efficient pyramiding of resistance genes, especially in combination with doubled haploids (Werner et al. 2005, 2007). With respect to BaMMV/BaYMV, pyramiding may become of special importance in the future, as most of the resistance genes (Table 5.1) have been overcome already by new pathotypes of BaYMV or BaMMV (Habekuss et al. 2008). This approach, which may be conducted following strategies involving one or two DH-line production steps (Werner et al. 2005, 2007), facilitates the extended use of respective resistance genes in barley breeding: for example, the combination of *rym5* being effective in Europe against BaMMV, BaYMV, and BaYMV-2, with *rym9* being effective against BaMMV, BaMMV-SIL and BaMMV-Teik, will result in complete resistance to all strains known in Europe until now.

Table 5.1. List of mapped major virus resistance genes in barley (modified and updated according to Ordon et al. 2009).

Resistance gene	Chromsomal location	Reference(s)
Barley stripe mosaic virus (BSMV)		
Rsm	7HS	Edwards and Steffenson 1996
Barley yellow dwarf virus (BYDV)		
Ryd2	3HL	Collins et al. 1996; Paltridge et al. 1998; Ford et al. 1998
Ryd3	6H	Niks et al. 2004
Ryd4^{Hb}	3HL	Scholz et al. 2009
Barley yellow mosaic virus (BaYMV), Barley mild mosaic virus (BaMMV)		
rym1	4HL	Okada et al. 2004
rym3	5HS	Saeki et al. 1999; Werner et al. 2003a
rym4	3HL	Graner and Bauer 1993; Ordon et al. 1995; Weyen et al. 1996; Stein et al. 2005; Kanyuka et al. 2005; Werner et al. 2005; Stracke et al. 2007; Tyrka et al. 2008; Sedláček et al. 2010
rym5	3HL	Graner et al. 1999b; Pellio et al. 2005; Stein et al. 2005; Kanyuka et al. 2005; Stracke et al. 2007; Tyrka et al. 2008; Sedláček et al. 2010
rym6	3HL	Kanyuka et al. 2005
rym7	1HS	Graner et al. 1999a
rym8	4HL	Bauer et al. 1997
rym9	4HL	Bauer et al. 1997; Werner et al. 2005; Werner et al. 2000
rym10	3HL	Graner et al. 1995
rym11	4HL	Bauer et al. 1997; Nissan-Azzouz et al. 2005; Werner et al. 2005
rym12	4HL	Graner et al. 1996
rym13	4HL	Werner et al. 2003b; Humbroich et al. 2010
Rym14^{Hb}	6HS	Ruge et al. 2003
rym15	6H	Le Gouis et al. 2004
Rym16^{Hb}	2HL	Ruge-Wehling et al. 2006
Rym17	3HS	Kai et al. 2012
rym18	4HL	Kai et al. 2012

Beside prolonging the usability of resistance genes, pyramiding may also result in a higher level of resistance as has been recently shown for BYDV (Riedel et al. 2011). In this study, *Ryd2*, *Ryd3* and the QTL located on chromosome 2H derived from cv. "Post" were combined using DH-Lines and molecular markers, and it turned out that those lines carrying a combination of *Ryd2* and *Ryd3* showed a significantly reduced virus titre; in other words, a combination of these loci has resulted in quantitative resistance to BYDV in contrast to tolerance encoded by these loci alone.

Isolation of Virus Resistance Genes in Barley

Isolation of resistance genes is of special interest, on the one hand, to get information on the structure and function of virus resistance genes in barley and on the other hand to facilitate directed allele-based selection procedures. To isolate such resistance genes in the complex genome of barley, a map-based cloning approach (cf. Stein and Graner 2004) turned out to be effective to isolate the *Rym4/Rym5* locus located on chromosome 3H and conferring resistance to BaMMV/BaYMV (Stein et al. 2005). For this purpose a high-resolution mapping population based on about 8,000 meioses was constructed and marker-based saturation was conducted employing 932 *Pst*I+2/*Mse*I+3, 2,048 *Eco*RI+3/*Mse*I+3 AFLP primer combinations, and 1,200 RAPD primer resulting in 11 markers within the target interval (Pellio et al. 2005). In the end, based on the closest marker, a BAC-contig was identified (Wicker et al. 2005) carrying the translation initiation

factor 4e (*Hv-eIF4E).* This was a perfect candidate gene as it co-segregated with *rym4/rym5* and it was shown that translation initiation factors are involved in recessive resistance against potyviruses in different pathosystems (Robagillia and Caranta 2006). By transformation of a resistant genotype with the wild-type allele, it turned out that the *Rym4/Rym5* locus comprises the translation initiation factor 4e (*Hv-eIF4E*, Stein et al. 2005; Kanyuka et al. 2005). Knowledge of such resistance genes facilitates on the one hand the design of allele-specific markers and on the other hand screening of large gene bank collections for new, maybe more efficient alleles, and opens the way to a directed access to the genetic diversity present with respect to resistance to BaMMV/BaYMV (Stracke et al. 2007; Hofinger et al. 2011). However, the isolation of resistance genes at that time was a very time-consuming process, which in the case of *Rym4/Rym5* took more than 10 years from constructing a high-resolution mapping population to the gene sequence. Today genomic tools are available in barley considerably enhancing marker development and isolation of virus resistance genes.

Genomics-Based Breeding for Virus Resistance in Barley

Genomic Tools

In barley, many genomic tools are available that can be efficiently used for improving virus resistance. These are: (1) dense maps consisting of non-genic markers (Ramsay et al. 2000; Hearnden et al. 2007; Varshney et al. 2007); (2) several high-density transcript maps (e.g., Stein et al. 2007; Sato et al. 2009; Close et al. 2009); (3) next-generation sequencing data and detailed fingerprints of several BAC libraries and respective physical maps (Wicker et al. 2008, 2009; Schulte et al. 2011); (4) a so-called genome zipper combining sequence information from rice, sorghum, and brachypodium (Goff et al. 2002; Yu et al. 2002; Paterson et al. 2009; Vogel et al. 2010) with chromosome-specific information and gene order in barley (Mayer et al. 2011); (5) several TILLING populations (e.g., Talame et al. 2008); and (6) efficient transformation protocols (e.g., Kumlehn et al. 2006). A combination of these tools may in the future lead to a faster marker development and isolation of virus resistance genes in barley followed by the estimation of allelic diversity concerning respective resistance genes and an allele-based breeding for virus resistance as outlined below.

Since the development of cheap high-throughput sequencing technologies, a major focus in barley genomics was on the generation of molecular markers from the expressed portion of the barley genome, namely the Expressed Sequence Tags (ESTs). In this respect, the first 1,000-loci transcript map combining Restriction Fragment-Length Polymorphisms (RFLP), Simple Sequence Repeats (SSRs, Thiel et al. 2003; Varshney et al. 2007), and SNPs (Kota et al. 2008) derived from three mapping populations was constructed by Stein et al. (2007). Independently, the first high-density single PCR-based map using SNPs and in/del markers was developed in a cross between *Hordeum vulgare sp. vulgare* cv. "Haruna Nijo" and the wild barley *Hordeum vulgare sp. sponatneum* "line 602" containing 2,890 markers (Sato et al. 2009). This map for the first time integrated a high number of PCR-based markers in a single population, thereby overcoming many weaknesses of the available consensus maps. From 2005 on, the Diversity Array Technology (DArTs) offered simultaneous analysis of up to 6,000 markers (Wenzl et al. 2004, 2006), which nowadays is rapidly replaced with the Ilumina platform–based SNPs (Close et al. 2009). SNP markers are today the most widely used markers in barley as they are most abundant and are easily prone to automation and high throughput (Ganal et al. 2009; Kilian and Graner 2012; Perovic et al. 2012).

Respective high-density SNP-markers facilitate the efficient detection of QTL and genes involved in virus resistance in barley in

bi-parental populations (Lüpken et al. 2012) but also applying genome-wide association studies (GWAS, e.g., Rode et al. 2012), as this has shown already for BYDV tolerance using SSRs and AFLPs (Kraakman et al. 2006). Furthermore, the availability of high-density genome-covering markers may also facilitate genomic selection procedures in barley, including selection for virus resistance (Heffner et al. 2009; Heslot et al. 2012).

High-quality genomic sequences of rice, the second sequenced plant genome (Goff et al. 2002; Yu et al. 2002), represent the ideal blueprint for re-sequencing and identification of markers across the *Triticeae*. More recently, the release of the sorghum (*Sorghum bicolor*) and *Brachypodium distachyon* genomes (Paterson et al. 2009; International Brachypodium Initiative 2010) has opened the way to use these species as models for comparative genomics of cereals with large genomes. The model-based strategy is supported by the extensive conservation of gene order and sequence homology among the *Poaceae* genomes (Bolot et al. 2009; Abrouk et al. 2010). Colinearity between rice and barley has been widely exploited with different purposes (Perovic et al. 2004; Faure et al. 2007; Ramsay et al. 2011). Comparative studies involving the other genomes were also conducted as the wild grass *Brachypodium* has emerged as an important model for wheat and barley (Bossolini et al. 2007; Higgins et al. 2010). Considering that synteny between species is not equally conserved across genomic regions (Turner et al. 2005), any strategy to search for genes in barley should benefit from surveying several related species, as their information may prove complementary. Initially, when the transcriptome sequence of barley was the main resource of barley genomics, a "reciprocal BLASTN search" to the sequenced rice genome (Perovic et al. 2004) turned out to be the most efficient procedure in the prediction of orthologs. Recent advances in barley genomics relying on barley genomic sequences from two cultivars open new horizons in comparative mapping of cereals for fine-mapping and marker saturation of loci of interest (Luepken et al. 2012). Today, the genome zipper available in barley combining sequence information from rice, sorghum, brachypodium, and genomic sequence of barley has led to the assignment of 86% of the estimated barley genes to individual chromosome arms and their organization in a putative linear order (Mayer et al. 2011). The genome zipper turned out to be a very efficient tool for marker saturation in barley (Shahinnia et al. 2011; Luepken et al. 2012; Silvar et al 2012).

Use of Genomic Resources in Marker Saturation

Beside the construction of high-resolution mapping populations, marker saturation of chromosomal regions affecting virus resistance is a prerequisite for isolating resistance genes. Whereas it took more than 10 years to isolate the resistance locus *Rym4/Rym5* (Pellio et al. 2005; Stein et al. 2005), using the genomic tools described above and the physical map partly available for the barley genome (Schulte et al. 2011) a candidate gene for the *rym11* locus (Bauer et al. 1997), located in the centromeric region of chromosome 4HL (Nissan-Azzouz et al. 2005), has been identified within about 3 years (Luepken et al. 2012). A brief overview on the procedure employing genomic tools in marker development and gene isolation is given for the BaMMV/BaYMV resistance gene *rym13* in Figure 5.1. Based on a low- to medium-resolution map, a high-resolution mapping population has been constructed and marker saturation was conducted using available high-density genetic maps and the genome zipper including next-generation sequencing data available in barley. Based on these markers, a BAC contig will be identified and sequenced harboring the resistance gene *rym13*. Using this approach, it is assumed that in the near future, many virus resistance genes in barley will be isolated, leading on the one hand to a deeper understanding of the structure and function of virus resistance genes and the development of allele

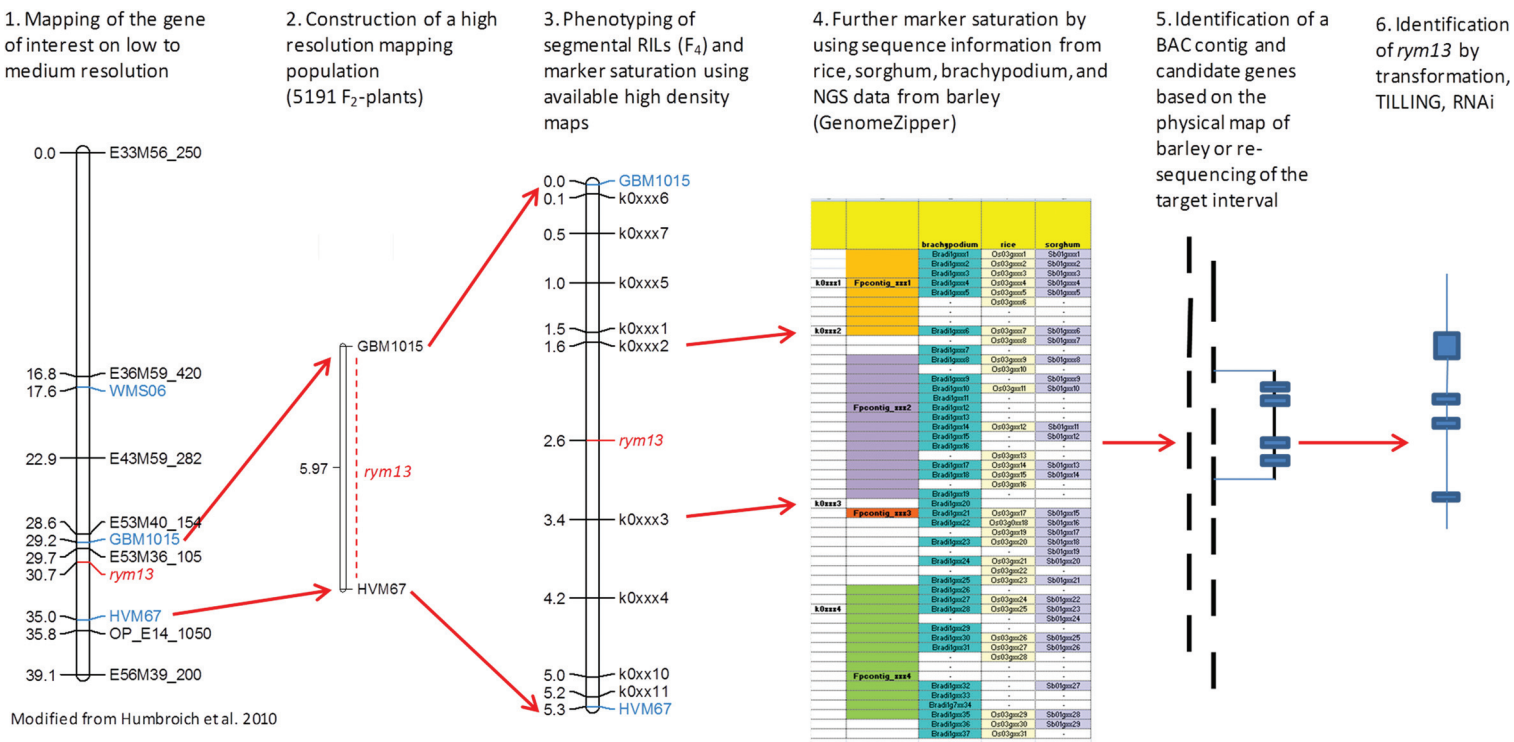

Fig. 5.1. Strategy for marker development and isolation of virus resistance genes in barley using genomic tools exemplified for *rym13* being effective against BaMMV/BaYMV (Lehmann et al. unpublished). For a color version of this figure, please refer to the color plate.

specific markers and on the other hand to the identification of new and perhaps more efficient alleles in TILLING populations or in germplasm collections, by allele mining.

Allele Mining and Future Prospects

Nowadays, the exploration of allelic diversity at the molecular level and the utilization of novel superior alleles using targeted molecular breeding ("precision breeding"; McCouch 2004) are of prime interest with respect to enhancing virus resistance. The analysis of natural or induced allelic variation and the identification of unknown valuable alleles at a locus of known function are called "allele mining" (Kaur et al. 2008). Usually, plant accessions from wild or locally adapted landrace gene pools contain a rich repertoire of alleles that have been left behind by the selective processes of domestication, selection, and cross-breeding that paved the way to today's elite cultivars. Allele mining involves the assembly of a germplasm collection or screening of a TILLING population for sequence variation in the target gene. Using this approach, 47 different alleles have been identified at the *Rym4/Rym5* locus in a barley world collection of about 1,000 genotypes (Hofinger et al. 2009, 2011). Respective studies can be conducted for all loci involved in virus resistance in barley (Table 5.1) as soon as respective genes have been isolated. The isolation of these virus resistance genes will transfer breeding to the allele level, facilitating the identification of novel alleles and their directed use in molecular breeding strategies (see above) in order to enhance virus resistance. The use of these alleles mainly derived from exotic germplasm can be fostered by marker-assisted backcrossing for the gene of interest simultaneously with the enhanced elimination of the donor background by genotyping using high-throughput SNP technologies, for example, the 9k iSelect chip in barley. However, respective alleles may also be transferred directly to high-yielding cultivars or be combined using new advances in gene technology like zinc-finger-nucleases (Shukla et al. 2009).

In summary, it may be concluded that based on the classical genetic and molecular marker analyses that resulted in mapping of many different virus resistance genes in barley, genomic tools available today and the complete sequence of barley that will be available in the near future, efficient breeding for virus resistance on the allele level will be facilitated, thereby broadening the genetic base of virus resistance in barley.

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Chapter 6

Molecular Breeding for *Striga* Resistance in Sorghum

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Abstract

Among the biotic stresses affecting dryland cereals, especially sorghum, *Striga hermonthica* is the most damaging obligate parasite, and is an important bottleneck to yield increases by smallholder farmers, yet it has been neglected by research in recent years. Integrated *Striga* management packages have been designed, but these will continue to require new cultural and chemical treatments, resistant varieties, and integrated approaches to manage both *Striga* and soil fertility. This review attempts to assess recent advances in bioassay development that are specific to resistance mechanisms, genomics such as New Generation Sequencing tools, RNA interference (RNAi) technologies in advancing knowledge of resistance and susceptibility to *Striga* including diversity in striga populations, and molecular marker technology in accelerating the development of *Striga*-resistant cultivars of sorghum. Recent advances in developing effective bioassays involving several modifications of rhizotrons and sand-packed titer plate assay will help dissect resistance mechanisms into component traits and increased understanding of the specific resistance mechanisms, which will directly help in efficient introgression and selection of several striga resistance mechanisms in breeding population. The current studies for identification of parasite genes specifically involved in haustorigenesis through transcriptomic and/or proteomic studies and more recently RNAseq studies will help understand susceptibility or resistance genes in striga. Release of improved version of cultivars resistant to striga developed by marker-assisted backcrossing of several striga resistance QTLs in Sudan had shown the power of integrating genomics and molecular breeding tools/techniques into routine breeding for tackling the complex constraint such as striga. Application and utilization of advance techniques in genomics and molecular breeding appropriately can further enhance the efficiency of integrated striga management practices, and thus crop productivity.

Introduction

Witchweeds (*Striga* spp.) are important pests of agricultural crops in much of Africa, especially East Africa, and had been a problem in parts of Asia and in the United States. These parasitic weeds have become the greatest biological constraint to cereal food production in resource-limited agricultural areas (Ejeta and Butler 1993; Gressel et al. 2004) as nearly 50 million hectares

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of field crops are infested with *Striga* annually, especially *S. hermonthica* and *S. asiatica*. These species infest maize, sorghum, pearl millet, finger millet, and upland rice, causing severe stunting. Yield loss attributable to *Striga* is acute, perhaps even exacerbated, ranging from 30% to 90% (Kroschel et al. 1999; van Ast et al. 2005; Ejeta 2007; Joel et al. 2007). Impacts are greatest on infertile soils, and the poorest subsistence farmers are the most severely affected. According to The Food and Agricultural Organization of the United Nations (FAO) (http://www.fao.org/), *Striga* infests over 40% of the cereal producing areas of sub-Saharan Africa, is continuing to spread, and accounts for US$7.4 billion in lost crops annually (Sharma 2006), with significant negative impact on the food supply of several million people. Effective control of *Striga* has been difficult to achieve through conventional agronomic practices, since the parasite exerts its greatest damage before its emergence above ground. Estimates on the extent of crop damage in a country or region in the African continent vary depending on the crop cultivar and degree of infestation (Parker and Riches 1993). However, *Striga* infestation is most severe in eastern Africa, especially in Ethiopia, where over 50% of sorghum production field are infested by several *Striga* species, and the invasion by the parasite is expanding at an alarming rate, often resulting in total crop losses annually on many farms. An expansion of *Striga* infestation is also occurring in West Africa. The impact of *Striga* in these regions is compounded by its predilection for attacking crops already under moisture and nutrient stress, conditions very acute in these regions and getting to be prevalent in much of the semiarid tropics.

Despite its agricultural importance, the molecular mechanisms controlling the establishment of parasitism are not well understood. The major species affecting sorghum in East Africa is *S. hermonthica*, and its life cycle is unique and well adapted to its parasitic lifestyle. The seeds need to be exposed to germination stimulants exudated from the host roots, such as strigolactones and ethylene; otherwise they can remain dormant in the soil for several decades (Bouwmeester et al. 2007). The seeds are tiny and possess limited amounts of nutrients, and this restricts their growth without a host connection. When a potential host is recognized through the sensing of strigolactones or other germination stimulants, the seeds that are close to the host roots (within 5 mm) can germinate. The germinated seedlings form haustoria—round-shaped organs specialized in host attachment and penetration (Yoder 2001). The formation of haustoria also requires host-derived signal compounds. The haustoria penetrate the host roots and finally connect with the vasculature to rob the host plant of water and nutrients. This dramatic developmental transition from an autotrophic to a heterotrophic lifestyle occurs within several days.

Intensive efforts in the scientific community, mainly in the United States during the 1960s, lead to the identification of some germination stimulants. This was followed by the development of a "suicidal germination" strategy to eradicate *Striga* weeds (Rispail et al. 2007). By this strategy, a germination stimulant (in this case ethylene) was mixed in the soil to trigger germination in the absence of the hosts. This approach was used successfully to eradicate *Striga asiatica* in North Carolina. The suicidal germination approach was not applicable for African farmers due to the high cost of the strategy and the much larger scale of infestation.

Integrated *Striga* management packages have been designed that include: *Striga* resistant varieties (Rodenburg et al. 2006); judicious and appropriate timing and application of fertilizers in combination with organic fertilizers, suitable crop rotations, and trap cropping (van Ast et al. 2005; Oswald 2005; Joel et al. 2007); intercropping with forage legume *Desmodium uncinatum* (Khan et al. 2007) and seed coating with amino acids, fusarium spps, and herbicides (Kanampiu et al. 2003); and water conservation measures. *Striga* management will continue to require cultural and chemical treatments,

resistant varieties, and an integrated approach to both *Striga* and soil fertility. Of these approaches, development of resistant crop cultivars has been recognized as the most effective and feasible method. To date, *Striga*-resistant sorghum cultivars – such as N13, SRN39, Framida, 555, ICSVs, SRN39 derivatives (P401 to P409), Soumalemba (IS15401), and Seguetana CZ and CMDT45 – have been identified and, as has been observed by Tabo et al. (2006) and ICRISAT (2009), these can be integrated with available crop management options to enhance productivity.

Success has been limited because *Striga* impacts host development shortly after attaching to the root, long before the parasite emerges above ground, and yield losses are not linearly related to parasite biomass (Gurney et al. 1999). However, a lack of knowledge of the genetic factors controlling host-parasite signalling at different stages of the life cycle, the paucity of resistant host germplasm, the polygenic nature of resistance coupled with complex genotype × environment (G × E) interactions, and insufficient knowledge of parasite race structure impede progress. Genomic approaches offer new opportunities to dissect polygenic resistance traits into their underlying genetic components (quantitative trait loci [QTL]), allowing breeders to utilize marker-assisted selection (MAS) for the transfer and pyramiding of useful alleles into locally adapted cultivars. Further, sequence information and genomic tools for forward and reverse genetics open the way for identification of key genes controlling parasitism (in both host and parasite) and their genetic manipulation. Here, we critically assess the current and future roles of genomic platforms such as New Generation Sequencing tools, RNA interference (RNAi) technologies, in advancing knowledge of resistance and susceptibility to *Striga* and molecular marker technology in accelerating the development of *Striga*-resistant cultivars of sorghum. Recent status of development of *Striga*-resistant cultivars is also discussed.

Development of Bioassays and Dissecting *Striga* Resistance Mechanisms

Additionally, such combinations would offer more buffering capacity to host plant population against building virulence in *Striga* populations, as it could only result from multiple mutations to overcome these obstacles resulting in durable host-plant resistance. To date, five specific *Striga* resistance mechanisms had been described, which include resistance associated with low germination stimulant (*LGS*) production, low production of the haustorial initiation factor (*LHF*), Germination inhibitors (*GI*), hypersensitive response (*HR*), and the incompatible response (*IR*) to parasitic invasion of host genotypes (Ejeta et al. 2000). Assessing these resistance mechanisms across several lines individually is very difficult, as most of times their effects are confounding and are difficult to separate. In this scenario, breeding for *Striga* resistance would be greatly assisted by in vitro methods that allow inspection of pre-attachment and early post-attachment phases of *Striga* interaction with host root systems. Such observations could reveal underlying resistance mechanisms in source germplasm and allow selection for specific resistance mechanism, alone or in combination, in breeding populations for future exploitation. Recent approaches such as RNA interference and microRNA assays to further characterize host-parasite interactions at nucleotide level offer new avenues for improving *Striga* resistance, but require more specific assays.

In case of S*triga*, it is particularly important that control is expressed early in the parasitic life cycle since severe consequences on host health are manifested within the first few days after parasitic attachment (Gurney et al. 1999). Traditionally, breeding for most of complex traits such as *Striga* resistance had been based on field selection/phenotypic performance of germplasm, and selection efficiency is based on a well-defined trait phenotype such as emerged parasite number or severity scores in artificially infested

plots over multiple years and locations (Omanya et al. 2004). However, these filed evaluation measures of *Striga* resistance fail to distinguish between resistance/tolerant traits and the associated interactive events between host and parasite. A potential host plant may have any of several defense responses to *Striga* infection (Ejeta 2007; Joel et al. 2007; Rich and Ejeta 2007). Tolerance to the ill effects of *Striga* is also known, whereby the crop is able to produce acceptable yields despite Striga infestation (Rodenburg et al. 2006).

Adequate genetic variation for a target trait and availability of effective selection tools are essential requisites for successful plant-breeding efforts, and unfortunately selection methods that work well for improving other desirable crop traits have not operated at the same level of efficiency for *Striga* resistance (Ejeta and Butler 1993). Similar to other major complex trait, field selection for *Striga* resistance had not been successful because of the difficulty in clearly identifying resistant variants, lack of understanding on the genetic control of field resistance, and the difficulty of establishing uniform infestation of the parasite population under varying environmental conditions. Lack of rapid and efficient screening techniques had been a major constraint in the progress of *Striga* resistance breeding, and hence development of bioassays received primary focus and consideration across all *Striga* resistance breeding programs.

Rhizotrons and other in vitro growth systems of various designs have been described for co-culture of weedy root parasites and their hosts (Rubiales et al. 2006). An agar-based system and its modifications had been quite useful in identifying sorghum seedlings with resistance based on low *Striga* germination stimulant production (Omanya et al. 2004), and sorghum accessions had been useful for their ability to trigger formation of the haustorium or appressorium in *S. asiatica* (Rich et al. 2004) and to observe early post-attachment reactions on sorghum expressed as hypersensitive response to *Striga* (Mohamed et al. 2003). These methods had inherent limitation of short time for observations and inconsistency. Co-culture of *Striga* on sorghum in Petri dishes stood on end and containing moistened paper topped with glass fiber with a hole to accommodate host shoot growth (Arnaud et al. 1999) allowed observations of parasitic associations over several weeks, but attachment frequency in these rhizotrons is also low if the growth medium is too wet. Larger rhizotrons using sand (Gurney et al. 2003) or rockwool (Gurney et al. 2006; Yoshida and Shirasu 2009) sandwiched between plastic plates have been well suited to co-culture of *S. hermonthica* on cereals. A fundamental problem with all the aforementioned methods, however, is that newly attached *Striga* are so small (millimeters or less) that a microscope capable of at least 10× magnifications is required to view newly attached parasites. This means that this entire activity becomes very cumbersome, low throughput, resource intensive, and time consuming. To be useful in a breeding program, in vitro methods should mimic natural conditions, occupy little space (particularly for containment facilities), and be low cost and relatively easy to set up and maintain. They should allow nondestructive and progressive observations, preferably at multiple times during co-culture, consistent, repeatable, and with high heritability.

A sand-based rhizotron for monitoring *Striga* parasitism with the aid of a scanner during the critical attachment and early post-attachment phases was recently developed (Amusan et al. 2011). The sand-packed titer plate assay (SPTPA) was used to examine *Striga*-susceptible and -resistant maize (Amusan et al. 2008) and sorghum, previously identified in field trials, to pinpoint the stage at which *Striga* seedlings stop growing or died on the host roots. These modifications and the ones to follow will help dissect the different *Striga* resistance mechanisms into the component traits, which in turn will allow for easier selection and introgression in breeding programs. Use of these assays had enhanced the ability more systematically to evaluate and exploit sorghum germplasm as sources

of *Striga* resistance by focusing on each stage of the parasitic process individually. The bioassays had also provided further insights to the interactive biological processes between *Striga* and the roots of host plants during the early stages of the infection process, giving an increased understanding of the specific mechanisms of resistance associated with each source of host genotype (Cai et al. 1993). Furthermore, defense responses triggered by infection could also be monitored and exploited via these assays. Hence, disrupting these interactions offers unique opportunities for controlling *Striga* through identification of genetic variants with single or multiple interventions at key critical stages throughout the life cycle, which are likely to be simply inherited and therefore easy to manipulate through conventional breeding or via other biotechnological approaches for the development of *Striga*-resistant crop cultivars.

Understanding Host-Parasite Biology: Exploring Pathway Stages as Entry Points for Breeding Resistance to *Striga*

Genetic resistance is a vital component of an effective integrated *Striga* management program (Ejeta 2007). Durable resistance for a trait like S*triga* is most securely based on multiple traits that block the establishment of weedy root parasites on their hosts (Rispail et al. 2007). This involves several metabolic pathways, both in host and parasite, that play a very dynamic role during expression of resistance or susceptibility for host and of virulence or avirulance for parasite. Successful parasitism is therefore a series of interactive processes between host and parasite conditioned by a large number of genetic and physiological events, each possibly influenced by additional array of environmental factors. Host plant resistance based on observation of emergence above ground of parasitic seedlings and level of infestation, therefore, is a complex, quantitatively inherited trait that is difficult to select for using conventional approaches of plant breeding. Characterization and dissection of *Striga* resistance into specific mechanisms based on a series of host-parasite signal exchanges should be the central focus and premise for any research approach and effort aimed at developing *Striga*-resistant cultivars (Ejeta et al. 1991).

The life cycle of *Striga* is intimately linked to that of its host and depends on a complex exchange of chemical signals; this poses a challenge and an opportunity for control, both before and after attachment of *Striga* to the host root. Understanding host-parasite biology at each life cycle stage is essential for the design of novel control strategies. The first committed step of the *Striga* life cycle is germination, which occurs only in response to specific secondary metabolites in the root exudates of host and some non-host plants. There are several classes of germination stimulants such as strigolactones (Bouwmeester et al. 2003), most commonly present in the exudates of many cereals species (Awad et al. 2006). Some low-germination-stimulant-producing sorghum cultivars have been identified and often perform well when used as part of an integrated control program (Joel et al. 2007). Manipulation of the production of germination stimulants requires further knowledge of their biosynthetic pathways. Forward and reverse genetic approaches may identify key genes involved in strigolactone synthesis such as in maize (Bouwmeester et al. 2003), and the use of maize mutants revealed that strigolactones are derived from the carotenoid pathway (Matusova et al. 2005; Bouwmeester et al. 2007) and can facilitate the identification of genes involved in the synthesis and regulation of strigolactones (Sun et al. 2007) in rice and sorghum as model crops.

Following germination, haustorial inducing factors (HIF) are required to trigger differentiation of the parasite haustorium (Keyes et al. 2000; Keyes et al. 2007). To initiate the identification of parasite genes specifically involved in haustoriogenesis, *Triphysaria versicolor*, a facultative parasite closely related to *Striga*, is being used as a model system. An EST database

(Pscroph) (http://pscroph.ucdavis.edu/) (Torres et al. 2005) has been developed representing transcripts differentially regulated in Triphysaria before and after contact with host roots, in response to host root exudates and to the HIF 2,6-dimethoxybenzoquinone (DMBQ) (Yodler et al. 2007). Approximately 40,000 ESTs from the different suppressive subtraction hybridization (SSH) libraries have been sequenced and annotated (http://www.plantgdb.org/prj/ESTCluster/progress.php). This approach is being extended to produce EST collections from *Striga* and *Orobanche* facilitating identification of potentially essential genes for germination stimulant perception, haustorial formation, and parasite development. Stable and transient transformation systems for *Triphysaria* (Tomilov et al. 2007) and transformation of *Striga* have also been successful, allowing the function of parasite genes to be tested by silencing or overexpression. These approaches may allow to design novel control strategies, for example, by utilizing gene-silencing vectors designed against essential parasite specific genes. If such vectors were transformed into cereals and the RNAi signal moved from host to parasite, silencing of the parasite gene could be lethal or inhibit parasite development. Post-transcriptional gene silencing (PTGS) has been demonstrated to work against viruses and nematodes, but its applicability for *Striga* control is unproven. Preliminary evidence suggests that this approach may work for the holoparasitic *Orobanche* where mRNA levels encoding the enzyme mannose-6-phosphate were reduced by 60–80% in the parasite when grown on transgenic tomato plants containing the appropriate silencing construct (Rady 2007). However, a key difference between *Orobanche* and *Striga* is that only the former establishes direct phloem connections with the host as the silencing signal is thought to be phloem mobile (Tournier et al. 2006).

Once the haustorium has attached to the host, *Striga* penetrates the cortex and endodermis to form a direct link with host xylem vessels. Identifying host genotypes that block penetration and identification of the genes involved is a major focus of *Striga* research. There are a few cultivated and many more wild sorghums that exhibit partial post-attachment resistance; several sorghum cultivars and a wild accession P47121 exhibit a hypersensitive response phenotype when infected with an ecotype of *S. asiatica* (Mohamed et al. 2003; Rich et al. 2004), and the sorghum cultivar N13 appears to exhibit "mechanical" resistance at the root endodermal barrier (Haussmann et al. 2004). The molecular basis and signalling pathways underlying resistance or susceptibility in plant-plant interactions are obscure, and rice offers the opportunity for molecular dissection of these traits. Preliminary analyses of changes in gene expression in susceptible and resistant cultivars using rice microarrays revealed that many of the genes upregulated in Nipponbare are those classically associated with defense responses to microbial pathogens, for example, pathogenesis related (PR) genes, cytochrome P450s, and WRKY transcription factors (Scholes et al. 2007).

Understanding the mechanistic basis of different types of resistance to *Striga* will facilitate pyramiding of resistance genes, via genetic engineering strategies or MAS with aim of providing durable polygenic resistance. Relatively few transcriptomic or proteomic studies to identify susceptibility or resistance genes in *Striga* are known to be under way. Increasing availability of microarrays for sorghum and newer expression-profiling tools such as RNAseq would address this in the near future.

Striga Diversity, Racial Differentiation, and its Implications on *Striga* Resistance Breeding

The issue of genetic diversity among *Striga* species and within species populations structured by geography, host crop, and other environmental factors affecting adoption plays an important role in host-plant interaction and expression of

resistance. It is clear that a much greater knowledge of *S. hermonthica* and *S. asiatica* diversity/race structure in relation to host species and cultivar specificity is urgently needed to inform breeding programs in different regions of Africa. There is growing evidence from field studies and molecular analysis that there is host specificity and adaptation in *S. asiatica* and *S. hermonthica* populations. Different *Striga* populations show specific genetic adaptation to host and host genotypes displaying variable virulence (Riopel and Timko 1995). In addition to variation in the genetic diversity between populations of a species of *Striga*, there is likely to be a difference between the species in within population diversity. *Striga hermonthica* is a self-incompatible outbreeder, whereas *S. asiatica* is autogamous, self-pollinating prior to floral opening, and, as such, it is highly inbred. The difference in reproductive biology is likely to have a significant impact on the within-population diversity of these species, *S. hermonthica* having a higher within-population genetic diversity than *S.asiatica* (Safa et al. 2006; Scholes et al. 2007). What implication this has for plasticity of populations regarding host specificity needs to be determined. Evaluation of the genetic diversity of *Striga* populations and determination of the influence of parasite genotype on virulence in differing hosts would better enable *Striga* researchers to ensure that potential control products are fully evaluated. This knowledge is key to the generation of more durable technologies and will enable better targeting of dissemination of control technologies to specific localities. A recent study by Estep et al. (2011) investigated genetic diversity of *S. hermonthica* populations collected from four different regions in Mali using SSR markers. The *Striga* populations were characterized by broadly distributed allelic diversity with little genetic differentiation and large amount of gene flow. It was also observed that population structure did not correlate with local environment or host species (sorghum versus pearl millet). These understandings can help plant breeders identify race/population specific resistance genes/genotypes, which can further be used to identify individual resistance genes in crop (host) germplasm and pyramid multiple resistant genes into a targeted crop plant. In order to fully characterize the existence of "races" and the factors driving their formation, further collections of *S. hermonthica* populations and their hosts are needed. The recent development of a high-throughput microarray-based Diversity Arrays Technology (DArT) (Wenzl et al. 2004) for several crop species, including sorghum, and Next Generation Sequencing (NGS) based Genotyping-by-Sequencing (GbS) tools could substantially accelerate knowledge of *Striga* diversity. The development of a *Striga*-specific assays would allow screening of thousands of molecular markers in parallel and facilitate comparison of *Striga* populations from micro (within a field) to macro (between countries) scales.

QTL Analysis and Marker-Assisted Selection for Improving *Striga* Resistance

The availability of sequence information from EST and genome-sequencing projects has led to the development of dense molecular genetic maps for many cereals including rice, sorghum, and maize (Varshney et al. 2004; Mace et al. 2009). Most resistance to *Striga* appears to be polygenic. The use of mapping populations, QTL analysis, and advanced backcross QTL analysis (AB-QTL) (for transferring important traits from wild relatives into a crop variety (Tanksley et al. 1996) combined with marker-assisted selection (MAS) is a promising approach that is beginning to yield results for the development of resistant cultivars. Several QTL and AB-QTL studies have been performed under laboratory conditions to identify the genetic basis of resistance in cultivated and wild relatives of sorghum (Haussmann et al. 2004; Grenier et al. 2007). An advanced backcross mapping population derived from a cross between PQ434 (low-HIF-producing wild sorghum) and Shanqui Red (cultivated,

high-stimulant-producing sorghum) allowed the *Lhf* (low haustorial factor) locus to be mapped to 19.3 cM from the microsatellite marker *Xtxp358* on linkage group nine (= sorghum chromosome SBI-09 short arm) (Grenier et al. 2007). Similarly, a cross between the wild sorghum species *S. arundinaceum*, which exhibits a hypersensitive like resistance response, with two cultivated sorghum species revealed that the resistance trait was controlled by two nuclear genes *HR1* and *HR2*, which were mapped at 7.5 cM from *Xtxp096* on SBI02 and 12.5cM from *SbKAFGK1* on SBI05, respectively (Mohamed et al. 2003; Grenier et al. 2007).

Analyzing gene expression profiles within a segregating population such as NILs or selected backcross inbred lines (BILs) or Recombinant Inbred Lines (RILs) allows the identification of both cis and trans acting QTL, thus providing information about factors that control the expression of a gene as well as its location on a genetic map (Schadt et al. 2003). Although it is often assumed that QTL do not accurately reflect the physical location of genes on the genome underlying a polygenic trait, in many cases, the gene was located within 1–2 cM of the QTL peak (Price et al. 2006). Combining QTL analysis with transcriptomic data can help determine whether genes that are differentially regulated within the QTL regions are putative candidate resistance genes.

Haussmann et al. (2004) reported a QTL analysis of field resistance to *Striga* using two mapping populations of RILs derived from a cross between IS9830, a low germination stimulant producer, and a E36-1, a susceptible genotype (RIL-1); and N13 (mechanical resistance) and E36-1 (RIL-2). Each mapping population was divided into two sets, which were tested in sequential years at five sites in Mali and Kenya (Haussmann et al. 2001). Composite interval mapping revealed some QTL in each RIL that were stable across years and environments and some that were not. In RIL-1, the most significant QTL corresponded to the *lgs* locus but other QTL also indicated the presence of other resistance mechanisms. In RIL-2, five QTL (derived from N13) were stable across years and environments and explained between 12% and 30% of the observed genetic variation for resistance indicating that flanking molecular markers would be excellent candidates for MAS. Recently Satish et al. (2012) fine-mapped the *lgs* locus on sorghum chromosome SBI-05 toward distal end. Four tightly linked SSRs were also tagged and validated for their linkage with *lgs* locus.

Recent Development in Marker-Assisted Backcrossing for Development of *Striga* Resistance Products

The QTLs identified by Haussmann et al (2004) in RIL-2 (based on cross N13 × E36-1) were subject to two MABC projects over last few years. Following the initial QTL mapping studies, a collaborative project of ICRISAT, IER, and the University of Hohenheim, two locally adapted, farmer-preferred sorghum varieties from Mali were introgressed with up to four of the five resistance QTL by marker-assisted backcrossing (MABC) (Muth et al. 2011). In this project, 32 of the resulting backcross-two lines (BC2S3) were field-evaluated for their *Striga* resistance under natural and artificial *Striga* infestation at three sites in Mali in 2009 and 2010. Together with yield data and agronomic properties, the number of emerged *Striga* plants per experimental plot was evaluated at regular intervals over the cropping season as an integrative measure of disease severity. In parallel, the presence/absence of the targeted genomic regions from N13 neighboring the QTL was tested in all lines using flanking SSR markers mapped to the vicinity of the targeted QTLs. Preliminary analyses of the data show a resistance of the best sorghum lines equal to or exceeding the resistance of the donor parent N13. However, yield of BC2-lines was on average inferior to the recurrent parents. A strong environmental influence on resistance between trial sites was observed in the field experiments.

The same project activities in Sudan were much more advance, and recently resulted in the release of four *Striga*-resistant varieties in the genetic backgrounds of popular, but *Striga*-susceptible, improved sorghum varieties "Tabat," "Wad Ahmed," and "AG8" (personal communication with Dr. Abdalla Mohamed, Sudan). The backcross/QTL validation project advanced to the second backcross generation (BC2) in several locally adapted farmer-preferred open-pollinated varieties. The resulting early-generation backcross progenies, although *Striga* resistant, were not agronomically elite enough to be submitted to national trials and considered for release. The national programs in Sudan, Eritrea, and Kenya, led by Dr. Abdalla Mohamed and with ICRISAT providing backstopping, then obtained funding through the regional agricultural science network (ASARECA Competitive Grant System for 2006) to fine-map the *Striga* resistance QTLs and complete the task of recovering recurrent parent eliteness for materials in the genetic backgrounds of farmer-preferred improved sorghum varieties from Sudan. Three more backcrosses were executed along with foreground selection, with QTL flanking SSRs at each stage and with background selection with DArT markers in BC4F1 progenies.

Standard variety trials were conducted in *Striga* sick plots over three rainy seasons (2009 to 2011) at the Gezira Research Station (GRS), Damazine, Sinnar, and Gedaref in Sudan. Results from these trials revealed that backcross-derived lines T1BC3S4, AG6BC3S4, AG2BC3S4, and W2BC3S4 were *Striga* resistant and agronomically superior, giving 180% to 298% increases in grain yield over their recurrent parents in the infested sick plots. These four experimental varieties in Sudan were approved by the National Crop Variety Release Committee, as "ASARECA.T1" (T1BC3S4), "ASARECA.W2 Striga" (W2BC3S4), "ASARECA.AG3" (AG2 BC3S4), and "ASARECA.AG4" (AG6BC3S4).

There were several hurdles, which in turn had led to significant new knowledge for handling the issues related to MABC of complex traits such as *Striga* resistance. Most of the *Striga* resistance QTLs targeted for this introgression work were characterized by large confidence interval between flanking markers, scant availability of flanking SSRs (Table 6.1), and lack of polymorphism between donor and recurrent parents. This had many implications for attempting introgression of *Striga* resistance QTLs in several target genetic backgrounds. The lack of enough SSRs at initial stage of project (till BC2-generation) spread across QTL region meant high probability of losing the QTL even after flanking marker confirmation because of the possibility of recombination occurring in the putative QTL regions, linkage drag with unfavourable traits, and ultimately lower recurrent parent recovery. This was evident from the first phase of the project were we had lower recurrent parent recovery in BC2-progenies. Before we can make any further progress toward this end, we were also stuck with unavailability of tightly linked SSRs with target QTLs and hence identification of confirmed QTL heterozygote(s). Also the underlying mechanisms/traits for each target QTL were not fully understood, which was linked to lack of proper phenotyping assays for those mechanism/traits. All of the phenotyping was done with field-level screening. These issues were addressed by advancing large BC-progenies until BC4 with foreground selection to reduce the probability of losing the QTL due to crossover between the large QTL interval. We simultaneously assayed the RIL population based on cross-(N13 $\times$ E 36-1), used for QTL identification, with additional SSRs and DArT markers used for MABC. This led to identification of additional markers for target QTL interval. These additional SSRs were subsequently used for foreground selection of BC4S4-population of >150 progenies, followed by background selection with DArT markers. We identified 31 BC4S4-progenies, which were screened across several years and locations, resulting in identification and release of the four varieties.

Table 6.1. Details of sorghum SSRs used for foreground selection for marker-assisted backcrossing of *Striga* resistance QTLs

Striga resistance QTLs	Sorghum chromosome[a]	Physical distance (Mbp)[b]	Linkage distance (cM)[c]
SSRs flanking to Striga resistance QTL on SBI-01			
Xtxp213	SBI-01	6.7	NA
XmsbCIR268	SBI-01	8.7	19.2
Xcup033	SBI-01	13.6	NA
SSRs flanking to Striga resistance QTL on SBI-02.1			
Xtxp197	SBI-02	1.5	2.2
Xtxp084	SBI-02	4.9	22.3
Xtxp080	SBI-02	3.9	18.9
XmbCIR223	SBI-02	4.7	19.7
SSRs between two *Striga* resistance QTLs on SBI-02			
Xtxp050	SBI-02	5.0	22.3
Xiabtp346	SBI-02	12.7	NA
Xiabtp500	SBI-02	18.8	NA
Xtxp072	SBI-02	27.9	74.8
SSRs flanking to Striga resistance QTL on SBI-02.2			
Xiabtp444	SBI-02	55.5	NA
Xtxp013	SBI-02	56.0	82.0
Xtxp298	SBI-02	57.1	92.9
Xtxp056	SBI-02	61.6	124.0
Xtxp296	SBI-02	71.1	171.8
SSRs flanking to Striga resistance QTL on SBI-05.1			
Xtxp115	SBI-05.1	NA	27.9
Xtxp268	SBI-05.1	1.7	NA
Xtxp065	SBI-05.1	1.9	14.4
Xiabtp420	SBI-05.1	3.2	NA
Xtxp112	SBI-05.1	3.9	NA
SSRs flanking to Striga resistance QTL on SBI-05.2			
Xtxp225	SBI-05.2	NA	59.4
Xtxp014	SBI-05.2	42.3	64.1
Xtxp015	SBI-05.2	42.0	64.2
Xtxp299	SBI-05.2	NA	64.9
Xtxp262	SBI-05.2	57.9	94.1
SSRs flanking to Striga resistance QTL on SBI-06			
Xtxp317	SBI-06	50.8	90.7
Xtxp097	SBI-06	66.1	92.9
Xtxp219	SBI-06	NA	91.9
Xiabtp130	SBI-06	53.9	NA
Xcup12	SBI-06	54.5	NA
Xtxp176	SBI-06	55.9	134.1
Xtxp057	SBI-06	57.4	141.0
Xcup37	SBI-06	61.9	165.2

[a]Sorghum chromosome nomenclature as per Kim et al. (2005).
[b]Physical map distance (in MbP) as estimated by BLAST search of primer pair sequence of individual SSR with sorghum genome sequence as described in Ramu and Deshpande et al. (2010).
[c]Linkage distance (in cM) of SSRs as estimated in consensus map developed by Mace et al. (2009).

Advances in Genomics and Applications for *Striga* Resistance Research

Simple sequence repeat (SSR) markers are still the preferred choice of markers in plant-breeding programs with interest in mapping and introgression of different traits in crop species. The amenability to simple assays, multiplexing, reproducibility, and more importantly codominant nature of SSRs works to the advantage of plant-breeding science to follow the segregating population as per principles of Mendelian and Population genetics for selection of best phenotypes. SSR markers were greatly exploited for mapping of different traits in sorghum, including *Striga* resistance (Haussmann et al. 2004, Satish et al. 2012). A major limiting factor for utilization of SSR markers is the resolution power. Recent advances in sorghum genomics including availability of sorghum genome sequence (Paterson et al. 2009), access to large number of markers including DArTs (Mace et al. 2009; Ramu and Deshpande et al. 2010), and alignment of major trait genes and quantitative trait loci (QTL) to integrated linkage and physical map (Mace et al. 2011) had strengthened the foundation for better integration of molecular marker technologies to dissect complex traits such as *Striga* resistance.

With the invention of Next Generation Sequencing (NGS) technologies, identification of a large number of markers, especially Single Nucleotide Polymorphism (SNPs), has become cheap as compared to the other marker systems. Utilizing Illumina NGS platform, Ed Buckler Lab at Cornell University in Ithaca, New York, developed a technically very simple and highly multiplexed (96-plex/384-plex) method for rapid sequencing and associated bioinformatics pipeline for genotyping the germplasm and is referred as Genotyping-by-Sequencing (GbS) (Elshire et al. 2011). These sequences produced are aligned to reference genome, BTx623 (Paterson et al. 2009) to identify SNPs with help of customized bioinformatics pipelines with appropriate computational power, which still remains the major challenge with handling the large datasets generated using NGS tools. This extensive coverage with a large number of SNPs across genome helps identify SNPs closest to or inside the genes associated with *Striga* resistance, and can be a part of customized SNPs assay using BeadXpress platform or CAPS markers at lower cost for further genotyping to transfer this trait to desired recurrent parent of sorghum. This will greatly improve the efficiency of introgression of component traits underlying different *Striga* resistance mechanisms of by reducing breeding cycles (for recurrent parent recovery) and further recombining these for development of improved *Striga*-tolerant varieties.

Application of NGS tools like GbS for dissecting complex traits such as *Striga* on DNA sequence level will capture most of the functional factors of genome related to trait expression. But other application of NGS tools in RNA sequencing (commonly referred as RNA-seq) will help capture the regulatory parts (Ozsolak and Milos 2011). For a complex trait such as *Striga* resistance, involving host-parasite interactions, many growth and development pathways from both host and parasite life cycles are involved in its expression. Application of RNA-seq platforms can help understand the role of regulatory and transcription factors (including small RNA, micro RNA) and their interaction with other pathways. There is big interest to utilize recent advances in RNA-seq technologies with the recombinants identified from fine-mapping exercise to move toward better understanding the *Striga* resistance in sorghum. As knowledge of QTL underlying resistance traits increases, comparative genomic approaches will aid detection of *Striga* resistance genes in syntenic regions of the rice, sorghum, maize, and pearl millet as sequence information becomes available.

Recently Yoshida et al. (2010) generated a full-length enriched cDNA library of *S. hermonthica* by sequencing over 37,000 clones and

identified over 17,000 unigenes. The comparative analysis of this unigene dataset with other plant genomes or ESTs revealed that approximately 80% of the unigenes had homologes in other dicotyledonous plants including *Arabidopsis*, poplar, and grape. Interestingly they found 589 unigenes that were conserved in the hemiparasitic *Triphysaria* species, a close relative of *Striga*, but not in other plant species. Furthermore they also identified 1,445 putative SSRs in the *S. hermonthica* unigene dataset. These recently developed extensive set of molecular resources using advanced molecular tools will help in studying *S. hermonthica* for genome annotation, gene discovery, functional analysis, molecular breeding, comparative mapping with different plant genomes, epidemiological studies, and studies of plant evolution. The recently started Parasitic Plant Genome Project (Westwood et al. 2012) aims to develop new tools for understanding the biology of *Orobanche* and *Striga*. This project had sequenced transcripts from three parasitic species and a nonparasitic relative in the *Orobanchaceae* with the goal of understanding genetic changes associated with parasitism. The species studied span the trophic spectrum from free-living nonparasite to obligate holoparasite. Parasitic species used included: *Triphysaria versicolor*, a photosynthetically competent species that opportunistically parasitizes roots of neighboring plants; *Striga hermonthica*, a hemiparasite that has an obligate need for a host such as sorghum; and *Orobanche aegyptiaca*, a holoparasite with absolute nutritional dependence on a host. *Triphysaria* is a genus of five hemiparasitic species that grow as common annuals throughout the Pacific Coast of the western United States (Hickman 1993). *Triphysaria* has a broad host range that includes maize, rice, and Arabidopsis, and is closely related to the agricultural pests *Striga* and *Orobanche*. *Triphysaria* has no agricultural significance and so can be grown without quarantine restrictions (Goldwasser et al. 2002). *Triphysaria* flowers are amenable to classical genetic manipulations and genomic resources are being developed, making *Triphysaria* a useful model species for parasite studies (Torres et al. 2005). For the genome project, tissues for transcriptome sequencing from each plant were gathered to identify expressed genes for key life stages from seed conditioning through anthesis. Importance of this project lies in that the two of the species studied, *S. hermonthica* and *O. aegyptiaca*, are economically important weeds and the data generated by this project are expected to aid in research and control of these species and their relatives. The sequences generated through this project will provide an abundant molecular resource for understanding population dynamics, as well as provide insight into the biology of parasitism and advance progress toward understanding parasite virulence and host resistance mechanisms. In addition, the sequences provide important information on target sites for herbicide action or other novel control strategies such as trans-specific gene silencing.

RNA interference (RNAi), or post-transcriptional gene silencing (PTGS), is a conserved mechanism in eukaryotes by which double-stranded RNA molecules (dsRNA), formed either by complementary base pairing of transgenic sequences or by fold-back of endogenous noncoding sequences, are processed by Dicer-like nucleases into short 21–24 nt interfering RNAs (siRNA) or micro-RNAs (miRNAs). These small RNAs are then incorporated, along with Argonaute-like proteins, into RNA-induced silencing complexes that direct the degradation of endogenous RNAs that are homologous to the siRNAs (Bartel 2004; Baulcombe 2004). When siRNAs are introduced into specific tissues of a plant by biolistics or agroinfection, siRNA moves through plasmodesmata into other tissues in a non-cell-autonomous fashion (Voinnet 2005). RNA-dependent RNA polymerase amplifies the primary siRNA, allowing further spread of the silencing signal (Himber et al. 2003).

RNAi signals can also enter the phloem and spread systemically throughout a plant, and even

across graft junctions from transgenic stocks to nontransgenic scions, although the nature of the translocated molecule is not known. *Agrobacterium*-based vectors have been developed to deliver siRNA precursors into plants in order to selectively target endogenous genes for inactivation. These vectors are designed so that the target RNA forms self-complementary, hairpin structures (hpRNA) that result in localized dsRNA regions that are cleaved into siRNA molecules by Dicer-like nucleases (McGinnis et al. 2005).

Tomilov et al. (2008) studied the transspecific gene silencing between host and parasite plants using transgenic roots of the hemiparasitic plant *T. versicolor* expressing the GUS gene to parasitize transgenic lettuce roots expressing a hairpin RNA containing a fragment of the GUS gene (hpGUS). These experiments described movement of RNAi molecules between parasitic and host plants. Using an *Agrobacterium rhizogenes*-mediated transformation system, Tomilov et al. (2008) developed root cultures of *Triphysaria* that express the GUS reporter gene (Jefferson et al. 1987). These roots retained their ability to develop haustoria in response to host factors and to invade host roots. The results of these experiments indicated that RNAi signals are translocated across haustorial junctions in both directions and mediate gene silencing in both parasite and host plants. Once genes critical to parasite growth and development are identified, these parasite-specific sequences could be cloned into hairpin vectors and transformed into plants for silencing of parasite genes by generating siRNA in the host. This could provide a novel strategy for controlling parasitic weeds.

Similarly, recent cloning and functional characterization of a race-specific R gene from cowpea (Timko et al. 2012) opens the door for further exploration of the mechanism of host resistance and provides a focal point for studies aimed at uncovering the molecular and genetic factors underlying parasite virulence and host selection.

Managing *Striga* in Sorghum: Current Technologies and Strategies

Farmers impacted by *Striga* occupy a very heterogeneous biophysical, cultural, social, economic, and political landscape with common key abiotic constraints of water and nutrient availability (Waddington et al. 2010). Failure to recognise this fact will invariably hinder the adoption of *Striga* control approaches. To harness the impact of variable efficacy of individual control practices, many advocate integrated *Striga* control (ISC) approaches – combinations of cultural and, where available and applicable, seed-based technologies (Schulz et al. 2003; Kamara et al. 2007). Nonetheless, just as there is no magic bullet for *Striga* control, there is no magic shotgun cartridge either (Douthwite et al. 2007). Technologies need to be packaged in such a way as to suit the abiotic, biotic, and market-access constraints the farmers experience. It is perhaps common sense that technologies that fit in with the current time-tested and culturally inherited farming systems are more likely to be rapidly adopted by the majority of farmers than those that demand significant modification to farming practices.

Information dissemination is key to the adoption of *Striga* control technologies. For example, weed management is the primary bottleneck to yield increases by smallholder farmers, yet has been neglected by researchers in recent years on the premise that this is a straightforward crop husbandry practice in which farmers should invest. However, because of labor shortages, most farmers prefer to use herbicides for controlling weeds other than *Striga*. As patents for key herbicides (such as glyphosate and atrazine) and others expire, their availability and use in cereal production areas are increasing, in many areas without technical guidance or understanding potential health risks involved. This information should include a simple explanation to farmers as to how control measures work; to explain, for example, that IR maize is a

combination of variety and herbicide, that both are needed to control the *Striga*, and that farmers should wash their hands after handling imazapyr-treatedmaize seed before planting other seed. Information should also be provided on complementary control technologies to increase awareness of ISC options and value for *Striga* control and for wider yield improvement (e.g., fertilization). Research support is thus needed to guide safe and efficient use and to develop alternative options for diverse dryland cereal production environments.

Integrated *Striga* management packages have been designed that include: *Striga* resistant varieties; judicious and appropriate timing and application of phosphate, nitrogen, and composite fertilizers in combination with organic fertilizers; and water conservation measures using tied ridges (or local alternatives). When demonstrating ISC technologies to farmers, including at least one method in all packages that gives rapid *Striga* control would facilitate sustained interest in ISC, allowing the sustained adoption of longer-impact technologies such as tools to improve soil fertility to continue (Douthwite et al. 2007). Of these approaches, development of resistant crop cultivars has been recognized as the most effective and feasible method. To date, *Striga*-resistant sorghum cultivars – such as N13, SRN39, Framida, 555, ICSVs, SRN39 derivatives (P401 to P409), Soumalemba (IS15401), Seguetana CZ, and CMDT45 – have been identified and, as has been observed by Tabo et al. (2006) and ICRISAT (2009), these can be integrated with available crop management options to enhance productivity. However, understanding the molecular nature of the plant-plant interactions is a major barrier. High genetic variability of parasite populations coupled with large, long-lived *Striga* seed banks makes it unlikely that single-gene resistance will be useful in the field. The development of durable polygenic resistance requires pyramiding of appropriate resistance genes and will depend on knowledge of the relationship between host resistance and parasite race structure. QTL studies and MAS certainly have the potential to aid the development of resistant cultivars in the short-to-medium term. Looking forward, a major challenge is to exploit genomic technologies to further advance our understanding of the biology of susceptible and resistant interactions allowing the development of novel control strategies that are appropriate for the agricultural and socioeconomic environment where this parasite is such a devastating problem.

Conclusion

Integrated *Striga* control remains the most effective way to manage *Striga* infestations in sorghum as a long-term approach. Increased understanding of the host-parasite interactions and possibility of identification of newer resistance factors by employing recent technologies such as NGS tools, RNA sequencing applications, RNAi technology, and precise phenotyping platforms will pave the way for developing cultivars with improved resistance. With molecular biology tools being practiced successfully in Africa and improved access to recent technologies such as GbS spreading to remote sorghum breeding programs, development of resistant cultivars with different resistance factors stacked together will form best short- and medium-term approaches toward *Striga* control.

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Chapter 7

Nematode Resistance in Soybean

Tri D. Vuong, Yongqing Jiao, J. Grover Shannon, and Henry T. Nguyen

Abstract

Soybean [*Glycine max* (L.) Merr.] is an important leguminous crop that produces the major sources of edible vegetable oil and protein closest to the optimum dietary essential amino acid profiles for human consumption and livestock feed. However, like many other crop plants, soybean can become infested with and severely damaged by three common plant-parasitic nematode species: cyst nematode (*Heterodera glycines*), root-knot nematode (*Meloidogyne* spp.), and reniform nematode (*Rotylenchulus reniformis*). Nematode parasitism results in economically significant yield losses for soybean producers worldwide. For decades, breeding for resistance variety has been an effective and practical method to control these pests. Soybean varieties with resistance to these nematodes have been successfully developed using traditional breeding and/or marker-assisted breeding approaches. Advancements in genetic marker technologies have greatly facilitated the identification and characterization of genomic regions or genes conveying resistance to different nematode species. Map-based cloning and microarray gene expression profiling studies in both soybean and nematodes reported a large number of transcription factors and candidate genes significantly associated with nematode parasitism and plant resistance. The findings enhanced an understanding of mechanisms of host resistance and also enabled scientists to elucidate the functionality of the genes involved in soybean-parasitic nematode interaction. In the past, traditional genetic mapping using bi-parental populations has been widely utilized to detect and characterize genomic locations associated with nematode resistance. However, this method also has disadvantages in discovering novel functional variations or estimating allelic effects because of the limitations of population structure. Recently, in conjunction with next-generation sequencing (NGS) technology and various genomics analysis approaches, traditional bi-parental genetic mapping has shifted to genome-wide association studies (GWAS), nested-association mapping (NAM), multi-parent advanced generation intercrosses (MAGIC), and so forth. It is anticipated that progress in new genetic mapping methods, coupled with the applications of omics-assisted analysis, significantly facilitate genomic-based selection and transgenic strategies, which can lead to the improvement of soybean resistance to these three important nematode species.

Introduction

Soybean [*Glycine max* (L.) Merr.] is an annual leguminous crop that supplies over half of the world's vegetable fats, oils, and protein meal (Wilson 2004). The origin of the soybean plant is in eastern Asia, where people have grown it for thousands of years. The value of

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soybean seed, which is recognized for its edible oil and protein, is the reason it has become a major oilseed crop during World War II and continually increasing into the present. This crop has been bred for adaptation to various latitudes and is now grown from temperate to tropical regions. North and South America presently account for 86% of worldwide soybean production. Among those, the United States, Brazil, and Argentina represent 81% of the total world supply (http://www.soystats.com). Soybean vegetative growth, reproduction, and maturity are daylength dependent (Carlson and Lersten 2004); therefore, soybean cultivars are assigned to 12 maturity groups (MG) varying from MGs 00 through X. Soybean MGs 00, 0, and I are adapted to the longer days of southern Canada and the northern United States. Succeeding MGs are adapted to lower latitudes as summer days progressively get shorter. Groups IX and X are adapted and grown under the shorter days of the subtropics and tropics (Carlson and Lersten 2004).

There are many diseases and pests that can infect and damage soybean plants, resulting in economically significant yield losses for soybean producers. One class of these pests is plant-parasitic nematodes, which are unsegmented roundworms that often cannot be seen by the naked eye. These nematode species are found in all soybean-growing areas (Niblack et al. 2004). The importance of each nematode species depends on the soybean-growing areas to which each species is most adapted. Among the nematode species, soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe) and root-knot nematode (RKN, *Meloidogyne* spp.) cause the greatest soybean yield losses and are the most important worldwide. Because of their agricultural importance, these two nematode species and their soybean host have been intensively investigated, leading to the identification and molecular characterization of new resistance sources, which then led to the development of new soybean varieties resistant to nematodes (Anand 1992; Boerma and Hussey 1992; Diers and Arelli 1999; Hartwig and Epps 1973a, 1973b). Another plant-parasitic nematode species, called reniform nematode (RN, *Rotylenchulus reniformis* Linford and Oliveira), also causes yield losses in soybean (Robbins et al. 1999). Efforts have been made to evaluate soybean germplasm for new sources of resistance to RN. However, compared to SCN and RKN species, information on RN species is limited and warrants further investigation of genetics and genomics of plant resistance.

These three parasitic nematode species differ in terms of their modes of plant parasitism. For example, unique structures called syncytia are formed following an initial penetration of SCN and RN through root tissue; in contrast, RKN penetration induces a giant cell near the root tips (Mitchum et al. 2012). Advances in molecular genetics and genomic methodologies of soybean and nematodes have provided efficient tools to identify and characterize transcription factors and genes at work during plant parasitism. These advances have also engendered a better understanding of the complex soybean-nematode phytosystem. Efforts have been made to identify the genes coding for nematodes' stylet secretions and to elucidate the functionality of the secreted proteins (Davis et al. 2004; Huang et al. 2003, 2004). Microarray gene expression profiling using Affymetrix GeneChip and laser capture microdissection (LCM) have been widely adapted to detect transcriptional changes that occur during plant parasitism in thousands of soybean and nematode genes (Ibranhim et al. 2011a; Ithal et al. 2007b; Klink et al. 2007b; Puthoff et al. 2007). These findings provided new insights into host plant responses to nematode infection, allowing soybean scientists to develop novel strategies to bioengineer crops with robust resistance to phytopathogenic nematodes (Mitchum et al. 2012).

For decades, breeding for soybean varieties resistant to parasitic nematodes has been shown to be an effective and practical method to manage these pests. However, developing new resistant cultivars has been challenging because of

the genetic variability of nematode species, as well as the complex involvement of resistance genes (Niblack et al. 2002; Williamson 1998). Advancements in molecular marker technologies have greatly facilitated the detection and characterization of genomic regions associated with broad resistance to nematodes (Concibido et al. 2004; Ha et al. 2004; Ha et al. 2007; Li et al. 2001; Tamulonis et al. 1997a; Vuong et al. 2010; Wu et al. 2009). Such genetic marker-trait associations can be efficiently utilized for marker-assisted selection (MAS) or genomic selection (GS), in which several resistance genes/genomic regions can be pyramided to select elite backgrounds, leading to the improvement of nematode-resistant varieties of soybean.

In this chapter, we summarize and highlight current knowledge about the parasitic biology of these three nematode species, genetic variation for virulence, candidate genes for host plant resistance, and host-nematode interactions. We also discuss soybean-breeding strategies and variety development for resistance and tolerance to nematodes. Finally we mention the prospects and applications of new genomic approaches and next-generation sequencing (NGS) technologies in future research to understand parasitic nematodes and their soybean host.

Overview of Nematode Problems in Soybean Production

Soybean Cyst Nematode

Among the plant-parasitic nematode species causing severe annual soybean yield losses, cyst nematode (SCN, *Heterodera glycines*) is the most devastating worldwide. It was estimated that this nematode species causes nearly $1 billion annually in yield losses in U.S. soybean production alone (Koenning and Wrather 2010). Once an SCN infestation is established in a soybean field, it is very difficult to eradicate it because of the genetic diversity of *H. glycines* field populations and their ability to eventually adapt to resistant soybean genotypes. SCN was first reported in Japan in 1915, in Korea in 1936, and in the United States in 1954 (Winstead et al. 1955). In the United States, it was reported that *H. glycines* field isolates in North Carolina were found to be different than isolates collected in Tennessee (Ross 1962). Since then, a number of studies have reported the genetic diversity among and within populations of SCN in various soybean production areas (Anand et al. 1994; Colgrove et al. 2002; Golden and Epps 1965; Niblack et al. 1993; Rao-Arelli et al. 1991; Riggs et al. 1968; Sugiyama et al. 1968; Zhang et al. 1998).

Regarding the inheritance of resistance to SCN, early studies indicated SCN resistance is genetically controlled by three recessive genes designated *rhg1*, *rhg2*, and *rhg3* (Caldwell et al. 1960). Later, Matson and Williams (1965) reported one dominant gene and designated it as *Rhg4*. Analyzing a new source of resistance, PI 88788, Rao-Arelli (1994) identified an additional dominant gene and designated it as *Rhg5*. However, further genetic studies of different sources of resistance showed that inheritance of SCN resistance was oligogenic and complex (Anand and Rao-Arelli 1989). Multiple alleles at a single locus could be involved in SCN resistance (Hancock et al. 1987; Hartwig 1985). For decades, advances in molecular genetics and biotechnology made it possible to identify and characterize genomic regions quantitatively conditioning SCN resistance, indicating that resistance to SCN is a complex trait conveyed by quantitative trait loci (QTL), with either small effects or epistatic interaction. Since then, many efforts have been made to screen more resistance sources, which were subsequently utilized for QTL mapping and characterization. QTL analysis and marker association are discussed in more detail later in this chapter.

Root-Knot Nematode

Root-knot nematodes (RKN, *Meloidogyne* spp.) belong to the genus *Meloidogyne*, which are

obligate parasitic pests of thousands of plant species and were first reported by Cornu (1879) and later by Neal (1889). The *Meloidogyne* genus includes more than 50 described species (Trudgill and Blok 2001) and has greater worldwide distribution than other major groups of plant-parasitic nematodes (Sasser 1980). Among these, four species—*Meloidogyne incognita* (Kofoid & White) Chitwood (Mi), *M. arenaria* (Neal) (Ma), *M. javanica* (Treub) Chitwood (Mj), and *M. hapla* (Chitwood) (Mh)—are the most common nematodes, causing 95% of the incidence of RKN parasitism on important crop plants worldwide (Doucet and Pinochet 1992; Hussey and Janssen 2002). In the southeastern United States, however, two RKN species, Mi and Ma, are the most concerning nematodes to soybean, tobacco, peanut, and cotton growers (Boerma and Hussey 1992; Rodriquez-Kabana et al. 1991), as they cause annual yield losses of an estimated $30 million (Sciumbato 1993). Once established in crop roots, using pest controls to prevent yield losses can be difficult. In past decades, due to the cancellation of permits for the use of fumigant nematicides, the development and deployment of RKN-resistant cultivars, in conjunction with crop rotation, became the most effective measure to control nematode damage (Boerma and Hussey 1992).

Results of a genetic study of the Forrest cultivar showed that resistance to Mi was controlled by a single gene, named *Rmi1* (Luzzi et al. 1994a). Since then, many efforts have been made to identify new sources of resistance, which have subsequently been utilized for further investigation of how soybeans inherit RKN resistance (Harris et al. 2003; Yates et al. 2010). However, genetic analysis in different soybean genotypes, when challenged with other RKN species (i.e., Ma, Mj, and Mi), indicated that the resistance to RKN was quantitatively inherited, with moderate to high heritability (Luzzi et al. 1994b, 1995a, 1995b). With the availability and accessibility of different molecular marker technologies, a number of QTL-underlying resistances to RKN species were identified and mapped (Ha et al. 2004; Li et al. 2001; Tamulonis et al. 1997a). Advances in microarray gene expression and transcriptional profiling enabled soybean researchers to characterize candidate genes for nematode parasitism, as well as plant host genes resistant to RKN infection (Huang et al. 2003, 2005; Ibrahim et al. 2011a, 2011b). Genetic mapping, host plant resistance genes, and candidate genes for parasitism are presented in more detail later in this chapter.

Reniform Nematode

Reniform nematode (RN, *Rotylenchulus reniformis* Linford and Oliveira) occurs primarily in tropical and subtropical regions in Australia, Southeast Asia, the Middle East, Africa, and South America, where it parasitizes a wide range of crop plants (Gaur and Perry 1991; Herald and Robinson 1990). This nematode species was first described in 1931 in Hawaii (Linford and Oliveira, 1940) and was first detected in the continental United States in Georgia in 1940 (Smith 1940). Since then, the nematode has been found throughout the southern United States in South Carolina, Arkansas, Louisiana, Mississippi, Florida (Herald and Thames 1982; Herald and Robinson 1990; Starr 1998), and as far north as Missouri (Wrather et al. 1992). Among important crop plants, cotton has been known to be a major host of RN, but many other vegetable and field crops, such as tomato, peanut, and soybean, are also infested by this nematode species (Davis et al. 2003; Herald and Robinson 1990). Parasitized plants exhibit varying degrees of stunting and chlorosis, resulting in moderate to heavy yield suppression (Koenning et al. 1999; Laurence and McLean 1996). In soybean, an average of 33% of yield reduction was reported in both moderately resistant and susceptible cultivars tested (Rebois et al. 1975).

Improving the genetic resistance of host plants has been demonstrated to be the most efficient approach to control the damage of this pest,

compared to nematicide application and rotation of non-host crops. The evaluations of soybean germplasm and varieties for resistance to RN identified many sources of resistance, such as PI 437654 (Davis et al. 1996), cultivars Picket and Dyer (Rebois et al. 1968), and Forrest and Hartwig (Davis et al. 1996; Robbins et al. 1994). A previous genetic study in soybean showed that resistance to RN was controlled by a recessive allele (Williams et al. 1981). However, in another study, Harville et al. (1985) reported that two loci, designated *Rn1* and *Rn2*, were associated with RN resistance with unequal effects. Similar to the studies of SCN and RKN, the advent of genetic marker technologies facilitated the identification and molecular characterization of QTL or genes for RN resistance. So far, several QTL for RN resistance and associated molecular markers were identified and mapped to chromosomes 11, 18, and 19 (LGs B1, G, and L, respectively) (Ha et al. 2007; Webb et al. 1995). Genetic mapping and host plant resistance genes is reviewed in more detail later in this chapter.

Nematode Biology and Host Response to Nematodes

Soybean Cyst Nematode

Life Cycle and Parasitic Biology

H. glycines nematode is a migratory and obligate sedentary endoparasitic pathogen. In the soil, its life cycle starts when a developed first-stage juvenile (J1) forms in a viable egg. An infective second-stage juvenile (J2) then emerges from the egg (Figure 7.1). The J2 nematode locates host plant roots by its attraction to diffusates. It then mechanically penetrates the root cell wall using its protrusible stylet, while secreting cell wall–hydrolyzing enzymes to facilitate migration toward the root vasculature. Once reaching the vasculature of the roots, the nematode selects an individual cell to initiate the

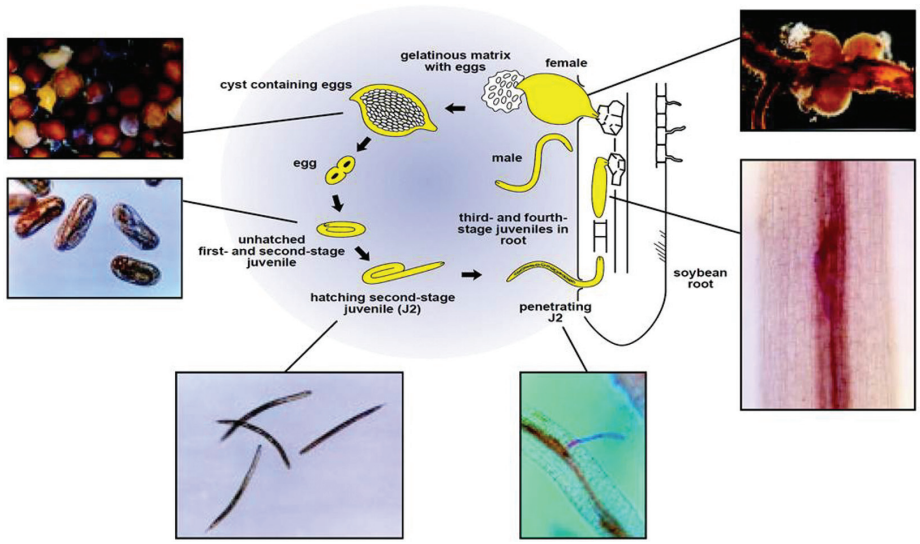

Fig. 7.1. A life cycle of *Heterodera glycines* nematode. Typically, a first-stage juvenile (J1) forms in an egg released from a cyst. A second-stage juvenile (J2) then hatches and emerges from the egg. Third- and fourth-stage juveniles (J3 and J4) develop in the roots of the host plant. An adult male fertilizes an adult female, which produces eggs externally. A dead body of a female serves as a cyst containing eggs (adapted from University of Minnesota Extension Publication "The Soybean Cyst Nematode" by S. Chen, et al.). For a color version of this figure, please refer to the color plate.

formation of a unique structure called a feeding cell or syncytium. A syncytium consists of hundreds of fused and highly metabolically active root cells on which the nematode feeds as it develops into a third- (J3) and fourth-stage (J4) juvenile. The life cycle of SCN depends on the successful formation of the feeding cells. In the roots, the juvenile continues the developmental process to the adult male or female life stages, which are vastly different in size and shape. The reproduction of SCN is completed by obligate amphimixis that requires an adult male to regain its vermiform shape and motility, migrating out of the plant's roots to fertilize an adult female protruding as a pearly white spheroid from the root's surface. The fertilized female can produce up to several hundred eggs. When the adult female dies, her large dead body serves as a cyst to protect the eggs from adverse environmental conditions in the absence of a host until conditions are favorable. More useful information of this subject was also presented in comprehensive reviews by Niblack et al. (2006) and Mitchum and Baum (2008).

For its parasitic development, the nematode is essentially dependent on the functions of parasitism proteins and plant host-SCN interaction. The nematode uses a stylet to secret proteins originating in its esophageal gland cells into the host's roots, where a syncytium is initially formed and serves as a major nutrient sink for the feeding nematode. Several studies have been made using genomic and proteomic approaches to identify SCN parasitism genes and host-SCN interactions (Davis et al. 2004; Gao et al. 2003; Ithal et al. 2007a, 2007b; Wang et al. 2001; Wang et al. 2005). It was reported that stylet-secreted proteins are encoded by nematode parasitism genes expressed in the nematode's three esophageal gland cells (Davis et al. 2004). Sequencing and bioinformatic analysis of the gland-enriched cDNA libraries led to the confirmation of proteins with predicted secretion signal peptides and the identification of more than 60 SCN parasitism gene candidates (Gao et al. 2001, 2003; Wang et al. 2001). Among these, a small subset of the parasitism proteins was shown to be involved in cell wall modification during infection, metabolic, and developmental reprogramming of host cells and manipulation of host defense mechanisms (Baum et al. 2007; Davis and Mitchum 2005). The roles and functions of several other parasitism proteins involved in metabolic reprogramming, molecular mimicry of host pathway components, avirulence and virulence proteins, and so forth were also reviewed in detail by others (Baum et al. 2007; Davis and Mitchum 2005; Mitchum and Baum 2008).

Genetic Variation for Virulence

Virulence of parasitic nematodes is the ability to evolve to either escape or overcome host resistance. Many studies showed that there is extensive genetic diversity among and within SCN populations in the field (Niblack et al. 2002; Niblack et al. 2006). Overuse of the same sources of SCN resistance in soybean has consequently resulted in genetic shifts of SCN populations and the loss of resistance among soybean cultivars. Young (1998) reported a synthetic nematode population called LY1, originating from a mass mating of races 2 and 3, which can reproduce on the broad-based resistant soybean cultivar Hartwig, as well as its primary resistance source, PI 437654. Results of a recent survey showed that most SCN populations collected from soybean fields in Missouri were virulent and could reproduce on indicator lines, such as PI 88788, PI 209332, PI 548316, and PI 548402, which are typically used in SCN bioassays and as resistance sources for soybean cultivar development (Mitchum et al. 2007).

Following the gene-for-gene hypothesis for plant-pathogen interaction, Golden et al. (1970) initially proposed a "races" classification scheme to describe SCN genetic variability and virulence using four soybean differentials: Pickett, Peking, PI 88788, and PI 90762, along with Lee, a susceptible check. The number of females produced by *H. glycines* population on each soybean

Table 7.1. Races of the soybean cyst nematode species *Heterodera glycines*, according to the race determination scheme proposed by Riggs and Schmitt (1988) (adapted from Niblack et al. 2006).

Number	Pickett	Peking	PI 88788	PI 90763
1	−	−	+	−
2	+	+	+	−
3	−	−	−	−
4	+	+	+	+
5	+	−	+	−
6	+	−	−	−
7	−	−	+	+
8	−	−	−	+
9	+	+	−	−
10	+	−	−	+
11	−	+	+	−
12	−	+	−	+
13	−	+	−	−
14	+	+	−	+
15	+	−	+	+
16	−	+	+	+

differential was counted and used to estimate female percentage (%), based on the number of females produced on the standard check cultivar Lee. Later, Riggs and Schmitt (1988) expanded the original scheme to a logical characterization of a new race designation by adding 16 new SCN races. For many years, this new system has been widely adapted by public and private soybean scientists to characterize *H. glycines* populations and resistance genes in soybean lines (Table 7.1).

However, due to incorrect characterization of SCN populations resulting in misinformation to soybean growers, a new classification and designation system was proposed (Niblack et al. 2002) that uses several sources of resistance as indicator lines (Table 7.2) to characterize and expand the genetic diversity of SCN. The term "HG Type" that stands for *Heterodera glycines* was adapted in place of "race" to classify SCN populations. This scheme is open-ended, meaning additional soybean indicators can be added, and it is easily adaptable to different geographical areas (Niblack et al. 2002).

In order to classify the HG Type of a *H. glycines* population, a bioassay is conducted according to a standard phenotyping protocol. An SCN population is named according to the number associated with the soybean indicator lines (Table 7.2) on which it is virulent. SCN virulence is measured as female index (FI, %) as described in the race determination system above. HG Type of an SCN population is then reported by numbers separated by periods. For example, the HG Type of a SCN field population is described as 2.5.7, meaning this SCN population is virulent on three indicator lines, PI 88788 (2), PI 209332 (5), and PI 548316 (7), with FI values greater than 10%. Recently, the HG Types classification scheme has been commonly used in various genetic studies of SCN (Afzal et al. 2012; Arelli et al. 2009; Vuong et al. 2010, 2011; Wu et al. 2009).

Table 7.2. Indicator lines for HG Type classification of genetically diverse populations of *Heterodera glycines* nematode (Niblack et al. 2002).

Number	Indicator line
1	PI 548402 (Peking)
2	PI 88788
3	PI 90763
4	PI 437654
5	PI 209332
6	PI 89772
7	PI 548316 (Cloud)

Sources of Resistance to SCN and QTL Mapping

Planting cultivars resistant to SCN and rotating soybean with non-host crops have demonstrated to be effective and practical measures for controlling SCN infestation that causes severe yield losses in soybean. For decades, soybean scientists in both public and private sectors have successfully developed several SCN-resistant cultivars, allowing the continuation of soybean production in large growing areas where soybean production could no longer be profitable due to SCN infestation (Concibido et al. 2004). For example, it was reported that the soybean

cultivar Forrest, which was developed from a SCN-resistant plant introduction PI 548402 (Peking), prevented yield losses worth $405 million from 1975 to 1980 (Bradley and Duffy 1982).

With over 19,000 plant introductions (PIs), the USDA Soybean Germplasm Collection (http://www.ars-grin.gov) has been a valuable resource in finding sources of SCN resistance. Efforts have been made to evaluate this collection for new sources of resistance to either single- or multi-race SCN populations (Anand and Gallo 1984; Anand et al. 1988; Arelli et al. 2000; Diers et al. 1997; Epps and Hartwig 1972; Rao-Arelli et al. 1997; Ross and Brim, 1957; Young 1990). In a field study conducted soon after SCN was reported in the United States, Ross and Brim (1957) reported PI 88788 and Peking (PI 548402) as new sources of SCN resistance. Subsequently, these two accessions were quickly incorporated into many breeding programs, in which PI 88788 became the predominant source of SCN resistance in U.S. soybean production areas (Diers and Arelli 1999).

Development of soybean varieties resistant to SCN has been an effective and practical method for controlling SCN. However, genetic shift of SCN populations due to the continuous cultivation of the same sources of resistance, coupled with a lack of diversity for SCN resistance genes in soybean varieties, has created a need for further investigation to discover new SCN genes from other sources of resistance. Recently, soybean scientists at the National Center for Soybean Biotechnology (NCSB) at the University of Missouri (MU), in cooperation with Dr. Nelson of USDA-ARS in Urbana, Illinois, have evaluated a subset of over 600 soybean accessions from maturity groups (MG) III to V for resistance to six SCN races, including a synthetic SCN population, LY1. Of these, more than 20 exotic PIs were identified and confirmed to be moderately or highly resistant to either single or multiple races of SCN (Nguyen Laboratory, unpublished data). These PIs could be useful as new broad-based SCN resistance sources in an effort to discover novel or rare alleles of SCN resistance using traditional QTL mapping or nested association mapping (NAM) approaches.

Advances in molecular genetic and genomic methodologies significantly facilitate the identification and mapping of QTL associated with resistance to SCN. In early mapping studies, Concibido et al. (1994) reported three RFLP markers significantly associated with SCN resistance and tentatively mapped these to soybean chromosomes 8, 9, and 18 (corresponding to molecular linkage groups (LGs) A2, K, and G) (http://www.soybase.org). Later, additional SCN resistance QTL have been detected and confirmed in several resistant sources of cultivated soybean (Guo et al. 2005; Guo et al. 2006; Meksem et al. 2001; Qiu et al. 1999; Vuong et al. 2010, 2011; Wu et al. 2009; Yue et al. 2001) and wild annual species (*Glycine soja*) (Wang et al. 2001; Winter et al. 2007). In a comprehensive review of a decade of QTL analysis efforts, Concibido et al. (2004) summarized 31 putative QTL for SCN resistance to various races, which were mapped to 17 of 20 soybean chromosomes (Chr.). Of these, the QTL on Chr. 18 has been well characterized and proven to be the most important QTL because it contains the *rhg1* locus, which underlies resistance to most of the existing SCN races: 1, 2, 3, 5, 14, and LY1 (Concibido et al. 1997; Guo et al. 2005; Guo et al. 2006; Vuong et al. 2011; Wu et al. 2009; Yue et al. 2001). A second important QTL located on Chr. 8 was also identified in other resistant PIs and corresponds to the dominant locus *Rgh4*, which was reported to play a distinct role in resistance to SCN race 3 (Concibido et al. 1994; Heer et al. 1998; Webb et al. 1995; Wu et al. 2009; Vuong et al. 2011). In addition to all of the QTL mapped to 17 soybean chromosomes in early studies (Concibido et al. 2004), two new QTL controlling resistance to multi-races of SCN were identified and mapped to Chrs. 18 and 10 in PI 567516C (Vuong et al. 2010). The new QTL on Chr. 18 was located at a new genomic region and was physically distant from the known *rhg1* locus. It was

tentatively named Chr. 18^{2nd} locus. The second QTL mapped on Chr. 10 has not been reported in other SCN resistance sources studies to date. Previous studies have reported that it is essential to have both genes from Chrs. 18- and 8-QTL associated with the *rhg1* and *Rhg4* loci, respectively, for the development of new varieties derived from any resistance source (Cregan et al. 1999; Meksem et al. 1999). However, results reported by Vuong et al. (2010) indicated that other than *rhg1* and *Rhg4* loci, comprehensive resistance to multiple SCN races can be conveyed by other genomic regions from different SCN-resistant sources, as shown in other studies (Guo et al. 2006; Winter et al. 2007; Wu et al. 2009).

Root-Knot Nematode

Life Cycle and Parasitic Biology

Root-knot nematode (*Meloidogyne* spp.) is an obligate sedentary endoparasitic pest and has a wide host range of at least 1,700 plant species (Cook 1991). Although SCN and RKN share many common features in their life cycles, they differ in many aspects of parasitism. In the soil, the life cycle of RKN starts with developed J1 juvenile in its egg. A second-stage J2 juvenile hatches from the egg and mechanically penetrates the root near the root tip at the zone of elongation (Hussey and Grundler 1998). The parasitic J2 juvenile migrates through the root intercellularly in the root cortex to reach the root vascular cylinder. The esophageal gland cell of the J2 nematode actively synthesizes and mobilizes secretions from its stylet during migration within tissues and subsequently initiates the formation of feeding cells (Hussey 1989). Unlike cyst nematode, the RKN J2 juvenile induces the formation of three or six multinucleate giant cells through repeated nuclear division uncoupled from cytokinesis. The giant cells can develop up to 100 times the size of normal root vascular parenchyma cells and serve as the feeding site, where the sedentary nematode goes through three successive molts to reach the reproductive adult stage (Davis and Mitchum 2005). In many host plants, pericycle and cortical root cells immediately surrounding the giant cells are stimulated to divide (hyperplasia), giving rise to the "gall" or "knot" characteristic of RKN infection.

However, similar to cyst nematode, RKN uses a protrusible style to secret parasitism proteins into the host roots, where giant cells are formed and required for its development and reproduction. The secretory parasitism proteins mediate the dynamic interaction of the RKN with the plant hosts. The broad host range of the RKN suggests that the RKN affects fundamental processes within plant cells (Davis et al. 2004). When using a gland-cell-specific cDNA of *M. incognita* (Mi), Huang et al. (2003) successfully isolated a parasitism gene encoding a putative signaling peptide, which significantly expressed itself in the subventral esophageal secretory gland cells. Further investigations of transgenic expression of this gene and its interaction with host plant transcription factors are also reviewed later in this chapter.

Genetic Variation for Virulence

Among four common RKN species, southern Mi (*M. incognita)* and peanut Ma *(M. arenaria)* are the most important because increasing levels of damage has been observed in the southeastern U.S., where soybean, peanut, and cotton are major crop plants.

For southern Mi nematode, four races have been recognized based on morphological characteristics of male head and female stylet shape (Sasser 1972). But various studies also reported that races of Mi nematode can be differentiated according to their parasitism on specific host plants, like tomato, soybean, alfalfa, and lima bean. Thus, it was essential to establish a race classification scheme for each crop (Canto-Saenz and Brodie 1986). In a study of two Mi populations originating from North Carolina and Georgia, these authors identified these nematode

populations as race 1 on the basis of reaction on host differential plants. However, these populations differed significantly in their parasitism on tomato (Canto-Saenz and Brodie 1986). In another study, Luzzi et al. (1987) used Mi race 3, established by combining three highly virulent populations from the southeastern United States, and successfully identified many new sources of resistance to Mi nematode.

Peanut Ma nematode has been shown to be very diverse in its morphology and cytological profiles (Cliff and Hirschmann 1985). Moreover, based on the host pathogenicity in peanut, two races of this nematode species were identified: race 1, which infects peanut, and race 2, which does not (Sasser 1972). In soybean, both Ma races can produce eggs, but race 2 is more aggressive, damaging, and fecund than race 1 (Pedrosa et al. 1994).

Sources of Resistance to RKN and QTL Mapping

In an evaluation of a subset of plant instructions from the USDA Soybean Germplasm Collection, Luzzi et al. (1987) identified many new sources of resistance to RKN species, in which PI 96354 was highly resistant to Mi nematode, while PI 200538 and PI 230977 were highly resistant to both Ma (race 2) and Mj nematodes. In a greenhouse test, although not different in gall index and Ma reproduction, PI 200538 was less damaged by Ma under field conditions than was PI 230977 (Pedrosa et al. 1994). In another evaluation, Harris et al. (2003) reported additional sources of resistance to Ma. Of these, PI 594403, PI 594427C, and PI 594651B were shown to be potentially unique resistance accessions to this nematode species. Recently, Yates et al. (2010) utilized three F2 populations developed from crosses of previously reported PI 200538 and newly identified PI lines, PI 594403 and PI 594651B, to characterize Ma resistance sources. The authors concluded that these PIs contained unique resistance genes that, when combined with known PI 200538, could improve the level of Ma resistance in soybean. Recently, among new exotic soybean germplasm identified to be highly resistant to multiple nematode species, PI 438489B was demonstrated to be resistant to three species, SCN, RKN, and RN (Shannon, per. comm.), and was subsequently employed in genetic mapping of resistance to RKN and RN (Vuong et al. in preparation; Xu et al. 2013).

In efforts to genetically locate genomic regions conferring resistance to RKN species, Tamulonis et al. (1997a) analyzed RFLP markers in an F2 mapping population developed from PI 96354, mapping two QTL for resistance to Mi nematode, a major one on Chr. 10 and a minor one on Chr. 18. These QTL explained 31% and 14% of the phenotypic variation, respectively. These two QTL were later confirmed by Li et al. (2001) by analyzing SSR markers in a mapping population derived from the same cross of PI 96354. Ha et al. (2004) reported that the flanking SSR markers on Chr. 10 also co-segregated with *Rmi1*, a gene conferring Mi nematode resistance. Subsequently, Ha et al. (2007) developed two SNP markers that have been shown to be highly effective in marker-assisted selection for Mi nematode resistance. In a separate genetic mapping study, Tamulonis et al. (1997b) analyzed an F2:3 population developed from PI 200538 and identified two genomic locations for resistance to Ma nematode, a major one on Chr. 13 and a minor one on Chr. 15. These QTL in combination accounted for 51% of the phenotypic variation in gall number.

Analyzing the 1,536 soybean SNP array (the USLP 1.0) (Hyten et al. 2010) in an F7 recombinant inbred line (RIL) population derived from PI 438489B, Vuong et al. (unpublished data) detected and mapped QTL for Mi resistance on Chrs. 8, 10, and 13, consistent with previously reported QTL (Tamulonis et al. 1997a; Li et al. 2001). Lately, with whole genome sequencing (WGS) technology applied to the same genetic population, these QTL were also detected and consistently mapped to the same genomic locations. Of these, a major QTL on Chr. 10 was

mapped in a 29.6-Kb interval, in which three candidate genes for Mi nematode resistance were identified and further investigated (Xu et al. 2013).

Work at the University of Missouri indicated resistance among exotic accessions to multiple nematode species, which include SCN, RKN, and RN, in the USDA germplasm collection will be valuable sources for developing resistance to important nematode species in soybean. The development and accessibility of molecular marker technologies enabled soybean researchers to accurately localize genomic regions and their associated DNA markers, which can be effectively employed in molecular breeding. Moreover, the WGS technology provided a powerful genomics tool to narrow genomic locations based on an ultra-high-density bin map (Huang et al. 2009; Xie et al. 2010) and to pinpoint candidate genes underlying resistance to nematode species, leading to study of gene cloning and functionality.

Reniform Nematode

Life Cycle and Parasitic Biology

Similar to other plant-parasitic nematodes, the RN's life cycle begins in the soil, with the J1 molt to the J2 stage occurring in the egg. The J2 juvenile hatches from the egg. However, unlike most endoparasitic nematodes, the RN J2 juvenile does not immediately infect the roots. Instead, it becomes inactive and progresses through three superimposed molts without feeding. The crescent-shaped and inactive J3 and J4 juveniles show slight movements in the head and tail regions, but the stylet, metacarpus, and valve are still not present (Gaur and Perry 1991). The final molt then produces approximately equal numbers of adult males or immature vermiform females (Robinson et al. 1997). While nonparasitic males do not feed, parasitic vermiform females penetrate the epidermis and cortical parenchyma of the root and establish a feeding site (syncytium). The syncytium quickly expands to encompass 100 to 200 cells through extensive cell wall dissolution and cytoplasmic fusion (Rebois et al. 1975). After fertilization by an adult male, a mature vermiform female, while feeding on a stellar syncytium, produces 30 to 200 eggs in a gelatinous matrix, which surrounds the swollen posterior portion of the female body producing from the root surface in a kidney-like shape (Robinson 2002).

It was observed that many cellular characteristics of reniform nematode-induced syncytium were similar to those formed by cyst nematodes, such as increased metabolic activity, hypertrophied nuclei, and granular, densely staining cytoplasm (Agudelo et al. 2005; Rebois et al. 1975; Vovlas and Lamberti 1990). Furthermore, in order to penetrate the epidermis and move through the root, RN uses mechanical force coupled with the activity of cell wall-degrading enzymes secreted by the esophageal gland cells (Baum et al. 2007). Among these enzymes, glycosyl hydrolase family 5 (GHF5) beta-1,4-endoglucanases, also known as cellulases, are well represented and have been isolated from many plant-parasites (Baum et al. 2007). Subsequently, this enzyme was further investigated and characterized when analyzing a cDNA library constructed from J2 juvenile females of RN (Wubben et al. 2010a, 2010b).

Genetic Variation for Virulence

Unlike other parasitic nematode species classified into different races based on their virulence levels in differentials of host plants, RN nematodes used in studies of crop plants so far were typically derived from inbred RN populations, which were locally maintained on host plants in a greenhouse. For instance, Davis et al. (1996) used a culture of field RN nematode population in North Carolina to evaluate soybean germplasm for resistance to this parasite. Similarly, Robbins et al. (2000, 2001, 2002) screened large numbers of both private and public soybean cultivars and lines for resistance to RN using inoculum from the same inbred RN populations, which

were long maintained on Braxton, a susceptible cultivar grown in a greenhouse. Recently, when mapping QTL for resistance to RN in soybean PI 437654, Ha et al. (2007) employed an RN population from a culture originating in Arkansas and maintained on soybean cultivar Braxton. Wubben et al. (2010a, 2010b) used a stock culture of an inbred RN population for their studies of cotton.

Evaluations of host plant reactions to the virulence of RN nematodes were normally conducted in a greenhouse with ambient temperature maintained at 28–34°C. Reproduction of RN indicated by a reproductive index (Pf/Pi) (final population/initial population) was estimated and widely used for the identification of resistance sources (Robbins et al. 2000, 2001, 2002). Alternatively, a rating method was also used earlier (Robbins et al. 1999). Overall, it is likely that genetic variation for virulence in RN nematode species is not as complex as other plant-parasitic nematodes, resulting in the simplicity and efficiency of the evaluation of host plants' reactions to the virulence of RN.

Sources of Resistance to RN and QTL Mapping

Similar to SCN and RKN management procedures, host plant resistance, in conjunction with non-host crop rotation, have been the highest research priorities and the most desirable measures to control RN damage for efficient soybean production, in particular following the cancellation of permits for the use of many nematicides (Boerma and Hussey, 1992). Similar to efforts being made for SCN and RKN, evaluations of soybean germplasm for resistance to RK have been conducted (Davis et al. 1996; Rebois et al. 1968, 1970; Robbins et al. 1999, 2001, 2002). It was determined that some soybean genotypes with resistance to SCN could potentially show levels of resistance to RN, possibly due to the similarities in the nematode-induced feeding site formation and parasitic mechanism (Rebois et al. 1970). However, further investigations also reported that multi-nematode resistance in soybean was dependent on the resistance source. For instance, Peking-derived soybean cultivars with resistance to SCN were potentially resistant to RN (Caviness and Riggs, 1976), whereas PI 88788-derived cultivars showed resistance to SCN but were not resistant to RN (Davis et al. 1996; Robbins et al. 1994; Robbins and Rakes 1996).

Earlier genetic studies showed that RN resistance was controlled by one locus with recessive alleles (Harville et al. 1985; Williams et al. 1981) or by two loci with unequal effects (Harville et al. 1985). However, in an unpublished study by Pioneer Hi-bred International (http://www.pioneer.com) in an RIL mapping population derived from PI 437654, two QTL for effective resistance to RN were identified and mapped to Chrs. 19 and 11, respectively. Interestingly, this PI was reported to be resistant to both SCN and RN (Robbins and Rakes 1996). Subsequently, soybean researchers have undertaken genetic mapping efforts leading to the identification and confirmation of many QTL underlying resistance to multi-races of SCN (Cregan et al. 1999; Webb et al. 1995; Wu et al. 2009). Employing the same RIL mapping population developed from a BSR101 x PI 437654 cross, Ha et al. (2007) refined previously reported QTL regions with SSR markers on Chr. 19 and identified additional QTL on Chr. 11 and 18, respectively. These two new QTL showed an epistatic interaction and provided the lowest reniform index (RI) in the presence of both favorable alleles from PI 437654.

In addition to studies of available sources of resistance to either single or both nematode species as previously reported, evaluations of new exotic soybean accessions have been conducted in the NCSB, MU. Among those, PI 438489B, PI 437690, and PI 404198B have been demonstrated to be highly resistant to both SCN and RN (Nguyen Lab, unpublished data). QTL analysis conducted using an F7 RIL population derived from a Magellan x PI 438489B cross confirmed a genomic location for resistance to RN on Chr. 18 (Vuong et al. in preparation),

which was previously reported to be associated with resistance to SCN (Vuong et al. 2011). Several genetic populations derived from new RN resistance PIs have been developed and can be utilized for the discovery of novel genomic regions or genes underlying resistance to RN using genotyping by sequencing (GBS) method.

Candidate Genes for Host Plant Resistance and Host-Nematode Interaction

Host Genes Involved in Nematode Resistance

For decades, the utilization of resistant cultivars in combination with non-host crop rotation and nematicide applications has been traditional methods to manage nematode problems in soybean. Meanwhile, geneticists and molecular biologists have been trying to identify the resistance genes from host plants and to elucidate underlying resistance mechanism, aiming to develop new varieties through advanced molecular biotechnologies. The technologies that have been demonstrated to be successful approaches for the identification and characterization of candidate genes are reviewed in this section.

Nematode Resistance Genes Cloned by Map-Based Cloning

Map-based cloning, or positional cloning, is an effective method to clone the genes controlling the important agronomic traits in crop plants. The most important genes conferring resistance to SCN in soybean, *rhg1* and *Rhg4*, have been claimed to have been cloned (Hauge et al. 2001; Lightfoot and Meksem 2002). Both of them encode the NBS-LRR protein, but no functional evidence was provided to indicate successful cloning of these genes. Recently, two groups reported that the *rhg1* and *Rhg4* genes previously cloned are not really the genes responsible for resistance to SCN in soybean (Liu et al. 2011; Melito et al. 2010). Moreover, Kim et al. (2010) identified the *rhg1-b* locus, which is different from the previously published *rhg1* from PI 88788. Lately, the *rhg1b* and R*hg4* loci have been claimed to be cloned with functional evidences. For *rhg1b*, three genes—Glyma18g02580, Glyma18g02590, and Glyma18g02610—are confirmed to be responsible for the *rhg1b* locus, which encode predicted amino acid transporter, predicted alpha-SNAP protein, and wound induced protein (Bent et al. 2012). For the *Rhg4* locus, the gene that does not encode any of the canonical classes of disease resistance proteins in plant was cloned (Mitchum et al. 2012). These remarkable findings enabled soybean scientists to elucidate the mechanism underlying nematode resistance in soybean and also provided valuable tools to improve soybean nematode resistance in the future.

Transcriptome Analyses of Nematode Resistances in Soybean

To elucidate how nematodes are established in hosts, scientists have conducted large-scale analyses of host or nematode gene expressions at the time of infection to identify differentially regulated genes and potential pathways involved. In an earlier investigation, Hermsmeier et al. (1998) used differential display of mRNA to detect host gene expression changes during the early compatible interaction between soybean and SCN. The authors identified 15 genes with different expressions in SCN-infected versus uninfected roots. Of those, the *ADR12* gene was identified to be involved in soybean auxin down-regulation, suggesting that the auxin pathway may be involved in soybean-nematode interaction (Hermsmeier et al. 1998).

Microarray gene expression profiling also enables scientists to detect and compare transcriptional changes of thousands of genes simultaneously when susceptible and non-host interaction happens. Using this method, a broader study of the transcriptional changes associated with both susceptible and non-host interactions was conducted, which revealed important details

about how the cyst nematode induces and maintains the syncytium (Alkharouf et al. 2006; Ithal et al. 2007a; Khan et al. 2004).

Khan et al. (2004) conducted a cDNA microarray with approximately 1,300 cDNA inserts targeted to identify differentially expressed genes during the compatible interaction of SCN using 2-d infected soybean roots. They found that the repetitive proline-rich glycoprotein, the stress-induced gene *SAM22, β-1,3-endoglucanase, peroxidase*, and those involved in carbohydrate metabolism, plant defense and signaling were upregulated two days after inoculation in inoculated roots, compared to the non-inoculated ones.

In order to understand the dynamic changes of gene expressions, Alkharouf et al. (2006) used microarray to study the changes of gene expressions within roots of susceptible soybean varieties infected by SCN at 6, 12, and 24 hours and 2, 4, 6, and 8 days post-inoculation (dpi). The microarray contained 6,000 cDNA inserts. The genes that were induced across most time points or at a specific time point were identified. They included the *WRKY6* transcription factor, *EIF4a*, and the stress-related gene *SAM-22*. Similar study was also conducted by Puthoff et al. (2007), in which a new platform of soybean Affymetrix GeneChip representing 35,593 soybean transcripts was adopted. A large proportion of differentially expressed genes identified were associated with cell wall structure, including *expansin* and *pectate lyase*. Others were involved in several pathways, like disease and defense, phytohormone metabolism, and histones.

In order to compare gene expression during both compatible and incompatible reactions, Klink et al. (2007a) started a time-course microarray analysis using the Peking soybean variety infected by incompatible (I) and compatible (C) populations of SCN. A substantial difference in gene expression was identified between I and C at 12 hours post-infection, indicating soybean can differentiate between I and C nematode populations even before the nematodes begin to select their feeding sites (Klink et al. 2007a).

It was observed that when nematode infections happen, expressions of genes change not only in the hosts but also in the nematodes (Itahal et al. 2007a). To find whether there were any correlations of gene expression between the hosts and the nematodes, Ithal et al. (2007b) analyzed the expression of gene transcripts in soybean and SCN, at the same time using the Affymetrix GeneChip soybean genome array. They identified 429 soybean genes that were significantly differentially expressed and were involved in various pathways, such as primary metabolism, stress and defense responses, cell wall modification, cellular signaling, and transcriptional regulation. Meanwhile, 1,850 significantly differentially expressed SCN genes were also identified, then grouped into nine different clusters (Ithal et al. 2007a). The study provided new insights into soybean responses to nematode infection compared with previous studies, because gene expressions of the nematodes and their host plants were studied at the same time.

Laser capture micro-dissection (LCM) is an effective means to isolate homogeneous cell populations with a high degree of precision and accuracy. Using this method, Klink et al. (2007b) assayed gene transcript abundance in syncytial cells in roots of cultivar Peking infected by incompatible and compatible populations of SCN. They observed that in syncytial cells from incompatible roots at 3 dpi (days post infection), expressions of genes encoding lipoxygenase (LOX), heat shock protein (HSP) 70, and superoxidase dismutase (SOD) were significantly elevated. However, in those formed during a compatible interaction, genes encoding prohibitin, the epsilon chain of ATP synthase, allene oxide cyclase, and annexin were more abundant. The results indicated that gene expression was different between syncytial cells from incompatible and compatible roots. Moreover, they found that genes that were differentially expressed in syncytial cells were not differentially expressed in the whole root analyses. Results suggested that

the identification of transcriptional events occurring within syncytial cells was obscured by the mass of transcriptional activity occurring in the whole root (Klink et al. 2007b).

To understand the effects of infection of roots by RKN, Ibrahim et al. (2011a) used the Affymetrix GeneChip to examine the expression of soybean genes in galls formed in roots by RKN 12 days and 10 weeks after infection. Results showed that genes encoding enzymes involved in carbohydrate and cell wall metabolism, cell cycle control, and plant defense were altered, providing insights into the interaction between RKN and soybean and the formation and maintenance of giant cells.

Host-Nematode Interaction

Cyst nematode and RKN are sedentary endoparasites that become sedentary at specialized feeding sites. Syncytium and giant cells are the feeding sites formed by SCN and RKN, respectively. A syncytium originates from the amalgamation of several hundred cells between the initial syncytial cell and adjacent cells, which is characterized by the breakdown of the cell walls. By contrast, giant cells originate from six cells that undergo repeated mitosis without intermittent cytokinesis, which is characterized by the induction of nuclear division. The giant cells are embedded in the plant root and form a gall or root-knot in the end (Gheysen and Mitchum 2011). Although giant cells and syncytia have different ontogeny, they have similar ultra-structurem such as dense cytoplasm and enlarged nuclei. They also have similar functions to support nematodes in finishing their life cycles, from the infective J2-stage juvenile to the J4 mature egg-laying female.

The nematode's effectors, which are generated in pharyngeal glands (Figure 7.2) (Lilley et al. 2005) and injected into plant cells through a protrusible hollow mouth spear called a stylet, play important roles in the formation of feeding sites (Gheysen and Mitchum 2011). The subventral glands are thought to be important for the early stages of parasitism because they produce a variety of plant cell-modifying proteins that facilitate nematode's migration through the plant root (Hewezi et al. 2008; Qin et al. 2004). Dorsal glands are important for the development and maintenance of feeding sites because the effectors that induce the plant's response to infection are mainly secreted from the dorsal gland (Bellafiore and Briggs 2010; Gheysen and Mitchum 2011). The effector proteins and their interactions with host proteins have been proved to coordinate many of the events happening during the formation of feeding cells, from early initiation of feeding cells to their complete development into a transfer cell-like nutrient sink (Figure 7.3) (Gheysen and Mitchum 2011).

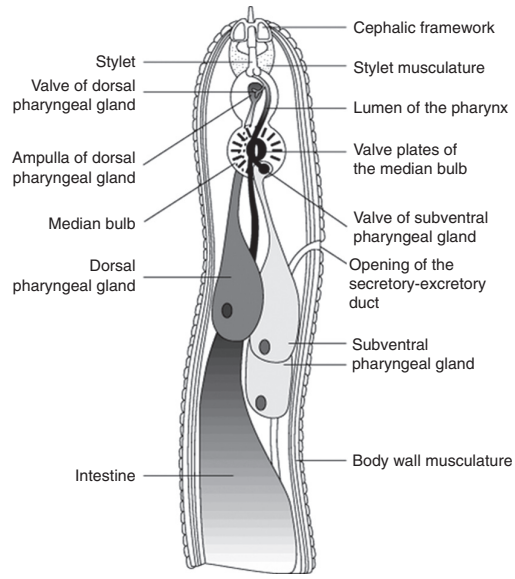

Fig. 7.2. Schematic representation of the anterior of a J2 cyst nematode showing the position of the pharyngeal glands (adapted from Lilley et al. 2005).

When the formation of SCN or RKN feeding cells is initiated, extensive cell wall architectural modifications such as dissolution, disassembly, and thickening take place. The activity of cell wall biosynthetic and cell-wall-degrading enzymes mediated these processes (Gheysen and Mitchum 2011). But whether the β-1,4-endoglucanase (EGases) of nematode or plant

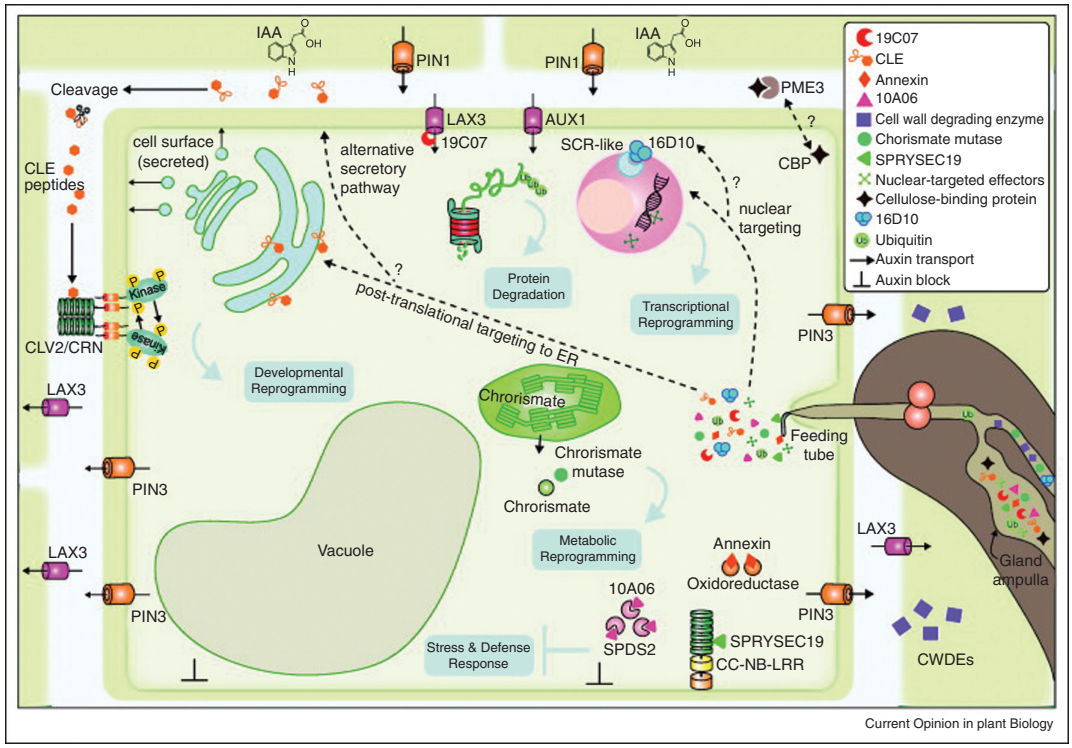

Fig. 7.3. Molecular mechanism of nematode effector protein action in host plant cells (adapted from Gheysen and Mitchum 2011). For a color version of this figure, please refer to the color plate.

origin were involved in this process is still unclear (Dropkin 1963; Jones 1981). The first characterized effectors are secreted from a cyst nematode esophageal encode EGases (Smant et al. 1998). EGases degrade polysaccharides possessing β-1, 4-glucan backbones, such as cellulose and xyloglucan. In their study, the authors isolated EGase that was synthesized in the esophageal glands of the cyst nematodes *Globodera rostochiensis* and *Heterodera glycines* (Smant et al. 1998). The homologous genes were also identified in RKN and in other cyst nematode species (Dautova et al. 2001; De Meutter et al. 2001). EGase of nematode origin was shown to be expressed primarily in J2 stage and again in adult males that migrate out of the root, supporting a role for it in cell wall degradation during the penetration and intracellular migration of the nematode through root tissue (Goellner et al. 2000). Nematode EGases are not expressed in later parasitic stages, during which syncytium continues to expand along the vasculature, suggesting cell wall-degrading enzymes of plant origin play a role in that process. Based on the study of five tobacco EGases that are upregulated upon infection by both root-knot and cyst nematodes, Goellner et al. (2000) proved that cell wall modification within plant-parasitic-nematode feeding cells arises from cell-wall-modifying enzymes of plant, rather than being of nematode origin (Goellner et al. 2000). Pectate lyase and polygalacturonase, two other cell-wall-degrading enzymes, have also been identified from RKN (Doyle and Lambert 2002; Jaubert et al. 2002; Popeijus et al. 2000). They likely play a role in nematode movement by softening the cell wall. An expansin gene was cloned from the plant-parasitic roundworm species *Globodera rostochiensis* (Qin et al. 2004). Expansin proteins have so far been identified only in plants,

inducing the extension of plant cell walls by weakening the noncovalent interactions that help maintain their integrity. Nematodes produced expansin to loosen cell walls when invading host plants (Qin et al. 2004).

Auxin, a plant hormone that is a major regulator of organogenesis, plays an important role in the successful establishment of nematode life. Increased auxin has been detected when galls and syncytia of RKN and SCN are initiated. Chorismate mutase was first cloned from RKN and also has been found in SCN (Bekal et al. 2003; Lambert et al. 1999). Chorismate mutase is a key branch-point regulatory enzyme in the shikimate pathway, in which auxin and salicylic acid were produced. Overexpression of nematode gene *MjCM-1* suppresses lateral root formation and the development of the vascular system by reducing auxin levels (Doyle and Lambert 2003). If the auxin signaling and transport pathway was interrupted, mutants had significantly lower rates of infection by nematodes (Grunewald et al. 2009a). Recently, the role of polar auxin transport has been elucidated (Grunewald et al. 2009b). PIN1-mediated auxin transport is needed to deliver auxin to the initial syncytial cell, whereas PIN3 and PIN4 distribute the accumulated auxin laterally and are involved in the radial expansion of the syncitum. In *pin3* mutant, syncytia and cysts are smaller and less developed. LAX3, an auxin influx transporter, is upregulated in the developing syncytia. Recent studies showed that SCN effector *Hs19C07* interacted with LAX3 and may increase LAX3 activity by binding to it (Lee et al. 2011). Overexpression of *Hs19c07* resulted in an increased rate of lateral root emergence, indicating an enhanced auxin influx. Previous studies showed LAX3 can upregulate cell-wall-degrading enzymes in developing syncytia, indicating different pathways jointly regulate the development of feeding cells (Lee et al. 2011).

Beside the cell wall and auxin pathways, ethylene and the SA pathway is also involved in the development of syncytia (Tucker et al. 2010). Arabidopsis ethylene-insensitive mutants are less susceptible to *H. schachtii*, while ethylene-overproducing lines attract more nematodes (Wubben et al. 2004). But no convincing evidence suggests ethylene is involved in root-knot infection because *M. incognita* affects ethylene-related mutants in a similar way to controls (Lohar and Bird 2003). Successful cyst nematode parasitism may involve a local suppression of SA signaling in roots. SA-deficient mutants (*sid2-1*, *pad4-1*, and *NahG*) exhibited increased susceptibility to *H. schachtii*. In contrast, SA-treated wild-type plants showed decreased *H. schachtii* susceptibility. The *npr1-2* and *npr1-3* mutants, which are impaired in SA signaling, also showed increased susceptibility to *H. schachtii*, whereas the *npr1-2* suppressor mutation *sni1* showed decreased susceptibility (Wubben et al. 2008).

The starch metabolic pathway is involved in the establishment of nematode feeding sites. The formation of feeding sites is accompanied by a massive solute import into syncytia, leading to highly elevated sucrose levels (Hofmann et al. 2008). The authors found that the syncytia use starch as intermediate carbohydrate storage to compensate for fluctuating sugar levels *in vivo*. Twenty of 56 genes known to be related to starch synthesis and degradation were upregulated in nematode-induced syncytia. Loss of function mutation of *Atss1* results in a decreased number of nematodes in nematode infection tests, indicating the importance of the starch metabolic pathway for the function of feeding sites.

Breeding Strategy and Variety Development for Nematode Resistance and Tolerance

Among several practices applied to control and prevent yield losses caused by these nematode species, use of nematode-resistant and -tolerant varieties is a primary and efficient tactic to suppress losses. Thus, breeding for resistance is a major goal of most soybean breeding programs. Resistant cultivars are advantageous by suppressing nematode reproduction, reducing the need for toxic nematicides and shortening

the length of rotations. Resistant cultivars do not require specialized equipment, and generally seed costs are similar to that of susceptible cultivars (Boerma and Hussey 1992). In addition, they may limit damage from other disease complexes associated with nematodes (Bond and Wrather 2004). Cultivars resistant to SCN have been shown to yield 10–50% more than susceptible cultivars (Shannon et al. 2004). Soybean cultivars resistant to RKN yielded five times more than highly susceptible cultivars (Kinloch et al. 1998).

Soybean Cyst Nematode

Developing cultivars resistant to SCN has been challenging because of the genetic variability of the pathogen, the exotic and unadapted nature of resistance sources, and the fact that several genes are involved in resistance. Resistance is defined on the basis of nematode reproduction on a genotype compared to a susceptible soybean standard. Reproduction on a range of genotypes or indicator lines is further used to classify susceptibility or resistance to different HG types (or races). The SCN female index (FI %), used to classify resistance, is defined elsewhere in this chapter. Four categories of resistance are designated to aid in classification of resistance (Niblack et al. 2002; Schmidt and Shannon 1992). The categories are FI < 10% (resistant), FI = 10–30% (moderately resistant), FI = 31–60% (moderately susceptible or slightly resistant), and FI >60% (susceptible).

Young (1998) summarized methods for evaluating resistance to nematodes that enable researchers to accurately evaluate numerous genotypes. Appropriate screening techniques are essential for the successful development of cultivars resistant to nematodes. Screening can be carried out in the field, but so far it is primarily performed in the greenhouse under controlled conditions. Screening is also complicated by the fact that SCN inheritance is complex, resistance to a specific HG type must be conducted in separate tests, and cyst counts must be made for reactions to each HG type. A standardized greenhouse method suggested by Niblack et al. (2002) provides a system to compare SCN reaction among genotypes based on the same phenotyping procedure. Recently, Brown et al. (2010) reported that an automated fluorescent-based imaging system is just as accurate ($r^2 \geq 0.95$) and more efficient (>50% faster) than manual counting under a microscope. This method can greatly improve the consistency and turnaround of data while reducing the time and labor commitment associated with SCN female counting.

Genetic marker technology has facilitated the identification, location, and characterization of QTL associated with SCN resistance. As already mentioned in the chapter, the genes *rhg1* located on Chr. 18 and *Rhg4* located on Chr. 8 are most frequently associated with SCN resistance from the various sources studied to date (Concibido et al. 2004). Resistance QTL have been shown to provide resistance to more than one HG type of SCN (Guo et al. 2006; Vuong et al. 2010, 2011; Wu et al. 2009; Yue et al. 2001). There are many additional examples of broad-based resistance provided by specific QTL, as well as resistance to only specific HG types. With the availability of genetic markers linked to SCN resistance genes, marker-assisted selection (MAS) promises to increase the efficiency and speed of the development of SCN-resistant cultivars (Mudge et al. 1997). Until recently, most breeders have selected resistant lines by inoculating plants in a greenhouse with eggs or cysts of SCN. The SCN greenhouse bioassay is labor-intensive, costly, and success is dependent on precise environmental conditions. Conventional SCN greenhouse screening can cost up to $6 or more per plant and still takes 30 days to complete a screening cycle. With MAS, breeders are able to select lines on the basis of alleles at genetic markers linked to SCN resistance. The lines with a high probability of having resistance genes will be selected, reducing the number of lines that need to be evaluated in the greenhouse (Concibido et al. 2004). MAS using SSRs is cost-effective, and single nucleotide polymorphism (SNP) markers are even lower in cost

compared with greenhouse screening. It is likely that the cost of MAS will further decrease as SNP markers become more widely available. As marker technologies continue to improve, the cost will be further reduced as new genotyping platforms become more available (Concibido et al. 2004). Pioneer Hi-Bred International and Monsanto Corporation routinely use marker-assisted breeding for SCN resistance.

There are 118 known sources of *G. max* accessions resistant to SCN that have been identified (Arelli et al. 2000; Rao-Arelli 1997). These sources are all unadapted exotic germplasm and have resistance to one or several SCN HG types. Of these, only eight have been commonly used to develop soybean cultivars or germplasm. These include Peking, PI 88788, PI 90763, PI 437654, PI 209332, PI 89772, PI 87631-1, and PI 438489B. There have been efforts to study whether a wild soybean species, Glycine soja, has novel SCN resistance genes (Wang et al. 2001; Winter et al. 2007). This species is widely distributed in China, Japan, Korea, Taiwan, and eastern Russia and is believed to be the ancestor of cultivated soybean (Hymowitz and Singh 1987). Wang et al. (2001) found a novel SCN resistance QTL from *G. soja* accession PI 468916 for resistance to HG type 0 (race 3). It has been shown that stacking resistance alleles from wild and domestic soybean sources results in a higher level of SCN resistance (Kim et al. 2011).

Developing high-yielding cultivars using PIs as SCN-resistant sources can be difficult. The difficulty is due to three or four major genes, plus minor genes for resistance, and the low recovery of high-yielding progeny. The process is complex because of the number of undesirable traits from SCN-resistant PIs, such as low yield, lodging, shattering, and susceptibility to other pathogens. Commonly, two or more breeding cycles are necessary to recover SCN-resistant genotypes with good productivity and other desirable agronomic traits (Shannon and Anand 1997). In addition, SCN resistance can become diluted up to a point due to the recombination of resistant and susceptible genes, as crosses and selection become more removed from the original resistance source (Shannon et al. 2004). This results in soybean cultivars or germplasm that lack the level of resistance of the original PI.

Productive varieties resistant to SCN are numerous; however, nearly all varieties with resistance trace to exotic lines Peking and PI 88788 (Concibido et al. 2004; Diers and Arelli 1999). With the widespread deployment of SCN-resistant genes from these two sources, nematode populations have responded with changes in parasitism causing HG-type shifts, resulting in these sources of resistance being less effective against SCN (Niblack et al. 2003). Broad-based resistance to different SCN HG types would be particularly beneficial in breeding to limit the damaging consequence of potential race shifts in nematode populations in the field. The HG type 1.2.5.7 (race 2), which can attack "PI 88788 type" resistance, is now a prominent SCN population in southeast Missouri and other U.S. states (Niblack et al. 2003). Soybean scientists at the University of Missouri screened the USDA Soybean Germplasm Collection and found that a black seed exotic accession, PI 437654, had the highest level of resistance to all HG types of SCN studied (Arelli et al. 1997). The SCN-resistant variety Hartwig, with broad resistance to SCN HG types from PI 437654, was developed and released (Anand 1992). Productive group V varieties, such as Anand (Anand et al. 2001), Stoddard, Jake (Shannon et al. 2007a, 2007b), and LD00-2719P (Diers et al. 2010), which trace to PI 437654, have been developed with broad resistance to SCN HG types. However, only a few cultivars trace to PI 437654 via cultivar Hartwig. In spite of this broad resistance, some populations of the nematode have been found that are capable of overcoming Hartwig-type resistance (Young 1999). Thus, it is evident that breeding for SCN resistance will remain a constant challenge because of parasitic variation in nematodes.

Some soybean germplasms show tolerance to SCN. A soybean line is considered tolerant to SCN if infected plants yield almost as well as

nematode-free plants (Young 1998). Tolerance is HG-type independent (Boerma and Hussey 1992), with plants being productive regardless of field SCN HG type. Ultimately, tolerant genotypes should be readily recognized by good performance in SCN-infested, untreated fields. High levels of tolerance have been reported in the Japanese cultivar Gendenshirazu (Ichnohe, 1988). PI 97100, MG VII collected from Korea and the germplasm line G88-20092 derived from this PI also show tolerance (Boerma and Hussey 1993). Boerma and Hussey (1984) suggest tolerant cultivars have an advantage over resistant cultivars by preventing yield losses without imposing selection pressure on SCN field populations. Growing resistant and tolerant cultivars in alternate years has been suggested to improve the longevity of resistance genes while providing stable yield performance without putting selection pressure on the nematode (Shannon and Anand 1997).

Root-Knot Nematode

Among common RKN species (*Meloidogyne* spp.), Mi, Ma, and Mj nematodes are the primary species that cause yield losses in soybean. Mi nematode is most prevalent, but the other species can be more prominent, such as Ma nematode in peanut-growing areas and Mj nematode in more tropical areas (Kinloch 1998). Resistant cultivars to date are not immune to RKN species, and 30–50% yield loss can occur if the nematodes are in high numbers in the field (Carter et al. 2004). Resistance to all three species can be found in the USDA Soybean Germplasm Collection. Publicly released southern U.S. cultivars Forrest, Bedford, Braxton, Gordon, Jackson, Kirby, and derivatives from some of these genotypes have resistance to all three RKN species (Carter et al. 2004).

Assessment of host plants' reactions to virulence of RKN is generally based on root gall index scores (Niblack et al. 2004). Gall index can be rated (1) by the percent of roots with galls on the entire root system (Stetina et al. 1997) on a 0–4 scale, with 0 being no galls to 4 with > 75% of the entire root system; and (2) on the basis of the number of galls on the root system on a 0-5 scale, where 0 means no galls and 5 equals ≥ 100 galls per root system (Ritzinger et al. 1998). In addition to root galling, nematode reproducibility was also assessed to identify resistant genotypes. Many studies using this screening scheme identified a number of soybean PIs with high levels of resistance to Mi and Ma nematodes (Harris et al. 2003; Luzzi et al. 1987; Luzzi et al. 1995a; Yates et al. 2010). Root-knot screening is most frequently carried out in greenhouse tests, but it can also be conducted in root-knot infested fields. A successful greenhouse system used at the University of Georgia-Atlanta (UGA) in many studies is detailed in research conducted by Hussey et al. (1991).

Many soybean varieties resistant to southern RKN (Mi) are readily available, and some cultivars have combined resistance to both RKN and SCN, primarily in MG V and later MGs (Young 1998). There are few genotypes available in MG IV and earlier that carry resistance to any species of RKN. However, a few productive cultivars in MGs II-IV were identified with resistance to Mi nematode. Cultivars with resistance to SCN and Mi RKN are needed for the midwestern United States (Kuger et al. 2008). Because MG IV and earlier genotypes are being grown in both North America and South America where these nematode species are abundant, there is more emphasis by soybean breeders to combine resistance to RKN and SCN in earlier maturities because they can often occur in the same field.

About 3,000 accessions from the USDA Soybean Germplasm Collection have been screened for higher resistance to RKN species (Harris et al. 2003; Luzzi et al. 1987). Among these, PI 96354, PI 200538, and PI 230977 were determined to have the highest levels of resistance to Mi, Ma and Mj nematodes, respectively. These PIs were found to carry unique resistant alleles with better resistance than in resistant U.S. cultivars (Luzzi et al. 1994a, 1995a, 1995b). Three breeding lines—G93-9009, G93-9106, and

G93-9223—were each developed and released from each of the three PIs above with resistance to Mi, Ma, and Mj, respectively (Luzzi et al. 1996a, 1996b, 1997). Harris et al. (2003) found additional PIs for resistance to Mi and Ma nematodes, which showed different patterns for egg production and galling, indicating contrasting mechanisms for resistance. Yates et al. (2010) found that PI 594403, PI 594427C, and PI 594651B contain useful resistance genes from those previously characterized in PI 200538. These PIs likely contain unique resistance genes that, when combined with PI 200538-derived Ma resistance, could improve the level of Ma resistance in soybean cultivars.

Molecular markers associated with PI 96354, PI 200538, and PI 230977 have been investigated (Tamulonis et al. 1997a, 1997b, 1997c). One QTL from PI 96354 was associated with the single gene *Rm1* previously found in Forrest (Luzzi et al. 1994b) for southern RKN resistance. SNP markers for two southern RKN genes that can be used in MAS have been identified (Ha et al. 2007).

Resistance to Multiple Nematode Species

Several species of nematodes can occur in the same field, causing damage and complicating control using host resistance. It has been mentioned that there are soybean cultivars that have resistance to both SCN and RKN. There are also cultivars that have shown resistance or tolerance to three or more nematode species. Examples are Forrest, Bedford, and Jake soybeans (Hartwig and Epps 1973a; Shannon et al. 2007) with resistance to SCN, RKN, and RN. It is evident that many of the 118 sources of SCN resistance also carry resistance to RKN, RN, or both (Shannon et al. 2007).

As mentioned earlier in this chapter, recent evaluations of more than 600 soybean germplasm accessions (MG III-V) showed that many new exotic soybean PIs were highly resistant to multi-nematode species (Nguyen Laboratory, unpublished data). Those PIs have been employed for further molecular characterization and for the development of new genetic materials, aiming to identify novel QTL or genes conveying resistance to these nematode pests. Genetic markers associated with the QTL or genes can be efficiently utilized for marker-assisted backcrossing or genomic selection (GS), which facilitate soybean molecular breeding programs.

New Genomics Approaches and Biotechnology

Genomics-Based Crop Improvement

In the past, QTL mapping was an important approach to identify loci of agronomic interest. This approach is typically conducted by analyzing the co-segregation of traits with markers in bi-parental populations (Hamblin et al. 2011). However, major disadvantages of traditional QTL mapping include the long time needed for population establishment, the limited inference made from alleles in just two parental lines, and the small number of recombinant events (Morrell et al. 2012).

In recent years, next-generation sequencing (NGS) technology has had a transforming effect on population-level studies linking genetic variation to gene function (Harrison 2012). NGS confers the ability to sequence the whole genome of many related organisms and makes feasible the development of high-throughput, dense genotyping (Metzker 2010). This progress has led to a shift from traditional QTL mapping to genome-wide association studies (GWAS). GWAS can identify more novel functional variation that may be deployed in cultivar improvement through MAS (Hamblin et al. 2011). Furthermore, GWAS enables a much greater genetic resolution than QTL mapping due to a long history of recombination events captured in most association panels. Combined with dense, genome-wide marker coverage, GWAS can potentially map causative loci to individual

nucleotide changes. However, there are limits to the precision available in GWAS, particularly in inbreeding organisms (Morrell et al. 2012).

To overcome many of the limitations of bi-parental QTL mapping and GWAS, a new generation of genetic-mapping populations has been designed, such as the nested association mapping (NAM) populations (Buckler et al. 2009), multi-parent advanced generation intercross (MAGIC) (Cavanagh et al. 2008) populations, and recombinant inbred intercross line (RIAIL) populations (Rockman and Kruglyak 2008). All of these populations involve the crossing of multiple parents and advancement of populations through several generations to improve resolution in genetic mapping compared with traditional bi-parental mapping (Morrell et al. 2012). The controlled nature of the crosses involved in next-generation design can overcome some of the difficulties of association mapping, including population structure and the unknown frequency of causative mutations. Next-generation design can also allow for better estimation of allelic effects because of the approximately even contribution of all parents (Cavanagh et al. 2008).

Today, genomics-based strategies for crop improvement, such as GWAS and GS, receive considerable attention among plant breeders (Morrell et al. 2012). Breeders can predict plant phenotypes by the use of genome-wide marker data rather than by direct phenotyping. As a result, these two methods can dramatically reduce the time and expenses involved in phenotyping breeding lines.

Other Omics-Based Crop Improvement

With the advancement of technologies, especially NGS and metabolite profiling technologies, all kinds of "omics" research have been conducted, namely transcriptomics, proteomics, metabolomics, and epigenomics. Combined with genomics information, some metabolic QTL (mQTL) and expression QTL (eQTL) have been identified (Fernie and Schauer 2009). It is possible that some protein and epigenome QTLs will also be gained. This information will provide an important addition to the tools currently employed in genomics-assisted selection for crop improvement in the future (Fernie and Schauer 2009).

Transgenic Approaches to Crop Improvement

Transgenic crops, including corn, soybean, cotton, and potato, are grown commercially in the United States. In addition, field trials of transgenics from at least 52 species are ongoing (Dunwell 2000). The trends of these trials are to progress from simple, single-gene traits, such as herbicide and insect resistance, toward more complex agronomic traits. These complex agronomic traits include photosynthetic enhancement, yield increase, modification of seed compositions, alteration in senescence, sugar and starch metabolism, and improvement in responses to abiotic and biotic stresses (Dunwell 2000). Along with this trend, transgenic approaches are also shifting from constitutive promoter and single-gene to tissue- or time-specific promoters and multiple genes stacking (Walker et al. 2002). One potato line being tested by Monsanto (APHIS Application 98–069–23N) contains seven transgenes, namely three selectable markers (gus, npt II, and CBI); a cry IIIA Bt gene to provide resistance to Colorado potato beetle; virus coat protein and replicase genes to give resistance to two viral diseases; another CBI gene associated with resistance to *Verticillium*; improvement in bruising resistance; and altered carbohydrate metabolism. These examples represent the future trend of transgenic crop developments (Dunwell 2000).

Combined with the development of transgenic technologies such as tasiRNA (Vaucheret 2005), virus-induced silencing (Lu et al. 2003), and ZF nuclease/TAL effector nuclease/Maganuclease technology (de Souza 2012), more agronomic interest genes will be identified and their functions elucidated. These progresses will facilitate

crop improvement by bioengineering, especially through transgenic approaches.

Future Prospects for Research on Soybean Nematode Resistance

Currently, only limited resources of resistance genes are available for soybean improvement through breeding or transgenic approaches (Vuong et al. 2010). Identifying and cloning more novel QTL relevant to nematode resistance will help solve this problem. In the past, little progress was made on the identification of novel nematode resistance QTL, especially the ones with large effects (Concibido et al. 2004). The possible reason is that soybean scientists selected soybean materials with close backgrounds in those experiments because no haplotypic data were available at that time. Now this difficulty can be overcome with the development of high-throughput genotyping technology. It can be predicted that more QTL-conferring soybean nematode resistance can be identified and further cloned in the near future.

Beside identifying new soybean resistance genes, elucidating how parasitic nematodes successfully establish their life cycle in hosts is another aspect of research on soybean nematode resistance. Until now, many nematode effectors and their interaction proteins in the host have been identified (Gheysen and Mitchum 2011). The primary mechanisms underlying soybean-nematode interaction have been revealed, such as the involvement of the cell wall pathway and the important role of the plant hormone auxin. However, those studies were mainly conducted using the model plant Arabidopsis. Whether the same mechanism exists in soybean is still not clear. Confirming the mechanism in soybean and directly identifying soybean genes involved in soybean-nematode interaction might be future objectives that soybean scientists want to achieve.

Scientists have gained more and more research results dealing with soybean nematode resistance. With the application of omics-assisted breeding or transgenic approaches, we anticipate we will soon make a breakthrough on improving nematode resistance in soybeans.

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Chapter 8

Marker-Assisted Selection for Biotic Stress Resistance in Peanut

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Abstract

Peanut is the second-most important legume grown worldwide. Cultivated peanut is a disomic tetraploid, 2n=4x=40, with limited genetic diversity due to a genetic bottleneck in formation of the polyploid from ancestors *A. duranensis* and *A. ipaënsis*. Consequently, resistance to biotic stresses is limited in the cultigen; however, wild species possess strong resistances. Transfer of these resistances is hindered by differences of ploidy, but production of synthetic amphidiploids, coupled with use of molecular markers, enables efficient gene transfer. Marker maps have been made from interspecific crosses, and SSR-based maps from cultivated parents have been developed recently. At least 410 resistance gene analogues have been identified. The first markers for biotic stress tolerance were for root-knot nematode resistance and introgressed from one *A. cardenasii* chromosome. These and improved markers have been used for marker-assisted backcrossing, contributing to release of three cultivars. Additional QTLs have been identified since. Early and late leafspots cause significant yield losses worldwide, and resistance depends on multiple genes. Using interspecific populations, five resistance QTLs for early leafspot were identified using greenhouse inoculations, and five QTLs for late leafspot were identified using detached leaf assays. Using cultivated species populations, 28 QTLs were identified for LLS resistance; all but one were minor QTLs; the major QTL was donated by an interspecific introgression line parent. Rust often occurs alongside leafspots, and rust resistance was characterized as one major QTL, plus several smaller QTLs. Marker-assisted backcrossing of this major QTL has been performed into different populations. QTLs for resistance to other biotic stresses have been identified, namely to groundnut rosette virus, Sclerotinia blight, aflatoxin contamination, aphids, and tomato spotted wilt virus. Marker-assisted breeding is still in early stages, and development of more rapid and inexpensive markers from transcriptome and genome sequencing is expected to accelerate progress.

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Significance of Peanut

Peanut (groundnut) ranks second to soybean in the world market trade of legume oilseeds both in area grown and tonnage produced. Peanut is grown in more than 100 countries (Nwokolo 1996), with a total production of 37.7 million tons from 24.1 million hectares in 2010 (FAO 2012), with a mean productivity of 1.56 t/ha. The five largest producers in the world in 2010, based on pod tonnage, were China, India, Nigeria, the United States, and Senegal. Crop yield per hectare varies from region to region with the United States having the highest (3.7 t ha^{-1}) among major producers, followed by China (3.4 t ha^{-1}), Brazil and Argentina with values of 2.7 tons per hectare, and Senegal, Nigeria, and India with yields of 1.0 to 1.1 tons per hectare (FAO 2012). The crop is a rich source of oil (36–54%), proteins (16–36%), and carbohydrates (10–20%) (Knauft and Ozias-Akins 1995). Peanut is used for its seed, which supplies essential minerals such as zinc, iron, phosphorus, and calcium and vitamins such as riboflavin, thiamine, niacin, and vitamin E; peanut is also a major source of oil with benefits for human health (The Peanut Institute 2004). In some countries, the haulm is used as a source of fodder. Peanut, as a member of the Fabaceae (Leguminosae), is capable of converting atmospheric nitrogen into ammonia by symbiotic nitrogen fixation. Thus, in addition to being a food crop, peanut is capable of increasing the fertility of the soil (Pimratch et al. 2004) as a rotation crop. Peanut is important industrially; the famous scientist George Washington Carver identified more than 300 uses for peanut and peanut products over a century ago.

Peanut is well suited to contribute significantly to poverty reduction in the developing world, with a potential to accelerate the achievement of the United Nation's Millennium Development Goal of halving world poverty by 2015, because more than 90% of world production is realized in developing countries (FAO 2012). In Africa, women typically have the responsibility for post-harvest processing and sale of peanut, and thus improvement in yields is expected to improve the economic well-being of women (Kaaya and Christie 2007).

Genetic Structure of Peanut (Groundnut)

Origin of the Genus *Arachis* and Sections within the Genus

In 1753, Linnaeus described the domesticated peanut as *Arachis hypogaea*, depicting peanut as a weed with underground fruits, unlike most angiosperms. The *Arachis* genus was placed within the Leguminosae (Fabaceae) family. Within this family, the major grain legumes are in the Papillionoideae, which is further subdivided into several clades, among them are the Phaseoloids (milletoids or warm season legumes) that include the genera *Glycine*, *Phaseolus*, and *Vigna*, Galegoids (cool season legumes) including *Pisum*, *Medicago*, *Lens*, and *Vicia*, and the Genistoids, which include *Lupinus* (Doyle and Luckow 2003; Lewis et al. 2005). *Arachis* is distinct from these, belonging to the Dalbergoids, which includes peanut and *Stylosanthes*.

According to Gregory and Gregory (1979) and Krapovickas and Gregory (1994), the genus developed in the southwestern part of Mato Grosso do Sul, Brazil or northeast Paraguay, because what appeared to be the morphologically most ancient species of the genus, *A. guaranitica* Chodat. and Hassl. and *A. tuberosa* Bong. ex Benth., are still growing in that area. This would be consistent with *Stylosanthes* being the progenitor genus, in agreement with molecular phylogenetic work (Lavin et al. 2001). Wild species of *Arachis* have been collected in Brazil, Bolivia, Paraguay, Argentina, and Uruguay (Krapovickas and Gregory 1994; Singh and Simpson 1994; Jarvis et al. 2003).

Based on morphological and cross-compatibility data and geographic distribution, it has been proposed that the genus has evolved into species that fit into nine taxonomic sections (Krapovickas and Gregory 1994), which include the morphologically most ancient section *Trierectoides* with its two species with three

leaflets, *A. tuberosa* and *A. guaranitica*. From these ancient progenitors developed the sections *Erectoides*, *Extranervosae*, *Triseminatae*, and *Heteranthae*. The species of these four sections have varying affinities to the primitive section, as reported by Gregory and Gregory (1979) and Krapovickas and Gregory (1994). The more advanced sections include the *Caulorrhizae*, *Procumbentes*, and *Rhizomatosae*. The affinities of these latter species groups are varied as well, but with very limited successes reported in crossing with species of the most advanced section, *Arachis* (Gregory and Gregory 1979; Krapovickas and Gregory 1994). Krapovickas and Gregory (1994) described 69 species and Valls and Simpson (2005) added descriptions of 11 more, for a total of 80 species. There are at least 11 more species that have been collected but not yet described (Valls 2011).

Section *Arachis*

The *Arachis* section is the most advanced of the 9 sections and encompasses 31 described species, including the cultigen, *A. hypogaea* and one other cross-compatible tetraploid species, *A. monticola* Krapov. & Rigoni, plus 29 diploid annual and perennial species (Krapovickas and Gregory 1994; Valls and Simpson 1994; Valls and Simpson 2005). All but one of these species can be crossed to *A. hypogaea* and *A. monticola* with varying degrees of difficulty (Krapovickas and Gregory 1994; Singh and Simpson 1994). The distribution of the *Arachis* section has overlapped that of the other sections in many areas. It is not unexpected that the most advanced species would be more adaptable and thus colonize a larger geographical area. Also, people have played a role in the distribution of several species, most of which belong to section *Arachis*, including *A. stenosperma* and *A. hypogaea*. This latter species is the most widely cultivated member of the genus.

The remaining species of section *Arachis* are diploid and had been grouped until recently into three genomes (A, B, and D) each having 20 chromosomes. To date, 20 A-genome diploid species have been described (Krapovickas and Gregory 1994); among these are perennials *A. cardenasii*, *A. diogoi*, *A. helodes*, *A. villosa*, and *A. correntina* and annuals *A. duranensis* and *A. stenosperma*. Based on cytological evidence and cross-hybridization data, *A. cardenasii* was considered originally to be the most probable A-genome ancestor of *A. hypogaea* (Smartt et al. 1978). More recently, it has been proposed that this genome type can be divided into three groups based on karyotype (Robledo and Seijo 2010).

Initially only one annual B-genome species, *A. batizocoi*, was identified (Smartt et al. 1978), the B genome being associated with the absence of a specific small pair of A chromosomes (Fernandez and Krapovickas 1994). Accordingly, *A. batizocoi* was first proposed as the B genome donor to the cultigen (Smartt et al. 1978). However, cytological measurements discounted this hypothesis (Stalker and Dalmacio 1986). Subsequently, cross-compatibility, molecular, and cytological studies provided evidence for up to 10 B-genome species (Krapovickas and Gregory 1994; Kochert et al. 1996; Milla et al. 2005b; Tallury et al. 2005; Valls and Simpson 2005; Burow et al. 2009). However, the low pollen fertility, sterility, and separate molecular phylogenetic groupings of *A. ipaënsis* and *A. batizocoi* led Burow and coworkers to question whether *A. ipaënsis* and *A. batizocoi* belong to the same genome (Burow et al. 2009). Based on FISH, GISH, and geographic origin, Robledo and Seijo (2010) proposed that the B genome classification is not accurate and should be split into three genome types. *Arachis ipaënsis*, *A. magna*, *A. gregoryi*, *A. vallsii*, and *A. williamsii* are B genome *sensu stricto*, *A. batizocoi*, *A. cruziana* and *A. krapovickasii* being reclassified as K genome, and *A. benensis* and *A. trinitensis* as F genome.

The D genome consists of one species, *A. glandulifera*. This species is characterized by extensive genome rearrangements relative to other section *Arachis* species, as observed cytologically (Stalker 1991).

In addition, there are three diploid species that possess 18 instead of 20 chromosomes. These

species have been described as *A. decora*, *A. palustris*, and *A. praecox* (Lavia 1996, 1998; Peñaloza and Valls 2005; Valls and Simpson 2005).

Origin of Arachis Hypogaea

A. hypogaea is a tetraploid (2n=4x=40) (Husted 1936), and the only other known tetraploid species in the section, *A. monticola*, is closely related to it. Hybridization between the cultigen and section *Arachis* diploids is possible, but no evidence has been found that this has contributed to ongoing gene flow into the cultigen in nature. Cultivated peanut is considered to be an AB tetraploid, arising from hybridization between A and B diploid species (Smartt et al. 1978).

Lack of marker polymorphism in the cultigen using RFLP and RAPD markers (Halward et al. 1991; Kochert et al. 1991) contributed to the hypothesis that all varieties and botanical types of *A. hypogaea* share common diploid progenitors (Kochert et al. 1996). RFLP analysis determined that *A. duranensis* had greater similarity to *A. hypogaea* than did *A. cardenasii* (Kochert et al. 1991, 1996), and *A. duranensis* is considered by many now to be the likeliest A-genome ancestor. However, subsequent marker analyses have also proposed *A. villosa* (Raina and Mukai 1999), *A. helodes*, and *A. simpsonii* (Milla et al. 2005b) as potential A-genome donors.

Evidence from archaeological data (Simpson and Faries 2001), molecular marker data (Kochert et al. 1991, 1996), fluorescent *in situ* hybridization analysis using rDNA as labeled probe (Raina and Mukai 1999; Seijo et al. 2004), and gene sequence data (Jung et al. 2003; Ramos et al. 2006) strongly supported *A. ipaënsis* instead of *A. batizocoi* as B genome donor. However, new discoveries of wild *Arachis* species are still being made (Valls and Simpson 2005), and it is possible that other candidates could be discovered.

As a result of explorations by the Spanish and Portuguese, peanut spread quickly from the Americas to other parts of the world since the 16th century. Wynne and Coffelt (1982) indicated the existence of an important secondary center of diversity within *A. hypogaea* in Africa, where a large amount of variation is thought to arise from hybridization and selection in different environments.

Krapovickas and Gregory (1994) classified *A. hypogaea* into two subspecies and six botanical varieties. *A. hypogaea* subsp. *hypogaea* is characterized by a spreading growth habit, alternating vegetative and reproductive nodes, lack of flowers on the mainstem, medium-to-large seeds, medium-to-late maturity, and includes the botanical varieties *hypogaea* (virginia and runner market types) and the less frequently cultivated *hirsuta*. Several genotypes cultivated among native Brazilian Indians from the Xingu region have been characterized morphologically and using molecular markers and are reported to group with the *hypogaea* subspecies (Freitas et al. 2007). The *fastigiata* subspecies is typified by erect growth habit, sequential reproductive nodes, flowers on the mainstem, small seeds, and early maturity. These include the botanical varieties *fastigiata* (valencia), *vulgaris* (spanish), *peruviana*, and *aequatoriana*. The latter two are not cultivated widely outside of Peru, northwestern Brazil, northern Bolivia, and Ecuador.

Introgression Pathways

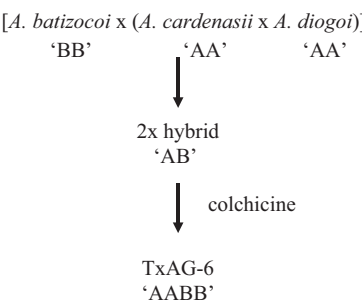
Attempts to utilize wild species as sources of new alleles have been met with limited success because of genomic (A and B genomes) and ploidy (diploid and tetraploid) barriers (Stalker and Moss 1987). Several pathways have been attempted with varying degrees of success, of which this chapter covers three: the hexaploid route, the autotetraploid route, and the allotetraploid route (the latter more commonly known simply as the tetraploid route).

The hexaploid route involves crossing a diploid wild species with *A. hypogaea* to generate a sterile triploid hybrid, followed by doubling

the hybrid chromosome number with colchicine to the hexaploid level (60 chromosomes). The progeny is backcrossed repeatedly to *A. hypogaea* until the progeny regained the normal chromosome number of 40. This method was used in crosses between *A. hypogaea* and seven diploid species, among them *A. cardenasii* (Smartt and Gregory 1967; Smartt et al. 1978; Stalker and Moss 1987; Wynne and Halward 1989). The progeny of the *A. hypogaea* x *A. cardenasii* cross have been used for development of marker maps, introgression populations, and germplasm releases (Stalker et al. 2002a, 2002b) or varieties with nematode, rust, and late leaf spot resistance, such as GPBD-4 (Gowda et al. 2002) (see discussion that follows).

The autotetraploid route involves the treating of two wild diploid species with AA and BB genomes types with colchicine to create synthetic autotetraploids. The synthetic auto tetraploids, with genomic composition AAAA or BBBB, are crossed to obtain plants with genotype AABB. Three autotetraploids were generated by Singh (1985) and crossed to *A. hypogaea*. Fertility of the autotetraploids varied, but fertilities of progenies backcrossed by *A. hypogaea* were higher.

The allotetraploid route involves the creation of synthetic amphidiploids by crossing two diploids of different genomes, followed by doubling with colchicine to the tetraploid level. This method was used to develop the TxAG-6 breeding line (Simpson 1991; Simpson et al. 1993), although by a slight variation of the procedure (Figure 1).



The hybrid TxAG-6 was backcrossed repeatedly to recover the cultivated phenotype to develop various varieties most notably incorporating resistance against root-knot nematode (see discussion later in the chapter).

Since the development of TxAG-6, a number of new synthetic amphidiploids have been created. For example, in a probable "resynthesis" of *A. hypogaea*, an amphidiploid was made from *A. ipaënsis* and *A. duranensis* (Fávero et al. 2006). From this amphidiploid, a series of structured introgression lines and agronomically adapted selected lines with some level of late leaf spot resistant have been made (Foncéka et al. 2009; Galhardo et al. 2011). Subsequently, additional amphiploids have been developed (Fávero et al. 2011; Leal-Bertioli et al. 2011; Santos et al. 2011). Almost all had greater resistance to leaf spot and rust than the cultivated species, with the most resistant amphiploids being *A. magna* x *A. cardenasii*, *A. magna* x *A. stenosperma*, *A. batizocoi* x *A. stenosperma*, and *A. gregoryi* x *A. stenosperma* (Fávero et al. 2011; Leal-Bertioli et al. 2011).

Genetic Linkage Maps of *Arachis*

Molecular Markers for *Arachis*

The development of molecular markers for peanut has followed the technical trends of the times. The first studies were based on isozymes and proteins (Krishna and Mitra 1988; Grieshammer and Wynne 1990; Lu and Pickersgill 1993), followed by Restriction Fragment Length Polymorphism—RFLPs (Kochert et al. 1991; Paik-Ro et al. 1992; Kochert et al. 1996), Random Amplified Polymorphic DNA—RAPDs (Halward et al. 1991, 1992; Hilu and Stalker 1995; Subramanian et al. 2000), Amplified Fragment Length Polymorphism—AFLPs (He and Prakash, 1997; Gimenes et al. 2000; He and Prakash 2001; Gimenes et al. 2002; Herselman, 2003; Milla et al. 2005a, 2005b; Tallury et al. 2005), and more recently microsatellite markers (Hopkins et al. 1999; Palmieri et al.

2002; He et al. 2003; Ferguson et al. 2004; Moretzsohn et al. 2004; He et al. 2005; Moretzsohn et al. 2005; Palmieri et al. 2005; Bravo et al. 2006; Budiman et al. 2006; Gimenes et al. 2007; Proite et al. 2007; Wang et al. 2007; Cuc et al. 2008; Naito et al. 2008; Liang et al. 2009; Moretzsohn et al. 2009; Song et al. 2010; Yuan et al. 2010; Koilkonda et al. 2012; Macedo et al. 2012; Pandey et al. 2012) and molecular markers based on MITE markers (Shirasawa et al. 2012 and unpublished data). Generally, these markers have shown a trend toward becoming more informative, and now microsatellites, being codominant and easy to score in the tetraploid genome, are considered the molecular marker of choice, with MITE markers also showing much potential.

Maps Based on Crosses Involving Wild Species

The very narrow genetic base of cultivated peanut has provided a substantial obstacle to genetic mapping using only cultivated germplasm. This meant that maps were initially generated using crosses involving wild species. Subsequently mapping in cultivated x cultivated crosses has advanced considerably (see discussion later in the chapter). In spite of this, mapping using crosses involving wild species is likely to continue to be important. Wilds are a source of new alleles for cultivated peanut conferring, for instance, strong disease resistances; the greater DNA polymorphism of the wilds allows for higher resolution mapping; also, diploid genetics simplifies genetic analysis and the use of some marker types (notably marker types based on single nucleotide polymorphisms, or SNPs).

The first genetic linkage map of peanut was developed using an F_2 population of a cross between A-genome diploids *A. stenosperma* and *A. cardenasii*. The 117 mapped RFLP markers were distributed among 11 linkage groups over 1,063 cM (Halward et al. 1993). A second map was constructed from a tetraploid cross of the cultivar Florunner × the synthetic amphidiploid TxAG-6 {*A. batizocoi* × [*A. cardenasii* × *A. diogoi*]}^{4x}. A total of 370 RFLP loci were mapped onto 23 linkage groups, for a map distance of 2,210 cM (Burow et al. 2001). The map was characterized by pairing of homoeologous linkage groups, consistent with a disomic nature of the cultigen. An AFLP-based A-genome map was generated from an F_2 population developed from the cross *A. kuhlmannii* x *A. diogoi*; 102 markers were mapped over 1,068 cM (Milla 2003). A RAPD-based map of *A. stenosperma* x *A. cardenasii* was developed by Garcia et al. (2005). This map contained 167 RAPD and 39 RFLP loci spanning 800 cM and 11 linkage groups.

The first microsatellite-based map of peanut was developed with an F_2 population derived from a cross between A genome diploids *A. duranensis* and *A. stenosperma*, and had 170 microsatellite markers on 11 linkage groups covering 1,231 cM (Moretzsohn et al. 2005). Subsequently a microsatellite map of the B genome based on a cross of *A. ipaënsis* and the closely related *A. magna* was produced (Moretzsohn et al. 2009). This map had 10 linkage groups, with 149 loci spanning a very similar total map distance of 1294 cM. The comparison of 51 shared markers between these two maps revealed high levels of synteny, with all but one of the B linkage groups showing a single main correspondence to an A linkage group. Foncéka et al. (2009) developed a map of 289 SSR markers using a BC_1 population between the cultivar Fleur 11 and a synthetic amphidiploid (*A. duranensis* x *A. ipaënsis*)^{4x}. This map again showed good colinearity between the A and B subgenomes in general, though several inversions of order were noted.

A higher-density version of the diploid map based on the cross of *A. duranensis* and *A. stenosperma* published by Moretzsohn et al. (2005) was reported by Leal-Bertioli et al. (2009). This map consisted of a total of 369 markers, including 188 SSRs and 80 legume anchor markers, 46 AFLPs, 32 NBS analogs, 17 SNPs, 4 RGA-RFLPs, and 2 RGA-SCAR

markers. Virtually all markers on this map were sequence characterized. This, in combination with the high proportion of low or single-copy gene markers allowed the map to be aligned to the fully sequenced genomes of *Lotus japonicus* and *Medicago truncatula* (Sato et al. 2008; www.medicago.org). These alignments revealed surprising degrees of synteny considering the time of species divergence (estimated at about 55 million years). Phylogenetically *Arachis* is an outgroup to *Medicago* and *Lotus*, and for this reason, comparisons are particularly informative for making evolutionary inferences. Using genome plots *Arachis* versus *Lotus*, *Arachis* versus *Medicago*, and comparing to a previously published plot between *Lotus* and *Medicago* genomes (Cannon et al. 2006; Bertioli et al. 2009), 10 distinct conserved synteny blocks and also non-conserved regions could be observed in all genome comparisons (Bertioli et al. 2009). This clearly implies that certain legume genomic regions are consistently more stable during evolution than others. It is notable that these regions are large scale, and apparently in some cases consist of entire chromosomal arms.

Intriguingly, an analysis of the retrotransposon distributions in *Lotus* and *Medicago* shed further light on these observations. Retrotransposons are unevenly distributed in both *Lotus* and *Medicago*, and retrotransposon-rich regions tend to correspond to variable regions, intercalating with the synteny blocks, which are relatively retrotransposon poor. Furthermore, while the variable regions generally have lower densities of single-copy genes than the more conserved regions, some harbor high densities of the fast-evolving disease resistance genes (Bertioli et al. 2009). For *Arachis* it was notable that LGs 2 and 4, which harbor the most prominent clusters of resistance gene analogs (RGAs) and QTLs for late leaf spot resistance, showed shattered synteny with both *Lotus* and *Medicago*. An association between RGAs and retrotransposons in *Arachis* has also been supported by studies on two peanut retrotransposons FIDEL and Matita (Nielen et al. 2010, 2011).

Genetic Maps Based on Cultivated x Cultivated Crosses

Screening of isozyme, RFLP, and RAPD markers on accessions of *A. hypogaea* identified only very low levels of polymorphism among cultivated peanut accessions (Kochert et al. 1991; Halward et al. 1992; Lu and Pickersgill 1993; Burow et al. 1996; Subramanian et al. 2000; Dwivedi et al. 2001). The partial first linkage map from a cross between accessions of *A. hypogaea* was constructed using an F_2 population (Herselman et al. 2004). Five linkage groups with 12 markers spanning 139 cM of the genome were reported. The first reasonably complete genetic maps of cultivated peanut were published by Hong et al. (2008) and Varshney et al. (2009). Hong et al. (2008) tested 1,048 SSR primer pairs and mapped 131 SSR loci onto 20 linkage groups for a total length of 670 cm on an RIL population between the cultivars Yueyou 13 and Zhenzhuhei. Varshney et al. (2009) screened 1,145 SSR markers and mapped 135 loci onto 22 linkage groups spanning 1,271 cM onto an RIL population developed from two parental genotypes, TAG 24 and ICGV 86031. Later a composite map containing 175 SSR markers in 22 linkage groups was developed from three cultivated crosses (Hong et al. 2010); of 901 primer pairs screened, 146, 124, and 64 were polymorphic. The most saturated map so far was recently published by Wang et al. (2012), containing 385 polymorphic SSRs covering 318 loci.

Attempts to develop maps with higher densities have required screening several thousand SSR markers. The SSR-based cultivated genetic map with 135 marker loci developed by Varshney et al. (2009) was then further saturated up to 191 SSR loci (Ravi et al. 2011). Two new partial genetic maps with 56 (TAG 24 × GPBD 4) and 45 (TG 26 × GPBD 4) marker loci (Khedikar et al. 2010; Sarvamangala et al. 2011) were constructed covering genome distances of merely 462.24 and 657.9 cM, respectively. These two maps were then saturated with enhanced genome coverage up to 188 (1,922.4 cM) and

181 (1,963 cM) marker loci, respectively, along with construction of a consensus map based on these two populations segregating for foliar disease resistance with 225 SSR loci and a total map distance of 1,152.9 cM (Sujay et al. 2011). In addition to the aforementioned three saturated maps, more recently two more genetic maps based on RIL populations segregating for traits related to drought tolerance, namely ICGS 76 × CSMG 84-1 (119 SSR loci) and ICGS 44 × ICGS 76 (82 SSR loci), were developed with genome coverage of 2,208.2 cM and 831.4 cM, respectively. Since the aforementioned three populations (TAG 24 and ICGV 86031, ICGS 76 × CSMG 84-1, and ICGS 44 × ICGS 76) were segregating for traits related to drought tolerance, a consensus map (2,840.8 cM) with 293 SSR loci was developed. All the parentals were cultivated genotypes, except for GPBD-4, which is predominantly cultivated with some *A. cardenasii* parentage derived through the hexaploid route (Smartt et al. 1978; Gowda et al. 2002). They observed 6-10% polymorphism for different marker types and mapped 652 markers into a high-density composite map based on all the five populations. More recently, Qin et al. (2012), after screening a total of 4,576 markers, identified 260 and 181 polymorphic markers, respectively, for the two RIL populations, namely Tifrunner × GT-C20 (T population) and SunOleic 97R × NC94022 (S population). Individual genetic maps were constructed for T and S populations with 236 and 172 marker loci, respectively. An integrated map was then constructed with 324 marker loci covering 1,352 cM genome distance (Qin et al. 2012). For the creation of the highest-density map of cultivated peanut to date, with more than 1,000 markers, screening was done by *in silico* analysis of DNA sequence data from the parentals (Shirasawa et al. 2012).

SNP-Based Maps of Peanut

Two significant SNP-based maps exist for peanut. The first is an extension of the A-genome diploid map of Moretzsohn et al. (2005) to 165 SSR, 78 anchor markers, 17 RGA, and 507 SNP markers (Gouvea 2012). A second SNP-based map of peanut has been reported by Nagy et al. (2010), wherein a high-density genetic map of the A genome was developed from an intraspecies cross within *A. duranensis*, and 971 SSRs, 221 single-stranded DNA conformation polymorphism (SSCP) markers, and 1,127 SNPs were mapped. Extension of SNP-based maps to the tetraploid has not been accomplished yet, and will require separation of A- and B-genome sequences, but is expected to greatly accelerate genetic mapping and marker-assisted selection when available.

Resistance Gene Analogs

Plant resistance genes have been found to fall into several classes, among which genes encoding the nucleotide binding site (NBS) are the most characterized. The NBS domain is thought to act in signal transduction pathways.

Using conserved amino acid motifs, degenerate primers can be designed that are able to amplify RGAs from any plant species (see Hammond-Kosack and Parker 2003). Using such primers, RGAs have been identified from wild and cultivated peanut. Seventy-eight nonredundant NBS-encoding regions were characterized by Bertioli et al. (2003). Phylogenetic analysis of these sequences with NBS encoding sequences from *Arabidopsis thaliana*, *Medicago truncatula*, *Glycine max*, *Lotus japonicus*, and *Phaseolus vulgaris* showed that most *Arachis* NBS sequences fall within legume-specific clades, and that sequences in some clades appear to have undergone extensive copy number expansions in the legumes. This underlines the apparent quickly evolving nature of resistance gene analogs. An additional 234 sequences were identified and mapped onto 250 nonredundant BAC clones containing NBS-encoding sequences (Yüksel et al. 2005). More recently, 401 RGAs were mined from a peanut

EST database, of which 2 were mapped (Liu et al. 2012).

As regards the genetic architecture of disease resistance genes, candidate genome regions that control disease resistance were identified by Leal-Bertioli et al. (2009). For this, 34 sequence-confirmed candidate disease resistance genes and five QTLs for resistance against late leaf spot were mapped in a diploid *A. duranensis* x *A. stenosperma* cross. Candidate genes and QTLs were distributed on all linkage groups except for the smallest, but the distribution was not even. Groupings were apparent on the upper region of linkage group 4 and the lower region of linkage group 2, indicating that these regions are likely to control disease resistances. As noted previously, these candidate regions showed shattered synteny with *Lotus* and *Medicago*, indicating that RGA-containing regions are probably faster evolving than some other genome regions. In a different study, resistance to root-knot nematode from the wild diploid *A. cardenasii* was mapped to the A genome linkage group 9 (Nagy et al. 2010). This region is particularly interesting genetically because it displays strongly suppressed recombination with the A genome of *A. hypogaea* and appears to cover about one-third to a half of a chromosome. Recently Ratnaparkhe et al. (2011) sequenced two peanut BACs containing six RGAs and concluded that synteny was not high with *Lotus*, *Medicago*, or *Arabidopsis*, and that there was evidence of intergenic and intragenic gene conversions and unequal crossing-over in this region in peanut.

Marker-Assisted Breeding of Peanut

Nematode Resistance: A Case Study in the Effectiveness of Markers in Breeding for a Simply Inherited Trait

Etiology

Meloidogyne species (root-knot nematode) are the most important nematode species limiting yield in peanut (Porter et al. 1984). Of these, the predominant pathogenic species to peanut are *M. arenaria* (Neal) Chitwood, *M. hapla* Chitwood, and *M. javanica* (Treub) Chitwood. *Meloidogyne haplanaria* (Eisenback et al. 2003) is a peanut parasite with limited distribution in the United States. Root-knot nematodes are found on the commercial peanut in many parts of the world, with *M. arenaria* being the predominant pathogenic species in the southern United States, especially in Alabama, Florida, Georgia, and Texas. *Meloidogyne javanica* is more common than *M. arenaria* on peanut in Africa and India (Tomaszewski et al. 1994). *M. hapla* has a cooler temperature optimum than *M. arenaria* or *M. javanica* and is referred to as the northern root-knot nematode. It is frequently found attacking peanut in the more northern areas of peanut production in the United States, specifically North Carolina, Oklahoma, and Virginia, and is also found on peanut in China. *Meloidogyne arenaria* and *M. javanica* are more aggressive pathogens than *M. hapla*, causing greater yield losses at lower nematode population densities (Koenning and Barker 1992; Abdel-Momen and Starr 1997).

The effects of *Meloidogyne* spp. are due to invasion of root tips cells by juvenile nematodes, followed by generation of giant cells in the roots as feeding sites, damaging the root system and impede nutrient transport in the plant (Caillaud et al. 2008). A plant gene conferring resistance to *M. incognita*, called *Mi*, was first isolated from tomato by positional cloning (Milligan et al. 1998), and encoded an NBS-LRR type protein. Other genes associated with response to nematode infection have been isolated by several researchers in different species (Lambert et al. 1999; Potenza et al. 2001). More recently, *M. arenaria*-challenged resistant species *A. stenosperma* (Guimarães et al. 2010; Morgante et al. 2011) identified many responsive genes. Two have been identified by RT-PCR to be upregulated upon infection.

High levels of resistance were identified in 11 of 15 diploid species tested against isolates of *M. arenaria*, and several accessions were also found with resistance to *M. hapla* (Nelson et al.

1989) and *M. javanica* (Stalker and Moss 1987). Since then, low to moderate levels of resistance were discovered in *A. hypogaea* (Holbrook and Noe 1992). Diploid crosses involving *A. cardenasii* demonstrated the presence of at least two dominant resistance genes (Starr and Simpson 1991). Resistance took the form of a hypersensitive response that inhibited the development of invading juveniles, and resulted in an almost total suppression of nematode reproduction. In *A. batizocoi* and *A. diogoi*, the mechanism of resistance could not be determined because of the difficulty in making interspecific test crosses with a susceptible parent, but resistance was associated with a lengthening in the time for juveniles to develop into adults and a decrease in the percentage of juveniles that reached adulthood (Nelson et al. 1990). Interestingly, the resistance of the resistant cultivar COAN, which is due to a single gene derived from *A. cardenasii*, is expressed as a failure of the invading nematode to initiate a functional feeding site in the vascular tissues, and many of the invading nematodes then emigrate from the roots or remain localized in the cortical tissues (Bendezu and Starr 2003). In the root-knot nematode-resistant *A. stenosperma*, penetration and development of the nematodes was dramatically reduced in comparison with that occurring in cultivated peanut. Neither giant cells nor nematodes developed beyond the second stage were found. Several cell features, including darkly staining cytoplasm and altered organelle structure, were observed in the central cylinder, indicating a hypersensitive-like response (HR) of infested host cells (Proite et al. 2008).

Breeding

Before introgression of resistance alleles from wild species, no root-knot nematode-resistant peanut cultivars were released, for lack of known sources of resistant germplasm. Root-knot nematode resistance was introduced into *A. hypogaea* from two crosses, that of *A. hypogaea* x *A. cardenasii* via the hexaploid route (Garcia et al. 1996) and by crosses among diploids followed by doubling with colchicine to the tetraploid level (Simpson 1991). The nematode-resistant cultivar COAN was the first peanut cultivar that contained a distinct trait donated from wild species (Simpson and Starr 2001). COAN was developed from the TxAG-6 amphidiploid, crossed to Florunner and advanced by five cycles of backcrossing followed by selfing and selection for root-knot nematode resistance (Simpson and Starr 2001).

Markers and Use in Selection

The first markers for an agronomically useful trait in peanut were for resistance to root-knot nematode (*M. arenaria*) from *A. cardenasii*. Two closely linked sequence characterized amplified region (SCAR) markers were identified for genes for reduced galling and egg number (Garcia et al. 1996). Simultaneously, three RAPD markers were associated with nematode resistance in several backcross breeding populations derived from the interspecific hybrid TxAG-6 [*A. batizocoi* × (*A. cardenasii* × *A. diogoi*)]^{4x} (Burow et al. 1996); however, these were all for the same gene, and although these did provide flanking markers, the one marker opposite the other two did not appear to be qualitatively inherited, but appeared to differ quantitatively in amplification, and was thus deemed too difficult to score accurately for marker-assisted selection (MAS). Instead, two (non-flanking) RFLP markers ca. 4cM from the resistance gene were developed by bulked segregant analysis (Church et al. 2000). The use of non-flanking markers was in part the result of a large gap (>30cM) (Burow et al. 2001) between markers on the other side of the gene.

MAS was used for the development of NemaTAM, the second nematode-resistant peanut cultivar (Simpson et al. 2003). The variety COAN had superior yield under disease pressure but had low yield under disease-free conditions. Two additional generations of backcrossing accompanied by the use of RFLP markers were used for the development of NemaTAM.

NemaTAM had the same markers for nematode resistance as were present in COAN, but it and other selected breeding lines had mean yields under disease-free conditions that were 135% to 160% higher than COAN had (Church et al. 2000). It was concluded that the linkage between resistance and low yield had been broken. However, scores of flanking markers were unavailable, and as such, it was never demonstrated whether the difference between COAN and NemaTAM resided on the chromosome containing the resistance gene or on a different chromosome.

Several benefits were observed in use of markers for development of the nematode-resistant variety NemaTAM (Choi et al. 1999; Church et al. 2000; Simpson et al. 2003; Cason et al. 2010). It was demonstrated that use of markers was more efficient than phenotypic selection, because plants selected by markers for the homozygous resistance gene bred true, unlike materials selected based on phenotype (which included heterozygous plants). Also, markers were more accurate, because phenotypic selection was accompanied by a certain amount of escapes. Although MAS would be affected by recombination between marker and the trait being scored, this rate of crossing over was less than the rate of assigning incorrect phenotypes. In addition, scoring could be performed on collected leaf tissue, eliminating the need to harvest the plant to perform nematode egg counts. However, the use of the RFLP marker was costly and required a large amount of DNA, radioisotope, and a long time (one to four weeks) before developing the X-ray film. Church et al. (2000) were able to determine the genotype of only 65-86% of the individuals attempted because of technical difficulties, such as the low quality or quantity of DNA, incomplete digestion of DNA, or poor hybridization or background on Southern blots. A nonisotopic method was used by Muitia et al. (2006), but this was more cumbersome and expensive than the use of radioisotope.

MAS was also used in development of a nematode-resistant, high-oleic variety from Tifguard. Tifguard is a nematode-resistant cultivar that also has resistance to tomato spotted wilt virus (Holbrook et al. 2008). Because of the cost, and difficulties associated with the RFLP marker technology, Tifguard was developed using standard phenotypic selection methods, using COAN as donor parent for nematode resistance (Holbrook et al. 2008). It would be desirable to have a high oleic peanut cultivar with the disease-resistant package available in Tifguard. Based on the development of improved molecular markers, it was decided to use MAS to develop Tifguard High O/L.

Improved markers were developed for the root-knot nematode resistance gene, and were used in development of Tifguard High O/L. Nagy et al. (2010) performed high-resolution mapping of nematode resistance with breeding material derived from the synthetic tetraploid pathway in comparison with an A-genome diploid species map. Twelve polymorphic markers and a previously published sequence characterized amplified region marker S197 (Chu et al. 2007a), developed from the published sequence of RAPD marker RKN440 (Burow et al. 1996), were found to be tightly linked with Rma in populations from two tetraploid crosses. During the breeding procedure to develop Tifguard High O/L, a dominant marker S197 (resistant allele), a dominant CAPS marker 1169/1170 (susceptible allele), and a codominant simple sequence repeat (SSR) marker GM565 (Nagy et al. 2010) were used (Chu et al. 2011). This allowed for the identification of homozygous resistant, homozygous susceptible, and heterozygous individuals.

Two homoeologous genes (*ahFAD2A* and *ahFAD2B*) encode for the key enzyme regulating the O/L ratio in peanut (Ray et al. 1993). Double recessive mutants are needed for the expression of the high O/L trait. A mutation in the *ahFAD2A* is prevalent in *A. hypogaea* subsp. *hypogaea* (Chu et al. 2007b), and all parents used in the development of Tifguard High O/L carry this mutant allele. A cleaved amplified polymorphic sequence (CAPS) marker 1101/1048 (Chu et al. 2009) was used to identify breeding lines

containing the mutant allele for the *ahFAD2B* gene. During the process of developing Tifguard High O/L, this CAPS marker was converted to a gel-free single nucleotide polymorphism (SNP) assay using HybProbe design (Chu et al. 2011).

An accelerated backcross breeding program with MAS was used to develop Tifguard High O/L. Tifguard was used as the recurrent female parent and two high O/L cultivars were used as donor parents for the high O/L trait. F_1, BC_1F_1, and BC_2F_1 individuals carrying the marker alleles for both nematode resistance and high O/L were selected for use as male parents in the next round of crossing. BC_3F_1 seedlings heterozygous for high O/L were selected and allowed to self. Homozygous BC_3F_2 seedlings were identified as Tifguard High O/L. Three cycles of backcrossing were deemed adequate based on the high coefficient of coancestry between recurrent and the donor parents (Chu et al. 2011).

The effectiveness of selection of nematode resistance has been the most successful use of MAS in peanut to date. However, the use of a single gene trait that confers near-immunity may be subject to breakdown of resistance under high selection pressure, and has been cause for concern even before the release of COAN. Therefore, new sources of resistance for nematodes, such as amphidiploids derived from *A. stenosperma*, which is highly resistant to fungi and nematodes (Proite et al. 2008, Leal-Bertioli et al. 2010; Santos et al. 2011), would be a useful resource for peanut breeding.

The previously mentioned markers for nematode resistance (Burow et al. 1996) were identified using bulked segregant analysis. This is efficient for identifying markers with major effects but is less successful at identifying markers with smaller effects. Evidence for presence of a second, recessive resistance gene was provided by Church et al. (2005). QTL analysis of a segregating BC_3F_1 population developed from the TxAG-6 x Florunner cross has revealed the presence of three additional QTLs, with QTLs now from both A and B genomes (Burow et al. 2012). The previously known marker contributes more to the explanation of phenotypic variance than the newer markers; however, newer markers may be of use to develop a variety with a more durable resistance. It is possible that the presence of these additional genes for resistance could explain in part the linkage drag for yield observed in COAN.

Leaf Spot Resistance: Two Complex Traits Controlled by Many Genes

Etiology

The foliar diseases of early leaf spot (caused by *Cercospora arachidicola* S. Hori) and late leaf spot (caused by *Cercosporidium personatum* [Berk. and Curtis] Deighton), also known as *Phaeoisariopsis personata* ([Berk. and Curt.] Deighton), are two of the most limiting biotic stresses in peanut production known worldwide (Shokes and Culbreath 1997), causing yield losses of up to 50% (Smith 1984; McDonald et al. 1985). In West Africa, yield losses can be as high as 70% (Waliyar et al. 2000). Both diseases often occur together in the same field, even though one may predominate (Hassan and Beute 1977). The result of the disease is defoliation, reducing yield through reduction of photosynthesis, death of the plant, and pod loss.

Although these diseases can be controlled using fungicides, their application is costly in the United States (Coffelt and Porter 1986). A study in Ghana (Naab et al. 2005) has confirmed that foliar application of fungicides can increase biomass and kernel yields in rainfed peanuts by 39% and 75%, respectively. However, the use of fungicides, though allowing to increase yields, is not feasible for many farmers in West Africa, where poverty is prevalent. Credit facilities for the purchase of inputs, as well as the input availability and delivery system, are not adequately developed. The most practical control method for these farmers would be the use of host plant resistance (Holbrook and Stalker 2003).

Breeding

Some wild peanut species have considerable resistance to leafspots. Subrahmanyam et al. (1989) evaluated 96 accessions of wild *Arachis* species for reactions to late leaf spot and observed that lesions were formed on all accessions, but lesions were small and nonsporulating on all accessions of sections *Erectoides*, *Triseminatae*, *Extranervosae*, *Rhizomatosae*, and *Caulorrhizae*. Lesions with diameters ranging from 0.16 to 1.0 mm were, however, found in section *Arachis*. In section *Arachis* it was further observed that 15 accessions had no sporulating lesions, and sporulation was slight to extensive in other accessions. In *A. stenosperma*, the failure of late leaf spot and rust pathogens to infect has been shown to be due to inability of the fungal hyphae to penetrate the stomata of peanut leaves (Leal-Bertioli et al. 2010). Rao et al. (2003) list seven wild species (held at The International Crops Research Institute for the Semi-Arid Tropics, ICRISAT) that have resistance to early leaf spot (ELS), nine to late leaf spot (LLS), and twelve to rust that have been exploited to develop breeding lines with some degree of resistance (Simpson et al. 1993; Stalker et al. 2002a). In Brazil, IAC-Caiapó, a runner-type cultivar, shows moderate resistance to foliar diseases and high productivity (Godoy et al. 1999).

All commercially grown cultivars used to be susceptible to some extent to both diseases (Shokes and Culbreath 1997), suffering yield losses of around 50% in the absence of fungicide sprays (Smith 1984; McDonald et al. 1985; Waliyar et al. 2000). It was not until 1984 that the first commercial U.S. cultivar (Southern Runner) with an appreciable level of resistance to late leaf spot was released (Holbrook and Stalker 2003). Cultivars with moderate levels of resistance such as Florida MDR 98 and C-99R (Gorbert and Shokes 2002a, 2002b) were later released. These have medium-to-late maturity. ICRISAT has also released several *A. hypogaea* accessions with some resistance to leafspots (Upadhyaya et al. 2001; Singh et al. 2003; Mathews et al. 2007), several with high yield as well as resistance to leafspots. Substantial progress has also been made in Ghana with collaboration between the Savanna Agricultural Research Institute (SARI) and ICRISAT, which has resulted in the release of three varieties, Edorpo-Munikpa, Nkatiesari, and Kpaniele (Frimpong et al. 2006; Padi et al. 2006). These are bunch-type, medium-to-late maturing (120 days) with resistance to both leaf spots.

Resistance has different components, including percent defoliation, incubation (time from inoculation until the appearance of lesions) period, latency (time from inoculation until sporulation) period, lesion number and diameter, sporulation, and pod yield (Green and Wynne, 1986; Chiteka et al. 1988a, 1988b; Anderson et al. 1993; Waliyar et al. 1993, 1995). Some have argued that as yield is the primary objective in any resistance program, selection based on defoliation would probably be the most appropriate due to its high heritability and the fact that reductions in yield are attributed in large part to premature defoliation in diseased fields (Anderson et al. 1991). Reports by Aquino et al. (1995) suggested that latency period and maximum percentage of lesions that sporulated were the components of resistance most highly correlated with late leaf spot disease development and suggested that using either component may facilitate more rapid selection of lines with improved levels of rate-reducing resistance in germplasm evaluations.

Wynne and Halward (1989) and Simpson (1991) suggested multigenic type resistance for the two leaf spot diseases with a strong possibility of each under the control of two or more genes. Available evidence indicates resistance to both early and late leaf spot to be quantitatively inherited (Sharief et al. 1978; Kornegay et al. 1980; Walls et al. 1985), with a large additive effect possibly with the involvement of cytoplasmic factors (Coffelt and Porter 1986). Resistance to late leaf spot is reported to be governed by five loci (Nevill 1982).

Some of the known components of resistance are subject to additive gene action (Sharief et al. 1978; Kornegay et al. 1980). Anderson et al. (1986) found significant differences among F_1 hybrids between relatively resistant and susceptible parents and argued that resistance to leaf spot may be controlled not only by recessive genes, but also by epistatic and additive alleles. In early leaf spot resistance studies, stability of resistance components has been found to vary across growing regions due to environmental interactions (Waliyar et al. 1993; Chiteka et al. 1997) as well as to differences in pathogen populations (Waliyar et al. 1993) or to both (Chiteka et al. 1997).

Heritability values for both diseases are reported to range from low to high depending on the resistance level of the parents used in the study, making selection in early generations ineffective in crosses resulting from parents with low heritabilities (Jogloy et al. 1987). Anderson et al. (1991) reported that values for broad-sense heritability for lesion number, sporulation, and defoliation rating for early leaf spot were 0.57, 0.16, and 0.56 while those for late leaf spot were 0.74, 0.54, and 0.88, respectively. For narrow-sense heritability for early leaf spot, the values were 0.18 and 0.53 for lesion number and sporulation. The corresponding values for late leaf spot were 0.74 and 0.26. Values for a second cross were generally lower in greenhouse studies, suggesting that dominance and epistatic genetic variance are substantial. In field studies, Iroume and Knauft (1987) obtained values of 0.16 to 0.26 for necrotic area and defoliation from segregating materials and attributed the variation between different crosses to relative differences in susceptibility levels of the parents used for each cross. Broad-sense (H_{bs}) and narrow-sense (h^2) heritability estimates for pod yield in peanut are reported to range from 28% to 82% and from 16% to 79%, respectively (Wynne and Gregory 1981; Wynne and Coffelt 1982). Combining ability estimates from a diallel cross indicated that GPBD-4 and ICG (FDRS) 79 were among the best parents for this trait. GPBD-4 was derived from a cross KRG 1 × ICGV 86855; the latter is an interspecific line (CS 16) developed previously from a cross between *A. hypogaea* and *A. cardenasii* (see Gowda et al. 2002; Stalker et al. 2002a).

High levels of resistance have also been associated with low yield, suggesting linkage or pleitropic effects (Iroume and Knauft, 1987), which means that breeding for high-yielding cultivars with resistance requires this linkage to be broken. Iroume and Knauft (1987) suggested this can be done in early generations under high disease pressure using an index that combines yield and disease severity traits. This was corroborated by Anderson et al. (1986) who reported effectiveness of selection in F_2 plants. Recently selection for leaf spot resistance, yield, and cultivated phenotype over four generations of progeny from a panel of BC_1s derived from *A. hypogaea* cv. IAC-Runner-886 x (*A. ipaënsis* x *A. duranensis*)^{4x} yielded 12 agronomically adapted lines with improved disease resistance compared to the recurrent parent (Galhardo et al. 2011). In this latter breeding scheme the apparently normal genetics of the progeny from this cultivated x wild cross was very helpful in obtaining the desired lines. In general, tapping useful alleles from wild species is hampered by linkage drag between desired and unadapted wild alleles requiring several cycles of backcrossing to recover most of the desirable agronomic traits. This is not only difficult but can be costly and time-consuming. Use of molecular markers can facilitate the identification, localization, and genetic dissection of loci that control quantitatively inherited traits such as yield (Tanksley and Nelson 1996) to speed up utilization of wild alleles.

Markers

Markers for additional traits have been developed. Stalker and Mozingo (2001) identified three RAPD markers associated with early leafspot lesion diameter in a peanut population derived from a cross between an *A. hypogaea*

x *A. cardenasii* introgression line and a cultivated variety. Two breeding lines developed from this material have been placed into advanced line trials.

Mapping of RFLP markers on BC_3F_1 lines in greenhouse studies identified five markers for leafspot resistance (Burow et al. 2008), including three QTLs for incubation period and one each for latency period, lesion number, and diameter. Those QTLs for latency period and lesion number were overlapping, suggesting linkage between the two or a QTL with pleiotropic effects. In addition, field evaluation of BC_3F_2 lines identified 29 markers for the domestication-related traits of main stem length, number of lateral branches, and pod and seed size (Burow et al. 2011).

Leal-Bertioli et al. (2009) reported the mapping of 34 RGAs and 5 QTLs for late leaf spot disease resistance on detached leaves of the F_2 plants of the A-genome mapping population derived from *A. duranensis* x *A. stenosperma*, and suggested additive or partial dominance gene action. One QTL explained almost half of the phenotypic variance observed. Two QTLs mapped near RGA markers. In a detailed QTL study based on cultivated genotypes, Khedikar et al. (2010) reported 11 QTLs for LLS; each QTL explained 2-7% of phenotypic variation in three environments, suggesting that the genes controlling LLS resistance in this cross are relatively minor. In maps from two populations, again using GPBD-4 as one parent, using a larger number (188 and 181) of markers and six trials, a major QTL for LLS was reported, which explained from 10% to 62% of phenotypic variance, depending on the environment; this appeared to give a bimodal (resistant/susceptible) distribution (Sujay et al. 2011). In all, 28 QTLs for LLS were identified.

These findings add to several others that leaf spot resistance in peanut is under the control of many genes and thus explains the difficulty in breeding for resistance. However, identification of a major QTL may allow for more rapid progress in transferring a significant degree of resistance from donor populations. Foncéka et al. (2009) concluded that the BC_1F_1 and BC_2F_1 interspecific hybrids resulting from their work should facilitate the development of advanced backcross and chromosome segment substitution breeding populations for the improvement of cultivated peanut, having used the putative progenitors of cultivated peanut from both the A and B genomes for the development of their interspecific amphidiploid. Combination of QTLs for agronomic and quality traits with those for leafspot analysis is expected to significantly accelerate breeding for resistance.

Rust Resistance: A Complex Trait that Could Be Simpler to Breed than Thought

Etiology

Rust (caused by *Puccinia arachidis* Speg.) is another important fungal disease that occurs widely in Africa and Asia and sporadically in North America and South America. It appears as a large number of small pustules on the underside of leaves, and in severe cases can cause significant defoliation and loss of yield. Overall, rust is generally less significant than leaf spots, even though occasionally outbreaks are severe and can cause severe losses. Rust frequently occurs in combination with leaf spots. Yield loss due to rust, in combination with early and late leaf spot diseases, can be particularly severe; in India, it is reported to be as high as 70% (Subrahmanyam et al. 1980, 1985).

Breeding

Resistance to rust, as also is the case for resistance to leaf spots, has been considered to be a quantitative trait. Resistance is measured as several components: leaf area damage percentage, infection frequency, incubation period, lesion diameter, and sporulation index. All measures were found to be positively correlated with one another, with the exception of incubation period, which was negatively correlated with the other measures (Mehan et al. 1994).

Resistance is present in *A. hypogaea*, with most of the resistant accessions being of subspecies *fastigiata* (Subrahmanyam et al. 1989). Inheritance studies indicated the presence of two or three recessive genes in some crosses, with evidence for epistatic interactions, and with resistance being accompanied by slowing down of disease development (Subrahmanyam et al. 1983b). Many wild peanut species were found to have strong resistance or immunity to rust, with evidence for dominance and additive epistatic interactions for resistance (Singh et al. 1984). In section *Arachis*, 11 diploid species accessions were immune and 3 were highly resistant; in sections *Erectoides*, *Extranervosae*, *Rhizomatosae*, and *Triseminate*, 37 of 38 accessions tested were immune (Subrahmanyam et al. 1983a). The tetraploid *A. monticola* was scored as susceptible. Further screening of 74 section *Arachis* accessions indicated that all had very low leaf damage, except for *A. monticola*, *A. ipaënsis*, and some accessions of *A. stenosperma* (Pande and Rao 2001). Breeding has resulted in release of some germplasm lines and varieties with improved rust resistance, sometimes also possessing resistance to late leaf spot (Gorbet and Shokes 2002a, 2002b; Singh et al. 2003).

Markers

Markers for rust in general have been discovered in the same populations analyzed for LLS mentioned earlier in this chapter. ICRISAT, in collaboration with University of Agricultural Sciences-Dharwad (UAS-D) in India, had identified and validated markers linked with these two foliar diseases. QTL analysis using a partial genetic map of a mapping population with 67 marker loci derived from the cross TAG 24 × GPBD-4 and multiple season phenotyping data on both the foliar diseases detected a total of 12 QTLs explaining between 1.7% and 55.2% of the phenotypic variation each (Khedikar et al. 2010). The SSR marker tightly linked to the major QTL (IPAHM103; QTLrust01) was then validated among a diverse set of genotypes as well as another mapping population (Sarvamangala et al. 2011) derived from the cross TG 26 × GPBD-4. Furthermore, the partial genetic linkage maps (TAG 24 × GPBD-4 with 67 marker loci and TG 26 × GPBD-4 with 53 marker loci) were both saturated to over 180 loci (Sujay et al. 2011). The populations were subjected to further phenotyping for seven to eight seasons. Final analysis detected a total of 15 QTLs for rust and 28 QTLs for LLS resistance (Sujay et al. 2011). These QTLs included a major QTL for LLS (QTLLLS01; linked markers GM1573 and pPGPseq8D09), which was detected across all the environments and explained between 10.27% and 62.34% of the phenotypic variation. In addition, three new SSR markers (GM1536, GM2301, and GM2079) significantly associated with the major rust QTL (QTLrust01) were identified (Sujay et al. 2011).

In parallel, the validated SSR marker (IPAHM 103) was deployed in initiating introgression of rust QTL into three elite groundnut varieties (ICGV 91114, JL 24, and TAG 24) using the donor GPBD-4 through marker-assisted backcrossing. Later, the newly identified linked markers (GM2079, GM2301, and GM1536) in the same QTL region have been used together with IPAHM103 for foreground selection to identify heterozygous plants at backcrossed F_1 generations (BC_1F_1, BC_2F_1 and BC_3F_1) and homozygous plants at backcrossed F_2 (BC_2F_2 and BC_3F_2) generations by S. Nigam and P. Janila of ICRISAT. As a result, 76 homozygous BC_3F_2 and 158 BC_2F_3 lines have been generated and screened for disease resistance during the rainy season of 2011 (Pandey et al. 2012). This initial screening has been encouraging and has lead to the identification of several promising lines showing remarkable reduction in disease symptoms.

Resistance to Other Diseases and Pests

Aphids

The aphid-transmitted groundnut rosette virus is an important pathogen of peanut in Africa and Asia. Groundnut rosette virus causes severe

stunting of the peanut plant and loss of yield. Until recently, there were no resistant cultivars, but resistant germplasm was identified (de Berchoux 1958, 1960; Subrahmanyam et al. 1998) and is being used for varietal development.

In an effort to identify markers for GRV resistance, Herselman et al. (2004) tested 308 AFLP primer combinations and were able to devise 5 linkage groups consisting of 12 markers; 1 marker was linked to aphid resistance.

Aflatoxin Resistance

Aflatoxin is a family of potent hepatotoxins and carcinogens that are also responsible for suppression of immune system function (Williams et al. 2004). Aflatoxin contamination occurs on several crops, including maize and peanut. The causative organism is *Aspergillus flavus*, which colonizes seeds and under certain conditions in the field and post-harvest storage may produce toxins.

Several attempts have been made to develop peanut varieties with low potential for developing aflatoxin, but this goal has been difficult to attain due to high variability in measurements, requiring up to 10 replications in the field. Eleven peanut accessions with at least a 70% reduction in aflatoxin have been identified (Holbrook et al. 2009), and advanced breeding lines have been developed. Additional materials have been identified (Nigam et al. 2009); however, high genotype x environment effects have been noted. Development of markers for this trait would be very useful in breeding.

Only one report exists to date on markers for resistance to aflatoxin contamination. Milla et al. (2005a) reported AFLP-based markers for *A. cardenasii*-derived resistance to aflatoxin contamination. Of 38 markers screened in the *A. hypogaea* x *A. cardenasii* population, 6 were found associated with aflatoxin concentration in the F_2 population at a low statistical threshold. Several proteins have been associated with infection of peanut with *Aspergillus* (Basha and Pancholy 1986). Luo et al. (2005) developed an EST-derived microarray of approximately 400 unigenes that were probed under different conditions. Twenty-five ESTs potentially associated with drought stress and response to *A. parasiticus* were identified. Subsequently, a microarray of 14,000 unigenes was developed from public peanut EST sequences (Kottapalli et al. 2009). Guo et al. (2011), using an oligoarray, profiled *Aspergillus flauvus* infection-responding genes in two contrast peanut genotypes. Additional work is still needed to find useful markers for aflatoxin resistance.

Tomato Spotted Wilt Virus (TSWV)

Tomato spotted wilt virus causes serious losses in the United States in fields where the virus is prevalent. The virus is transmitted by tobacco thrips (*Frankinellia* sp.) A segregating population of F_2 plants of an A-genome diploid cross *A. kuhlmannii* x *A. diogoi* was screened for resistance to TSWV, and five linked AFLP markers on one chromosome were associated with resistance at a high statistical threshold (Milla 2003; Milla et al. 2004). In Brazil, interspecific populations and wild species have also been found as promising for introgression of resistance to the thrips *Enneothrips flavens* (Janini et al. 2010).

Recently, one QTL each in Tifrunner x GT-C20 (T population) and SunOleic 97R x NC94022 (S population) crosses, explaining 12.9% and 35.8% phenotypic variance, respectively, was reported (Qin et al. 2012). The linked markers (IPAHM287 and Seq12F7) provide hope for marker-assisted improvement of this disease, but validation of markers as well as QTLs are required as these were identified based on single environment data.

Sclerotinia Minor

Sclerotonia blight (*Sclerotinia minor* Jagger) is a major problem in U.S. areas with cool autumns. The fungal form, sclerotia, can survive in the field for many years. Yield losses are typically about 10% but have been reported to be as high as 50% (Melouk and Backman 1995). Several resistant cultivars have been developed, but this

requires field screening; growth chamber screening can be done (Melouk et al. 1992) but does not work with all market types (Wilson 2008). By association analysis of 39 genotypes with 16 SSR markers, 1 SSR marker was found to be associated with resistance (Chenault et al. 2009). This marker was found to work in runner, spanish, and valencia market types but not in the virginia market type (Chamberlin et al. 2010). Using a transgenic approach, Livingstone et al. (2005) obtained peanut plants with increased resistance to *S. minor* by expressing a barley oxalate oxidase gene.

Conclusion

MAS in peanut has lagged behind other major crops. This is due in good part to the genetic bottleneck that occurred at tetraploidization, resulting in a limited amount of molecular variability detectable among accessions of the cultivated species. However, marker maps have been developed from wild species, and, to an increasing extent, the cultivated species using new marker types. It is expected that, with the increase in number of SSR markers and development of SNP-based markers, there will be greater use of MAS in both interspecific and cultivated accession crosses.

MAS has already proven itself to be useful in developing cultivars possessing resistance to the root-knot nematode, and is being used for selection for resistance to late leaf spot and rust, as well as for the high-oleic-acid trait. It is to be expected that, as the power of molecular tools increases and the cost decreases, MAS will be used to an increasing degree in this crop.

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Chapter 9

Organization of Genes Conferring Resistance to Anthracnose in Common Bean

Juan José Ferreira, Ana Campa, and James D. Kelly

Abstract

Anthracnose, caused by the fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.- Scrib, is one of the most economically important diseases of common bean (*Phaseolus vulgaris* L.). The plant-pathogen interaction is very specific and essentially follows the gene-for-gene model. Many pathogenic variants or races have been described in this pathogen using the same set of twelve differential cultivars, standardized resistance tests, and a standardized nomenclature system to name the races. Since the first anthracnose resistance gene was described early in the twentieth century, twenty resistance genes (named as Co-) that condition resistance to specific isolates or races of anthracnose pathogenic on common bean have been described. Although not all characterized resistance genes have been mapped, those genes that have been mapped reside on seven of eleven bean linkage groups: Pv01, Pv02, Pv03, Pv04, Pv07, Pv08, and Pv11. Many of these regions are gene clusters that include genes conditioning resistant reactions to other pathogens such as angular leaf spot, bean rust, bean common mosaic virus, and halo blight. Genetic and molecular evidence indicates that anthracnose resistance loci are organized in gene clusters in which individual genes confer resistance to one specific isolate or race. In this chapter the inheritance patterns of this pathogen are reviewed in detail, and the limitations of classical genetic analysis based on allelism tests are discussed. Direct or indirect mapping using molecular markers linked to specific genes or genomic regions is recommended in the characterization of new resistances genes. In addition, a new system to name the anthracnose resistances gene(s) considering their position on the genetic map and the specific fungus genotype-bean genotype interaction is suggested. The *C. lindemuthianum-P. vulgaris* interaction can be used as a model to investigate and explain other race-specific resistance interactions in plants.

Introduction

Colletotrichum lindemuthianum (Sacc. & Magnus) Lams.- Scrib. is a hemibiotrophic fungal pathogen that attacks common bean (*Phaseolus vulgaris* L.) causing the disease known as anthracnose (Bailey et al. 1992). The plant-pathogen interaction is very specific and it essentially follows the gene-for-gene model (Flor 1955). A large number of physiological races

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of the pathogen have been identified worldwide based on reaction on a standard set of twelve host differential cultivars (Pastor-Corrales 1991). Races are assigned a binary code according to their pathogenicity on members of the differential series (Melotto et al. 2000). The use of the same twelve differentials by scientists has resulted in a uniform and useful characterization of pathogenic variability of *C. lindemuthianum* in different countries and continents. Knowledge of pathogenic variability has allowed for a planned approach to breeding for resistance (Kelly et al. 1994). The genetics of anthracnose resistance has been studied in many bean genotypes since Barrus (1911) described a differential response in the host-pathogen interaction. Several resistance genes that condition resistance against specific isolates or races have been described in common bean. These include the *Co-1*, *Co-2*, *Co-3*, *Co-4*, *Co-5*, *Co-6*, *Co-7*, *Co-8*, *Co-9*, *Co-10*, *Co-11*, *Co-12*, *Co-13*, *Co-14*, *Co-u*, *Co-v*, *Co-w*, *Co-x*, *Co-y*, and *Co-z* genes. Multiple resistance alleles were also reported at the *Co-1*, *Co-3*, *Co-4*, and *Co-5* loci. The majority of these loci were described based on the interpretation of allelism tests challenged against different anthracnose races. However, recent molecular and genetic evidence suggests that the classical interpretation of the *C. lindemuthianum – P. vulgaris* interaction should be reconsidered because:

1. Resistance to different isolates or races of anthracnose in bean genotype(s) can be controlled by different genes although monogenic segregation ratios are commonly observed in response to different races.
2. Anthracnose resistance genes are located in specific regions of genome and are organized in groups or clusters of loci in which individual gene(s) confers resistance to one specific isolates or race.
3. Molecular analysis revealed an organization of gene clusters that encode for proteins containing leucine-rich repeat motifs involved in the response to pathogens.

This chapter reviews limitations of the classical genetic analysis of resistance based on allelism tests and the current state of knowledge about resistance to anthracnose in common bean.

The Pathogen, *Colletotrichum lindemuthianum*

Colletotrichum lindemuthianum (Sacc. & Magnus) Lams.- Scrib., the causal agent of bean anthracnose, is a serious seed-borne pathogen of common bean (*P. vulgaris*) that has also been reported to attack other species of *Phaseolus* (Zaumeyer and Thomas 1957; Sicard et al. 1997). Bean anthracnose has worldwide distribution, but it occurs in temperate regions and at higher elevations in the tropics where beans are grown in cooler, more humid environments (Pastor-Corrales and Tu 1989).The pathogen is seed borne and is particularly problematic for small seed producers who save their own seed. Clean-seed programs are the most effective means to control the disease, but these require organized seed production in areas where the disease is not endemic. Local spread of the pathogen can occur by wind-driven rain and by movement of animals and machinery through infected fields. Long-distance spread in infected seed has been reported to occur both within and between countries (Tu 1994). Disease control measures that include seed and foliar treatment with fungicides, as well as crop rotation, are not very effective. Genetic resistance can offer a long-term solution, but the appearance of new physiological races of *C. lindemuthianum* continuously poses a challenge to breeders and producers making resistance appear elusive and short-lived.

The symptoms of anthracnose are most distinctive on bean pods appearing as round black shrunken lesions containing flesh-colored spores (Figure 9.1). Lesions will expand and coalesce to cover the entire pod under extreme conditions (Schwartz and Pastor-Corrales 2005). Seed harvested from infected pods will exhibit similar lesions particularly obvious on light-colored seeds. Foliar lesions are dark brown/black

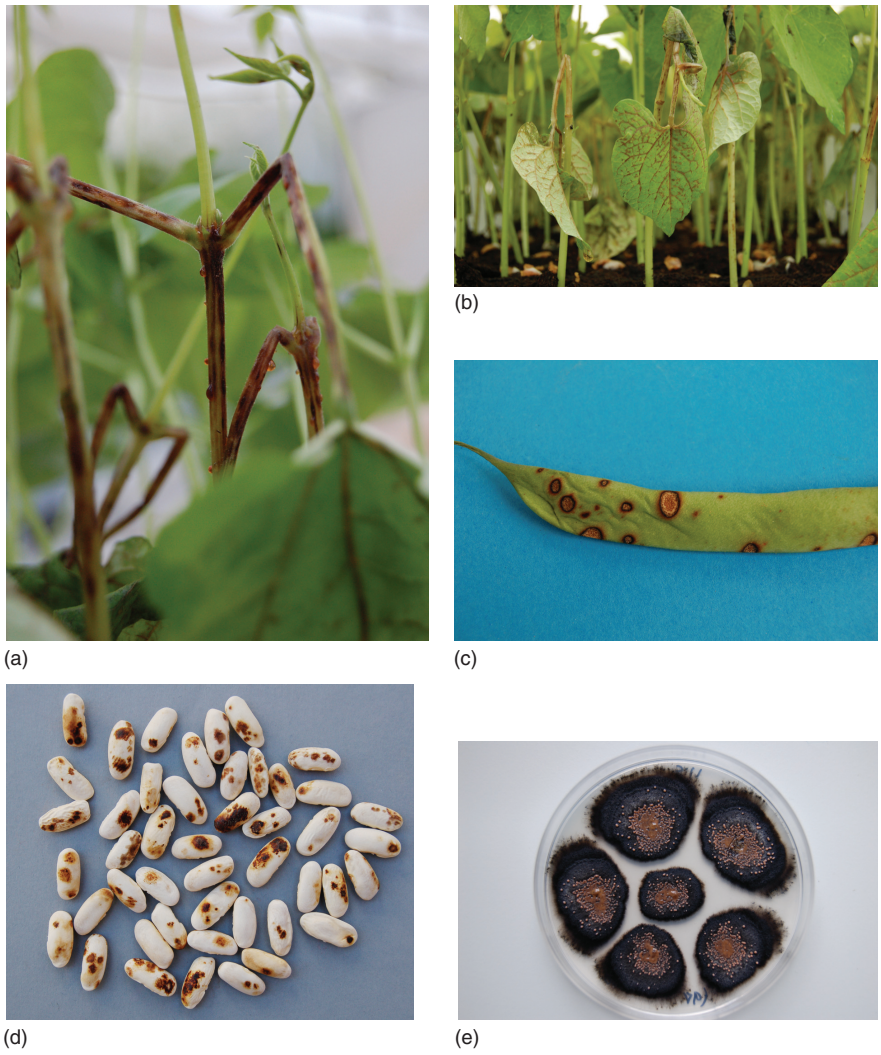

Fig. 9.1. Anthracnose lesions on common bean leaf, seedling, pod, seed, and spores growing on infected bean leaf on agar. For a color version of this figure, please refer to the color plate.

shrunken lesion on leaf veins, stems, and hypocotyls of seedling plants. Lesions expand to cause leaf flagging and wilting that exhibit typical chlorotic tissue not dissimilar to that observed for other foliar pathogens. Infected plants produce infected seed and seed is the major means of dispersal of the pathogen locally and across international boundaries and is the source of infection next season. Plant debris infected with anthracnose is not recognized as a major source of infection assuming farmers rotate crops, but infected debris has survived 22 months in northern temperate zones. Planting resistant cultivars is the most effective control method.

Methods for Test Response to Pathogen

Resistance Tests

Plant diseases are a result of the interaction between a susceptible host and a virulent pathogen in favorable environmental conditions.

Variables such as temperature, relative humidity, time, and physiological state of the plant or pathogen can modify the outcome of the *P. vulgaris–C. lindemuthianum* interaction (Tu and Aylesworth 1980). Use of standardized methods to test response to pathogen is relevant to compare results among different studies. Different methods were used to investigate the resistance of bean genotypes in controlled conditions. Drijfhout and Davis (1989) dip germinated bean seeds in suspensions of conidia prior to planting in moist river sand. Champion et al. (1973) and Menezes and Dianese (1988) immersed seedlings in a conidia suspension and wrapped the stems of inoculated seedling in wet paper. Tu and Aylesworth (1980) applied a suspension of conidia with a paintbrush on primary leaves and maintained inoculated seedlings in high relative humidity using a transparent plastic bag. Currently, the most widespread inoculation method is based on spraying a suspension of 10^6 conidia ml^{-1} onto seedling plants. Plants are maintained at moderate temperature (20–24 °C) under high relative humidity (>80%). Symptoms are observed 8–10 days after inoculation and they are reliable and precise and resemble reaction in the field. Reaction of the same check genotypes to different inoculation methods should be considered before comparing results of different studies using different types of resistance tests.

Reaction Score

In order to describe the response of bean genotypes to *C. lindemuthianum*, three main types of qualitative scales have been reported in the literature. Several studies classified the reaction of bean genotypes as resistant or susceptible (Barrus 1911; Cardenas et al. 1964; Krüger et al. 1977). Other classical studies used scales with five levels of response (Yerkes and Ortiz 1956; Bannerot 1965; Leakey and Simbwa-Bunnya 1972; Muhalet et al. 1981; Drijfhout and Davis 1989; Menezes and Dianese 1988) where the resistant reaction is considered as plants with no disease symptoms or a few small lesions (0 to 1 or 1 to 2). Currently, a 1-9 scale based on the type of lesion and proportion of seedling with disease symptoms is commonly used to describe disease reaction on seedlings (Balardin et al. 1997). Plants with no visible symptoms or with few very small lesions mostly on primary leaf veins are recorded as resistant (values from 1 to 3). Plants with numerous small or enlarged lesions, with sunken cankers on leaves and seedling stem, or death are recorded as susceptible (values from 7 to 9). Plants with small lesions on leaves and seedling stem are recorded as intermediate reaction and classified as susceptible or moderate resistance. Use of scales including wide range of reactions helps identify genes conferring moderate resistance that appears to behave as a quantitative trait. The most common method is to classify reaction types into two broad categories, which implies qualitative resistance that further assists in race characterization.

Pathogenic Variability

Pathogenic variation can be described as the differential response of a plant genotype to different isolates of a pathogen. Barrus (1911) reported the first evidence of pathogenic variation in the interaction of P. vulgaris- C. lindemuthianum. Using bean cvs. Detroit, Imperial, Blue Pod Butter, Navy Pea, and China Red Eye, Barrus (1911) found variation in the response of two local isolates of *C. lindemuthianum*. Later, the same author (Barrus 1918) tested 70 bean genotypes against ten isolates of *C. lindemuthianum* and classified the ten isolates into two pathogenic groups: strains alpha and beta based on reaction profiles. In 1923, Burkholder described a third strain, gamma. Since then, many studies have reported pathogenic variants or races of *C. lindemuthianum* and at least 14 races have been described prior to 1988; alpha, beta, gamma, delta, Mex I, II, and III, epsilon, alpha mutant later designated as lambda, Brazil I and II, ebnet later designate as kappa, alpha Brazil, and C236 (reviewed by Drijfhout and Davis 1989). Unfortunately, many of these studies used different

sets of bean genotypes to describe pathogenic variants and different designation codes such as Greek letters or specific codes. Cultivars Michelite, Dark Red Kidney, and Perry Marrow were typically used to differentiate races alpha, beta, gamma, Mex I, II, and III (Yerkes and Teliz 1956); cvs. Imuna, Kaboon, Coco a la créme, Widusa and Cornell 49242 were used in differentiation of the isolates PV_6, D_{10}, E_8b, I4, alpha1, and alpha 5 (Bannerot 1965); cvs. Dark Red Kidney, Widusa, Kaboon, Michelite, Sanilac, Prelude, and Cornell 49242 were used in differentiation of the races alpha, alpha-Brazil, beta, gamma, delta, kappa, lambda, and epsilon (Tu et al. 1984; Tu 1994). However, virulence of isolates classified as one of these races, or pathogenic groups in other bean genotypes can reveal additional variation (Leakey and Simbwa-Bunnya 1972). For example, races alpha and alpha-Brazil differ in the reaction on cultivar Cornell 49242 (Tu et al. 1984; Tu 1994).

The standardization of a differential set of common bean cultivars for the purpose of characterizing the pathogenic variability of *C. lindemuthianum* and comparison of research results was first proposed by Drijfhout and Davis (1989). However, the proposal did not include a standardized method to name new pathogenic variants. Pastor-Corrales (1991) suggested a standardized method to characterize and designate new races of *C. lindemuthianum*. Isolates are characterized based on the response to twelve differential cultivars (Table 9.1), many of them included in a previous set of differential cultivars. Races are designated numerically according to standardized nomenclature system. A binary value is assigned to each differential cultivar and an isolate is classified as the sum of values of all susceptible differential cultivars. For example, the majority of isolates collected in northern Spain (Ferreira et al. 2008) attacked the following cultivars: Michigan Dark Red Kidney (MDRK, binary value 2), Perry Marrow (binary

Table 9.1. Set of twelve differential common bean cultivars, gene pool origin, and binary nomenclature system used to characterize and name pathogenic variability of *Colletotrichum lindemuthianum*. Reaction of twelve differential bean cultivars against nine different isolates of *C. lindemuthuanum* is shown.

			Isolate								
Differential cultivars	Host Gene Pool	Binary value	CL 124	CL 63	CL 18	CL. RW 2.0	CL. USA 2.1	CL. USA 8.1	CL. CA 1.0	CL. CR 2.0	CL. Hond 1.1
Michelite	MA	**1**	S	R	R	S	S	S	S	S	S
MDRK	AND	**2**	S	S	S	S	R	R	R	R	R
Perry Marrow	AND	**4**	R	S	S	S	R	R	R	R	R
Cornell 49242	MA	**8**	R	R	R	R	R	S	S	S	S
Widusa	AND	**16**	R	R	R	S	R	R	R	S	R
Kaboon	AND	**32**	R	R	S	S	R	R	S	R	R
Mexico 222	MA	**64**	R	R	R	R	S	S	S	R	S
PI 207262	MA	**128**	R	R	R	R	R	R	R	S	R
TO	MA	**256**	R	R	R	R	R	R	R	S	R
TU	MA	**512**	R	R	R	R	R	R	R	R	S
AB136	MA	**1024**	R	R	R	R	R	R	R	S	S
G2333	MA	**2048**	R	R	R	R	R	R	R	S	S
Race			**3**	**6**	**38**	**55**	**65**	**73**	**105**	**3481**	**3657**
Race Gene Pool			Mx	AND	AND	AND	MA	MA	Mx	Mx	MA

CL, *Colletotrichum lindemuthianum*; First 3-isolates from Spain (Ferreira et al. 2008); RW, Rwanda; CA, Canada; CR, Costa Rica; Hond, Honduras; AND, Andean; MA, Mesoamerican; R, resistant; S, susceptible, Mx Mixed; References: Pastor-Corrales 1991; Balardin et al 1997; Kelly et al 1994; Kelly and Vallejo 2004.

value 4), and Kaboon (binary value 32). In consequence, local isolates were classified as race 38. Fifteen isolates described in the literature and denominate using Greek letters or specific codes were re-characterized using this set of differential cultivars and named according to this numerical method (Balardin and Kelly 1997; Melotto et al. 2000): race alpha corresponds to race 17 in binary nomenclature system, race beta to race 130, and race gamma to race 102.

At least 100 pathogenic variants or races have been reported among isolates of *C. lindemuthianum* collected worldwide using this set of twelve differential cultivars and the standardized method to name the races (Sicard et al. 1997; Balardin and Kelly 1998; Gonzalez et al. 1998; Sharma et al. 1999; Mahuku and Riascos 2004; Ansari et al. 2004; Pathania et al. 2006; Ferreira et al. 2008). A significant finding resulting from the use of the differential series was that some races such as race 73 are widely dispersed and exist from Argentina to Canada while other races are found only in specific countries or regions (Balardin et al. 1997). Either these races have evolved locally in response to the genetic makeup of local host cultivars or lack the fitness typified by race 73 to survive in a broader range of climatic conditions. The pathogenic variability in C. lindemuthianum was also shown to follow the gene pool origins of the host which offered new approaches in breeding for more durable resistance (Balardin and Kelly 1998).

Limitations in the use of this differential set in characterization of pathogenic variants have also been described. For example, isolates classified as race 65 differ in the reaction to several bean genotypes. Rodríguez-Suárez et al. (2005) observed that race 65 from Brazil showed greater virulence on breeding lines A321 and A493 than race 65 from the collection maintained at the Michigan State University (Balardin et al. 1997). Gonçalves-Vidigal and Kelly (2006) used an isolate of race 65 that overcame resistance in BAT93, and differed from the one used by Alzate-Marin et al. (2007) for which BAT93 showed a resistant response. An intra-race molecular variability between isolates of race 65 was demonstrated using RAPD markers (Thomazella et al. 2004). Despite these anomalies, the differential set has been invaluable in comparing studies of pathogenic variability between labs and countries and has led to better strategies for resistance breeding.

Evolution of Pathogenic Variability

There is no documented evidence on the rate of evolution of pathogenic variability in *C. lindemuthianum* as new races may be imported in the seed or be present and have not been detected previously. New production areas provide a unique opportunity to study the evolution of pathogenic variability, since the pathogen is not previously present in these areas. In southern Manitoba Canada, beans are a relatively new commodity, and production expanded dramatically from 20,000 hectares in 1995 to more than 80,000 hectares in 2000. In the absence of a local breeding program, seed of new bean cultivars was imported from other Canadian provinces, mainly Ontario and a few production states in the United States, to meet the need of farmers. Initial reports on the appearance of anthracnose on susceptible bean cultivars in the Manitoba surfaced in 2002 and 2003 (Conner et al. 2004). This was not surprising as conditions were generally favorable for the development and spread of the disease and certified seed was being imported from Ontario where no tolerance standards for anthracnose levels existed in certified seed (Conner et al. 2006). Seed treatments were used but they are not fully effective in the control of seedborne anthracnose. In 2003, race 73 was reported to be present in Manitoba (del Rio et al. 2003), which was not surprising since the same race had been reported earlier in Michigan, Ontario, and North Dakota (Kelly et al. 1994; Tu 1994; del Rio et al. 2002). One of the cultivars shown to be resistant to race 73 was Envoy (del Rio et al. 2003) and this navy bean cultivar became very popular and was widely grown since 2003 on in Manitoba. Envoy carried the same *Co-1*^{2}

resistance gene as is present in the differential cultivar Kaboon, a widely recognized resistance source that provided resistance to race 73. Within five years the new race 105 (reported in 2008; Dongfang et al. 2008) emerged that attacked Envoy and overcame the *Co-1*^{2} resistance gene. The most likely scenario was that race 73 acquired additional virulence gene(s) to overcome the *Co-1*^{2} resistance gene that evolved into race 105 (Table 1). Race 105 has never been reported in North America and is relatively rare in South and Central America (Balardin et al. 1997). Fortunately, many of the resistance genes that control race 73 are also effective against race 105. The speed with which race 73 evolved into race 105 that defeated the *Co-1*^{2} gene was rapid as all the conditions favorable for the development of a new race of anthracnose existed in Manitoba. These included favorable cool wet weather conditions, planting of infected seed of susceptible cultivars that produced an abundance of inoculum of race 73, and the widespread planting of a highly resistant host variety in adjacent fields in the localized production area. The potential of this pathogen to rapidly evolve underscores the risk of planting resistance cultivars with single resistance genes, and breeders should be encouraged to develop cultivars with multiple resistance genes to ensure more durable resistance to anthracnose in the future bean cultivars.

Genetic Resistance to Anthracnose

3.1 Modes of Action of Genes Conferring Resistance

Genetic analysis of resistance to *C. lindemuthianum* generally follows a qualitative mode of inheritance where resistant and susceptible reactions are clearly differentiated. Alleles at a locus can interact in several ways either through complete dominance, partial dominance, additive effect, or overdominance. The majority of described genes conferring resistance to bean anthracnose show complete dominance where the dominant allele conditions resistance reactions. Genetic evidence for one dominant gene is generally deduced from resistant reaction in F_1 plants or 3R:1S or 15R:1S observed ratios in F_2 segregating populations derived from R x S crosses. Recessive resistances genes were also described in the literature based on susceptible reaction of F_1 plants or 1R:3S or 13R:3S observed ratios in descendents derived from R x S crosses. Genes conferring recessive resistance were described for race beta in the crosses Tuscola x Montcalm and Tuscola x Swedish Brown (Muhalet et al. 1981) or in the reaction of cultivar AB136 to race 73 in the population derived from the cross AB136 x Cornell 49242 (Alzate-Marin et al. 1997). Recessive resistance genes to race beta were also described in the cultivars Michelite or Brazilian Red (Cardenas et al. 1964). In those instances where recessive resistance was reported these observations were later explained as a reversal of dominance between different resistance alleles at the same locus when challenged with different pathogenic races (Melotto and Kelly 2000).

The majority of resistance genes to anthracnose exhibit an epistatic interaction of duplicate dominant loci controlling resistant reactions to a specific pathogenic variant of *C. lindemuthianum*. Complementary mode of action between two genes was described and deduced in F_2 populations from observed ratios such as 9R:7S, 57R:7S or 249R:7S (Cardenas et al. 1964; Muhalet et al. 1981). Genetic mechanism conditioning resistance to beta, gamma, and delta races conferring by duplicate and complementary genes was described in F_2 populations involving cultivar Kaboon (Muhalet et al. 1981; Melotto and Kelly 2000). Two dominant and complementary genes conditioning resistance to race 31 were also found and mapped in cv. Kaboon (Campa et al. 2011). Expression of this complementary mode of action depends on genetic background and is more common in Andean genotypes. If two genotypes only differ in one complementary gene, a more common monogenic dominant segregation pattern would be expected.

Historical Review of the Genes Conditioning Resistances to *C. lindemuthianum*

In addition to the standardization of the pathogenic variability of *C. lindemuthianum* using a uniform differential host series, it became clear that there was a need to standardize the naming of gene symbols in the host that conditioned resistance to individual races of the pathogen. To address this issue, Kelly and Young (1996) proposed the use of the symbol "*Co*" to identify genes conditioning resistance to races of *C. lindemuthianum* in common bean. Five original independent genes—*A*, *Are*, *Mexique 1*, *2*, and *3*—were renamed *Co-1* to *Co-5*, respectively and a total of 20 Co-genes have since been identified and named (Table 9.2). In addition, different alleles conferring resistance to different resistance profiles were described in for the loci *Co-1*, *Co-3*, *Co-4*, and *Co-5* (see Table 9.2). A complete description of resistance genes *Co-1* to *Co-10*, their mode of action, race specificity, map location, and value to breeders was reviewed by Kelly and Vallejo (2004). Since then, seven additional resistance genes have been reported (Table 9.2). The *Co-11* gene, described in differential cultivar Michelite using the races 8 and 64 (Gonçalves-Vidigal et al. 2007), is not effective against many races but does not exhibit resistance to some highly virulent Andean races such as race 38 from Spain. The gene *Co-u* conferring resistance to isolates E4 and E42b was identified in the cultivars BAT93 (Geffroy et al. 2008). The *Co-12* gene was described in cultivar Jalo Vermelho using the races 23, 55, 89, and 453 (Gonçalves-Vidigal et al. 2008) and the *Co-13* gene was described in cultivar Jalo Listras Pretas using the races 9, 64, 65, and 73 (Gonçalves-Vidigal et al. 2009). Finally, the presence of the *Co-14* gene has been postulated in the bean cultivar Pitanga using race 2047 (Gonçalves-Vidigal et al. 2012).

Within *P. vulgaris* host, two major gene pools, Andean and Middle American, exist (Gepts 1988), and resistance genes are classified according to gene pool. Genes *Co-u*, *Co-12*, *Co-13*, and *Co-14* represent new sources of resistance in the Andean gene pool that lacked high levels of resistance in contrast to the genes of Mesoamerican origin. However, in limited testing to date, the new gene *Co-12* in Jalo Vermelho only conditions resistance to races 9, 23, 31, 55, 65, 81, 89, 95, and 453, the *Co-13* gene in Jalo Listras Pretas conditions resistance to Mesoamerican races 9, 64, 65, and 73, and the *Co-14* gene in Pitanga conditions resistance to Mesoamerican races 23, 64, 65, 73, and 2047. Only the *Co-12* gene conditions resistance to the virulent Andean race 55, whereas the *Co-14* conditions to the highly virulent race 2047 that defeats many of the resistance genes in the differential series. Interestingly the resistance pattern of the *Co-14* gene in Pitanga is very similar to that of reported for the $Co\text{-}1^4$ gene in the Andean genotype AND 277 (Gonçalves-Vidigal et al. 2011). The apparent similarity of both genes could be tested using markers tightly linked to the $Co\text{-}1^4$ gene. The potential of these new genes in providing resistance to highly virulent races 3481 and 3657 from Central America should be evaluated as these races are strongly Mesoamerican in origin as they attack many of the Middle American differential cultivars, so new Andean genes could offer potential for resistance.

Classical Genetic Analysis of Resistance to *C. lindemuthianum*

Classical genetic analyses of resistance to *C. lindemuthianum* are based on the interpretation of results obtained from F_2 segregating populations derived from two types of crosses: R x S or R x R (complementation or allelism tests). Results observed in R x S crosses are used to infer the number and mode of action of genes conferring resistance to *C. lindemuthianum*. Burkholder (1918) analyzed the resistance in a F_2 population derived from the cross between the cvs. White Marrow (resistant to race alpha) and Wells' Red Kidney (susceptible to race alpha) and probably described the first analysis of resistance to

Table 9.2. Genes conditioning resistance to specific races or isolates of *C. lindemuthuanum* described in common bean. Bean genotype where the resistance genes was described and the isolates or races used in the genetic analysis are also indicated.

Gene Symbols		Bean	Gene	Isolates or races used	First Reference
New	Original	Genotype	pool	in genetic analysis	
	A	Wells Red Kidney		A^{11}, *F*	Burkholder 1918
Co-1	*A*	MDRK	AND	*alpha, beta, gamma*	Cardenas et al. 1964
Co-1^2		Kaboon	AND	*7, 73*	Melotto and Kelly 2000
Co-1^3		Perry Marrow	AND	*7, 73*	Melotto and Kelly 2000
Co-1^4		AND 277		*65, 81, 453*	Alzate-Marin et al. 2003
Co-1^5		Widusa		*7, 65, 73, 453*	Gonçalves-Vidigal et al. 2006
Co-2	*Are*	Cornell 49242	MA	*alpha, beta, gamma*	Mastenbroek 1960
Co-3	*Mexique 1*	Mexico 222	MA	*14, 51*	Bannerot et al. 1971
Co-3^2		Mexico 227	MA	*gamma, delta 2*	Fouilloux 1976
Co-3^3		A493		*38*	Méndez de Vigo et al. 2005
Co-4	*Mexique 2*	TO	MA	*gamma, delta 2*	Fouilloux 1976
Co-4^2		SEL1308		*64, 73, 521*	Young et al. 1998
Co-4^3		PI 207262	MA	*65, 73, 79*	Alzate-Marin et al. 2002
Co-5	*Mexique 3*	TU	MA	*gamma, delta 2*	Fouilloux 1976
Co-5^2		SEL1360		*7, 23, 64, 73, 3481*	Young and Kelly 1996b Vallejo and Kelly 2009
Co-6	Q	Catrachita	MA	23, 64, 73	Gonçalves-Vidigal et al. 1997
Co-7	NA	G2333	MA	521, 1545	Young et al. 1998
co-8	NA	AB 136	MA	64, 89, 73	Alzate Marin et al. 1997
Co-9	NA	BAT 93	MA	38, C531, E29b E33c	Geffroy et al. 1999
Co-10	NA	Ouro Negro	MA	23, 64, 73, 81, 89	Alzate-Marin et al. 2003
Co-11	NA	Michelite	MA	8, 64	Gonçalves-Vidigal et al. 2007
Co-12	NA	Jalo Vermelho	AND	23, 55, 89, 453	Gonçalves-Vidigal et al. 2008
Co-13	NA	Jalo Listras Pretas	AND	9, 64, 65, 73	Gonçalves-Vidigal et al. 2009
Co-14	NA	Pitanga	AND	2047	Gonçalves-Vidigal et al. 2012
Co-u	NA	BAT93	MA	E4, E42b	Geffroy et al. 2008
Co-v	NA	BAT93	MA		Geffroy 1997
Co-w	NA	JaloEEP558	AND	E25	Geffroy 1997; Geffroy et al. 2008
Co-x	NA	JaloEEP558	AND	100, 40, 3616, 82	Geffroy 1997; Geffroy et al. 2008
Co-y	NA	JaloEEP558	MA	38, C531, E29b, E33c	Geffroy et al. 1999
Co-z	NA	JaloEEP558	MA	38, C531, E29b, E33c	Geffroy et al. 1999

C. lindemuthianum. He found 362 resistant F_2 plants and 111 susceptible plants suggesting that a single dominant gene was involved in resistance (named as A factor).

Results observed in R x R crosses are used to identify the specific genes involving in the reaction to pathogen. Genotype (A) with unknown resistance gene(s) is crossed with genotypes with known resistance genes (B, C, D, . . .) and the respective progenies evaluated against an avirulent race. The resistance genes possessed by genotype A are deduced from observed

segregation responses to *C. lindemuthianum*. No segregation is interpreted as both genotypes sharing the same resistance gene or locus. This interpretation was used in classical studies reported by Cardenas et al. (1964), Fouilloux (1976), and Muhalet et al. (1981). For example, Fouilloux (1976) investigated the resistance to two races (gamma and delta 2) in the cvs. TO, TU, TV, TX, TY, and TW. He analyzed the responses in F_2 populations derived from crosses between the four cvs. (TO, TU, TX, TY) and the susceptible cv. Tenderette and from 3R:1S observed ratios deduced that the four genotypes carry a resistance gene. He also studied the responses in F_2 populations derived from crosses among the several resistant cultivars Silvert (carrying the *Are* gene) and MY132 (carrying the *Mexique 1* gene). From 15R:1S observed segregation ratios, he concluded that cvs. TO, TU, TX, and TV carry independent gene(s) to the *Are* and *Mexique 1* genes. Finally, from observed segregation in the F_2 population derived from the cross TO x TU, he concluded that the cvs. TO and TU carried different resistance genes, *Mexique 2* and *Mexique 3*, respectively. Both genes conferred resistance to the races gamma and delta 2. Recent evidence indicates that cultivar TU has at least three independent loci-conferred resistance to anthracnose (Campa et al. 2009). Now the question is which loci confer resistance against races used by Fouilloux in cultivar TU. This question is relevant because classical allelism tests consider that cultivar TU carried only one resistance gene (*Mexique 3* or *Co-5*).

Classical analysis generally assumes that the resistance to different races/isolates in a genotype is conferred by the same locus. Based on this hypothesis, classical studies also assumed that different resistance spectra in cultivars carrying the same resistance gene are due to different alleles residing at the same locus. For example, resistance in cvs. MDRK and Kaboon was shown to be controlled by the *Co-1* gene (Melotto and Kelly 2000). Since both genotypes differ in the response to race 7, this difference was interpreted as being conditioned by two different alleles at the *Co-1* locus: *Co-1* in MDRK (susceptible to race 7) and $Co\text{-}1^2$ in Kaboon (resistant to race 7). However, recent evidence suggests that resistance to races 7 and 73 in Kaboon is controlled by different loci, which mapped at the end of linkage group Pv04 in the relative position of the *Co-3* locus (Campa et al. 2011). The confirmation that the resistance to race 73 in MDRK is controlled by a locus located in the Co-3 region is very important because new *Co*-genes have been described recently (Alzate-Marin et al. 2003; Gonçalves-Vidigal et al. 2009), based on the results of allelism tests in which it was assumed that resistance to race 73 in MDRK was conditioned by the *Co*-1 gene on linkage group Pv01.

Allelism tests based on analysis of F2 segregating populations have additional limitations: plants can only be evaluated once against a single race. A single evaluation can lead to errors due to escapes. In addition, more complex segregation patterns involving two or more loci with different types of gene interaction are difficult to identify correctly in F2 populations. For example, larger populations are needed to differentiate between the segregation ratios for a dominant gene and the segregation for two genes where the resistance is conferred by a combination of a dominant gene and a recessive gene (expected ratio 13R:3S). These limitations of F_2 populations can be solved by using other types of segregating populations such as recombinant inbred lines (RIL) or $F_{2:3}$ families where it is possible to repeatedly test families or RILs against multiple races.

Linkage Analysis in Resistance to Bean Anthracnose

A very important step in the characterization of a gene is the knowledge of its relative position in the genetic map of the species and its relationship with other genes (epistasis). Linkage disequilibrium between resistance genes and various molecular markers has been identified (Kelly and Vallejo 2004) and now provides opportunities to map these genes. Currently, the availability of numerous molecular markers, many of them

included in genetic maps, offers the possibility of locating new genes.

Molecular Markers

Molecular markers detect DNA polymorphism both at the level of specific loci and at the whole genome level. Different methods allow the analysis of DNA sequence variation. Currently, PCR-based molecular markers such as simple sequence repeat or microsatellites (SSR), sequence characterized amplified regions (SCAR), or single nucleotide polymorphism (SNP) are the most commonly used in plant breeding and genetics. Numerous SSR markers have been described in common bean (Blair et al. 2003; Grisi et al. 2007), although not all have been mapped. SCAR markers linked to specific genes have also been described and many of them mapped in common bean (http://www.css.msu.edu/bic/Genetics.cfm). Sequencing projects of different bean genotypes are in progress. The number of markers is expected to expand dramatically with the recent publication of the common bean genome sequence (http://www.phytozome.net/commonbean_er.php). In conclusion, numerous molecular markers are available in common bean and they offer the possibility to carry out studies such as genetic analysis and development of genetic linkage maps to saturate specific regions, as well as to locate new loci or to investigate interactions between loci.

Tagging of Co-Genes with Molecular Markers

Two linked loci are transferred together to the progenies, in a proportion that depends on the genetic distance between them. Genetic distance between two loci is estimated from the frequency of recombination between both loci. The major Co-genes conditioning resistance to anthracnose in common bean were among the first traits to be used in the discovery of the molecular markers linked to resistance genes. The first anthracnose resistance gene, *Co-2 (Are)*, was tagged independently in two labs using RAPD markers (Adam-Blondon et al. 1994b; Young and Kelly 1994, 1996a). Since that date, more refined SCAR, AFLP, and SSR markers linked to at least 9 of the 14 major Co-genes (Table 9.3) have been reported (reviewed by Kelly and Vallejo 2004). In the absence of a saturated map of *P. vulgaris*, researchers first screened segregating populations with RAPD markers to detect linkage with many of the major Co-genes (Young and Kelly 1997a). Markers that proved the most useful were converted to more robust SCAR markers to facilitate their utilization across breeding programs (Young et al. 1998). In some instances, the markers were used in fine-mapping regions around the gene (Melotto and Kelly 2001), but in most cases they were used in marker assisted selection (MAS) or marker-assisted backcrossing (Garzón et al. 2008; Ferreira et al. 2012). Many markers were linked to the Co-genes at distances of less than 5cM, which is sufficient for MAS, but a few were more tightly linked at less than 1 cM (Kelly and Vallejo 2004). When RAPD markers failed to produce useful polymorphisms between parents, researchers turned to AFLP and eventually to SSR markers to find useful linkages with new anthracnose genes. Markers linked to many of the major Co-resistance genes are shown in Table 9.3. More recently sequence-based STS markers from the bean genome are being used to tag resistance genes (Gonçalves-Vidigal et al. 2011) and these will continue to become more important in future studies.

Genetic Linkage Map of Common Bean

Genetic linkage maps show the relative position of loci established from recombination fraction. Linkage maps are important tools for geneticists and breeders because they show the linkage relationship among loci.

In recent years, building saturated genetic maps has been possible due to the availability of techniques exploring the size or sequence polymorphism at the DNA level (molecular markers). Different genetic maps have been

Table 9.3. Major molecular markers linked to resistance genes conferring resistance to specific isolates or races of *C. lindemuthianum* localized in different Co-gene clusters. The specific race of *C. lindemuthianum* and host genotype in which the linkage relationship was observed are indicated.

Cluster	LG	Linked marker	Race /isolate	Genotype	Reference
Co-1	Pv01	$OF10_{530}$	1545	MDRK	Young and Kelly 1997; Rodríguez-Suárez et al. 2007; Campa et al. 2009, 2011
			31, 81, 1545	Kaboon	
			73, 65	Xana	
			65	Andecha	
		CV542014	65, 73, 2047	AND277	Gonçalves-Vidigal et al. 2011
		TGA1.1	65, 73, 2047	AND277	Gonçalves-Vidigal et al. 2011
		SE_{ACT}/M_{CCA}	unspecified	Kaboon	Vallejo and Kelly 2008
		PvM97*		-	Hanai et al. 2010; Pérez-Vega et al. 2012
Co-2	Pv11	SCH20	1	Cornell 49242	Adam-Blondon et al. 1994
		OQ41400 (SQ4)	6, 31 38, 39, 65	A252	Young and Kelly 1996; Rodríguez-Suárez et al. 2007
		SCAreoli	6, 31, 38, 39, 65	A252	Geffroy et al. 1998; Rodríguez-Suárez et al. 2007
		$OH13_{480}$ (SH13b)	6, 31, 38, 39, 65	A252	Rodríguez-Suárez et al. 2007
		PV-ag001*		-	Blair et al. 2003; Pérez-Vega et al. 2012
Co-3	Pv04	SW12	19, 31, 38	Mexico222	Miklas et al. 2000; Rodríguez-Suárez et al. 2007, 2008; Méndez-Vigo et al. 2005
			65, 73, 102, 449	Widusa	
			6, 38, 39, 357	A252	
			38	A493	
		SB12	38	A493	Méndez-Vigo et al. 2005
		Pv-ctt001	19, 31, 38	Mexico 222	Blair et al. 2003; Rodríguez-Suárez et al. 2008; Campa et al. 2011
			65, 73, 102, 449	Widusa	
			7, 3, 19, 31, 449, 453, 1545	Kaboon	
		$OAH18_{1100/600}$	19, 31, 38	Mexico 222	Rodríguez-Suárez et al. 2007, 2008; Méndez Vigo et al. 2005
			65, 73, 102, 449	Widusa	
			6, 38, 39, 357	A252	
			38	A493	
		SB10	7, 3, 19, 31, 449, 453, 1545	Kaboon	Campa et al. 2011
		BM161*		-	Blair et al. 2003; Pérez-Vega et al. 2012
Co-4	Pv08	SAS13	73, 1545	SEL1308	Young et al. 1998
		SH18	unspecified	SEL1308	Awale and Kelly 2001
		SBB14	unspecified	SEL1308	Awale and Kelly 2001
		$OPY20_{830}$ (SY20)	65	TO ^{65}	Arruda et al. 2000
Co-5	Pv07	$OAB3_{450}$ (SAB3)	Alpha, 73	SEL1360	Young and Kelly 1997; Vallejo and Kelly 2001
		g1233	64	MSU7-1	McConnell et al. 2010; Sousa et al. 2012
		Phs	38, 3, 7, 6, 39, 102, 449, 31	TU	Kami et al. 1995; Campa et al. 2009
		SCARAZ20	38, 3, 7, 6, 39, 102, 449, 31	TU	Queiroz et al. 2004; Campa et al. 2009
		BM210	38, 3, 7, 6, 39, 102, 449, 31	TU	Campa et al. 2009
Co-13	Pv03	$OPV20_{680}$	73	Jalo Listras Pretas	Gonçalves-Vidigal et al. 2009
Co-u	Pv02	*I* gene	E4, E42b	BAT 93	Geffroy et al. 2008
		SW13*		-	Melotto et al. 1996

LG: linkage group. *Molecular markers indirectly deduced from their relative map position.

reported in common bean. The first saturated bean genetic maps, based on RFLP and RAPD markers, were developed by Vallejos et al. (1992), Nodari et al. (1993), and Adam-Blondon et al. (1994a). The correspondence between these maps was later established in an integrated linkage map (Freyre et al. 1998; http://www.css.msu.edu/BIC/PDF/Bean_Core_map_2009.pdf) spanning 1,226 cM and including more than 550 markers assigned to 11 linkage groups (LG) (named Pv01 to Pv11; Pedrosa-Harand et al. 2008). A large number of linkage maps, complete or partial, had been subsequently developed to study a wide array of economic traits in common bean. These maps differ in the parents used, the type of segregating populations analyzed, and the number and type of molecular markers included. Establishing relationships between different genetic maps is sometimes possible using common anchor markers. Comparison of genetic maps can help in the identification of new markers located at specific relative positions where interesting loci are mapped.

Use of Linkage Relationship

Linkage relationship can be used to identify a gene from the expression of another closely linked gene. This characteristic is used for the indirect selection in the development of breeding programs (Collard and Mackill 2008) and can also be used in genetic analysis to investigate the inheritance of traits. Implication of a gene in the expression of a specific trait can be inferred from the expression of other closely linked genes.

Linkage analysis can be used for the identification of genes involved in a specific resistance reaction through direct or indirect mapping. Inclusion of a gene controlling the expression of specific genotype in a genetic map allows its location to be determined directly. This strategy was used to map the two genes (*Are* and *RVI*) involved in the genetic control of resistance to two anthracnose races (1 and 21) in the resistant genotype Ms8Eo2 (Adam-Blondon et al. 1994a) and the loci controlling the resistance against six races in the genotypes A252 and Andecha (Rodriguez-Suárez et al. 2007). However, each mapping population may lack polymorphisms, and direct mapping is not always possible. Identification of robust linkage among an unmapped gene and markers previously included in a genetic map also allows the location of new gene to be determined through indirect mapping. This method was used to locate the resistance gene *Co-5* on Pv07 (Campa et al. 2005). The *Co-5* gene (previously named *Mexique 3*) conferred resistance to races gamma and delta 2 and was originally described in the bean genotype TU (Fouilloux 1976). SCAR marker SAB3 linked to *Co-5* (Vallejo and Kelly 2001) was used to locate the *Co-5* gene on Pv07 using four anchor loci (BM183, SAS8, Phs, and Sp4/Sp5).

Another application of markers has been the discovery of gene clusters not only conditioning resistance to anthracnose but linked to other gene clusters conditioning resistance to other pathogens. The SQ4 marker linked to the *Co-2* gene on Pv11, for example, appears to be linked to the *Ur-3/Ur-11* gene cluster conditioning resistance to bean rust (Awale et al. 2008). Markers on Pv01 were used to confirm the presence of a gene cluster consisting of the *Co-1*^{4} gene and the *Phg-1* gene that conditions resistance to race 63-23 of the *Pseudocercospora griseola* pathogen in the Andean genotype AND 277 (Gonçalves-Vidigal et al. 2011).

Characterized Resistance Genes to *C. lindemuthianum*

Resistance Specificities Directly or Indirectly Located in the Genetic Map

Genes conferring specific resistance to different races or isolates of *C. lindemuthianum* have been located on the genetic map of common bean. Seven main chromosome regions possessing these resistance specificities were identified on linkage groups Pv01, Pv02, Pv03, Pv04, Pv07, Pv08, and Pv11. Figure 9.2 shows the

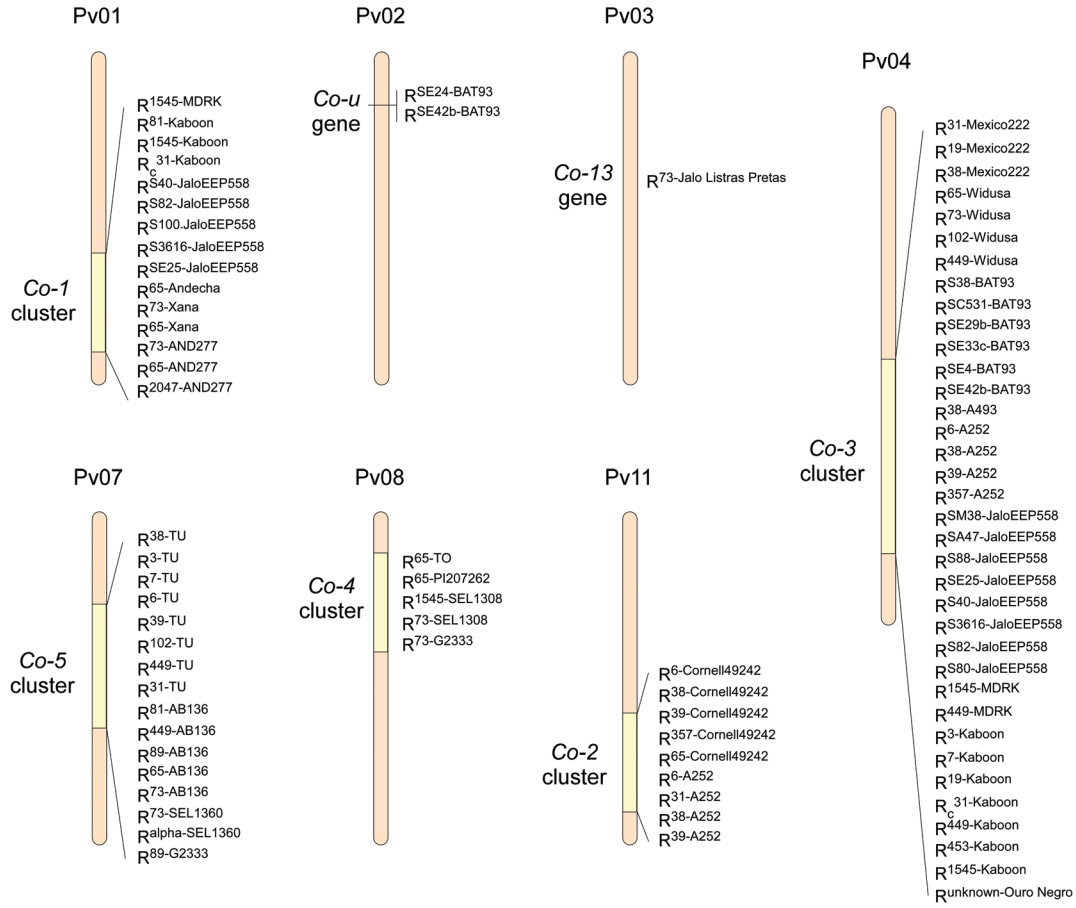

Fig. 9.2. Linkage groups showing anthracnose race-specific resistance genes directly mapped using different common bean genotypes. Specific resistance genes are named considering the relative position of seven gene clusters (*Co-1, Co-2, Co-3, Co-4, Co-5, Co-13*, and *Co-u*), the isolate or race of *C. lindemuthianum*, and bean genotype used in the genetic analysis to describe the corresponding resistance gene(s) are indicated by superscript. For a color version of this figure, please refer to the color plate.

race-specific resistance genes located in the genetic map in different genotypes included in the Andean and Mesoamerican gene pool.

Linkage Group Pv01

Race-specific *Co*-resistance genes from Andean bean genotypes MDRK, JaloEEP558, Kaboon, Andecha, Xana, and AND277 were mapped to Pv01 (Figure 9.2).

In the differential cultivar MDRK, a gene conferring specific resistance to race 1545, linked to $OF10_{530}$ marker, was mapped to Pv01 using a $F_{2:3}$ population derived from the cross TU x MDRK (Campa et al. 2009). The $OF10_{530}$ fragment was previously linked to the anthracnose resistance locus *Co-1* in the $F_{2:3}$ population derived from the cross between the near-isogenic lines N85006 S and N85006 R (Young and Kelly 1997a) and later mapped to Pv01 (Rodríguez-Suárez et al. 2007; Geffroy et al. 2008; Vallejo and Kelly 2008). The *Co-1* gene first described as the *A* factor in the cultivar Wells' Red Kidney (Burkholder 1918; McRostie 1919). It was assumed that the *A* gene is the same gene present in the differential cultivar MDRK (Melotto et al.

2000). The specific resistance gene in MDRK effective against race 1545 most likely corresponds to *Co-1* gene.

Genotype JaloEEP558 was derived from the cross of two Andean landrace cultivars, Roxinho x Pão from Brazil, and is one of the parents used to construct the bean integrated linkage map (Freyre et al. 1998). Two closely linked anthracnose resistance genes from JaloEEP558 were mapped at the end of Pv01 in the RIL population BAT93/JaloEEP558 (B/J). One of these genes confers specific resistance against *C. lindemuthianum* isolates 40, 82, 100, and 3616, and the other gene confers specific resistance against strain E25 (Geffroy 1997; Geffroy et al. 2008). These genes named as *Co-w* and *Co-x*, respectively, may correspond with the *Co-1* gene based on their relative positions in the genetic map. The correspondence between these genes cannot be confirmed because the anthracnose strains used by Geffroy et al. (2008) are unknown as it was not characterized on the uniform differential set. One of the quantitative trait loci (QTL) associated with resistance in B/J population (Geffroy et al. 2000) is most likely associated with the *Co-1* cluster from the JaloEEP558 parent.

In the cultivar Kaboon, a second allele at the *Co-1* locus, *Co-1*^{2}, was reported by Melotto and Kelly (2000). Three closely linked race-specific resistance genes were mapped at the end of Pv01 analyzing the $F_{2:3}$ population derived from the cross Kaboon x Michelite: two dominant genes conferring specific resistance to races 81 and 1545 and a dominant gene with a complementary mode of action for the resistance to race 31 (Campa et al. 2011). The organization observed suggests that the *Co-1* gene is actually a cluster of closely linked race-specific resistance genes. This resistance cluster is linked to the $OF10_{530}$ marker, located in the same relative position as the specific resistance gene against race 1545 mapped in MDRK.

Cultivar Andecha is a very valuable large white-seeded cultivar originating from a selection of landraces from Asturias, Spain (Ferreira et al. 2012). This cultivar carries a single dominant gene conferring intermediate resistance to race 65, linked to $OF10_{530}$ marker on Pv01 (Rodríguez-Suárez et al. 2007). Based on their relative map position, this resistance gene can be part of the Co-1 cluster.

Cultivar Xana is a bean variety developed at SERIDA (Spain) from a cross between the two Andean genotypes, Andecha and V203. Using the RIL population derived from the cross Xana x Cornell 49242 (Pérez-Vega et al. 2010), two closely linked genes from Xana conferring specific resistance to races 73 and 65, respectively, were mapped in Pv01 linked to $OF10_{530}$ marker (Campa et al. unpublished data). Based on their relative map position, these resistance genes correspond to cluster Co-1.

Genotype AND277 is an important resistance source used in breeding programs in Brazil and Southern Africa (Carvalho et al. 1998; Aggarwal et al. 2004). Resistance to races 65 and 2047 has been mapped to Pv01 in F_2 and $F_{2:3}$ populations, respectively, derived from the cross AND277 (R) x Ouro Negro (S). A gene conferring resistance to race 73 in AND277 was also located on Pv01 using a F_2 population derived from the cross AND277 x Rudá (Gonçalves-Vidigal et al. 2011). This resistance gene, corresponding to *Co-1*, was named as the *Co-1*^{4} allele, closely linked to the CV542014 and TGA1.1STS markers (Gonçalves-Vidigal et al. 2011).

The presence of a race-specific resistant gene *Co-1*^{5} on Pv01 was proposed in the differential cultivar Widusa (Gonçalves-Vidigal and Kelly 2006) based on the lack of segregation in the crosses Widusa x MDRK and Widusa x Kaboon against race 65 (an isolate of race 65 that overcomes BAT93). The location of this resistance specificity on Pv01 should be confirmed by analyzing its linkage relationship between the resistance gene and markers linked to the *Co-1* locus on Pv01.

Cultivar Perry Marrow was crossed with MDRK and Kaboon to test for allelism inoculating with race 73 (Melotto and Kelly 2000; Mendoza et al. 2001). No segregation was observed in either of the two F_2 populations. Considering

the knowledge at that time about the resistance profile of cultivars MDRK and Kaboon, the authors proposed that resistance to race 73 in Perry Marrow was conditioned by a third allele of the *Co-1* locus, *Co-1*^{3}. However, current results support evidence that the resistance to race 73 from MDRK and Kaboon is not located in Pv01 (Campa et al. 2011), and consequently the results concerning Perry Marrow should be reinterpreted. To date, resistance genes from Perry Marrow had not been mapped in the Co-1 cluster, on linkage group Pv01.

Linkage Group Pv02

A gene conferring specific resistance to *C. lindemuthianum* strains E4 and E42b (showing complete co-segregation) was directly mapped to the end of Pv02 in the Mesoamerican genotype BAT93 (Figure 2). This gene was named as *Co-u* and located in the vicinity of the *I* locus (Geffroy et al. 2008), a complex resistance cluster effective against potyviruses (Vallejos et al. 2006). The anthracnose isolates E4 and E42b used to detect the *Co-u* gene are unknown as they were not characterized on the international differential set. For this reason the presence of the *Co-u* gene was not tested in other genotypes. The possibility that some of the undefined anthracnose resistance QTL mapped near the *I* locus on Pv02 (Geffroy et al. 2000), in a relative similar position corresponding to *Co-u*, has never been considered. Molecular markers located in the chromosome region corresponding to *Co-u* should be tested in co-segregation analyses before characterizing a new anthracnose resistance gene to avoid synonymy between *Co-* genes.

Linkage Group Pv03

A single major gene controls the specific resistance against each one of the races 9, 64, 65, and 73 in the Brazilian landrace cultivar Jalo Listras Pretas (Gonçalves-Vidigal et al. 2009). The gene effective against race 73 was linked in coupling at 1.8cM to the marker $OPV20_{680}$, previously reported on Pv03 (Figure 2), in an F2 population obtained from the cross of Jalo Listras Pretas (R) x Cornell 49242 (S) (Lacanallo et al. 2010). It was assumed that this resistance gene located in Pv03 was the same locus that confers resistance against races 9, 64, and 65 and it was named as *Co-13* gene, independent from the previously *Co*-genes described (Gonçalves-Vidigal et al. 2009). The identity of this resistance gene(s), effective against races 9, 64, and 65, should be confirmed by analyzing its co-segregation with the resistance against race 73, or by confirming its map position on Pv03 with marker analyses. Incidentally the same marker appears to colocalize with a QTL for anthracnose resistance in the B/J mapping population (Geffroy et al. 2000). Another QTL CL20 conditioning resistance to race 521 had been reported previously in DOR364/G19833 (D/G) mapping population (Beebe et al. 1998). It is not possible to associate the *Co-13* gene with these QTL since different mapping populations were used in these studies but the resistance sources in the QTL studies were the Andean parents, Jalo EEP558 and G19833, also known as Chaucha Chuga, a landrace cultivar from Peru. G19833 provides resistance to highly virulent races 3481, 3977, and 3993 from Costa Rica. As has been demonstrated in other bean genotypes, Jalo Listras Pretas could carry more than one major anthracnose resistance gene, apart from *Co-13*, which could affect these interpretations.

Linkage Group Pv04

Genes conferring specific resistance to different *C. lindemuthianum* strains were mapped to the distal portion of Pv04 in bean genotypes Ms8EO2, Mexico 222, Widusa, BAT93, A493 JaloEEP558, A252, MDRK, Kaboon, and Ouro Negro (Figure 9.2).

The first anthracnose resistance gene *RVI* to be mapped to Pv04 came from the Ms8EO2 breeding line (Adam-Blondon et al. 1994a, 1994b). This gene conferred resistance to *C. lindemuthianum* race 21 from Colombia

(identity unknown). Using a backcross population (Ms_8Eo_2/Corel), the *RVI* gene was mapped at the end of linkage group P4 (Adam-Blondon et al. 1994b), which corresponds to Pv04 of the bean core map (Freyre et al. 1998). The association of the *RVI* gene with other characterized resistance genes on Pv04 has not been possible due to the lack of molecular markers linked to this gene common to both linkage maps, and the absence of the original resistance source and anthracnose race.

The single dominant anthracnose resistance gene *Mexique 1* (renamed as *Co-3* gene) was first described in the Mesoamerican cultivar Mexico 222 by Bannerot et al. (1971) as a gene conferring resistance against isolates 14 and 51. The resistance to race Ebnet (corresponding to race Kappa or 31) present in Mexico 222 was analyzed by Fouilloux (1976) and assumed to be conferred by the *Co-3* gene. Resistance to race 31 present in Mexico 222 was directly mapped in Pv04, closely linked to specific anthracnose resistance genes effective against races 19 and 38 in the $F_{2:3}$ population, Mexico 222 x Widusa (Rodríguez-Suárez et al. 2008). This suggests that the original *Co-3* gene from Mexico 222 is in fact a cluster of linked resistance genes each conditioning resistance to different races of *C. lindemuthianum*. This resistance cluster is flanked by the markers SW12, and Pv-ctt001, and $OAH18_{1100/600}$.

The differential cultivar Widusa was considered to belong to the Andean gene pool, but pedigree evidence suggests a stronger relationship with Mesoamerican gene pool (Gonçalves-Vidigal and Kelly 2006). Using the $F_{2:3}$ population Mexico 222 x Widusa, a gene conferring specific resistance to races 65, 73, 102, and 449 (no recombination was observed) was located on Pv04, tightly linked to the resistance genes from Mexico 222 protecting against races 19, 31, and 38 mapped in this same $F_{2:3}$ population (Rodríguez-Suárez et al. 2008). This indicates that cultivars Mexico 222 and Widusa carry different haplotypes of the same Co-3 resistance cluster on Pv04.

Breeding line BAT93 (derived from [Veranic 2 x PI207262] x [Jamapa x Great Northern Tara]) is the parent of the B/J mapping population. The *Co-9* gene conferring resistance to *C. lindemuthianum* strains 38, C531, E29b, E33c, E4, and E42b was first described in BAT93 and was directly mapped to the distal position of Pv04 using the RIL population B/J (Geffroy et al. 1999, 2008).

The *Co-z* gene from JaloEEP558 conferring resistance against *C. lindemuthianum* strain 80 was mapped to Pv04 in the B/J RIL population. A second resistance gene from JaloEEP558, the *Co-y* gene conferring resistance against *C. lindemuthianum* strains M38, A47, 88, E25, 40, 3616, and 82, was mapped to Pv04, tightly linked to *Co-z* (Geffroy et al. 1999, 2009). The *Co-y* gene co-segregate with the *Co-9* gene mapped in BAT 93 in this same RIL population, indicating that *Co-y*, *Co-z*, and *Co-9* are closely linked race-specific genes of the Co-3 resistance cluster located on Pv04.

A gene present in breeding line A493 (obtained from Alubia x BAT 93) that conferred resistance against race 38 was mapped to Pv04 using the $F_{2:3}$ population Andecha x A493 (Méndez-Vigo et al. 2005). This resistance gene is closely linked to the SW12 and $OAH18_{1100}$ markers, suggesting that its relative position on Pv04 corresponds to the Co-3 cluster in Mexico 222. From the results of different allelism tests, Méndez-Vigo et al. (2005) demonstrated that the previously considered independent genes *Co-3* and *Co-9* could be alleles at the same locus or, based on the current knowledge about the organization of the anthracnose resistance loci, could be two closely linked race-specific genes of the same resistance cluster. The *Co-9* gene present in BAT93 was renamed *Co-3*^{3} by the Genetics Committee of the Bean Improvement Cooperative.

Resistance to races 6, 38, 39, and 357 in breeding line A252 (Carioca x Guanajuato 31) was investigated in a $F_{2:3}$ population derived from the cross Andecha (S) x A252 (R). A cluster of four genes conferring specific resistance against

the four *C. lindemuthianum* races was mapped at the end of Pv04, tightly linked to the markers SW12 and $OAH18_{1100}$ (Rodríguez-Suárez et al. 2007), which flank the Co-3 resistance cluster.

In the Andean cultivar MDRK, two genes conferring specific resistance against races 449 and 1545 were mapped to Pv04 in a $F_{2:3}$ population derived from the cross TU x MDRK (Campa et al. 2009). These resistance loci are probably part of the Co-3 cluster based on their closely linked relationship with the Pv-ctt001 marker (Campa et al. 2009; Rodríguez-Suárez et al. 2008). The presence of specific resistance genes from MDRK at the Co-3 resistance cluster is of considerable interest as this differential cultivar has been generally used in allelism tests as a resistance source of the *Co-1* gene. According to current research, monogenic segregation observed from the differential cultivar MDRK could be controlled by a gene located at either the Co-1 or the Co-3 cluster, and is necessary to select a specific anthracnose race to compare before conducting allelism tests with MDRK.

In the cultivar Kaboon, a cluster formed by at least seven genes conferring specific resistance to races 3, 7, 19, 31, 449, 453, and 1545 were mapped to Pv04 in the $F_{2:3}$ population Kaboon x Michelite (Campa et al. 2011). The gene conferring specific resistance against race 31 shows a complementary mode of action, so the presence of other complementary resistance genes is necessary for the resistance response. This resistance cluster present in Kaboon is tightly linked to Pv-ctt001 marker, so based on its map location it would correspond to *Co-3* cluster. As with MDRK, Kaboon had been used in allelism tests as resistance source of the *Co-1* gene, so researchers are cautioned to know which genes they are comparing based on judicial selection of anthracnose races used for screening.

The *Co-10* gene was described in the commercial cultivar Ouro Negro as a single gene conferring resistance against races 23, 64, 73, 81, and 89 (Alzate-Marin et al. 2003) and, to date, it has been only been identified in this genotype. A gene in Ouro Negro conferring resistance against one of these anthracnose races (not specified by the authors; Alzate-Marin et al. 2003) was located on Pv04 based on its linkage relationship with the SCAR marker SCF10. The RAPD marker $OF10_{1072}$, from which the SCAR SCF10 was developed, was mapped on Pv04 in a relative position corresponding to the Co-3 resistance cluster (Kelly et al. 2003; Rodríguez-Suárez et al. 2005; Pérez-Vega et al. 2012). However, *Co-10* was reported as independent from *Co-9* (included in cluster Co-3 on Pv04) based on the segregation ratio observed against race 89 corresponding to the presence of three independent resistance genes in the F_2 population Ouro Negro (R) x PI 207262 (R): $Co\text{-}4^3$ and *Co-9* genes reported in PI207262 (Alzate-Marin et al. 2007) and a gene independent from those in Ouro Negro. In the interpretation of the allelism tests conducted by Alzate-Marin et al. (2003), the possibility that the monogenic segregation observed against races 23, 64, 73, 81, and 89 could be conferred by different single genes—one linked to the SCF10 SCAR in Pv04 and the other independent from *Co-3* gene—was not considered. To confirm the assumption that monogenic segregation observed against different races are controlled by the same gene in a genotype, co-segregation analyses should be done. The independence between the *Co-10* gene and resistance genes included in the Co-3 cluster should be investigated.

Linkage Group Pv07

Genes conferring specific resistance to different races of *C. lindemuthianum* were mapped to the central position of Pv07 in the Middle American genotypes TU, SEL1360, G2333, and AB136 (Figure 9.2). In the anthracnose differential cultivar TU, eight closely linked genes conferring specific resistance to races 3, 6, 7, 31, 38, 39, 102, and 449 were mapped to a central position of Pv07 using the $F_{2:3}$ population TU x MDRK (Campa et al. 2009). This resistance cluster appears to correspond to the *Co-5* gene, first named as *Mexique 3* and described

in the genotype TU as a single gene conferring resistance against *C. lindemuthianum* strains gamma and delta 2 (Fouilloux 1976). This cluster is linked to the SCAR marker SAB3 in TU, developed from the RAPD $OAB3_{450}$, previously described as linked to *Co-5* gene in the genotype SEL1360 (Young and Kelly 1997a; Vallejo and Kelly 2001).

Breeding line SEL1360 was developed at CIAT through backcrossing to the differential cultivar G2333, shown to possess three resistance loci: *Co-4*, *Co-5*, and *Co-7* (Young et al. 1998). The RAPD marker $OAB3_{450}$ was linked to a gene in the genotype SEL1360 conferring specific resistance to race alpha (race 17) in the $F_{2:3}$ population Black Magic (S) x SEL 1360 (R), and linked to a gene conferring specific resistance to race 73 in the $F_{2:3}$ population Blackhawk (S) x SEL 1360 (R) (Young and Kelly 1997a). This resistant gene was assumed to be the same—the *Co-5* gene derived from G2333. The lack of segregation reported by Young and Kelly (1996b) in the allelism test conducted with race 7 in the F_2 population TU (R) x SEL1360 (R) was used to suggest that both genotypes carried the same resistance gene, probably included in the Co-5 cluster reported in TU. More recent studies have shown that TU is resistant to race 3481 whereas SEL 1360 is susceptible to it (Vallejo and Kelly 2009). Based on the presence of a differentiating race, the authors proposed that the Co-5 allele in SEL 1360, MSU 7-1, and G2333 parent be named *Co-5*^{2} (Table 9.2). Additional confirmation for the location of the *Co-5* locus comes from screening of cross of MSU7-1 breeding line with STS markers developed by McConnell et al. (2010). In the F_2 population of MSU 7-1 crossed with Mexico 222 and screened with race 64, the g1233 marker located on Pv07 (McConnell et al. 2010) was shown to be linked at 1.2cM from *Co-5* gene (Sousa et al. 2012).

The resistance to race 89 in the anthracnose differential cultivar G2333 was reported to be controlled by two independent dominant genes using a F_2 population derived from the cross Rudá (S) x G2333 (R) (Alzate-Marin et al. 2001). One of these resistance genes was dissected in a $BC_1F_{2:3}$ population obtained from this cross and was linked in coupling phase to RAPD marker $OPAB3_{450}$, described as linked to *Co-5* gene.

In the differential cultivar AB136, a monogenic segregation against races 73, 81, and 89 was observed in the F_2 population Michelite (S) x AB136 (R) (Alzate-Marin et al. 1999). Each one of these resistance specificities was linked to the $OPZ04_{560}$ marker. The same authors identified a resistance gene conferring resistance against race 64 in the F_2 population Mexico 222 (S) x AB136 (R), also linked to the $OPZ04_{560}$ marker. This marker and the corresponding SCAR SZ04 marker (Queiroz et al. 2004) were located on Pv07 (Freyre et al. 1998; Kelly et al. 2003; Rodríguez-Suárez et al. 2007; Pérez-Vega et al. 2010). A resistance gene against race 89 from AB136 was also identified in the F_2 population Rudá (S) x AB136 (R) linked to RAPD marker $OPAZ20_{940}$ (Alzate-Marin et al. 2000). SCARZ20 marker developed from the $OPAZ20_{940}$ RAPD marker (Queiroz et al. 2004) located on Pv07 was closely linked to SZ04 marker (Campa et al. 2009). In agreement with these results, two linked genes from AB136 conferring specific resistance against races 81 and 449 were mapped on Pv07 using the $F_{2:3}$ population AB136 (R) x Michelite (S), linked to SCARZ20 and SZ04 markers (Campa et al. 2007). All these results suggests that AB136 carries an anthracnose resistance cluster located on Pv07 probably corresponding, based on its relative map position, with the Co-5 resistance cluster identified in this linkage group (Campa et al. 2007, 2009).

The *Co-6* gene was first described in cultivar Catrachita, linked to RAPD marker $OAK20_{890}$, as a single gene conferring specific resistance against races 23, 64, and 73 (Young and Kelly 1996b, 1997a). Since Catrachita was derived from the cross BAT1225 x AB136 (anthracnose-resistant donor), it was assumed that the anthracnose resistance present in AB136 was also, and exclusively, due to the *Co-6* gene described in Catrachita (Young and Kelly 1996b, 1997a).

Using a F_2 population derived from the cross of TU (R) x Catrachita (R), a segregation ratio corresponding to two independent resistance genes for race 64 was described, and these results were taken as a evidence of independence between the resistance gene present in TU (*Co-5*) and the one present in Catrachita (*Co-6*) (Young and Kelly 1996b). Independent segregation between the $OAK20_{890}$ marker linked to *Co-6* gene and the *Co-5* resistance cluster mapped in TU on Pv07 has been observed (Campa et al. 2009). The presence of more than one resistance gene in AB136 has been reported (Campa et al. 2007), so the presence of *Co-5* in AB136 does not exclude the possibility of the presence of *Co-6* resistance gene first described in Catrachita. To date, the location on the genetic map of the *Co-6* gene described in Catrachita as linked to the $OAK20_{890}$ marker has not been reported.

Linkage Group Pv08

Genes conferring specific resistance to different races of *C. lindemuthianum* were directly mapped to the distal position of Pv08 from Middle American genotypes TO, PI207262, SEL1308 and G2333 (Figure 9.2). In differential cultivar TO, a gene conferring specific resistance against race 65 was mapped to Pv08 using a $F_{2:3}$ population developed from the cross Rudá (S) x TO (R), closely linked in coupling to $OPY20_{830}$ marker (Cardoso de Arruda et al. 2000). The SCAR marker SY20 was developed from the RAPD marker $OPY20_{830}$ (Queiroz et al. 2004) and its location on Pv08 was confirmed (N. Trabanco, per. com.). This gene probably corresponds to the *Mexique 2* gene, conferring specific resistance against pathotypes gamma and delta 2, that was first described in the differential cultivar TO (Fouilloux 1976) and was later renamed as *Co-4* gene (Kelly and Young 1996).

A single dominant gene conditioning resistance to race 65 was reported in the anthracnose differential cultivar PI 207262 investigating the F_2 population derived from the cross Rudá (S) x PI 207262 (R) (Silva et al. 2007). A lack of segregation for the resistance to race 65 in the F_2 population derived from the cross PI 207262 (R) x TO (R) (Alzate-Marin et al. 2001a) suggests that the gene effective against this race in PI 207262 is located on Pv08, corresponding to *Co-4* locus. That gene was later interpreted as a third allele $Co\text{-}4^3$ at the *Co-4* locus (Alzate-Marin et al. 2007).

Breeding line SEL1308 was developed at CIAT through backcrossing with the differential cultivar G2333 known to possess the $Co\text{-}4^2$, $Co\text{-}5^2$, and *Co-7* genes (Young et al. 1998). The *Co-4* gene was reported to be present in the SEL1308 line, identified at that time, as the $Co\text{-}4^2$ allele based on the different resistant patterns against in G2333 and SEL1308 genotypes. A gene from SEL1308 conferring resistance against race 1545 was linked to the SCAR marker SAS13 in the $F_{2:3}$ population, Catrachita (S) x SEL1308 (R). Another gene conferring resistance against race 73 was also closely linked to the SCAR marker SAS13 in the $F_{2:3}$ population, Black Magic (S) x SEL1308 (R). In both populations, no recombinants between the resistant gene and the marker were detected (Young et al. 1998). Two independent dominant genes conferring resistance to race 73 in the donor line G2333 were described in the $F_{2:3}$ population, Rudá (S) x G2333 (R). One of these resistance genes was dissected in a $BC_3F_{2:3}$ population and was also linked in coupling phase to RAPD marker $OPAS13_{950}$ (Alzate-Marin et al. 2001b), from which the SCAR SAS13 was developed. The SAS13 marker was mapped to Pv08, in a relative position similar to the mapped *Co-4* gene in TO (Rodríguez-Suárez et al. 2005), confirming the presence of the *Co-4* gene in the genotypes SEL1308 and G2333. In agreement with this result, the *Co-4* resistance locus linked to the SAS13 was physically mapped to the short arm of bean chromosome 3, which corresponds to Pv08, using a BAC library constructed from the snap bean cultivar Sprite (Melotto et al. 2004). Further support for map position of the *Co-4* locus comes from the reported linkage of *Mexique 2 (Co-4)* gene to the pod anthocyanin, *Anp*

gene (Gantet et al. 1991) known to be a member of the complex C locus on Pv08.

Linkage Group Pv11

Genes in the Middle American genotypes Cornell 49242 and A252 that confer specific resistance to different strains of *C. lindemuthianum* have been directly mapped in a distal position of Pv11 (Figure 9.2). Cultivar Cornell 49242 is a bean genotype from Venezuela in which the *Co-2* gene, originally known as the *Are* gene, was first described as a single dominant gene conferring resistance against strains alpha, beta, gamma, and delta (Mastenbroek 1960). The *Are* gene was introgressed from Cornell 49242 in the derived cultivar Ms_8EO_2 (Adam-Blondon et al. 1994a, 1994b). Using a BC_1F_1 population obtained from the backcross (Ms_8EO_2/* Corel), the *Are* gene was mapped at the end of linkage group P1 of the Paris bean map (Adam- Blondon et al. 1994b), which corresponds to Pv11 on the bean core map. Five different race-specific genes from Cornell 49242 protecting against races 6, 38, 39, 65, and 357 were mapped tightly linked, forming a cluster, in a distal position of Pv11 in the RIL population Xana x Cornell 49292 (Campa et al. unpublished data). This suggests that the original *Are* gene from Cornell 49242 is in fact a cluster of linked resistance genes each conditioning resistance to different races of *C. lindemuthianum*. This cluster is linked to the SCAreoli and SQ4 markers (Campa et al. unpublished data).

An organization in cluster of the *Co-2* locus was also reported in the breeding line A252 developed at CIAT. Using the $F_{2:3}$ population derived from the cross Andecha (S) x A252 (R) five genes conferring specific resistant against *C. lindemuthianum* races 6, 31, 38, 39 and 65 were mapped, closely linked, at the end of Pv11 (Rodríguez-Suárez et al. 2007). This cluster is linked to the SCAR SCAreoli and the RAPD $OQ4_{1400}$ (from which the SCAR SQ4 was developed; Awale et al. 2008), corresponding to the Co-2 cluster from Cornell 49242.

Incompletely Characterized Resistance Genes

Gene Co-7

The presence of the *Co-7* gene was first described in the genotype G2333 (Young et al. 1998). In the absence of linked markers, its relative position in the genetic map has not been reported. The resistance spectrum of *Co-7* gene in MSU7 breeding lines derived from G 2333 was studied by Vallejo and Kelly (2009), but the lines did not demonstrate the expected resistance reaction to races 515, 521, and 1545. A second genotype, H1 carrying only the *Co-7* gene, was reported by Pereira and Santos (2004) to possess resistance to race 1545 (S to 73, 2047) and could be used for future studies to better characterize and map this gene.

Gene co-8

The *co-8* gene was first described in the genotype AB136 (Alzate-Marin et al. 1997) and is the only recessive anthracnose resistance gene reported in common bean. Later, the same authors (Alzate-Marin et al. 1999) were unable to support the presence of this recessive gene in AB136 and other authors put into question the existence of a recessive gene in AB136 (Gonçalves-Vidigal et al. 1997, 1999, 2001; Poletine et al. 2000).

Gene Co-11

The *Co-11* gene was described in the differential cultivar Michelite as a dominant gene conditioning resistance against race 64, independent from the previously described *Co-1*, *Co-2*, *Co-3*, *Co-4*, *Co-5*, *Co-6*, *Co-7*, *Co-9*, and *Co-10* loci (Gonçalves-Vidigal et al. 2007). The *Co-11* gene was characterized by means of allelism tests conducted with F_2 populations derived from crosses between Michelite and the cultivars AB136, AND277, BAT93, Cornell 49242, G2333, Kaboon, Mexico 222, MDRK, Ouro Negro, Perry Marrow, PI 207262, TO, TU, and

Widusa. However, based on the current knowledge about the anthracnose resistance present in the cultivars used in these allelism tests, the independence between the gene present in Michelite and some of the Co-genes described should be reconsidered. For instance, the independence between the resistance gene present in Michelite and *Co-9* was tested in the cross Michelite x BAT93 (R x R) against race 64. In BAT93, the presence of the *Co-9* gene was described (Geffroy et al. 1999) and recently, other independent anthracnose resistance locus, named as *Co-u* and located in Pv02, was reported in this cultivar (Geffroy et al. 2008). The 15R:1S segregation ratio found in the F_2 population Michelite x BAT93 suggests that one dominant gene confers resistance to race 64 in BAT93, but it is unknown if these resistance gene corresponds to *Co-9* or to *Co-u* locus, so the possibility that *Co-11* correspond with *Co-u* should not be discarded. The existence of the *Co-11* gene as a new anthracnose resistance gene should be confirmed, given the existence of more than one resistance gene in bean genotypes MDRK, Kaboon, Widusa, TU, and AB136 (Campa et al. 2007, 2009, 2011; Rodríguez-Suárez et al. 2008) used to test independence with the *Co-1*, *Co-1*^{2}, *Co-1*^{5}, *Co-5*, and *Co-6* genes.

Gene Co-12

The *Co-12* gene was described in the landrace Jalo Vermelho as a gene independent from the previously described *Co-1*, *Co-2*, *Co-3*, *Co-4*, *Co-5*, *Co-6*, *Co-7*, *Co-9*, *Co-10*, *Co-11*, and *Co-13* loci (Gonçalves-Vidigal et al. 2008). The resistance in Jalo Vermelho against races 23, 55, 89, and 453 is controlled by one dominant gene in each case, as is deduced from the 3R:1S segregation ratios obtained in F_2 populations in R x S crosses, being Jalo Vermelho the resistant parent. Additional analysis should be carried out to verify that the same locus is involved in the resistance reaction to the races 23, 55, 89, and 453 and its independence from previously described resistance genes.

Gene Co-14

The presence of the *Co-14* gene has been postulated in the bean cultivar Pitanga, landrace cultivar from Brazil, following inoculation of segregating populations with races, 23, 64, 65, and 2047 (Gonçalves-Vidigal et al. 2012). Independence from previously reported Co-genes is based segregation in 14 F_2 populations inoculated with various races listed above. The fact that some of the differentials are now known to carry more than a single gene on different LGs, independence will need to be tested using family-derived populations challenged against a number of differentiating races capable of detecting all known genes in the differentials. Since the resistance pattern of the *Co-14* gene in Pitanga is very similar to that of reported for the *Co-1*^{4} gene in the Andean genotype AND 277 (Gonçalves-Vidigal et al. 2011), the apparent similarity of both genes should be tested using markers tightly linked to the *Co-1*^{4} gene before the independence of the Co-*14* gene can be fully accepted by the bean community.

Genomic Organization of Resistance to Anthracnose

Genes with different function are involved in the response to *C. lindemuthianum* (Borges et al. 2012; Oblessuc et al. 2012). Many plant disease resistance genes (R genes) encode proteins that are part of the plant-pathogen interaction. R proteins can have two functions: they work as sensors detecting pathogens or they activate the reaction to pathogens (Chisholm et al. 2006). The most characterized R genes encode proteins containing a leucine-rich repeat motif (LRR). The vast majority of genes cloned so far can be classified in three families considering the conserved motif in R gene products: NBS-LRR (Nucleotide-Binding Site- LRR), eLRR (extracytoplasmic LRR), and LRR-Kinase (McDowell and Woffenden 2003). The NBS-LRR family has also been classified in two types based on the N-terminal domain of the R protein:

Coiled-coil-NBS-LRR and Toll-Interleukin receptor NBS-LRR. Resistance gene analogs (RGAs) were identified in several linkage groups of common bean using degenerated primers that targeted the conserved domains of R genes (Lopez et al. 2003). Some RGAs were located in the relative position of anthracnose resistance genes. A molecular characterization in detail was carried out in several regions including anthracnose resistance loci. Genomic analyses revealed the presence of R genes organized in clusters including several nucleotide sequences that code for R proteins. In plants, genes encoding NBS-LRR are often located at cluster of tightly linked loci (Dangl and Jones 2001; McDowell and Woffenden 2003). This molecular organization of genes in complex clusters, encoding resistances to diverse pathogens or to different races of the same pathogen, is supported by genetic evidence from different species (Kanazin et al. 1996; Michelmore and Myers 1998; Sharma et al. 2004).

Linkage Group Pv02

A large cluster of NBS-LRR sequences associated within the *I* locus that is involved in the response to several related potyviruses was described in the bean genotype Sprite (Vallejos et al. 2006). The *I* locus was located at the end of Pv02 in several genetic maps. The Co-u resistance gene conferring resistance to anthracnose isolates E4 and E42b was located in the relative position of the *I* gene (Geffroy et al. 2008). Polygalacturonase-inhibiting proteins (PGIPs) family was mapped in the Pv02 linkage group, although its relative position was not specified (Freyre et al. 1998). PGIPs are extracellular plant inhibitors of fungal endopolygalacturonases (PGs) that belong to the family of LRR proteins. PGIP family in the bean genotype BAT93 comprises four clustered genes that span a 50-kb region (D'Ovidio et al. 2004). The relation of this cluster of genes with resistance to anthracnose should be verified.

Linkage Group Pv04

A complex cluster of resistance genes including the resistant genes to numerous races or isolates are located at the end of Pv04, cluster Co-3, or B4 resistance (R) gene cluster. Four expressed resistance gene candidates that map at the Co-3 cluster and co-localize with R-specificities or R-QTLs effective against *C. lindemuthianum* were analyzed in the bean genotypes BAT93 and JaloEEP558. These candidate genes encode NBS-LRR proteins (Ferrier-Cana et al. 2003).

Six bacterial artificial chromosome clones (BAC), derived from genotype BAT93 and located in same relative position as the Co-3 cluster, were sequenced. The analyzed region approximately corresponds to 29 cM in the genetic linkage map. A total of 97 genes, 26 of which correspond to coiled-coil-NBC-LRR proteins, were annotated. These 26 NBS-LRR genes were identified in the approximately 650 kb sequence and they are organized in four subclusters. Fluorescent in situ hybridization of meiotic pachytene chromosomes revealed that these sequences are located in the subtelomeric region of the short arm of chromosome 4 (Pv04). Phylogenetic analysis showed a closely relationship of this Co-3 (Pv04) cluster and the Co-2 cluster (Pv11) suggesting an ectopoic recombination event in the legume (David et al. 2009, 2010; Geffroy et al. 2009). DNA sequences from cluster Co-3 and derived from the genotypes BAT93 and JaloEEP558 were compared and different numbers of genes encoding for NBS-LRR proteins were found. In addition, the sequencing and annotation of one BAC clone located at the Co-3 cluster revealed three genes encoding for formate dehydrogenase (FDH), an enzyme involved in the oxidation of formate into CO_2. FDH protein accumulation and increased activity has been correlated with abiotic stress in different plant species. FDH genes are differentially up-regulated after infection with isolate M126 of *C. lindemuthianum* (David et al. 2010).

Linkage Group Pv08

A BAC from the bean genotype Sprite harboring part of the *Co-4* gene was sequenced and assembled in a single contig of 106.5 kb. Five copies of the COK-4 gene that encodes for a LRR-Kinase and 19 novel genes with no similarity to any previously identified genes were described (Melotto et al. 2004).

Linkage Group Pv11

A gene family encoding for LRR sequences located in the vicinity of the Co-2 locus was characterized in the genotype EO_2 (Geffroy et al. 1998; Creusot et al. 1999). The PvH20 clone contains six sequences for LRR motifs, suggesting that the PvH20-related sequences may be candidate genes conditioning resistance for anthracnose on Pv11. Recent analysis of the genomic region containing the Co-2 resistance locus in the BAT93 genotype revealed the presence of clusters of sequences coding for Coiled-coil-NBS-LRR (Chen et al. 2011).

Conclusions

Evidence about *C. lindemuthianum*-common bean interaction suggests:

1. Anthracnose resistance in bean genotype(s) is generally controlled by major genes exhibiting a race-specific mode of action.
2. Interaction of C. *lindemuthianum* genotype with the bean genotype seems to be very specific.
3. Classification of the variation of C. *lindemuthianum* into races is limited. Additional variation can be found within races or pathogenic variants.
4. Resistance response against a specific isolate/race can be controlled by different loci in common bean.
5. Genetic control of anthracnose resistance against different isolates or races in a bean genotype can be controlled by different genes, although a monogenic segregation is observed in the response to specific races.
6. Anthracnose resistance genes are located in specific regions of genome and they are organized in groups or gene clusters in which each gene confers resistance to one specific isolate or race.
7. Description of alleles at locus based on resistance profiles should be reconsidered because different and linked loci can be involved in the resistance reaction against different races or isolates in a bean genotype.
8. Many resistance loci are organized in clusters of closely linked genes since recombination between resistance genes has been detected.

Implications

Implication in Genetic Analysis of Anthracnose Resistance

The authors suggest that the following guidelines are to be followed in the future characterization of all genes conditioning resistance to *C. lindemuthianum* in common bean.

- Researchers should base future characterization of new resistance sources on multiple inoculations of families ($F_{2:3}$ or later generations) or RIL populations, instead of relying on single races inoculated on single F_2 plants.
- Researchers should choose a number of different pathogenic races/isolates to screen both the RxS and RxR populations. The authors recognize that the choice of races will depend on reaction of new resistance sources, but every attempt should be made to select a number of specific races and bean genotypes at each gene cluster on the seven known linkage groups in order to truly characterize new resistance source (tagging of clusters using races). Since different races and bean genotypes were used to characterize specific genes on specific linkage groups, some of these key races need to be selected when conducting complementary testing. Different races inoculated on the same

resistance source have been shown to identify specific genes on different linkage groups, so the random use of races in complementary testing is not recommended.

- Researchers should also consider verifying the co-segregation or linkage between molecular markers linked to known genes and the new resistance locus in order to verify that new resistance genes identified in a bean genotype do not reside at a known resistance cluster (tagging of clusters using molecular markers).

Implication for Plant Breeding

Classification of *C. lindemuthianum* into pathogenic races using a set of different bean genotypes may mask true pathogenic variation. Breeders should use their local isolates and should identify resistance sources against the local isolates in the development of their plant-breeding programs. Genetic analysis of resistance against new isolates is also recommended in known and unknown resistance sources to verify the genes involved in the resistance reaction.

The existence of intra-cluster recombination can be of considerable practical importance. Race-specific resistance genes present in different haplotypes of the same cluster could be pyramided by genetic recombination within the cluster. For example, intra-cluster recombination was used to pyramid resistance loci located on cluster Co-3 from Widusa (S19;R65;R73;R102;R449;S31) and Mexico 222 (R19;S65;S73;S102;S449;R31) into the line MexW1 (R19;R65;R73;R102;R449;R31). After accumulating the resistance specificities, the enhanced new recombinant haplotype could be introgressed into susceptible varieties, practically as a single locus (A Campa unpublished data). Gene pyramiding is essential for the long term control of this highly variable pathogen.

Implication for Gene Nomenclature

The current nomenclature system only recognizes major Co-genes, which may result in imprecise description of this plant-pathogen interaction. A new system for naming Co-genes is being proposed that provides critical additional information to describe the specific interaction between *C. lindemuthianum- P. vulgaris* and the relative position in the bean genetic map. The authors suggest that the new system of nomenclature should consider the relative position on the genetic map (resistance cluster), the genotype of the pathogen (isolates or race), and the bean genotype in which the resistance gene was identified. The regions containing anthracnose resistance genes would be named following the classical nomenclature based on the Co-genes: clusters Co-1 (Pv01), Co-2 (Pv11), Co-3(Pv04), Co-4 (Pv08), Co-5 (Pv07), Co-13 (Pv03), and Co-u (Pv02). The specific resistance gene included in each resistance cluster would be named, using a superscript indicative of the isolate or race and the bean genotypes in which the gene was identified. For example, the genes conferring resistance to isolates CL18 and CL63 (races 38 and 6) in the Cornell 49242 bean genotype located in the relative position of Co-2 cluster would be named as genes $Co\text{-}2^{38\ CN}$ and $Co\text{-}2^{6\ CN}$, respectively. The gene conferring resistance to isolate CL18 (race 38) in the Widusa bean genotype located in the relative position of the Co-3 cluster (Rodríguez-Suárez et al. 2008) would be named as $Co\text{-}3^{38\ WD}$ and variety abbreviations would be provided.

Future Prospects

Genomic sequence projects and analysis of expressed sequence tags (ESTs) are providing an enormous enrichment of databases (http://phaseolusgenes.bioinformatics.ucdavis.edu/; Langridge and Fleury 2011). Advances in "omics" technologies are also providing new tools for breeders and geneticists such functional molecular markers or fine genetic maps. Functional markers (FMs) derived from polymorphic sites within gene-coding sequences causally affecting phenotypic trait variation are more reliable for identification and selection of

favorable alleles, as absence of recombination between marker and target locus increases the diagnostic power and eliminates marker-based miss-selection. To date, only a limited number of genes have been isolated for FM development in common bean, but considerable efforts are being carried out in bean genomic and transcriptomics. Bean genotypes G19833 and BAT93 have been sequenced (http://www.phytozome.net/commonbean_er.php). In consequence, numerous FM for important traits may become available over the next decade. Polymorphisms among alleles for genes of interest may be due to single nucleotide polymorphisms (SNPs, CAPs, dCAPS), small insertions or deletions (Indels), partial or complete loss of the gene, and on some occasions due to different numbers of repeat motifs within simple sequence repeats (SSRs). FM have advantages over other markers because they are completely linked to the desired trait allele. The future availability of FM for anthracnose resistance genes will offer the opportunity to identify new alleles or other resistance genes by exploring germplasm, tagging specific alleles that confer resistance to anthracnose for use in MAS or genetic analyses, and through detailed analysis of the molecular mechanism involving in the response(s) of *C. lindemuthianum* to common bean. The present challenge is to correlate phenotypes with gene discovery and coding regions at the molecular level, and to explore the variation and genetic interaction between genes to further enhance anthracnose resistance in future bean cultivars worldwide.

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Chapter 10

Enabling Tools for Modern Breeding of Cowpea for Biotic Stress Resistance

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Abstract

This chapter summarizes recent advances in genomic resources, technologies, breeding platforms, and molecular markers that are being used to expedite delivery of cowpea cultivars with resistance to important biotic stresses. The genomic resources available in cowpea include high-throughput single nucleotide polymorphism (SNP) genotyping platforms, a high-density consensus genetic map with more than 1,100 markers, a whole genome sequence assembly, bacterial artificial chromosomes (BACs), and a physical map anchored to the genetic map. Markers linked to important biotic resistance traits, including resistance to foliar and flower thrips, Fusarium wilt, root-knot nematode, bacterial blight, ashy stem blight (*Macrophomina*), *Striga*, and viruses are described, together with tools developed to integrate SNP genotypes, genetic maps, and trait information to guide breeding. A genetic transformation system has been developed for cowpea and breeding lines developed with resistance to cowpea weevil and Maruca pod borer. Some examples of initial work in evaluating and optimizing marker-assisted backcross and marker-assisted pedigree breeding are described. Major challenges that must be overcome to allow for the adoption of modern marker-assisted breeding (MAB) in cowpea include human and precision phenotyping capacity issues. Further, as essentially all cowpea breeding is being conducted by the public sector with modest resources, efficient strategies are needed to minimize running costs.

Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is perhaps the most widely adapted, versatile, and nutritious grain and fodder legume for warm and dry agro-ecologies of the tropics and subtropics. Worldwide there are more than 14 million ha of cowpea being cultivated, of which about three-fourths of the area and two-thirds of the production occur in the semi-arid Sudan savanna and Sahelian zones of the African continent running from west to east, south of the Saharan desert, and in more dispersed but extensive pockets of similar agro-ecologies in eastern and southern Africa.

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Substantial quantities of cowpea also are produced in similar agro-ecologies in South America, with Brazil being the second-largest producing country in the world after Nigeria. Although of less importance today than in the recent past, cowpea is also grown in the southern and western parts of North America and is a 'traditional' crop of several indigenous tribes of lower-elevation central Mexico. A form of cowpea (subspecies *sesquipedialis*) known as 'long bean' or 'asparagus bean' is cultivated as a vegetable crop throughout East Asia for its long fleshy green pods that can be one meter in length. Long bean is fully fertile with other cultivated and some wild subspecies of cowpea and comparisons of genetic maps show essential identity (Xu et al. 2010). Cowpea was documented from before Roman times in Europe and the presence of significant phenotypic and genotypic diversity exists among landrace germplasm from Italy (Tosti and Negri 2002) and other southern European countries, suggesting a long history of use in this region. As a general rule, cowpeas are grown in hotter low-elevation equatorial and subtropical areas, often being replaced by common bean (*Phaseolus vulgaris L.*) at altitudes above 1,300 m, although cowpeas are grown at altitudes up to 1,500 m in Kenya. Commercial production of the crop extends as far north as 40° latitude in California, and experimental plantings of early maturing breeding lines have been successful as far north as Minnesota (45° N latitude) in the United States (Davis et al. 1986).

While cowpea is both responsive to favorable growing conditions and tolerant to drought, high temperatures, and poor soils (Hall 2004; Fery 1990), biotic stresses from pests and diseases inflict heavy losses and are key factors underlying why on-farm cowpea yields of traditional varieties in Africa are 3- to 5-fold lower than potential yields (Ehlers and Hall 1997).

Development of cowpea cultivars that resist or tolerate biotic stresses would result in dramatic yield improvements. Breeding resistant cultivars is a particularly desirable strategy for this crop because it is grown mostly by resource-poor farmers, many of whom are women who lack access to capital for application equipment, pesticides, and protective wear, as well as to expertise in the efficacious and safe use of these products.

Breeding for resistance to biotic stresses has been undertaken by the International Institute for Tropical Agriculture (IITA) and by African National Research Systems (NARS), in some cases supported by the USAID Collaborative Research Support Program (CRSP) and other donors. These breeding programs have achieved a number of successes in the last 20 years, having bred cultivars resistant to some of the key pests such as cowpea aphid, cowpea weevil, the parasitic weed *Striga gesneroides,* bacterial blight, root-knot nematodes, and cowpea aphid-borne mosaic virus (CABMV) (Hall et al. 1997; Hall et al. 2003; Timko et al. 2007) and thereby contributing to raised on-farm yields. These breeding efforts employed wholly conventional breeding approaches and typically took more than ten years from concept and crossing to release.

Similar to breeding programs in other crops, the need to employ sequential and repeated phenotypic evaluations and performance trials is the most resource-intensive and time-consuming aspect of the process. In many cases, these evaluations require complex, specialized conditions, techniques and expertise to assess phenotypes for selection. These evaluation resources are difficult to assemble and costly to operate, and in many cases it is not possible to conduct a complete breeding program consisting of teams of scientists of all necessary disciplines because of budget and manpower limitations for 'orphan' crops (Delmer 2005) such as cowpea. Selection based on molecular markers linked to key biotic resistance traits or quantitative trait loci (QTL) can be used to reduce the phenotyping burden typically required through conventional breeding efforts and thereby help speed the

delivery of improved varieties. Neutral, 'genetic background' molecular markers that are well distributed through the genome are also part of molecular marker-assisted selection (MAS) schemes such as marker-assisted backcrossing (MABC), where the goal is to reassemble the genetic background of the recurrent parent with the addition of one to a few target traits. Accordingly, MAS provides a powerful and potentially cost-saving avenue for increasing the rates of genetic gain in plant breeding programs (Xu and Crouch 2008).

In the recent past, cost and technology limitations meant that MAS was restricted to major crops and to one or a very few high priority traits, where phenotyping was costly or otherwise problematic. The development of relatively low cost high-throughput SNP genotyping platforms in many crops and the availability of high-density genetic linkage maps have greatly enhanced what is now possible with MAS. These capabilities mean new breeding strategies can now be considered that utilize molecular marker information at tens to thousands of points in the genome, encompassing selection for multiple traits and/or multigenic traits simultaneously. Selection targets include easy-to-phenotype agronomic and grain quality traits such as grain size, texture, and color, in addition to multiple biotic stress tolerance and resistance traits that are more difficult to phenotype.

The effectiveness of MAS breeding schemes depends on high quality phenotyping and precision genotyping for assembly of robust marker-trait associations and QTL estimates, closeness of marker-trait determinant linkages, and relative cost of genotypic selection compared to traditional selection protocols based on phenotypic selection. MAS can increase the efficiency of breeding in several other ways, through the selection of desirable progeny for crossing before flowering, providing year-round, environmentally independent selection capability, and through simultaneous selection of multiple traits and for a particular genetic background. In addition, it is possible to deduce useful marker-trait associations and marker effects in early generations of breeding cycles that can then be used for selection in later generations if genotypic information is available at the later stage.

Pioneering the Use of SSR Markers for Introgression of Striga Resistance

To date only limited deployment of molecular markers in cowpea breeding programs has occurred. MAS using simple sequence repeat (SSR, or microsatellite) markers to develop cowpea varieties resistant to the parasitic weed *Striga gesnerioides* (Striga) was initiated in 2006 by the national research organizations of Senegal, Burkina Faso, Nigeria, and Mali, and at IITA in Nigeria in collaboration with the University of Virginia (Timko et al. 2007). These projects have focused on the use of a limited number of SSR markers with the goal of introgressing Striga resistance into improved or local susceptible varieties. This approach eliminates the need for phenotypic evaluation of Striga resistance at each stage of the breeding process. Also, by incorporating resistance genes effective against multiple races of Striga prevalent across national boundaries, MAS eliminates the need for meeting quarantine requirements associated with the movement of Striga seeds of different races across national boundaries that would be required for centralized phenotypic evaluations of resistance. However, the approach uses only trait-linked 'foreground' markers, meaning that recovery of the recurrent parent 'background' genotype will occur at nearly the same rate as with conventional backcross breeding. SSR genotyping with numerous background markers on multiple individuals to facilitate recovery of the recurrent parent would be cost prohibitive. Another major drawback of MAS using SSR markers in backcross breeding is that high quality gels are difficult to produce consistently, thus

progress can be hampered by misclassified individuals. This is in contrast to single nucleotide polymorphisms, or SNPs, which show very high fidelity of allele calling in repeated rounds of genotyping.

SNPs in Cowpea Breeding for Biotic Stress Resistance

Genotyping platforms based on SNPs are preferred over other marker types in plant breeding applications because they are amenable to high-throughput yet highly precise assays (Rafalski 2002). SNPs derived from expressed sequence tags (ESTs) are especially useful because they are derived ultimately from mRNA transcripts and thereby reflect allelic differences between individuals and present opportunities for cloning genes through comparisons with annotated genome sequences of other plant species. SNP genotyping is highly developed from a technical and equipment perspective because of the large investments made in this area in human and animal genetics that are being leveraged for crop improvement. The private crop breeding sector was quick to grasp this opportunity and develop these systems for major crop plants such as maize and soybean, having employed them in breeding programs for more than a decade (Eathington et al. 2007). It appears that the private sector has found marker-assisted breeding (MAB) with SNPs to be an effective approach, as evidenced by the fact that SNP genotyping platforms continue to be developed for more and more crops bred in this sector. In cowpea, a 1536-Illumina GoldenGate SNP genotyping platform was developed in 2009 (Muchero et al. 2009) and is described briefly below.

High-Throughput SNP Genotyping Platform for Cowpea Breeding

Several key resources are needed for the comprehensive implementation of modern cowpea breeding. First among these is high-throughput genotyping capability, for rapid and dense fingerprinting of parents and breeding progenies at a reasonable cost. This multiplexing approach is a critical advance over low-throughput systems where genotyping is conducted on a marker-by-marker, individual-by-individual basis. For cowpea, a high-throughput genotyping platform was developed between 2007 and 2009 as an important outcome of a 'Tropical Legumes I' project based at the University of California, Riverside, and funded by the Consultative Group on International Agricultural Research (CGIAR) Generation Challenge Programme (GCP). The genotyping platform was developed as an Illumina GoldenGate Assay for 1536 SNP loci. This platform can genotype simultaneously 96 DNA samples at 1,536 SNP loci. The genotypic information is provided to users with specialized 'Illumina GenomeStudio' software, which allows rapid visual inspection and data summarization. The development and technical details of this platform were presented in detail in the work of Muchero and colleagues (2009). Briefly, this platform consists of 1,536 EST-derived SNPs chosen from a selected subset of about 10,000 SNPs identified from comparisons of 183,000 EST sequences from 13 cowpea genotypes. Of these, 1,375 SNPs performed technically well in the assay. Among the several technical considerations in choosing the 1,536 SNP subset, high polymorphic information content among African germplasm was primary, in order to ensure that the chosen SNPs were highly polymorphic in African breeding materials. Examples of the numbers of polymorphic markers observed in crosses between breeding lines both within and between several African breeding programs following genotyping with this platform are shown in Table 10.1. These results show a high average level of polymorphic markers between any two parents, ranging from 134 to 391 and indicating that the SNPs selected for inclusion on the current 1,536 Illumina GoldenGate platform represent an effective set for MAS in West African breeding programs.

Table 10.1. Examples of the number of SNP markers that are polymorphic between two parental lines of potential combinations for intra- (e.g., IITA x IITA) and inter- (e.g., IITA x INERA) breeding program crosses.

Parent 1	Parent 2	Cross Type	No. Polymorphic markers
IT84S-2246	IT93K-503-1	IITA x IITA	134
IT89KD-288	Suvita-2	IITA x INERA	245
KVx 525	KVx 396-4-5-20	INERA x INERA	257
KVx 442-3	KVx 396-4-5-20	INERA x INERA	261
Suvita-2	Melakh	INERA x ISRA	274
IT97K-499-35	Mouride	IITA x ISRA	289
Suvita-2	Calif. Blackeye No. 27	INERA x UCR	291
IT84S-2246	IT00K-1263	IITA x IITA	294
IT97K-499-35	IT00K-1263	IITA x IITA	309
KVx 525	Bambey 21	INERA x ISRA	313
Bambey 21	Melakh	ISRA x ISRA	325
IT84S-2246	IT82E-18	IITA x IITA	329
KVx61-1	Calif. Blackeye No. 50	INERA x UCR	339
Melakh	Calif. Blackeye No. 27	ISRA x UCR	343
Yacine	Calif. Blackeye No. 50	ISRA x UCR	345
IT84S-2246	Mouride	IITA x ISRA	347
IT89KD-288	Calif. Blackeye No. 50	IITA x UC R	372
IT93K-503-1	Bambey 21	IITA x ISRA	376
IT82E-18	KVx 525	IITA x INERA	381
IT93K-503-1	Calif. Blackeye No. 27	IITA x UCR	391

High Quality Consensus Genetic Map

Breeders have often conducted their marker breeding programs based on map information derived from a single cross. Two major limitations of using a single-cross map for the analysis of marker or gene segregation in a population are (1) the limited number of traits of economic importance that will segregate in any one population and (2) the limited number of polymorphic markers. Hence, a second key resource needed for modern cowpea breeding is a high quality consensus genetic map. Consensus maps are formed from merging multiple individual linkage maps. By the term 'high quality' we mean marker positions determined with precision and of sufficient density across the genome. Merging markers from several populations increases marker density and the precision of marker position estimation, as well as the quantity of mapped traits. Consensus genetic linkage maps provide breeders with a resource for analyzing the inheritance of traits and marker-trait associations needed for marker-assisted breeding. The usefulness of a genetic linkage map to plant breeders is determined largely by its thoroughness of genome coverage, with markers that are highly polymorphic in target breeding germplasm.

The first version of the cowpea consensus genetic linkage map was the product of merging individual genetic maps built from 6 recombinant inbred line (RIL) populations that had been genotyped with the 1,536 Illumina GoldenGate Assay (Muchero et al. 2009). This map included 928 markers spanning 11 linkage groups over a total map distance of 680 centiMorgans (cM). Map refinement has continued since then, with the genotyping of five additional RILs and two F_4 breeding populations, construction of individual maps for these populations, and integration of these maps into an improved consensus map (Figure 10.1) (Lucas et al. 2011). These new map inputs resulted in improved marker order and density and fewer gaps, with the addition of 179 SNP markers (bringing total markers to 1,107) with an average distance between markers of about 0.6 cM. Further

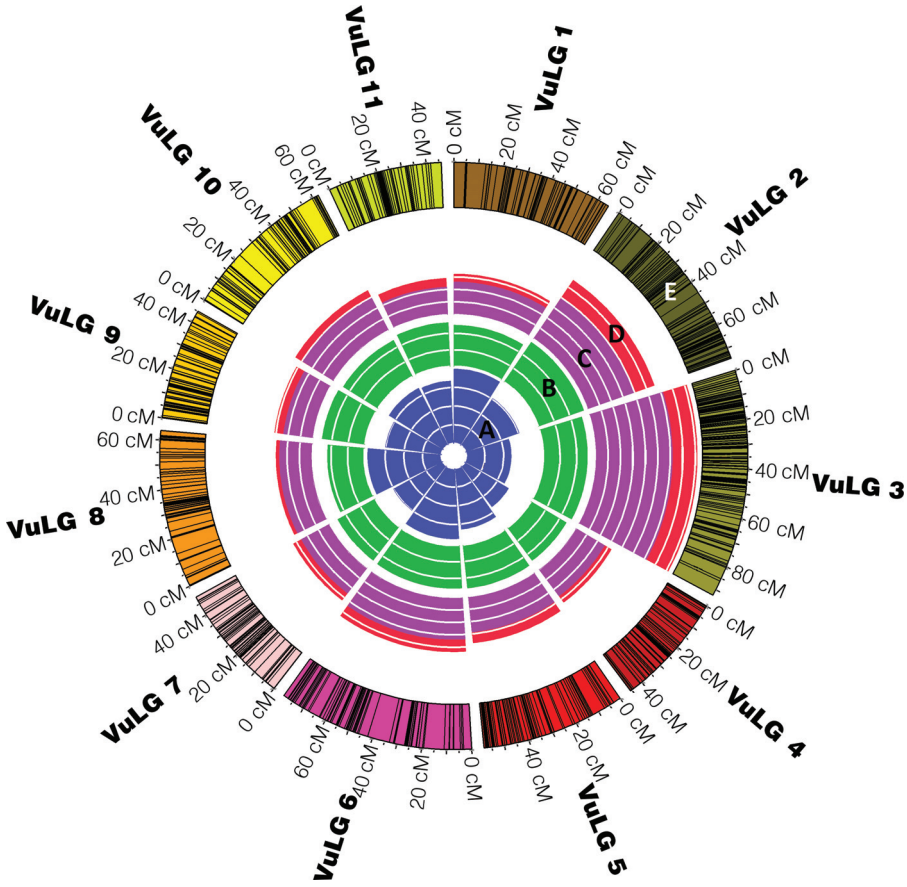

Fig. 10.1. Consensus genetic map of cowpea and parameters depicting map characteristics (Lucas et al. 2011). (A) Average distance between bins (0.25 cM). (B) Average number of markers per bin (0.5 units). (C) Number of bins (25 units). (D) Number of markers (25 units). (E) Bin locations. C and D begin at the same radial position. For a color version of this figure, please refer to the color plate.

refinements since then have been achieved through comparison with the common bean whole-genome assembly and a partially assembled cowpea whole-genome assembly. The current SNP-based cowpea consensus genetic linkage map is included in a publicly available browser called HarvEST:Cowpea, which can be downloaded as Windows software from http://harvest.ucr.edu, or operated through an online portal at www.harvest-web.org. This map will continue to be updated periodically as additional genotyping and other types of information become available. With this current consensus map, discovery of useful trait-linked and genetic background markers in cowpea can be made with a high probability of success by phenotyping for any trait segregating in any of the 11 RIL populations and overlaying the existing genotypic data.

Fingerprinting of Cultivars and Other Prospective Parents

Genotyping a large sample of current varieties and advanced breeding lines with a platform of mapped markers is valuable and convenient for several applications. For breeding, this information supplies quick decision making for the selection of lines for crossing, while a database of information on parental genotypes, coupled

with historical phenotype information, can result in the discovery of haplotypes associated with traits. These genotyping profiles can also lead to the discovery of duplicates and help uncover hidden, accumulated 'off-types' in breeding programs (Lucas et al. 2013). In work conducted by the Tropical Legumes Project, ~600 cultivars, breeding lines, and accessions were genotyped with the 1,536 Illumina GoldenGate Assay. This SNP fingerprinting information was combined with map coordinates from the consensus map described earlier, providing a rich resource for breeding and haplotype analysis, as discussed in the following sections.

Biotic Stresses and Genetic Marker Resources

Until recently, finding trait-linked molecular markers for cowpea breeding was slow and laborious. Now, with the availability of the high-throughput SNP genotyping platform and the consensus genetic map, coupled with extensive phenotyping of RIL populations and panels of germplasm within the last five years, SNP markers or QTL have been identified for a range of biotic stress-resistance traits and examples are presented in Table 10.2. Among these are trait determinants for resistance to foliar thrips (*Thrips tabaci*) (Lucas et al. 2012), flower thrips (*Megalurothrips sjostedti*) Trybom (M. Lucas et al., unpublished data), ashy stem blight (*Macrophomina phaseolina*) (Muchero et al. 2011), bacterial blight (*Xanthomonas axonopodis* pv. *vignicola*) (Agbicodo et al. 2010), root-knot nematodes (*Meloidogyne* spp.) (Roberts et al. unpublished data), and to Fusarium wilt (*Fusarium oxysporium* Schlechtend.:Fr. f. sp. *tracheiphilum* (E. F. Smith) W. C. Snyder & Hansen) (Pottorff et al. 2012a; 2012b). Additional information on these biotic stresses and their associated SNP markers is presented in Table 10.2 and the following sections.

Several bacterial and fungal diseases can significantly affect cowpea production, and their incidence and severity vary with the amount and distribution of rainfall (Williams 1975). Septoria leaf spot (*Septoria vignae* P. Henn and *S. vignicola* Vasat Rao, S. Kozopolzanii Nikolajeva), scab (*Sphaceloma* sp.), brown blotch (*Colletotrichum capsici* (Syd.) Butler and Bisby and *C. truncatum* (Schwein.) Andrus & W. D. Moore), and cercospora leaf-spot are important in the Guinea savanna, while cercospora leaf-spot, bacterial blight, and ashy-stem blight (or charcoal rot) caused by *Macrophomina phaseolina* (Tassi.) Goid. are important in the Sudan savanna, and ashy stem blight in the Sahel. Scab, cercospora leaf spot, powdery mildew (*Erysiphe polygoni* DC.), Fusarium wilt, and ashy stem blight are significant diseases in Brazil (Lin and Rios 1985). To date, molecular markers have been developed only for a few of these diseases, and this section will focus on those with near-term prospects for MAS.

Bacterial blight is a major disease of cowpea affecting most production areas. Severe outbreaks occur regularly and can completely devastate crops when environmental conditions are conducive to disease development. Earlier inheritance studies suggested resistance was simply inherited (Patel 1985). More recently molecular markers have become available that could be valuable for MAB. Agbicodo and colleagues (2010) identified three SNP-based QTLs, *CoBB-1*, *CoBB-2,* and *CoBB-3,* on linkage groups LG3, LG5, and LG9, respectively (Lucas et al. 2011), which co-segregated with resistance to two Nigerian strains of bacterial blight, *Xav18* and *Xav19*. *CoBB-1*, *CoBB-2,* and *CoBB-3* explained 22.1, 17.4, and 10% of the genetic variation in the RIL discovery population. SNP markers linked to the QTL include 1_0853 on LG3, 1_0037 on LG5, and 1_1202 on LG9. Validation of these markers in an actual breeding program and across multiple populations remains to be done.

In semi-arid agro-ecologies, the soil-borne fungus *Macrophomina phaseolina* (Tassi.) Goid. causes ashy-stem blight of cowpea (known in soybean as charcoal rot). This ubiquitous disease is exacerbated under drought conditions.

Table 10.2. Trait-linked SNP markers for marker-assisted breeding for biotic resistances. Marker positions are based on the consensus genetic map (Lucas et al. 2011). Details of trait-linked SNPs are described in the text. SNP markers for virus resistance are located near AFLP markers (Ouédraogo et al. 2002a) linked to the trait.

Marker	LG	Position (cM)	Trait
1_0277	2	18.92	Foliar Thrips
1_0589	2	18.92	Foliar Thrips
1_0698	2	18.92	Foliar Thrips
1_0829	2	19.45	Foliar Thrips
1_0492	2	19.53	Foliar Thrips
1_0253	2	20.16	Foliar Thrips
1_0337	2	20.35	Foliar Thrips
1_0164	2	21.00	Foliar Thrips
1_1086	2	22.96	Foliar Thrips
1_0284	2	25.15	Foliar Thrips
1_1406	2	26.12	Foliar Thrips
1_1139	2	26.86	Foliar Thrips
1_1061	2	29.22	Foliar Thrips
1_1048	2	29.78	Foliar Thrips
1_0482	2	49.92	Macrophomina
1_0709	2	67.32	Macrophomina
1_0071	3	9.28	Cowpea mosaic virus
1_0080	3	9.91	Cowpea mosaic virus
1_0853	3	10.82	Bacterial Blight
1_0853	3	10.82	Macrophomina
1_0604	3	64.78	Macrophomina
1_0345	3	77.55	Cowpea severe mosaic virus
1_0964	3	77.55	Cowpea severe mosaic virus
1_1413	4	18.38	Foliar Thrips
1_0774	4	20.16	Foliar Thrips
1_1221	4	20.16	Foliar Thrips
1_1242	4	20.72	Foliar Thrips
1_1242	4	20.72	Foliar Thrips
1_0678	4	27.60	Macrophomina
1_0804	4	40.51	Macrophomina
1_0032	5	45.28	Macrophomina
1_0037	5	49.10	Bacterial Blight
1_0919	6	25.68	Blackeye cowpea mosaic potyvirus
1_1496	6	26.10	Blackeye cowpea mosaic potyvirus
1_0897	6	47.25	Fusarium Wilt
1_1363	6	47.41	Fusarium Wilt
1_1202	9	0.00	Bacterial Blight
1_1137	9	42.46	Striga
1_0276	9	42.84	Striga
1_0958	9	42.84	Striga
1_0840	10	43.86	Foliar Thrips
1_0161	10	44.86	Foliar Thrips
1_0754	10	45.04	Foliar Thrips
1_0281	10	46.25	Foliar Thrips
1_1453	10	46.55	Foliar Thrips
1_0354	10	46.70	Foliar Thrips
1_0952	10	47.26	Foliar Thrips
1_1062	10	47.26	Foliar Thrips
1_1041	10	47.63	Striga
1_0003	10	49.05	Striga

Muchero and colleagues (2011) identified nine quantitative trait loci (QTLs) that accounted for between 6.1 and 40.0% of the phenotypic variance (R^2). Maturity effects on the expression of resistance were indicated by the co-location of *Mac-6* and *Mac-7* QTLs with maturity-related senescence QTLs *Mat-2* and *Mat-1*, respectively. The multigenic nature of resistance and interaction with crop maturity will complicate the use of these markers in MAB, but may lead to greater precision in identifying resistant individuals in segregating populations. As breeding targets, combinations of pairs of QTLs, such as *Mac-2* and *Mac-5*, can provide significant protection from this disease (Muchero et al. 2011). Efforts are under way to combine QTL for Macrophomina resistance and drought tolerance for cowpea protection in rain-fed, drought-prone production areas (Muchero et al. 2011).

Cowpea rust caused by *Uromyces vignae* is a severe problem in humid ecologies across Asia and Africa that is best managed by the use of resistant varieties (Emechebe and Shoyinka 1985). Li and colleagues (2007) reported the identification of an amplified fragment length polymorphism (AFLP) marker converted to a sequence characterized amplified region (SCAR) marker for resistance to *Uromyces vignae* in China.

Fusarium oxysporum f.sp. *tracheiphilum* (Fot) is a soil-borne fungal pathogen that causes vascular wilt disease in cowpea. Fot race 3 is one of the major pathogens affecting cowpea production in the subtropical regions where the crop is grown. Resistance to Fot race 3 was studied in the RIL population 'California Blackeye 27' (resistant) x 24-125B-1 (susceptible). Biparental mapping identified a Fot race 3 resistance locus, Fot3-1, which spanned 3.56 cM on linkage group 1 of the CB27 × 24-125B-1 genetic map. A marker-trait association narrowed the resistance locus to a 1.2 cM region and identified SNP marker 1_1107 as co-segregating with Fot3-1 resistance (Pottorff et al. 2012a). Two QTLs governing resistance to Fot race 4, which attacks cowpeas with Fot race 3 resistance, have also been identified using other cowpea mapping populations developed from crosses between resistant and susceptible parents (Pottorff et al. 2012b).

Cowpea is attacked by a wide spectrum of other fungal diseases. Those of major importance include anthracnose (*Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib.), cercospora leaf spot (*Cercospora canescens* Ellis & G. Martin), web blight (*Rhizoctonia solani* Kuhn), ascochyta (*Ascochyta phaseolorum* Sacc.), brown blotch (*Colletotrichum capsici* (Syd.) Butler and Bisby and *C. truncatum* (Schwein.) Andrus & W. D. Moore), and powdery mildew (Emechebe and Shoyinka 1985). Provision of molecular markers for resistance to these diseases should be an important goal for cowpea breeding programs in areas where these organisms are problematic.

Markers for Resistance to Viruses

Development of virus-resistant cultivars is an important objective of several cowpea breeding programs in Africa and in Brazil. The main seed-borne viruses affecting cowpea production include cowpea aphid-borne mosaic virus (CABMV), blackeye cowpea mosaic virus (BlCMV), cucumber mosaic virus (CMV), cowpea mosaic virus (CPMV), cowpea severe mosaic virus (CPSMV), southern bean mosaic virus (SBMV), and cowpea mottle virus (CMV). Cowpea golden mosaic virus (CGMV) and cowpea chlorotic mottle virus (CCMV) are important non-seed-borne viruses. The seed-borne viruses are particularly damaging because they spread via infected seed. Seed-borne infections can result in early season spread of the virus to neighboring plants. Sources of resistance to all of these viruses have been identified, and cultivars with resistance to several viruses have been developed at IITA (Singh 1993). The presence in Senegal and Nigeria of different strains of CABMV that have overcome resistance complicates breeding efforts, but breeding lines have been developed that are resistant to multiple strains of this virus as well as to other viruses. IT86D-880,

developed at IITA, is exceptional in that it is resistant to four strains of CABMV, cowpea yellow mosaic virus (CYMV), and cowpea mild mottle virus (CMMV). A severely damaging disease of cowpea known as 'stunt' is caused by a dual infection of BlCMV and CMV (Kuhn 1990). Ouédraogo and colleagues (2002a) were able to map determinants of resistance to CPMV, CPSMV, B1CMV and SBMV in the RIL population 524B x IT84S-2049 using AFLP markers. Subsequently, this population was genotyped with the 1,536 Illumina GoldenGate Assay allowing for the localization of SNP markers for these resistance loci (Table 10.2).

Markers for Resistance to the Parasitic Weed Striga

The parasitic weed *Striga gesnerioides* (Wild.) Vatke causes considerable damage to cowpea in the Sudan savanna and Sahelian regions of Africa. Several sources of resistance to this parasite have been identified and resistance has been incorporated using conventional approaches into advanced breeding lines by IITA (Timko et al. 2007) and by Cissé and colleagues (1995) into cultivars for Senegal (Hall et al. 2004). Striga exhibits strain variation such that cultivars that are resistant in one location may be susceptible in another (Lane et al. 1993). Genetic studies have shown that three dominant, non-allelic genes confer resistance to different Striga isolates but that the mechanisms differ (Singh 1993).

Useful markers for implementation of MAS are available only for some of the *Striga* resistance genes, and these were the first candidates for broad application in cowpea breeding programs. Ouédraogo and colleagues (2002a, 2002b) found three AFLP markers linked to *Rsg2-1*, a gene that confers resistance to *Striga* Race 1 (SG1) present in Burkina Faso, and six AFLP markers linked to gene *Rsg4-3* a gene that provides resistance to *Striga* Race 3 (SG3) from Nigeria. Two of the AFLP markers were associated with both *Rsg2-1* and *Rsg4-3*. Ouédraogo and colleagues (2002a) were able to convert one of these markers to a SCAR (sequence characterized amplified region) that has proven to be an effective and remarkably reliable marker for resistance to *Striga* SG1 and SG3 conferred by *Rsg2-1* and *Rsg4-3*. This SCAR marker, designated 61R (E-ACT/M-CAA), detects a single polymorphic band linked to SG1 and SG3 resistance in the resistant cultivars B301, IT82D-849, and Tvu 14676, and is being tested for use in breeding trials. Subsequently, two AFLP markers were identified that are closely linked to *Rsg1-1*, a gene that also confers resistance to SG3 in Nigeria (Boukar et al. 2004). One of those AFLP markers, designated E-ACT/M-CAC_{115} and determined to be 4.8 cM from *Rsg1-1*, was converted to a SCAR marker for ease of use in breeding programs (Boukar et al. 2004). Because the mapping population 524B x IT84S-2049 used in the studies undertaken by Ouédraogo and colleagues (2002b) and Boukar and colleagues (2004) was genotyped with the 1,536 Illumina GoldenGate array, we were able to identify SNP markers corresponding to the AFLP markers identified earlier (Table 10.1).

Markers for Resistance to Cowpea Aphid

Cowpea aphid (*Aphis craccivora*) is one of the most destructive pests of cowpea in Africa and worldwide. Myers and colleagues (1996) reported the identification of an RFLP (restriction fragment length polymorphism) marker linked to the resistance to cowpea aphid, but MAS for aphid resistance was not implemented. More recently we have identified SNPs associated with aphid resistance in California in an RIL population developed from the cross 'California Blackeye 27' (susceptible) x IT97K-556-6 (resistant). IT97K-556-6 has also been shown to be resistant to cowpea aphid in Texas and Nigeria (B. B. Singh, personal communication, 2010), but its efficacy relative to possible aphid

biotype differences is unknown in other parts of Africa.

Markers for Resistance to Other Insects

Insect resistance is a good candidate for MAS in cowpea since assessments of host plant resistance to insects are often difficult to conduct in the field or greenhouse. However, other than for aphid, insect-resistance factors in cowpea discovered thus far do not provide immunity to the pest and often have low heritability under field conditions. Field screenings that rely on natural insect infestations are subject to natural fluctuations in pest pressure. When such variability is combined with incomplete resistance, field screens can lead to misclassification and selection of lines lacking the strongest resistance. For example, this has been the case with screening cowpea breeding lines and accessions for resistance to Lygus bug (*Lygus hesperus*) and pod sucking bugs (such as *Nezara viridula, Clavigralla tomentosicollis, Riptortus dentipes*). In addition, colonies of insects may be difficult to rear without specialized facilities and trained entomologists to monitor insect growth and screening tests. Such resources have not been available to cowpea breeding programs historically. Resistance to the pod bug *C. tomentosicollis* has been identified in the wild cowpea (ssp. *dekindtiana*) germplasm line TVNu 151 (Koona et al. 2002). MAS could be used to introgress resistance factors from such wild cowpea into cultivated forms using a rapid backcrossing approach, based on simultaneous selection for the resistance genes (markers) and against markers associated with unwanted wild germplasm characteristics such as small seed size and pod shattering.

The cowpea storage weevil (or cowpea Bruchid) (*Callosobruchus maculatus* Fabricius) is a devastating pest of stored cowpea in Africa, Asia, and the Americas. Resistance to the cowpea storage weevil has been identified (Singh and Jackai 1985) and incorporated into a number of breeding lines and cultivars by IITA, using conventional breeding approaches (Singh 1993), and by ISRA (Institut Sénégalais de Recherches Agricoles) (Cissé et al. 1995). The resistance provides effective protection from weevil damage for about two months, but levels of damage in resistant cultivars approach that found in susceptible cultivars after six months of infestation. This resistance is useful in developing countries but when used alone provides insufficient protection to ensure that the grain retains its market value and seed quality characteristics. It would be helpful to identify SNP markers for resistance to cowpea weevil and to look for options that provide higher levels of protection.

Genetically-Modified Cowpea for Control of Maruca Pod Borer and Cowpea Weevil

Cowpea was one of the last major grain legume species for which genetic transformation was achieved (Popelka et al. 2006). This technology now presents an additional option for addressing constraints that have not yielded to other approaches.

Maruca pod borer (*Maruca testulalis*) is one of three major insect pests affecting cowpea production in Africa and parts of Asia (Singh et al. 1996). Major efforts to screen both wild and cultivated cowpea for resistance to this pest have not revealed meaningful levels of resistance. Effective transgenes have been identified for control of cowpea weevil and the Maruca pod borer, and these genes have been used to transform cowpea (Higgins et al. 2012). 'Bt cowpea' is currently undergoing yield and efficacy testing in confined yield trials in Burkina Faso and Nigeria. Since the distribution of wild cowpea species overlaps with areas of cowpea cultivation in Africa and since many of these wild cowpeas can form fertile hybrids with cultivated cowpeas, the escape of the 'Bt gene' into wild cowpea is a probable outcome of release of *Bt*-cowpea. An expert panel formed to address this 'geneflow' issue

and other Bt-cowpea safety concerns concluded that the gene would escape into the wild, but that the chance of negative environmental consequences would be negligible (Murdock, personal communication, 2012). Efforts have been initiated in several West African countries to use the marker-assisted breeding tools cited in this chapter to introgress the *Bt* gene into locally adapted varieties. If this MABC effort is successful and effective seed delivery and resistance management plans can be implemented, *Bt*-cowpea can help dramatically increase grain yields of cowpea in regions of Africa with Maruca infestations, while reducing insecticide inputs and the negative health risks and environmental damage associated with insecticide use.

The most important pest of stored cowpea is the bruchid beetle, or cowpea weevil. Even low initial infestation rates can cause significant grain damage because of the high fertility and short generation periods of this pest. As noted above, only low to moderate levels of resistance have been found, which provide only limited storage protection (Murdock et al. 2008). Genetic engineering has been used to transfer the gene coding for the α-amylase inhibitor αAI-1, a bruchid resistance factor from the common bean, into cowpea (Solleti et al. 2008). The α-amylases, the target of αAI-1, are key enzymes for starch digestion and vital for bruchid development; transformed cowpea carrying αAI-1 exhibits near immunity to the cowpea weevil (Solleti et al. 2008). However, food safety concerns have constrained the development and release of this valuable product.

Tools and Genetic Resources for Cowpea Breeders

A computer program called 'BreedIt© SNP Selector' (www.BreedIt.org) has been developed at the University of California, Riverside, which integrates fingerprint information, marker-trait association information, and the genetic map. It allows breeders to choose a set of custom markers based on markers that are polymorphic between parental lines, marker interval distance, and marker association with target traits.

Eleven RIL populations have been described in the international cowpea community. Most populations were developed with the goal of mapping specific high priority traits, and these population resources are described in Table 10.3.

Challenges for Adoption of Modern Breeding Tools in Cowpea Improvement

A suite of prospective molecular markers and genomic tools for modern breeding are now available in cowpea. Will these resources be

Table 10.3. Cowpea RIL populations available for marker-trait association and partial list of biotic stress resistance traits segregating.

Population	Individuals	Segregating biotic traits
CB27 x IT97K-556-6	92	Cowpea aphid
CB27 x IT82E-18	160	Foliar thrips
CB27 x UCR 779	56	Cowpea aphid
CB46 x IT93K-503-1	114	Macrophomina, foliar thrips
524B x IT84S-2049	85	Multiple, described in text
Dan Ila x TVu-7778	79	Bacterial blight
Yacine x 58-77	97	Flower thrips
Sanzi x Vita 7	122	Flower thrips
TVu14676 x IT84S-2246-4	136	Striga
CB27 x 24-125B-1	87	Root knot nematode
LB30#1 x LB1162 #7	90	Rust and powdery mildew

used to develop improved varieties in a cost-effective manner? Major challenges constraining adoption include lack of capacity in the use of these tools, motivation on the part of breeders to learn these technologies and then make the necessary changes in the general workflow and cost structure of their breeding programs to accommodate MAB activities, and realizing a favorable cost/benefit ratio compared to conventional breeding. Part of achieving cost-effectiveness will be to develop breeding strategies that minimize the amount of genotyping while maximizing overall marker information. Almost all cowpea breeding programs worldwide are public enterprises. Thus, the question arises as to who will absorb the costs of capacity building and, in some cases, infrastructure development for these efforts. With the increased potential of MAB there are costs beyond those for genotyping. These include learning costs for the implementation of new breeding plans and methods. More detailed planning and logistics are needed, as well as new ways of operating, greater design complexity, including knowledge about genotyping, marker-trait association, marker platforms, and a several-fold increase in the amount of genotypic and phenotypic data that need to be managed effectively. Training in modern cowpea breeding must be a key future activity, since so few breeders are prepared now to utilize effectively all the new tools of modern breeding. Cowpea breeders have developed a number of cultivars with 'stacked' resistance for traits such as resistance to cowpea weevil, cowpea aphid, and Striga, along with resistances to bacterial blight, CABMV, and other pathogens. The challenge is to incorporate all of these desirable traits into individual cultivars with acceptable grain quality and adaptation to a diverse array of farming systems and environments. MAB can be a key enabling tool to realize this goal.

Phenotyping

Accurate phenotyping for biotic stress resistance or tolerance is central to success with modern breeding as well as with conventional breeding. Inaccurate or failed phenotyping negatively impacts modern breeding programs more than conventional breeding programs, in that poor phenotyping outcomes consume resources in generating genotypic information that cannot be used effectively. SNP genotyping of cowpea is now straightforward and, from a technical standpoint, virtually error-free. For nearly all traits, phenotyping is, by comparison, much more difficult. Yet each process must be conducted with a level of precision adequate to capitalize on the information content of the other for modern breeding to be efficient and effective. Therefore, breeders must make the necessary infrastructure and time investments in phenotyping capability to ensure a correct balance consistent with the goals of the breeding program.

Improved phenotyping capability is needed in conventional as well as modern breeding programs, but the way in which it is applied may differ in the different types of breeding programs. A comprehensive review of classical cowpea breeding and phenotyping for critical biotic and abiotic production constraints that is still relevant was published by Hall and colleagues in 1997. New or modified protocols have been published more recently for phenotyping for resistance to bacterial blight (Agbicodo et al. 2010), rust (Li et al. 2007), ashy-stem blight (Muchero et al. 2011), and foliar thrips (Muchero et al. 2010).

MAS for Pyramiding Multiple Pest and Disease Resistance Traits

One of the major goals of most cowpea breeding programs is to combine resistances to numerous pests and diseases and other desirable traits such as those governing maturity, photoperiod sensitivity, plant type, and seed quality. Until recently these programs have lacked modern breeding tools and an array of elite parental lines that are fixed for most of the desired resistance factors. The elite lines can be used to recombine traits and generate lines with multiple pest and disease resistance, high yield, appropriate

maturity, and desirable seed quality. Now, different cultivars and advanced breeding lines are available with desirable traits, such as resistance to cowpea weevil, cowpea aphid, and Striga, along with resistances to bacterial blight, CABMV, and other pathogens. The challenge is to incorporate suites of these desirable traits into individual cultivars with acceptable grain quality and adaptation to a diverse array of farming systems and environments. The array of markers now available for biotic stress resistance and other traits will be very useful in expediting this process.

Outsourced Genotyping

A limitation of high-throughput genotyping systems has been the initial investment required for platform development, especially the sequencing costs related to SNP discovery. However, as sequencing costs have fallen dramatically over the last several years, this is becoming less an issue. Another consideration is the high cost of genotyping equipment and locating dedicated bench space and lab personnel for genotyping operations. On the positive side, several public and private 'fee-for-service' SNP genotyping providers are now available using a range of SNP genotyping platforms, so there is little need for cowpea breeding programs to invest in in-house genotyping infrastructure. In developing countries, issues related to availability and high costs of imported consumables and repairs make the case for out-sourced genotyping even more compelling. In our marker-assisted recurrent selection (MARS) breeding projects, genotyping was done via outsourcing from two African locations. Fresh leaf tissue disks from 6 plants per line were bulked, placed in 96-well plates provided by the genotyping service company, the plates were enclosed with silica gel packs (to dehydrate the tissue) inside a plastic bag, and mailed via a courier service to the genotyping provider. The samples were genotyped for a subset of markers polymorphic in each parental pair using the Sequenom platform after marker conversion from the Illumina platform. Genotypic information was received from the genotyping provider in about two weeks, which is considered normal turnaround time. Since we had genotyped the same lines with the same SNPs using Illumina GoldenGate Assay, we compared the genotyping results from the two assays and found identical genotype calls for all alleles of all individuals.

The Illumina GoldenGate Assay platform is a fixed array assay for 1,536 SNPs. This feature presents advantages in that data handling can be standardized and time and effort are not needed to choose subsets of markers for individual populations. However, the fixed array design means paying for assays on a large number of markers that are not informative between a given pair of parent genotypes. As a general rule, the fixed array sets will be less expensive on larger numbers of markers, but more expensive when fewer markers are being used, which is typically the case for genotyping a breeding population. However, in these calculations, the informatics costs related to choosing and analyzing specific marker sets for specific populations also must be considered.

In order to make an SNP genotyping platform more available, flexible, and affordable to the broader cowpea breeding community, the CGIAR Integrated Breeding Platform has contracted with KBiosciences (LGC Genomics) in the United Kingdom to convert a subset of ~1,000 of the mapped SNPs from the Illumina GoldenGate Assay Platform for use with the single-plex KBiosciences KASP (Kompetitive Allele Specific PCR) genotyping platform. This platform allows users to choose customized subsets of SNPs for genotyping on particular sets of germplasm. Hence a breeder can choose a suite of trait-linked polymorphic SNPs and well-distributed 'background' SNPs for genotyping, with the number chosen partially determined by budget considerations and the trade-off between numbers of markers and numbers of genotypes. For example, assume the breeder is working to pyramid four resistance traits for which SNP markers have been identified and which are

polymorphic in the target population. A suite of SNP markers for these resistance traits (flanking markers) would be chosen along with a group of evenly distributed polymorphic 'background' markers every 5 to 20 cM along each linkage group, depending on the desired marker density, available polymorphism, breeding strategy, and budget. Access to reliable, precise, and reasonably priced genotyping services will be essential if MAB is to become a routine methodology in cowpea breeding programs.

Summary and Conclusions

In less than seven years, cowpea has moved from the status of an 'orphan crop' (Delmer 2005) with only scarce genomic resources to a situation where modern breeding is being implemented. In the near future, other advanced breeding approaches such as genome-wide selection (Bernardo and Yu 2007) should be explored if these are shown to be effective in other crops. While cowpea breeding is poised to become much more effective with the new tools that are available, at the same time it is increasingly more complex. Significant increases in training and funding for developing country NARS cowpea breeding programs will be required for meaningful adoption of new genetic tools and acceleration of genetic improvement.

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Chapter 11

Disease Resistance in Chickpea

Teresa Millán, Eva Madrid, Muhammad Imtiaz, Mohamed Kharrat, and Weidong Chen

Abstract

Chickpea is a grain legume with valuable nutritional characteristics; it is a basic aliment in Asian countries such as India and Pakistan as well as a traditional ingredient in Mediterranean diets. Biotic stresses such as ascochyta blight and fusarium wilt, together with other diseases, such as botrytis gray mold and rust, are major constraints on stable chickpea production. Marker-assisted selection (MAS) is a tool that is significantly augmenting the efficacy and efficiency of chickpea breeding programs. This chapter reviews the current status and future potential of genomic tools for chickpea breeding aimed at countering biotic stresses. It also provides an overview of causal agents, host resistance inheritance, gene or quantitative trait locus (QTL) mapping, genetic resources, and progress in introgression of resistance genes to cultivated chickpea as well as integrated disease management.

Introduction

Chickpea (*Cicer arietinum* L.) is the only cultivated species within the genus *Cicer*. It is a self-pollinated, annual diploid ($2n = 2x = 16$) with a relatively small genome size (740 Mbp, Arumuganathan and Earle 1991). It is included in the tribe Cicereae within the Galegoid clade together with the tribes Viceae and Trifolieae (Choi et al. 2004). The accepted wild progenitor of chickpea (*Cicer reticulatum* Ladz.) was discovered and collected in an area of southeastern Turkey (Ladizinsky and Adler 1976).

This grain legume is used mainly for human consumption and is cultivated all over the world. India is the main producer, followed by Turkey and Pakistan, which contribute 67.5%, 7%, and 5%, respectively, of the total world production of 10.5 M tonnes (FAOSTAT 2011). On the basis of seed type, chickpea has been divided into two groups: pink to purple flowers and dark angular seeds are characteristics of the Desi type, while white flowers and large beige to white colored seeds mark the Kabuli type. These types differ also in agronomic traits such as growth habit and resistance/tolerance to biotic and abiotic stresses.

Compared to other legumes, chickpea is more tolerant to heat and drought, and it is considered an important low-input crop in cropping systems of arid and semi-arid regions around the world (Saxena 1987). Even though world chickpea yield has improved during the past decades (world average 905 kg/ha in 2009, FAOSTAT 2011) there are still major production constraints to overcome, particularly under current changing climatic conditions.

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MAS is a tool that could increase the efficacy and efficiency of chickpea breeding programs, accelerating development of new varieties adapted to different environmental conditions or market requirements. In recent years, improvement of genetic maps and identification of markers targeting genes or quantitative trait loci (QTLs) has progressed rapidly in chickpea. Thus, numerous sequence tagged microsatellite site (STMS) markers have been developed, allowing for unified nomenclature for different linkage groups (LGs) and the establishment of reference maps (Winter et al. 2000; Millan et al. 2010; Gaur et al. 2011). Additionally, gene-based single nucleotide polymorphisms (SNP) marker loci from transcript sequences have been included in chickpea genetic maps, providing anchor points for comparison of chickpea LGs with chromosomes of the model species *Medigaco truncatula* (Nayak et al. 2010; Gujaria et al. 2011; Thudi et al. 2011). In addition, bacterial artificial chromosome (BAC) libraries and the generation of BAC-end sequence markers are facilitating the availability of genome-wide physical maps in *C. arietinum* (Rajesh et al. 2004; Lichtenzveig et al. 2005; Zang et al. 2010; Thudi et al. 2011). Integration of genomic tools for marker-assisted breeding, high-resolution mapping, and positional cloning of genes and QTLs are expected to enhance genetic and genomic research and breeding applications in chickpea.

This chapter will focus on the current status and future potential of genomic tools for MAS in chickpea for resistance to major biotic stresses such as ascochyta blight (AB) and fusarium wilt (Foc), which currently impose major constraints on stable productions. Botrytis gray mold (BGM) and rust diseases, biotic stresses that are becoming increasingly important, will be also considered.

Ascochyta Blight (AB)

Causal Agent

Ascochyta blight of chickpea is caused by the fungal pathogen *Ascochyta rabiei* (Pass) Lab. (teleomorph: *Didymella rabiei*). The disease affects all aerial parts of the plant, causing brown lesions with concentric rings of black pycnidia on stems, leaves, flowers, pods, and seeds (Bayaa and Chen 2011) (Figure 11.1). *A. rabiei* propagates both asexually through conidia (pycnidiospores) and sexually through ascospores produced in pseudothecia. It requires two mating types for sexual reproduction and both mating types have been found in many chickpea production areas. Sexual reproduction requires a cold and moist period and usually occurs off season (between chickpea crops) on chickpea debris (Trapero-Casas and Kaiser 1992). The sexual spores mature and are ready for release when the next chickpea growing season starts. During storm and moist conditions, the ascospores, which serve as the primary inoculum, are ejected from pseudothecia and carried by wind to chickpea plants, where they initiate primary infection. Conidia on overwintering debris and on seeds may also serve as the local primary inoculum and initiate infection (Navas-Cortes et al. 1995). Secondary spread of the pathogen within chickpea fields is through rain splash of conidia.

The pathogen as well as the disease are favored by cool (15–25° C) and moist conditions (Bayaa and Chen 2011). The disease is particularly severe in growing areas with a prolonged rainy season. *A. rabiei* causes economic losses only on chickpea, but it readily infects wild chickpea relatives (*Cicer* spp.), and it may infect a range of other plant species occasionally or under artificial inoculation.

Considerable variation in *A. rabiei* has been reported, both in terms of virulence and in neutral genetic markers. Several systems are available to classify or categorize the virulence forms, ranging from 3 pathotypes to 14 virulent forms. The current trend is to reduce the number of categories of virulence forms (Chen et al. 2004). More virulent isolates have been reported recently (Imtiaz et al. 2011). It should be noted that no genetic bases for virulence differences in the systems have been found, although genes or QTLs in chickpea for resistance to different

Fig. 11.1. Typical symptoms of major chickpea diseases (a) ascochyta blight (b) fusarium wilt (c) botrytis (d) rust. For a color version of this figure, please refer to the color plate.

virulence levels have been reported (Udupa and Baum 2003; Cho et al. 2004).

Host Resistance Inheritance and QTL Mapping

Resistance to AB is either partial or incomplete, and owing to difficulties in phenotype evaluation it is mostly considered to be a quantitative trait. The genetic inheritance of resistance reaction has been variously described as controlled by one dominant, one recessive, two complementary recessive, or two complementary dominant genes, plus minor modifier genes that could determine the degree of resistance (Pande et al. 2005a). More recently, Danehloueipour and

colleagues (2007) studied the genetics of AB resistance by diallel analysis using three different sources of resistance, revealing the involvement of both major and minor genes and the dominant nature of susceptibility. High narrow-sense heritability indicated that additive gene effects were more important than nonadditive gene effects in this trait. Bhardwaj and colleagues (2010), studying crosses with four different sources of resistance, concluded that AB resistance was conferred either by a single dominant gene (C) or by two minor recessive genes (a, b) that independently have a very low effect. Thus resistant genotypes should be: aa bb - - or - - - - C-.

Santra and colleagues (2000) and Tekeoglu and colleagues (2000) published the first study relating to the identification and mapping of two quantitative trait loci (QTL-1 and QTL-2) conferring resistance to AB. They analyzed a recombinant inbred line (RIL) population derived from an interspecific cross. QTL-1 accounted for more than 40% of the variation in blight reaction and was associated with two randomly amplified polymorphic DNA (RAPD) markers (ubc733b and ubc181a). QTL-2 was flanked by the RAPD ubc836b and the isozyme diaphorase, and explained approximately 20% of phenotypic variation. Saturation of both genomic regions with co-dominant STMS markers allowed identification of the loci as two distal regions of LGIV of the chickpea genetic map (Millan et al. 2010). Iruela and colleagues (2006) also identified two QTLs (QTL_{AR1} and QTL_{AR2}) in the same genomic regions (LGIVa and LGIVb). Three SCARs (sequences characterized amplified region) markers – $SCK13_{602}$, $SCA19_{336}$, and $SCY17_{590}$ – linked to QTL_{AR2} were included in LGIVb. Both QTLs (QTL-1=QTL_{AR1} and QTL-2=QTL_{AR2}) have been widely reported using different genetic backgrounds (Table 11.1) and may be associated with resistance to pathotype II of AB (Udupa and Baum 2003; Cho et al. 2004; Aryamanesh et al. 2010). In addition, a third QTL (here named QTL_{AR3}) has been consistently reported on LGII (Table 11.1) and seems to be more associated with *A. rabiei* pathotype I (Udupa and Baum 2003; Cho et al. 2004). QTL_{AR2} (in LGIVb) and QTL_{AR3} (in LGII) are located in genomic regions with a high density of markers, whereas QTL_{AR1} (in LGIVa) is located in a loosely mapped genomic region. Other QTLs related to AB have been located in LGIII, LGVI, and LGVIII (Table 11.1).

Attempts have been made to isolate genes involved in chickpea defense responses to AB located in different QTLs. Iruela and colleagues (2009) studied the genomic sequences surrounding the SCAR marker $SCK13_{603}$, linked to QTL_{AR2}, using a genome walking approach and deduced that this marker was located in a region of a putative retrotransposon. Screening of BAC libraries with markers linked to QTL_{AR1} (Rajesh et al. 2008) allowed identification of a sequence (*CaETR-1*) coding an ethylene insensitive (EIN4)-like gene (Madrid et al. 2012). This locus is the first potentially functional sequence identified under a QTL for AB resistance (Table 11.1). A multiplex PCR assay based on this gene sequence was developed. The PCR not only discriminated resistance and susceptible phenotypes of chickpea to ascochyta blight, but also easily detected heterozygous genotypes (Madrid et al. 2013). Moreover Madrid and colleagues (2013) demonstrated that using this marker in combination with a previously developed co-dominant SCAR marker ($SCY17_{590}$), linked to QTL_{AR2} (Iruela et al. 2006), detected resistant alleles in 90% resistant accessions among a chickpea germplasm collection.

Fusarium Wilt (Foc)

Causal Agent

Fusarium vascular wilt of chickpea is caused by the fungal pathogen *Fusarium oxysporum* Schlechtend: Fr. f. sp. *ciceris*. It is a soil-borne disease and is widespread in most chickpea growing areas. Two pathotypes are accepted, based on symptoms (yellowing and wilting), and eight races are recognized, based on host

Table 11.1. Location of markers associated with QTLs or genes conferring resistance to key chickpea diseases.

Disease	Gene/QTL	LG	Indicative markers	References
Ascochyta blight	QTL_{AR1}	LGIV	GAA47, SCAR733b, *CaETR-1*	Flandez Galvez et al 2003; Iruela et al 2006; Tar'an et al 2007; Rajesh and Muehlbauer 2008, Anbessa et al 2009; Kottapalli et al 2009, Madrid et al 2012
	QTL_{AR2}	LGIV	TA72, TA146, SCY17, TA176, TA2, TS54, STMS11, TAA170, H2G20, H3D09, H1A12	Tekeoglu et al 2002; Udupa and Baum 2003; Iruela et al 2006; Lichtenzveig et al 2006, Tar'an et al 2007; Kottapalli et al 2009; Aryamanesh et al 2010
	QTL_{AR3}	LGII	GA16, TA194, TR19	Udupa and Baum 2003; Cho et al 2004; Cobos et al 2006; Lichtenzveig et al 2006, Iruela et al 2007; Anbessa et al 2009
	QTL_{AR4}	LGIII	STMS28, TS12b, TA64, TR58, TA34, TA142	Flandez Galvez et al 2003; Tar'an et al 2007; Anbessa et al 2009; Kottapalli et al 2009; Aryamanesh et al 2010
	QTL_{AR5}	LGVI	TA176	Anbessa et al 2009
	QTL_{AR6}	LGVIII	TA3, TS45, H3C11a	Flandez-Galvez et al 2003; Lichtenzveig et al 2006; Anbessa et al 2009
Fusarium wilt	*foc-0*$_1$	LGV	$OPJ20_{600}$, TR59	Cobos et al 2005
	foc-0$_2$	LGII	TA59	Halila et al 2009
	foc-1	"	ASAPCs27, H3A12, TA110	Mayer et al 1997; Gowda et al 2009
	foc-2	"	TA96, H3A12	"
	foc-3	"	TA96, TA27, CS27, H1B06y, TA194	Sharma et al 2004, Gowda et al 2009
	foc-4	"	TA96, CS27,	Winter et al 2000
			R-2609-1	Benko-Iseppon et al 2003
	foc-5	"	TA59, TA96, TA27	Winter et al 2000, Cobos et al 2009; Iruela et al 2007; Halila et al 2009
Rust	*Uca1/uca1*	LGVII	TA18, TA180	Madrid et al 2008
Botrytis grey mould	QTL-1	LGVI	SA14-TS71rts36r	Anuradha et al 2011
	QTL-2	LGVIII	TA25, TA144	"
	QTL-3		TA159,TA118	

[1] Aryamanesh et al 2010 identified two different QTL in this genomic region of LGIV.

specificity (0, 1A, 1B/C, 2, 3, 4, 5, and 6) (Jimenez-Díaz et al. 2011). The yellowing pathotype includes races 0 and 1B/C, whereas the wilting pathotype includes the other six races (races 1A through 6). A pathogenicity assay on a set of chickpea differentials is required to identify the races and pathotypes (Sharma et al. 2005). Molecular markers are available for identifying certain races (Jiménez-Gasco et al. 2003), however, it is recommended that races identified solely by molecular markers, especially in areas where the disease is first reported, should be confirmed by pathogenicity assays on chickpea differentials. The pathogenic races also differ in their geographic distribution: races 2, 3, and 4 are found in India, whereas races 0, 1B/C, 5, and 6 are found mainly in the Mediterranean region and the United States. Race 1A is more widespread and has been reported in India, the Mediterranean region, and the U.S.

Chickpea plants may be infected at any growth stage. High temperature is critical for development of fusarium wilt, and disease severity is higher at 25-30° C. Nevertheless, optimal temperatures vary for different race and cultivar combinations (Jimenez-Diaz 2011). Typical wilting symptoms include drooping of the petioles and leaflets, followed by a dull-green discoloration, desiccation, and collapse of the entire plant (Figure 11.1). Sometimes partial wilting of a few branches is seen, owing to restriction of the pathogen within infected vascular bundles. An important diagnostic feature of the disease is internal discoloration of xylem in root and stems, without external root discoloration or mycelial growth on the surface.

In the absence of host plants the pathogen can survive for several years as chlamydospores in infected chickpea debris in soil. It can also infect seeds and become seed borne. Infected seeds are the primary source for introduction of the pathogen into new production areas. Infested soil and infected debris serve as inoculum for local spreading through cultivation and other human activity.

The pathogen is host-specific and is known to infect *Cicer* species only. The pathogen invades feeder roots directly without wounds. After reaching the xylem tissue, the fungus spreads up in the vessels and to adjacent vessels. The development of the pathogen clogs the xylem vessels, restricting movement of nutrients and water through the vascular tissue.

In addition to chickpea genotypes (cultivars), pathogen races, and inoculum density, the other important factor affecting development of the disease is soil temperature. High temperature is critical for development of Foc.

Host Resistance

Fusarium wilt resistance in chickpea has been reported as race-specific and controlled by a maximum of three major genes, which are recessive for the majority of favorable alleles. Upadhyaya and colleagues (1983) and Singh and colleagues (1987) found that resistance to race 1 was governed by three independent genes. The combination of any two of the three genes (*h1h1* or *h2h2* or *h1H3* or *h2H3*) was required for complete resistance to race 1. Nevertheless, Sharma and colleagues (2005) and Gowda and colleagues (2009) reported a single gene governing resistance to race 1, using the resistant lines WR315 and Vijay, respectively. The presence of one favorable allele in the genotype causes the symptom of late wilting (reviewed by Sharma and Muehlbauer 2007).

Similarly, as many as three genes have been described as controlling resistance to race 2; a combination of recessive alleles of two of these genes confers resistance, whereas when only one gene is present in a recessive state, late wilting reaction occurs (Kumar 1998; Sharma and Muehlbauer 2007). Sharma and colleagues (2005) suggested that a single recessive gene in WR315 also governs resistance to this race, similar to that reported in the RIL population derived from JG62 x Vijay, Vijay being the source of resistance (Gowda et al. 2009).

Resistance to race 3 has been found to be monogenic (Sharma et al. 2005; Gowda et al. 2009), but its dominant or recessive nature remains unknown. Resistance to race 4 was observed to be controlled by either one or two genes, with the resistant allele being recessive (Tullu et al. 1998, Tullu et al. 1999). In the case of resistance to race 5, several authors have proposed monogenic inheritance using different sources of resistance (Tekeoglu et al. 2000; Sharma et al. 2005; Iruela et al. 2007; Cobos et al. 2009). Assays developed at the University of Córdoba (Spain) revealed the recessive nature of resistance to race 5 (Dr. P. Castro and Dr. J. Gil, personal communication). Two genes were reported to control resistance to race 0, one in the genotype JG62 (the same as ICC 4951) and another in CA2139 (Rubio et al. 2003).

The slow wilting resistance reaction in chickpea seems to be controlled by minor genes (Sharma and Muehlbauer 2007). Different

studies using both an RIL population and near-isogenic lines (NILs) suggest that in race 5, apart from a major gene, additional genomic areas influence slow wilting resistance (Cobos et al. 2009; Castro et al. 2010). Larger segregating populations are required to clarify the nature of genetic control of slow wilting. Likewise, further studies are necessary to identify the genetics of resistance to races 1B/C and 6.

Gene Mapping

Markers identified early on as linked to a Foc reistance gene were RAPD UBC-170_{550} and CS-27_{700}, which each target one of the three loci controlling resistance to race 1 (*foc-1*) (Mayer et al. 1997). An allele-specific associated primer (ASAP) was designed, based on CS-27_{700}. Later, Tullu and colleagues (1998) demonstrated that *foc-1* was linked to *foc-4* and suggested the presence of a gene cluster for Foc resistance sited in LGII of chickpea map. Winter and colleagues (2000) mapped a RIL population segregating for resistance to Foc-4 and Foc-5 located in the same gene cluster on LGII previously mentioned, with ASAP CS27, STMS TA27, TA59, and TA96 included as indicative markers. These markers have been validated in different genetic backgrounds (Table 11.1). Similarly, single genes for *foc-0*, *foc-2,* and *foc-3* have been mapped in the same genomic area (Table 11.1). Apart from this gene cluster, a different gene controlling resistance to race 0 (*foc-0_1*) was previously mapped on LGV (Table 11.1).

Near isogenic lines recently developed for Foc resistance confirmed the efficacy of the STMS marker TA59 for targeting resistance genes for races 1A, 2, 3, 4, and 5 (Castro et al. 2010). On the other hand, TR59 (targeting *foc0_1* on LGV) and TA59 (targeting *foc0_2* on LGII) were useful in determining which of those genes were segregating in five chickpea RIL populations (Halila et al. 2010). Nevertheless, there are still genes for different races that have not been mapped; knowledge of their locations in the chickpea map is essential to enable breeders to pyramid genes in a single variety and thus provide durable resistance.

Botrytis Gray Mold (BGM)

Causal Agent

Botrytis gray mold of chickpea is caused by *Botrytis cinerea* Pers. Ex. Fr. Its teleomorph (sexual stage) is *Botryotinia fuckeliana*, although the sexual stage has not been reported on chickpea. The pathogen attacks all the aerial parts of the plant but most frequently the growing tips and flowers (Davidson et al. 2004). The disease initially appears as water-soaking lesions, which turn gray or dark brown (Figure 11.1). Sometimes tiny black sclerotia may develop on the dead infected tissues (Pande et al. 2011b). Infected chickpea pods result in no seeds at all or only shriveled seeds.

B. cinerea has a very wide host range of more than 100 plant species including many economically important vegetable and field crops, ornamentals, and pre- and post-harvest fruits (Davidson et al. 2004).

The pathogen survives between crops in infested soil and infected plant debris as mycelia, chlamydospores, and sclerotia. It may also survive in infested or infected seeds. Seeds carrying the fungus may not show any visible symptoms. Because of its wide host range, other alternative hosts are also important inoculum sources (Pande et al. 2011b).

Relative humidity, leaf wetness, and temperature are the most important factors for disease infection and development. The effects of the disease on yield depend on the growth stage of the crop at onset of the disease and its severity (Davidson et al. 2004). Isolates show variation in virulence on chickpea, but no host-specialization has been reported.

Host Resistance Inheritance and QTL Mapping

Information available on the genetic inheritance of resistance to BGM suggests that it is

oligogenic in nature. Thus, as many as three different genes have been described that occasionally show epistatic interaction (reviewed by Pande et al. 2006a).

The locations of genomic areas controlling resistance in the chickpea genetic map have been identified by Anuradha and colleagues (2011). Three QTLs were identified, with QTL1 sited on LG6 (or LGVI), and QTL2 and QTL3 mapped on LG3 (corresponding to LGVIII in the chickpea consensus map, Table 11.1). QTL1 and QTL2 explained 12.8% and 9.5% of the total phenotypic variation for BGM. The strongest QTL for BGM resistance was QTL3, explaining 48% of the phenotypic variation, with a logarithm of odds (LOD) score of 17.74. When BGM disease scores of 126 RILs were grouped into two classes, a ratio of 54 (resistant):72 (susceptible) was found, not significantly different ($P = 0.1$) from 1:1 distribution. This result suggests the presence of a major gene for BGM resistance corresponding to QTL3. Markers linked to different QTLs are summarized in Table 11.1. Further analysis using different sources of resistance will facilitate the use of MAS to pyramid BGM resistance genes in commercial varieties in order to obtain higher levels of resistance.

Rust

Causal Agent

Several rust species can infect grain and forage legumes, most of them belonging to the genus *Uromyces* (Rubiales et al. 2001). In particular, chickpea rust is caused by *Uromyces ciceris-arietini* (Grogon) Jacz. & Beyer, and has been described as a problem in central Mexico and Italy (Ragazzi 1982; Díaz-Franco and Pérez-García 1995) and also in India (Hiremath et al. 1987). Chickpea rust develops in cool and moist weather conditions, although rain is not essential for its development. Environmental conditions favoring rust occurrence are similar to those for AB. Consequently, rust has been reported in many of those countries where AB of chickpea is a problem (Reddy et al. 1990).

Rust symptoms are large pustules on leaves that appear initially as small, round, brown spots (Figure 11.1). In the mature pustule, when the epidermis ruptures, uredospores are released from the center of the spots (Singh et al. 2007). The fungus can affect various plant stages (vegetative, flowering, or fruiting) and different organs, including leaves, stems, pods, or even seeds. In severely affected plants, lesions coalesce, causing premature defoliation and considerable reduction in yield (Jones 1983).

Host Resistance and QTL Mapping

The genetic basis of rust resistance in most cool-season legumes is largely unknown. Evaluation of germplasm collections reveals moderate levels of incomplete and partial resistance in *C. arietinum* and some resistant accessions in wild *Cicer* relatives (Rubiales et al. 2001). Madrid and colleagues (2008) postulated that resistance is controlled by one major gene located on LGVII; oligogenic control of rust resistance is in agreement with studies conducted on other legumes. For example, recent mapping studies have yielded identification of major QTLs for resistance to *U. viciae-fabae* (Vijayalakshmi et al. 2005; Rai et al. 2011) and to *U. pisi* (Barilli et al. 2010). In lentil, monogenic resistance to *U. viciae-fabae* has been described (Erskine et al. 1994), and a sequence related amplified polymorphism (SRAP) marker, F7XEM4a, has been identified at 7.9 cM, apart from the gene for resistance (Saha et al. 2010). Nevertheless, more studies are necessary to elucidate the recessive/dominant nature of this gene.

Only one study to date has been focused on rust resistance in chickpea (Madrid et al. 2008). This research describes a QTL located on LGVII of the chickpea genetic map, explaining 31% of the total phenotypic variation in seedlings and 81% of the area under the disease progress curve (AUDPC) in adult plant. It was hypothesized that a single dominant gene (*Uca1/uca1)* controlled resistance to rust, with resistance regarded as a qualitative character.

This locus was mapped between STMS markers TA18 and TA180 (3.9 cM apart) in the same genomic region of the QTL (Table 11.1). These STMS markers are close enough to *Uca1/uca1* to allow reliable marker-assisted selection for rust resistance.

Genetic Resources and Breeding Progress

To make progress in breeding against evolving biotic stresses, it is imperative for chickpea breeding programs worldwide to search and deploy new sources of resistance for these stresses. These sources can be mined from wide collections of chickpea landraces and wild relatives. The two CGIAR (Consultative Group on International Agricultural Research) centers – ICARDA (International Center for Agricultural Research in the Dry Areas) and ICRISAT (International Crop Research Institute for the Semi-Arid Tropics) – conserve more than 34,085 accessions, in addition to those available in the USDA-ARS (United States Department of Agriculture-Agricultural Research Service) and the ATFCC (Australian Temperate Field Crops Collection). Of the CGIAR centers' collections, 578 accessions are of wild relatives, which are a rich reservoir of useful genes/alleles unavailable in the cultivated gene pool (Kumar et al. 2011). The *Cicer* genus encompasses one cultivated species – chickpea (*C. arietinum*) – 34 wild perennial and 8 wild annual species. The 8 wild annuals include *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum*, *C. judaicum*, *C. bijugum*, *C. cuneatum*, *C. chorassanicum,* and *C. yamashitae* (Singh et al. 2008; Kumar et al. 2011). Basing their work on crossability, molecular diversity, and karyotype analyses, Croser and colleagues (2003) classified the annual *Cicer* species into three major gene pools. The primary gene pool consists of the cultivated species (*C. arietinum*), the wild progenitor *C. reticulatum*, and the closely related *C. echinospermum*. The secondary gene pool consists of *C. bijugum*, *C. pinnatifidum*, and *C. judaicum*, while the remaining three annuals were assigned to the tertiary gene pool.

The cultivated chickpea has a narrow genetic base, and sources of resistance to several stresses are often lacking within cultivated germplasm (Abbo et al. 2007). Diversification and broadening of the genetic base through the use of wild relatives and primitive landraces is therefore pivotal for making progress in the development of new cultivars. Some efforts have been made in the past to screen germplasm collections under field and controlled conditions in order to identify useful genes for resistance to AB, Foc, BGM, rust, and other diseases. These efforts have resulted in identification of valuable sources of resistance for these key diseases (Table 11.2). However, concerted efforts are needed to mine these collections further to identify new sources for resistance.

Ascochyta Blight (AB)

Breeding cultivars with durable resistance to AB is a challenging task because of the continuous evolution of the pathogen and the appearance of new virulent pathotypes (Atik et al. 2011; Imtiaz et al. 2011). This makes resistance short-lived and consequently limits the effectiveness of resistant cultivars. Accordingly continuous efforts are required to identify new sources of resistance for deployment in chickpea breeding programs. One of the approaches that breeders are adopting is to find and pyramid different genes into the same cultivar to improve its resistance level and durability. Considerable progress has been made in identifying resistant germplasm and breeding for resistance to AB (Malhotra et al. 2010; Pande et al. 2011a). Climatic conditions at ICARDA are very conducive for AB development, and each year an area of approximately 6 ha is planted with 20,000 to 25,000 lines, under artificial epiphytotic field conditions, in order to select resistant lines, mostly in the Kabuli background. Reddy and Singh (1984) reported 11 Kabuli and 6 Desi genotypes resistant to AB. However, among

Table 11.2. Useful sources of resistance/tolerance for key diseases in chickpea.

Disease	Sources of resistance	References
Ascochyta blight	ILC 72, ILC 191, ILC 196, ILC 201, ILC 202, ILC 2506, ILC 2956, ILC 3274, ILC 3279, ILC 3346, ILC 3856, ILC 3956, ILC 3996, ILC 4421, ICC 3634, ICC 4200, ICC 4248, ICC 4368, ICC 5124, ICC 6981, ILWC 7-1, ILWC 33/S-4, 03039, 03041, 03053, 03115, 03131, 03133, 03143, 03159, 93A-086, 93A-111, 93A-3354	Malhotra et al 2003 Ilyas et al 2007 Kumar et al 2011a
Fusarium wilt	JG 16, JG 62, ILC 482, C 104, GJ 74, WR 315, K-850, KWR 108, L-550, BG 212, BG 215, Ghaffa, CPS-1, UC 27, Vardan, Vijay, Vishal, Annigeri, ILWC 7-1, ILWC 33/S-4, CM 368/93, CM 444/92, FLIP 00-17C, FLIP 02-7C, FLIP 02-9C, FLIP 02-40C, FLIP 02-47C, FLIP 03-26C, FLIP 03-29C, FLIP 03-57C, FLIP 03-108C, FLIP 03-127C, FLIP 05-28, FLIP 05-68C, FLIP 05-72C, FLIP 05-85C, FLIP 05-106C, FLIP 90-131C, FLIP 99-66C	Sharma et al 2005 Infantino et al 2006 Sharma and Muehlbauer 2007 Singh et al 2009 Ali et al 2011 Kumar et al 2011a
Botrytis grey mould	ICCV 2, Pusa 209, Gaurav	Singh et al 2009
Rust	FLIP05-74C, PI 593072, PI 642748	Rubiales et al 2001 Rubio et al 2006

those, the resistance of ILC 72, ILC 196, and ILC 2956 was eroded under field conditions because of the appearance of new pathotypes (Atik et al. 2011; Imtiaz et al. 2011). Malhotra et al. (2003) reported the development of more than 3,000 lines with moderate AB resistance. Furthermore, 2,576 resistant lines (rating < 4 on a 1-9 scale) derived from 8,660 bulk populations evaluated from 2000 to 2009 at ICARDA were added to this list (Dr. M. Imtiaz, personal communication).

Because the pathogen is evolving and with the presence of highly virulent pathotype IV (Imtiaz et al. 2011), it is mandatory to search for new sources of resistance, especially from the wild gene pool.

Resistance to AB was identified in different accessions of wild *Cicer* species (*C. bijugum, C. judaicum, C. pinnatifidum, C. echinospermum, C. reticulatum, C. cuneatum,* and *C. montbretti*) (Singh et al. 1981; Singh and Reddy 1993; Singh et al. 1998; Collard et al. 2001, Collard et al. 2003; Nguyen et al. 2005). Danehloueipour and colleagues (2007) reported that wild *Cicer* accessions in *C. reticulatum* and *C. echinospermum* may have major or minor resistant genes different from those in the cultivated chickpea. The identification of resistant accessions from *C. reticulatum* and *C. echinospermum* is of particular importance because they are in the same primary gene pool (Knights et al. 2008). Reports show that high resistance to AB is more frequent in *C. judaicum* than in other annual wild *Cicer* species. Unfortunately this species belongs to the secondary gene pool, making it difficult to introduce resistance genes into the cultivated chickpea, although Verma and colleagues (1995) obtained fertile F1 from a cross between *C. arietinum* and *C. judaicum*. To our knowledge no commercial varieties carrying genes of resistance to AB from wild *Cicer* species have been registered. However, a few breeding lines recently developed by ICARDA and distributed to National Agriculture Research System (NARS) partners as part of the International AB Nursery (CIABN) include lines containing genes from wild *C. reticulatum* accessions.

Despite the fact that the pathogen is highly variable, good progress in breeding cultivars with improved AB resistance has been made by NARS in many countries (Chen et al. 2004; Sabaghpour et al. 2006; Siddique et al. 2007a; Siddique et al. 2007b; Singh et al. 2009).

Fusarium Wilt (Foc)

Possibly because of climate change, particularly the rise in soil temperature related to global warming, Foc is becoming more significant even in areas where it was not a severe problem in the past. To design a breeding strategy for the development of wilt-resistant varieties, knowledge of genetic variations for virulence in the pathogen is important. A number of resistant sources to Foc have been reported under field and controlled epiphytotic conditions in both cultivated and wild chickpea germplasm (Haware et al. 1992; Halila and Strange 1997; Sharma et al. 2005). Cost-effective field, greenhouse, and laboratory methodologies for resistance screening have been developed (Gaur et al. 2007; Hamwieh et al. in preparation; http://icardanews.wordpress.com/2011/07/31/new-screening-method-for-wilt-resistance-in-chickpea/). Since development of a wilt-sick plot for field screening is comparatively easy, such plots are available at many research stations around the world. However, with time these sick plots become contaminated with other soil-borne pests such as nematodes and other root-rot pathogens, thus the racial picture and availability of uniform inoculum is becoming a challenge when evaluating materials in sick plots.

A collection comprising more than 8,231 germplasm and improved lines was evaluated against a mixture of races of *F. oxysporum* at ICARDA, and 2,645 lines with less than 10% mortality (resistant) were identified (Imtiaz, personal communication). Similarly, in cooperative research between INRAT, Tunisia, and ICARDA, 650 lines were tested at Beja in 2011 in a wilt-sick plot infested by race 0. In that test, 131 entries were found to be resistant (less than 10% mortality), and 25 of those resistant lines presented 0% mortality. Haware and colleagues (1992) screened more than 13,500 germplasm accessions from 40 countries for race 1 at ICRISAT and identified 160 resistant accessions (150 Desi and 10 Kabuli).

Evolution by stepwise mutation, as suggested for the pathogen, justifies bringing additional sources of resistance from wild *Cicer* into cultivated germplasm. Fortunately, various species of annual wild *Cicer* possess a high level of resistance to Foc (Rao et al. 2003). Indeed, Nene and Haware (1980) reported resistance to Foc in five of six annual wild *Cicer* species. All *C. bijugum* tested accessions and some accessions of *C. judaicum*, *C. reticulatum*, *C. echinospermum,* and *C. pinnatifidum* showed resistance. Moreover, some accessions of *C. bijugum*, *C. judaicum,* and *C. reticulatum* were completely free of wilt damage. Resistance to Foc was also reported in accessions from the eight annual wild *Cicer* species (Kaiser et al. 1994; Infantino et al. 1996; Rao et al. 2003). Results showed that most of the highly resistant accessions belong to species of the first and second gene pools, and this facilitates introgression of resistance genes into cultivated chickpea.

Significant progress has been made in Foc research and germplasm and cultivars are now available that possess resistance to multiple races. For example, WR315 is widely recognized as possessing resistance to all known races, while JG 74 is resistant to all races except races 2 and 5 (Haware 1998). Cultivars with stable Foc resistance have been released in many countries (Malhotra et al. 2007, Singh et al. 2009). Simple genetic control of Foc resistance favors success in the selection of resistant lines using marker-assisted backcrossing methods (Dr. J. Gil, personal communication).

Other Diseases

Few accessions belonging to either cultivated or wild *Cicer* are tolerant of BGM and the search for higher levels of disease resistance is continuing (Pande et al. 2006a). Higher levels of resistance to BGM were identified in some accessions of *C. judaicum, C. bijugum, C. echinospermum,* and *C. pinnatifidum* (Singh et al. 1982; Singh et al. 1991; Haware et al. 1992; Haware 1998; Rao et al. 2003). Moreover, at ICRISAT Pande

and colleagues (2006b) evaluated 136 accessions belonging to seven wild *Cicer* species and found 23 accessions of *C. judaicum*, 3 of *C. reticulatum,* and 3 of *C. bijugum* that showed resistant reactions, although none were immune. Kaur and colleagues (2007) found inter- and intra-accession variations for resistance to BGM in six wild *Cicer* species, and resistant plants maintained their resistance in subsequent evaluation. The attempt to introduce the resistance genes from wild species to cultivated chickpea produced only a few interspecific hybrids with moderate levels of resistance to BMG and desirable agronomic traits (Pande et al. 2006a).

Although rust in chickpea is considered a minor disease, it may become more important with climate changes. A limited number of studies to identify rust-resistant genotypes have been carried out, and resistance was found in wild *Cicer* spp. (*C. reticulatum, C. echinospermum, C. judaicum,* and *C. bijugum*) (Rubiales et al. 2001). Recently a severe rust epidemic was observed in northern India (Dhaulakuan), where 57 entries of CIABN were evaluated, but only one line – FLIP05-74C – survived the epidemic and thus carries potential resistance to rust (Dr. M. Imtiaz, personal communication). Rust-resistant RILs from a cross between *C. arietinum* (ILC72), as susceptible parent, and *C. reticulatum* (Cr5-10 = PI593062), as resistant parent, were obtained by Spanish breeders. One of those RILs was selected and registered as PI642748, which also is resistant to AB (Rubio et al. 2006, Table 11.2).

In the case of Phytophthora root rot caused by *Phytophthora medicaginis,* an important disease of chickpea in Australia, through targeted field screening, two accessions of *C. arietinum* and *C. echinospermum* were found resistant (Brinsmead et al. 1985; Knights et al. 2008). Introgression of these resistance genes into the cultigen allowed for a substantial yield increase in resistant cultivars when compared with susceptible ones (Singh et al. 1994). Some progress has been made in breeding, but nevertheless, substantial concerted efforts are needed to enhance breeding for resistance to diseases that are increasingly becoming a threat in the chickpea industry.

Progress in Introgression of Resistance Genes

Multiple resistances to diseases in wild *Cicer* are frequently reported (Singh et al. 1998; Yadav et al. 2003; Nguyen et al. 2005; Kumar et al. 2011). Many interspecific crosses have been made in recent years, mainly at ICARDA, ICRISAT, and some national research institutions or programs. However, none of these efforts has resulted in the release of new cultivars and subsequent use by farmers (Chaturvedi and Nadarajan 2010). There is still a gap between producing breeding lines derived from interspecific hybridization and releasing high yielding cultivars with good agronomic performance and quality, especially for the large-seeded Kabuli type. However, some examples of successful transfer have been reported. Singh and colleagues (2005) obtained four Desi and two Kabuli lines possessing a high degree of resistance to wilt, foot rot, and root rot diseases, from interspecific crosses between varieties of cultivated chickpea with a *C. reticulatum* accession, and recorded 6-17% seed yield increase over the best check cultivars. In India, variety BG 1103 (PUSA 1103) of Desi type was developed from a cross (Pusa 256 x *C. reticulatum*) x Pusa 362 and has significantly outyielded all the well adapted checks in multilocation yield testing during 2000 and 2003. The variety BG 1103 has multiple resistances against soil-borne diseases and waterlogging. It is suitable for late planting in north India (International Chickpea Genomics Consortium; http://www.icgc.wsu.edu/iara.html).

Integrated Disease Management

Although developing and planting resistant cultivars is the most economical means of managing chickpea diseases, in the absence of completely resistant cultivars, integrated disease

management must be employed. Even if complete resistance is available as in fusarium wilt resistance, we still need to develop and employ integrated disease management in order to preserve and prolong the effectiveness of the resistant cultivars.

Integrated disease management should be developed locally because cultural practices, climate conditions, social-economic conditions, and regulations vary. For instance, there are mostly small and subsistence farmers in North Africa, the Middle East, and the Indian subcontinent, while there are mostly large-scale and modernized farmers in developed and industrialized countries. Consequently many management practices cannot be applied globally.

Ascochyta blight can be managed using moderately resistant cultivars in combination with a number of cultural practices and fungicide application (Pande et al. 2005a; Gan et al. 2006; Davidson and Kimber 2007; Singh et al. 2007). Shtienberg and colleagues (2000) demonstrated that a contribution of up to 70% of AB control was achieved by using moderately resistant cultivars when weather conditions supported severe epidemics; fungicides improved control efficacy significantly, to >95%. Using non-infected seeds and seed treatment with appropriate fungicides is also critical. Foliar protection is mainly recommended, particularly during the reproductive stage (Chongo and Gossen 2001). Shtienberg and colleagues (2000) concluded that winter sowing of chickpea is feasible in the Middle East if adequate suppression of AB is achieved. Other practices, including adjusting (delaying) sowing dates (Dusunceli et al. 2007), rotation of chickpea production fields (Reley et al. 2011), deep plowing (van Gastel et al. 2007), and appropriately reducing plant population density (Gan et al. 2007) could also be recommended.

Management of Foc relies mainly on genetic resistance, crop rotation, and use of disease-free and treated seeds. Nowadays, there are several Kabuli and Desi chickpea varieties combining resistance to AB and Foc that could be recommended for use in Foc-infested fields. Additionally, biological control using *Trichoderma viride* (Nikam et al. 2007), soil amendment with groundnut and neem cakes, and seed treatment with BION have also been reported to be efficient against wilt (Sarwar et al. 2010).

Integrated management of BGM relies mainly on cultural practices to ensure good aeration by using erect varieties, increasing inter-row spacing and reducing the plant population density (Reley et al. 2011), and late sowing in certain areas, since genetic resistance to BGM in chickpea is partial and scarce. Indeed few varieties with partial resistance have been developed. In Nepal, moderate resistance to BGM was combined with resistance to Foc (Pande et al. 2005b), and with resistance to AB (Pande et al. 2006b). Seed dressing and crop rotation are also very important and reduce the risk of early infection. Foliar fungicides can ensure some protection to the crop when the disease is detected at its early stage by frequent crop monitoring. In Nepal, an integrated disease management (IDM) program increased grain yield by 400% and farmers' net income by 300% (Pande et al. 2006a).

Conclusions and Future Prospects

Chickpea breeding programs need to be focused on selection of high yielding varieties for different cropping systems, taking into consideration the specificity of this crop and the need for varieties resistant to several major biotic and abiotic stresses. Genetic gain using conventional breeding methods is limited in this crop, but molecular breeding presents new prospects for the future. Chickpea is one of the pulses where major progress has been achieved in the use of marker-assisted selection, numerous simple sequence repeat (SSR) and SNP resources have been developed, and the chickpea genetic map density has been considerably increased (Varshney et al. 2010, Kumar et al. 2011). In previous sections of this chapter it has been shown that there are markers available for targeting resistance for major biotic stresses, and efforts

are being made to find molecular markers associated with resistance in minor diseases to improve pyramiding genes in breeding programs.

However, chickpea molecular breeding for biotic stresses still has some limitations. Not all the genes or QTLs for major diseases have been finely mapped and new sources of resistance remain to be genotyped. Markers currently targeting resistance genes or QTLs (Table 11.1) are mostly of the microsatellite type, but high-throughput SNP genotyping platforms are overtaking SSR as the marker type of choice for screening germplasm collections (Zhu et al. 2008). Minor diseases have been scarcely studied and require much more attention, as is the case with root-rot diseases, because of the difficulty in resistance screening and in differentiating the effect of various resistance sources. The lack of genetic variability in the cultivated species requires chickpea breeders to use wild *Cicer* species, which contain a higher degree of resistance to many stresses. Unfortunately most of the resistance sources are present in the secondary and tertiary gene pools. Transferring resistance and desirable gene complexes from unexploited wild annual species to cultivated species by hybridization is often confronted with reproductive barriers, which may be overcome by using novel biotechnological approaches. In addition, further insights into the genetic bases of virulence, resistance mechanisms, and plant-pathogen interactions are required in order to increase the efficacy in breeding for biotic stress in chickpea.

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Chapter 12

Resistance to Late Blight in Potato

Jadwiga Śliwka and Ewa Zimnoch-Guzowska

Abstract

This chapter presents a summary of research on potato's resistance to its most devastating pathogen, *Phytophthora infestans*. Beginning with the infamous emergence of potato late blight in Europe, the chapter continues with the history of breeding potatoes resistant to this disease that began soon afterwards. We discuss changing trends in breeding, from the introduction of qualitative resistance, through the elimination of qualitative resistance in favor of quantitative resistance, then to the coming back into fashion of major resistance (R) genes. Also reviewed are the species from the *Solanum* genus that are exploited in potato breeding as sources of resistance to late blight. We then focus on R genes, their origins, identification, localization, and sequence, if known. R genes, as described in the literature to date, are grouped according to their position in ten loci on the potato genetic map. Their effectiveness in tubers and in the foliage, as well as the spectrum of provided resistance are described whenever data are available. In the case of some R genes, the corresponding *P. infestans* effectors have already been identified. We mention also quantitative resistance loci (QTLs), when they overlap with the positions of the R genes. Recently the number of identified R genes for late blight resistance has grown quickly. Both *P. infestans* and potato genomes have been sequenced, providing vast resources for studying interactions between these organisms, which researchers hope will result in more efficient and successful breeding of late blight resistant potatoes.

Introduction

Potato *Solanum tuberosum* L. is one of the main strategic crops worldwide. It is the fourth most important staple crop after rice, wheat, and maize. Potato is produced in more than 100 countries and it is an important food for more than a billion people. The global total crop production exceeded 329 Mt in 2009. Major potato producing countries with more than 30 Mt of potato harvested are China, India, and the Russian Federation (www.faostat.fao.org). The level of potato consumption is stable in Western Europe and North America but is increasing in Africa and Asia. In the last five decades, growth in the potato production area in developing countries has exceeded that of all other food crops. Potato is a significant element in the food security chain in developing countries in South America, Africa, and Southeast Asia. For that

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reason, the year 2008 was officially announced as the International Year of the Potato by the Director-General of FAO (http://www.fao.org/agriculture/crops/core-themes/theme/hort-indust-crops/international-year-of-the-potato/en/).

Potato as a vegetatively propagated crop is more exposed to disease not only through attacks of pathogens during vegetation season, but also through the transmission of pathogens to the next generation by infected seed tubers.

Late blight, today the most important potato disease worldwide, is caused by *Phytophthora infestans* (Mont.) de Bary. This disease affects potato foliage and tubers. Total destruction of potato crops occurred in 1845 and 1846 and it was distastrous. The decimation of the basic food crop in Ireland caused the death of one million people and emigration of another million, in what is known as the Irish Potato Famine (Bourke 1991). Until now, the problem of late blight control has not been solved through use of the genetic resistance of the host plant. Despite more than 100 years of concerted efforts worldwide toward breeding resistant potato varieties and, in recent decades, releasing efficiently resistant cultivars, chemical control is still the predominant method employed to protect the potato crop. Current management practices rely on multiple fungicide applications for disease control (up to15 sprays per season, as in the U.S. state of Maine), a practice that is harmful to the environment and to people (Schepers and Spits 2006; Forbes 2009). Accordingly efforts are being directed toward improving preventive control strategies by reducing doses or reducing the number of protective fungicide sprays on more resistant potato cultivars (Kessel et al. 2006; Spits et al. 2007).

In 1999 annual losses in global potato production caused by late blight were estimated at a cost of US$3 billion by Duncan (1999). Ten years later Haverkort and colleagues (2008) estimated the annual losses caused by late blight in European Union countries, attributable to the cost of crop damage and chemical control, at €1 billion, while the value of potato production in EU countries was estimated at €6 billion. The same authors assessed the global cost of losses caused by late blight, assuming global potato production on 20 Mha with an average yield of 16 T/ha. They calculated M€4,800 as a very conservative loss annually caused by late blight.

Effective solution of the late blight problem in potato production by utilization of resistant cultivars would efficiently increase income and food security on a world scale.

History of Late Blight Resistance Breeding

In modern potato breeding programs, resistance to late blight was and still is an important issue. In the nineteenth century potato cultivars selected within *S. tuberosum* sources varied in susceptibility to late blight. In this period, instead of several popular cultivars, there were hundreds of them in cultivation, but of very local importance (Toxopeus 1964). It was noted during the potato famine in the 1840s, that a few of the cultivars grown in Europe exhibited some resistance while other cultivars were totally damaged (Glendinning 1983). However, *S. tuberosum* was not a sufficient source of resistance variation for selection of highly resistant cultivars.

Thus, in the beginning of the twentieth century the resistant Mexican species *S. demissum* and *S. stoloniferum* were introduced in breeding for resistance to late blight (Salaman 1937). Since the discovery around 1920 of the hypersensitive reaction of *S. demissum* to *P. infestans*, attempts have been made to transfer the R genes responsible for this type of resistance into *S. tuberosum* by backcrossing *S. demissum* with potato cultivars (Toxopeus 1956). Breeding centers in the USA, England, the Netherlands, Germany, and Russia were actively engaged in enhancing late blight resistance in bred cultivars. Soon it was found that this resistance in new cultivars having single R genes was not stable and was overcome by new virulent races of *P. infestans* (Müller and Black 1952). At this time breeders had recognized four completely

dominant R genes originating from *S. demissum* (R_1, R_2, R_3, R_4) (Black 1952; Black 1954). In 1953 an international nomenclature of *P. infestans* races and of genes controlling immunity in *S. demissum* was established by scientists from Scotland, the Netherlands, and the USA (Black et al. 1953). A theory of a gene-for-gene interaction between host (*R* gene) and pathogen (*p*– strain) was proposed by Toxopeus (1956) following Flor (1956), based on research on flax and the flax rust.

Race-specific resistance to late blight is mediated by dominant R genes and is associated with hypersensitive response leading to cell death and rapid localization of the pathogen, preventing further colonization of the host tissue. This is a result of the interaction between plant receptors encoded by the respective R genes, which, directly or indirectly, recognize pathogen elicitors encoded by the respective avirulence gene *Avr* (Flor 1971). A rapid mutation of the *Avr* gene leads to loss of recognition by the R gene product and results in loss of resistance governed by the R gene. Soon it was evidenced that *S. demissum* contains more than four R genes in their different combinations (Black and Gallegly 1957). There have been 11 R genes identified in breeding lines having *S. demissum* in the origin: R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9 (Malcolmson and Black 1966), R_{10}, and R_{11} (Skidmore and Shattock 1985), and two R genes in those originating from *S. stoloniferum* (Schick et al. 1958; Schick and Schick 1961). Some R genes from S. *demissum* were relatively easy introgressed into many potato cultivars owing to their monogenic, dominant nature. Świeżyński (1988) compiled a list of 66 European cultivars outstanding in foliage or tuber resistance, out of the 600 described by Stegeman and Schnick (1985). The majority of them had *S. demissum* in their origin. Resistance based on groups of R genes from *S. demissum* introgressed into potato cultivars grown on large scale was soon overcome by the pathogen (van der Planck 1957; Ross 1986).

Although tuber resistance is an essential element of potato resistance to *P. infestans*, not much attention was paid to it in research and breeding (reviewed by Świeżyński and Zimnoch-Guzowska 2001). Tubers are infected under field conditions by infected foliage (Hirst et al. 1965) or during tuber handling (Dowley and O'Sullivan 1991). The rate of tuber resistance changes with progressive tuber age. The level of tuber resistance also changes with the storage period (Malcolmson 1981; Darsow 1983; Lebecka et al. 2006). In general the resistance of lenticels, tuber eyes, and cortical and medullar tissues may differ (Lapwood and McKee 1961; Lapwood 1965). Overwintering of pathogen in tubers or by means of oospores is possible, however overwintering in tubers seems to be prevalent (Flier et al. 1998). In cultivars with R genes, an incompatible reaction is expressed both in foliage and tubers; however, in the case of some R genes, only a small effect of delayed tuber infection might be observed. Expression of R genes in tubers might be dependent on potato genotype and testing conditions (Lapwood and McKee 1961; Doke 1982). Positive, but rather loose correlation was noted by several authors between foliage and tuber resistance, which was related to the type of materials tested and evaluation methods applied (Świeżyński and Zimnoch-Guzowska 2001).

The *P. infestans* populations that dominated in Europe and the United States (mainly US-1 lineage) until the mid-1970s were of the A1 mating type and asexually reproduced. The old populations were displaced worldwide by new populations of both the A1 and A2 mating types, capable of sexual reproduction with increased aggressiveness, fitness, and virulence against host resistance (Spielman et al. 1991). R genes from *S. demissum* introgressed into widely grown cultivars were not able to provide durable resistance to a changing pathogen and its virulent races. Thus, several breeding centers switched to selection for field resistance, quantitatively inherited with the use of races of *P. infestans* compatible with the R genes present in the breeding pool (Toxopeus 1964; Umaerus 1970).

Race-non-specific type of resistance was advocated as weaker but durable and more stable than race-specific resistance (Colon 1994). Horizontal resistance is a quantitative trait, similarly effective against a wide spectrum of races of *P. infestans.* When R gene-based resistance was broken down by new races of *P. infestans,* breeders switched to the development of a broad genetic base and identification of differences in expression of single components of field resistance (i.e., infection efficiency, lesion growth rate, latent period, and sporulation capacity) (Thurston 1971). Sources of horizontal resistance were found in numerous *Solanum* species, including *S. demissum, S. stoloniferum, S. verrucosum*, *S. phureja,* and many others (Ross 1986).

Breeding for field resistance resulted in selection of late maturing genotypes, as this type of resistance is strongly associated with lateness (Toxopeus 1958; Świeżyński 1990). Later, Visker and colleagues (2005) confirmed that the most important QTL for foliage maturity type is located on chromosome V near molecular marker GP21, at the same location where the most important QTL for field resistance is present. As a consequence, the combination of late blight resistance in early maturing genotypes can be selected using a QTL other than that located on chromosome V. One of the important breeding programs focused on horizontal resistance has been under way at the International Potato Center (CIP) since 1990. The goal was to improve potato populations by increasing frequencies of alleles that enhance horizontal resistance to late blight in the absence of R genes. An important goal of the program was to broaden the genetic diversity of desired traits from potato wild relatives (Landeo et al. 2000). Several promising clones resistant under short-day conditions have been selected from the B3C1 population, with complex genetic backgrounds believed to contain horizontal resistance to late blight (Li et al. 2012). The enhancement of field resistance to *P. infestans* was found to be less effective especially in European breeding due to its truly polygenic nature and needed backcrossing to *S. tuberosum.*

In the past twenty years, researchers, disappointed by the long vegetation periods of materials and the slow progress in field resistance breeding, switched back to R genes found in different *Solanum* wild and cultivated species.

Bonierbale and colleagues (1988) noted high colinearity of potato and tomato genomes and a conservation of gene order. The use of comparative genomics tool indicated the conserved map positions of R loci within *Solanaceae* family, located in hot spots for resistance in the potato genome (Gebhardt and Valkonen 2001). Both comparative genomics and positional cloning are approaches useful in R gene isolation in potato.

With increasing knowledge on resistance to *P. infestans*, the differences in the nature of race-specific and race-non-specific resistances were found to be smaller, owing to the presence of R genes providing partial resistance to *P. infestans* and to residual resistance expressed by overcome R genes (Stewart et al. 2003). A similar hypothesis was formed by Allefs and colleagues (2005), proposing that under long-day conditions any level of foliage resistance higher than expected for a given maturity class is R gene based. The expression of R gene might be complete or partial depending on the virulence spectrum of the late blight pathogen. The QTLs for race-non-specific resistance were found to be located on all 12 potato chromosomes, confirming the polygenic nature of the trait (Simko 2002, Simko et al. 2006, Danan et al. 2011). Among the conserved QTLs found across different genetic materials, those on chromosomes III, IV, V, and VI became good candidates for both gene cloning and marker-assisted selection (MAS) (Gebhardt and Valkonen 2001). The authors underlined the importance of a candidate gene approach for MAS and the need for molecular identification of universal diagnostic markers, obtained by linkage disequilibrium mapping in wide gene pools.

The clustering of genes controlling monogenic and polygenic resistance to pathogens has

been observed in the potato genome. Some of the genes underlying the resistance QTL in their structure may be related to R genes responsible for resistance to the same or different pathogen and may be encoded by the same classes of genes (Gebhardt and Valkonen 2001).

In 2002 the Global Initiative on Late Blight (GILB) organized a survey of research and breeding for resistance to late blight in potato, in which 39 centers from 25 countries participated. Resistance to late blight was a high priority trait for more than 60% of the questioned centers. The majority of responding breeding centers (70%) indicated that the most preferred type of resistance was the one in which both R genes and race-non-specific resistances operate together. For 26% of respondents, exclusive race-non-specific resistance was the most valuable (Zimnoch-Guzowska and Flis 2002).

Progress in identifying and cloning R genes within the *Solanum* species allowed for the proposal of new breeding methods for durable late blight resistance based on genetic modification with cisgenes in order to breed marker-free cultivars with different cassettes of R genes of *Solanum* origin. In this proposal management of the resistance control, based on temporal and spatial composition of R genes in the cassette set, adapted to the changing pathogen potential (Haverkort et al. 2008).

Sources of Resistance to *P. infestans*

According to the recent taxonomic and molecular studies, there are 188 known *Solanum* species of wild potato along with one cultivated species in section *Petota* and three species in section *Etuberosa* (Spooner and Salas 2006). Part of these species are the original ancestors of today's potato cultivars. Spooner et al. (2005) hypothesized that potato was domesticated in Peru, on the basis of an amplified fragment length polymorphism (AFLP) markers study.

Wild relatives are a rich source of natural resistances to diseases, pests, and climatic stresses. Wild *Solanum* species grow from the Southwest of United States to the south of Chile, with most species found in Peru and Bolivia. Their habitats include diverse climates and soil conditions. There are more than 4,300 varieties of native potatoes, mostly found in the Andes.

At the International Potato Center (CIP) more than 7,000 accessions of native, wild, and improved varieties are maintained in the gene bank – the largest collection in the world. This gene bank is supplemented by the potato collections in the U.S. (NRSP-6), Germany (CGN and GLKS), Scotland (CPC), Russia (VIR), and Argentina (INTA). The Association of Potato Inter-genebank Collaborators (APIC) developed the Inter-genebank Potato Database (TPD), containing in total data of 11,819 wild potato accessions conserved in seven potato gene banks (Huaman et al. 2000). The database is available at www.potgenebank.org.

For a large number of wild potato accessions preserved in world gene banks, the information on resistance to diseases and pests has already been made available (Hanneman and Bamberg 1986; Zoteyeva 1986; Darsow and Hinze 1991; Colon 1994; Bradshaw et al. 1995; Bamberg et al. 1996; Angeli et al. 2000; Huaman et al. 2000). In 2002, the Global Initiative on Late Blight organized a survey on late blight resistance research and breeding, in which 34 breeding programs and 5 research centers participated. Cultivars, breeding lines, and wild species were indicated as explored sources of resistance. In total, 44 cultivars were listed as resistance sources utilized in late blight resistance breeding. The cultivar Stirling was applied as a source by five centers; cultivars such as Jacqueline Lee, Kuras, Lugovska, and Zarevo were used by three centers; and Bionta, Cara, Innovator, and Torridon were used by two centers. In several centers breeding lines were explored as sources of resistance supplementing cultivars. The most popular were AWN 86514-2, A90586-11, and BO718-3. However, clones from the *Neotuberosum* program of Cornell University, ABPT hybrids from the Netherlands, and several selections from CIP, INIFAP, VIR, IHAR, and INTA were also listed.

The group of exploited wild species was led by the more often used *S. demissum* (16 centers), *S. bulbocastanum* (13), *S. microdontum* (9), *S. stoloniferum* (8), *S. andigena* (6), *S. berthaultii* (6), *S. hougasii* (6), *S. phureja* (6), and *S. verrucosum* (6). The rest of the group was represented by 25 wild species. Among them were *S. chacoense* (5), *S. pinnatisectum* (4), *S. vernei* (4), *S. iopetalum* (3), *S. acaule* (2), *S. circaeifolium* (2), *S. fendlerii* (2), *S. papita* (2) *S. polytrichon* (2), *S. stenotomum* (2), *S. sucrense* (2), and *S. tarijense* (2). An additional thirteen wild species were investigated only in single centers (Zimnoch-Guzowska and Flis 2002).

R genes

In the 1990s, the first major genes encoding resistance (R genes) to late blight were located on potato genetic maps. They all originated from *S. demissum*, and although they no longer provide effective resistance in the field, there has been renewed interest in vertical resistance. Many scientists started the search within the collections of wild potato relatives for new R genes, potentially more durable ones than those from *S. demissum*. Updated, detailed data on R genes mapped and cloned and implications of the mode of operation of R genes in potato are described in this section. R genes for late blight resistance (resistance to *P. infestans*, *Rpi* genes) that have been mapped so far are summarized in Figure 14.1. It was postulated that the R genes not only for late blight resistance but also encoding resistance to other diseases and even pests tend to be located together, in several segments of the potato genome called resistance hot spots (Gebhardt and Valkonen 2001). Later, after many more resistance genes were identified and mapped, this postulate remained valid, especially for the *Rpi* genes that, although from different *Solanum* species, are all located in ten hot spots or clusters on eight potato chromosomes (Figure 12.1). These clusters usually contain genes of high sequence similarity.

R1

The first genetic maps of potato were constructed of restriction fragment length polymorphism (RFLP) markers, as was the map that located the first late blight resistance gene, *R1*, to potato chromosome V between the markers GP21 and GP179 (Leonards-Schippers et al.1992). Apart from the application of molecular markers, the use of potato dihaploids, which made genetic analyses and mapping feasible, was a breakthrough in the research on *Rpi* genes (Leonards-Schippers et al.1992). Later, the position of the *R1* gene was shown to overlap with the position of a quantitative trait locus (QTL) for late blight resistance (Leonards-Schippers et al. 1994; Sandbrink et al. 2000) and in other studies, with a QTL for foliage and tuber resistance to *P. infestans* as well as a QTL for foliage maturity (Oberhagemann et al. 1999; Collins et al. 1999; Bradshaw et al. 2004; Śliwka et al. 2007). More detailed studies devoted to the relation between maturity and late blight resistance also support the importance of the *R1* region for both traits but have not revealed so far if it is a pleiotropic effect of the same genes or rather a result of different genes genetically linked (Visker et al. 2003; Bormann et al. 2004). Meta-QTL for both traits were also detected in this region (Danan et al. 2011).

The *R1* gene was also the first *Rpi* gene that was cloned from a diploid potato line (Ballvora et al. 2002). This was done by positional cloning combined with a candidate gene approach. The *R1* gene was shown to belong to the R gene family with characteristic domain composition containing a coiled coil (CC), a nucleotide-binding site (NBS), and a leucine-rich repeat domain (LRR). The functional *R1* gene was present as a large insertion only in a resistance allele (Ballvora et al. 2002). However, the complex nature of the *R1* gene cluster was described in three genomes of allohexaploid *S. demissum*, where numerous and diverse *R1* homologues, including an *R1* sequence identical to the one mentioned above (Ballvora et al. 2002), formed three NBS-LRR families (Kuang et al. 2005).

Knowledge of the *R1* sequence was exploited in several ways. A PCR marker specific to the *R1* gene sequence and a closely linked anonymous marker were used in an association study in a gene bank collection of 600 potato cultivars. The *R1* was present in approximately 33% of them. In the association analysis, a highly significant QTL for resistance to late blight and maturity was again detected in the *R1* region, and moreover, the marker alleles associated with increased resistance and later plant maturity were traced to an introgression from *S. demissum* (Gebhardt et al. 2004). Beketova and colleagues (2006) tested 70 potato cultivars bred mostly within the former USSR (of which 26 were included in the study by Gebhardt et al. 2004) for the presence of *R1,* and they also found a significant relationship between the presence of *R1*, late blight resistance, and late maturity. The *R1* gene could be more widespread than expected, as it was shown in the case of Black's differentials R5, R6, and R9, which contained *R1* in addition to *Rpi* genes indicated by their names (Trognitz and Trognitz 2007; Kim et al. 2011). *R1* analogs were analyzed by PCRs specific to the *R1* NBS sequence in *S. caripense* (Trognitz and Trognitz 2005). They have been found recently in some accessions of *S. demissum*, but also in several species of series *Demissa*, *Longipedicellata,* and *Tuberosa,* although none of them has been shown to be functional so far (Sokolova et al. 2011). Although a majority of contemporary *P. infestans* isolates are virulent on R1 plants, in breeding the *R1* could be still a useful marker for the late blight resistance QTL on chromosome V and therefore, *R1*- and *R2*-specific markers were included in a multiplex PCR for marker-assisted selection (MAS) of potatoes combining five disease and pest resistance genes (Mori et al. 2011). The *R1* gene can also be useful in that it was shown to act both in foliage and in tubers of potato (Park et al. 2005c). The *Avr1* gene encoding the *P. infestans* effector corresponding to the *R1* gene product has been isolated by positional cloning and shown to have the RXLR motif as well as to be highly induced during the biotrophic phase of potato infection (Vleeshouwers et al. 2011a).

R2

R2 was the first *Rpi* gene mapped in a tetraploid potato population and with use of amplified fragment length polymorphism (AFLP) markers. One of the genes introgressed from *S. demissum* was mapped to chromosome IV (Li et al. 1998). Although less widespread than *R1*, the *R2* gene is present in some European potato cultivars such as Eden, Fresco, Naturella, Pentland Dell, and Rector. The *R2* was shown to provide a delay in disease onset in field trials in France even though virulent isolates were found in the French population of *P. infestans* (Pilet et al. 2005).

The second gene mapped to the same locus, *R2-like*, could not be phenotypically distinguished from the *R2* but was identified in the mapping population that did not contain *S. demissum* in the pedigree (Park et al. 2005d). Another two genes, *Rpi-blb3* and *Rpi-abpt*, originated from *S. bulbocastanum*, a 1 EBN (endosperm balance number) species that could not be crossed directly with the potato (Park et al. 2005a, b, d). However, while the *Rpi-blb3* was mapped in an intraspecific *S. bulbocastanum* cross, the *Rpi-abpt* was mapped in advanced breeding material, in which the resistance – most likely from *S. bulbocastanum* – was introgressed into the potato gene pool by bridge crossing four species: *S. acaule* (A), *S. bulbocastanum* (B), *S. phureja* (P), and *S. tuberosum* (T) (Hermsen and Ramanna 1973). These materials were involved in the potato breeding programs that yielded at least five cultivars: Biogold, Bionica, Kibama, Kisoro, and Suprema (Web site for Potato Pedigree Database, Wageningen University: http://www.plantbreeding.wur.nl/potatopedigree/). The *Rpi-abpt* gene was shown to function only in potato foliage (Park et al. 2005c).

Rpi-blb3 and *Rpi-abpt* have been isolated by map-based cloning, and *R2* and *R2-like* by allele mining; and they all belonged to the leucine zipper (LZ)-NBS-LRR gene family. Something

else they have in common is that their products are involved in recognition of the same RXLR effector PiAVR2 (Lokossou et al. 2009). Presence/absence polymorphisms and differential transcription of this effector explain the virulence of *P. infestans* isolates on R2 plants (Gilroy et al. 2011). A method based on the polymorphism within the *Rpi* NBS domain sequences called NBS-profiling resulted in identification of a new member of the *R2* family, *Rpi-snk1*, within the species *S. schenckii* (Jacobs et al. 2010). This finding was further confirmed by effectoromics, that is, screening *Solanum* genotypes with *P. infestans* effectors for the hypersensitive reactions and *R2* allele mining. Apart from *Rpi-snk1*, *Rpi-snk1.2* from the same species, *Rpi-edn1.1* from *S. edinense* and *Rpi-hjt1.1-1.3* from *S. hjertingii* were localised to the *R2* cluster, cloned, and shown to recognize PexRD11 and PiAvr2 effectors (Champouret 2010; Verzaux 2010).Within *S. brachistotrichum*, an *Rpi-bst1* gene was mapped to the Rpi cluster on chromosome IV and the cloning of this gene was begun (Hein et al. 2009).

Several QTLs for late blight resistance spanning the same region have been discovered in different mapping populations (Leonards-Schippers et al. 1994; Oberhagemann et al. 1999; Collins et al. 1999; Sandbrink et al. 2000; Śliwka et al. 2007; Danan et al. 2009), and this resulted in locating there a meta-QTL for this trait (Danan et al. 2011). QTLs for maturity were detected in the same region in two studies (Collins et al. 1999; Bormann et al. 2004). A QTL for resistance to *P. infestans* on chromosome IV originated from many wild species and two of them repeat in independent studies: *S. spegazzinii* (Leonards-Schippers et al. 1994; Danan et al. 2009), and *S. microdontum* (Sandbrink et al. 2000; Śliwka et al. 2007). The resistance from *S. microdontum*, although quantitative and expressed in the field conditions, was later defined more precisely as encoded by a single locus, $R_{Pi\text{-}mcd1}$ (Tan et al. 2008). The $R_{Pi\text{-}mcd1}$ gene was then used in gene pyramiding together with $R_{Pi\text{-}ber}$. Its effect was rather weak, that is, it caused a delay of three days to reach 50% infection. However, there was an additive effect, suggesting that $R_{Pi\text{-}mcd1}$ can be still useful for potato breeding programs (Tan et al. 2010). The last gene of the *R2* cluster to be identified so far is *Rpi-dmsf1*, originating most likely from *S. demissum* and shown to provide quantitative and potentially durable resistance that was also tested in the field (Hein et al. 2007; Hein et al. 2009).

Rpi-blb2

The *Rpi-blb2* gene was first mapped in tetraploid backcross populations derived from ABPT materials mentioned above (Hermsen and Ramanna 1973; van der Vossen et al. 2005). Its position on chromosome VI corresponded to the position of the tomato *Mi-1* gene for resistance to nematodes, aphids, and white flies. A more precise map was constructed using F1 progeny of intraspecific *S. bulbocastanum* cross, and the *Rpi-blb2* gene was positionally cloned and shown to be a close homolog of the *Mi-1* gene, within the CC-NBS-LRR gene family (van der Vossen et al. 2005). It was detected in ABPT-derived cultivars Toluca and Bionica (Vleeshouwers et al. 2011a). Alone or together with the *Rpi-blb1*, the *Rpi-blb2* gene is also being introduced into potato cultivars via cisgenesis (Haverkort et al. 2009). One such cisgenic cultivar, Fortuna, with both *Rpi-blb1* and *Rpi-blb2* genes, is now being tested in advanced field trials by BASF (Web site: http://www.basf.com/group/corporate/en_GB/function/conversions:/publish/content/products-and-industries/biotechnology/images/Fortuna_VC.pdf). However, *P. infestans* isolates able to infect plants with each of these two genes separately or even stacked together were found in a Dutch population of this pathogen in the years 2007-2008 (Förch et al. 2010). *Avrblb2* effector is an RXLR gene and belongs to the Avrblb2 family, which is highly variable and under diversifying selection (Oh et al. 2009). It accumulates around haustoria and significantly enhances susceptibility of the host plant to *P. infestans* (Bozkurt et al. 2011).

Another late blight resistance gene, *Rpi-ver1,* from *S. verrucosum,* was located on chromosome VI by NBS-profiling (Jacobs et al. 2010). The only marker linked to this gene in two rather small mapping populations (Jacobs et al. 2010), on the potato consensus map is located 4 cM away from the *Rpi-blb2* gene (Danan et al. 2011). A finer mapping is needed to resolve the question of whether the *Rpi-ver1* is a member of the *Rpi-blb2* cluster or a separate one.

Rpi1

The *Rpi1* gene encoding resistance against *P. infestans* was identified in the *S. pinnatisectum* and mapped to potato chromosome VII using an interspecific cross with *S. cardiophyllum* (Kuhl et al. 2001). Later, as a starting point for cloning this gene, two BAC (bacterial artificial chromosome) libraries were constructed from *S. pinnatisectum,* and four markers linked to the *Rpi1* gene were hybridized to 14 BAC clones of these libraries (Chen et al. 2004). So far, the sequence of this gene has not been published. Within *S. michoacanum*, a species that is believed to be a natural hybrid of *S. bulbocastanum* and *S. pinnatisectum*, the *Rpi-mch1* gene was recently mapped to a similar location using Diversity Array Technology (DArT) (Śliwka et al. 2012a).

RB/Rpi-blb1

The first R gene for *P. infestans* resistance located on chromosome VIII was identified and sequenced independently by two research groups in *S. bulbocastanum*. Under the name *RB*, it was mapped (Naess et al. 2000) and cloned (Song et al. 2003) using BC2 populations derived from somatic hybrids (Helgeson et al. 1998). The same gene, although named *Rpi-blb1*, was cloned using an F1 population of the intraspecific *S. bulbocastanum* cross. This CC-NBS-LRR gene was found in a cluster of four resistance gene analogs with the same domain composition, and analyses of their diversity suggested that *Rpi-blb1* could be relatively old and subject to balancing selection (van der Vossen et al. 2003). A highly similar gene originating from the same species, *Rpi-bt1*, was also mapped to the potato chromosome VII and then cloned (Oosumi et al. 2009). The *RB* is the best characterized Rpi gene. Its transcription level was shown to correspond to gene copy number (Bradeen et al. 2009) and it was correlated with late blight resistance of the transgenic plants (Kramer et al. 2009). Factors such as temperature, physiological age of the plant, and genetic background did not alter the transcription of the *RB* gene (Iorizzo et al. 2011). The *Sgt1* gene (suppressor of the G2 allele of *skp1*) was reported to be essential for the RB-mediate resistance (Bhaskar et al. 2008). Allele-specific PCR and RT-PCR markers for the presence of this gene were developed and applied in breeding programs (Colton et al. 2006; Millet and Bradeen 2007). Field tests of transgenic *RB* lines indicated their high foliar resistance and susceptibility of tubers (Halterman et al. 2008). A proteomics study was also done during the process of RB-mediated plant defence (Liu and Halterman 2009).

Several studies were dedicated to the search for *RB/Rpi-blb1*-like sequences in diverse *Solanum* species. Several *RB* orthologs were found in *S. verrucosum* and one of them proved to be functional after transfer into susceptible potato (Liu and Halterman 2006). Allele mining and effector genomics allowed for discovery of three more functional homologs of *Rpi-blb1*: *Rpi-sto1* from *S. stoloniferum*, *Rpi-pta1* from *S. papita,* and *Rpi-plt1* from *S. polytrichon* (Wang et al. 2008; Vleeshouwers et al. 2008). Screening of 196 different taxa from *Solanum* section *Petota* resulted in detection of an *Rpi-blb1* fragment in *S. cardiophyllum* subsp. *cardiophyllum* and *S. stoloniferum*, apart from various *S. bulbocastanum* accessions, and showed geographical confinement of these homologs to Central America (Lokossou et al. 2010). *RB/Rpi-blb1* homologs were also detected in several other species (Sokolova et al. 2011; Pankin et al. 2011).

Although both *RB* and *Rpi-blb1* were described as providing broad-spectrum

resistance to *P. infestans* (Song et al. 2003; van der Vossen et al. 2003), compatible isolates have already been noted (Förch et al. 2010). A virulence factor corresponding to the *RB/Rpi-blb1* gene is an RXLR effector from a highly diverse *ipiO* gene family (Vleeshouwers et al. 2008; Champouret et al. 2009). It causes a hypersensitive response also in plants with *Rpi-pta1* and *Rpi-sto1* genes (Vleeshouwers et al. 2008). *P. infestans* isolates lacking whole class I of *ipiO* genes were virulent on *RB/Rpi-blb1* plants (Champouret et al. 2009). Diversity of ipiO was also described by Halterman et al. (2010), and evidence was reported of *P. infestans* inhibiting recognition of the effector protein and in that way defeating the plant's resistance.

Rpi-phu1

The first Rpi gene from this cluster on chromosome IX was identified in complex interspecific potato hybrids but most likely it originated from *S. phureja*. The *Rpi-phu1* gene provided very high level of broad-spectrum late blight resistance both in potato foliage and in tubers and it was not linked with a long vegetation period (Śliwka et al. 2006). It is now being used in conventional potato breeding programs together with a molecular marker suitable for marker-assisted selection (Śliwka et al. 2010). In later studies, an Rpi gene from *S. venturii* was mapped to the same position and then cloned and sequenced by two research teams (Foster et al. 2009; Pel et al. 2009). Three variants of this CC-NBS-LRR gene – *Rpi-vnt1.1*, *Rpi-vnt1.2,* and *Rpi-vnt1.3* – were detected in different *S. venturii* accessions (Foster et al. 2009; Pel et al. 2009) and the sequence of the *Rpi-phu1* gene was shown to be identical with the *Rpi-vnt1.1* (Foster et al. 2009). Cisgenic plants containing *Rpi-vnt1* have been in field trials in the UK, Belgium, and the Netherlands (GMO notifications: B/BE/10/V1 and B/GB/10/R29/01).

So far, only *P. infestans* isolates belonging to the clonal lineage of EC1 from Ecuador have been shown to be capable of infecting *Rpi-vnt1* plants (Foster et al. 2009; Pel et al. 2009). The corresponding RXLR effector was identified using effectoromics and named *Avrvnt1,* and only four variants of this sequence were detected in a broad set of *P. infestans* isolates. In virulent EC1 strains the *Avrvnt1* coding sequence was intact but its transcript was not detected (Pel 2010; Vleeshouwers et al. 2011a).

A large effect QTL for late blight resistance and a conditional QTL acting only at a certain stage of infection have recently been described in the *Rpi-phu1* region of chromosome IX (Li et al. 2012).

Rpi-mcq1

There is one more cluster of Rpi genes on chromosome IX, located approximately 15 cM away from the *Rpi-vnt1* genes (Danan et al. 2011), at the distal end of the long arm of the chromosome (Smilde et al. 2005). *Rpi-moc1* was first mapped in *S. mochiquense* using an intraspecific cross (Smilde et al. 2005), but later it was renamed with a proper acronym of this species name, that is *Rpi-mcq1* (Hein et al. 2009). The sequence of this gene has not been published but it was patented (Patent number WO2009013251) and it is being used in cisgenic potatoes in the UK (GMO notification B/GB/10/R29/01). Another Rpi gene was mapped to the same location in European accessions of *S. dulcamara*, a species that is distantly related to *S. tuberosum* and that does not produce tubers. The *Rpi-dlc1* gene was shown to provide incomplete resistance to *P. infestans* (Golas et al. 2010). *Rpi-edn2* was mapped with the use of NBS-profiling in *S. edinense,* and although the mapping data were not conclusive, the author postulated that the gene was most likely homologous to *Tm-2*^{2} and located in the *Rpi-mch1* cluster. The gene was naturally stacked with two others that together provided a wide spectrum of late blight resistance (Verzaux 2010). Until now, *R8* from *S. demissum* has been the last Rpi gene mapped to this cluster and not to the chromosome XI as suggested previously (Jo et al. 2011). The R8 differential plant that served as

a parent of the mapping population was shown to be stacked with four Rpi genes (*R3a, R3b, R4,* and *R8*), while the *R8* gene was also present in the R9 differential. However, the contribution of the *R8* gene to resistance was remarkable in recent field trials (Kim et al. 2011).

R_{ber}

The first Rpi gene mapped on chromosome X originated from *S. berthaultii* (Ewing et al. 2000). The same gene, later named $R_{Pi\text{-}ber}$, was mapped more precisely to a distance of 4.6 cM from the marker TG403 (Rauscher et al. 2006). When isolates of *P. infestans* compatible with $R_{Pi\text{-}ber}$ were used for inoculation, a smaller but significant resistance effect was detected in the same map position as the R gene. This could be explained by the residual effect of the gene or/and a resistance QTL located in the same position (Rauscher et al. 2010). The $R_{Pi\text{-}ber}$ gene proved to be effective in both foliage and tubers (Mayton et al. 2011). In a breeding experiment on the effects of pyramiding of the R genes, mentioned in the *R2* section of this chapter, $R_{Pi\text{-}ber}$ was used, together with the $R_{Pi\text{-}mcd1}$ gene, and both genes showed an additive effect on resistance to late blight in a field test. A larger effect was provided by the $R_{Pi\text{-}ber}$, which produced a three-week delay in infection reaching 50% of the leaf area (Tan et al. 2010). In the same species, *S. berthaultii,* two more R genes, *Rpi-ber1* and *Rpi-ber2*, were identified and mapped to chromosome X (Park et al. 2009). Park and colleagues speculate that, on the basis of location and origin, *Rpi-ber1* (Park et al. 2009) may be identical to $R_{Pi\text{-}ber}$ (Rauscher et al. 2006). *Rpi-ber2* is most likely a different gene, although located in a similar position 12 cM north of the marker TG403 (Park et al. 2009). Recently, an *Rpi-rzc1* from *S. ruiz-ceballosii* (syn. *S. sparsipilum*) was mapped to the same location with use of Diversity Array Technology. It provided a high level of resistance to *P. infestans* in detached leaflet, tuber slice, and field tests. Remarkably it was linked to the violet flower color encoded by the *F* locus, which can serve as a phenotypic marker in addition to molecular ones (Śliwka et al. 2012b).

Apart from R genes, a number of QTLs for resistance to *P. infestans* have been mapped to potato chromosome X (Oberhagemann et al. 1999; Śliwka et al. 2007; Danan et al. 2009). These QTLs originate from various *Solanum* species, including *S. sparsipilum*, which is a synonym of *S. ruiz-ceballosii* (Danan et al. 2009). A QTL meta-analysis that allowed more precise comparisons of different genetic maps has identified two meta-QTLs for late blight resistance on potato chromosome X. The *Rpi-ber1*/$R_{Pi\text{-}ber}$ is located between them and the *Rpi-ber2* localization overlaps with a meta-QTL named MQTL_2_Late_blight (Danan et al. 2011).

R3

This gene from *S. demissum* was the first one mapped to chromosome XI (El-Kharbotly et al. 1994). It was soon followed by *R6* and *R7,* originating from the same species (El-Kharbotly et al. 1996). High-resolution mapping of *R3* combined with precise phenotyping with specific *P. infestans* isolates brought a discovery that there are actually two closely linked genes with distinct specificities, *R3a* and *R3b* (Huang et al. 2004). These two genes were subsequently shown to be CC-NBS-LRR genes with 82% of nucleotide identity (Huang et al. 2005; Li et al. 2011). Genes *R5, R6, R7, R8, R9, R10,* and *R11* were all suspected to be alleles of the same locus on chromosome XI (Huang 2005). Apart from *R6* and *R7* mentioned above, the localization of *R10* and *R11* was also confirmed by mapping (Bradshaw et al. 2006), but *R8* has more recently been mapped to chromosome IX (Jo et al. 2011). A functional homolog of *R3a* – *Rpi-sto2* – was identified in *S. stoloniferum* (Champouret 2010). The region on chromosome XI that contained the described Rpi genes was shown in several studies to also harbor the QTL for resistance to *P. infestans* and for maturity, introduced from diverse

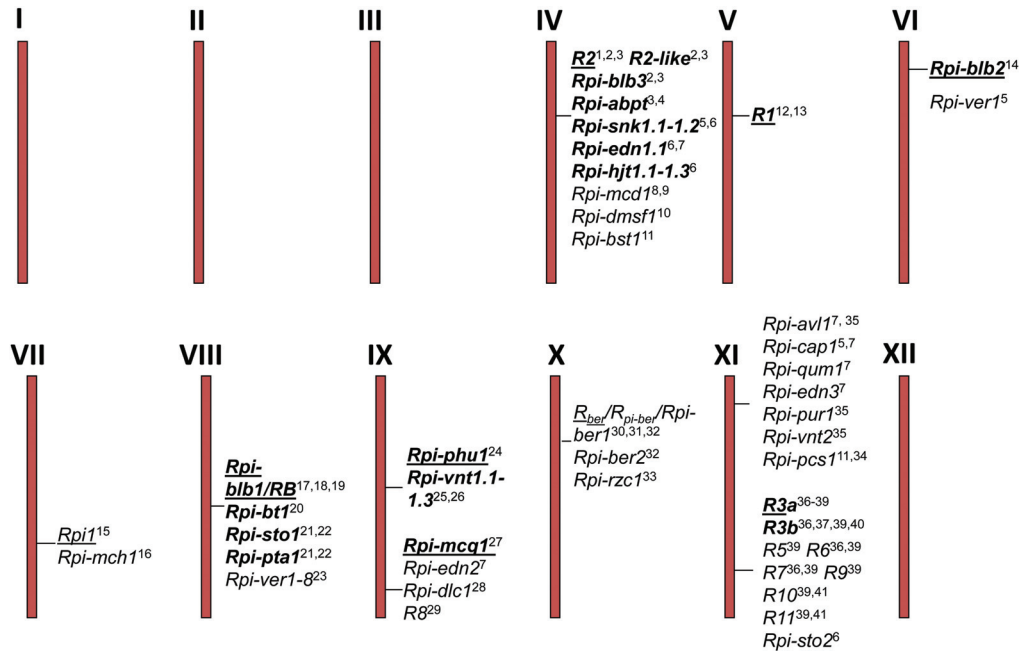

Fig. 12.1. Potato *Rpi* genes on the genetic map. Genes that were cloned are bolded, while those that are underlined are the gene cluster names that provide the section titles of this chapter. [1]Li et al. 1998, [2]Park et al. 2005a, [3]Lokossou et al. 2009, [4]Park et al. 2005b, [5]Jacobs et al. 2010, [6]Champouret 2010, [7]Verzaux 2010, [8]Sandbrink et al. 2000, [9]Tan et al. 2008, [10]Hein et al. 2007, [11]Hein et al. 2009, [12]Leonards-Schippers et al. 1992, [13]Ballvora et al. 2002,[14]Van der Vossen et al. 2005, [15]Kuhl et al. 2001, [16]Śliwka et al. 2012a, [17]Naess et al. 2001, [18]Song et al. 2003, [19]Van der Vossen et al. 2003, [20]Oosumi et al. 2009, [21]Wang et al. 2008, [22]Vleeshouwers et al. 2008, [23]Liu and Halterman 2006, [24]Śliwka et al. 2006,[25]Pel et al. 2009, [26]Foster et al. 2009, [27]Smilde et al. 2005, [28]Golas et al. 2010, [29]Jo et al. 2011, [30]Ewing et al. 2000, [31]Rauscher et al. 2006, [32]Park et al. 2009, [33]Śliwka et al. 2012b, [34]Villamon et al. 2005, [35]Rietman 2011, [36]El-Kharbotly et al. 1994, [37]Huang et al. 2004, [38]Huang et al. 2005, [39]Huang 2005, [40]Li et al. 2011, [41]Bradshaw et al. 2006. For a color version of this figure, please refer to the color plate.

origins such as wild relatives and potato accessions (Leonards-Schippers et al. 1994; Collins et al. 1999; Bormann et al. 2003). Meta-QTLs for both traits were detected here as well (Danan et al. 2011).

R3a is widely present in potato cultivars and the compatible *P. infestans* strains are also widespread (Vleeshouwers et al. 2011). *Avr3a* is a quite well characterized RXLR effector that has two alleles – virulent and avirulent – differing only in two amino acids (Armstrong et al. 2005). Products of both alleles are able to suppress the cell-death response in potato, although the avirulence allele has a stronger effect. The Avr3a was shown to suppress immunity by binding and stabilizing one of the host ubiquitin ligases (Vleeshouwers et al. 2011a).

A new cluster of Rpi genes has been recently discovered in a different region of chromosome XI using NBS profiling and effectoromics. It contains genes from *S. avilesii, S. capsicibaccatum, S. circaeifolium ssp. quimense, S. edinense, S. venturii, and S. piurae* (Figure 12.1) (Jacobs et al. 2010; Verzaux 2010; Rietman 2011). The gene *Rpi-avl1*, from *S. avilesii* was later mapped with higher resolution and its spectrum was characterized with the use of various *P. infestans* isolates (Verzaux et al. 2011). Late blight resistance of *S. paucissectum* was also mapped to this region (Villamon et al. 2005), and it was described as an *Rpi-pcs* locus by Hein and colleagues (2009).

In a recently revealed genome sequence of potato, there were 408 NBS-LRR-encoding

genes detected. Among genes involved in various resistances, some must play a role in interactions with *P. infestans*. Homologs highly related to the late blight resistance genes *R1, RB, R2, R3a, Rpi-blb2,* and *Rpi-vnt1.1* were present in the assembly (Potato Genome Sequencing Consortium 2011). A more detailed study of the same genome revealed 438 NB-LRR genes organized within 63 clusters. Among them there were 107 genes with CC domain and 77 containing TIR domain (Jupe et al. 2012). Significant sequencing efforts have been devoted to another potato genome, RH89-039-16 (*S. tuberosum* ssp. *tuberosum*), where 738 partial and full-length NB-LRR sequences have been identified (Bakker et al. 2011). The number of known functional Rpi genes and their homologues identified by allele mining in various wild species is fast growing. Recently, an online tool for cataloguing and analyzing the R genes from *Solanum* section *Petota* in relation to their phylogeny, has been developed (Vleeshouwers et al. 2011b, http://www.plantbreeding.wur.nl/SolRgenes/index.php).

On the other hand, the corresponding arsenal of *P. infestans* was predicted to contain 563 RXLR effectors, located in fast mutating, gene sparse, and repeat rich regions of the genome (Haas et al. 2009). Access to genome sequences of both host and pathogen will, one hopes, bring a better understanding of their interaction and with that knowledge, progress in the application of genomics in potato breeding.

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Chapter 13

Late Blight of Tomato

Marcin Nowicki, Elżbieta U. Kozik, and Majid R. Foolad

Abstract

Late blight (LB), caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most destructive diseases of tomato (*Solanum lycopersicum* L.) and potato (*S. tuberosum* L.) worldwide, causing significant economic losses annually. The success of *P. infestans* as a pathogen originates from its effective asexual and sexual life cycles, as well as its remarkable capacity to rapidly overcome plant resistance genes, a result of its high evolutionary potential. The most sustainable strategy to manage tomato LB would be to deploy an integrated system including cultural practices, fungicide application, and the use of cultivars with broad-spectrum genetic resistance against LB. Prior to the reemergence of LB in the late 1980s, cultural practices in combination with fungicide applications were highly effective measures to control the tomato LB. However, with the appearance of new and more aggressive isolates of *P. infestans*, many of which are resistant to LB-specific systemic fungicides, the greatest contribution to tomato LB control in the future will have to be through the development of cultivars with improved genetic resistance. Thus far, a number of major LB-resistance genes and quantitative trait loci (QTLs) have been identified in tomato and several breeding lines and cultivars, with improved resistance developed. Research is also underway to identify additional resistance genes or QTLs and to pyramid multiple resistance factors in order to develop stronger and more durable resistance. Further, as exemplified by the fast progress in potato LB research and conservation of LB signaling pathways between potato and tomato, detailed knowledge of the pathogen effectors in combination with high-throughput genomics technology will facilitate a better understanding of the LB disease and host-pathogen interactions, which in turn may lead to development of tomatoes with more durable resistance.

Introduction

Tomato, *Solanum lycopersicum* L. (formerly known as *Lycopersicon esculentum* Mill.) is a major vegetable crop worldwide (FAOSTAT 2011). Tomato is thought to have originated in the Andean region of South America, now encompassed by parts of Peru, Chile, Colombia, Ecuador, and Bolivia (Jenkins 1948; Rick 1978), and domesticated in Mexico (Rick 1976). Although a tropical plant, it is grown in almost every corner of the world (see below). The broad

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spectrum use of tomato, including its importance in a healthy and balanced diet, has resulted in increased production of both processed and fresh varieties worldwide in the recent past.

Disease is the number one concern to both fresh-market and processing tomato industries throughout the world, and economic losses resulting from crop damage or disease control measures are significant (http://faostat.fao.org/). More than 200 pests and diseases have been identified as tomato pathogens hampering its production (Lukyanenko 1991). Among these, many are frequently occurring diseases caused by fungi, oomycetes, bacteria, viruses, and nematodes (listed in Foolad 2007). Late blight (LB), caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most destructive diseases of tomato as well as potato (*Solanum tuberosum* L.) worldwide, causing significant economic losses annually (reviewed in Foolad et al. 2008 and Nowicki et al. 2012). The pathogen is best known for its role in the Irish potato famine, where it caused the loss of more than a million lives (Andrivon 1996).

When left uncontrolled, *P. infestans* can destroy a tomato or potato crop within several days. The success of *P. infestans* as a pathogen originates from its effective asexual and sexual life cycles as well as its remarkable capacity to rapidly overcome plant resistance genes (Foolad et al. 2008; Nowicki et al. 2012). The latter feature has led researchers to describe *P. infestans* as a pathogen with a "high evolutionary potential" (Raffaele et al. 2010b). Evolutionary and comparative analyses of the *P. infestans* genome reveal the peculiar architecture that underpins the pathogen's accelerated adaptation to host plants (Raffaele et al. 2010a; Vleeshouwers et al. 2011). The ability of *P. infestans* to propagate asexually and through sexual mating results in rapid reproduction, fast moving epidemics, and increased genetic diversity and survival (Fry 2008). A sustainable control of the LB disease requires integration of cultural practices, fungicide applications, and use of resistant cultivars (Fry and Goodwin 1997; Nowicki et al. 2012).

At the first stage of infection, *P. infestans* penetrates the plant and translocates effector proteins inside the host cells. Specific effectors can act as avirulence (*Avr*) factors and activate corresponding host-plant resistance genes (*R*-genes) according to the gene-for-gene resistance model (Hardham and Blackman 2010; Vleeshouwers et al. 2011; Nowicki et al. 2012). Upon recognition of the effector by the host-plant resistance protein (R-protein), effector-triggered immunity (ETI) is activated, often resulting in the hypersensitive response (HR). Thus far all cloned *R*-genes against *P. infestans* are of potato origin (Jia et al. 2010; Vleeshouwers et al. 2011), although a conservation of the LB-signaling pathways has been proven for both potato and tomato (Jia et al. 2009; Faino et al. 2010; Jia et al. 2010). Despite the current lack of cloned tomato *R*-genes, several sources of LB-resistance genes have been identified, and a few have been mapped to tomato chromosomes and successfully introduced into several tomato breeding lines and hybrid cultivars via plant breeding (Foolad et al. 2008; Gardner and Panthee 2010a; Gardner and Panthee 2010b; Panthee and Gardner 2010; Merk and Foolad 2011, Merk et al. 2012). Considering the resurgence of LB disease in the recent past, along with the decreasing effectiveness of fungicide treatments, it is prudent to conduct further research to identify and characterize new sources of resistance and develop new resistant cultivars by pyramiding multiple resistance genes. Currently, several research groups around the world are working towards this goal. In this chapter we summarize and discuss the current understanding of *P. infestans*, its effects on tomato production, and the genetics and breeding of LB resistance in tomato.

Significance of Tomato

Origin of the Species and Crop Production

The cultivated tomato (*S. lycopersicum* L.) originated from the Andean region, part of modern-day Chile, Boliva, Ecuador, Colombia, and Peru.

The exact time and place of tomato domestication is not clearly known; however, by the fifteenth century the crop had reached a fairly advanced stage of domestication. Tomato was exported to Europe during that time, and subsequent further intensification of the process occurred. Since the twentieth century, human beings have created a huge array of morphologically different cultivars and forms from the single species *S. lycopersicum* via plant breeding (Foolad et al. 2008). Worldwide efforts aimed at domestication, research, and breeding activities have resulted in modern tomato varieties (mostly hybrids) developed in all shapes, colors, and sizes. Modern tomato genomics knowledge has transformed breeding from an individually based activity to multidisciplinary teamwork, focused on exploiting genes from a tremendous tomato germplasm, for high efficiency breeding (Bai and Lindhout 2007).

Improvement in tomato cultivars is necessitated by increased tomato production in response to global demand for this commodity. In 2009, the total net economic value of tomato was ~Int.$56 billion, which ranked it fourth among all crop species (after rice, wheat, and soybeans) and first among all vegetable crops (FAOSTAT 2011). Although a tropical plant, tomato is grown in almost every region of the world, from the tropics to within a few degrees of the Arctic Circle. When outdoor production is restricted because of cold temperatures, the tomato is grown in greenhouses. Major tomato-producing countries (in descending order of tonnage as of 2009) include China, the United States, India, Turkey, and Egypt, followed by Italy, Iran, Spain, Brazil, Mexico, Russian Federation, and Uzbekistan (FAOSTAT 2011). In North America, production takes place in the U.S., Canada, and Mexico, comprising a total of approximately 310,000 ha.

Tomatoes are an important part of a diverse and balanced diet. In addition to tomatoes that are consumed as raw vegetables or added to other food items, a variety of processed products such as pastes, juices, sauces, and soups are mass-produced and marketed. Although tomato does not rank high in nutritional value, by virtue of volume consumed, it contributes significantly to the dietary intake of vitamins A and C, essential minerals, and other nutrients. For example, tomato ranks first among all fruits and vegetables as a source of vitamins, minerals, and phenolic antioxidants in the U.S. diet (Rick 1980; Vinson et al. 1998). In addition, fresh and processed tomatoes are the richest sources of the antioxidant lycopene, which arguably protects cells from oxidants that have been linked to cancer (Ashrafi et al. 2011). Market demands require high global production output of tomato. In order to provide growers and consumers with high quality tomatoes, breeders have to overcome a number of issues threatening tomato production.

Low Genetic Diversity within the Cultivated Species

Crop plant genomes have evolved under human selection, which often has led to loss-of-function mutations, such as loss of seed dispersal through shattering in grains, loss of seed dormancy, and loss of long branches (Sim et al. 2009). At the same time, some agriculturally desirable characteristics result from gain-of-function mutations. Examples include disease and pest resistance, high fruit nutritional quality, and large size of fruit and seed (Foolad 2007; Sim et al. 2009). During and following its domestication, the cultivated tomato has undergone intensive selection and a few genetic bottlenecks occurred, resulting in narrow genetic variation within the cultigen. For example, tomatoes that were originally introduced to Europe by Spanish explorers furnished the entire genetic base for the modern tomato cultivars throughout the world (Foolad 2007). The low genetic diversity in the cultivated tomato is reflected by a low level of isozymes and DNA markers polymorphism across tomato breeding lines and commercial cultivars (Labate and Baldo 2005). It is estimated that only about 5% of the total genetic variation within Solanum section Lycopersicon (i.e.,

all tomato species) can be found within the cultivated species, *S. lycopersicum*, and that genes for many desirable agricultural characteristics do not exist in this species (Miller and Tanksley 1990; Foolad 2007). The related wild tomato species, however, are a rich source of desirable genes and characteristics for crop improvement, though they remain largely under-exploited. For instance, a panel of 31 *S. lycopersicum* lines was approximately as polymorphic as one population of *S. pimpinellifolium*, the closest wild relative of the cultivated tomato (Labate et al. 2009). The species with the greatest variability are *S. chilense*, *S. haborchaites*, *S. peruvianum,* and *S. pennellii*, whereas the least variable species are *S. cheesmanii* and *S. pimpinellifolium* (Foolad 2007). Recent advancements in molecular markers and MAS (marker-assisted selection) technology are expected to make tomato improvement via introgression from wild species more feasible. Further, recent investigations based on single nucleotide polymorphism (SNP) suggest the existence of several tomato genome regions with much higher diversity relative to other tested regions (Labate and Baldo 2005; Sim et al. 2009; Hamilton et al. 2012; Sim et al. 2012). These regions are associated with introgressions from wild relatives and their identification will be useful for both tomato improvement and germplasm conservation. Moreover, the recent identification of SNPs within the tomato cultigen (Sim et al. 2009; Hamilton et al. 2012; Sim et al. 2012) facilitates effective use of marker technology in breeding programs that mainly exploit elite tomato germplasm.

Significance of Late Blight

Historical Significance of the Disease

Late blight (LB) has been identified as a major disease of tomato and potato and is one of the most devastating plant diseases of all time. An unprotected tomato field can suffer yield losses reaching up to 100% because of LB infection (Nowicki et al. 2012). *Phytophthora infestans* – literally, "plant destroyer," in Greek – has been traced back to the same origin as tomatoes and potatoes, that is, the Andean region (Foolad et al. 2008; Vleeshouwers et al. 2011). Common origin for both the host and pathogen populations, initially suggested in the nineteenth century shortly after the Irish potato famine (de Bary 1876), has been recently confirmed by isozyme and DNA studies as well as pathogenicity similarities among Peruvian, U.S., and European isolates of *P. infestans* (reviewed in Foolad et al. 2008; Vleeshouwers et al. 2011; and Nowicki et al. 2012). Briefly, the pathogen incited the first recorded instance of potato LB in Philadelphia and New York City, in the U.S., in 1843. Because of weather patterns, winds spread the dehiscent pathogen sporangia to neighboring states, dispersing them, and thus increasing the area affected by LB. By 1845, LB was affecting crops from Illinois to Nova Scotia and from Virginia to Ontario. The disease then crossed the Atlantic Ocean from the U.S. to Europe in 1845 with a shipment of infested seed potatoes. Once *P. infestans* reached Ireland, a country that was strongly dependent on potatoes as a main source of food and predisposed to adverse political, social, and economic factors, widespread potato LB resulted in a near-complete destruction of the crop. This led to the death of one million people and the displacement of another million refugees, many of whom immigrated to the United States. Continued spread of the pathogen in subsequent years resulted in a worldwide distribution of LB by the beginning of the twentieth century and global devastation of potato and tomato crops.

Recent observations confirm virulence of the pathogen towards potato, but also tomato: Once an unprotected crop (field, greenhouse, and/or plastic-cover cultures) is infected by *P. infestans*, the whole crop can be devastated within seven to ten days (Foolad et al. 2008). Economic losses may be in the form of reduced yield, lower quality of the fruit (such as low specific gravity), diminished storability, and increased cost

associated with fungicide applications (Nowicki et al. 2012).

Disease Cycle and Development

The severe fiscal and social impact of the pathogen increased scientific interest in LB research. Discoveries in the disease biology from potato-driven research have also benefited understanding of tomato LB. *P. infestans* can be particularly destructive in areas where both tomatoes and potatoes are grown year-round, for example in the highland tropics of Africa, the Americas, Asia, and Europe (Nowicki et al. 2012). The unhampered success of *P. infestans* as a pathogen originates from its effective asexual and sexual reproduction. The asexual form serves as the major vehicle driving epidemics during the season. In this form, *P. infestans* produces thousands of sporangia per lesion on sporangiophores (Figure 13.1C, D). Sporangiophores are the indeterminate structures that aid in air dispersal of the sporangia through wind, rain, or wind-blown rain transportation. The disease cycle begins when sporangia land on host plant tissue, which must be covered with a film of water, which is necessary for the motile, germinated spore movement towards a penetration site (Hardham and Blackman 2010). Sporangia germination occurs either through the direct extension of germ tubes or by zoosporogenesis. The latter is stimulated by cool and moist conditions, and it is important in that it extends the range of weather conditions in which infections can occur. Direct germination of sporangium on host tissue occurs at temperatures above 21° C (optimally at 25° C) in a process taking between 8 and 48 hours. The sporangium can germinate directly at temperatures above 15° C and quickly develops mycelial growth. At temperatures below 21° C, up to eight biflagellate zoospores are released from the sporangia, with optimal zoospore formation occurring at 12° C. Motile zoospores penetrate through the film of water, detach their flagella, and encyst until they produce germ tubes (Figure 13.1A, B). This occurs after approximately two hours at an optimum temperature (12 to 15° C). Germ tubes differentiate into appressoria that invade the host through the leaf cuticle, or less frequently, the stomata (Figure 13.1A, B). The optimal germ tube differentiation temperature is between 21 and 24° C. Intercellular hyphae develop and travel inside the host between cells, using haustoria to form biotrophic feeding relationships in the mesophyll (Figure 13.1A, B). Rapid colonization occurs between 22 and 24° C. Hyphae spread and sporangiophores eventually emerge from stomata (Figure 13.1C, D). Soon thereafter, LB symptoms become apparent, generally between five and ten days after inoculation. Sporulation occurs to produce 2N sporangia, which eventually release zoospores to promote aerial transmission of LB and continue the disease cycle (Judelson 1997). Disease development ceases if temperatures increase above 35° C, although *P. infestans* can survive in living host tissue and the disease can progress when conditions again become favorable (Scott and Gardner 2007).

Sporangia develop many organelles that are absent in hyphae, such as large peripheral vesicles, encystment vesicles, kinetosomes, and flagella. Zoosporogenesis involves cleavage of the multinucleate sporangial cytoplasm by nucleus-enveloping membranes. Subsequently, assembly of flagella, dissolution of the sporangial papilla, and expulsion of uninucleate zoospores take place. Several of the early-induced genes encode participants in vesicle movement that may aid in assembling these structures. Other early-induced genes encode components of the spore-specific vesicles or organelles themselves, including more than 70 flagella-associated, as well as a thrombospondin-like encystment proteins. Under favorable field conditions, sporangia can maintain viability for as long as a week, although levels of mRNAs enabling germination and affecting its dynamics usually decrease during this period (Judelson 1997; Hardham and Blackman 2010; Nowicki et al. 2012).

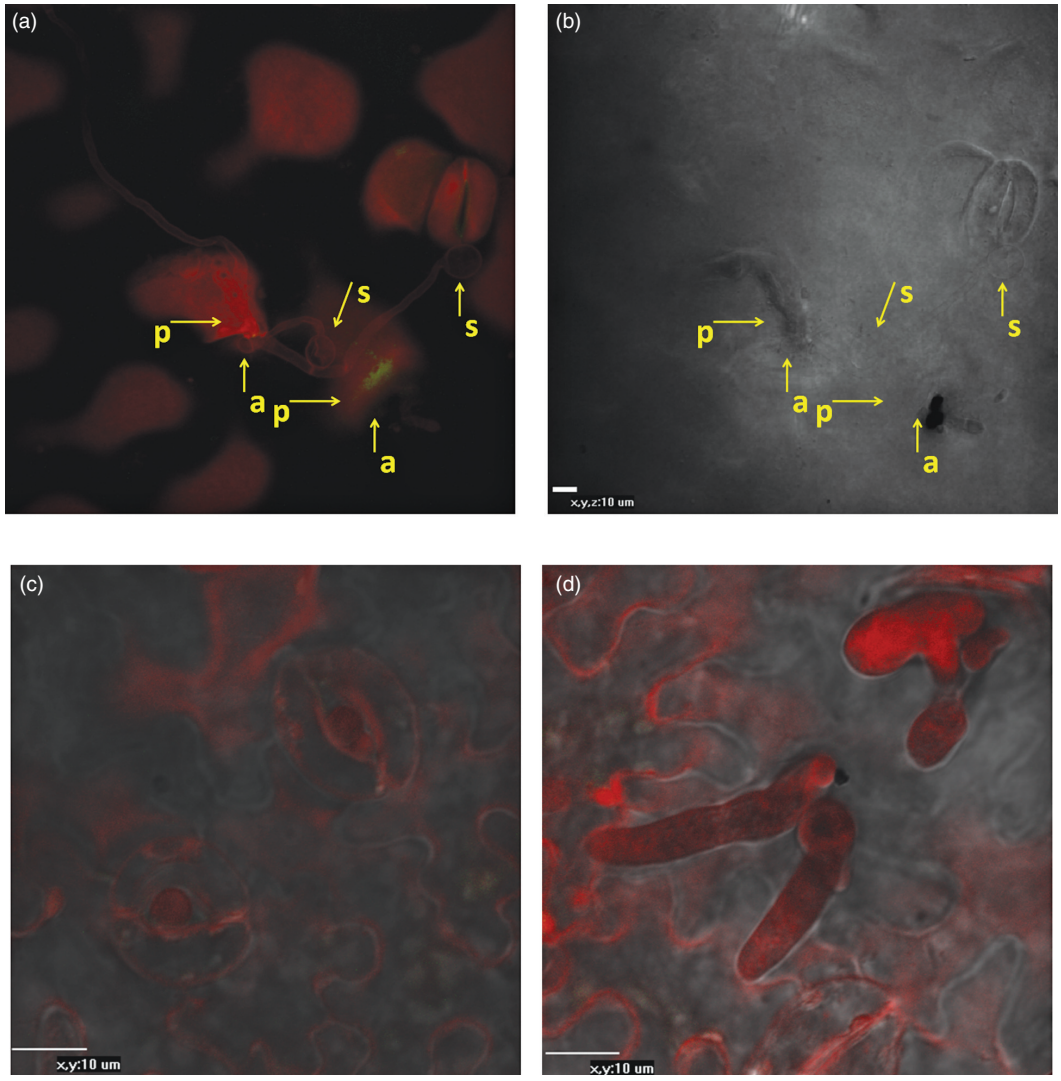

Fig. 13.1. *Phytophthora infestans* infection cycle on susceptible tomato cv. Rumba, as visualized by dual stain and confocal laser microscopy (Nowicki et al. 2012). Red channel: pathogen-specific stain and plant autofluorescence; green channel: callose-specific stain; black and white: DIC. (a) Germinating spore [s] develops infection tube and attempts leaf tissue penetration by formation of appressorium [a]. Host plant's primary line of defense is rapid formation of callose-rich papilla [p] directly underneath the attempted penetration area. (b) Same as A, but visible in DIC. (c) Upon successful infection, the pathogen grows in the leaf mesophyll, as denoted by the numerous hyphae. (d) At the end of the infection cycle, the hyphae emerge through the abaxial leaf-side stomata, followed by sporangial development. Rapid field infestation is achieved through successful colonization; descendant sporangia will drive the epidemics through the season. For a color version of this figure, please refer to the color plate.

The sexual life cycle of *P. infestans* requires the mating of individuals with opposite mating types, known as A1 and A2 (Gallegly and Galindo 1958; Gisi et al. 2011). The mating types are not dimorphic forms of *P. infestans*, but are compatibility types differentiated by mating hormones (Judelson 1997). When mycelia of the two mating types interact, mating hormones induce gametangial formation in the opposing mating types, resulting in sexual reproduction. In

the course of gametangia formation, vegetative, diploid mycelia undergo meiosis to form haploid antheridia and oogonia. During the sexual life cycle, an antheridium fuses with an oogonium to form a diploid oospore. Unlike sporangia, which are airborne, fragile, and need live plants for survival, oospores are large, thick-walled spores that enable pathogen survival for extended periods of time in harsh conditions outside the living host plant. Oospore survival in plant debris or soil outside the living host can serve as a persistent source of inoculum in the field, similar to potato tuber-borne inoculum (Nowicki et al. 2012). Oospores can germinate under environmentally favorable conditions and release diploid progeny of A1 or A2 mating type (Judelson 1997). Sexual recombination occurs in the presence of both mating types, giving rise to new and possibly more aggressive isolates, thus making LB disease management more challenging (Fry and Goodwin 1997; Fry 2008).

There are several reasons LB remains a major threat to Solanaceae crop species. As stated above, *P. infestans* reproduces very rapidly and can destroy an unprotected tomato or potato crop within several days of occurrence. The asexual disease cycle consisting of pathogen penetration, colonization, sporulation, and dispersal can occur in fewer than five days. Each LB lesion can produce as many as 300,000 sporangia per day (Figure 13.1C, D), contributing to a rapid spread of the disease. Further, plants of a given crop are not simultaneously affected, making the early stages of disease easy to miss. By the time the disease is detected it is often too late to save the crop through fungicide application.

Prior to the LB resurgence (see below), only the A1 mating type was observed in potato growing areas outside Mexico, so sexual reproduction did not play a significant role in the disease cycle. Migration of the A2 mating type outside Mexico in the 1980s created the opportunity for pathogen sexual reproduction and DNA recombination, resulting in the creation of new, more aggressive isolates (Fry and Goodwin 1997; Fry 2008). The resultant oospores enable the organism to survive for long periods in plant debris or soil outside the living host plant (Judelson 1997), and may play a key role in the epidemiology of LB and serve as a persistent source of inoculum in the field, similar to potato tuber-borne inoculum.

The final concern associated with LB reemergence is that many new, complex *P. infestans* lineages exhibit resistance to metalaxyl, the predominant systemic fungicide used to control the pathogen. Improper applications of phenylamide fungicides have created a selective pressure on the pathogen, leading to the establishment of phenylamide-resistant isolates of *P. infestans* in many regions throughout the world. The occurrence of metalaxyl resistance nearly coincided with the observation of the A2 mating type outside Mexico, however, no genetic correlation has been documented between mating type and metalaxyl resistance (Gisi and Cohen 1996). Metalaxyl resistance poses a great threat to tomato and potato growers, as these are the main systemic fungicides against LB. The presence of metalaxyl resistance suggests that once LB is observed, it is likely to be too late to use protectant fungicides to save the crop. Despite different means to circumvent this issue (e.g., distribution of these fungicides only in combination with at least one other fungicide with a different mode of action, or metalaxyl replacement by its optical isomer, metalaxyl-m [mefenoxam]), the concern with fungicide resistance further stresses the need for identifying sources of host genetic resistance against the pathogen (discussed below).

P. infestans Pathogenesis

The life cycle of *P. infestans* involves differentiation into as many as 11 different cell types. These cell types are highly specialized for life cycle stages involved in sexual and asexual reproduction, propagule dispersal, spore germination, host penetration, and biotrophic or necrotrophic phases of infection (Nowicki et al. 2012). *P. infestans* adopts a two-step infection mode, typical of hemibiotrophs. During the early

biotrophic growth phase, nutrients are obtained from living plant cells. This is achieved by forming a penetration peg, which pierces the cuticle and enters an epidermal cell to form an infection vesicle (Figure 13.1A, B). Formation of digit-like haustoria (i.e., appressoria-like structures) by the branching hyphae, and the subsequent secretion of enzymes that degrade components of the plant cell wall further facilitate the nutrient access. Haustoria, specialized for nutrient uptake from the host cell, develop within the boundary of the plant cell wall, although they remain outside the host cell plasma membrane (Hardham and Blackman 2010). This is followed by extensive necrosis of host tissue resulting in colonization and sporulation. During necrotrophic growth, nutrients required for pathogen growth and reproduction are obtained from dead and dying cells in the necrotic lesions that develop as the pathogen colonizes the plant. As the infected tissue necrotizes, the mycelium develops sporangiophores that emerge through the stomata to produce numerous asexual sporangia (Hardham and Blackman 2010; Nowicki et al. 2012) (Figure 13.1C, D).

P. infestans preferentially sporulates *in planta* during dark periods. Studies have demonstrated that continuous light inhibits sporulation (Nowicki et al. 2012). As there is no evidence for a light-regulated clock in the pathogen (Raffaele et al. 2010b), this suggests that a plant-derived signal generated by the day-night cycle influences sporulation. Similar phenomena are common among the oomycetes (Judelson et al. 2009; Hardham and Blackman 2010). At the plant surface, *P. infestans* zoospores maneuver such that their ventral surface faces the plant before the flagella are detached and proteins from three different cortical vesicles are secreted onto the zoospore surface (Hardham and Blackman 2010). By doing so, the spores form walled cysts, which germinate from the center of the ventral surface, allowing the hyphae to grow along the anticlinal walls between epidermal cells to form appressoria for breaching plant cells (Figure 13.1A, B). Under favorable conditions, the multinucleate sporangia can also initiate infections. Mature sporangia can preemptively synthesize the proteins involved in zoosporogenesis and encystment (Nowicki et al. 2012).

In general, during infection, phytopathogens secrete peptides and proteins, broadly known as effectors (Raffaele et al. 2010b; Vleeshouwers et al. 2011). Effectors can be of two types: apoplastic effectors, which target the extracellular space, and cytoplasmic effectors, which target subcellular compartments. Effectors include enzymes involved in degrading the plant cell wall and suppressing extracellular plant defenses (Vleeshouwers et al. 2011; Hardham and Blackman 2010; Nowicki et al. 2012). In-depth discussion of these proteins in *P. infestans* pathogenesis is presented in the potato LB chapter (Chapter 12 in this volume), as major advances in this field have been achieved in research on this host plant.

Disease Symptoms and Progression

P. infestans can quickly devastate tomato and potato crops at any time during plant ontogeny. The entire plant may collapse in five to ten days. LB can infect all aboveground parts of the plant, causing leaf and stem necrosis, fruit rot, and eventual plant death (http://www.nysipm.cornell.edu/publications/blight/). The pathogen can also infect tomato seed and potato tubers (Rubin et al. 2001; Nowicki et al. 2012). Initial infection symptoms, including small lesions on leaf tips and plant stems, are visible only after three to four days, and in some cases reach only 1 to 2 mm in diameter. The purple, dark brown, or black water-soaked lesions often have a pale yellowish-green border that blends into the healthy tissue. As the pathogen penetrates the plant tissue, lesions enlarge in size (http://www.longislandhort.cornell.edu/vegpath/photos/lateblight_tomato.htm). Fluffy, white sporangia may grow on the lower (abaxial) leaflet surface in moist weather. As the disease progresses, plant leaflets shrivel and die and the disease spreads to the rest of the foliage, leading to extensive defoliation. Dark brown LB lesions first appear at the

top of the stem or at a node and may progress down the stem. Firm, brown, and greasy tomato fruit lesions are often located at the stem end and sides of green fruit, rendering them unmarketable. Infected tomato fruit may be invaded by secondary pathogens, causing soft-rot disease.

Host Plant Defense

Plants are finely tuned to detect the presence of pathogens on their surface. They perceive both chemical and physical signals of pathogens and react rapidly to the attempted infection (Hardham and Blackman 2010; Nowicki et al. 2012). Possible plant responses to oomycete invasion vary from invaded cells with no visible response, to more localized reactions preventing formation of pathogen intra-cellular structures, to hypersensitive response (HR) and programmed cell death, the latter occurring only after pathogen growth had proceeded to the formation of a clearly identifiable haustorium (Hardham and Blackman 2010; Hardham et al. 2007).

The primary line of induced defense in tomato plants against *P. infestans* is resistance against penetration at the leaf surface. This is a highly effective defense strategy rapidly mobilized by host plants to attempted penetrations (Hardham et al. 2007). Recent studies have demonstrated the direct participation of plasma membrane-bound receptor proteins in the recognition of apoplastic pathogen elicitors and activation of host defenses (Haas et al. 2009). Additionally, the physical pressure exerted by the invading penetration peg may act as a signal to plant surface detection systems. Triggering of this system normally leads to reversible formation of microfilaments beneath the pressure point within minutes of the acting stimulus. Both stimuli may set off the downstream signaling pathways, including salicylic acid-regulated induction of cell death, ethylene-dependent production of phytoalexin, and reactive oxygen species (ROS)-regulated gene expression and induction of HR (Haas et al. 2009; Shibata et al. 2010; Nowicki et al. 2012). In response to the perceived pathogen attack, plant cells secrete a broad spectrum of compounds to combat the disease, including constitutively expressed and inducible defense-related proteins. These include defense-related proteins, which function in the degradation of microbial cell walls and inhibiting pathogen-released elicitors (Foolad et al. 2008; Hardham and Blackman 2010). Early localized epidermal cell reactions include major rearrangements within the cytoplasm leading to its aggregation beneath the pathogen site, synthesis of ROS, and accumulation of callose beneath the appressoria within 14 hours after inoculation or natural pathogen attack (Hardham et al. 2007; Hardham and Blackman 2010) (Figure 13.1A, B). The rapidly occurring site-directed cytoplasmic streaming is achieved by reorganization of cortical and vascular actin arrays, such that actin microfilaments and cables form a radial array focused on the infection site. Subsequently, because of the actin-dependent focus of cellular secretory apparati (endoplasmic reticulum, Golgi bodies, and peroxisomes) beneath the infection site, a wide variety of molecules are delivered to the developing cell wall apposition (Hardham et al. 2007; Hardham and Blackman 2010). Callose, a major component of the cell wall apposition (Figure 13.1A, B), alongside other cell wall apposition components, such as phytoalexins, phenolics, silicon, H_2O_2, and enzymes such as peroxidases and enzyme inhibitors, is likely to inhibit pathogen hyphal penetration. Subsequent defense responses include changes in gene expression and production of phytoalexins and pathogenesis-related proteins (Hardham et al. 2007; Shibata et al. 2010).

Only after primary host-plant defenses fail and hyphae successfully penetrate with the formation of haustorium does the plant invoke a second system of resistance against *P. infestans*, which involves recognition of specific pathogen molecules and induction of programmed cell death. HR constitutes a highly effective means of restricting pathogen growth and its containment before transition to the necrotrophic phase

occurs (Vleeshouwers et al. 2000; Hardham et al. 2007). Elicitins and effectors are the two main groups of molecules that induce HR. Upon their being recognized by host plants, elicitins induce HR. For instance, *P. infestans* INF1 elicitin initiates cell death upon interaction with the lectin-like receptor kinase NbLRK1 in *N. benthamiana* (Hardham et al. 2007; Kanzaki et al. 2008). In total, genes coding for seven avirulence effector proteins of *P. infestans* have been cloned at present (Vleeshouwers et al. 2011; van Damme et al. 2012), and all of them are recognized by cytoplasmic nucleotide-binding site/leucin-rich repeat (NBS-LRR) receptors (Jia et al. 2010; Vleeshouwers et al. 2011) encoded by the plant resistance (*R*)-genes. Race-specific and broad-spectrum *R*-genes in host plants induce a similar set of defense reactions as a response to recognition of their corresponding *P. infestans* avirulence factors, the effector proteins (Shibata et al. 2010; Nowicki et al. 2012). In tomato, a wide range of proteins secreted by *P. infestans* and the host plant during a compatible interaction was identified in an *in planta* secretome study using the yeast secretion trap technique (Lee et al. 2006). Almost half the secreted proteins in the tomato plants had known associations with defense, including pathogenesis-related (PR) proteins, structural proteins, glycosyl hydrolases (which target glucans, typical constituents of microbial cell walls), and a putative peroxidase. Other secreted proteins had less defined roles in defense, including those induced by wounding or elicitors. Additionally, about one third of the induced genes had no obvious functional domains or homology to known genes (Lee et al. 2006).

To date, no mechanisms for LB resistance have been proposed in either tomato or potato. A preliminary study was conducted to elucidate the mechanism of vertical LB resistance conferred by the *S. bulbocastanum R*-gene, *RB*, by investigating the effects of *RarI* and *SgtI* on LB resistance (Bhaskar et al. 2008). Unlike the resistance conferred by most major genes, *RB* slows disease progress but does not eliminate disease symptoms (see Chapter 12 of this volume). *RarI* and *SgtI* are known to regulate *R*-genes' expression. Although proposed to be involved in forming or stabilizing R-protein associated recognition complexes, silencing *RarI* using RNAi had no effect on *RB* resistance, indicating that it is not required in the LB-resistance response. Conversely, silencing *Sgt1*, known to be involved in NBS-LRR and Pto-kinase mediated resistance, resulted in disease susceptibility, indicating that SGT1 plays a role in LB resistance (Shibata et al. 2011). Another study on phenotypic characterization of potato LB resistance conferred by *RB* suggests that expression of *HR* and *PR* genes may play a critical role in the resistance response, with callose deposition being negatively correlated with resistance levels in tested plants (Chen and Halterman 2011).

Collectively, multiple defense mechanisms seem to be involved in LB resistance and alteration of metabolic pathways may be one of the most important disease defense responses (Bhaskar et al. 2008; Foolad et al. 2008; Chen and Halterman 2011). Nevertheless, innate tomato-derived LB-resistance mechanisms alone may not suffice to defend the plants against the aggressive pathogen, hence the need to integrate various protection methods into crop production.

Late Blight Disease Control

Tomato and potato LB are of significant fiscal importance to growers and consumers worldwide, costing approximately US$5 billion annually, including the cost of disease control and crop losses (Foolad et al. 2008). In the United States, in 2009 total yield losses for fresh-market and processing tomato industries reached US$46 million and US$66 million, respectively (USDA 2011). Of these losses, up to half could be attributed to crop losses resulting from LB (Haverkort et al. 2008; Nowicki et al. 2012). In tomato, fruit infection may range from 41 to 100% in unprotected fields and from 12 to 65% in plots protected with systemic fungicides (Nowicki et al. 2012). Polish tomato production has

suffered significantly from rampant outbreaks of LB over the past several years, often causing yield losses of up to 100% (Nowicki et al. 2012). Social impact of LB is illustrated by the significant media and internet coverage (Vleeshouwers et al. 2011). Losses resulting from tomato LB are accompanied by higher fungicide applications worldwide, exemplifying the need for crop protection-related expenditures and environmental safety concerns. Estimated cost of fungicides and crop losses resulting from LB in the U.S. exceeds $210 million annually (Foolad et al. 2008). Predictions that fungicide applications needed to control LB in the future would increase by 20 to 25%, compared to that required from 1977 to 2008 (Nowicki et al. 2012), further add to concerns over the fiscal and environmental costs related to LB control.

Prior to the reemergence of LB in the late 1980s, cultural practices in combination with fungicide applications were highly effective measures for controlling LB in tomato. These approaches, however, are not expected to provide sustainable control of the disease in the future, as discussed below. It seems the greatest contribution to tomato LB control in the future will have to be through the development of cultivars with improved genetic resistance via modern plant breeding approaches.

Cultural Practices

Cultural practices are important components of growers' strategy in disease management, and they can impact disease development and control. The aims of cultural control of LB are to minimize inoculum buildup, prevent introduction of inoculum from nearby potato cull piles or from tomato transplants, minimize infection rate, and generate conditions unfavorable for disease development and spread. Specific cultural practices usually employed to control LB include crop rotation and fallow, elimination of volunteer tomato and potato plants, planting noninfected seedlings and tubers, and elimination of LB sources such as potato cull piles. The latter is of particular importance because cull piles can serve as a living host on which *P. infestans* mycelia can survive over the winter and produce tremendous amounts of airborne spores at the beginning of the new field season. If this occurs, the next year's crop is at risk of LB devastation (Foolad et al. 2008). Recent reemergence of *P. infestans* with its enhanced ability to develop more virulent isolates through sexual recombination makes LB control via cultural practices alone very challenging, and the pathogen could be particularly destructive in areas where both tomatoes and potatoes are grown year-round, as for example in the highland tropics of Africa, South America, Asia, and Europe (Nowicki et al. 2012).

Fungicide Application

Chemical control measures can be effective in managing LB, especially when guided by disease forecast systems, and have been increasingly utilized in recent years. Two main groups of fungicides have been used routinely, including protectants (e.g., chlorothalonil, dithiocarbamates, and triphenyl tin hydroxide), which are usually applied before or upon disease development, and systemic fungicides (a.k.a. therapeutic fungicides; e.g., phenylamides such as metalaxyl/mefenoxam, aliphatic nitrogen fungicides such as cymoxanil, and morpholine fungicides such as dimethomorph), which inhibit or reduce disease progress once symptoms are apparent. Current chemical practices to control LB include a mixture of fungicides designed to slow the disease progress. Metalaxyl fungicides, a class of systemic fungicides, have been widely employed to control LB; they inhibit ribosomal RNA (rRNA) polymerases in fungi by reducing incorporation of uridine (Gisi et al. 2011). These treatments, however, can be ineffective, particularly when the environmental conditions are highly conducive to disease development. Moreover, improper applications of phenylamides have created a selective pressure on the pathogen, leading to the spread of fungicide resistance controlled

by a single, incompletely dominant gene present in natural *P. infestans* populations. Establishment of metalaxyl-resistant isolates of *P. infestans* was observed in many countries as early as 1980 (Gisi et al. 2011). In addition to becoming increasingly ineffective as a result of the development of pathogen resistance, fungicides are expensive, harmful to the environment and humans, and must be applied at the proper time. With the use of at least partially resistant cultivars, the number of fungicide applications and/or the rate of application can be significantly reduced, particularly when combined with LB forecasting (Foolad et al. 2008). It should be noted that copper fungicides have also been used in organic fresh-market tomato production, but they have been shown to only suppress the LB symptoms, while not stopping the disease. In a 2012 field investigation of the effects of different fungicide programs on controlling tomato LB, the organic fungicide program consisting of copper hydroxide tank mixed with *Bacillus subtilis* only helped reduce LB infection but did not control the disease (Gugino and Foolad, unpubl. data). Overall, the issues surrounding the use of fungicides, as well as economic and environmental safety considerations, necessitate careful adoption of effective and sustainable disease control measures, including the development and integration of commercially acceptable cultivars with genetic resistance against LB. The more resistant the cultivar, the greater its potential for reduction of fungicide application (Foolad et al. 2008).

Genetic Resistance against LB in Tomato and the Importance of Breeding for Resistance

Since the Irish famine of the 1840s, there has been a great deal of interest in developing LB-resistant potato and tomato cultivars. In the course of this process, it has been observed that resistance against *P. infestans* could be classified into race-specific resistance (a.k.a. pathotype-specific, vertical, or "gene-for gene" interaction) and race-non-specific resistance (a.k.a. field, horizontal, or partial resistance), with races defined by the disease interaction with different host plant genotypes. In tomato, similar to potato, vertical as well as horizontal resistances against LB have been reported. Developing plants that display disease resistance conferred by major genes (i.e., vertical resistance) have been effective (at least initially) in preventing infection and protecting the crop from LB (see below). The host resistance gene product, also known as the *R*-gene product, interacts with the pathogen's pathogenicity gene product, also known as the *Avr*-gene product. Single gene resistance typically confers complete resistance against one or a limited number of pathogen races. Due to rapid evolution of pathogen effectors and sexual reproduction of *P. infestans* leading to more aggressive lineages, vertical resistance against the pathogen could ultimately fail. For example, durability of major LB-resistance genes in potato has proven variable (Vleeshouwers et al. 2011), and isolates of *P. infestans* have been identified that basically overcome all 11 *R*-genes identified in potato wild species *S. demissum* (Chen and Halterman 2011). Similar situation may be stipulated for major LB-resistance genes in tomato (Nowicki et al. 2012), though this has not been documented (discussed below).

In contrast to vertical resistance, race-non-specific resistance is often controlled by several genes or quantitative trait loci (QTLs), and potentially could be more durable. Horizontal resistance usually confers partial resistance against multiple isolates/races of the pathogen. This type of resistance often slows, but does not stop progress of the disease. The polygenic nature of horizontal resistance makes it more difficult to breed for, compared with vertical resistance, however, its greater durability may render the breeding efforts worth undertaking. In the case of tomato LB, it has been argued that horizontal resistance is likely of limited value because of the pathogen's short reproductive cycle and heavy spore production, as well as the ability of the disease to spread quickly

by airborne spores over long distances (Scott and Gardner 2007). Conversely, race-specific major resistance genes have been useful in breeding tomatoes for resistance against LB, and in fact against many other tomato diseases (Foolad 2007). Further, many major resistance genes have shown durability and been very useful for breeding purposes in other crop species. The use of major disease resistance genes, however, has been shown to be most effective when multiple resistance genes are pyramided; this approach often increases resistance strength and durability (Kim et al. 2012; Luo et al. 2012). Thus, it is prudent to identify additional major resistance genes against tomato LB, a task that is underway in many tomato-breeding programs around the world (Merk and Foolad 2011, Merk et al. 2012; Nowicki et al. 2012).

Identification of Resistance Resources and Breeding for LB resistance in Tomato

Overview of Early Studies of LB Resistance

Following an LB outbreak in the U.S. in 1946, which affected potatoes and tomatoes, a substantial amount of research was initiated to locate sources of genetic resistance in tomato (Bonde and Murphy 1952; Gallegly and Marvel 1955; Gallegly and Galindo 1958; Gallegly 1960). Such research led to the discovery of resistant accessions within wild tomato species, in particular *S. pimpinellifolium* (Gallegly and Marvel 1955). The first reported tomato LB-resistance gene, *Ph-1*, a completely dominant gene conferring resistance against *P. infestans* tomato race-0 (T_0), was originally located in *S. pimpinellifolium* accessions known as West Virginia 19 and 731 (Bonde and Murphy 1952; Gallegly and Marvel 1955; Pierce 1971). An LB-resistant cultivar containing *Ph-1*, Rockingham, was released in 1962 (Rich et al. 1962). This cultivar was subsequently used to map the gene conferring LB resistance to the distal end of chromosome 7 using morphological markers (Pierce 1971). Subsequently this resistance trait was incorporated into the old processing tomato cv. 'Nova' and the old fresh market tomato cv. 'New Yorker'. Since T_0 is no longer the predominant race of *P. infestans*, and since *Ph-1* has been long overcome by new aggressive pathogen lineages, this LB-resistance source is no longer considered useful for tomato breeding (Foolad et al. 2008; Nowicki et al. 2012). Currently, cultivars containing *Ph-1* exhibit complete susceptibility to LB.

A second tomato LB-resistance gene named *Ph-2* was identified in an *S. pimpinellifolium* accession known as West Virginia 700 (Gallegly and Marvel 1955) and subsequently mapped to the long arm of tomato chromosome 10 between markers CP105 and TG233 (Moreau et al. 1998) (Figure 13.2A). There has not been any further effort to fine map or clone *Ph-2* (N. Grimsley, CNRS-INRA, personal communication). Incompletely dominant LB resistance conferred by *Ph*-2 provides only partial resistance against several pathogen isolates and confers only a reduction in the rate of disease development, rather than blocking the disease. Furthermore, *Ph-2* often fails in the presence of more aggressive *P. infestans* isolates. Characterization of this resistance has been hampered because its expression is partially dependent upon environmental conditions, plant physiological age, the organ assessed, and the pathogen isolate used (Moreau et al. 1998). Despite these shortcomings, *Ph-2* has been successfully incorporated into a number of named fresh-market and processing tomato varieties, including Legend, Centennial, Macline, Pieraline, Herline, Fline, Flora Dade, Heinz 1706, Campbell 28, and Europeel (Gallegly 1960; Nowicki et al. 2012). Recently a few PCR based markers associated with *Ph-2* have been identified (M. Mutschler, personal communication), which are being used for development of breeding lines and hybrid cultivars of tomato containing *Ph-2*. Nevertheless, virulence variability observed in *P. infestans* has made this source of tomato resistance less

effective for protection against LB when used alone (see below).

Resurgence of LB and Identification of Additional Resistance Genes and QTLs

Until the late 1970s, LB was relatively well controlled through the use of cultural practices, heavy application of fungicides, and deployment of somewhat resistant cultivars of potato. LB reemerged in the 1980s as an important disease of both potato and tomato. This has been in part because of several independent global migrations of the pathogen from its Andean origin (Fry and Goodwin 1997) and potentially the occurrence of sexual reproduction in populations of *P. infestans*. The latter has resulted in generation of new and more aggressive isolates of the pathogen, many of which exhibit resistance to known systemic fungicides (Gisi and Cohen 1996; Gisi et al. 2011). After several years of observation, it has become clear that these new isolates of the pathogen constitute a greater threat to potato and tomato production than did the previous dominant lineage, US-1. The new *P. infestans* lineages differ in pathogenicity factors (specific virulence, aggressiveness, and fungicide specificity) and ecological factors (responses to physical factors such as temperature; Fry and Goodwin 1997). Moreover, host preference change has been observed. For example, prior to the 1980s many of the potato isolates were not pathogenic to tomato, whereas new isolates with greater pathogenicity to tomato have appeared since (Fry 2008). During the 1980s and 1990s, perhaps the most significant consequence with respect to tomato LB was the breakdown of resistance conferred by *Ph-1* and *Ph-2* in tomato. Such a breach of resistance as well as the appearance of new and more aggressive isolates of *P. infestans* resulted in worldwide increased occurrence of LB in tomato (Fry and Goodwin 1997), reaching as far as Taiwan, Nepal, Indonesia, and the Philippines. This prompted further screening of tomato wild species, in particular accessions within *S. pimpinellifolium*, to find new sources of LB resistance. Subsequent research led to the discovery of a strong LB-resistance gene named *Ph-3* in *S. pimpinellifolium* accession L3708 (a.k.a. LA1269 and PI 365957) (AVRDC 1993).

Ph-3 confers resistance against a wide range of *P. infestans* isolates that overcome *Ph-1* and *Ph-2*. A bulked segregant analysis (BSA) using AFLP (amplified fragment length polymorphism) markers resulted in the identification of DNA markers associated with *Ph-3* and enabled mapping of *Ph-3* to the long arm of chromosome 9, close to RFLP (restriction fragment length polymorphism) marker TG591A (Chunwongse et al. 2002) (Figure 13.2B). Subsequently a number of DNA markers, including RFLP- and PCR-based markers, closely linked to *Ph-3* have been reported (Zhu et al. 2006; Qiu et al. 2009; Park et al. 2010; M. Mutschler, personal communication) (Figure 13.2B). Recently several public tomato breeding programs in the U.S., including programs at North Carolina State University (R.G. Gardner and D.R. Panthee, personal communication), Cornell University (Kim and Mutschler 2005; M. Mutschler, personal communication), and the Pennsylvania State University (M.R. Foolad unpublished results), have developed fresh-market and/ or processing tomato lines possessing *Ph-3*. It has been determined, however, that LB resistance in L3708 is controlled by more than just the *Ph-3* locus on chromosome 9 and that LB-resistant breeding lines developed at the Asian Vegetable Research and Development Center, in Taiwan (AVDRC) were probably lacking at least one of those additional hypostatic genes and were not as resistant against LB as was L3708 (Kim and Mutschler 2005; Lee et al. 2006). The authors further concluded that inbred lines or hybrids that contain the *Ph-3* gene alone in either homozygous or heterozygous condition would not be highly desirable commercially, as they would not exhibit strong resistance against aggressive isolates such as US-7 and US-17. The presence of the yet undetermined additional hypostatic gene(s) in homozygous or heterozygous

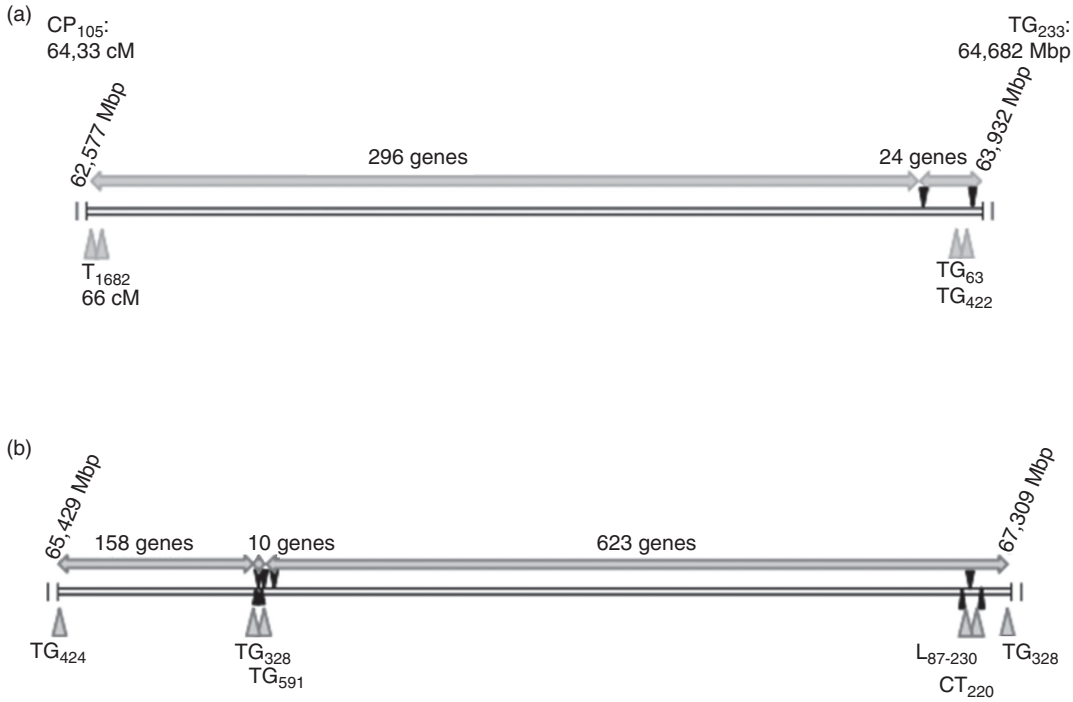

Fig. 13.2. Partial physical maps of tomato chromosomes 10 and 9, to which tomato late blight resistance genes have been mapped. Red triangles indicate the approximate positions of respective markers and black arrowheads indicate the approximate positions of *NBS-LRR* genes present in the two regions. (a) A fragment of chromosome 10 to which *Ph-2* has been mapped between markers CP105 at 64.33 cM and TG233 at 64.82 cM (Moreau et al. 1998; http://solgenomics.net/); the fragment is narrowed down to 320 genes between markers T1682 (Marczewski, Nowakowska, Nowicki, Kozik unpublished results) and TG63 and TG422 (Foolad and Panthee 2012). (b) A fragment of chromosome 9 to which *Ph-3* has been mapped (Chunwongse et al. 2002); this fragment covers 791 genes and contains markers reported in the literature to be associated with *Ph-3* (see Park et al. 2010 and Foolad and Panthee 2012).

condition is necessary to provide full resistance (Kim and Mutschler 2005). Nevertheless, despite the superiority of the *Ph-3* complex, as compared with *Ph-1* or *Ph-2*, *P. infestans* isolates have emerged that overcome this resistance (Chunwongse et al. 2002; AVRDC 2005; R.G. Gardner, personal communication).

It has been observed, however, that Ph-3 is most effective when combined with *Ph-2* (R.G. Gardner, personal communication; M.R. Foolad unpublished results). Most recently, these two resistance genes have been pyramided in a few tomato breeding lines (e.g., NC1 CELBR, NC2 CELBR) and hybrid cultivars (e.g., Mountain Magic and Mountain Merit), which exhibit much stronger LB resistance than breeding lines or cultivars that have only *Ph-2* or *Ph-3* (Gardner and Panthee 2010a; Panthee and Gardner 2010; MR Foolad, pers. observations). Scott and Gardner (2007) reported, however, that under ideal environmental conditions for LB, including heavy inoculum pressure and presence of other lines with single gene resistance in the same plantings, the lines with pyramided *Ph-2* and *Ph-3* genes in either homozygous or heterozygous state were overcome by LB in two field locations in North Carolina, USA, in the summer of 2004. In contrast, in the absence of lines carrying either *Ph-2* or *Ph-3* resistance genes alone, the resistance in lines or cultivars with both *Ph-2* and *Ph-3* held up throughout the season (Scott and Gardner 2007). In comparison, in a 2012

multi-location trial of numerous tomato genotypes containing *Ph-2* and/or *Ph-3* in Pennsylvania, USA, it was determined that lines and cultivars containing both *Ph-2* and *Ph-3* exhibited very strong resistance under high LB pressure throughout the season (M.R. Foolad and B.K. Gugino unpublished results).

In addition to the abovementioned major LB-resistance genes identified in tomato wild species *S. pimpinellifolium*, several quantitative trait loci (QTLs), potentially conferring race-non-specific resistance, have been reported in different accessions of the green-fruited tomato wild species *S. habrochaites*. The first accession of *S. habrochaites* discovered with LB resistance was LA1033 (Lough 2003), and the LB-resistance gene/QTL it carried was designated by AVRDC as *Ph-4* (AVRDC 1993; 2005). In *S. habrochaites* accession LA2099, QTLs conferring LB resistance were identified on all 12 tomato chromosomes (Brouwer et al. 2004). In subsequent research, three near-isogenic lines (NILs) were developed, each containing one of three major LB-resistance QTLs (Brouwer and St Clair 2004). Unfortunately, severe linkage drag has thus far prevented the NILs from being useful for breeding purposes. Further inspection of these NILs has determined that they also contain genes/QTLs for other characteristics, including plant type and maturity, canopy density, and fruit size and yield in the same introgressed regions. In these accessions, QTLs for LB resistance were associated with late maturity and large plant size (Brouwer and St Clair 2004). In a more recent study, five LB-resistance QTLs were identified in *S. habrochaites* accession LA1777 and one new QTL for LB resistance in LA2099 (Li 2010). Nevertheless, the actual value of these QTLs for breeding purposes is yet unknown. Until these QTLs are refined and NILs are developed with individual QTLs in the background of the cultivated tomato, no judgment can be made as to their breeding value. Furthermore, it is important to determine whether the combination of major LB-resistance genes, such as *Ph-2* and *Ph-3*, with LB-resistance QTLs would increase the level or durability of resistance against LB. Although it is difficult to predict the outcome, it would be a worthwhile effort to develop such gene combinations. Regardless, we are not aware of any tomato program that is conducting such a research at this time.

Recent Screening and Identification of New LB-Resistance Genes

The recent occurrence of newer, more aggressive isolates of *P. infestans*, which have been highly pathogenic to tomato, necessitated a search for further sources of LB resistance in tomato. During the past several years, a comprehensive effort towards identification, mapping, and pyramiding LB-resistance genes has been undertaken at the Pennsylvania State University. This effort was initiated with screening of a large collection of *S. pimpinellifolium* accessions for LB resistance under different conditions, including field, high tunnel, greenhouse, and growth chamber conditions. The screening trials resulted in the identification of several new accessions with strong resistance against tomato LB (Foolad et al. 2008; M.R. Foolad unpublished results). Among the newly identified accessions, one accession (PI 270443) that exhibited strong resistance against at least 7 isolates of *P. infestans* was chosen for further characterization, including inheritance studies and identification and mapping of resistance gene(s). A parent-offspring correlation analysis, employing F_2 and F_3 populations of a cross between PI 270443 and a susceptible tomato breeding line (NC EBR-2), determined that the LB resistance conferred by PI 270443 was highly heritable ($h^2 = 0.86$) and estimated to be controlled by two genes (Merk and Foolad 2011). Subsequently, a selective genotyping approach, using various molecular markers (RFLP, SSR, EST, CAPS), led to the identification of two genomic regions on tomato chromosomes 1 (tentatively named *Ph-5-1*) and 10 (tentatively named *Ph-5-2*) associated with LB resistance in PI 270443 (Merk et al.

2012). The genomic location on chromosome 10 (*Ph-5-2*) is co-localized with *Ph-2*, however, it is unknown whether it is the same as *Ph-2*, an allele of the same gene, or a separate but tightly linked resistance gene. Currently, efforts are underway to further delineate the two regions on chromosomes 1 and 10 associated with LB resistance (M.R. Foolad unpublished results). Furthermore, LB resistance from PI 270443 is being introduced into Penn State fresh-market and processing tomato lines by a combination of MAS and traditional breeding protocols (M.R. Foolad unpublished results). Once the individual value of these new genes is determined, it is imperative to combine them with *Ph-2* and/or *Ph-3* to determine whether such gene combinations would increase the strength or durability of tomato resistance against LB. In particular it is important to determine whether combinations of such genes increase the resistance against additional *P. infestans* isolates.

Other Challenges in Breeding for LB Resistance in Tomato

Despite encouraging progress toward overcoming the low genetic diversity of the cultivated tomato, which has been achieved through frequent gene introgressions from the related wild species of tomato, there are other concerns related to LB-resistance breeding. Among them, for example, is the lack of clear standards within the cultivated tomato for *P. infestans* race assessment. Researchers have tried to circumvent this difficulty by determining various characteristics of the pathogen isolates, including mating type, fungicide sensitivity, isozyme genotype, and mitochondrial and nuclear DNA fingerprints (reviewed in Nowicki et al. 2012; also see Chapter 12). Furthermore, some researchers have tried to characterize/classify *P. infestans* isolates based on their pathogenicity on established resistant lines carrying known LB-resistance genes, which to some extent is analogous to the Black's standards in potato. For example, tomato researchers at the AVRDC started analyzing their *P. infestans* isolate collections in this manner in the early 1990s, when *S. pimpinellifolium* accession L3708 was believed to carry a single resistance gene, *Ph-3*, and *S. habrochaites* accession LA1033 was designated as the source of *Ph-4* resistance gene (AVRDC 1993; 2005). Follow-up investigations revealed complex resistance involving multi-genes in L3708 (Kim and Mutschler 2005) or multi-QTLs in LA1033 (Lough 2003). Classifications such as these constituted a mere first step toward development of a more comprehensive and useful classification system of the races of tomato-virulent *P. infestans* strains. With the availability of *P. infestans* genomic sequence data and identification of additional sources of LB resistance in tomato, more information regarding the various races of the pathogen may soon become available. It should be noted, however, that if pyramided major LB-resistance genes hold effectively, knowledge of races may not be critical from a breeding perspective.

Implications of Potato LB Resistance Research in Tomato

As discussed above, LB resistance, both vertical and horizontal, has been reported in several related wild species of potato. For example, at least 11 race-specific LB-resistance genes (*R1* to *R11*) have been identified in potato wild species *S. demissum*. Several of these have been mapped or cloned and incorporated into various potato cultivars, as discussed elsewhere (Foolad et al. 2008; Nowicki et al. 2012). Currently, most new cultivars of potato carry one or more LB-resistance genes and demonstrate a good level of field resistance. All of the hitherto cloned *R*-genes encode the NBS-LRR class of plant resistance proteins (Jia et al. 2010; Vleeshouwers et al. 2011) and confer race-specific HR responses. It is noteworthy, however, that such race-specific resistance genes may lose their effectiveness as new and more aggressive *P. infestans* strains appear that could overcome the resistance. For example, isolates of

P. infestans have been identified that overcome all 11 *S. demissum*-derived R-genes in potato (Chen and Halterman 2011). Nonetheless, pyramiding various race-specific resistance genes often results in stronger and more durable resistance, as has been observed in many plant species, including potato and tomato (Tan et al. 2010; Kim et al. 2012; Luo et al. 2012).

Pyramiding of LB-resistance genes using conventional breeding approaches may not always be possible or it may be laborious and time-consuming. An alternative approach to introducing single or multiple LB-resistance genes is genetic transformation. This approach, however, requires prior knowledge of the gene(s), including identification, cloning, and characterization. In this respect, a recent strong body of evidence that tomato and potato share the conserved pathway for LB signaling is very beneficial for LB-resistant tomato breeding (Jia et al. 2009; Faino et al. 2010; Jia et al. 2010). The conserved LB-signaling pathway phenomenon has been demonstrated by several research groups (see below), which heterologously over-expressed the cloned potato LB *R*-genes in tomato plants and showed increased LB resistance in the transgenic plants.

An attempt with the transgenic approach using potato *R1* gene heterologously expressed in tomato indicated the activation of the transgene in the isolate-specific, *R1*-incompatible reaction during *P. infestans* attack (Faino et al. 2010). Increased expression of the transgene, but also of the native tomato *PR-1* gene, has been recorded as early as three hours post infection (hpi); additionally, expression of *PR-1* increased by one-fold between three and six hpi in the transgenic plants (incompatible reaction), while in the native tomato plants remained at the three hpi level (Faino et al. 2010). In another study, *Agrobacterium*-mediated transformation and heterologous expression in tomato of the coding sequences of the *R3a*, *R1* (*S. demissum*), or *RB* (*S. bulbocastanum*) genes was employed to obtain transgenic tomato plants (Jia et al. 2009). When challenged with *P. infestans* isolates, the transgenic plants displayed a strong resistance response. The results demonstrated that *R3a* and *R1* conferred resistance against some tomato-virulent isolates, while *RB* granted resistance against all five isolates examined (Jia et al. 2009). Follow-up studies proved that the transgenic tomatoes carrying the potato *R3a* developed HR specific to *P. infestans* strains with the corresponding avirulence gene, *Avr3a* (Jia et al. 2010). This seems to indicate that the signaling pathway from the *R3a-Avr3a* recognition to HR is conserved between potato and tomato. The transgenic tomatoes carrying both *R3a* and *Avr3a* genes, with the latter under the control of a glucocorticoid-inducible promoter, exhibited a localized HR under dexamethasone-induced *R3a-Avr3a* interaction (Jia et al. 2010).

A similar transgenic approach employing novel LB *R*-genes from potato wild species *S. okadae* (*Rpi-oka1*) and *S. mochiquense* (*Rpi-mcq1.1*, *Rpi-cmq1.2*) was employed to enhance LB resistance in potato and tomato cultivars (Jones et al. 2010). In this study, eight of the nine recovered transgenic potato plants of susceptible cultivar Desiree exhibited resistance against the tested *P. infestans* isolates. Furthermore, when cloned under their native promoter and terminator sequences and stably transformed into potato or tomato cultivars, expression of all three genes induced resistance against a wide spectrum of *P. infestans* isolates tested. Interestingly, the spectrum of LB resistance exhibited for each construct was in agreement with that exhibited by the original wild potato accessions harboring these genes. In particular, *Rpi-oka1*–derived LB resistance was retained in the transgenic plants, and the resistance phenotype was not a result of constitutive activation of defense pathways by the transgene (Jones et al. 2010). Similarly, LB susceptible potato and tomato cultivars exhibited resistance when transformed with resistance gene *Rpi-vnt1.1* from *S. venturii* (Foster et al. 2009). In this study, the transgenic potato and tomato plants exhibited resistance against 10 of

the 11 isolates tested; only isolate EC1 from Ecuador was able to overcome RPI-VNT1.1 and cause disease on the inoculated plants (Foster et al. 2009).

Because potato and tomato are closely related and share high genome similarity, the LB-resistance genes and QTLs identified in potato may have relevance in tomato or correspond to tomato LB-resistance genes and QTLs (Foolad 2007; Vleeshouwers et al. 2011). By analyzing the tomato genome regions to which LB R-genes have been mapped and taking into account that all such potato genes thus far cloned possess the NBS-LRR structure, and also considering the conservation of LB signaling pathways between potato and tomato, candidate genes for LB resistance can be rather easily traced down. For example, the ~1.5 Mbp region of chromosome 10, to which Ph-2 has been mapped, contains only two of the 155 genes annotated as 'similar to resistance-like' (Mueller et al. 2005) or containing the aforementioned NBS-LRR domains (Moreau et al. 1998; Vleeshouwers et al. 2011) (Figure 13.2A). On the other hand, ambiguous reports exist regarding the *Ph-3* in tomato: Within the originally mapped *Ph-3* region of ~2.5 Mbp, two NBS-LRR 'hotspots' exist, and different groups have announced discovering the *Ph-3*-related molecular markers (Chunwongse et al. 2002; Zhu et al. 2006; Park et al. 2010; Foolad and Panthee 2012) (Figure 13.2B). Comprehensive comparison of potato and tomato genetic maps containing information on the location of LB-resistance traits may guide future searches for the tomato *R*-genes, previously mapped to corresponding chromosomal regions (Foolad 2007; Nowicki et al. 2012). Further information on potato LB-resistance genes is reported in Chapter 12.

Future Prospects

More than 165 years ago, LB made its mark as a destructive disease of potato. Although control of the disease was eventually attained in the 1950s and managed relatively well through the use of fungicides and semi-resistant cultivars (Fry 2008; Vleeshouwers et al. 2011), *P. infestans* remains one of the most devastating plant pathogens of all time. In potato, the identification, characterization, and introgression of new LB-resistance genes and QTLs within the past two decades have significantly contributed to the effective control of the disease. Somewhat similar but to a lesser extent, efforts have been made in tomato and some progress has been made. For example, pyramiding of the known major resistance genes *Ph-2* and *Ph-3* and recent identification of novel resistance resources and genes has increased the prospect for development of tomatoes with strong resistance against LB. Nevertheless, because of the risk of the pathogen breaching the resistance conferred by the available resistance genes, future efforts are imperative to identify new and more desirable sources of resistance and new resistance genes and QTLs. With the availability of genome sequencing data and the advent of modern high-throughput genomic methods exemplified by potato LB effectoromics, identification, cloning, characterization, and deployment of yet undiscovered tomato LB-resistance genes will not be unexpected.

Genomic Resources in Tomato

The recently sequenced potato genome of ~844 Mbp (~39,000 genes, mostly located within the ~570 Mbp of euchromatin) (Mueller et al. 2009; Xu et al. 2011) and the tomato genome of ~950 Mbp (~40,000 genes, >90% of which lie within the ~220 Mbp of euchromatin) (Mueller et al. 2005; Wang et al. 2006; http://solgenomics.net/organism/Solanum_lycopersicum/genome) significantly contribute to a larger research initiative known as the International Solanaceae Genome Project (SOL): Systems Approach to Diversity and Adaptation (http://www.sgn.cornell.edu/solanaceae-project/index.pl). The SOL Genomics Network

(SGN) database contains biological data for species in the Solanaceae family and their close relatives. Data ranges from chromosomes, maps, markers, ESTs, microarray data, and genes, to phenotypes and accessions. SGN hosts a pre-release of the tomato (*S. lycopersicum* cv Heinz 1706) reference genome sequence. It is also an open source software project, continuously developing and improving a complex system for storing, integrating, and analyzing data. The SGN curation model is community-driven, allowing researchers to add and edit up-to-date information using simple Web tools (Bombarely et al. 2011). This database facilitates a systems approach, investigating the basis of adaptation and phenotypic diversity in the Solanaceae family and other species in the Asterid clade such as coffee (*Coffea arabica*), Rubiaceae, and beyond (Mueller et al. 2005). Such a rich and broad repertoire of bio-informatic tools is an invaluable resource for both fundamental and applied science scholars. In combination with the current cutting-edge high-throughput plethora of data, SGN provides tremendous potential for research progress.

An auxilliary resource to forward- and reverse-genetics approaches in tomato studies constitutes the tomato mutant database, distributing Micro-Tom mutant collections (Saito et al. 2011). Their freely accessible database, TOMATOMA, contains 1,048 individual ethylmethane sulfonate (EMS) tomato mutant lines classified into 15 major categories and 48 subcategories, with a total of 1,819 phenotypic categories. Of these mutants, 549 were pleiotropic, whereas 499 were non-pleiotropic. A combination of the two approaches – genome comparison studies and use of forward/reverse genetic screens – may significantly enhance the tomato LB *R*-gene studies, especially when employing the modern high-throughput molecular methods such as transcriptomics and second-generation sequencing (e.g., microarray analyses). By using these technologies, researchers will be able to accrue a more complex and comprehensive view of the resistance mechanisms by which tomato defends against LB. This field of research can be strengthened even further by the input of the pathogen-related genomic resources.

Genomic Resources in *P. infestans*

Tremendous progress has been made recently in the pathogen genomics area through the availability of the second-generation sequencing of *Phytophthora* genomes. With more than 240 Mbp encoding a mere 18,155 genes in *P. infestans* (Haas et al. 2009; Raffaele et al. 2010b), its genome exhibits extremely discontinuous distribution of gene density. More than 1,400 putative disease-effector genes localize to the expanded, repeat-rich, and gene-sparse regions of the genome, which constitute ~74% of the genome size. In contrast, the housekeeping 'core ortholog' genes occupy the repeat-poor and gene-dense regions of the genome (Haas et al. 2009; Raffaele et al. 2010b). Additionally, both seem to have evolved at different paces, with the gene-sparse regions experiencing accelerated rates of evolution. Distribution patterns of the pathogen genes, which are induced during preinfection and infection stages, indicate enrichment in genes located in these gene-sparse regions (Avrova et al. 2003; Raffaele et al. 2010a; Raffaele et al. 2010b). In marked contrast, the slowly evolving gene-dense regions are enriched in genes induced in sporangia (Haas et al. 2009; Raffaele et al. 2010a; Raffaele et al. 2010b). This distinct genomic environment is thought to contribute to *P. infestans*' evolutionary potential by promoting genome plasticity, thus enhancing genetic variation of effector genes leading to host adaptation (Raffaele et al. 2010a; Raffaele et al. 2010b).

Pathogen-related genomic resources are less abundant than those for host plants, although the modern high-throughput analyses contribute a wealth of data to be utilized. An example of such input is the *Phytophthora infestans* Database, spanning the Broad Institute

laboratories from several countries, in the collective effort aiming at multiple coverage of the pathogen genome. Chromosomal and mitochondrial (haplotypes Ia, IIa, and IIb) genome sequences are available for download/analysis at http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html (*Phytophthora infestans* Sequencing Project, Broad Institute of Harvard and MIT). Use of such resources extends the LB-related research and contributes to fundamental research as well, in terms of comparative genomics, genetics, gene/protein annotation, plant resistance investigations, and many others.

An example of speeding-up the race with the highly variable *P. infestans* is the recent strategy for effectoromics, that is, cloning the genes, whose products display the characteristics typical for other known *P. infestans Avr* genes. Thus far, all cloned effector genes of this pathogen encode one class of proteins: The modular secreted proteins with a RXLR motif for translocation into the host cell, followed by diverse, rapidly evolving C-terminal effector domains (Vleeshouwers et al. 2011). Effectoromics, a high-throughput functional genomics approach that uses effectors to probe plant germplasm for specific recognition by R-proteins, has recently emerged as a powerful tool for identification of *Avr* and *R* genes (Jia et al. 2010; Vleeshouwers et al. 2011). The availability of genome sequence resources for *P. infestans* has enabled the generation of effector libraries cloned in vectors designed for *in planta* expression (Haas et al. 2009). Effectors are transiently expressed in *Solanum* germplasm by agro-infiltration with *Agrobacterium tumefaciens* and/or a virus vector such as Potato virus X (PVX), and plants are monitored for the occurrence of macroscopic cell death responses to the individual effectors (Vleeshouwers et al. 2011). Effectors triggering cell death represent candidate *Avr* genes. These are subsequently validated for *Avr* activity by genetic analyses. Co-segregation of the cell death response to the effectors correlates with HR-based resistance against *P. infestans* isolates in genetic populations. If the matching *R*-gene has been cloned, additional verification of R-AVR pairs can be obtained by co-expression of *R*-gene and *Avr*-gene candidates in leaves of tester plants such as *Nicotiana benthamiana* (Jia et al. 2010; Vleeshouwers et al. 2011). Similar studies with tomato are yet to be undertaken. Nevertheless, as exemplified by potato, the potential exists for the R-AVR pairs discovery of tomato proteins interacting with the pathogen AVR protein being over-expressed, for instance by means of fixation, immunoprecipitation, and subsequent analyses. Such a strategy should considerably enhance the tomato-related modern LB-resistance studies.

Conclusions

The most sustainable strategy for managing tomato LB is to deploy an integrated system including cultural practices, fungicide application, and the use of cultivars with broad-spectrum genetic resistance against LB. Unfortunately, traditional disease resistance breeding approaches can be slow and may take little advantage of the emerging knowledge of pathogenicity mechanisms. Tomato breeders now have access to at least a few LB-resistance genes of tomato origin with associated genetic markers, which can be introduced into breeding lines and commercial cultivars via marker-assisted breeding. Accumulating evidence suggests that sustainable disease resistance constitutes successful pyramiding of multiple resistance genes within a single cultivar. In tomato, the combination of *Ph-2* and *Ph-3* has been shown to confer LB resistance superior to the resistance conferred by either of the two genes alone. This superiority has been observed and confirmed by several tomato researchers. It is noteworthy, that a possible breach in the pyramided *Ph-2* and *Ph-3* was reported by R. Gardner (North Carolina State) in tomato plantings in North Carolina under highly LB-conducive conditions at the end of the season

and only in the presence of other lines with single gene resistance in the same plantings (Scott and Gardner 2007). In contrast, under severe LB infestation in multiple locations in Pennsylvania, we have observed that the resistance conferred by the combination of *Ph-2* and *Ph-3* held very well. Nonetheless, there exists a possibility for breakdown of the resistance conferred by the combination of *Ph-2* and *Ph-3*, and thus the continued search for identification and subsequent introduction of additional LB-resistance genes in tomato remains of crucial importance. Fortunately, research in this direction is underway, which will provide opportunities for pyramiding multiple resistance genes and developing tomatoes with stronger and more durable resistance against LB. This is a strategy that only will gain more significance, since predicting the extent to which an *R*-gene will continue providing resistance against a highly-variable pathogen remains problematic. Another promising research area is to combine single resistance genes with QTLs that may confer moderate but more broad-spectrum field resistance against tomato LB. Similar to work in potato, considerable efforts have been made in tomato to identify QTLs for LB resistance in different wild species, and the findings may prove to be useful for development of tomatoes with more durable resistance against LB. Furthermore, as exemplified by the fast progress in potato research and conservation of LB-signaling pathways between potato and tomato, detailed knowledge of the pathogen effectors, in combination with high-throughput genomics, may significantly contribute to identification and deployment of tomato LB-resistance sources.

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Chapter 14

Marker-Assisted Selection for Disease Resistance in Lettuce

I. Simko

Abstract

Lettuce (*Lactuca sativa* L.) is the most popular leafy vegetable cultivated mainly in moderate climates. Consumers demand lettuce with good appearance and free of disease. Improved disease resistance of new cultivars is achieved by combining desirable genes (or alleles) from existing cultivars or by introgressing novel resistance genes from wild *Lactuca* species sexually compatible with cultivated lettuce. Development of elite lettuce cultivars is a lengthy process that involves cross-pollination, several rounds of selection, development of homozygous genotypes, and testing of material performance. Use of molecular markers linked to the disease resistance genes allows for rapid and frequently more accurate selection of desirable genotypes than phenotype-based selection. This marker-assisted selection (MAS) is used by both public and private lettuce-breeding sectors. At the present time, MAS for disease resistance in lettuce is limited to simply inherited traits. Assays have been developed to detect genes and alleles for resistance to downy mildew, corky root, lettuce mosaic, and lettuce dieback (a description of three assays based on the high-resolution DNA melting approach is provided in this chapter). Resistance genes against Verticillium wilt, turnip mosaic, root downy mildew, powdery mildew, big-vein, Fusarium wilt, and anthracnose have been mapped on the molecular linkage map of lettuce, and assays for MAS are under development for several of these genes. The lettuce genome has been sequenced recently, paving the way to speedier mapping, cloning, and functionally validating genes for disease resistance and for more efficient development of molecular markers used in MAS.

Introduction

Lettuce (*Lactuca sativa* L.) is commercially produced in many countries around the world, making it the most popular vegetable from the group of leafy vegetables. The total world production of lettuce in 2010 was more than 23 million tons (FAOSTAT 2012). Asia produced about 65% of the total, followed by North and Central America (19%), and Europe (13%). Substantially less lettuce was grown in South America, Africa, and Oceania, each producing around 1% of the world's total. The largest producer of lettuce as a salad crop is the United States, where production is centered in California (70–75% of the lettuce grown in the U.S.) and Arizona (18–20%) (Ryder

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1997). China is the largest producer of stem lettuce, which is consumed raw, cooked, pickled, dried, or stir-fried, in soups or as a sauce vegetable (Mou 2008; Simoons 1991). In Egypt, oilseed lettuce is used to produce oil (Lebeda et al. 2007; Ryder 1986). From the main lettuce producing regions, the largest per capita production in 2010 (FAOSTAT 2012) was in Spain (17.5 kg), followed by Italy (13.9 kg), and the USA (13.1 kg).

Cultivated lettuce is a self-fertilizing diploid ($2n = 2x = 18$ chromosomes) species from the family of *Compositae* (*Asteraceae*). Lettuce cultivars are usually classified into seven horticultural types based on head and leaf shape, size, and texture, and stem length (Figure 14.1): crisphead, butterhead, romaine (cos), leaf (cutting), Latin (grassé), stem (stalk), and oilseed (de Vries and van Raamsdonk 1994; de Vries 1997; Ryder 1999; Lebeda et al. 2007; Křístková et al 2008; Mou 2008). The crisphead lettuce produces a firm spherical head. This type is frequently split into two subtypes – iceberg, with a dense head, and Batavia, with a less dense and smaller head. Butterhead lettuce produces a smaller, less compact head than the crisphead lettuce. Romaine type lettuce tends to have an upright stature and forms a loaf-shaped head. Leaf lettuce forms a bunch or rosette of leaves. This type shows a large phenotypic variation in leaf size, shape, color, and margin. Latin lettuce somewhat resembles romaine lettuce, but the leaves are shorter and less crispy. Stem lettuce produces a long, erect stem, with either narrow or broad leaves. Oilseed lettuce to some degree resembles stem lettuce with its long stem and narrow, elongated leaves. The stem of this type is frequently branching and the seeds are larger than those formed on other types of lettuce.

Lettuce suffers from several economically important diseases. To introgress novel resistance genes into the cultivated lettuce, crosses between *L. sativa* and its wild relatives are carried out. Of the approximately 100 species belonging to the genus *Lactuca* (Feráková 1977)

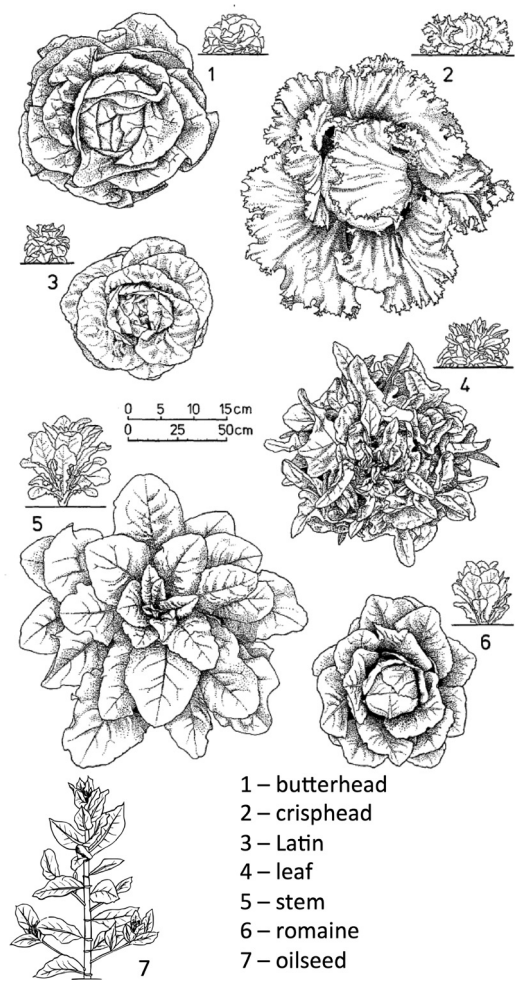

Fig. 14.1. Drawing of seven lettuce types: (1) butterhead, (2) crisphead, (3) Latin, (4) leaf, (5) stem, (6) romaine (de Vries and van Raamsdonk 1994), and (7) oilseed (illustration by Nina Simkova).

only a few species are completely or partially sexually compatible with cultivated lettuce. Prickly lettuce (*L. serriola*) belongs to the primary gene pool that is closely related to cultivated lettuce and does not show any crossing barrier; willowleaf lettuce (*L. saligna*) belongs to the secondary gene pool that exchanges genes with *L. sativa* to a limited degree; while bitter lettuce (*L. virosa*) belongs to the tertiary gene pool that is difficult to cross with *L. sativa*. Combined

taxonomic and genetic evidence indicates that another three species (*L. aculeata* Boiss. & Ky., *L. dregeana* DC., and *L. altaica* Fisch. & C.A. Mey.) are likely to be interfertile with *L. sativa* and together with *L. serriola* constitute a primary gene pool of wild lettuce species (Zohary 1991). Sequencing of the internal transcribed spacer (ITS-1) has confirmed the proposed classification (Koopman et al. 1998) of these three species, while analysis with molecular markers separated *L. aculeata* from the other species of the primary gene pool (Koopman et al. 2001). Based on the taxonomic analysis, Zohary (1991) speculates that *L. scarioloides* Boiss., *L. azerbaijanica* Rech., and *L. georgica* Grossh. also belong to the primary gene pool, but the information on the genetic affinities of these species with *L. sativa* is incomplete.

Molecular Markers and Marker-Assisted Selection

Development of cultivars with improved resistance is a slow and labor-intensive process that takes several years and requires extensive phenotypic evaluation of breeding material. To increase the reliability of selection methods and to shorten the time needed for development of new cultivars, many breeding companies and research institutions apply molecular marker techniques in plant breeding programs (Cahill and Schmidt 2004; Holland 2004; Foolad 2007; Collard and Mackill 2008). Use of molecular markers linked to the genes of interest enables moving from selection based only on phenotype toward selection based on genotype (or a combination of the two). This marker-assisted selection (MAS) is particularly useful if the phenotypic evaluation of a target trait is time-consuming, difficult, or costly (Simko et al. 2007). MAS allows the breeder to conduct selection on a large number of individuals in early stages of their development (or seeds). A rapid selection of desirable material can be carried out off-season without depending on the natural occurrence of the pathogen. Another advantage of MAS is that selection for resistance to multiple pathogens can be performed simultaneously, unlike a phenotype-based selection that frequently requires a number of separate trials. MAS also makes possible rapid pyramiding of multiple resistance genes against a pathogen (Peleman and Rouppe van der Voort 2003; Simko et al. 2007; Collard and Mackill 2008).

Although advantages of using molecular markers in resistance breeding programs are considerable, practical application of MAS lags behind expectations. One limiting factor of MAS is the initial capital investment required for the development of marker assays, including cost of equipment, consumables, labor, and (sometimes) licensing of patents. It has been demonstrated that MAS offers the greatest benefit for quantitative traits with low heritability (Moreau et al. 1998) as these are the most difficult to assess in field conditions. However, development of marker assays for such traits is particularly difficult and costly because of the extensive phenotyping required (Peleman and Rouppe van der Voort 2003). Another factor preventing a broader application of MAS in breeding programs is possibly a limited reliability of marker assays, particularly those developed for quantitative trait loci (QTLs) of complex traits. Reliability of MAS can be diminished by lesser accuracy of mapping studies, insufficient linkage between marker and gene(s), low polymorphism of markers in breeding material, effect of genetic background, and gene(s) $\times$ environment interaction (Collard and Mackill 2008).

MAS is most accurate if the molecular markers used in the assay are residing directly within the coding region of the resistance gene. When markers are linked to the gene of interest but not residing within the gene, a linkage observed in a specific cross may not be observed universally in the wide gene pool. There are several examples of markers being tightly linked to resistance genes in the specific mapping population, but whose use presents problems in a

different genetic background (Niewöhner et al. 1995; Moreno-Vázquez et al. 2003). To ensure universal applicability of a marker in MAS, the marker-gene association has to be tested in different genetic backgrounds, that is, in different horticultural types of lettuce.

Although the potential for use of MAS in plant breeding programs is extensive, application varies widely among crop species. In crops such as maize, rice, wheat, or barley, use of MAS is well established, while in others, MAS is used less frequently. Historically, a number of different marker-systems have been applied for lettuce genotyping, such as isozymes (Kesseli and Michelmore 1986); restriction fragment length polymorphism – RFLP (Kesseli et al. 1991); random amplified polymorphic DNA – RAPD (Kesseli et al. 1994); sequence characterized amplified regions – SCAR (Maisonneuve et al. 1994); retrotransposon-based sequence specific amplification polymorphism – SSAP (Syed et al. 2006); amplified fragment length polymorphism – AFLP (Hill et al. 1996); simple sequence repeats – SSR (Van de Wiel et al. 1999; Rauscher and Simko 2013); target region amplification polymorphism – TRAP (Hu et al. 2005); expressed sequence tag based SSR – EST-SSR (Simko 2009); single-stranded conformational polymorphism – SSCP (McHale et al. 2009); single nucleotide polymorphism – SNPs (Kwon et al. 2012); and single position polymorphism – SPP (Stoffel et al. 2012). The majority of markers currently used in plant genetics are molecular markers that allow high-throughput genotyping. These markers have been used to construct several molecular linkage maps of lettuce (Truco et al. 2007) to map resistance genes (Paran and Michelmore 1993; Meyers et al. 1998a; McHale et al. 2009), and to develop assays for MAS (Moreno-Vázquez et al. 2003; Simko et al. 2009). The following chapter describes progress in mapping disease resistance genes and use of MAS in lettuce breeding. Although most of the information comes from public breeding programs, a brief description of MAS in the private sector will also be provided.

Marker-Assisted Selection for Disease Resistance

Precise assessment of a phenotype is critical for development of assays for MAS. Therefore, it is generally easier to develop marker assays for monogenic traits that can be scored with high confidence than for QTLs of complex traits. At the present time MAS for diseases resistance in lettuce is limited to simply inherited traits. Assays were developed to select desired genes and alleles for resistance to downy mildew, corky root, lettuce mosaic virus, and lettuce dieback (Figure 14.2).

Downy Mildew

Downy mildew, caused by the oomycete *Bremia lactucae* Regel, is probably the most frequent and destructive disease of cultivated lettuce worldwide. The pathogen can infect lettuce at any developmental stage, from young seedlings to mature plants. Infected plants show yellow to pale green areas on the adaxial side of leaves that eventually become necrotic. When conditions are favorable for growth of the pathogen, sporulation appears predominantly on the abaxial part of the leaves (Figure 14.2A).

Two types of resistance to downy mildew are known in cultivated lettuce: qualitative – based on single dominant genes or resistance factors (termed *Dm* and *R,* respectively), and quantitative – based on multiple genes with minor effect. More than thirty race-specific *Dm* genes and *R* factors have been introgressed into lettuce cultivars from wild species, and several of the genes were mapped (McHale 2008; Michelmore et al. 2010). Most of the mapped *Dm* genes are clustered at three linkage groups (LGs); *Dm5/8*, *Dm10*, *Dm17*, *Dm43*, and *Dm45* at LG 1; *Dm1*, *Dm2*, *Dm3*, *Dm6*, *Dm14*, *Dm15*, *Dm16*, and *Dm18* at LG 2; and *Dm4*, *Dm7*, *Dm11*, *Dm44*, *Dm48*, and *Dm49* at LG 4 (Table 14.1). A single *Dm* gene, *Dm13*, is located at LG 3 (Paran et al. 1991; Maisonneuve et al. 1994; McHale 2008; McHale et al. 2009; Michelmore et al. 2010;

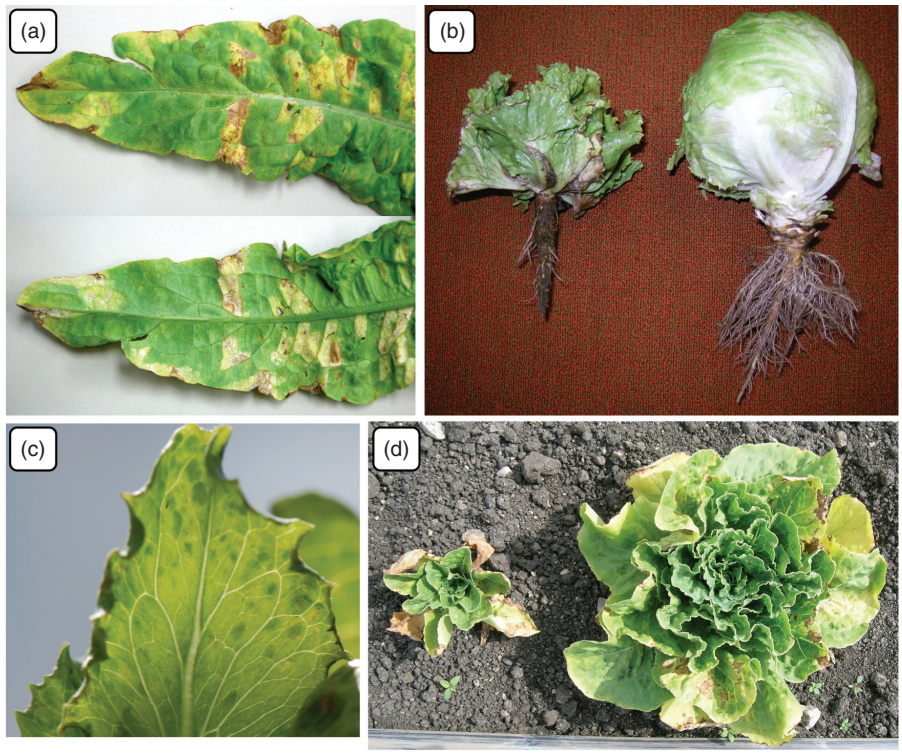

Fig. 14.2. Symptoms of four lettuce diseases for which resistance breeding is aided by marker-assisted selection. (a) adaxial (top) and abaxial (bottom) surface of a leaf infected with downy mildew; (b) plant with corky root symptoms (left) and a healthy plant (right) (photo courtesy of Beiquan Mou); (c) detail of a leaf inoculated with LMV; (d) plants with dieback symptoms that were infected with TBSV and/or LNSV in very early (left) or later (right) stages of development. For a color version of this figure, please refer to the color plate.

Paran and Michelmore 1993). Molecular markers that are closely linked to several of these resistance genes were converted into SCAR markers suitable for MAS (Paran and Michelmore 1993; Maisonneuve et al. 1994; McHale 2008; Michelmore et al. 2010). Cloning and analysis of the *Dm3* gene revealed that the gene belongs to the NBS-LRR (nucleotide binding site and a leucine-rich repeat region) group of resistance genes (Meyers et al. 1998a; Meyers et al. 1998b). However, the *Dm3* gene alone, similar to many other *Dm* genes, is not suitable for the development of cultivars with durable resistance against downy mildew because the gene is defeated by many of the current isolates of the pathogen (Michelmore et al. 2011). Because single-gene resistance is frequently overcome with new races of *B. lactucae* in a short time, pyramiding of multiple *Dm* genes by MAS is currently used to develop cultivars with resistance to the races of downy mildew appearing in specific geographic regions.

Quantitative resistance (sometime also called field-based, horizontal, or polygenic) of lettuce to downy mildew has been documented (Norwood et al. 1983; Crute and Norwood 1981; Grube and Ochoa 2005). This type of resistance is usually race non-specific and is not overcome by new races of the pathogen. Genotypes with quantitative resistance to downy mildew typically get infected with most races of the pathogen, but lesions are small and sporulation of the pathogen is limited. Batavia-type cultivars Iceberg and Holborn Standard, and leaf-type

Table 14.1. List of mapped resistance genes and QTLs and their location on the molecular linkage map of lettuce.

Disease	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9
Downy mildew*	*Dm5/8* *Dm10* *Dm17* *Dm43* *Dm45*	*Dm1* *Dm2* *Dm3* *Dm6* *Dm14* *Dm15* *Dm16* *Dm18*	*Dm13*	*Dm4* *Dm7* *Dm11* *Dm44* *Dm48* *Dm49*					
Corky root			*Cor*						
Lettuce mosaic	*Mo-2*			*mo-1*					
Lettuce dieback		*Tvr1*							
Verticillium wilt									*Vr1*
Turnip mosaic	*Tu*								
Root downy mildew	*Plr*								
Powdery mildew	*pm-1.1*	*pm-2.1* *pm-2.2*					*pm-7.1*		
Big-vein			*BV1*	*BV2* *BV3*	*BV4*	*BV5*			
Fusarium wilt	*FUS1*	*FUS2* *RRD2*					*FUS3*		
Anthracnose		*ANT2*						*ANT3*	

Location of resistance genes was compiled from journal articles, books, and book chapters, Ph.D. dissertations, conference proceedings, and research reports cited in the chapter.
*Only locations of *Dm* genes are shown. QTLs for resistance to downy mildew were detected on all linkage groups.
LG – linkage group.

cultivar Grand Rapids have shown a high level of resistance to different races of the pathogen for a number of decades (Crute and Norwood 1981; Norwood et al. 1983; Hand et al. 2003; Grube and Ochoa 2005; Simko et al. 2012). Low susceptibility to downy mildew was reported in cv. Iceberg as early as 1923 (Milbrath 1923), in cv. Grand Rapids in 1960 (Verhoeff 1960), and in cv. Holborn Standard in 1961 (Chapman and Finch 1961). These cultivars still retain low susceptibility to downy mildew (Simko et al. 2012), although both Grand Rapid and Iceberg possess only the *Dm13* resistance gene, which is defeated with current isolates of the pathogen (Grube and Ochoa 2005). Although the quantitative resistance in cvs. Iceberg, Grand Rapids, and Holborn Standard has long been recognized, the resistance has not yet been transferred to other lettuce types (e.g., iceberg, butterhead, or romaine). This may be a result of the difficulty of screening for quantitative resistance in field conditions when the resistance is based on several QTLs. To identify the mechanism of resistance to downy mildew in cv. Iceberg, a mapping population was developed from a cross with highly susceptible iceberg-type cv. Saladin (synonym for cv. Salinas). Initial analysis of the phenotypic data identified putative QTLs on several linkage groups, however their position was not disclosed (Hand et al. 2003). Progeny from a cross between cvs. Grand Rapids and Iceberg was used for genetic analysis of quantitative resistance. A high estimate of narrow-sense heritability suggested simple inheritance of the trait and distribution of resistance in the population indicated that at least one unique resistance allele is present in each of the two cultivars (Grube and Ochoa 2005). Recent analysis of the Grand Rapids × Iceberg

mapping population identified QTLs on LG 2, and LG 5 (Simko, unpublished results).

Complete resistance to downy mildew was reported in several *L. saligna* accessions that stayed disease-free in all stages of plant development after inoculation with a number of different isolates of *B. lactucae* (Norwood et al. 1981; Bonnier et al. 1992; Petrželová et al. 2011). The molecular basis of nonhost resistance of *L. saligna* to *B. lactucae* was investigated in a progeny developed from a cross between susceptible *L. sativa* cv. Olof and resistant *L. saligna* accession CGN05271 (Jeuken and Lindhout 2002; Jeuken et al. 2008; Zhang et al. 2009a; Zhang et al. 2009b; den Boer et al. 2011). Nonhost resistance in *L. saligna* was explained by multiple QTLs, rather than *R*/*Dm*-genes. Disease evaluation at the young and the adult plant stage revealed several QTLs, some of them reducing infection in the young stage only, or the adult stage only, or both developmental stages (Zhang et al. 2009a). Fifteen resistance QTLs originating from *L. saligna* were mapped on all linkage groups with the exception of LG 3, and LG 5 (Jeuken and Lindhout 2002; Jeuken et al. 2008; Zhang et al. 2009a; Zhang et al. 2009b). Tests with multiple races of *B. lactucae* did not provide evidence that any of the QTLs is race-specific. Nonhost resistance in *L. saligna* thus appears to be conferred by a cumulative effect of many QTLs, frequently operating at different developmental stages (Zhang et al. 2009a; den Boer et al. 2011). The majority of mapped QTLs did not coincide with the known *Dm*-gene clusters, indicating that these QTLs are not likely alleles of known *Dm* genes. Combination of three QTLs, (*rbq4*, *rbq5*, and *rbq6+11*) was sufficient to confer complete resistance to downy mildew at the young plant stage and almost complete resistance at the adult plant stage (Zhang et al. 2009b). In addition to QTLs, specific *L. sativa* × *L. saligna* hybrids also harbor a downy mildew resistance resulting from a digenic hybrid incompatibility between an *L. saligna* target protein, *RIN4*, and a potential *L. sativa R*-gene (Jeuken et al. 2009). None of the detected QTLs was reported to be used in MAS.

Corky Root

Corky root of lettuce is caused by the bacterium *Sphingomonas suberifaciens* (Yabuuchi et al. 1999), formerly known as *Rhizomonas suberifaciens* (van Bruggen et al. 1990). Plants susceptible to the disease show yellow-green lesions and corky-like texture on the taproot and main lateral roots. Since function of the infected roots is impaired, plants may wilt and/or become stunted, leading to production of small, unmarketable heads (Figure 14.2B). Testing for corky root resistance is difficult even under controlled conditions because of the large environmental effect on expression of symptoms (Brown and Michelmore 1988). Screening of germplasm for resistance to *S. suberifaciens* strain CA1 that causes corky root disease in California, identified a single, recessive resistance gene designated *cor* (Brown and Michelmore 1988), which is located on LG 3 (Moreno-Vázquez et al. 2003) (Table 14.1). The resistance gene was identified in several accessions of *L. sativa*, *L. serriola*, and *L. saligna*. The difficulty of testing combined with the recessive nature of the resistance gene makes corky root resistance a prime candidate for MAS (Moreno-Vázquez et al. 2003). When markers in the proximity of the *cor* gene were tested for their predictive value, the best results were obtained with marker *SCO07* (Appendix). Analysis of 124 unique accessions and breeding lines with this molecular marker revealed about 90% accuracy of *SCO07* in predicting correct phenotypic reaction (combined results from Moreno-Vázquez et al. 2003; Dufresne et al. 2004; Simko and Mou, unpublished results). Development of new molecular markers with higher accuracy in predicting plant response to the pathogen is in progress (Michelmore et al. 2010). To identify novel sources of resistance, more than 1,000 accessions were screened for their reaction to the pathogen (Mou and Bull 2004). Three *L. serriola* accessions and one *L. virosa* accession highly

resistant to corky root were identified. However, it has not yet been determined if these accessions harbor a new resistance gene, because genotyping with markers (*SCY15* and *SC853*) flanking the *cor* gene was not conclusive.

Lettuce Mosaic

Lettuce mosaic is caused by *Lettuce mosaic virus* (LMV) from the family *Potyviridae*. LMV is one of the most serious viruses attacking lettuce crops worldwide. The virus is seed-borne and highly transmissible by aphids (Candresse et al. 2006). Leaves of infected plants show symptoms of mosaic (Figure 14.2C) and their growth is greatly reduced. Severely infected and stunted plants do not form heads, thus making them unmarketable. Prevention of disease outbreak is mainly based on the use of virus-free seeds, management of aphids, and growing resistant cultivars. Four genes have been reported that confer resistance to LMV; one recessive gene (*mo-1*) and three dominant genes (*Mo-2*, *Mo-3*, and *Mo-4*) (Candresse et al. 2006). The dominant genes are currently not used in breeding programs because of their limited durability (*Mo-2*) (Pink et al. 1992), or the difficulty of introgressing genes from *L. virosa* (*Mo-3* and *Mo-4*) into cultivated lettuce (Le Gall et al. 1999; Maisonneuve et al. 1999; Candresse et al. 2006). The position of the *Mo-3* and *Mo-4* genes on the molecular linkage map is not known; the *Mo-2* gene that was first identified in crisp-type cv. Ithaca (Pink et al. 1992) is located on the LG 1 (McHale et al. 2009) (Table 14.1).

The recessive *mo-1* gene is usually associated with tolerance or resistance to LMV (Dinant and Lot 1992), depending on the virus isolate and genetic background of the plants (Pink et al. 1992; Revers et al. 1997). Two alleles of the *mo-1* gene that is located on LG 4 (Nicaise et al. 2003) were originally identified in cv. Gallega de Invierno (*mo-1*^{1}) (von der Pahlen and Crnko 1965) and the *L. sativa* accession PI 251245 (*mo-1*^{2}) (Ryder 1970). The *mo-1*^{1} allele is frequently used in the European breeding programs (Pink et al. 1992), while the *mo-1*^{2} allele was used to develop LMV-resistant cultivars in the USA (e.g., Salinas 88, and Vanguard 75) (Ryder 1979; Ryder 1991). Sequencing of the *mo-1* gene confirmed that the *mo-1*^{1} and *mo-1*^{2} are alleles of the same gene that is coding the eukaryotic translation initiation factor (eIF4E) (Nicaise et al. 2003). Nucleotide difference between the two resistant alleles and the susceptible *Mo-1* allele allowed development of molecular markers that distinguish among all three alleles and can be used in MAS (Appendix). Strains of LMV (pathotype IV) have been identified that overcome resistance based on *mo-1*^{1} and *mo-1*^{2} alleles (Pink et al. 1992). This LMV pathotype from Spain has never been reported in the USA. The *Mo-3* gene from *L. virosa* appears to provide efficient protection against all LMV isolates breaking resistance based on *mo-1*, including pathotype IV (Maisonneuve et al. 1999). A gene conferring a mild systemic reaction to LMV in its dominant stage was described and named *Mi* (Ryder 2002). Combining the *mo-1* gene and the *Mi* gene raises the level of lettuce resistance to nearly complete (Ryder 2002). The position of the *Mi* gene on the molecular linkage is not known, and an assay for MAS has not been developed.

Lettuce Dieback

Lettuce dieback is a soil-borne disease caused by two closely related viruses from the family *Tombusviridae – Tomato bushy stunt virus* (TBSV) and *Lettuce necrotic stunt virus* (LNSV) (Obermeier et al. 2001). The mechanism of virus transfer to plants is yet unknown. The disease is widespread in all types of commercially grown lettuce with the exception of modern iceberg-type cultivars. Symptoms of this disease include mottling, yellowing, and necrosis of older leaves, and stunting and death of plants (Figure 14.2D), leading to complete loss of the crop in a severely infected field. The disease has been observed

throughout the main producing areas of California and Arizona (Grube et al. 2005; Simko et al. 2009). The virus was also detected on lettuce in Slovakia (Novák et al. 1981) and Portugal (Blancard et al. 2006), but no widespread distribution of the disease and significant yield loss was reported outside the U.S. Since there are no known methods to prevent the disease when lettuce is grown in an infested field, the only option to control the disease is development of resistant cultivars. Analysis of modern iceberg cultivars that are resistant to this disease has led to the discovery of a single dominant gene (*Tvr1*) located on LG 2 (Grube et al. 2005) (Table 14.1). Additional analysis indicates that the resistance genes originating from iceberg-type cv. Salinas, romaine accession PI 491224, and *L. serriola* accession UC96US23 are either allelic versions of *Tvr1* or closely linked loci (Grube et al. 2005; Simko et al. 2009). Testing of molecular markers from the *Tvr1* region identified *Cntg10192* as the best marker for MAS (Simko et al. 2009; Simko et al. 2010b) (Appendix). Sequencing of the *Cntg10192* marker from four *Lactuca* species revealed six haplotypes at this locus. Five of the haplotypes from *L. sativa*, *L. serriola*, *L. saligna*, and *L. virosa* accessions were associated with disease resistance, while a single haplotype from both *L. sativa* and *L. serriola* accessions was associated with susceptibility to the disease (Simko et al. 2010b). When more than 1,000 accessions from all horticultural types of lettuce were analyzed with the *Cntg10192*–based assay, the accuracy of detecting resistant and susceptible phenotypes was 100% (Simko, unpublished results).

Marker-Assisted Selection in the Private Sector

Use of MAS in the private sector may be more common than in public breeding programs (Foolad 2007), but detailed information is usually not published because of competing interests among companies (Collard and Mackill 2008). To assess the use of MAS in lettuce breeding programs, a mini-survey was performed among lettuce breeding companies and companies offering genotyping services. Based on the information provided by the U.S. and European companies, molecular markers are routinely used in cultivar fingerprinting, marker-assisted breeding, and marker-assisted selection. MAS is applied to develop cultivars with resistance against downy mildew, corky root, LMV, and dieback. Assays for MAS were developed from publicly available information about markers linked to the resistance genes. In addition, assays have been developed for pyramiding publicly known *Dm* genes and proprietary resistance genes against downy mildew.

Mapped Resistance Genes

Genomic locations of resistance genes against Verticillium wilt, turnip mosaic virus, root downy mildew, powdery mildew, big vein, Fusarium wilt, and anthracnose have been mapped on the molecular linkage map of lettuce (Table 14.1). Assays for MAS are under development for several of these genes. Mapping of the genes for resistance to lettuce drop and bacterial leaf spot is in progress.

Verticillium Wilt

Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* Kleb, is a relatively new but highly devastating disease of lettuce. It was first discovered in three fields in October 1995 and gradually spread to other lettuce producing areas of coastal California (Subbarao et al. 1997). The first symptoms are yellowing and wilting appearing on basal leaves and progressing acropetally, eventually leading to plant collapse and death. In infected plants, a dark, brown-green discoloration is visible in vascular tissue of the taproot. Disease progress is quick on plants near market maturity, causing a high yield loss (Subbarao et al. 1997; Hayes

et al. 2007). Seven cultivars from four diverse lettuce types were identified as having a complete resistance to the race 1 of the pathogen (Hayes et al. 2007). One of the resistant cultivars (La Brillante, a Batavia-type lettuce) was used to develop a mapping population when crossed with Salinas 88, a susceptible iceberg-type cultivar. The dominant *Vr1* gene for resistance to Verticillium wilt race 1 was mapped on LG 9 (Hayes et al. 2011b). The gene is closely linked to the EST marker *QGD8I16.yg.ab1,* which can be considered for development of a MAS assay. The sequence of the *QGD8I16.yg.ab1* marker is similar to the sequence of the tomato *Ve* gene that confers resistance to *V. dahliae* race 1 (Kawchuk et al. 2001; Hayes et al. 2011b).

In tomato, introduction of cultivars with the *Ve* gene led to widespread distribution of the race 2 strain of the pathogen that defeats the *Ve* gene-based resistance (Pegg and Brady 2002). Because *V. dahliae* pathogenicity on lettuce is similar to that described in tomato (the pathogenicity of isolates from lettuce and tomato is strongly correlated) (Maruthachalam et al. 2010), it is expected that growing lettuce cultivars with the *Vr1* gene will increase the frequency of the race 2 strains of the pathogen. So far no accession with the complete resistance to the race 2 strain was identified, although four accessions with a partial resistance were detected (Hayes et al. 2011a). Locations of the genes involved in the partial resistance are not yet known.

Turnip Mosaic Virus (TuMV)

Turnip mosaic virus belongs to the *potyvirus* family of plant viruses. Symptoms of disease on lettuce leaves progress from small, light green lesions to asymmetrical distortion of the leaf blade. Disease is observed only on crisphead varieties having in their pedigree the *L. serriola* accession PI 91532 (Witsenboer et al. 1995); butterhead, leaf, and romaine lettuce types are all resistant (Koike and Davis 2012). Resistance to the disease is conferred by a single dominant gene (*Tu*), located on the LG 1 (Robbins et al. 1994; Montesclaros et al. 1997).

Root Downy Mildew

Plasmopara lactucae-radicis (Stang. & Gilbn.), the root downy mildew pathogen, is an oomycete related to *B. lactucae*. While *B. lactucae* predominantly infects lettuce leaves, *P. lactucae-radicis* infection is restricted to the root system (Stanghellini et al. 1990). The pathogen was identified while analyzing root necrosis of hydroponically grown plants. The recessive resistance gene (*plr*) originating from butterhead cv. Cobham Green (Vandemark et al. 1991) was mapped to the LG 1 (Kesseli et al. 1993).

Powdery Mildew

Powdery mildew of lettuce is caused by the fungus *Golovinomyces cichoracearum sensu stricto* (formerly *Erysiphe cichoracearum* DC) (Braun 1987; Lebeda and Mieslerová 2011). The disease principally affects older, outer leaves that become slightly yellow, then brown, and eventually die. The fungus develops on both leaf surfaces, producing white, powdery spores (Paulus 1997). Powdery mildew usually occurs on plants grown in warm, relatively dry climates or under greenhouse conditions. Most crisphead and nonheading leafy types of lettuce are susceptible to the disease (Dixon 1981), although a monogenic dominant resistance was reported in the crisphead cv. Imperial 850 (Whitaker and Pryor 1941). The *Pm* symbol was proposed for this resistance gene (Robinson et al. 1983). However, the *Pm* gene-based resistance is apparently race-specific as evidenced by the fact that cv. Imperial 850 is easily infected by the current races of *G. cichoracearum* from California and Arizona (Simko et al. 2013). Resistance and moderate resistance were reported in some butterhead cultivars (Lebeda 1985) and several wild species (Lebeda 1994). From seven wild *Lactuca* species (*L. serriola*, *L. saligna*, *L. virosa*, *L. viminea*, *L. perennis*, *L. tenerrima*, *L. tatarica*) and *Mycelis*

muralis (syn. *L. muralis*) that were tested for natural infection with *G. cichoracearum*, *L. serriola* was the most susceptible one. While all *L. serriola* accessions were highly susceptible to the disease, *L. saligna* accessions showed variable levels of resistance, and accessions of other *Lactuca* species were mostly resistant or only moderately susceptible (Lebeda 1994). Analysis of the mapping population developed from the cross between iceberg-type cv. Salinas and the highly susceptible *L. serriola* accession UC96US23 revealed a significant QTL on LG 2 (*pm-2.2*), linked to the EST-SSR marker *SML-22* (Simko et al. 2013). The resistance QTL explained up to 40% of the total phenotypic variation of the trait. Other QTLs were detected on LG 1 (*pm-1.1*), LG 2 (*pm-2.1*), and LG 7 (*pm-7.1*) (Simko et al. 2013).

Big-Vein

Big-vein is a viral disease caused by *Mirafiori lettuce big-vein virus* (MLBVV) vectored by the soil-borne fungus *Olpidium brassicae* (Jagger and Chandler 1934). This economically damaging disease is distributed worldwide, especially in areas where lettuce is grown under cool conditions in moist soil (Falk 1997). A partial resistance to big-vein disease was identified in butterhead cv. Margarita and Latin-type cv. Pavane, while *L. virosa* accession IVT 280 appeared to be immune (Hayes et al. 2006). However, RT-PCR (reverse transcription polymerase chain reaction) analysis demonstrated the presence of the virus in asymptomatic plants of all three accessions (Hayes et al. 2008c). A population developed from crossing susceptible butterhead cv. Parade with cv. Pavane allowed identification of one chromosomal region on LG 3 and two regions on LG 4 that contribute to resistance against big-vein. The three QTLs together explained 56% of the observed phenotypic variation (Hayes et al. 2010a; Michelmore 2010). Two different QTLs were mapped on LG 5 and LG 6 in a population developed from a cross between resistant iceberg-type cv. Thompson and susceptible iceberg-type cv. Cisco (Michelmore 2010), indicating that cvs. Pavane and Thompson represent different sources of resistance to big-vein. Resistance genes in accession IVT 280 and cv. Pavane are different (Hayes and Ryder 2007), thus a higher level of resistance could be achieved by combining QTLs from multiple sources.

Fusarium Wilt

Fusarium wilt of lettuce is caused by the fungus *Fusarium oxysporum*. The disease was first observed in Japan (Matuo and Motohashi 1967), but later reported also in Iran, Italy, Taiwan, and the U.S. (Matheron et al. 2005; McCreight et al. 2005). The first symptoms of the disease are observed as early as two weeks after seeding when young plants wilt and die. The incidence of disease increases as the crop develops. Infected plants exhibit a distinctive red-brown streak extending from the taproot into the plant crown (Hubbard 1997). Three races of the pathogen are known in Japan, but only race 1 has been detected in the U.S. Resistance to race 1 of the pathogen was observed in the iceberg-type cvs. Salinas and Salinas 88, and the romaine-like cv. Costa Rica No. 4, while iceberg-type cv. Vanguard was highly susceptible (McCreight et al. 2005). Analysis of F_1 and F_2 populations indicated that the resistance in both Costa Rica No. 4 and Salinas is recessive, and that iceberg-type cv. Calmar is the likely source of resistance in cvs. Salinas and Salinas 88 (McCreight et al. 2005). Resistance against *F. oxysporum* race 1 was mapped in a population derived from the (Valmaine × Salinas 88) × Salinas cross (Michelmore et al. 2010). Three resistance QTLs were detected on LG 1, LG 2, and LG 7. Resistance alleles of QTLs on LG 1 and LG 2 originated from the romaine cv. Valmaine, while resistance on LG 7 was conferred by the 'Salinas' alleles. The QTL on LG 2 is linked with the *Tvr1* gene conferring resistance to dieback (Simko et al. 2009, Simko et al. 2010b), however the two resistances do not co-locate absolutely (Michelmore et al. 2010). Resistance to race 2 of *F. oxysporum*

was also mapped to LG 2 (Aruga et al. 2012). The *RRD2* gene was detected in the F_2 populations originating from a cross between two crisphead accessions, a resistant accession VP1013 and a susceptible cultivar Patriot. A SCAR marker developed from RAPD markers closely linked to the *RRD2* gene will allow selection of material resistant to race 2 of the disease (Aruga et al. 2012). The chromosomal location of *RRD2* was not compared to other resistance genes because specific RAPD markers were used in this study.

Anthracnose

Lettuce anthracnose is caused by the fungus *Microdochium panattoniana*. Loss of yield resulting from the disease is usually low, but severe losses can occur when high rainfall creates conditions conducive for disease spread. Infection starts as small, water-soaked spots that rapidly expand and then became chlorotic and necrotic (Ochoa et al. 1987). Masses of pinkish-white spores are visible on lesions in moist conditions. When more than 400 *Lactuca* accessions were tested for their reaction to five races of the pathogen, a single accession of *L. saligna* (UC83US1) was resistant to all isolates. Among *L. sativa* accessions the highest resistance was observed in the leaf-type cv. Salad Bowl, that was resistant to three isolates (Ochoa et al. 1987). Genetic mapping of resistance genes was carried out on the F_2 mapping population derived from a cross between cv. Salad Bowl and the *L. serriola* accession CGN 14263. The population was tested with two isolates of the pathogen (McHale et al. 2009). Accession CGN 14263 was resistant to both isolates (Ant 99-1 and Ant 83-5) of *M. panattoniana*, while cv. Salad Bowl was resistant to only one of them (Ant 83-5). Testing of the mapping population with the Ant 83-5 isolate identified two QTLs located on LG 2 (*ANT2*), and LG 8 (*ANT3*). In both cases the resistance allele originated from *L. serriola*, accounting for about 40% and 30% of the variance, respectively. Only the QTL on LG 2 was significant when the mapping population was screened with the Ant 99-1 isolate of the pathogen. The QTL accounted for 55% of the phenotypic variance (McHale et al. 2009).

Lettuce Drop

Lettuce drop is caused by two species of the *Sclerotinia* fungus, *S. minor* Jagger and *S. sclerotiorum* (Lib.) de Bary. One of the species may predominate in certain areas, but both species may occur in the same field (Subbarao 1997). The mode of infection by the two species differs, but infection with *Sclerotinia* can occur at any stage of plant growth. The symptoms progress from initial wilting of the outermost leaf layers, to the wilting of the entire plant and its collapse, frequently within a few days (Subbarao 1997). Despite extensive germplasm screening, no lettuce accession has been identified as having complete immunity to the infection by the two *Sclerotinia* species. A reduction in disease incidence after inoculation with *S. minor* was detected in several genotypes, including the oilseed lettuce accession PI 251246 (Grube and Ryder 2004). In the F_2 population developed from a cross between PI 251246 and susceptible cv. Salinas, a strong correlation was observed between lower disease incidence and an erect growth habit, early bolting, and the narrow leaf shape typical for PI 251246 (Grube 2004). These correlations indicate a possibility that resistance in PI 251246 may result from a plant morphology that promotes avoidance or escape from infection. Analysis of resistance to lettuce drop that takes into consideration earliness of bolting identified eight accessions with higher resistance than predicted by their rate of bolting (Hayes et al. 2010b). Resistance in cv. Eruption (Latin type) and accession SAL 012 (*L. virosa*) was confirmed by their reaction to inoculation with *Sclerotinia* ascospores, whereas PI 251246 may have only a partial resistance to the pathogen. Resistance in cv. Eruption is independent of the short plant stature, allowing development of resistant romaine cultivars from crosses with this

source of resistance (Hayes et al. 2011c). Mapping of QTLs involved in the resistance to lettuce drop has begun (Hayes et al. 2008b; Michelmore 2010).

Bacterial Leaf Spot

Bacterial leaf spot usually has a minor effect on lettuce crops, but damage might be significant under conducive environmental conditions. The disease, caused by the bacterium *Xanthomonas campestris* pv *vitians,* was reported in the U.S., Europe, Australia, and Asia (Koike and Gilbertson 1997). All types of lettuces are susceptible to this disease. Infected plants show small water-soaked leaf spots on outer leaves that later become necrotic. If infected plants are harvested, secondary decay organisms may colonize spot lesions, leading to significant post-harvest losses (Koike and Gilbertson 1997). Based on disease incidence, two leaf-type cultivars (Grand Rapids and Waldmann's Green) were identified as being the least susceptible (Carisse et al. 2000). Further testing revealed even lower disease incidence and/or disease severity in the Latin-type cv. Little Gem and the Batavia cv. Batavia Reine des Glaces (Bull et al. 2007). A high level of resistance was reported also in cvs. Salad Crisp (iceberg), Iceberg (Batavia), La Brillante (Batavia), and seven iceberg-type breeding lines (Hayes et al. 2008a). The mapping population developed from a cross between cvs. La Brillante and Salinas 88 is being used to map a single large-effect QTL (Hayes and Trent, unpublished results).

Study of the Lettuce Genome

In plant genomes, resistance genes are frequently clustered (Michelmore and Meyers 1998). A typical example of the resistance gene cluster in lettuce was detected on LG 2, which harbors at least seven known *Dm* genes, including *Dm3* (Meyers et al. 1998a), which belongs to the NBS-LRR group of resistance genes (Shen et al. 2002). Post-transcriptional gene silencing (PTGS, RNAi) showed that within the same gene cluster different domains of the resistance gene silence different subsets of resistance specificities. For example, a fragment of the *Dm3* gene LRR domain silenced *Dm16* but not the *Dm6* gene. Conversely, a fragment of the NBS domain of the *Dm3* gene silenced *Dm6*, but not the *Dm16* gene (Wroblewski et al. 2007; Michelmore 2010). Analysis of the lettuce EST database identified more than 700 candidate resistance genes that have sequences similar to the known disease resistance genes (McHale 2008). These candidate resistance genes can be classified into four broad groups: resistance gene candidates (*RGCs*), signaling pathway genes, defense response associated genes, and susceptibility factors (McHale et al. 2009). Significantly more *RGCs* co-localized on the molecular linkage map with the mapped resistance genes than did other categories of candidate resistance genes. The candidate resistance genes that co-localize with resistance phenotypes provide molecular markers that can potentially be used in MAS. In addition to candidate resistance genes, the genetic determinants of reactions to effector molecules from bacterial plant pathogens were mapped on LG 1, LG 8, and LG 9 (McHale et al. 2009). The genomic locations of determinants provide potential targets of pathogen effector proteins.

The reference molecular linkage map of lettuce was developed using RILs (recombinant inbred lines) from the interspecies mapping population of *L. sativa* cv. Salinas × *L. serriola* accession UC96US23 (Truco et al. 2007). This map was originally constructed from more than 700 AFLP and SSR markers and was later saturated with approximately 14,000 loci to an ultrahigh density genetic map (Truco et al. 2013). The 14,000 loci are based on transcribed sequences from the microarray chip (Michelmore 2010; Michelmore et al. 2010; Stoffel et al. 2012). The chip contains sequences for detecting polymorphism in approximately 35,000 lettuce unigenes and facilitates high-throughput,

massively parallel genotyping, linkage mapping, and gene expression analysis (Stoffel et al. 2012). Recently, the whole genome of cv. Salinas has been sequenced and assembled into scaffolds (Reynes-Chin-Wo et al. 2012). Approximately 45,000 gene models have been predicted in scaffolds (Michelmore et al. 2010; Reynes-Chin-Wo et al. 2012) that are being assigned to the reference molecular map using genotyping-by-sequencing (GBS) (Elshire et al. 2011) of RILs from the mapping population. The lettuce genome sequence was released publically in 2012. The most comprehensive and recent information regarding the lettuce genome project is available at the Compositae Genome Project (CGP) website (http://cgpdb.ucdavis.edu). The Salinas 88 × La Brillante mapping population was recently used to test the sequence-based genotyping (SBG) approach, a technology for simultaneous marker discovery and co-dominant scoring (Truong et al. 2012). More than 1,000 SNP markers for lettuce were developed with SBG and used to construct a de novo linkage map.

Association mapping technique allows the linking of plant resistance phenotypes to genotypes through analysis of biodiversity in a wide gene pool (Simko 2004). In lettuce, only the *Tvr1* gene was mapped through association mapping (Simko et al. 2009, Simko et al. 2010b). However, the test of association in this study was limited to only a few molecular markers from the specific genomic region. The availability of an ultrahigh-density molecular linkage map combined with high-throughput genotyping will have a substantial effect on mapping and tagging lettuce resistance genes. It will allow genome-wide association studies (GWAS) (Atwell et al. 2010) to detect genetic variations associated with a particular disease. The sequenced, assembled, and mapped transcriptome of lettuce will be used to dissect the signaling pathways controlling the plant–pathogen interaction. Because GWAS requires a genomic map with marker density higher than is the extent of linkage disequilibrium (LD) (Brachi et al. 2011), it will be necessary to estimate the extent of LD in the lettuce genome. Analysis of the *Tvr1* genomic region indicates extensive LD (Simko et al. 2009). However, this genomic region may not be representative of the overall LD in the lettuce genome, because considerable variation in the extent of LD exists within an individual genome (Tenaillon et al. 2001). Moreover, the extent of LD is highly dependent on the population in which it is measured (Rafalski and Morgante 2004). In cultivated lettuce, the population structure is profound and generally in good agreement with horticultural types (Simko and Hu 2008; Simko 2009). It was observed that the decay of LD within crisphead cultivars is slower than the LD decay within a group of romaine cultivars (Simko et al. 2009).

MAS is used in both public and private lettuce-breeding sectors for selecting material with a desirable combination of resistance genes. Although MAS has been used for only four diseases (downy mildew, corky root, LMV, and dieback), current progress in the analysis of the lettuce genome indicates that development of assays for MAS will accelerate in the near future. Besides using MAS for disease resistance, assays already exist or are in development for selecting material with resistance to lettuce aphid (*Nasonovia ribisnigri* Mosley), late bolting, male sterility, and slow deterioration of minimally processed salad. Use of MAS for polygenic traits remains problematic. To capture the effects of polygenic traits, a selection based on a large number of molecular markers distributed throughout the whole genome was proposed. This selection method was termed genomic selection (GS) (Meuwissen et al. 2001). Simulation (Bernardo and Yu 2007) and empirical (Heffner et al. 2011a) studies revealed that accuracies of the prediction of phenotypes using GS were significantly greater than with MAS. The accuracy of the GS was tested in both multi-family prediction models (Heffner et al. 2011b) and bi-parental plant populations (Lorenzana and Bernardo 2009). Use of GS in lettuce breeding has not yet been reported.

Appendix: Assays for Marker-Assisted Selection

Three high-resolution DNA melting (HRM) assays were developed at the USDA-ARS in Salinas, California, for detecting alleles associated with resistance to corky root, lettuce dieback, and LMV (Figure 14.3). All assays were developed for a LightScanner system (96-well tray) and analysis of melting profiles with LightScanner software v. 2.0.0.1331 (both from Idaho Technology, Salt Lake City, UT).

Corky Root

The assay was developed to distinguish alleles of the *SCO07* marker. This marker is linked to the *cor* gene located at LG 3 (Moreno-Vázquez et al. 2003). The *SCO07* marker sequence from resistant cv. Green Lake (AY207423) and susceptible cv. Diana (AY207424) were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov). The 480bp-long fragment from cv. Green Lake differs from the 481bp-long fragment from cv.

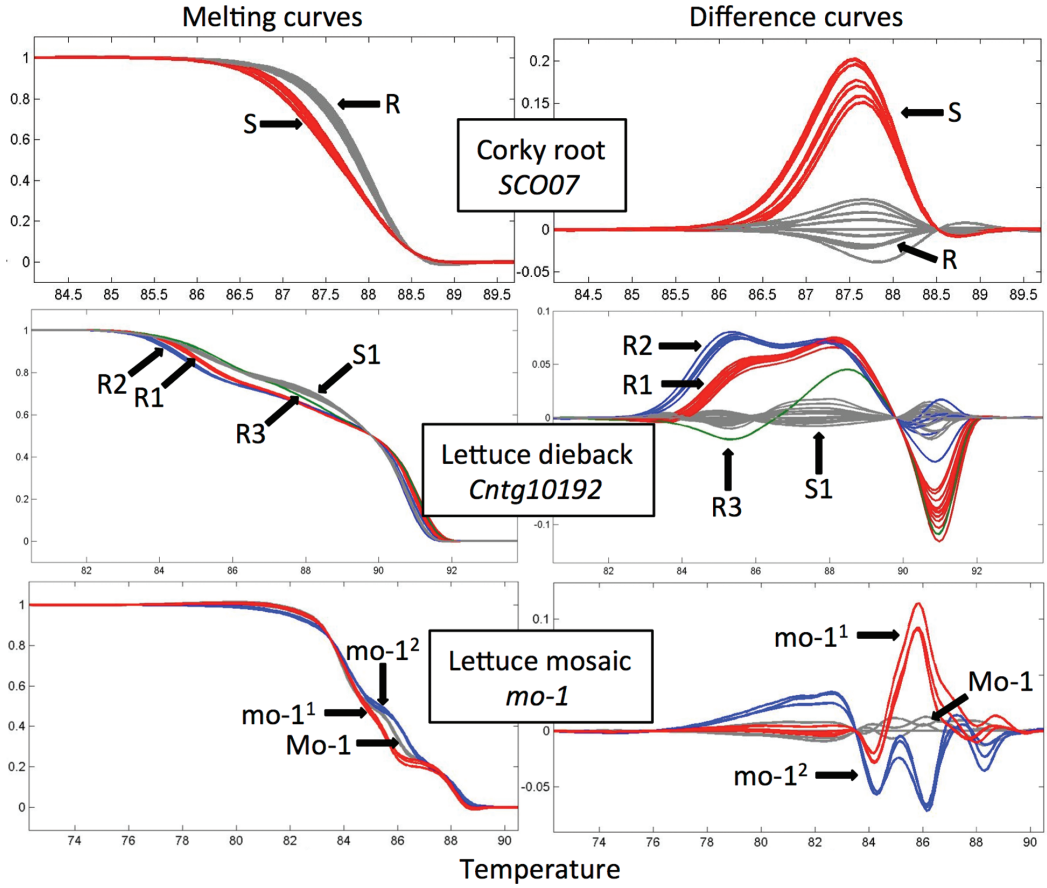

Fig. 14.3. Examples of MAS with high-resolution DNA melting (HRM) assays. Top row – the *SCO07* marker linked to the *cor* gene for resistance to corky root (R – allele linked to resistance; S – allele linked to susceptibility). Middle row – the *Cntg10192* marker linked to the *Tvr1* gene for resistance to lettuce dieback (R1 – allele linked to the 'Salinas' haplotype; R2 – allele linked to the 'PI 491224' haplotype; R3 – allele linked to the 'UC96US23' haplotype; and S1 – allele linked to the susceptible haplotype). Bottom row – alleles of the *mo-1* gene for resistance to lettuce mosaic (*Mo-1* is the susceptible allele, *mo-1*^{1} and *mo-1*^{2} are resistant alleles). The *mo-1* assay is based on an unlabeled probe. In each assay only melting curves of homozygous genotypes are shown, however heterozygous genotypes were also identified by HRM. For a color version of this figure, please refer to the color plate.

Diana in eight SNPs and a single nucleotide deletion. The HRM assay that differentiates between alleles associated with resistance and susceptibility is based on the 397bp-long region encompassing seven of the SNPs (Figure 14.4).

PCR amplification was performed in a 10 μl reaction volume containing 10 ng of genomic DNA as a template, 1× PCR buffer, 0.75 U *Taq* polymerase, 0.125 mM of each dNTP (all from New England Biolabs, Ipswich, MA), 1× LCGreen Plus Melting Dye (Idaho Technology, Salt Lake City, UT), and 0.25 μM of each primer (AY207423b-F: 5'- TCC TGA ATT CCC AAA ATA CCC -3', and AY207423b-R: 5'- TAG CCC ATT TAA TGC CAT GC -3'). The reaction was filled to the final volume with nuclease-free water; 15 μl of mineral oil was added to prevent evaporation during HRM analysis. PCR was performed with an initial denaturation of 95° C for 2 min, followed by 45 cycles of 95° C for 30 s, 66° C for 30 s, and 72° C for 30 s, with final extension of 72° C for 5 min. To facilitate heteroduplex formation, samples were heated to 95° C for 30 s, followed by cooling to 25° C for 30s (this step can be omitted if only homoduplexes are analyzed). Figure 14.3 (top row) shows different melting profiles of the two alleles.

Lettuce Dieback

The *Cntg10192* marker locus on LG 2 is linked to the *Tvr1* gene that provides complete resistance against lettuce dieback (Simko et al. 2009; Simko et al. 2010a; Simko et al. 2010b). This locus was sequenced from 73 accessions of four *Lactuca* species (*L. sativa*, *L. serriola*, *L. saligna*, and *L. virosa*) and the sequences were deposited into the NCBI database under numbers GQ341366 to GQ341438. The 349bp-long fragment contains 11 SNPs that differentiate six haplotypes (Simko et al. 2010b). The HRM assay is based on the 185bp-long fragment that encompasses three of the SNPs and can be used to distinguish four haplotypes identified in *L. sativa* and *L. serriola* (Simko et al. 2009, Simko et al. 2010b) (Figure 14.4). One of the haplotypes is always associated with susceptibility to the disease, while three haplotypes are associated with disease resistance. The three resistant haplotypes were termed R1 (from cv. Salinas), R2 (from accession PI 491224), and R3 (from *L. serriola* accession UC96US23).

PCR amplification was performed according to published protocol (Simko et al. 2009). A 10 μl reaction volume contained 10 ng of genomic DNA as a template, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.6 U *Taq* polymerase, 0.2 mM of each dNTP (all from New England Biolabs, Ipswich, MA), 1× LCGreen Plus Melting Dye (Idaho Technology, Salt Lake City, UT), and 0.25 μM of each primer (Cntg10192-F: 5'- CTC GTT TTC AAC ACC GAC AA -3', and Cntg10192-R: 5'- TAG GTG GGT CCG ACT TTG AG -3'). The reaction was filled to the final volume with nuclease-free water; 15 μl of mineral oil was added to prevent evaporation during HRM analysis. PCR was performed with an initial denaturation of 95 °C for 2 min, followed by 45 cycles of 95° C for 30 s, 61° C for 30 s, and 72° C for 30 s, with final extension of 72° C for 5 min. To facilitate heteroduplex formation, samples were heated to 95° C for 30 s, followed by cooling to 25° C for 30s (this step can be omitted if only homoduplexes are analyzed). Different melting profiles of the four haplotypes are shown in Figure 14.3 (middle row).

Lettuce Mosaic

The HRM assay was developed to distinguish two resistant (*mo-1*^{1} and *mo-1*^{2}) and one susceptible (*Mo-1*) allele of the *mo-1* resistance gene located on LG 4 (Nicaise et al. 2003). The resistant alleles were first identified in cv. Gallega de Invierno (von der Pahlen and Crnko 1965) and accession PI 251245 (Ryder 1970), respectively. The full-length sequence from susceptible cv. Salinas (NCBI database number: AF530162) is 1,032bp long (Nicaise et al. 2003). The *mo-1*^{2} allele differs from *Mo-1* in two SNPs, while *mo-1*^{1} differs from *Mo-1* in two different SNPs and one 6bp-long deletion. The HRM

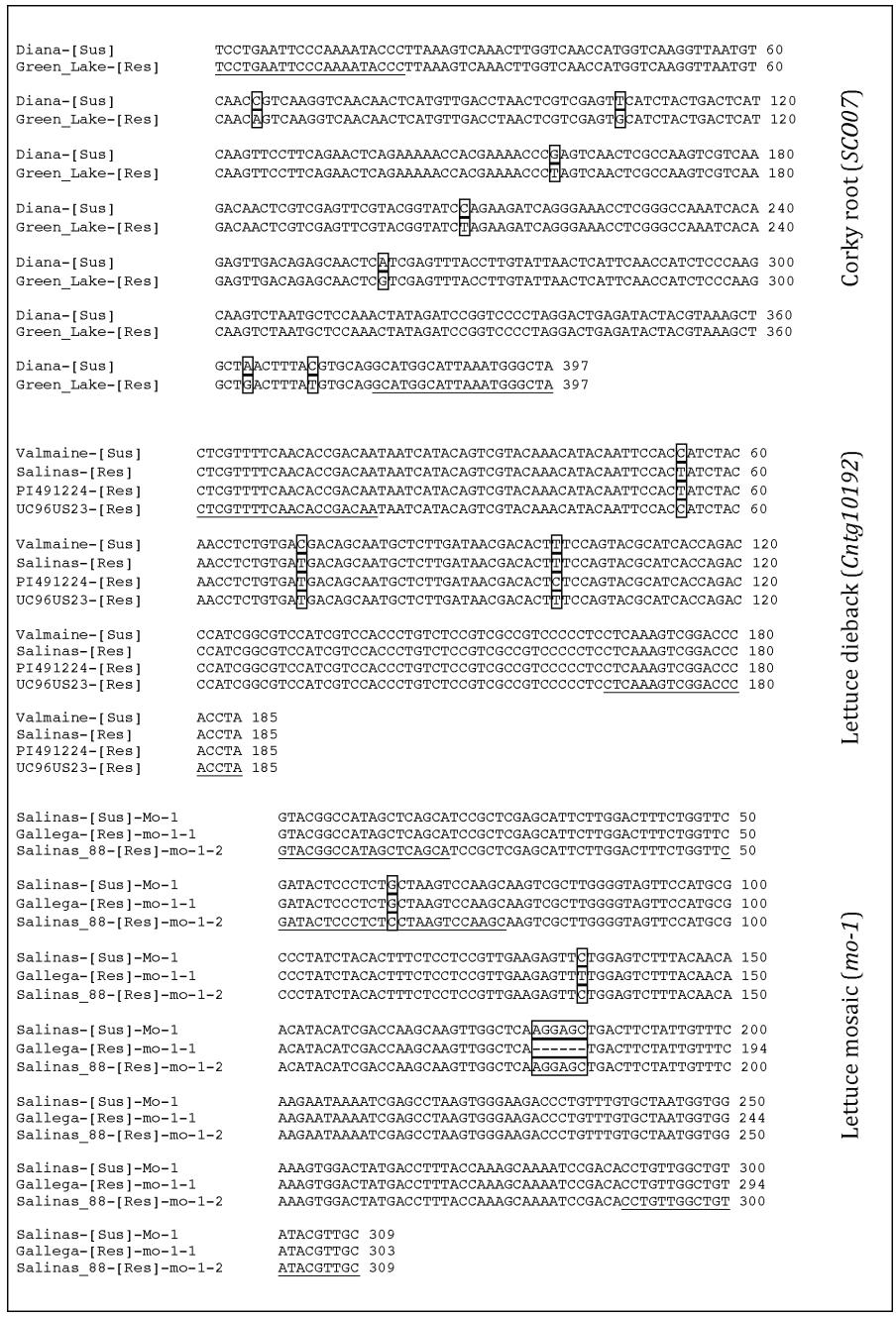

Fig. 14.4. Sequence of markers used for high-resolution DNA melting (HRM) assay. Sequence data are shown for alleles of the *SCO07* marker linked to the corky root resistance gene *cor*; the haplotypes of the *Cntg10192* marker that is linked to the dieback resistance gene *Tvr1*; and the alleles of the *mo-1* gene for resistance to lettuce mosaic. Origin of alleles (cultivar name or plant introduction number) and their association with disease resistance (Res) or susceptibility (Sus) is indicated before sequence data. Positions of the primers and the probe used in HRM assays are underlined; SNPs and deletions present in the amplicons are boxed.

assay to distinguish the three alleles is based on the 309bp-long fragment that encompasses one SNP for the *mo-1*^{2} allele, and one SNP and the deletion for the *mo-1*^{1} allele (Figure 14.4). To increase resolution, this assay uses an unlabeled probe that overlaps the single SNP differentiating *Mo-1* and *mo-1*^{2} alleles.

Asymmetric PCR amplification was performed in a 10 µl reaction volume containing 10 ng of genomic DNA as a template, 1× PCR buffer, 1 mM $MgCl_2$, 0.75 U *Taq* polymerase, 0.2 mM of each dNTP (all from New England Biolabs, Ipswich, MA), 1× LCGreen Plus Melting Dye (Idaho Technology, Salt Lake City, UT), 0.1 µM of forward primer (Mo-1-F: 5'- GTA CGG CCA TAG CTC AGC A -3'), 0.5 µM of reverse primer (Mo-1-R: 5'- GCA ACG TAT ACA GCC AAC AGG -3'), and 0.5 µM of probe (Mo-1-probe: 5'- CGA TAC TCC CTC TCC TAA GTC CAA GC /3SpC3/ -3' with a carbon spacer at the 3' end to block extension on that end). The reaction was filled to the final volume with nuclease-free water; 15 µl of mineral oil was added to prevent evaporation during HRM analysis. PCR was performed with an initial denaturation of 95° C for 2 min, followed by 55 cycles of 95° C for 30 s, 63° C for 30 s, and 72° C for 30 s, with final extension of 72° C for 5 min. To facilitate heteroduplex formation, samples were heated to 95° C for 30 s, followed by cooling to 25° C for 30s. Melting profiles of the three alleles are shown in Figure 14.3 (bottom row).

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Chapter 15

Marker-Assisted Breeding for Cassava Mosaic Disease Resistance

E. Okogbenin, I. Moreno, J. Tomkins, C. M. Fauquet, G. Mkamilo, and M. Fregene

Abstract

Cassava, grown primarily for its starchy tuberous root, is the third most important source of calories in the tropics. Cassava mosaic disease (CMD), caused by cassava mosaic geminiviruses (CMGs), is the most important viral disease and a major constraint for cassava production in Africa and in India. The emergence of new viral diseases and the increasing evolutionary capacity of the viruses and their vectors to adapt and remain highly virulent is a huge challenge to breeding programs. Molecular markers are valuable tools for understanding the genes and genomes underlying natural variation. Three sources of CMD resistance have been tagged with the aid of markers (*CMD1*, *CMD2,* and *CMD3*). They have now been deployed via marker-assisted breeding (MAB), with *CMD2* being the most widely used in breeding recently. MAB for CMD resistance has been used for breeding in the absence of the pathogen in the Neotropics, broadening the germplasm base in Africa, exploring heterosis, transfer of novel traits, and pyramiding of resistance genes, as well as germplasm screening for parent selection in CMD-resistance breeding in Asia. Initiatives involving BAC (bacterial artificial chromosome) sequencing for cloning of the *CMD2* gene have also been initiated. The ex ante impact assessment of MAB-developed varieties combining CMD resistance with other key farmer-desired traits is estimated to be between US$2.89 and US$4.3 billion in Africa alone. Recent genome sequencing projects in cassava are expected to enhance understanding of the genetic bases of CMD resistance and allow for their effective deployment toward development of superior CMD-resistant varieties. This chapter highlights the advances and challenges of marker-assisted breeding in the last decade, since the discovery of genetic markers for CMD beginning in 2002, and the future prospects for revolutionizing cassava breeding by combining advanced molecular technologies, high throughput platforms, and innovative breeding strategies.

Introduction

Cassava (*Manihot esculenta* Crantz, Family Euphorbiaceae), which originates in Latin America, is a major source of food for more than 800 million people, mostly in sub-Saharan Africa, Asia, the Pacific, and South America. It is the third most important source of calories in the

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tropics (FAO 2010) and sixth most important crop, in terms of global annual production (FAO 2010). It is cultivated on more than 20 million hectares, with a total production of 241 million tonnes (FAO 2009), with 50% of total production in Africa, 30% in Asia, and 20% in Latin America. Under favorable experimental conditions, cassava as a monocrop can yield as much as 80 tons of fresh roots per hectare. However it is usually grown in adverse marginal environments of poor soils and harsh climates (Ekanayake and Lyasse 2003), and under such conditions average yields in tons of fresh roots per hectare are much lower: 12.4 tons per hectare worldwide; 10.2 tons in Africa; 12.5 in Latin America; and 17.3 in Asia.

Cassava is grown primarily for its starchy tuberous roots, which consist of 20 to 40% dry matter. The root contains mostly carbohydrates, but it is also rich in vitamin C, carotenes, calcium, and potassium, although poor in protein. However, cassava leaves, consumed in some parts of the world as a vegetable, contain high levels of protein, in addition to being rich in vitamins and minerals. Although cassava is mostly valued for its role as a food security crop in sub-Saharan Africa, it is gradually gaining importance as a cash crop on the African continent, and it is grown as an industrial crop in Thailand, China, India, and Brazil. The cassava roots are processed into a diverse spectrum of products including starch, flour, animal feed, and potentially biofuel, all of which are derived from a crop with the ability to grow in poor soils (Thresh 2006).

The inherent potential of cassava to grow in marginal environments, the recent success in identifying high-value traits, and the incorporation of new tools for genetic enhancement offer bright prospects for the crop and the people who depend on it (Ceballos et al. 2010). But it suffers from a plethora of pests and disease, including at least 20 different viral diseases to which it is highly vulnerable. The cassava mosaic disease (CMD), caused by cassava mosaic geminiviruses (CMGs) (Family *Geminiviridae*: Genus *Begomovirus*), transmitted by the whitefly, *Bemisia tabaci*, is the most important viral disease and a major constraint for cassava production in Africa and in India (Legg and Fauquet 2004; Legg et al. 2006). Currently, CMD is managed mainly through the use of conventional resistance breeding. Significant efforts, however, have been made to supplement this basic approach with alternatives, including vector management, cross-protection (to help to minimize transmission and symptom development), marker-assisted selection, and genetic transformation (Legg and Fauquet 2004). Although management practices are useful, the viruses' high rate of recombination and co-infection capabilities have caused CMD to be one of the most detrimental diseases affecting food supply in Africa (Patil and Fauquet 2009).

Breeding in cassava with the goals of yield increases, root quality improvement, and disease resistance has been slowed considerably by the biological characteristics of the crop, which include a long growth cycle, a heterozygous genetic background, and a crop diversity organization that is as yet poorly understood by scientists. These factors severely hamper the speed and ease of effective deployment of useful genes in cassava. The consequences are that cassava production fails to keep up with demand, especially in regions where more than 90% of yield is consumed as food, as in Africa, leading to an increase in acreage of cassava fields, mostly expanding into marginal lands. Molecular markers, genome studies, and plant genetic transformation have provided ways around breeding obstacles in long growth-cycle and heterozygous crops. In the 1990s, genomics studies in cassava were initiated, leading to the development of markers and the development of the first genetic map in cassava (Fregene et al. 1997), followed by gene-tagging studies. A number of these tools – including molecular genetic maps, markers linked to disease-resistance genes, and marker-aided studies of complex traits – now exist.

In this chapter, we consider the impact of innovative approaches to marker-assisted

breeding in the genetic improvement of cassava for resistance to CMD and its huge impact in shortening the breeding cycle, achieved through a fast-track breeding scheme hastening the release of novel cassava cultivars for increased production and commercialization of cassava. Marker-assisted breeding encompasses different breeding strategies, such as marker-assisted selection (MAS) – the selection of specific alleles at a few loci; marker-assisted breeding backcross (MABC) – the transfer of a limited number of loci from one genetic background to another; marker assisted recurrent selection (MARS) – the identification and selection of several genomic regions involved in the expression of complex traits in order to assemble the best performing genotype within a single or across related populations (Ribaut et al. 2010); and genome-wide selection (GWS) – selection based on markers without significance testing and without identifying a prior subset of markers associated with the traits (i.e., without quantitative trait locus (QTL) mapping) (Bernardo 2007).

In this chapter we report on the significant advances made in the last decade in the integration of molecular markers for CMD-resistance breeding. The challenges of combining CMD resistance with other key traits in marker-assisted breeding in a heterozygous genetic background are also highlighted. Also covered in this chapter are the future prospects and opportunities for cassava breeding based on access to advanced molecular technologies, high throughput platforms, and innovative paradigms.

Cassava Breeding and Genetics

Plant breeding has one of the highest rates of return among investments in agricultural research. Cassava has benefitted from breeding (Kawano 2003), resulting in the development of new cultivars in Africa, Asia, Latin America, and the Caribbean, bringing US$440 million in the late 1990s, with immense benefit to rural households. Recent estimates indicate that in Thailand and Vietnam, adoption of improved varieties has reached 90%, generating gains worth US$12 billion in the last two decades (CGIAR 2011). New technologies in the area of tissue culture and molecular biology have further strengthened this, resulting in more positive contributions (Schopke et al. 1996; Fregene et al. 1997, Fregene et al. 2000; De Vries and Toenniessen 2001; Taylor et al. 2001; Taylor et al. 2011).

Cassava is allogamous and most cultivars are therefore highly heterozygous. Cassava genetic improvement starts with the production of new recombinant genotypes derived from selected elite clones. Hybridization of select parents is necessary to create source populations with high genetic variability. With large separate male and female flowers, which do not open at the same time, cassava flowers are very easy to hand-pollinate. One successful pollination yields a maximum of three seeds – a very low yield compared to cereal crops – but this is seldom obtained, and in most situations one seed per pollinated flower is a good average. Seeds mature 2.5-3 months after pollination. The success rate of hand-crossing seems to vary widely. Fukuda (1980) reports that 80% of pollinated flowers set fruit. The breeding scheme often involves elaborate evaluation stages, which require a longer time for output delivery (Figure 15.1).

Genetic improvement of clonally propagated non-inbred crops such as cassava is helped by the fact that a superior genotype can be fixed at any stage in the breeding scheme. Nonadditive gene actions, including dominance and epistasis, are important components of the genetic variance, which can be manipulated by the breeder (Jaramillo et al. 2005; Calle et al. 2005; Perez et al. 2005). The multiplication rate of cassava planting materials is low, as five to ten cuttings can be obtained from one plant (Blair et al. 2007). A typical selection scheme cycle in cassava begins with the crossing of elite clones and finishes when the few clones surviving the selection process reach the stage of regional trials across several locations (Figure 15.1). As the number of plants representing each genotype increases, the weight of selection criteria

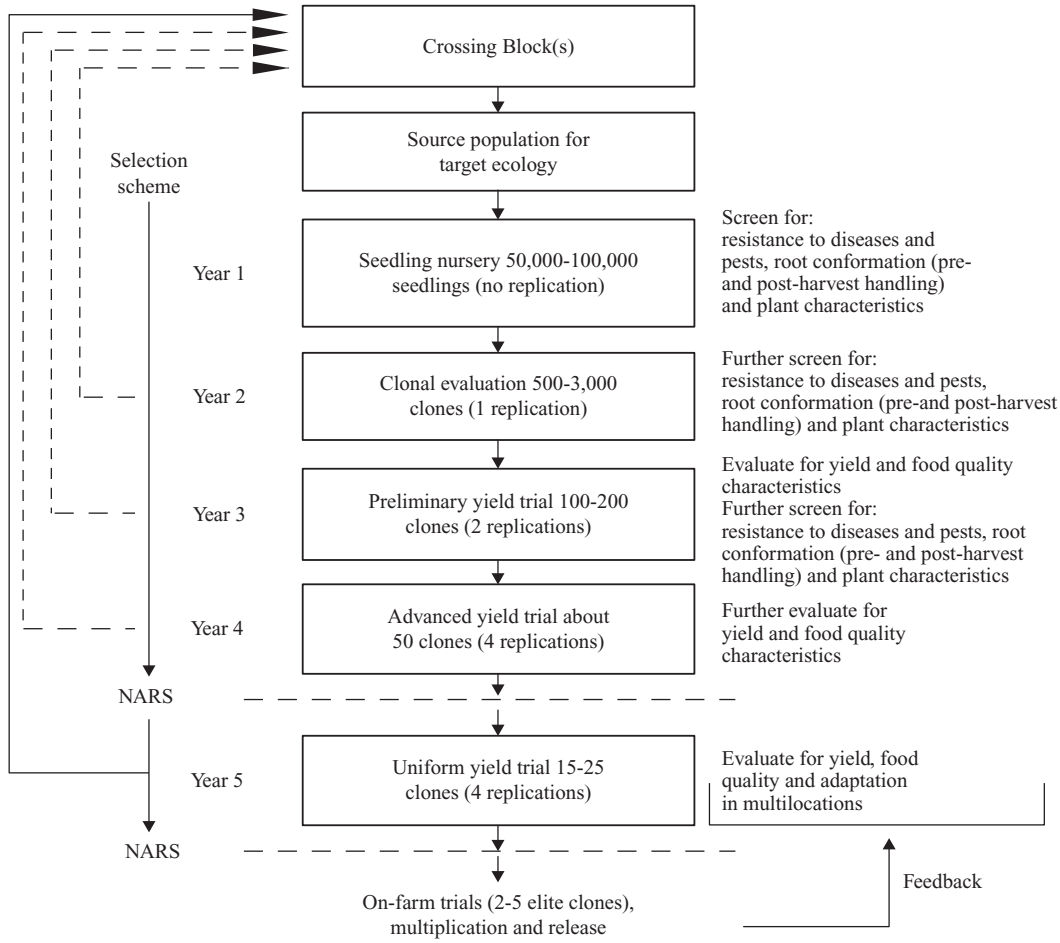

Fig. 15.1. Cassava breeding scheme at the International Institute of Tropical Agriculture, IITA used mainly in African national breeding programs.

shifts to low heritability traits such as root yield. The clones that show outstanding performance in the regional trials are released as new cultivars, and eventually incorporated as parents in the crossing nurseries. The long selection cycle that results in the release of few cultivars implies that the adaptation of molecular tools for cassava genetic improvement is required to make the process more efficient in order to reduce the long process involved in classical breeding.

Breeding objectives depend on the ultimate use of the crop. Genetic variability is limited in *M. esculenta* and, therefore, interspecific crosses with other *Manihot* species to introgress useful alleles have often been attempted. Stability of production is associated with resistance or tolerance to major biotic stresses, and the emphases vary with the target environments. Cassava is normally propagated vegetatively, with stem cuttings. This process easily disposes the plant to pests and disease, as planting materials are reused in the subsequent growing season, leading to higher inoculum pressure, especially in susceptible cultivars, which have the tendency to accentuate the rapid spread of diseases. In Africa and India the key biotic constraint is CMD. From the early years of research into CMD, it was apparent to workers that cultivars varied in their

response to the disease. Resistant cultivars are much less readily infected than are susceptible cultivars. While in resistant cultivars the localized distribution of the virus and overall concentrations tend to be low, they are nevertheless a potent source of inoculum from which spread can occur (Fargette et al. 1988).

The earliest resistance-breeding programs, initiated almost simultaneously in the 1930s in Madagascar and at the Amani station in northeast Tanzania, used both intraspecific and interspecific crosses with a cultivar of cassava wild species to produce progeny with increased levels of CMD resistance. Most success was achieved with the interspecific *Manihot glaziovii* Muell. Arg. crosses, and the Amani group then used a series of backcrosses to restore root quality whilst retaining resistance. High levels of resistance were obtained, and the program was terminated in the late 1950s, but seeds from one of the most resistant clones, 5318/34, was used to reinitiate the work at the International Institute of Tropical Agriculture, IITA, Nigeria, beginning in 1970. One of the clones derived from this seed, designated 58308, had a good combination of CMD resistance and root quality and formed the basis for much of the resistance breeding work that followed at IITA (Hahn et al. 1980b). Some of the most important clones from the Tropical *Manihot* Species (TMS) series that resulted from this work have been widely distributed across the continent, with sizeable production of the varieties in some countries, including Nigeria, Uganda, and Ghana (FAO 2005).

Benefits of Molecular Markers for Genetic Improvement

The pressure to meet the food and industrial requirements of the world's population has necessitated the need to explore and develop new tools to complement conventional breeding practices for efficient development and rapid delivery of superior cultivars to stakeholders. Molecular markers are tools that could easily be amenable to and integrated into breeding programs to rapidly increase the precision of selection and reduce the breeding cycle required for the development of improved cultivars for the dynamic demands of trade.

The pace of development of molecular markers has been phenomenal in recent years. Markers are very useful and available to breeders for gene discovery and crop improvement via MAS. With conventional approaches, genetic improvement can be expected to increase over time, but it could be slow and up to a defined limit. However, with molecular markers, results can be achieved much sooner, and obstacles to success can be overcome by the introduction of novel genes and through the combination of more positive alleles.

The increased potential for breeders to use genetic rather than phenotypic selection in their programs means that many of the previous approaches may be effectively redesigned. The ways in which the new breeding tools will directly alter returns from the program are: (1) enhanced productivity, through novel genes and traits, including the pyramiding of existing genes more rapidly; (2) improved cultivars, resulting from more thorough testing before commercial release; and (3) accelerated breeding, by shortening the breeding cycle, and genes that promote the development of new products or provide new options for producers. Desirable breeding technologies are those that allow a significant change in the number of tests that can be carried out in a given stage of the program, permit the evaluation of traits(s) that was/were not previously possible, or provide information on lines at an earlier stage of the program. In some cases, this can simply mean an increase in the number of lines that can be assessed in the early stages because of the improved selection efficiency for the late stages of the program (Bennan and Martin 2007). Such tools will be highly beneficial to CMD-resistance breeding, which under normal conventional breeding schemes, requires active intensive screening and selection for stable and durable CMD resistance at the early stages, after which, at about the third year of the scheme, the

evaluation of complex agronomic traits such as yield then begins (Figure 15.1).

Cassava Mosaic Disease

CMD is the most important disease threatening cassava production in Africa. CMGs have been reported from many cassava-growing countries in Africa and India and the CMD induced by them constitutes a formidable threat to cassava production (Legg and Fauquet 2004; Patil and Fauquet 2009). The CMD begomoviruses are unknown in the Americas, the center of diversity for cassava.

Geminiviruses are a large family of plant viruses with circular, single-stranded DNA (ssDNA) genomes packaged within geminate particles. Members of the genus *Begomovirus* have caused significant yield losses in many crops worldwide (Varma and Malathi 2003). The genome of CMGs consists of two DNA molecules, DNA-A and DNA-B, each of about 2.8 kbp (Stanley et al. 2004), which are coding for different proteins responsible for different functions in the infection process.

Nine species of CMGs have been identified between Africa and South Asia based on their genomic sequence and phylogenetic analysis. They include representatives of seven African and two South Asian species, namely *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), and *South African cassava mosaic virus* (SACMV), all from Africa, as well as the *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) in Asia (Fauquet et al. 2008; Patil and Fauquet 2009). This number will probably grow, resulting from a high rate of natural recombination between geminiviruses and a high transmission rate of whitefly vectors (Patil and Fauquet 2009).

There is a high level of molecular diversity in the CMGs and evidence that certain CMGs, when present in mixtures, employ pseudo-recombination or reassortment strategies and recombination at certain hot spots, such as the origin of replication (Stanley 1995; Deng et al. 1997; Zhou et al. 1997; Fondong et al. 2000; Pita et al. 2001), resulting in the emergence of 'new' viruses with altered virulence. Such examples are EACMV-UG2, an ACMV-EACMV recombinant component A, as well as EACMV-UG3, which is a pseudo-recombinant component B, (Pita et al. 2001). These recombinant viruses combine parts of the genomes of both ACMV and EACMV and were implicated in the pandemic of severe CMD that devastated cassava in much of East and Central Africa (Legg and Fauquet 2004).

The severity of CMD is impacted by human and environmental factors, as it is transmitted through cuttings by the farmers and through the natural whitefly vectors. If the transmission through cuttings has been the prevalent mode of transmission for a long time (Fauquet and Fargette 1990), recently it has been observed that transmission through whiteflies played a major role in the recent pandemic in East Africa (Legg and Fauquet 2004; Patil and Fauquet 2009). Given that CMGs are transmitted by whiteflies, the spread of the virus is going to depend largely on the vector. Temperature is the most important environmental factor controlling the size of the vector population (Fauquet and Fargette 1990). Vector preferred temperature estimates vary from 20 to 30° C (Fargette et al. 1994) to 27 to 32° C (Thurston 1998), but generally high temperatures are associated with high fecundity, rapid development, and greater longevity in whitefly (Fargette et al. 1994). Increased light intensity has also been shown to increase activity of the whitefly vector (Thurston 1998). High rainfall – more than 1200 mm –favors more spread than in locations that are drier and have a shorter growing season and where less cassava is grown (Bock 1983; Fargette et al. 1994). CMD is generally more virulent in hotter and more humid regions, while the incidence of

Fig. 15.2. (Left) Cassava plant infected with African cassava mosaic virus (ACMV), in Ghana, showing mosaic, chlorosis, and distorted leaves. (Right) Cassava plant infected with ACMV and East African cassava mosaic Cameroon virus (EACMCV), in Ghana, showing a synergistic effect of the two viruses with extreme "candle stick" symptoms. For a color version of this figure, please refer to the color plate.

infection diminishes with altitude. Disease is much less prevalent in altitudes greater than 1000 m above sea level (Storey 1936; Cours-Darne 1968). Virus incidence increases where cassava is grown intensively (Fargette et al. 1994), and thus plant density impacts the spread of the virus, with low density fields encouraging faster disease propagation than high density fields (Fauquet and Fargette 1990). High CMD infection leads to high chlorosis and distorted leaf patterns that severely affect plant growth (Figure 15.2)

CMD Epidemic

The first report of cassava mosaic disease (CMD) was from East Africa in 1894 (Warburg 1894; Zimmerman 1906). Since then, epidemics have occurred throughout the African continent, resulting in great economic loss and devastating famine (Legg and Fauquet 2004). The first reported epidemics occurred in many parts of Africa in the 1920s, followed by further epidemics in Uganda and Madagascar in the 1930s and 1940s (Cours 1951). Records associated with the "first encounter" epidemics were from Sierra Leone, Ivory Coast, Ghana, Nigeria, Madagascar, and Uganda. It was apparent that by the 1930s, CMD had spread to virtually all cassava-growing environments of the African mainlands and its islands (Cours 1951). In second encounter epidemics, the detailed description of symptoms provided by Cours (1951) highlighted the intense chlorosis, reduction in leaf size, and stunted architecture of plants infected by the severe CMD associated with the epidemic. There were no further reports of epidemics in East Africa until the 1990s, when severe CMD epidemic resurged in Uganda (Gibson et al., 1996; Otim-Nape et al. 1997). However, we have brief descriptions of epidemics in Cameroon (Fondong et al. 2000), Ghana (Fauquet pers. com.), Ivory Coast (Pita et al. 2001), Nigeria, and the Cape Verde Islands (Calvert and Thresh 2002), but none of these

situations seems to have developed beyond the local level.

The epidemics of severe CMD that occurred in the 1990s in Uganda (Gibson et al. 1996; Otim-Nape et al. 1997) spread subsequently to Kenya and Tanzania by the late 1990s (Legg and Thresh 2000). The epidemic of severe CMD in Uganda devastated the country's cassava production, causing losses valued in excess of US$60 million annually between 1992 and 1997 (Otim-Nape 1993; Thresh et al. 1994; Otim-Nape et al. 1997; Legg 1999). Farmers literally abandoned the crop in large parts of the country, and in eastern districts widespread food shortages led to some famine-related deaths (Thresh and Otim-Nape 1994). During the second half of the 1990s, the epidemic spread to the neighboring countries of Sudan and eastern Democratic Republic of Congo (DRC), with a similar impact on cassava cultivation (Legg 1999). Key characteristics of what was by this stage known as the CMD pandemic were high incidences of severe CMD (Gibson et al. 1996), association with a synergistic interaction between ACMV and EACMV (Harrison et al. 1997b; Pita et al. 2001), presence of an EACMV recombinant strain (Deng et al. 1997; Zhou et al. 1997), rapid vector-borne spread (Otim-Nape et al. 1997), and superabundant *B. tabaci* populations (Legg and Ogwal 1998). Although it is not possible to attribute a single causal element for this pandemic, it is possible that the conjunction of these different elements worked together to promote the viral pandemic (Legg and Fauquet 2004).

The first deployed resistance came from *M. glaziovii,* a source that was used to develop cultivars, providing effective control of the disease for many years. However, the emergence in Uganda in the late twentieth century of a new and highly virulent strain further threatened cassava production in that country and spread quickly to other parts of East and Central Africa (Legg and Fauquet 2004). First it was discovered that the pandemic was associated with a novel recombinant virus, the EACMV Uganda strain (EACMV-UG2) (Patil and Fauquet 2009). This novel geminivirus strain has been found to be associated with the CMD pandemic in Uganda and Kenya (Harrison et al. 1997a; Harrison et al. 1997b; Deng et al. 1997; Zhou et al. 1997). EACMV-UG2 was identified as the dominant virus in pandemic-affected areas of Kenya and Tanzania (Legg 1999), Rwanda (Legg et al. 2001), and Burundi (Bigirimana et al. 2003). The EACMV-UG2 is reported as expanding further in Rwanda-Burundi and in the entire Congo basin, extending up to Gabon and Cameroon (Neuenschwander et al. 2002; Legg et al. 2006). The virus has also been found in what is now South Sudan (Harrison et al. 1997a). EACMV-UG2 has the serological properties of ACMV but from nucleotide sequencing data, it has been shown to have most of the features of the EACMV genome. Sequence analyses confirmed the presence of a recombinant fragment in the coat protein gene, a feature later found to be common for begomoviruses (Padidiam et al. 1999).

Synergism between cassava mosaic geminiviruses – that is, mixed ACMV and EACMV infections – have also been implicated in the widespread epidemic reported in Uganda and subsequently in neighboring countries (Harrison et al. 1997b; Legg 1999; Pita et al. 2001). Studies of the relationship between CMGs and the pandemic, with the specific primer PCR diagnostics developed by Zhou and colleagues (1997), revealed a consistent association of the recombinant EACMV-UG with ACMV (Harrison et al. 1997; Pita et al. 2001; Deng et al. 1997; Zhou et al. 1997). Significantly this biological phenomenon has been attributed to the emergence of new geminivirus diseases and has been hypothesized to be a key factor in the genesis and spread of the CMD pandemic in East and Central Africa (Harrison et al. 1997; Legg 1999; Fondong et al. 2000; Pita et al. 2001). Plants infected with EACMV-UG and ACMV expressed more severe symptoms than those infected with EACMV or ACMV alone (Harrison et al. 1997b; Pita et al. 2001). In Uganda, it has been demonstrated that while plants infected with mild (less aggressive) strains of EACMV-UG yielded only 12% less

than CMG-free plants, yields of plants infected by ACMV were reduced by 42%, those infected by severe strains of EACMV-UG by 68%, and those with mixed ACMV + EACMV-UG infections by 82% (Owor 2002), highlighting the impact of the synergistic interaction between these two geminiviruses.

CMG Situation in Africa

Despite the demonstrated presence of EACMV-UG in West Africa (Tiendrebeogo et al. 2009; Akinbade et al. 2010), we have not yet witnessed the emergence of a CMD pandemic in this part of the African continent. The dominant viruses infecting cassava in West Africa are ACMV and EACMCV (Fondong et al. 2000*;* Ogbe et al. 2006; Alabi et al. 2007). On the other hand, it is clear that these viruses have the capacity to synergize in cassava fields and cause the drastic "candle stick symptom" (Figure 15.2; Fondong et al. 2000; Alabi et al. 2007). It is probable that the explanation for why they have not yet done this resides in the fact that the superabundant whitefly populations from East Africa have not been able to move and become established in West Africa (Legg and Fauquet 2004). This does not mean that this could not happen in the coming years, and the co-prevalence of ACMV and EACMCV, capable of a synergistic interaction, in many West African countries suggests that the viral components for a possible pandemic are already present in West Africa. This also supports the breeding of cassava CMD-resistant cultivars to prevent such a pandemic.

Breeding for CMD Resistance

In the last 75 years cassava mosaic disease has severely affected cassava production in Africa. The continued threat of a CMD pandemic in Africa could undermine production gains achieved in the recent past. The economic impact of CMD, as estimated by studies investigating possible yield loss worldwide has been calculated at something between 20% and 95% (Fauquet and Fargette 1990). Legg and Thresh (2003) estimated an annual economic loss of US$1.9-2.7 billion, making CMD, in economic terms, one of the most globally important plant virus diseases.

Productivity and stability of cassava are key major objectives of breeding. Stability of production is highly associated with resistance and tolerance to major biotic stresses. CMD is one of the major biotic stresses that severely limits its productivity and stability in cassava, and accordingly CMD resistance is a primary objective and of paramount importance to successful cassava breeding programs in Africa and India. The aim of breeding for resistance is to produce cultivars with improved resistance to several CMGs and high yields that persist under a wide range of environmental conditions. Fortunately genetic resistance to important diseases of cassava has been identified (Hillocks and Wydra 2002; Ceballos et al. 2004).

The emergence of new viral diseases and the increasing evolutionary capacity of the viruses and their vectors to adapt and remain highly virulent is a huge challenge to breeding programs. Durability of resistance is therefore a priority as a breeding objective in Africa and India. Identifying and pyramiding different virus-resistance genes will therefore provide stable resistance against a broad spectrum of CMGs. This is very important and much more pertinent given the recent discovery that CMGs can synergize and cause unusually severe symptoms leading to almost total yield loss in infected plants. Gene pyramiding in cassava will require molecular marker-assisted breeding to expedite the process.

Cassava's center of origin is Latin America, where huge cassava genetic resources are available for genetic improvement. Wild *Manihot* germplasm offers a wealth of useful genes for the cultivated *M. esculenta* species (Hahn et al. 1980; Chavarriaga et al. 2004). The International Center for Tropical Agriculture (CIAT, the Spanish acronym) has utilized wild *Manihot* species for genetic improvement of key novel traits for which genetic variation is highly

limited in cultivated gene pools, including high protein, delayed post-harvest physiological deterioration, and acyanogenesis. The introgression of this genetic diversity has been successfully initiated through pre-breeding activities prior utilization in breeding programs in South America and to some extent in Asia (e.g., Thailand). However, because of the susceptibility of the Latin American (LA) germplasm to CMD, this germplasm cannot be used directly in Africa or India unless it is improved for CMD resistance (Okogbenin et al. 1998; Okogbenin et al. 2007). A key breeding objective is continued improvement of LA germplasm for CMD resistance to give it greater utility in CMD-prone regions of the world. Given that CMGs do not yet exist in LA (and the accidental introduction of the CMGs to LA is a frightening potential!), pre-emptive breeding for CMD resistance in the absence of the pathogen is critical to protecting the vast wealth of genetic resources of cassava in Latin America.

Vital breakthroughs have been made in recent years in understanding the nature, biology and interactions of the CMGs, *B. tabaci,* and the cassava host. The information gleaned from such efforts will help to further refocus key breeding objectives for CMD resistance. Only determined, well coordinated, and comprehensive breeding objectives addressing both research and development needs will allow the true potential of cassava to be unlocked, enabling this most versatile crop to provide food security, income, and new commercial opportunities for a growing world population, especially in Africa.

Sources of CMD Resistance

A very small number of natural sources of geminivirus resistance have been identified in wild and cultivated plants. In many instances, genetic analysis indicates these sources of resistance to geminiviruses are controlled by multiple loci, making their use in a breeding program very difficult. Attempts were made in the 1930s and 1940s to breed cultivars with greater levels of resistance (Jennings 1994). The earliest resistance breeding programs, initiated in the 1930s in Madagascar and at the Amani station in northeast Tanzania, used interspecific crosses with *Manihot glaziovii* Muell.-Arg. to produce progenies. Interspecific hybrids were then backcrossed to cassava, leading to highly resistant cultivars that have been developed and used in East Africa. Seeds from one of the most resistant clones, 5318/34, were sent to Nigeria, where selections made in the 1960s were then used in the 1970s as parents in the initial cassava improvement program at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria (Hahn et al. 1980b). The program resulted in the development of resistant clones and seeds, which were distributed to African countries (Mahungu et al. 1994). This is the most widely deployed source of resistance and is represented principally in African farmer fields by clones of the Tropical *Manihot* Species (TMS) series (TMS 4(2) 142, TMS 30337, TMS 91934, TMS 30001, TMS 60142, and TMS 30572) (Hahn et al. 1980b).

Resistance derived from *M. glaziovii* was found to be multigenic with a recessive component. The mechanisms of action of the multigenic *M. glaziovii*-derived resistances include: prevention of initial virus infection via resistance to insect vectors, reduction in the rate of virus replication, restriction in the movement of the virus within the plant, and tolerance to the virus (reduction in the effects of a given virus titer on growth and development of the plant) (Fargette et al. 1996). Cassava breeding is complicated by the biology of the crop and its heterozygosity, which in the absence of inbred lines has imposed limitations to the successful use of this multigenic source of resistance in cassava genetic improvement.

The second major source of resistance was identified in several Nigerian cassava landraces that have consistently shown strong resistance to CMD. High levels of resistance to CMD have been described in a group of closely related Nigerian cassava landraces (Akano et al. 2002). These landraces, TME3 to TME7 and TME14,

could not be differentiated with a genetic similarity greater than 95% using 76 AFLP (amplified fragment length polymorphism) or 36 SSR (simple sequence repeat) markers (Fregene et al. 2000; Fregene et al. 2003). The Nigerian landraces are highly resistant in the field to ACMV (Akano et al. 2002), and resistant to EACMV-UG (CIAT 2003), ICMV (CIAT 2005), and EACMV (CIAT 2006). Resistant plants showed little or no symptoms during the entire life cycle of the plant in Nigeria, Uganda, and India. However, in Tanzania, resistant plants developed symptoms; these symptoms were followed by a complete recovery (CIAT 2006). These field observations have been confirmed by laboratory infection of TME3, TME7, and TME14, validating these landraces as completely resistant to ACMV and partially resistant to EACMV, which can induce fairly strong systemic symptoms followed by complete recovery 45 days after infection (Fauquet pers. com.).

Although, the source of CMD resistance in the Nigerian landraces is currently not known, it has been speculated that, given the large number of closely related landraces, they are 'lost' sibling lines from a CMD breeding program run by British breeders in Nigeria in the 1950s and 1960s (Beck 1980). However, the possibility that the resistant landraces arose via a spontaneous mutation in the CMD resistance gene(s) in a single line, followed by selection and dissemination by farmers, cannot be disregarded. In contrast to the recessive multigenic inheritance of resistance from the *M. glaziovii* source (Hahn et al. 1980b) genetic studies demonstrated that this CMD resistance was single locus and dominant (Akano et al. 2002). Since the 1990s, the IITA and African National Agricultural Research Systems (NARS) have been exploiting this resistance derived from Nigerian landraces (Akano et al. 2002). Under the CMD resistance project of the IITA, resistance profiles of cassava cultivars were tested in multilocal trials in Nigeria, revealing the top highly CMD-resistant lines, which are being considered as possible additional sources of CMD resistance. This set of cultivars is being evaluated with molecular markers to understand the genetic basis of resistance and to identify the loci associated with CMD resistance.

Genetic Mapping of *CMD* Resistance Genes

Molecular markers are valuable tools for understanding the genes and genomes underlying natural variation. Although the genetics of cassava is the least understood of the major staples in the world, increasing research investment in the last two decades has resulted in the development of ample genomic resources, which are readily available to enhance the genetic improvement of the crop. Since the development of the first genetic map of cassava (Fregene et al. 1997) other new maps have since followed (Mba et al. 2001; Okogbenin et al. 2006; Kunkeaw et al. 2010; Sraphet et al. 2011). The first genetic map (Fregene et al. 1997) was constructed based mainly on RFLPs (restriction fragment length polymorphisms), RAPDs (random amplified polymorphic DNA), isozyme, candidate genes, and AFLPs (Amplified Fragment Length Polymorphism). The need for change to a more polymorphic, easily accessible, and user-friendly marker system led to the development of SSR markers and construction of SSR-based genetic maps, between 2001 and 2005 at CIAT (Mba et al. 2001; Okogbenin et al. 2006; Zarate 2004). However, since 2008, efforts in genomic resources development in cassava have rapidly shifted to SNPs (single nucleotide polymorphisms), which are more abundant in genomes compared to SSRs. SNPs are also well suited to high-throughput genotyping platforms, making them the markers of choice for application and successful implementation of efficient marker-assisted breeding.

Considering the importance of CMD, one of the primary objectives of the first genetic mapping studies conducted in cassava at CIAT was to use molecular markers to tag genomic regions controlling CMD resistance, first begun under a Rockefeller Foundation (RF) initiated project, in

order to understand the molecular basis of CMD resistance and to improve cassava genetic resistance to the disease via MAS. Following that initial step, and over the last decade, three CMD genomic regions have since been identified (Fregene et al. 2000; Akano et al. 2002; Okogbenin et al. 2012).

(a) *CMD1*

The first genetic map of cassava was developed using an intraspecific cross between TMS30572, an improved cultivar from IITA, Ibadan, Nigeria, and CM2177-2, an elite line from CIAT, Cali, Colombia (Fregene et al. 1997). The female parent has CMD resistance derived from *M. glaziovii*. A bulk segregant analysis (BSA) approach was used to identify a marker linked to *M. glaziovii* resistance. An SSR marker, SSRY40, on linkage group D of the genetic map of cassava, was found to be associated with CMD resistance and was designated *CMD1* (Fregene and Puonti-Kaerlas 2002); it explains 48% of the phenotypic variance of CMD resistance. *CMD1* appears recessive, as its effect is detected only in backcross progeny and not in the F_1. The recessive nature of this source of resistance, however, makes it less attractive, given cassava's outcrossing and heterozygous nature. Considering the fact that resistance in *M. glaziovii* is based on classical genetic analysis of crosses, which indicate that the trait is polygenic with a component that is recessive, this result implies therefore that more loci from those sources of disease resistance have yet to be mapped with markers. The differential resistance profile lines developed at IITA from the *M. glaziovii* source supports its multigenic character.

(b) CMD2

Classical genetic analysis and mapping of the resistance phenotype in landraces was used to identify the inheritance and genetic location of *CMD2* on a genetic map of cassava. The mapping population was an F_1 progeny from a cross between the CMD-resistant landrace TME3 and a susceptible improved line TMS 30555. The progeny, comprising 158 individuals, was established in vitro, sub-cloned, and evaluated in a replicated field trial in two locations in Nigeria with high CMD pressure in June 1998 (Akano et al. 2002). The ratio of resistant to susceptible plants was 1:1, fitting with the expected segregation ratio for a single dominant locus heterozygous in TME3. Bulk segregant analysis (BSA) was used to identify simple sequence repeat (SSR) markers linked to *CMD2* (Figure 15.3). Results of single marker analysis showed that the SSR marker explained 70% ($P < 0.0001$) of the phenotypic variance of CMD resistance, confirming the hypothesis of single-gene inheritance of this CMD resistance. Subsequent genetic mapping located the gene on linkage group G of the cassava genetic map between an RFLP (GY1) and an SSR marker, SSRY28, at a distance of 9 cM and 8 cM respectively (Akano et al. 2002; Beck 1980).

After the first mapping of the gene, further activities aimed at identifying molecular

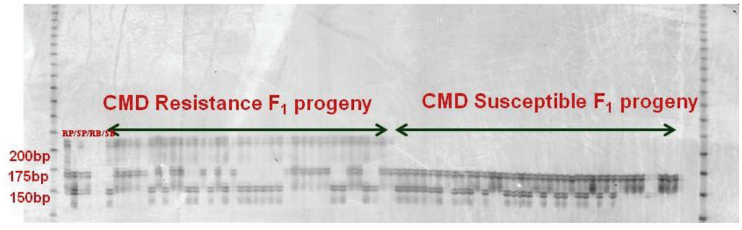

Fig. 15.3. Polyacrylamide gel electrophoresis for *CMD2* marker SSRY28 between resistant and susceptible genotypes. For a color version of this figure, please refer to the color plate.

markers closer to the gene were initiated, resulting in the identification of another SSR marker, NS158. With this result, two SSR markers: SSRY28 and NS158 were selected as the closest to the *CMD2* gene, at genetic distances of 9 and 6.7 cM respectively (Akano et al. 2002; CIAT 2002). To improve on these markers, fine mapping of the *CMD2* region of the genome was initiated by searching for recombinants between *CMD2* and the two SSR markers closely linked to the gene (SSRY28 and NS158), using a large full-sib population followed by an analysis of the recombinants with thousands of readily assayed polymorphic markers to identify additional markers more closely linked to the gene. The fine-mapping population consisted of 1,690 individuals from a cross between TME3, the source of *CMD2,* and the improved cultivar TMS30572. The population had initially been intended to improve CMD resistance by combining both sources of resistance but was found to be suitable for fine mapping studies, given that cassava is highly heterozygous and as expected segregated for CMD expression as revealed by the phenotypic data for the disease. Beyond the need to improve resolution of genetic mapping of markers in the genomic region of interest and to identify new markers tightly linked to the *CMD2* gene, which, being a dominant gene, was easily evaluated for MAS efficiency in the population. The cross was evaluated for CMD resistance at IITA, Nigeria, under heavy natural pressure of the disease. DNA was isolated from the individuals of the cross, using the method formulated by Dellaporta and colleagues (1983). The population was then evaluated with SSRY28 and NS158, described by Mba and colleagues (2001), and 112 recombinants between the markers and *CMD2* were identified.

DNA from ten resistant recombinants and ten susceptible recombinants was combined to form two bulks, which were then evaluated with several marker systems, including AFLPs, ISTRs (inverse sequence-tagged repeats), RAPDs, SCARs (sequence characterized amplified regions), and SSRs, using a modified bulk segregant analysis (BSA) method (Michelmore et al. 1991). Evaluation with AFLP markers (Vos et al. 1995) was done using a commercial AFLP (Invitrogen Life Technologies, Gaithersburg, MD), following the manufacturer's instructions. All 64 possible combinations were used in the evaluation. For ISTRs (inverse sequence-tagged repeats), the method described by Rohde and colleagues (1996) was used, with all possible 64 combinations of the 8 F and 8 B universal retro-elements (retrotransposons) sequence primers. Evaluation with RAPD markers was done using 768 commercial primers (Operon Technologies Inc, CA) and a modified protocol of that developed by Williams and colleagues (1990). Markers that were polymorphic in the recombinant bulks were then analyzed in individuals of the bulks. Analysis with RAPD markers produced three polymorphic candidate markers, AC-15, RME-1, and RME-2, that remained consistent in the individuals of the bulks (Figure 15.4). Evaluation of the three markers in the entire fine-mapping progeny revealed that the *CMD2* gene is flanked by NS158 and RME-1, with RME-1 being the closest marker, at 4.5 cM. The polymorphic fragment in RME-1, a band of 800 bp was cloned into the pGEMT-easy and sequenced. A SCAR primer pair was designed from the cloned sequence in order to permit the application of the new marker in marker-assisted selection (MAS) programs. The above result provided a molecular marker with a closer link to the *CMD2* to be used for screening of a cassava BAC library and construction of BAC contigs, toward positional cloning of the gene.

(c) CMD3

TMS97/2205 is a top elite line developed at IITA and has high resistance to CMD, with severity symptoms being low or near immunity. Disease incidence in this cultivar is also very low (<1%) in high CMD pressure zones in Nigeria. The resistance profile provided a basis to evaluate this cultivar with molecular markers for

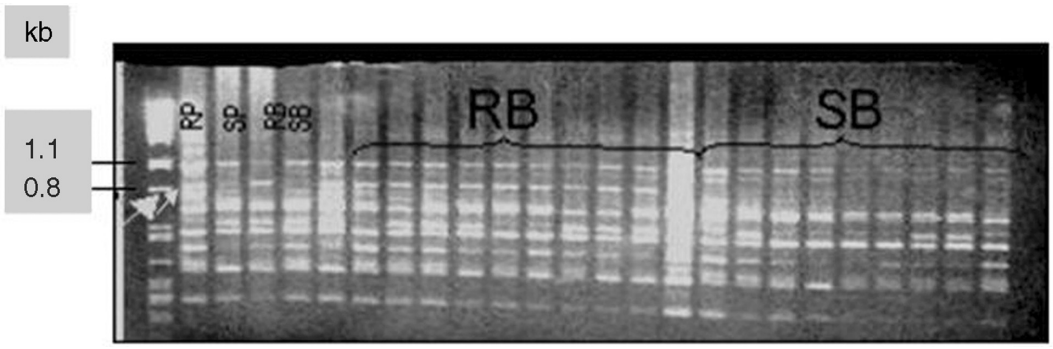

Fig. 15.4. Ethidium bromide stained agarose gel of individuals from the recombinant bulks, evaluated with the RAPD marker RME-1. A fragment around 800bp (arrow) can be observed in the resistant parent (RP) and resistant bulks (RB), and it is absent in the susceptible parent (SP) and susceptible bulk (SB). For a color version of this figure, please refer to the color plate.

CMD resistance. Given its near immunity-level resistance to CMD, two levels of molecular analysis were conducted. The first was to test the cultivar for the *CMD2* gene and to use a cross of this parent to do a BSA test to identify further genes or QTLs that may be involved in the high CMD resistance observed in this cultivar. Initial molecular analysis of TMS97/2205 with *CMD2* markers identified the presence of the *CMD2* gene in this cultivar, showing that the *CMD2* gene was involved in the CMD resistance of this cultivar. Further genetic analysis of this cultivar as a source of high resistance to CMD using a segregating F_1 population derived from a TMS97/2205 x NR8083 cross was initiated involving 530 SSR markers to identify QTLs for CMD resistance.

The F_1 population from the cross between TMS97/2205 and NR8083 was analyzed to identify markers linked to gene(s) associated with resistance to CMD via bulked segregant analysis (BSA) as described by Michelmore and colleagues (1991). Two bulked (pooled) DNA samples corresponding to the extreme groups of resistant and susceptible individuals, based on the phenotypic data from a two-year evaluation, were prepared. Markers that are polymorphic between the two pools are expected to be genetically linked to the trait used to construct the pools (Michelmore et al. 1991). The bulks were made by mixing equal volumes of DNA solution from each of the genotypes in each bulk (resistant vs. susceptible). The composition of the resistant bulk was 24 individuals and 12 individuals were used for the susceptible bulk. DNA extraction and genotyping was as described above. For the BSA, a total of 530 SSR markers, with wide coverage of the cassava genome, were used for genotyping. The parents of the selected cross were screened for polymorphic markers. The polymorphic markers between the two parents were then used to analyze the contrasting bulks to identify SSR markers with possible association with genes (QTLs) for resistance. Results of BSA analysis identified a marker (NS198) associated to a QTL for CMD resistance explaining 11% of the phenotypic variance observed. This QTL was designated *CMD3*.

The combined effect of this QTL (*CMD3*) and *CMD2* may account for the high resistance of TMS97/2205. The SSR marker is located on the same linkage group as the *CMD2* gene but is at least 36cM away from the *CMD2* marker loci (SSRY28, SSRY158, NS169) (Whankaew et al. 2011). The SSR marker SSRY106 in the genomic region with NS198 and in the interval with *CMD2* markers was not significantly linked to the *CMD2* gene, indicating that NS198 is associated with a different QTL. The synergistic effect of the *CMD2* gene and this QTL (*CMD3*) may have accounted for the high resistance to CMD observed in TMS97/2205.

Marker-Assisted Breeding for *CMD2* Resistance in Cassava

Cassava genetic improvement can be made more efficient through the use of easily assayable molecular markers, which allow for the precise identification of genotype without the confounding effect of the environment, thereby increasing heritability. The selection of progenies based on genetic marker data substantially increases the rate of genetic gain, especially if the number of cycles can be reduced (Meuwissen et al. 2001).

An ideal target for MAS is the breeding for disease resistance, since one or only a few genes are often involved. The discovery of CMD resistance in TME3, a landrace from Nigeria, and the major nature of the gene also means that a genetic marker for marker-assisted selection (MAS) can be easily identified. MAS could thus become an invaluable tool for breeding CMD resistance in Africa, where the disease is most prevalent, as well as in Latin America, where preemptive breeding for CMD disease resistance has assumed importance, with the aim of mitigating possible accidental introduction of the disease to the region. The occurrence in the Americas of *B. tabaci* biotype B (*Bemisia argentifolia*), which has a wide range of hosts, including cassava, makes the threat of the virus more frightening for cassava production in the Neotropics (Polston and Anderson 1997). Using the *CMD2* gene, MAS has been applied for CMD resistance breeding in Latin America and Africa. The *CMD2* markers are gradually being used for breeding and genetic analysis of CMD resistance in Asia (Biu 2010). MAS in cassava has been applied in breeding for CMD resistance in the absence of the gene, in the Americas, by using *CMD2* markers to introgress CMD resistance into valuable LA susceptible germplasm. The LA germplasm were selected with CMD markers and then introduced into CMD-infected environments in Africa and Asia. The other benefits of MAS include improved potential to reduce large population sizes early in the breeding scheme, fast-tracking the long breeding scheme typical in cassava, and screening for new sources of resistance through negative selection for identified known sources of resistance in African germplasm. Gene pyramiding through selection of parents with different sources of CMD resistance (to improve stability and durability of resistance in cassava) and fixing of CMD genes in a clonally propagated crop such as cassava, through selection for homozygosity at loci of interest, are other added advantages of MAS. The *CMD2* markers have been applied in MAB basically in the following major areas of cassava breeding, as outlined in the sections that follow.

(a) Breeding in the Absence of the Pathogen in the Americas

CMD is a threat to South America, where the disease has not been reported but where the vector of CMD viruses has recently become widespread on other hosts, although not on cassava (Polston and Anderson 1997), increasing the possibility that these vectors could adapt to cassava and that the CMGs could cross over to cassava in the Neotropics. Latin American cassava is highly susceptible to CMD (Okogbenin et al. 1998). The absence of the pathogen in the Neotropics has imposed limited breeding capacity to develop LA cultivars with CMD resistance. In the absence over a period of decades of the proper environment that would support CMD-resistance breeding in LA, it is not unexpected that elite LA lines would be highly susceptible to the disease.

Even in instances where CMD resistance breeding was initiated, the environment was not appropriate to test the materials. In the 1990s, the use of the polygenic source for improving CMD resistance in cassava of LA genetic background was not successful because of its recessive nature. Several hundreds of thousands of seeds from CIAT developed from crosses involving donor lines for the *M. glaziovii* source of resistance succumbed to the disease when tested in Africa. The large quantities tested were partly a result of the inability to preselect for the

polygenic resistance in CIAT before field-testing in Africa. The lack of pathogen also implies that cassava in Latin America was not able to evolve with the virus as in the case of Africa, where disease pressure has aided evolutionary changes of the crop with enhanced genetic capacity for survival, as evidenced in the new sources of resistance available in the secondary center of diversity. The dominant gene nature of *CMD2* means that CMD resistance can now easily be transferred and tracked by molecular markers. Breeding for resistance to CMD in Latin America, where the disease does not exist, therefore essentially requires the tools of MAS. The use of markers for CMD resistance is enhancing the ability of the CIAT breeding program in Latin America to breed and select in the absence of the disease, which is now the case in the New World.

CIAT initiated MAS, using TME3 as the donor parent for *CMD2*. Progenies of TME3 were established from embryo axes and imported to CIAT from IITA. They were crossed extensively into elite LA cassava parents. Two sets of germplasm were developed. The first set consists of F_1 germplasm derived from crosses of elite CIAT lines with CMD-resistant donor parents, followed by MAS for the *CMD2* gene. These lines developed from this process were denoted as CR lines. The second set of genotypes was obtained by crossing CMD-resistant lines to backcross derivatives of CIAT lines having resistance to cassava green mite (CGM). This second set combines resistance to CMD and CGM and these were designated as AR lines. Seeds harvested from the crosses were germinated in vitro from embryo axes according to standard protocols for cassava (Fregene et al. 1997; CIAT 2002) to allow testing of these genotypes in Africa. Each plantlet was multiplied and allowed to grow for several weeks. Leaves of all plants were removed for molecular analysis and the plants were multiplied again to obtain sufficient plantlets.

DNA isolation was done by the rapid mini preparation method developed for rice (Noboyuki et al. 2000). PCR amplification,

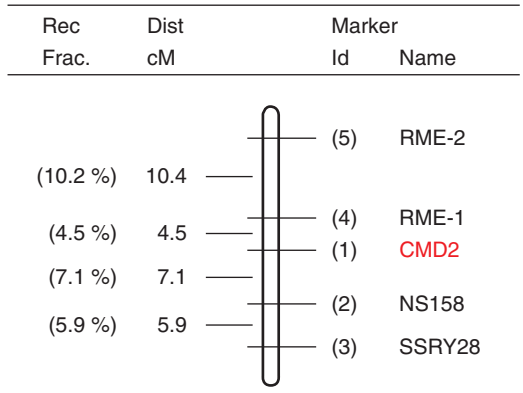

Fig. 15.5. Multiple flanking markers of the *CMD2* gene normally used for marker-assisted selection. For a color version of this figure, please refer to the color plate.

polyacrylamide gel electrophoresis (PAGE), or agarose gel analysis of SSR markers and SCAR markers were as described by Mba et al. (2001). Multiple flanking markers of the *CMD2* gene (Figure 15.5) were used for the molecular analysis. After molecular analysis, genotypes that carry the marker allele associated with *CMD2* were further multiplied to obtain enough plants for testing in Africa. The flow chart for MAS used is shown in Figure 15.6. Field evaluations have indicated that RME1 and NS158 were excellent prediction tools for CMD resistance. A recent validation study indicates that MAS efficiency with these markers was around 68% (Okogbenin et al. 2007). The dominant nature of the gene and its effectiveness against a wide spectrum of the viral strains make its deployment very appealing for protecting cassava against the CMD threat (Blair et al. 2007).

(b) Broadening of the Germplasm Base in Africa

The presence of CMD in Africa and India and its absence in the Americas limits the value of cassava germplasm from the crop's center of diversity in Africa because CMGs potentially carried from Africa would have a devastating effect on LA germplasm. This situation has imposed huge limitations on the utilization of germplasm from

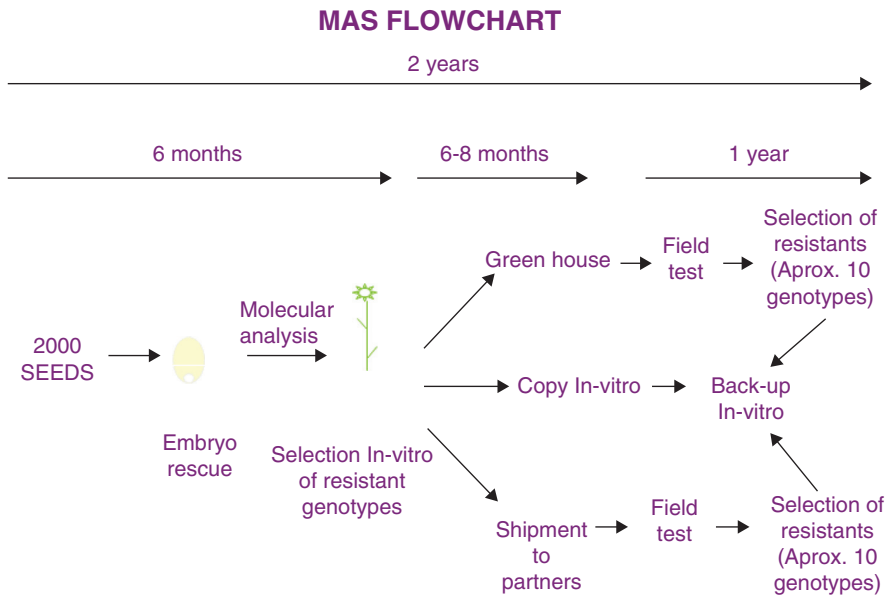

Fig. 15.6. Schematic presentation of MAS workflow followed in introgressing CMD resistance in LA germplasm and shipment to partners in Africa and Asia. For a color version of this figure, please refer to the color plate.

the crop's center of diversity in the Neotropics for other key cassava productions regions of the world, where CMD is highly endemic (Blair et al. 2007).

CIAT has the largest cassava collection in the world, with more than 6,000 accessions. CIAT gene pools have elite cassava lines with very high yield, high starch content, good dry matter content, and good plant architecture. The gene pools for breeding cassava in Africa currently contain only a fraction of the existing genetic variation found in Latin America, where the crop originates. One key area of application of MAS is in facilitating the introduction of LA germplasm introgressed with CMD for survival and adaptation in Africa as an initiative in broadening the germplasm base of cassava in Africa through access and use of LA germplasm. Cassava is vegetatively propagated and because of the high susceptibility of LA germplasm, heavily infected LA genotypes are easily and readily wiped out by the second season, as a result of the build-up of the virus load on the vegetative planting materials by the second year. CMD affects plant vigor and flowering, making it difficult to utilize unadapted introduced LA germplasm in crosses with African germplasm. For this reason, previous attempts to introduce LA germplasm for evaluation and release as cultivars in Africa through collaborative a partnership between IITA and CIAT in the 1990s was not very successful. No LA genotypes were selected for on-farm testing from the IITA breeding scheme (Figure 15.1) because of their high susceptibility to the disease.

Under a collaborative project between CIAT and African NARS, elite CIAT lines developed for CMD resistance with the aid of markers have been developed and shipped to Africa. The use of *CMD2* and MAS has rapidly changed the setback experienced in the 1990s, leading to the successful introduction of LA germplasm with broad genetic variation for traditional breeding traits (yield and other agronomic traits) for the African continent. Following the same procedure as described earlier, more than 1,400 genotypes from F_1 and BC_2 populations having CMD parent lines and CIAT

Table 15.1. CIAT shipment of cassava mosaic disease (CMD) resistant genotypes in combination with other traits to Africa and Asia

Country	Trait	Number of Genotypes shipped
Tanzania	r-CMD, r-CGM	530
Nigeria	r-CMD, r-CGM, RQ	765
Uganda	r-CMD, r-CGM	530
Ghana	r-CMD, r-CGM, RQ	765
Kenya	r-CMD, r-CGM, Dr	850
Mozambique	r-CMD, r-CGM, RQ	150
South Africa	r-CMD, r-CGM	80
India	r-CMD, r-CGM	530
Thailand	r-CMD, r-CGM	50

r-CMD - resistance to Cassava Mosaic Disease; r-CGM - resistance to cassava green mite; RQ-root quality; Dr - dry matter

elite materials in its pedigree were developed between 2003 and 2005. Subsets of the materials developed after MAS were shipped to several countries in Africa and Asia. The shipments are listed in Table 15.1. The low number of genotypes shipped to each country is a sharp contrast to the huge number (hundreds of thousands) of genotypes shipped as seeds in the 1990s, reflecting a huge reduction in cost and management in a breeding program through MAS.

Field evaluations in Africa have resulted in the successful selection of LA genotypes in the breeding scheme, through seedling nursery to regional trials, leading to a collection of top performing elite lines assembled in the gene pool of NARS in Africa in Ghana, Nigeria, Tanzania, and Uganda. These materials have also been integrated into the recurrent selection activities of the breeding program aimed at combining CMD resistance with other farmer-preferred traits in these countries, in order to develop lines better suited to meet consumer needs (culinary qualities).

The LA CMD-resistant lines were integrated into the breeding scheme of African NARS, and then evaluated, leading to release or use in crosses with local African farmer-preferred lines to develop better adapted lines. The LA germplasm shipped was developed for characteristics such as high yield, vigor, high dry matter content, and high starch content. A good number of these materials have been selected and incorporated into gene pools of these African countries for continuous use in their breeding programs, a step intended to reduce genetic gaps in African germplasm. This is a landmark breakthrough that has largely facilitated the broadening of the germplasm base. A good number of the LA materials, which have been evaluated in the breeding scheme, have been selected by farmers under the participatory varietal selection (PVS) and plant participatory breeding (PPB) activities of NARS. For example CR41-10 was preferably selected for its culinary and root quality characteristics at on-farm testing stages. Results indicated that a high proportion of the materials are showing good resistance to CMD in the different countries. Through the success of MAS using CMD markers, several elite genotypes of Latin America were successfully introduced into Africa (Nigeria, Ghana, Tanzania, and Uganda) under the CGIAR Generation Challenge Program (GCP).

The successful use of the CMD markers also implies that they could be used by breeders to breed for CMD resistance in parts of Africa were disease pressures are low, or in seasons of low disease pressure where heritability is low or near zero, and could minimize the cost associated with maintaining several screening sites for breeding programs. It also means that breeders can quickly downsize on the number of

genotypes being tested for CMD resistance via MAS thereby reducing breeding cost.

(c) Exploring Heterosis

Genetic diversity studies using molecular markers have shown genetic differentiation in cassava between the primary center of diversity in Latin America and the secondary center of diversity in Africa (Fregene et al. 2000; Fregene et al. 2003; Kizito et al. 2005). This genetic divergence is believed to represent heterotic groups that may be explored in breeding to enhance cassava production.

A marker-assisted selection (MAS) project was initiated in Tanzania in 2003 with funding support from the Rockefeller Foundation to improve gene pools for resistance to disease and pests (CMD and CGM) and for high yield and dry matter content (Blair et al. 2007; Fregene et al. 2006). This initiative was later supported with funding by the CGIAR GCP and Alliance for Green Revolution in Africa (AGRA) with technical assistance from IITA. The project sought to explore heterosis, in order to improve yield and dry matter content, by carrying out extensive crosses between the LA germplasm (introgressed with CMD resistance via MAS) and Tanzanian germplasm. While the CR and AR genotypes from LA have CMD resistance that was developed via MAS, the AR has CGM resistance introgressed from wild relative *M. flabelifolia,* offering sources of resistance to the cassava green mite in addition to the resistance *CMD2* provides.

The marker-assisted breeding scheme used in connection with MAS developed in LA germplasm and implemented in East African NARS is different from that used in West Africa resulting from the prevalence of cassava brown streak disease (CBSD) in East Africa. CBSD, which is a highly devastating viral disease, has yet to be reported in West Africa. LA germplasm is susceptible to the disease (CIAT 2005; Mkamilo pers. com.). A total of 503 LA genotypes were shipped and only one genotype (AR40-6) was tolerant of CBSD (Mkamilo pers. com.). LA germplasm selected with *CMD2* markers that were shipped into East Africa were first evaluated on the field for CMD resistance. A selection based on CMD resistance, harvest index, and total biomass was made and the best genotypes (more than 80) were selected from the 503 LA genotypes received by Tanzania. The CMD-resistant LA genotypes were then introgressed into Tanzanian germplasm with CBSD tolerance. They were planted in controlled crossing blocks together with 54 local genotypes (with resistance to CBSD) from the Eastern and Southern zones of the country. More than 40,000 crosses were made between the improved genotypes and the local cultivars, producing more than 60,000 seeds. The resultant progenies developed from the cross between LA genotypes and Tanzania lines were screened with *CMD2* markers (the second round of MAS selection for CMD resistance in the scheme) to reduce population size, and then field tested to select those combining CMD and CBSD resistance. The genotypes having both CMD and CBSD resistance were selected and then integrated into the normal breeding scheme for the evaluation of agronomic traits. The materials were then planted and evaluated through the breeding scheme. The genotypes were tested extensively in the breeding scheme, leading to the identification of ten best genotypes with vigor, high yield, good dry matter, and combining both CMD and CBSD resistance.

Yield obtained for the best 10 genotypes were between 50 and 87 tons per ha (Table 15.2). These are very high yields – roughly four to eight times higher than the normal yield average of local cultivars – suggesting the possibility of heterosis from cross combination between LA germplasm and Tanzania local germplasm. Similar activities are underway in other African countries that have received LA germplasm from CIAT. While it is possible to release in West Africa the MAS-developed CMD-resistant LA genotypes produced by CIAT, it is not possible to do so easily in East Africa because of the need

Table 15.2. Root yield and dry matter content of cassava genotypes bred for CMD resistance via MAS and nominated for release in Tanzania

Clones	Pedigree	Root yield (t/ha)	Dry matter (%)
CI85-4	AR37-38 x Kifumulo	63.8	31.5
C119-3	CR52A-19 x Namikonga	50	30.8
C7-46	Kalolo x AR9-44	57.5	33.9
C61-1	Amani x CR20A-6	53	32.4
C17-27	Namikonga x AR9-18	77.5	29.4
B201-97	Mkiwa	73	34.1
B2013-44	AR9-18	75	35.5
C170-11	AR30-4 x Kifumulo	86.9	31.5
C211-49	Namikonga x AR30-3	53.8	32.4
C179-4	AR17-25 x Kifumulo	54	34.2
C150	AR38-30 x Muzege	76.9	52.1
Kiroba	Local check	14	36.4

to improve the introduced germplasm for CBSD resistance. The marker-assisted breeding scheme used is summarized as follows:

1. CIAT cassava elite lines that are high yielding and resistant to CMD (screened using markers for *CMD2*) were introduced from CIAT into Tanzania.
2. Tanzania's farmer-preferred germplasms that are generally low yielding but tolerant/resistant to CBSD were selected.
3. Field evaluation of germplasm from CIAT and Tanzania to select parents for hybridization program.
4. Introgression of genes from CIAT germplasm to Tanzania's elite germplasm.
5. Screening of F_1 progenies from Tanzania for *CMD2* resistance at CIAT, using markers for CMD.
6. Field evaluation of CMD-resistant clones and phenotypic selection of clones with dual resistance to CMD and CBSD.
7. Intensive farmer-participatory research conducted to identify farmer preferences and criteria used in selecting suitable cassava clones. Also phenotypic selection for high yield and dual resistance to CMD and CBSD.
8. Field resistance to CMD and CBSD confirmed (phenotypically) by intensive testing of the clones at many locations with high CMD and CBSD pressure.
9. In collaboration with the seed regulatory body in Tanzania, national performance trials, and distinctiveness, uniformity and stability tests were conducted.
10. Release of cultivars.

(d) Transfer of Novel Traits to Africa

Cassava has been transformed from a food crop into a cash and industrial crop, and breeding to improve its quality and commercial traits has assumed high importance in cassava growing countries. Improvement for quality traits in cassava is mainly targeted at enhancing the low cyanogenic potential in the roots; increasing protein content in the roots, the waxy starch content, beta carotene content, and dry matter content; reducing post-harvest physiological deterioration; and reinforcing other consumer preferences related to cooking quality. Unfortunately, the genetic variation for these traits is highly limited in *M. esculenta.* Wild *Manihot* species provide a wealth of useful genes for the cultivated species, *M. esculenta*.

M. esculenta sub spp. *flabellifolia*, *M. peruviana* and *M. tristis* have been identified as sources of high levels of nitrogenous compounds (with possible implications for high protein

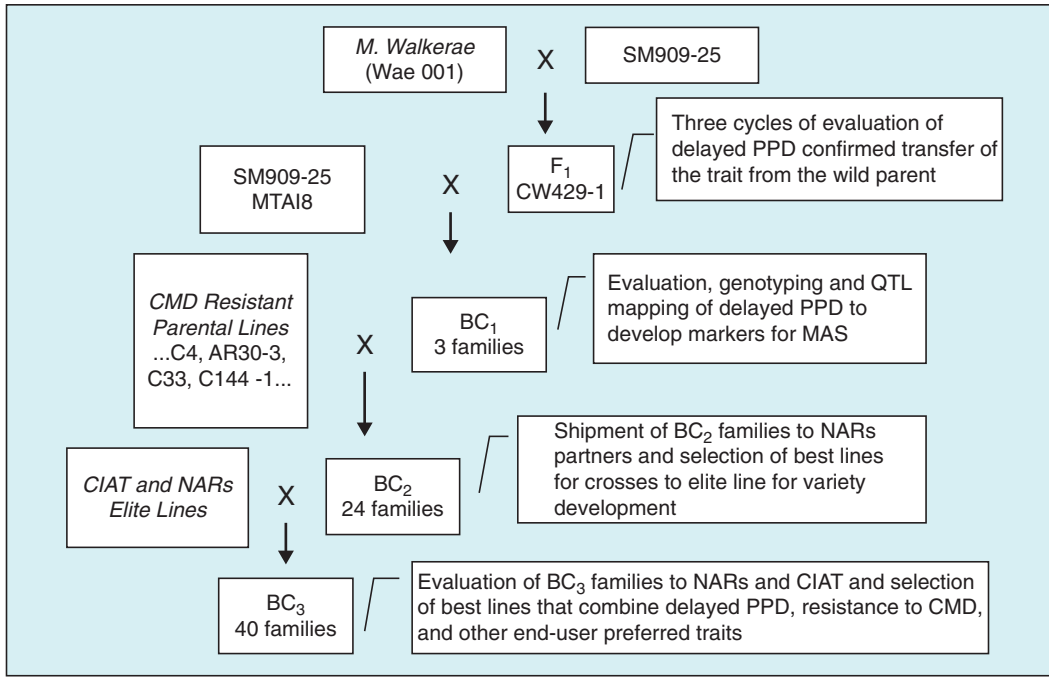

Fig. 15.7. Modified advanced backcross scheme used for introgressing delayed post-harvest deterioration from *M. walkerae* into *M. esculenta* species in combination with MAS for CMD resistance. For a color version of this figure, please refer to the color plate.

content) (Asiedu et al. 1992; CIAT 2004; Akinbo et al. 2011). The only source of post-harvest physiological deterioration (PPD) has been identified in an interspecific hybrid between cassava and *M. walkerae* (Bertram 1993). Backcross derivatives have been developed from *M. walkerae* for delayed PPD and for protein content (Blair et al. 2007). The only source of resistance to the cassava hornworm was identified in fourth backcross generation progenies of *M. glaziovii* (Jennings 1976; Chaviarriaga et al. 2004). These genetic resources hold immense opportunities for the cassava crop, but their full utilization is dependent on their being improved for adaptation in targeted ecologies and countries. Pre-breeding activities to transfer genes from wild relatives into cassava is being done and progenies showing the traits of interest are utilized to transfer those traits to partners.

Gene mining of wild relatives of cassava in order to introgress useful genes from the wild gene pool into LA cassava was done at CIAT using an advanced backcross scheme (Blair et al. 2007). A modified advanced backcross scheme (Figure 15.7) was used to introgress high protein and delayed PPD into cassava. The accessions of wild relatives of cassava with high protein or with delayed PPD were crossed to elite cassava parental lines and traits were then evaluated. The first backcross generations (BC_1) developed as shown in the scheme (Figure 15.7) were found to show the target traits. These accessions were then backcrossed again to cassava with CMD resistance, which were then selected with *CMD2* markers before shipment to Africa. About 140 genotypes of protein-rich accessions developed for CMD resistance via MAS, designated as CRCR lines, were shipped to Ghana, Nigeria, Tanzania, and Uganda. Similarly, about 500 genotypes of delayed PPD and MAS-bred for CMD resistance were shipped to the same African countries under the cassava-breeding Community of Practice (CoP) project supported by the CGIAR GCP.

(e) Pyramiding CMD resistance

Pyramiding genes is vital for increasing disease resistance durability in cassava cultivars and provides the most potent strategy for reducing the threat of CMGs. A key target in breeding is to combine the different sources of CMD resistance with the aid of markers. In the cassava breeding CoP, F_1 populations developed using parents of two sources of CMD genes (*CMD2* and *CMD3*) have been developed. Six genotypes of the *CMD2* source (CR36-5, CR42-4, AR12-45, CR52A-41, AR15-5, and AR36-2) were used in crosses with TMS97/2205. Another clone, 96/1089A, identified as a high-resistance cassava cultivar and described as a likely different source of CMD resistance by IITA, is being used to pyramid CMD-resistance genes. TMS96/1089A has been used to develop a mapping population to tag CMD QTLs in this parent in the cassava research initiative of the CGIAR GCP. It is expected that once additional markers for new sources of CMD resistance are identified, MAS can be effectively used to select for multiple sources of CMD resistance in breeding populations.

(f) Germplasm Screening for Parent Selection in CMD Resistance Breeding in Asia

CMD is a potential threat to cassava cultivation in Southeast Asian (ASEAN) countries. Plantlets of 18 cassava cultivars collected from China, Thailand, and other ASEAN countries were infected with CMD by means of agro-inoculation mediated infection, and these plantlets developed various levels of CMD symptoms, indicating a lack of resistance to CMD. There was a positive association between symptom severity scores and accumulation levels of viral DNA in the different cultivars tested. Molecular marker analysis of these cultivars, using markers RME1, SSRY28, and/or NS158, tested positive for the marker allele associated with the *CMD2* gene in only three of the cultivars (11Q, T7, and N13), which showed moderate resistance to CMD. The three cultivars are believed to be of African origin. To reduce the risk of introducing susceptible germplasm into Asia, it has been suggested that CMD-resistance germplasm from Africa should be introduced with the aid of MAS (Bi et al. 2010).

CMD2 Resistance Profile

The best LA genotypes (64 in total), selected after initial analysis of CMD resistance in Nigeria, were reevaluated and integrated into the breeding scheme of the National Root Crops Research Institute, Nigeria. The genotypes were evaluated in Nigeria in order to validate their CMD resistance profile and their stability at three to four years. The LA genotypes were evaluated using a symptom severity index (SSI) of 1- 5 (where 1 is symptomless and 5 is highly severe). The results indicate that resistance levels and response patterns vary among the genotypes. While some genotypes were relatively stable in CMD resistance, maintaining low symptom expression (SSI 1 or 2) throughout the growing season, some genotypes were found to show a significant increase (to SSI 3) in symptom expression (moderate) at disease peak period and then recovered from the disease rapidly with less symptom expression levels later. The highly resistant genotypes often showed good resistance at the peak pressure of the disease (often midway into the season) and were therefore relatively stable. Of the 64 genotypes validated, 48 genotypes were found to show good resistance whereas the other 18 genotypes showed moderate resistance to the disease (Okogbenin et al. 2012).

The 64 genotypes evaluated showed good resistance to the disease, although with differential response. Three types of disease response were observed in the *CMD2* genotypes: (1) the highly resistant genotype, (2) the resistant genotype, and (3) moderately resistant genotype. Both the highly resistant and the resistant genotypes are generally low in disease symptoms with the former almost symptomless for almost all of the growing season and therefore with a lower

severity index than the latter. The disease response profile of the moderately resistant genotypes suggests evidence of good and efficient recovery mechanism in the disease response pattern of the *CMD2* gene.

The results also indicate that the resistance status of these genotypes was influenced by their different genetic backgrounds, considering that the 64 genotypes evaluated are of different cross combinations representing 29 families. However, disease symptom expression observed among the CRs was not distinctly different from those of the ARs. Resistance mechanisms for the *CMD2* source appear to be related to constricted long-distance movement or gene silencing of the virus, as plants may be infected but ultimately recover (Akano et al. 2002). *CMD2* has been shown to provide resistance for all CMGs, including those from India, indicating that the mechanism of resistance is probably generic for all CMG and pointing to a single virus avirulence protein as a resistance (R) protein interacting elicitor of resistance (Fauquet pers. com.).

The CMD-resistant TME3, with the resistant locus *CMD2*, was challenged with different species of CMGs along with the CMD susceptible cultivar 60444 (Fauquet pers. com.). TME3 showed a very high level of resistance to ACMV, with very mild symptoms in the inoculated leaves, and it did not show systemic infection. In contrast EACMV-like viruses showed varied levels of infection in TME3, which later recovered completely. There is a very strong correlation between the visual symptom recovery and the virus content (Fauquet pers. com.). Although EACMV-UG did not produce any visible symptoms in TME3 at 35dpi, PCR analysis showed the presence of virus in the systemic leaves. Thus TME3 does not confer immunity to CMGs, but does result in drastically reduced virus accumulation and symptoms in comparison to susceptible cultivars for all tested geminiviruses. Viral DNA accumulation was positively correlated to symptom severity for ACMV and EACMV-like viruses, as in cultivar 60444 (Chellappan et al. 2004).

These studies clearly demonstrate that the participation of a virologist is necessary in a CMD and CBSD resistance program. Understanding the response of each type of resistance to each virus is essential in order to breed better cultivars and to evaluate the response of the lines under selection to make the best choice possible. Too many times the exact nature of the viruses in the fields influencing resistance selection is unknown and grouped under the term CMD, and this is not sufficient. Such grouping leads to misinterpretation of data, to wrong hypotheses, or to wrong explanations. It is clear that *CMD2* provides an extremely high level of resistance to ACMV – close to immunity – but only moderate resistance to EACMV-like viruses. Until the nature of the triggering viral protein(s) for resistance is/are known, response of plants to these two different types of viruses has to be considered separately. A synergistic response of the plant to a dual infection can easily be interpreted as a resistance breakdown, while it could be an immediate response to the mixture of the two types of viruses. A flush of symptoms to EACMV-like viruses can easily be interpreted as a susceptible response, while the plant may recover after some time. These field data could mask and somewhat undermine the MAS data. Evidently the situation could be more complex if new CMG recombinants emerge, and here too the participation of a virologist would be essential. A record of the vector populations in the breeding fields could also be a very good indicator and is highly recommended, as it seems obvious, albeit for unknown reasons, that pandemics in East and Central Africa greatly depend on the population build up.

BAC Sequencing Reveals New Markers for *CMD2* Gene

To elucidate the function of *CMD2,* further attempts were made to clone the gene via positional cloning, using a large full-sib cross-developed from noninbred parents and used for fine mapping (as described above) and a

constructed bacterial artificial chromosome (BAC) library from TME3. A BAC library was constructed in order to generate BAC contigs around *CMD2*. The BAC library construction was carried out at the Clemson University Genome Institute (CUGI) and the screening was conducted at CIAT. Construction of the library was as described by Tomkins and colleagues (2004). To estimate the distribution and average size of the clones, a total of 370 clones from the TME3 library were picked randomly and grown overnight in 3 ml of liquid LB medium + 12.5 μg /μl chloramphenicol. Plasmid DNA was isolated, digested with *Not* I restriction enzyme, and inserts were separated from the vector by pulsed-field electrophoresis.

A BAC library arrangement of plate, column, and row pools was created, namely 'plate pools' (PP), 'column plates' (CP), and 'row plates' (RP). All 192 384-well plates were duplicated using a 384-pin replicator and allowed to grow in the LB/chloramphenicol (12.5 μg/ml) medium at 37° C overnight. For the BAC plate pool, all the bacteria culture in a 384-well plate was combined into an omnitray and 200 μl of this transferred into a single well in a 96-well plate, to yield 2 'BAC pool' plates. Simultaneously, every ten plates of the library were inoculated into a single 384-well plate using a 384-pin replicator to give twenty 384-well plates. Each row of each of the twenty plates was inoculated, using a sterile toothpick, into a single well containing the LB/chloramphenicol (12.5 μg/ml) medium, to form 'row plates (RP),' i.e., five RPs of 96-well plates in total. The same was done for each column of the twenty 384-well plates, combined into a single well to form four 96-well 'column plates (CP).' A total of eleven PPs, RPs, and CPs was obtained. Contig construction was conducted by PCR amplification of 'BAC pools.' For PCR amplification, 5 μl of the bacteria culture was transferred, using a multi-pipette, to a clean 96-well plate and the bacteria pelleted at 4,500 rpm for ten minutes in a Sorvall centrifuge. The supernatant was discarded and the pellet re-suspended in 5μl of sterile water and used as template for PCR amplification under standard conditions.

The BAC library from TME3 has a 10.1 haploid genome equivalent, providing a 99% chance of finding any particular sequence. The BAC library was screened by PCR amplification of 'BAC pools' (Moreno 2005). Results of the 'BAC pools' screening with NS158 yielded 2 positive clones, whereas screening with RME1 yielded 14 positive clones. NS158 is a single copy SSR marker, whereas RME1 was developed from a multiple-copy RAPD marker. The clones were digested with 20 U of *Hind*III overnight and run for 24 hours on 1.2% agarose gel to obtain a BAC-clone fingerprint (Figure 15.8). Positive clones were located using plate, column, and row coordinates. The 16 positive BAC clones were selected, fingerprinted, and assembled into contigs associated with each marker. The chromosome-walking approach to the *CMD2* gene started with the contig construction, using the FPC ('Fingerprinting Contig') program (Marra et al. 1997), followed by two methodologies (allele-specific primers and SSCP-SNP) to design specific primers from the BAC ends in order to develop new molecular markers closer to the *CMD2* gene region. This process includes consecutive rounds of fine mapping and BAC-library screening toward the *CMD2* gene.

The positive BAC clones were digested with the *Hind*III enzyme and restriction profiles were analyzed with the FPC program, using an image.tif of the agarose gel. The astringent parameters to define clone overlapping recommended for the author were tolerance = 7 and cutoff = $1e^{12}$. The clones located at the ends of each contig were identified and their BAC ends were sequenced at the Iowa State University facility sequencing center. The sequences were edited for vector contamination and analyzed in the genome database with the Blastx algorithm (Altschul et al. 1997). Primers were designed on each sequence, anticipating an amplification product between 300-350 pb using the Primer 3.0 software (Rozen and Skaletsky 2000). The

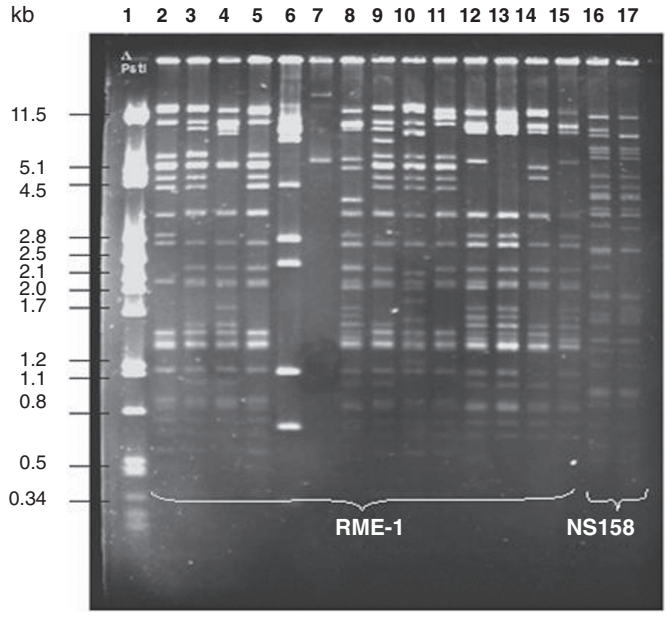

Fig. 15.8. Fingerprinting of positive clones. RME-1 and NS158 clones were digested with *Hind*III and resolved on 1.2% agarose gel.

PCR was conducted under standard conditions. To develop allele-specific primers, the amplified bands from parentals and bulks were eluted, cloned into the commercial vector pGemT-easy, and sequenced at the Iowa State University facility center. Sequences obtained with each primer were aligned using ClustalW (Thompson et al. 1994) to find single nucleotide polymorphism (SNP) regions, and then allele-specific primers were designed using the SNAPER 3 program. To develop SSCP-SNP markers, the PCR amplification products of the parentals and bulks were denatured and separated by single-strand conformational polymorphism (SSCP) gels using a mutation detection enhancement (MDE) gel solution, following the conditions recommended for the author (Bertin et al. 2005). The candidate markers were evaluated in each individual of each bulk (opened bulk) and the recombinant susceptible individuals.

Two contigs from RME-1 positive clones and one contig from NS158 positive clones were obtained with the FPC program. Six BAC clones (# 9, 18, 23, 33, 35, and 36) were identified as the contigs' ends. BAC clones at the extremes of the two contigs were end-sequenced and converted into SSCP markers. The Clustal W analysis of all sequences obtained for each primer permitted us to identify an allele-specific region that showed differences between resistant and susceptible individuals. This region was identified when the sequences from clone # 9 were analyzed, and eight pairs of primers were designed on this region. During the SSCP-SNP primers evaluation, a clear difference between resistant and susceptible bulks with the BAC#33b SSCP-SNP was obtained (Figure 15.9). The SSCP markers were then screened in resistant bulk, susceptible bulk, and the parents of the mapping population, and then used in another round of BAC pool screening. In addition, a sublibrary of the BAC#33 was constructed (Figure 15.10) by digestion with *Hind*III and cloning into the pBluescript vector using standard protocols. The sequencing was conducted at The Institute for Genomic Research (TIGR). Low copy sequences were analyzed in the genome public database and the biotechnology cassava

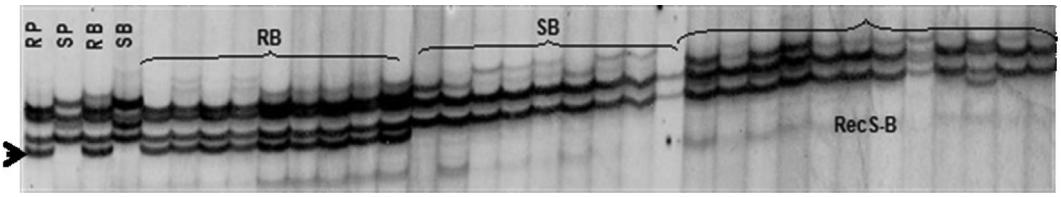

Fig. 15.9. Segregant Bulk Analysis with the BAC33b SSCP-SNP primer. The sample order is: RP (resistant parent), SP (susceptible parent), RB (resistant bulk), SB (susceptible bulk), and RecS-B (recombinant susceptible-bulk). The arrow points to the polymorphic band.

database at CIAT with the Blastx algorithm (Altschul et al. 1997); primers were designed on each sequence selected, with expectation of an amplification product of 300-350 pb, using the Primer 3.0 software (Rozen and Skaletsky 2000). The amplification products were analyzed as SSCP-SNP markers, following the protocol explained previously. A clear difference between resistant and susceptible individuals with the new S-BAC33c SSCP-SNP marker that was consistent in each individual of the bulk (opened bulk) and without amplification in the recombinant susceptible Bulk (RecS-B) was also obtained. Selected BACs were again fingerprinted and assembled into contigs; a new set of SSCP markers were identified based on the BAC end-sequence. Results of successive screenings led to the identification of two markers, BAC33b and SBAC33c, tightly linked to *CMD2*, at genetic distance of 1 cM and 1.5 cM respectively. Additional screening of the BAC library with the SBAC33c SSCP-SNP primer led to the construction of a BAC contig that stretches for about 500kb around *CMD2*. Toward final cloning of the *CMD2* gene, the five BAC clones that traverse a 500 kb region around the gene will be sequenced and annotated to identify candidate R genes.

Significance and Impact of MAB for CMD Resistance

Breeders desire technologies that can lead to improved utilization of genetic resources, improved selection methods, improved productivity in targeted environments, and enhanced potential for more rapid development of new cultivars. MAB is quickly changing the structure and operations of breeding programs in Africa, Latin America, and Asia, as breeders introduce MAS technology into their selection process. Increasing numbers of breeders on the three continents are now rapidly integrating molecular markers into their work, no longer considering it an "add on" but, rather, an integral part of the breeding process. Breeders in countries where MAS is now being used are beginning to combine both genetic and phenotypic selection to maximize breeding effectiveness and efficiency.

In conventional breeding programs, the first two to three years are devoted to screening for CMD resistance before commencement of

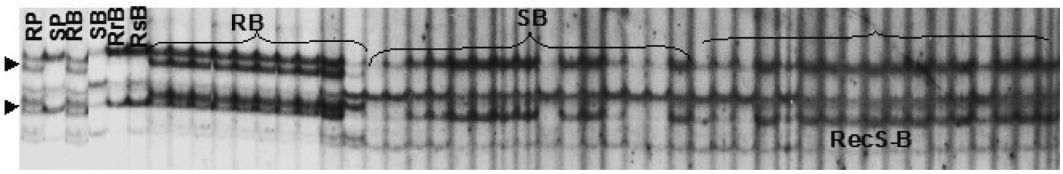

Fig. 15.10. Segregant Bulk Analysis using the S-BAC33c SSCP-SNP primer. The sample order is: RP (resistant parent), SP (susceptible parent), RB (resistant bulk), SB (susceptible bulk), and RecS-B (recombinant susceptible-bulk). The arrow points to the polymorphic band.

advanced yield trials. MAS for CMD permits rapid screening, thereby shortening the process and fast-tracking the use of Neotropical germplasm materials and their possible release as new cultivars in Africa. The advantage with this strategy is that markers are used to preselect Neotropical cassava genotypes for CMD resistance. The advantage of MAS for CMD-resistance breeding is that the breeder can, in early stages, eliminate CMD-susceptible genotypes in parts of the world where the disease is either low or does not exist. In hot-spot zones for the disease, susceptible genotypes can easily be discerned within the first few months (two to six months). However, the challenge for the breeder is often not in identifying the susceptible genotypes but in selecting genotypes with durable and stable resistance, which basically requires field screening for three years (seedling nursery into preliminary yield stages). Because cassava is vegetatively propagated, genotypes with mild or moderate resistance might appear resistant in the first year, but as the innoculum builds up in the vegetative planting materials, there is the tendency for disease severity to increase for such genotypes. Eliminating such genotypes in the first year, thereby reducing costs, would be best achieved with the aid of markers. As new sources of CMD resistance are identified, the need to select for high resistance and gene pyramiding would make marker-aided breeding for CMD resistance the best bet option and almost inevitable. Cassava, as a clonal propagated crop, is often evaluated upon a single plant at the early stages of the selection cycle. MAS can facilitate the efficient reduction of large breeding populations at the seedling stage based on minimum selection criteria. The need for modestly sized breeding populations makes MAS for CMD resistance a powerful tool for accelerating cassava improvement, even in Africa. In the case of a heterozygous CMD-resistant donor parent, elimination would be 50%, reducing the costs of disease evaluation by half and increasing selection efficiency. The breeder can then concentrate on fewer genotypes at the seedling and crucial single-row trial stages, where progenies are reduced by as much as 95%. Identification of markers for other traits in addition to CMD resistance can be used to choose more efficiently parents that combine the different traits. By fixing genes, segregation for CMD resistance can be minimized through careful selection of parents, and, where crosses are made either with susceptible parents or with those that are heterozygous at the CMD loci, segregation at the early stages in breeding is also minimally reduced.

Through the use of CMD markers, the breeding scheme can be fast-tracked, meaning that cultivars could be released in five to six years. The fast-track breeding scheme shown in Figure 15.11, involving MAS for CMD, is currently being used at NRCRI, Nigeria. In East Africa, CBSD is a major viral disease and improved cultivars must essentially show resistance to both CMD and CBSD. An additional step is thus required to introgress CBSD to MAS-developed materials for CMD. At least an additional year is required to release materials compared with the strategy in West Africa. Following the fast-track method, high yielding CMD-resistant LA genotypes have been identified and evaluated in multilocal farmer-participatory trials in Africa. Through MAS, an LA cassava cultivar, CR41-10 (UMUCASS 33), was developed and released in 2010 in Nigeria (within a time frame of six years) and represents the first LA cultivar released in Africa. The cultivar was selected by farmers for its culinary quality, resistance to CMD and tolerance for other pests and disease, and good architecture well suited to the cropping systems used by smallholder farmers. Similarly, in Tanzania four cultivars combining CMD resistance and CBSD tolerance were released in January 2012. The cultivars have a yield of between 30 and 51 tons/ha on farmers' fields. The yield by the new cultivars developed in Tanzania is much higher than yield levels of the local cultivars and old improved varieties under low disease conditions, indicating the possible effects of heterosis on the performance of these cultivars.

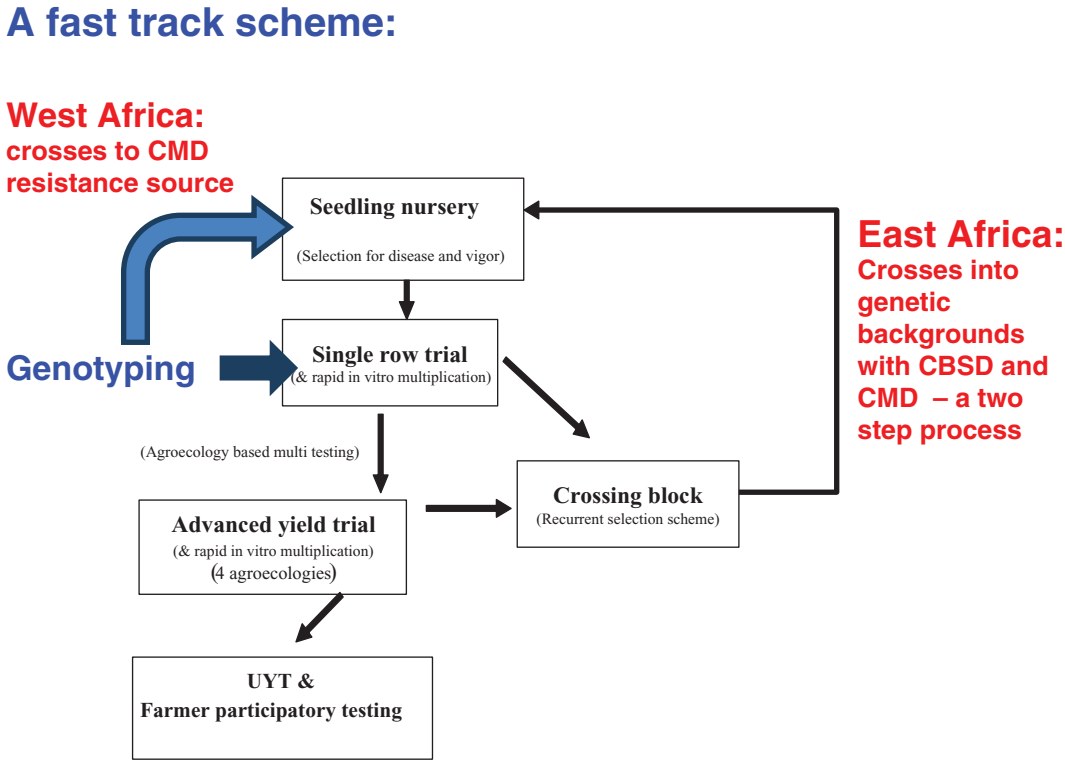

Fig. 15.11. A Marker-Assisted Selection-based fast-track evaluation scheme implemented in the cassava breeding community of practice in Africa, leading to release of cultivars in 5-6 years. For a color version of this figure, please refer to the color plate.

MAS has facilitated the introgression of CMD into backcross derivatives of wild relatives developed for novel traits, which have now been introduced into Africa to add value to the crop. The novel traits include high nitrogenous compound (potentially reflective of high protein levels) and dry matter contents, delayed post harvest physiological deterioration, and drought tolerance. The next wave of LA genotypes to be released as cultivars in Africa will come from the set of germplasm selected for root quality traits (delayed-PPD and high protein content). Following success with MAS, Nigeria is now developing capacity in MARS (marker-assisted recurrent selection) in identifying complex traits such as drought tolerance, with technical support of the CGIAR GCP.

To improve access to genomic studies and MAB, the GCP has initiated the Integrated Breeding Platform (IBP) to provide service support to developing countries to improve their capacity in modern breeding through the cassava breeding CoP. The platform provides online one-stop shopping with centralized and functional access to MAB technologies, value-added germplasm, cost-effective marker services laboratories, data management, and analysis tools. Through efficient marker technologies offered by the platform, NARS are able to overcome technological bottlenecks in MAB (Ribaut et al. 2010).

Ex ante impact assessment studies indicate that cultivars developed with marker-assisted breeding that incorporates CMD and CGM resistances and delayed PPD are worth US$2.89 billion in Nigeria, US$854 million in Ghana, and US$280 million in Uganda over 20 years. If these cultivars were to be developed with resistance to CMD and green mites alone they would be

worth US$1.49 billion in Nigeria, US$675 million in Ghana, and US$52 million in Uganda, if developed through MAS. If developed solely by conventional breeding they would be worth about US$676 million in Nigeria, US$304 million in Ghana, and US$18 million in Uganda. The difference is mostly due to the faster timing of release for the cultivars developed with MAS and the higher probability of success. Several sensitivity analyses were conducted and benefits for MAS range from US$1.7 billion to US$4.3 billion for all three traits, depending on assumptions. In all cases, the research investment is highly profitable from a societal standpoint (Rudi et al. 2010).

The benefits from improved cultivars through breeding have been widely demonstrated, particularly in international agricultural research centers (Heisey et al. 2002; Everson and Gollin 2003) and also in national programs (Brennan et al. 2004). In any plant-breeding program, breeders inevitably have more potential selection traits than can be feasibly targeted with their current resources. As a result, the pressure on all breeding programs to be more efficient and to utilize the latest technologies has increased enormously. Many breeding programs must demonstrate commercial success if they are to continue. Given the circumstances, there are powerful incentives for all breeders to introduce new breeding technologies and to do so to maximum effect (Brennan and Martin 2007).

Future MAB Targets for CMD Resistance

Abiotic stresses and especially CMD account for major fraction-of-yield losses on farmers' fields. Breeding for CMD resistance is therefore critical to boosting productivity in Africa and Asia. Farmers and consumers desire virus-resistant cultivars that meet their criteria. However, yield increase alone is not sufficient for addressing the myriad of problems contended with by cassava farmers, most of whom are poor resource holders. Their criteria include factors related to taste and palatability of the tuberous roots, growth habit, and overall suitability for use in the cropping system.

Improving cassava to meet the dietary needs of consumers for various nutritional attributes aimed at balancing diets and improving the health status of millions is a top-priority breeding target. The expansion of the cassava industry has meant that the crop has to be improved for commercial traits if it is to enhance the value chain and improve income generation for farmers and processors. Effective MAB strategies that enhance the combination of CMD resistance with high yield and value-added traits in a fast-track scheme will be crucial to the successful development of next-generation cassava cultivars that will drive the economies of developing countries. Exploring robust marker-based breeding strategies such as MARs and, in the near future, genome-wide selection (GWS) (Bernardo and Yu 2007) is critical to this step. Once the draft sequence of the cassava genome becomes available, rapid improvement in the use of genomic resources as in genomic selection is likely to be the future direction for improvement of complex traits.

For unknown reasons cassava has evolved with CMG resistance in wild species, and these and African cassava germplasm have been identified as sources of high CMD resistance, the latter probably under the heavy pressure of CMGs in West Africa. The discovery of highly resistant CMD cultivars provides hope for the detection of new genes or QTLs associated with CMD resistance. These will be explored to identify more genomic regions involved in CMD disease resistance. The synergistic relationships among CMGs that lead to higher virulence would probably require such multiple sources of resistance stacked into one cultivar to build a strong resistance that would be more difficult to break by newly emerging CMGs. The identification by the IITA breeding team of additional sources of resistance to CMD opens up possibilities for pyramiding these genes, thereby assuring durability.

The early breeding stages are often devoted to screening for CMD and CBSD (as in the case of East Africa, for two to three years). If genes at targeted loci for CMD are fixed, segregation could be minimized or eliminated altogether, and therefore preliminary MAS for CMD at early stages could be avoided. This fast-tracking would allow breeders the opportunity to rapidly generate planting material and start conducting trials for yield and complex traits, thereby significantly reducing the breeding cycle. The scheme proposed in Nigeria gives preference in selection for parents with fixed genes at targeted loci. MAS for CMD would become necessary only where heterozygous parents for the targeted CMD loci are unavoidably used as donor parents for key traits or to recombine complementary genes for other traits being bred in combination with CMD resistance.

MAB in cassava is currently based on the SSR markers. Although SSRs, are generally multi-allelic, that is, multiple alleles at a locus (Syvänen 2001), and thus highly informative, SNPs are better, because of their high density coverage in the genome and suitability for ultra-high-throughput genotyping techniques (Appleby et al. 2009; Rafalski 2002) required for large scale MAB. The identification of high density of SNPs in cassava should lead to discovery of genic SNPs that could be used to enhance trait predictability in breeding programs. Recently, the GCP converted 1,740 SNPs in cassava for use on the KASPar platform. This system is extremely flexible in terms of the combination of numbers of markers and samples that can be genotyped and, therefore, is particularly suitable for molecular breeding applications, such as MAS or MARS (Morag et al. 2011). The developed SNPs are being deployed in several genetic mapping studies including those for CBSD, CMD, and drought tolerance.

With genome sequencing initiatives in cassava, the development of genomic resources and breeding for cassava is expected to improve rapidly in the years ahead. The first pilot project produced a little less than 1x coverage from more than 700,000 Sanger shotgun reads, using plasmid and fosmid libraries providing insights into the overall characteristics of the cassava genome. Much of the utility of the genome sequence will come from the development of breeding tools. Researchers at 454 LifeSciences and the Joint Genome Institute (JGI) produced the first draft of the cassava genome from a CIAT accession at the end of 2009. The assembly remains highly fragmented (12,000 scaffolds) but is believed to cover 69% of the predicted genome size and to contain 97% of known coding loci (http://www.phytozome.net/cassava.php). The predicted 30,666 genes and 3,485 alternate splice forms are supported by 1.4 M expressed sequence tags (ESTs). Nearly 61 million 454 reads (single and paired-end) were generated and combined with the Sanger data from the pilot project as input for genome assembly. Plans are underway to expand and improve upon the initial cassava genome sequence and to aid SNP discovery via resequencing of many varieties of cassava. These efforts should stimulate major advance in applying modern genomics technology and analytics for improving disease resistance as well as for understanding the genetic basis for disease resistance (Russell et al. 2011).

Having whole-genome sequences allows for the exploration of genomic variations associated with traits of interest. A large number of genomic sequences from a broad array of CMD-resistant varieties of genotypes will enhance marker-assisted CMD breeding in cassava. The rapid advance of genomics, especially large-scale genome resequencing technology, will accelerate the improvement of cassava resistance to pests and diseases and improve other key traits such as yield and quality. These advances will bring a much better understanding of the resistance genes available from various sources and how they can be best deployed in breeding and will also address the methodologies for transferring high resistance to grower-accepted varieties.

Over the past 20 years we have witnessed CMD pandemics in Africa where all the plants already infected by a geminivirus have become

infected with a second geminivirus or by one of the two CBSD viruses. Understanding in detail the molecular mechanisms involved may help us prevent such human catastrophe by deploying new resistant plants that will not succumb to CMGs or other viruses. The molecular mechanisms involved in the control of geminiviruses are not yet known. The challenge for the cassava research and development community will be to bring adequate resources to bear in strengthening efforts to tackle CMD and CBSD scourges in Africa and other disease hotspots.

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Chapter 16

Genetics and Gene Mapping of Disease Resistance in Brassica

Genyi Li and Peter B.E. McVetty

Abstract

The deployment of cultivars resistant to major diseases such as sclerotinia, blackleg, and clubroot in Brassica crop production is a cost effective and environmentally friendly method to reduce losses caused by these diseases. To develop cultivars with high levels of disease resistance, it is necessary to understand the genetics of resistance genes in plants and avirulence genes in pathogens and the mechanism of interactions between host and pathogens. As the technologies in plant genomics are advanced, it becomes feasible and effective to clone and characterize both resistance genes and avirulence genes. There are several avirulence genes of *L. maculans* that have been characterized and the analysis of the whole genome sequence of *L. maculans* reveals more than a hundred of effectors similar to cloned avirulence genes. Gene mapping of resistance genes to clubroot and blackleg have been extensively performed and more than a dozen of resistance gene loci have been identified. As the whole genome sequence of *B. rapa* is available, these mapped genes might be cloned in the near future. Since there is no source with dominant resistance to sclerotinia, it is very challenging to breed Brassica cultivars with high levels of resistance to sclerotinia. QTL (quantitative trait locus) mapping for sclerotinia medium resistance or field tolerance is reported in several publications, and this mapping would help introduce QTLs from cultivar to cultivar, if a QTL can be constantly detected. Because of their relatively small economic impact, other diseases in Brassica crops are not as well characterized as sclerotinia, blackleg, and clubroot. However, the situation may change if a disease becomes severe in brassica crop production.

Introduction

The Brassica genus consists of numerous species that are important agricultural and horticultural crops used as oilseeds, vegetables, and condiments. There are three major diploid and three amphidiploid Brassica species and their relationship is described as the triangle of U, on the basis of their genomes and evolution (Figure 16.1). *B. napus* canola (or double zero rapeseed) is one of the most important oilseed crops worldwide and, based on its fatty acid profile, it is considered to be a healthy vegetable oil for humans. Additionally, canola oil is a feedstock for biodiesel.

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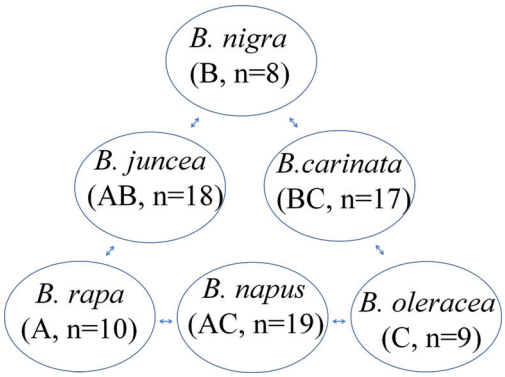

Fig. 16.1. The U-triangle in Brassica species (U 1935). For a color version of this figure, please refer to the color plate.

Therefore, canola oil is in high global demand, which encourages steadily higher canola production. *B. juncea* and *B. rapa* also produce edible oil, consumed in India, China, and other countries. *B. oleracea* vegetables, including cabbage, broccoli, cauliflower, Brussels sprouts, collard greens, kale, Chinese kale, and kohlrabi are cultivated globally, and similarly *B. rapa* vegetables such as Pak choi, Chinese cabbage (Napa cabbage), and turnip are popular in Asia. Two Brassica species including *B. juncea* and *B. nigra* are used for condiment mustard production. Consequently, the Brassica genus plays an important role in the human diet and in the global economy.

Various bacterial, fungal, and viral diseases pose significant risk to Brassica crop production. There are many diseases that affect Brassica species, for example, bacterial soft rot (*Erwinia carotovora*), bacterial leaf spot (*Pseudomonas syringae*), sclerotinia stem rot (*Sclerotinia sclerotiorum*), clubroot (*Plasmodiophora brassicae*), blackleg (*Leptosphaeria maculans*), fusarium wilt (*Fusarium avenaceum* and *F. oxysporum*), verticillium wilt (*Verticillium alboatrum*), light leaf spot (*Pyrenopeziza brassicae*), downey mildew (*Peronospora parasitica*), powdery mildew (*Erysiphe polygoni*), and turnip mosaic virus.

Canola has become one of the most important oilseed crops in the world, second only to soybean. Some as diseases, such sclerotinia, blackleg, and clubroot, cause heavy yield losses worldwide, while some diseases, such as verticillium wilt, light leaf spot, and fusarium wilt are regional problems in canola production. In addition, diseases such as clubroot, downy mildew, bacterial soft rot, and turnip mosaic virus also causes heavy yield losses in Brassica vegetables.

Development and deployment of resistant cultivars is the most commonly used strategy to control diseases in crop production. For most diseases of Brassicas, such as clubroot and blackleg, sources of resistance are identified easily and the resistance can be successfully transferred from variety to variety and from species to species. On the other hand, for other diseases of Brassicas, such as sclerotinia stem rot and bacterial soft rot, it is very difficult to identify sources with high levels of resistance so it is very challenging to develop resistant cultivars for these diseases.

Genetic and genomics research is currently being conducted for many diseases of Brassicas, especially clubroot, blackleg, sclerotinia, downy mildew, and turnip mosaic virus. Molecular markers such as restriction fragment length polymorphism (RFLP); simple sequence repeats (SSR), also known as microsatellite repeats; inter-simple sequence repeat (ISSR); single nucleotide polymorphism (SNP): amplified fragment length polymorphism (AFLP); sequence-related amplified polymorphism (SRAP); sequence-characterized amplified region (SCAR); sequence-tagged sites (STS); and random amplified polymorphic DNA (RAPD) are used for gene mapping, gene cloning, and marker-assisted selection in Brassica crops.

In the amphidiploid species *B. napus*, *B. juncea,* and *B. carinata*, it is necessary to develop genome-specific molecular markers, since most polymorphic loci occur in one genome while their corresponding loci in another genome are monomorphic. To detect all homozygous and heterozygous genotypes in breeding lines and populations, genome-specific primers are necessary in order to amplify the alleles of interest in the genomic DNA. Fortunately, the whole genome sequence of *B. rapa* is available (Wang

et al. 2011) and sequencing of other Brassica genomes is underway. Consequently, all major Brassica genomes will be sequenced in the near future, which will facilitate molecular marker development, gene mapping, and cloning, as well as marker-assisted selection in Brassica crop breeding.

Sclerotinia

Searching for Resistance to Sclerotinia Stem Rot

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most devastating diseases of Brassica oilseed crops. Since *S. sclerotiorum* is non-host specific, necrotrophic, and very aggressive, it can cause heavy yield losses in canola production. Unfortunately, there is no Brassica species with a high level of resistance to sclerotinia stem rot although there are some accessions in *B. napus*, *B. oleracea*, *B. juncea,* and *B. carinata* that confer partial resistance or field tolerance. For example, Zhao and colleagues (2004) evaluated resistance to sclerotinia stem rot in *B. napus,* and CK821 showed a high level of resistance in all tested accessions. Mei and colleagues (2011) did testing of sclerotinia resistance with 68 accessions from six Brassica species, and their data showed that wild species of *B. oleracea* such as *B. insularis* and *B. villosa* are resistant sources to sclerotinia stem rot. Additionally, Garg and colleagues (2010) identified introgression lines developed from wild crucifers and from *B. napus* and *B. juncea* with high levels of resistance to sclerotinia stem rot. Navabi and colleagues (2010) detected B-chromosome carrying *B. napus* lines, derived from an interspecific cross of *B. napus* and *B. carinata,* with a high level of resistance to sclerotinia stem rot.

Optimization of Testing Methods for Sclerotinia Stem Rot Resistance

It is critical to develop an adequate testing method to detect sclerotinia partial resistance. In previous reports, several testing methods, such as cotyledon, detached true leaf, and petiole and stem inoculation, have been developed (Table 16.1). Zhao and colleagues (2004) used a petiole inoculation technique to test 47 accessions of oilseed *B. napus* collected from ten countries and found that most cultivars showing relatively high levels of resistance to sclerotinia stem rot were from China. Bradley and colleagues (2006) tested 19 canola cultivars from Canada and the United States, using three indoor inoculation methods – the petiole inoculation technique (PIT), the detached leaf assay, (DLA), and the oxalic acid assay (OAA) – and also screened a few canola cultivars under field conditions. The results showed that the PIT and the OAA methods significantly differentiated levels of sclerotinia stem rot resistance, while the disease incidence of tested cultivars collected under field conditions over a period of four years was

Table 16.1. Testing methods for sclerotinia resistance in Brassica species*

Inoculation methods	Full Description	Inoculum	Inoculation tissues	Scoring methods	References
CT	Cotyledon test	Mycelial suspension	Cotyledon	Lesion size	Garg et al. 2008
PIT	Petiole inoculation technique	Mycelial agar plug	petiole	Days to wilt	Zhao et al. 2004
DLT	Detached leaf assay	Mycelial agar plug	leaf	Lesion size	Zhao et al. 2003
IPI	Infected petal inoculation	Infected petals	leaf	Lesion size	Yin et al. 2010
MTI	Mycelial toothpick inoculation	Mycelial agar plug	Stem	Lesion length	Zhao et al. 2003
MPI	Mycelial plug inoculation	Mycelial agar plug	Stem	Lesion length	Yin et al. 2010
OAA	Oxalic acid assay	Oxalic acid	Stem	OA concentration	Bradley et al. 2006

*Fungal inoculum is cultured in potato dextrose agar medium (PDA).

not consistent from year to year or from location to location. Li and colleagues (2008) used a stem inoculation method under field conditions to screen 93 genotypes of *B. napus* and *B. juncea* from China and Australia. They found that most resistant accessions were from *B. napus* while most *B. juncea* accessions showed relatively low levels of resistance to sclerotinia stem rot. Detached leaf and stem inoculations were used to test 68 accessions from six other Brassica species, of which 47 belonged to *B. oleracea* and its wild types such as *B. rupestris*, *B. incana*, *B. insularis*, and *B. villosa* (Mei et al. 2011). These *B. oleracea* wild types showed higher levels of resistance to sclerotinia stem rot, suggesting that wild types of *B. oleracea* might be potent sclerotinia resistance sources in canola breeding. In other research, cotyledons were inoculated with a drop of macerated mycelium and this cotyledon inoculation was used to test 32 *B. napus* genotypes (Garg et al. 2008). These authors identified a hypersensitive response and compared the cotyledon testing data with the previously collected field testing data, from which they determined that there was a significant correlation between stem field testing and indoor cotyledon inoculation.

All sclerotinia stem rot resistance screening results reported previously are a quantitative assessment to illustrate that the resistance is genetically controlled and can be adequately measured. In reality, the resistance is not easily or consistently identified. Environmental conditions and inoculation methods play a major role in the detection and quantification of sclerotinia stem rot resistance. In general, stem inoculation is commonly accepted as an effective and reliable method, which is therefore extensively used in genomic analyses.

QTL Mapping of Sclerotinia Stem Rot Resistance

Since sclerotinia stem rot resistance is quantitative, QTL mapping is commonly used to analyze the genetic basis of resistance. Zhao and colleagues (2003) used the detached-leaf and stem inoculation methods to collect phenotypic data from the F3 lines of a segregating population in *B. napus*. Three QTLs for resistance were identified using the leaf inoculation, and three QTL for resistance were identified using the stem inoculation. However, none of the QTLs detected using the detached-leaf technique overlapped with the QTLs identified using the stem inoculation technique. Later, Zhao and colleagues (2006) performed QTL mapping in two doubled haploid (DH) line populations of *B. napus* using two inoculation methods, petiole and stem, for collecting phenotypic data. They aligned their genetic maps with the commonly used genetic map that has the linkage groups N1 to N19 with their corresponding chromosomes (Parkin et al. 1995). In total, eight genomic regions or QTLs in one population, and one region or QTL in another population were identified in four evaluations with two inoculation methods. However, only one QTL on N16 was detected in three evaluations and most other QTLs were detected only once. With regard to the testing methods, three QTLs – on N3, N12, and N16 – were detected in one evaluation. These QTLs explained relatively small portions (6% to 22.7%) of phenotypic variation. In another QTL-mapping report, stem inoculation under field conditions was applied to phenotype a DH population of *B. napus* over a period of four years (Yin et al. 2010). Among all treatments, 17 QTLs were detected. Most of these QTLs were mapped in only one treatment, while some QTLs on N3, N10, and N12 were detected in two to four treatments. The maximum phenotypic variation explained by a single QTL was 36%, but this QTL was detected only three times in two of the four years, and the mapped position of this QTL varied among different treatments and years.

QTL mapping of sclerotinia stem rot resistance suggested that the resistance is controlled at least in part by genetic factors, while environmental conditions play an important role in the development of disease symptoms, which

leads to the challenges of collecting adequate and consistent phenotypes with the same genetic stocks. As described previously, most QTLs were detected only once even if multiple inoculation methods, evaluations, replicates, years, and locations were used. Moreover, most QTLs explained relatively small portions of phenotypic variation, frequently less than 20%. Therefore, it is very difficult to use the mapping information to conduct marker-assisted selection in canola breeding. In the future, it will be necessary to optimize inoculation methods and screening conditions to identify major QTLs consistently with high proportions of explained phenotypic variation, which would lead to efficient and effective marker-assisted selection to improve sclerotinia stem rot resistance.

Clubroot

In *B. napus*, most canola and rapeseed accessions are highly susceptible to *P. brassicae* while in one subspecies – Swedes, or rutabaga – most accessions are resistant to clubroot disease. Recently some canola varieties such as 'Mendel' and 'Tosca' were bred. *P. brassicae* displayed extremely diverse pathogenicity that was reported in many reports (Williams 1966; Voorrips and Visser 1993; Voorrips et al. 1997). To establish a standard classification of pathogen isolates, the European Clubroot Differential (ECD) series, including a total of fifteen accessions, five each of *B. rapa*, *B. napus,* and *B. oleracea*, respectively, were tested in 299 independent experiments, with data collected from 236 tests used to set up 894 ECD triplet codes. The triplet codes were also used to distinguish pathogen isolates in several other publications (Laurens and Thomas 1993; Voorrips and Visser 1993; Voorrips et al. 1997). In contrast, in other research, pathogen isolates were classified into race 1 to 9 according to the interaction between host and pathogen (Williams 1966). Ayers and Lelacheur (1972) used race 2 and 3 to study the genetic basis of clubroot resistance in rutabaga and Figdore and colleagues (1993) used race 7 to analyze clubroot resistance in a cross of broccoli and cauliflower.

Availability of Clubroot Resistant Sources

Clubroot is a devastating disease in Brassica crop production when the cultivars in production are susceptible. This disease causes heavy losses in canola production in European countries and Chinese cabbage production in East Asia. This disease was discovered a few years ago in a single canola field in Alberta, Canada; however, it spread quickly from the original infection site to surrounding areas and has now spread to Saskatchewan, the neighboring province. Currently, most Canadian canola cultivars do not contain clubroot-resistance genes and are therefore highly susceptible to this disease. Consequently, clubroot disease poses a major threat to canola production in the Canadian prairies.

Clubroot resistant cultivars are considered the most feasible solution for controlling this disease effectively. To develop clubroot resistant Brassica crops, knowledge of available genetic sources of resistance is critical. Knowledge of the inheritance of the clubroot-resistance genes is also essential. The range of available clubroot-resistance genes and their inheritance will influence what strategies are used to combine different clubroot-resistance genes. There is genetic complexity in both disease resistance in the host and virulence in the pathogen (Some et al. 1996), suggesting that the development of durable clubroot-resistant Brassica crops will be challenging.

Clubroot resistance in *B. oleracea*, *B. rapa*, *B. napus* and other Brassica species has been extensively screened and tested. For example, Tjallingii (1965) tested several Brassica species, radish, and *Sinapis alba* in 11 locations where infected Brassica crops occurred. In this intensive testing, most turnip and radish accessions were resistant, while cauliflower, cabbage, *B. napus* accessions, and *S. alba* accessions were susceptible in most cases. In another report, *B.*

napus canola cultivars were tested and only a few lines showed low levels of resistance in Quebec, Canada (Vigier et al. 1989).

In *B. oleracea*, Portuguese cole landraces were screened for clubroot resistance, and among 44 accessions, two cabbage cultivars and one kale showed the highest clubroot resistance under field conditions (Dias et al. 1993). Moreover, of 71 accessions of *B. oleracea* evaluated using indoor testing, several cabbage, broccoli, and kale that had high levels of clubroot resistance were identified (Voorrip and Visser 1993). Similarly, Manzanares-Dauleux and colleagues (2000b) tested 240 kale, 38 cabbage, and 126 winter cauliflower from the *B. oleracea* French landrace gene pool. Among these 404 accessions, two kale lines were identified with high levels of clubroot resistance that, it was suggested, could be valuable sources for breeding clubroot-resistant Brassica vegetables such as broccoli and cauliflower. In yet another report, clubroot resistance in 48 *B. oleracea* accessions together with a few *B. rapa* and *B. napus* accessions were tested, in which one *B. rapa* turnip and one *B. napus* displayed the highest levels of resistance (Carlsson et al. 2004). Clubroot resistance in *B. oleracea* is commonly identified. Some accessions of kale, cabbage, broccoli, and Brussels sprouts have displayed resistance to clubroot disease, whereas most of these sources of resistance have a relatively lower level of clubroot resistance than that possessed by European fodder turnips.

In *B. rapa*, most accessions, especially conventional Chinese cabbage cultivars, are highly susceptible to *P. brassicae*. Fortunately, as described previously, European fodder turnips (*B. rapa ssp rapifera*) are strongly resistant to clubroot disease (Crute et al. 1983). The European fodder turnip resistance sources have been used successfully to introduce resistance genes into Chinese cabbage in order to develop clubroot-resistant hybrid cultivars. These clubroot-resistant hybrid cultivars were first developed and extensively used to control clubroot disease in Chinese cabbage production in Japan. Currently, Chinese cabbage cultivars with the turnip resistance genes are commonly used to control clubroot disease in Asian countries such as Japan, South Korea, and China.

Genetic Analysis of Clubroot Resistance

Understanding the genetic basis of resistance to *P. brassicae* in Brassica species is vital, and dozens of reports on the genetics of clubroot-resistance are available in *B. oleracea*, *B. rapa*, *B. napus,* and other Brassica species (Chiang and Crete 1970; Crute et al. 1980; Voorrips and Visser 1993; Voorrips et al. 1997). Early genetic studies on clubroot resistance were done mainly in *B. oleracea* (Chiang and Crete 1970; Crute et al. 1980). Since the resistance in *B. oleracea* varies with regards to the materials used, a multiple-gene model for resistance genes was commonly reported. In contrast, turnip resistance is strong and dominant, with a single gene or a few major genes commonly detected and reported in segregating populations. Voorrips and Visser (1993) found that the hybrids of highly resistant cabbage and kale, and highly susceptible cabbage were totally susceptible; suggesting that the clubroot resistance in these cabbage and kale lines was controlled by recessive genes. In a diallel analysis of clubroot resistance among several cabbage cultivars, additive effects were found to be stronger than dominant effects (Chiang and Crete 1976). In contrast, six kale lines were used to make crosses in a diallel mating design and analysis of the clubroot-resistance data showed that incomplete dominant effects were more important than additive effects (Laurens and Thomas 1993).

Genetic analysis is generally used to detect resistance gene loci based on the segregation of the clubroot-resistance phenotype. Using two clubroot-resistant accessions of *B. napus* rutabaga, a single major dominant gene locus was detected in both rutabaga accessions, whereas another dominant resistance gene locus was identified in one of these two rutabaga

parental lines (Ayers and Lelacheur 1972). The inheritance of clubroot resistance in crosses of cabbage also showed that the resistance was determined by two unlinked complementary genes in cabbage (Voorrips et al.1997). *B. napus* is an amphidiploid species and originally evolved from the interspecific hybridization of the two parental diploid species, *B. rapa* and *B. oleracea*. Similar to the reports in the parental diploid species, a few genes were commonly identified as conferring clubroot resistance in *B. napus* (Manzanares-Dauleux et al. 2000b).

Gene Mapping of Clubroot Resistance

As genomics research advances, molecular markers become increasingly useful tools for studying clubroot resistance. In *B. oleracea*, Figdore and colleagues (1993) associated RFLP markers with a major clubroot-resistance locus in broccoli. Grandclement and Thomas (1996) used RAPD markers to identify two QTLs for clubroot-resistance genes in kale. Voorrips and colleagues (1997) constructed a genetic map with AFLP markers that was used to identify two clubroot-resistance QTLs, pb-3 and pb4, in cabbage. Similarly, a genetic map constructed with several types of molecular markers and four single-spore isolates was used to identify nine genomic regions anchoring clubroot-resistance genes in kale (Rocherieux et al. 2004).

In *B. napus*, Landry and colleagues (1992) mapped two clubroot-resistance loci to *P. brassicae* race 2 isolates. Manzanares-Dauleux and colleagues (2000a) mapped a major gene and QTL that conferred resistance to clubroot disease in *B. napus*. In another report, seven different isolates were used to detect nineteen QTLs in one *B. napus* DH-mapping population and all QTLs were found to be race specific (Werner et al. 2008). Additionally, the results in this report indicated that the broad resistance in the diploid parental line of *B. oleracea* disappeared in newly resynthesized *B. napus* lines, a result of epistatic interactions when clubroot-resistant *B. oleracea* was used to produce these synthetic *B. napus* lines and their corresponding mapping populations.

In *B. rapa*, early genetic studies of resistant turnips suggested that dominant race-specific genes conferred clubroot resistance in the European fodder turnips. These results were confirmed through gene mapping in Chinese cabbage cultivars in which clubroot-resistance genes were introgressed from the European fodder turnips. For example, Kuginuki and colleagues (1997) found RAPD markers linked to a clubroot-resistance gene. Similarly, Suwabe and colleagues (2003, 2006) used dozens of simple sequence repeats (SSRs) in *B. rapa* to identify the SSR markers linked to two major clubroot-resistance genes *Crr1* and *Crr2*. Hirai and colleagues (2004) added another dominant clubroot-resistance gene, *Crr3,* to the list. Additionally, *Crr4* was detected as a QTL that confers resistance to clubroot disease (Suwabe et al. 2006).

Similarly, Matsumoto and colleagues (1998) identified a major dominant clubroot-resistance gene locus, *CRa,* on linkage group LG3 of an RFLP genetic map of *B. rapa*. Another major locus, *CRb* in *B. rapa,* conferring resistance to *P. brassicae,* was mapped and closely linked molecular markers to this major locus were developed (Piao et al. 2004). More recently, Sakamoto (2008) detected two major clubroot-resistance gene loci, *CRk* and *CRc,* on linkage groups R3 and R2 in *B. rapa*. *CRk* was mapped to the region similar to where *Crr3* was located, suggesting that *CRk* and *Crr3* might be the same gene or different genes in the same genomic region of *B. rapa*.

Comparative Genomics in Clubroot Resistance

Comparative mapping of clubroot-resistance genes in Chinese cabbage has progressed dramatically because *B. rapa* is a diploid species and its whole genome sequence is available (Wang et al. 2011). According to the previous description, eight dominant clubroot-resistance

genes – *Crr1, Crr2, Crr3, Crr4, CRa, CRb*, *CRk,* and *CRc* – in Chinese cabbage, introduced from different European fodder turnips were mapped on different linkage groups in work done in Japan (Hirai et al. 2004; Hirai 2006; Matsumoto et al. 1998; Piao et al. 2004; Saito et al. 2006; Suwabe et al. 2006). Furthermore, most of the mapped clubroot-resistance genes were integrated on the corresponding chromosomes. *Crr3*, *CRa, CRb,* and *CRk* were mapped to chromosome R3, and it seems that *Crr3* and *CRk* might be allelic, while *CRa* and *CRb* are located at other genomic regions of the same chromosome. *Crr1*, *Crr2*, *Crr4,* and *CRc* were mapped to chromosomes R8, R1, R6, and R2, respectively.

For understanding the complexity of clubroot resistance in Brassica species, gene cloning will provide answers to many questions about gene locations, functions, and interactions between clubroot-resistance genes and pathogen isolates. Since *B. rapa* contains dominant Mendelian clubroot resistance, it would be expected that the clubroot-resistance genes would be cloned from *B. rapa* first. Saito and colleagues (2006) fine mapped clubroot-resistance gene *Crr3* and used the sequence-tagged site (STS) markers to perform comparative genomics with the Arabidopsis genome. They suggested that the *Crr3* gene on R3 in *B. rapa* is located in a genomic location sharing sequence similarity to a region of chromosome 3 in Arabidopsis. However, flanking SCAR markers of *CRb* share sequence similarity to a region of chromosome 4 in Arabidopsis, suggesting that *CRb* is located in a position that is different from that of the *Crr3* gene, since both the genes were mapped on the R3 chromosome (Piao et al. 2008).

Successful Use of Clubroot-Resistance Genes in Chinese Cabbage

Clubroot-resistance genes from European fodder turnips have been successfully used to develop clubroot-resistant Chinese cabbage cultivars in Japan, and these clubroot-resistant cultivars have been successfully used to control clubroot disease in Japanese production of Chinese cabbage for many years. However, after the clubroot-resistant Chinese cabbage cultivars were in production for a few years, it was found that some of the clubroot-resistant Chinese cabbage cultivars had become susceptible, suggesting that in Japan the resistance of some clubroot-resistance genes had been overcome (Kuginuki et al. 1999). Fortunately, most mapped clubroot-resistance genes are located in different chromosomes or in different genomic regions of the same chromosome, which would allow gene pyramiding to combine multiple clubroot-resistance genes into a cultivar. Pyramiding of different clubroot-resistance genes from various sources may facilitate the development of durable clubroot resistance in the future.

Marker-assisted selection is essential for the successful pyramiding of disease-resistance genes. The abundant repertoire of clubroot-resistance genes in Brassica species offers some opportunities to produce clubroot-resistant cultivars. On the other hand, it also poses challenges to exploiting different clubroot-resistance sources. In particular, gene introgression from species to species is often necessary, and multiple genes are required for good resistance. In interspecific gene transfer, the flanking regions of resistance genes are often incorporated into the new species as a result of linkage drag, and some traits in the introgressed lines become problematic, especially in chromosome regions where few recombination events occur.

For pyramiding multiple resistance genes where it is not easy to distinguish the phenotypic differences among individuals with one, two, or more resistance genes, it becomes difficult to transfer these genes using conventional breeding. Actually, most disease-resistance genes interact in this way, and each gene gives only a relatively small contribution to the phenotypic variation. Therefore, molecular marker-assisted selection (MAS) becomes mandatory in order to transfer multiple resistance genes simultaneously.

To achieve effective and efficient MAS, gene effects and gene locations in genomes have to be well characterized. Meanwhile, easily detected and closely linked molecular markers should be developed to implement MAS in breeding.

Blackleg

Pathogenicity Groups and Differentiation Hosts

Blackleg disease is caused by *L. manculans*, a fungal pathogen with extensive pathogenicity differentiation. The interaction of the blackleg pathogen and plant resistance, first tested using a cotyledon inoculation method (Williams and Delwiche 1979), is commonly used to detect sources of resistance and pathogenicity groups. The first differential set including three canola cultivars – 'Westar,' 'Glacier,' and 'Quinta' – was established to classify pathogen isolates into three pathogenicity groups, PG2, PG3, and PG4 (Mengistu et al. 1991). 'Westar' is susceptible to all virulent isolates and accepted as the best check that is used in most blackleg-resistance studies. 'Glacier' is resistant to PG2 and susceptible to PG3 and PG4 isolates. 'Quinta' is resistant to both PG2 and PG3 but susceptible to PG4. With a different set of canola cultivars including 'Lirabon,' 'Glacier,' 'Quinta,' and 'Jet Neuf,' pathogen isolates were classified into A1 to A6 groups (Badawy et al. 1991). Actually in the A-group classification, the susceptible canola cultivar 'Westar' was replaced with 'Lirabon' and PG2, PG3, and PG4 were subdivided into two groups using 'Jet Neuf' as a host differential cultivar. Later, the PGT group was added to the PG groups. The PGT isolates were virulent to 'Quinta' but avirulent to 'Glacier.'

Genetics of Blackleg Disease Resistance

There are two types of blackleg disease resistance detected from the seedling (or cotyledon) stage to the adult stage. Seedling resistance is analyzed using cotyledon inoculation, whereas adult-stage resistance is measured at a late developmental stage approaching maturity, especially the development of the stem canker in greenhouses or under field conditions (Rimmer and van den Berg 1992).

In genetic studies, blackleg-resistance segregation in a doubled haploid (DH) line population derived from a cross between 'Westar' and 'Cresor' was tested under field conditions with a dominant major locus identified (Dion et al. 1995). Similarly, Pang and Halloran (1996) detected a single dominant resistance gene locus in 'Maluka' conferring adult stage resistance. Blackleg resistance is commonly identified in most Brassica species, for example, *B. rapa* (AA), *B. napus* (AACC), *B. juncea* (AABB), *B. nigra* (BB), and *B. carinata* (BBCC), while all tested accessions in *B. oleracea* (CC) are susceptible, suggesting that this diploid Brassica species does not contain blackleg disease resistance genes (Monteiro and Williams 1989). Since *B. juncea*, *B. nigra,* and *B. carinata* show strong resistance to *L. maculans*, the previous reports suggest that resistance genes in these three species may exist in the B genome of Brassica species (Roy 1984; Chevre et al. 1997; Struss et al. 1996).

Genetic analysis of the resistance genes in the B genome has been performed, and gene introgression via interspecific hybridization has been extensively used to move the B genome resistance genes into canola. For example, Roy (1984) reported that blackleg resistance to *L. maculans* from *B. juncea* had been introduced into *B. napus*. Moreover, *B. napus-B. nigra* and *B. oleracea-B. nigra* additional lines were used to pinpoint the B genome chromosomes that carry blackleg-resistance genes (Chevre et al. 1996, Chevre et al. 1997). Struss and colleagues (1996) reported that two or three B chromosomes anchored blackleg-resistance genes and Chevre and colleagues (1997) illustrated that blackleg-resistance genes

are located on the B4 and B8 chromosomes of *B. nigra*.

Gene Mapping of Blackleg Resistance

Gene mapping allows the detection of individual disease-resistance genes and the comparison of major dominant resistance genes from different resistance sources. The earlier reports used very limited numbers of RFLP, AFLP, and RAPD markers and the linkage groups that were reported to anchor blackleg-resistance genes were unique in each publication. A major resistance gene locus in 'Cresor' was mapped as a quantitative trait locus (QTL), explaining 72% of phenotypic variation in all tested environments, suggesting that it might be a dominant resistance gene in this cultivar (Dion et al. 1995). Ferreira and colleagues (1995) mapped a single major locus controlling cotyledon resistance (*LEM1*) in 'Major' to linkage group 6. RAPD and AFLP markers were used to pinpoint a major resistance gene locus *Lmr1* and *cRLMm* in 'Shiralee' and 'Maluka,' respectively (Mayerhofer et al. 1997). All these three resistance genes, *LEM1*, *LmR1,* and *cRLMm* were linked on the same linkage group N7 (Rimmer 2006).

Genetic interaction between pathogen isolates and host cultivars was used to name the resistance genes in the host that corresponded to their avirulence gene in the pathogen (Ansan-Melayah et al. 1995, 1998). According to the interaction between *L. maculans* and hosts, two dominant resistant genes, *Rlm1* in 'Quinta' and *Rlm2* in 'Glacier,' were described. In addition to these two resistance genes, another dominant resistance gene in 'Quinta' was also inferred. Later, *Rlm3* in 'Glacier' was separated from the previously reported *Rlm2* (Balesdent et al. 2006). Similarly, *Rlm4* was identified as linked with *Rlm1*, but mapped to different positions in 'Quinta.' *Rlm4* also was detected in 'Net Jeuf' (Balesdent et al. 2001). In addition, *Rlm7* in a breeding line 23.1.1 and *Rlm9* in 'Yudal' were mapped (Balesdent et al. 2001). *Rlm1*, *Rlm3*, *Rlm4*, *Rlm7,* and *Rlm9* formed a resistance gene cluster on linkage group 10, according to comparative linkage analysis (Delourme et al. 2004).

As molecular marker development advances, mapping of blackleg-resistance genes will facilitate the detection of similar linkage groups and chromosomes in different laboratories. Several mapped genes such as *LEM1*, *cRLMm,* and *LmR1* on linkage group 6 in Dr. Rimmer's group were suggested to be on linkage group N7 of the commonly used genetic map (Parkin et al. 1995). Similarly, all resistance genes mapped on linkage group 10 in France are believed to be on the same linkage group N7, and *Rlm2* on linkage group 16 should be on linkage group N10 on the Parkin genetic map (Delourme et al. 2006). Gene cloning has been attempted but it was found that it is too difficult to clone a blackleg-resistance gene on linkage group N7 because of the complexity of this chromosome (Mayerhofer et al. 2005). However, until all the previously mentioned resistance genes on linkage group N7 are cloned and sequenced, their linkage and allelic relationships will remain undetermined.

Mapping Blackleg-Resistance Genes Introduced from Related Species

All the previously described resistance genes on N7 and N10 have been identified in *B. napus*. Actually, resistance genes from other Brassica species such as *B. rapa*, *B. nigra,* and *B. juncea* have been introduced into *B. napus* through interspecifc hybridization, and these resistance sources are commonly used to develop commercial canola cultivars. For instance, *Rlm5* and *Rlm6* were introduced from *B. juncea* to *B. npaus* and Rlm8 from *B. rapa,* although these gene loci have not been characterized (Delourme et al. 2006). Most domesticated strains of *B. rapa* are susceptible to *L. maculans*, but a wild-type accession, *B. rapa* subsp. sylvestris, is resistant to *L. maculans* and this accession has been used to introgress several blackleg-resistance

genes, such as *LepR1*, *LepR2*, *LepR3,* and *LepR4,* into *B. napus* (Yu et al. 2005, 2008, 2012). The gene introgression was first performed in the development of the canola cultivar 'Surpass 400' and this canola cultivar was then used to develop a series of commercial canola cultivars in Australia. All sylvestris-derived canola cultivars theoretically contained blackleg-resistance genes introduced from the wild *B. rapa* subsp. sylvestris. Currently, 'Surpass 400' is commonly used in canola breeding programs by Canadian breeding organizations. Yu and colleagues (2008) mapped a dominant blackleg-resistance locus, named *LepR3* on linkage group N10 in 'Surpass 400.' More recently, in the same region on linkage group N10 in 'Surpass 400,' two separate resistance genes, *BLMR1* and *BLMR2,* were identified, and *BLMR1* was fine mapped (Long et al. 2010). Using a map-based cloning strategy, *BLMR1* was successfully cloned and functionally confirmed through complementary transformation of the susceptible cultivar 'Westar.' This is the first blackleg-resistance gene that has ever been cloned in *B. napus* (Li et al. 2010 and unpublished data).

The B genome Brassica species, including *B. nigra*, *B. juncea,* and *B. carinata*, have a high level of resistance to *L. maculans*. Gene introgression from the B genome to *B. napus* has been extensively performed with the blackleg-resistance genes introduced into canola. Using RAPD markers and *B. napus*-*B. nigra* additional lines, the B8 chromosome of *B. nigra* was shown to carry blackleg-resistance genes. A genetic map constructed with RFLP molecular markers was used to illustrate that a blackleg-resistance gene locus is located on linkage group JR13, suggesting that the blackleg-resistance gene in *B. juncea* belongs to the B genome. In general, genetic mapping of blackleg-resistance genes introgressed from the B genome into the A and C genomes in *B. napus* has not been successful, since the B genome is quite different from the A and C genomes and recombination between the B and A, B, and C genomes is very low.

Mapping and Cloning of Avirulence Genes in *L. maculans*

Based on a gene-for-gene interaction theory, several avirulence genes, such as *Avrlm1* to *Avrlm9,* have been successfully identified using cultivars and breeding lines harboring resistance genes to blackleg (Balesdent et al. 2006), and *Avrlm1*, *Avrlm6,* and *Avrlm4-7* have been characterized (Gout et al. 2006, Fudal et al. 2007, Parlange et al. 2009). These cloned avirulence genes are coded for secreted small proteins (SSPs) as fungal effectors in the interactions of plant host and pathogen. More recently, after the whole genome of *L. maculans* was assembled, 651 SSP genes in the genome of *L. macunlans* were predicted (Rouxel et al. 2011). The authors suggested that 122 SSP genes in AT-blocks might correspond to avirulence genes to blackleg or other biotic stresses, and those SSPs in GC-blocks might not belong to avirulence genes, since they lack the features of known effectors (Rouxel et al. 2011). Obviously, the numbers of SSP genes in the genome of *L. maculans* are more than those of the mapped resistance-gene loci described previously in Brassica species; it is highly possible that not every SSP gene belongs to an avirulence gene. Otherwise, one blackleg-resistance gene might interact with more than one SSP gene. Therefore, after resistance genes to blackleg are cloned in the future, the interactions of effectors and resistance genes can be investigated further.

QTL Mapping for Blackleg Resistance

Disease resistance is commonly classified into qualitative and quantitative traits that are controlled by major and minor genes respectively. All mapped blackleg-resistance genes described previously are major genes, which display a typical Mendelian segregation ratio in mapping populations. Since these dominant or recessive resistance genes are considered to be race specific, this resistance might be overcome by

new virulence genes evolved in the pathogen based on the gene-for-gene interaction model. In contrast, quantitative resistance is conferred by race nonspecific minor genes, the resistance is thought to be more durable even though quantitative resistance genes are not individually as strong as race-specific resistance genes, and quantitative resistance is much more difficult to manipulate.

Quantitative trait loci (QTLs) are analyzed through QTL mapping. Ferreira and colleagues (1995) detected two QTLs for blackleg disease resistance in *B. napus* under field conditions. Similarly, Pilet and colleagues (1998) described ten field resistance QTLs in one cross and later ten QTLs in another cross; however, only four QTLs were common to the previously reported ones in *B. napus* (Pilet et al. 2001). In a recent report, more than a dozen QTLs of resistance to blackleg have been identified (Kaur et al. 2009). The genomic regions controlling race nonspecific QTLs and race-specific resistance genes are not commonly co-located, but there is one QTL sharing a similar genomic region where a race-specific resistance gene Rlm2 is mapped on linkage group N10 (Pilet et al. 2001, Delourme et al. 2006) and another QTL was co-located with a dominant resistance gene locus *Rlm4* (Raman et al. 2012).

Genetic Mapping of Resistance to Other Brassica Diseases

Compared with the previously described major diseases in canola, other Brassica diseases are not well characterized, since the losses due to these diseases in Brassica species are, in general, not as great as those caused by sclerotinia, clubroot, and blackleg in canola. Ren and colleagues (2001) performed genetic analysis of resistance to bacterial soft rot caused by *Erwinia carotovora* with 25 accessions of *B. rapa* vegetable and oilseed types. The results showed that the resistance was quantitative, whereas the narrow-sense heritability of the resistance ranged from 42% to 60% in a Griffing's diallel analysis.

Downy mildew is a serious disease in broccoli and Chinese cabbage, and resistance sources have been identified in Brassica vegetables. Farnham and colleagues (2002) observed a single dominant resistance gene in broccoli that segregated in a Mendelian fashion in F2 and BC1 populations.

For turnip mosaic virus disease, Hughes and colleagues (2002) screened 42 *B. rapa* and *B. napus* accessions to identify resistance to turnip mosaic virus (TuMV) isolates representing the three major pathotypes in Europe. Most tested accessions were found to be resistant to TuMV disease, and 14 out of 42 accessions were resistant to all three used pathogen pathotypes. Genetic analysis in individual accessions indicated that the resistance might be controlled by single or multiple, dominant or recessive resistance gene loci in various accessions, based on the interaction between genotypes and pathotypes observed.

Genetic mapping of resistance genes has been performed for several Brassica diseases such as downy mildew, TuMV. In *B. napus* and *B. rapa*, several dominant and recessive resistance gene loci have been mapped with RFLP and SSR markers (Walsh et al. 1999; Hughes et al. 2003; Rusholme et al. 2007). Two gene loci, *ConTR01* and *retr01* were located on chromosome R4 and R8 (Rusholme et al. 2007). Another TuMV-resistance gene locus, *TuRB03*, was assigned to a region on chromosome N6 where two other gene loci, *TuRB01* and *TuRB01b,* were mapped, suggesting that these three resistance gene loci might be allelic (Walsh et al. 1999; Hughes et al. 2003).

In genetic mapping of resistance genes for downy mildew, a genetic map constructed with RFLP, SSR, ISSR, and RAPD markers was used to map a major dominant resistance-gene locus in a cross of rapid cycling kale and broccoli (Farinhó et al. 2004). In Chinese cabbage, Yu and colleagues (2009) used SSR, STS, SRAP, and enzyme markers to perform QTL mapping of downy mildew resistance and identified a major downy mildew resistance QTL on chromosome A8.

Marker-Assisted Selection for Disease Resistance in Brassica Crop Breeding

Mapping and cloning of disease-resistance genes will facilitate the practical use of molecular markers in Brassica crop breeding through marker-assisted selection. Currently, major blackleg- and clubroot-resistance genes are commonly used in canola, Chinese cabbage, and other Brassica vegetable crops, and MAS is successfully integrated with conventional breeding selection at many breeding organizations. However, most major blackleg- and clubroot-resistance genes have not been cloned yet, pyramiding of multiple resistance genes is randomly performed, and the results are quite elusive. After resistance genes are cloned, resistance gene-specific molecular markers will be developed easily which will facilitate the pyramiding of different race-specific resistance genes. Multiple race-specific dominant resistance genes are combined with quantitative resistance genes, which may result in stronger and longer lasting blackleg and clubroot resistance.

Genome-specific molecular markers are prerequisite for efficient and effective MAS in allotetraploid Brassica crop species. As described in the triangle of U, Brassica species are genetically classified into diploid and allotetraploid groups. In general, development and detection of molecular markers in diploid species is relatively easier than that in allotetraploid species. In the diploid Brassica species, most allelic variants can be used to develop high quality molecular markers, although sequence similarity of homologs in gene and segmental duplications in the genome may interfere with the detection of a few allelic variants. In the allotetraploid species, intrasubgenome and intersubgenome polymorphism co-exist, whereas only allelic variants in intrasubgenomes are useful for the development of genome-specific molecular markers. Since there is a high level of sequence similarity between the A, B, and C subgenomes of the allotetraploid Brassica species, most allelic variants in one subgenome correspond to monomorphic loci in another subgenome. For example, 18,066 out of 23,037 SNPs on two genetic maps in *B. napus* were polymorphic in one subgenome and monomorphic in another one, and these molecular markers were identified as hemi-SNPs (Bancroft et al. 2011). Although hemi-SNPs can be detected through next-generation sequencing, it is not practical to directly sequence hemi-SNPs for marker-assisted selection in crop breeding, where thousands of individuals are commonly included. To deal with hemi-SNPs, a strategy for developing genome-specific molecular markers as was done for the *FAE1.1* gene for marker-assisted selection of the erucic acid trait in crosses of rapeseed and canola might be used (Rahman et al. 2008). In this case, genome-specific primers flanking allelic variants are used to amplify the targeted allelic variants in one subgenome and then PCR products containing the targeted SNPs can be detected with all the commonly used SNP detection methods.

Marker-assisted selection is very useful for eliminating linkage drag in introgression of disease-resistance genes from related species or wild species in Brassica. In general, most agronomic traits in wild species are not as good as those in cultivated species. Moreover, most agronomic traits in Brassica vegetables are quite different from those in Brassica oilseed crops. When disease-resistance genes are introgressed from wild species to cultivated species, or from Brassica vegetables to oilseed species or vice versa, linkage drag may occur if the genes underlying important agronomic traits are flanking these disease resistance genes. For example, two blackleg-resistance genes on chromosome N10 in *B. napus* were introgressed from wild species *B. rapa* subsp. sylvestris L. with a genetic distance of 20 cM between these two resistance genes (Long et al. 2011). In canola breeding, these two genes from wild *B. rapa* and the genes in the middle between these two blackleg-resistance genes is retained in genotypes when a high level of blackleg resistance is selected. Recently, new canola lines, in which the wild

B. rapa chromosome fragment between these two blackleg-resistance genes was eliminated, were developed using molecular markers for one cloned gene and one fine-mapped blackleg-resistance gene (unpublished data).

Summary

Disease resistance is a very important trait in plant breeding, since deployment of resistant cultivars is almost always an effective and efficient method for reducing losses caused by different diseases. Therefore, resistance to most major diseases such as sclerotinia stem rot, clubroot, and blackleg in Brassica species has been extensively studied and significant progress towards the development of resistant cultivars achieved. Although it is not easy to identify sources with high levels of resistance to sclerotinia stem rot, some accessions such as Chinese canola cultivars display partial resistance or field tolerance, and these may facilitate the development of canola hybrid cultivars with an effective level of resistance to sclerotinia stem rot. Indeed, most breeding organizations worldwide have this goal in their breeding programs. Clubroot is so devastating that clubroot resistance becomes a prerequisite trait for releasing new cultivars in the regions of the world where the soil-borne clubroot pathogen prevails. Fortunately, excellent sources with high levels of resistance to *P. brassicae* have been identified in several Brassica species, and gene mapping of dominant resistance genes in *B. rapa* have been performed and gene cloning is underway. Blackleg is another important disease in canola and extensive research of pathogenicity, mapping of major dominant resistance genes, and introgression of resistance genes from allied species into *B. napus* canola has been accomplished. In fact, this disease has been controlled effectively and efficiently in most canola-production areas in the world. Moreover, cloning of a blackleg-resistance gene has been successfully done and more blackleg-resistance genes will be cloned in the near future, which will provide a solid basis for the pyramiding of different resistance genes into single lines and this may facilitate the development of durable resistance to blackleg disease in canola breeding. As described previously, good progress has also been made in controlling other diseases, such as downy mildew and turnip mosaic virus, via the development of resistant cultivars, since sources with high levels of resistance to these diseases are available. Finally, genome sequencing will facilitate gene mapping and gene cloning and in turn, promote the development of high quality and high throughput molecular markers. Undoubtedly, molecular marker-assisted selection is playing and will increasingly play a very important role in modern breeding in Brassica species.

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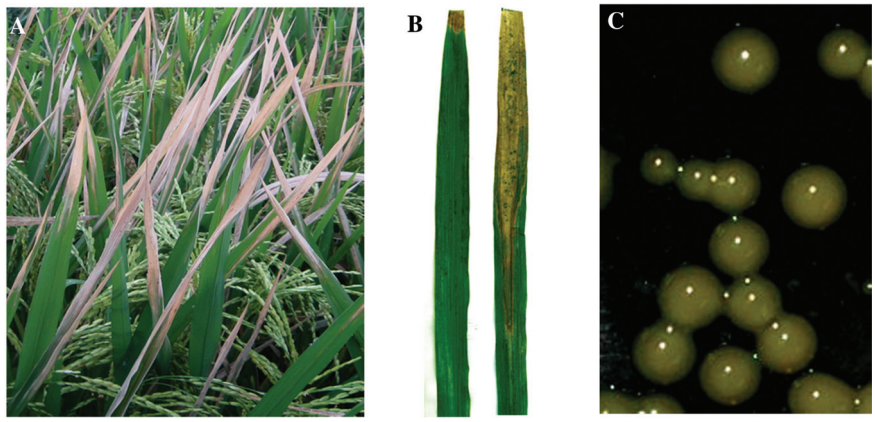

Plate 2.1. Bacterial blight disease of rice. (A) Rice cultivar infected by *Xoo*. (B) Infected rice leaves after artificial inoculation of *Xoo*. (C) *Xoo* colonies.

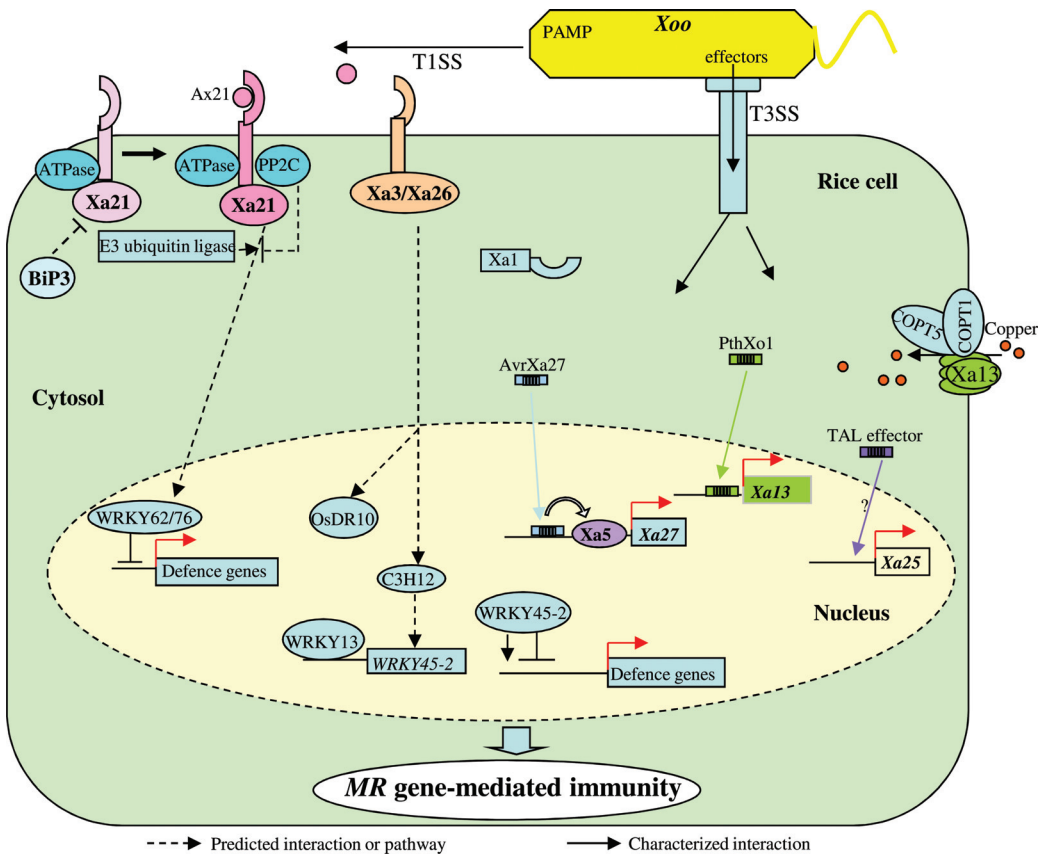

Plate 2.2. Molecular mechanisms of characterized major disease resistance gene-mediated resistance to *Xoo*.

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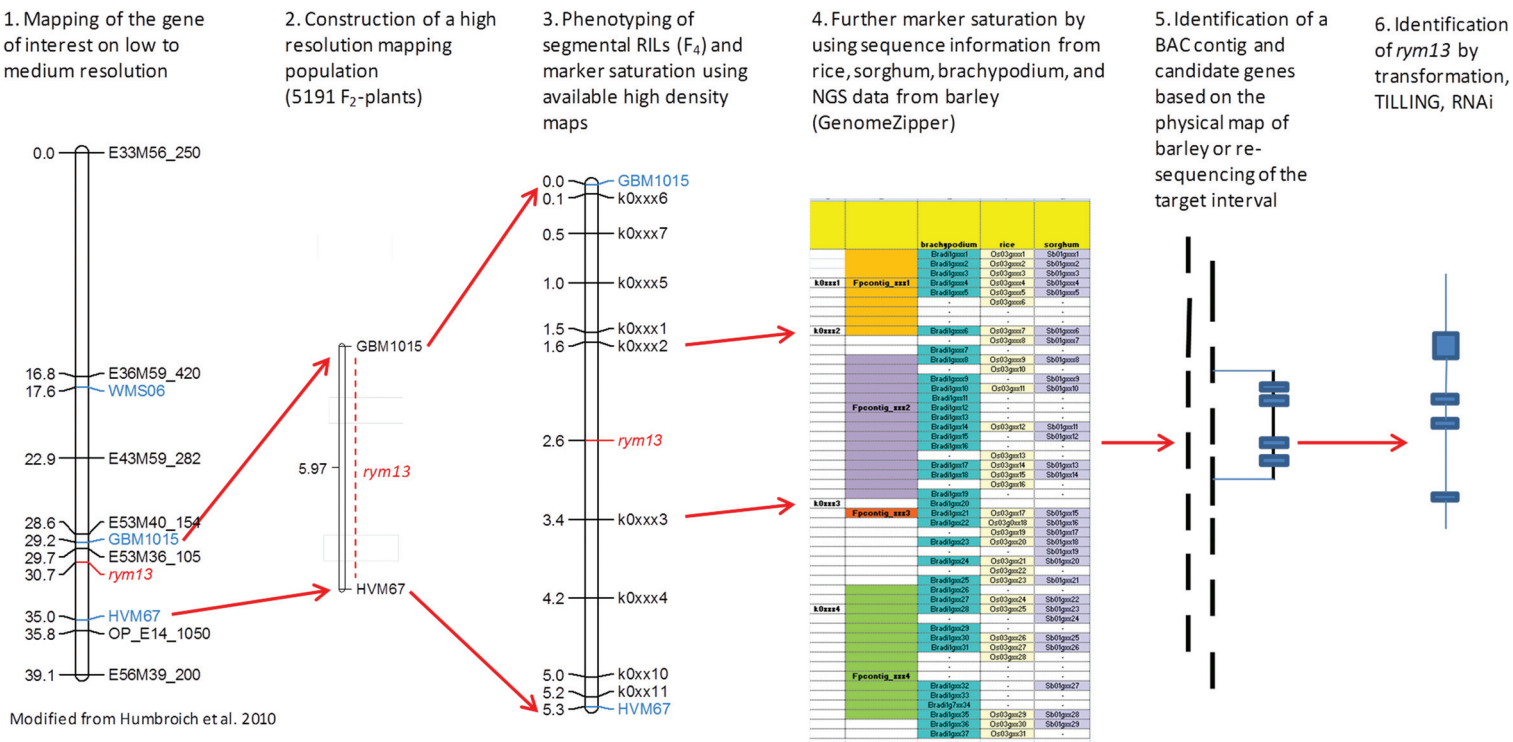


Plate 5.1. Strategy for marker development and isolation of virus resistance genes in barley using genomic tools exemplified for *rym 13* being effective against BaMMV/BaYMV (Lehmann et al. unpublished)

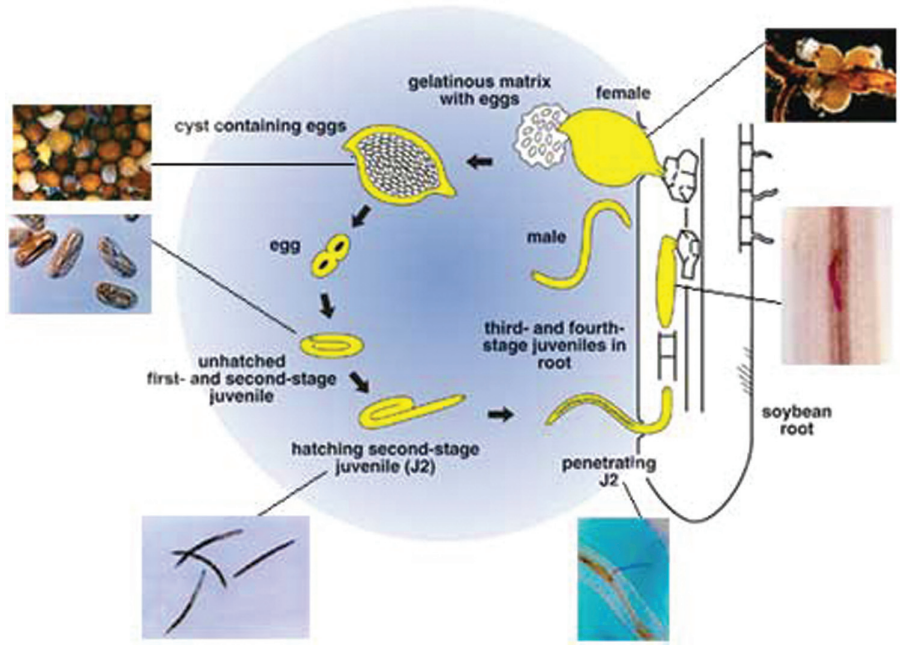


Plate 7.1. A life cycle of *Heterodera glycines* nematode. Typically, a first-stage juvenile (J1) forms in an egg released from a cyst. A second-stage juvenile (J2) then hatches and emerges from the egg. Third- and fourth-stage juveniles (J3 and J4) develop in the roots of the host plant. An adult male fertilizes an adult female, which produces eggs externally. A dead body of a female serves as a cyst containing eggs (adapted from http://www.extension.umn.edu).

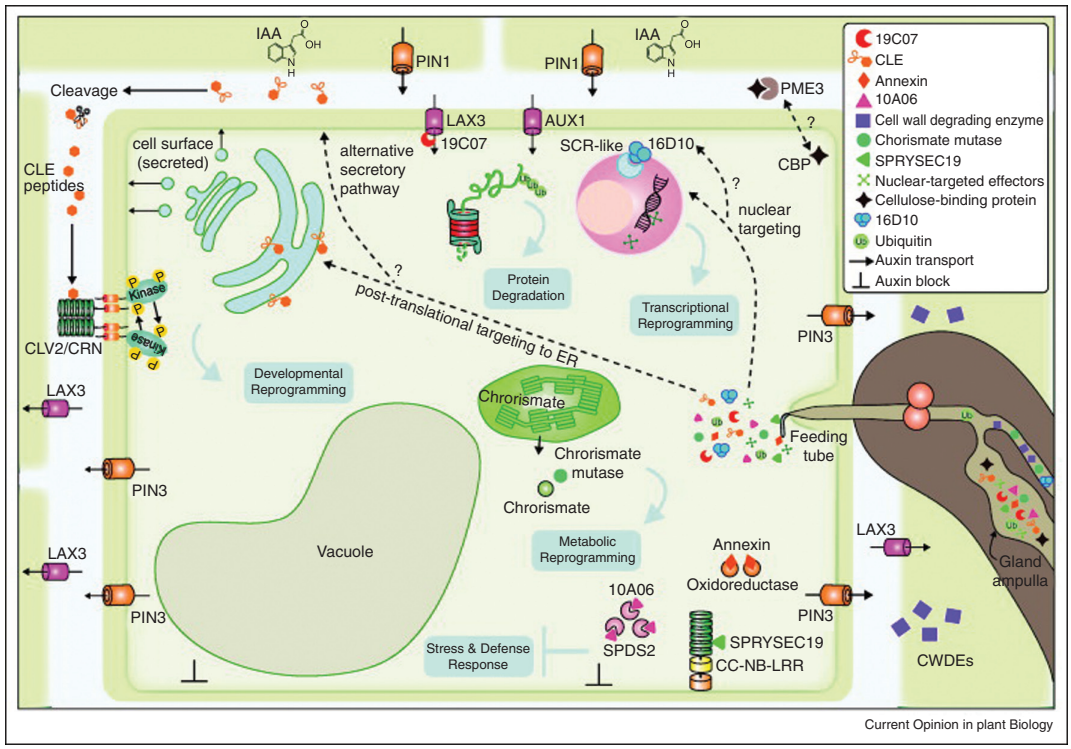
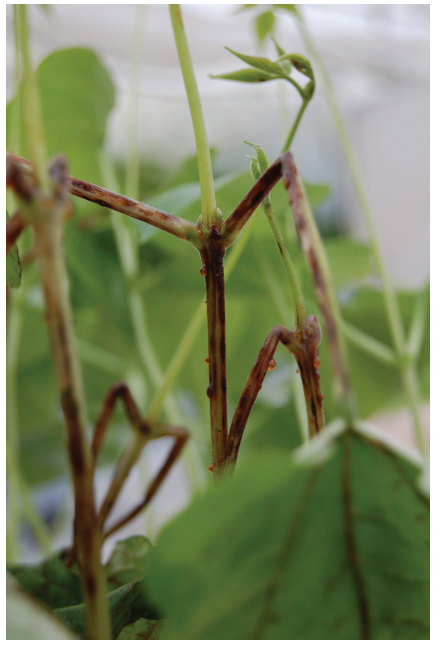


Plate 7.3. Molecular mechanism of nematode effector protein action in host plant cells (adapted from Gheysen and Mitchum 2011).

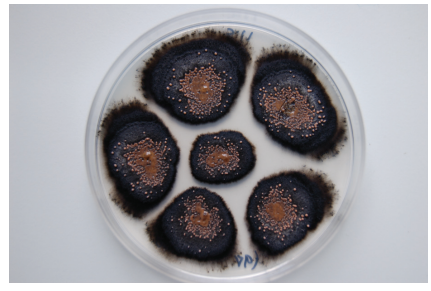


(a)

(b)

(c)

(d)



(e)

Plate 9.1. Anthracnose lesions on common bean leaf, seedling, pod, seed, and spores growing on infected bean leaf on agar.

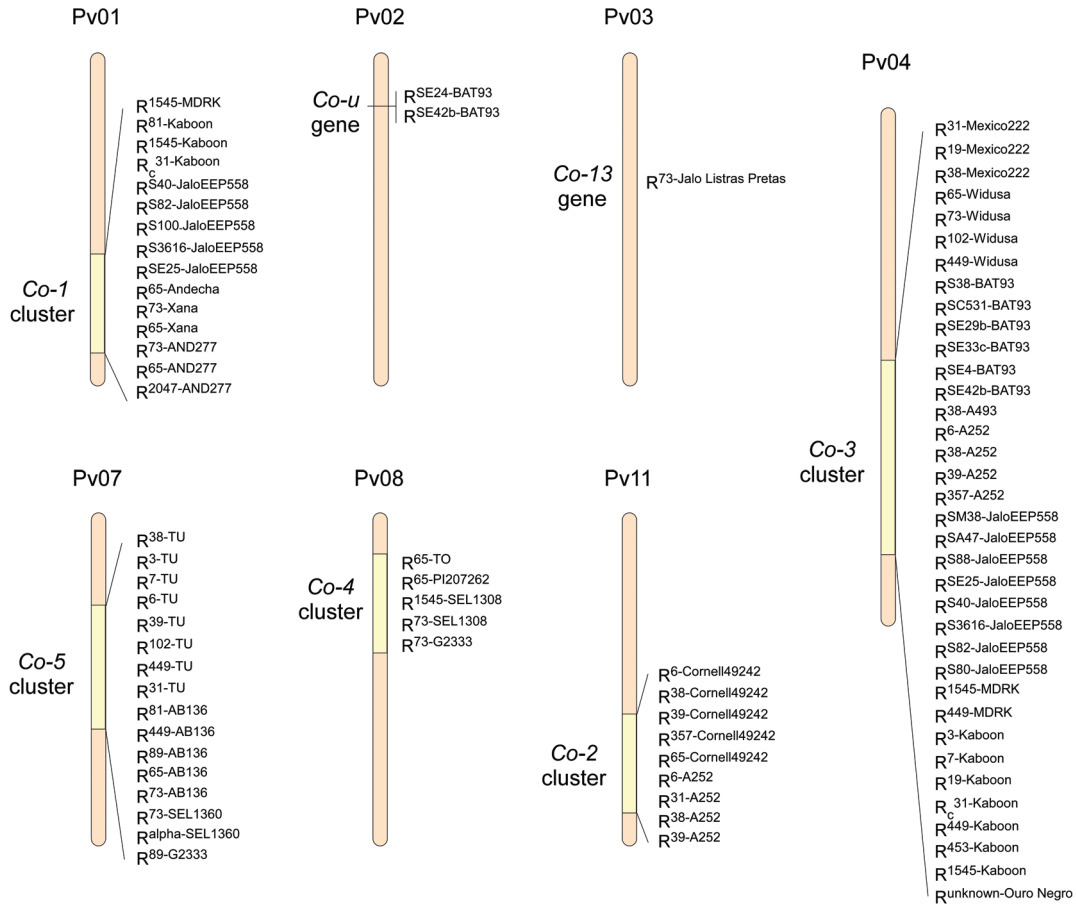


Plate 9.2. Linkage groups showing anthracnose race-specific resistance genes directly mapped using different common bean genotypes. Specific resistance genes are named considering the relative position of seven gene clusters (*Co-1, Co-2, Co-3, Co-4, Co-5, Co-13*, and *Co-u*), the isolate or race of *C. lindemuthianum*, and bean genotype used in the genetic analysis to describe the corresponding resistance gene(s) are indicated by superscript.

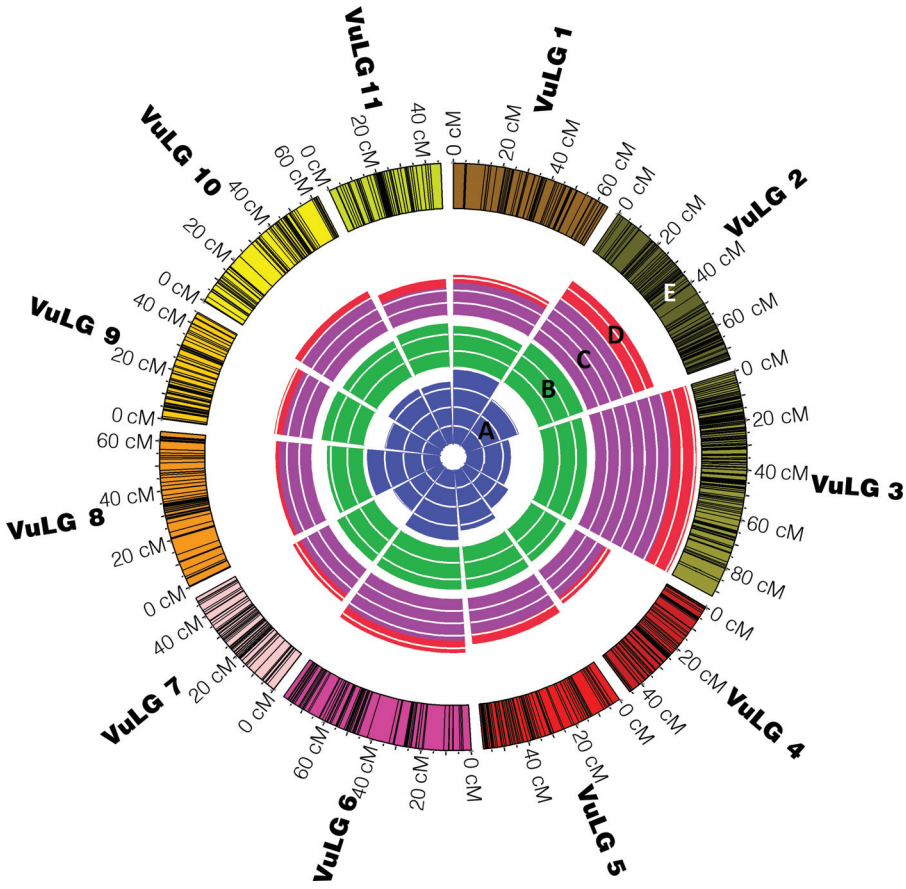


Plate 10.1. Consensus genetic map of cowpea and parameters depicting map characteristics (Lucas et al. 2011). (A) Average distance between bins (0.25 cM). (B) Average number of markers per bin (0.5 units). (C) Number of bins (25 units). (D) Number of markers (25 units). (E) Bin locations. C and D begin at the same radial position.

Plate 11.1. Typical symptoms of major chickpea diseases (a) ascochyta blight (b) fusarium wilt (c) botrytis (d) rust.

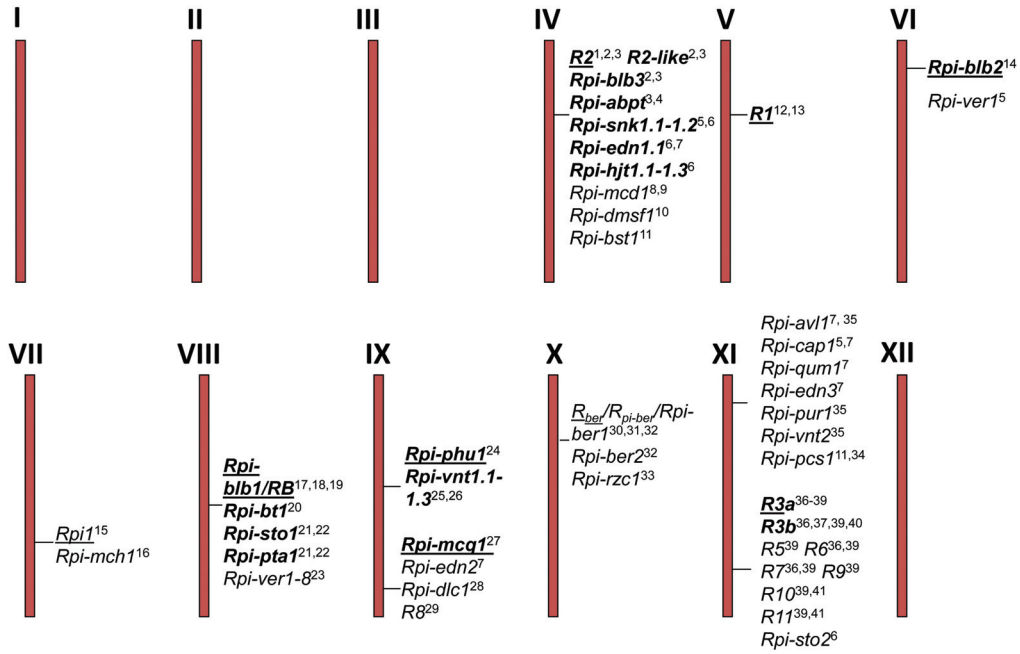


Plate 12.1. Potato *Rpi* genes on the genetic map. Genes that were cloned are bolded, while those that are underlined are the gene cluster names that provide the section titles of this chapter. [1]Li et al. 1998, [2]Park et al. 2005a, [3]Lokossou et al. 2009, [4]Park et al. 2005b, [5]Jacobs et al. 2010, [6]Champouret 2010, [7]Verzaux 2010, [8]Sandbrink et al. 2000, [9]Tan et al. 2008, [10]Hein et al. 2007, [11]Hein et al. 2009, [12]Leonards-Schippers et al. 1992, [13]Ballvora et al. 2002,[14]Van der Vossen et al. 2005, [15]Kuhl et al. 2001, [16]Śliwka et al. 2012a, [17]Naess et al. 2001, [18]Song et al. 2003, [19]Van der Vossen et al. 2003, [20]Oosumi et al. 2009, [21]Wang et al. 2008, [22]Vleeshouwers et al. 2008, [23]Liu and Halterman 2006, [24]Śliwka et al. 2006,[25]Pel et al. 2009, [26]Foster et al. 2009, [27]Smilde et al. 2005, [28]Golas et al. 2010, [29]Jo et al. 2011, [30]Ewing et al. 2000, [31]Rauscher et al. 2006, [32]Park et al. 2009, [33]Śliwka et al. 2012b, [34]Villamon et al. 2005, [35]Rietman 2011, [36]El-Kharbotly et al. 1994, [37]Huang et al. 2004, [38]Huang et al. 2005, [39]Huang 2005, [40]Li et al. 2011, [41]Bradshaw et al. 2006.

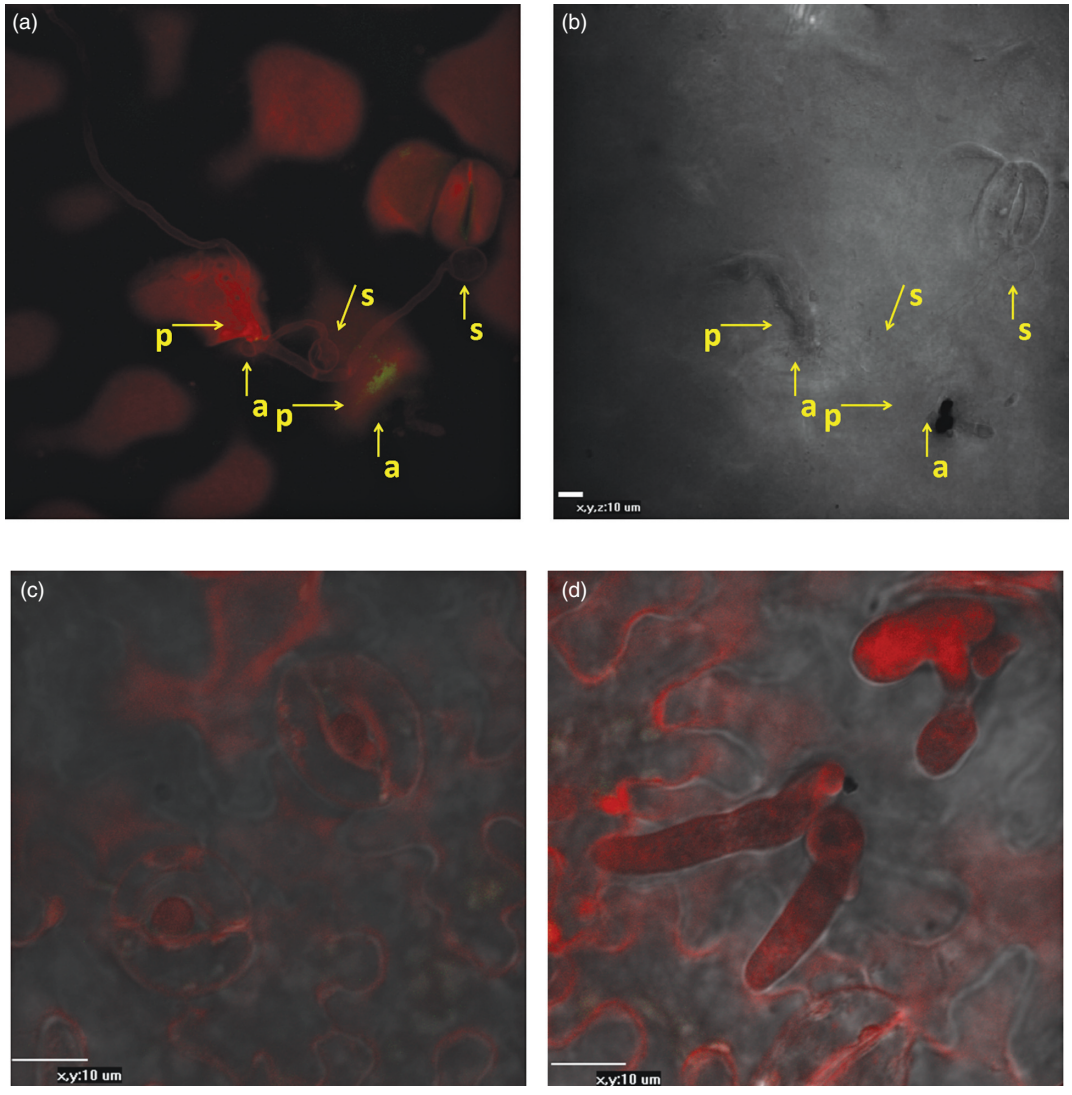


Plate 13.1. *Phytophthora infestans* infection cycle on susceptible tomato cv. Rumba, as visualized by dual stain and confocal laser microscopy (Nowicki et al. 2012). Red channel: pathogen-specific stain and plant autofluorescence; green channel: callose-specific stain; black and white: DIC. (a) Germinating spore [s] develops infection tube and attempts leaf tissue penetration by formation of appressorium [a]. Host plant's primary line of defense is rapid formation of callose-rich papilla [p] directly underneath the attempted penetration area. (b) Same as A, but visible in DIC. (c) Upon successful infection, the pathogen grows in the leaf mesophyll, as denoted by the numerous hyphae. (d) At the end of the infection cycle, the hyphae emerge through the abaxial leaf-side stomata, followed by sporangial development. Rapid field infestation is achieved through successful colonization; descendant sporangia will drive the epidemics through the season.

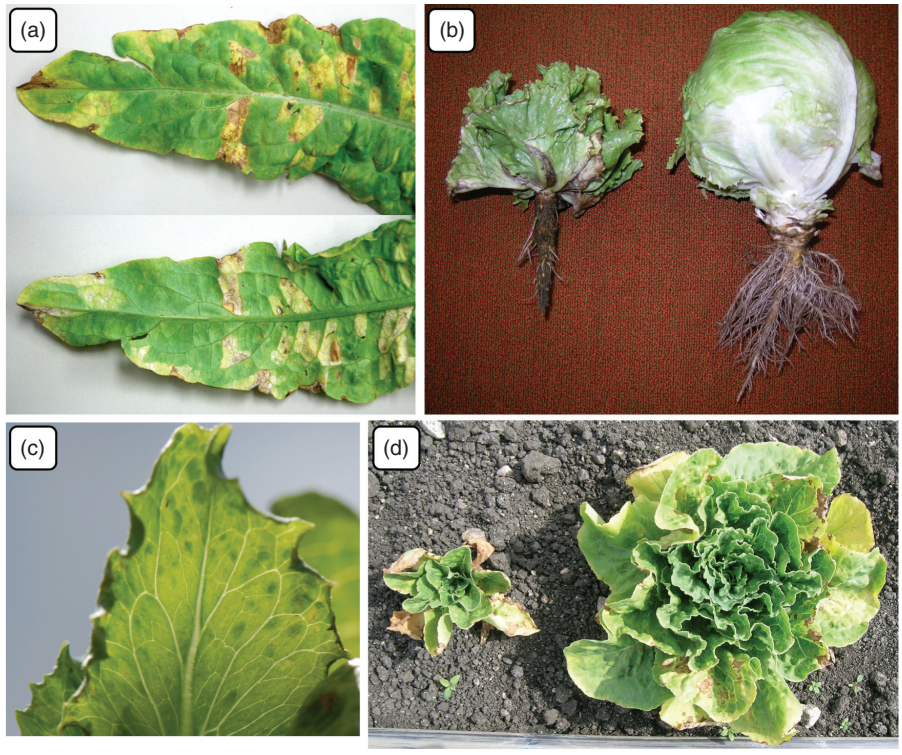


Plate 14.2. Symptoms of four lettuce diseases for which resistance breeding is aided by marker-assisted selection. (a) adaxial (top) and abaxial (bottom) surface of a leaf infected with downy mildew; (b) plant with corky root symptoms (left) and a healthy plant (right) (photo courtesy of Beiquan Mou); (c) detail of a leaf inoculated with LMV; (d) plants with dieback symptoms that were infected with TBSV and/or LNSV in very early (left) or later (right) stages of development.

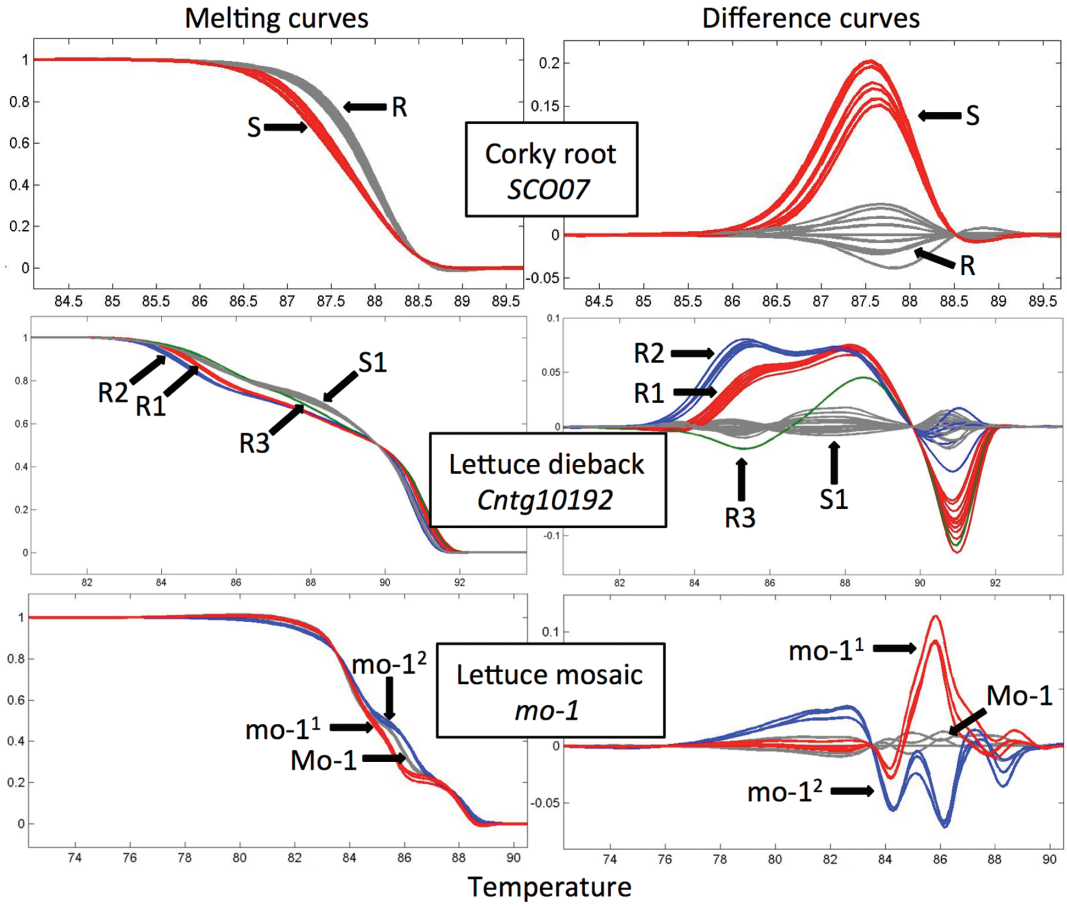


Plate 14.3. Examples of MAS with high-resolution DNA melting (HRM) assays. Top row – the *SCO07* marker linked to the *cor* gene for resistance to corky root (R – allele linked to resistance; S – allele linked to susceptibility). Middle row – the *Cntg10192* marker linked to the *Tvr1* gene for resistance to lettuce dieback (R1 – allele linked to the 'Salinas' haplotype; R2 – allele linked to the 'PI 491224' haplotype; R3 – allele linked to the 'UC96US23' haplotype; and S1 – allele linked to the susceptible haplotype). Bottom row – alleles of the *mo-1* gene for resistance to lettuce mosaic (*Mo-1* is the susceptible allele, *mo-1*^{1} and *mo-1*^{2} are resistant alleles). The *mo-1* assay is based on an unlabeled probe. In each assay only melting curves of homozygous genotypes are shown, however heterozygous genotypes were also identified by HRM.

Plate 15.2. (Left) Cassava plant infected with African cassava mosaic virus (ACMV), in Ghana, showing mosaic, chlorosis, and distorted leaves. (Right) Cassava plant infected with ACMV and East African cassava mosaic Cameroon virus (EACMCV), in Ghana, showing a synergistic effect of the two viruses with extreme "candle stick" symptoms.

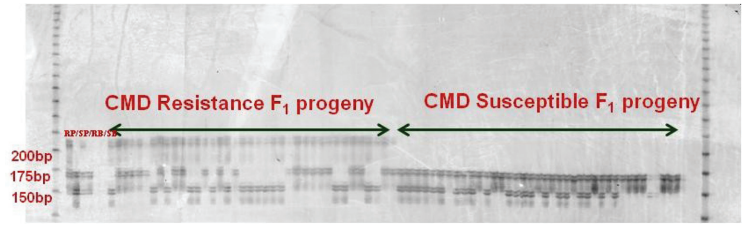


Plate 15.3. Polyacrylamide gel electrophoresis for *CMD2* marker SSRY28 between resistant and susceptible genotypes.

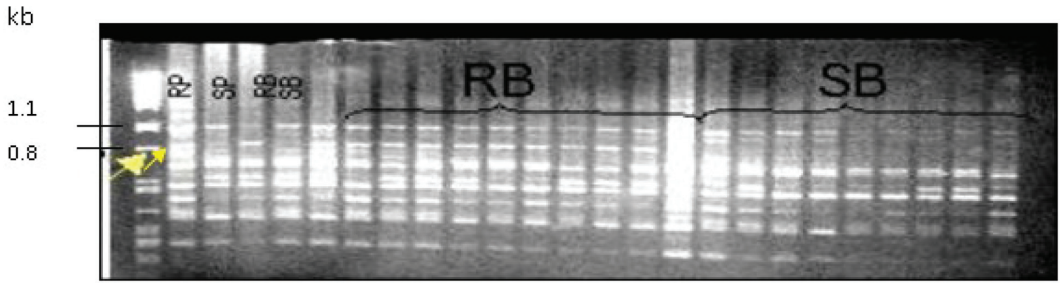


Plate 15.4. Ethidium bromide stained agarose gel of individuals from the recombinant bulks, evaluated with the RAPD marker RME-1. A fragment around 800bp (arrow) can be observed in the resistant parent (RP) and resistant bulks (RB), and it is absent in the susceptible parent (SP) and susceptible bulk (SB).

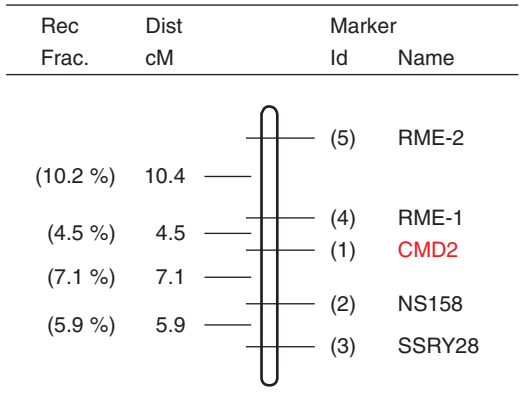


Plate 15.5. Multiple flanking markers of the *CMD2* gene normally used for marker-assisted selection.

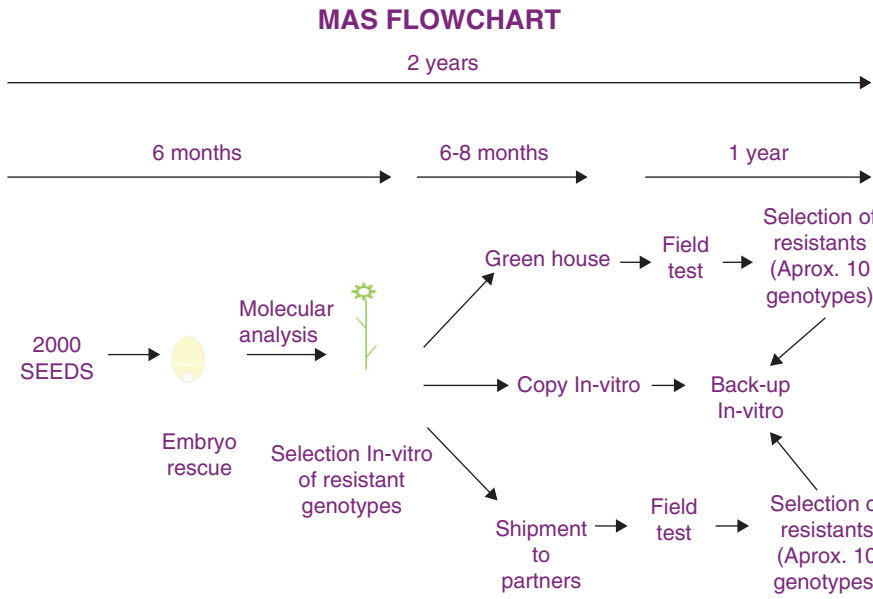


Plate 15.6. Schematic presentation of MAS workflow followed in introgressing CMD resistance in LA germplasm and shipment to partners in Africa and Asia.

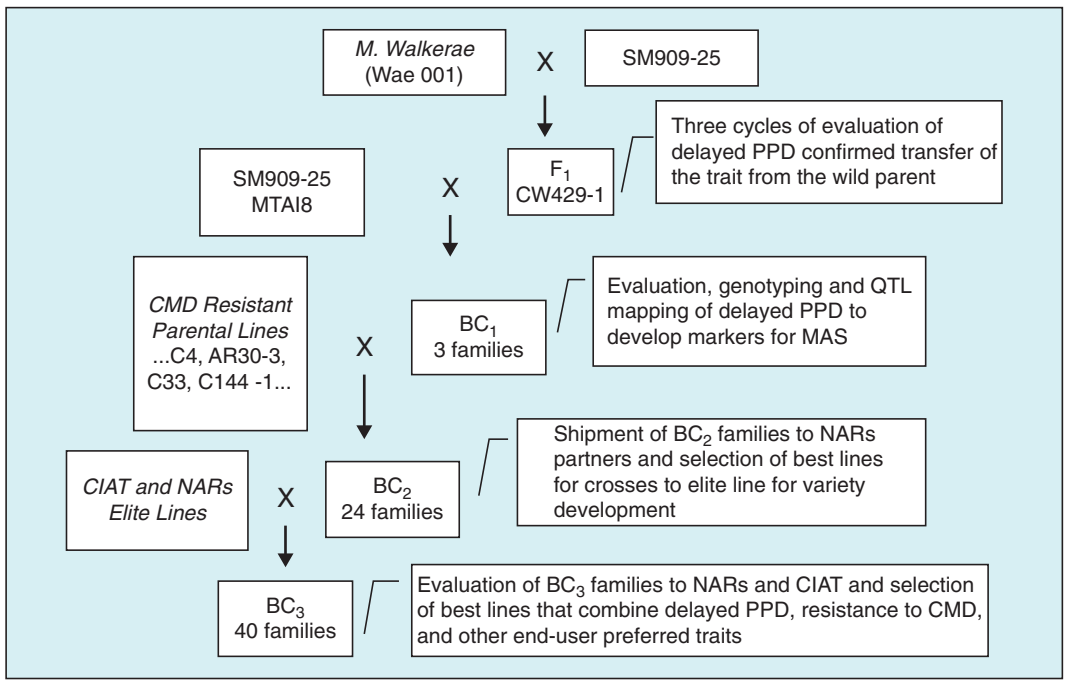


Plate 15.7. Modified advanced backcross scheme used for introgressing delayed post-harvest deterioration from *M. walkerae* into *M. esculenta* species in combination with MAS for CMD resistance.

A fast track scheme:

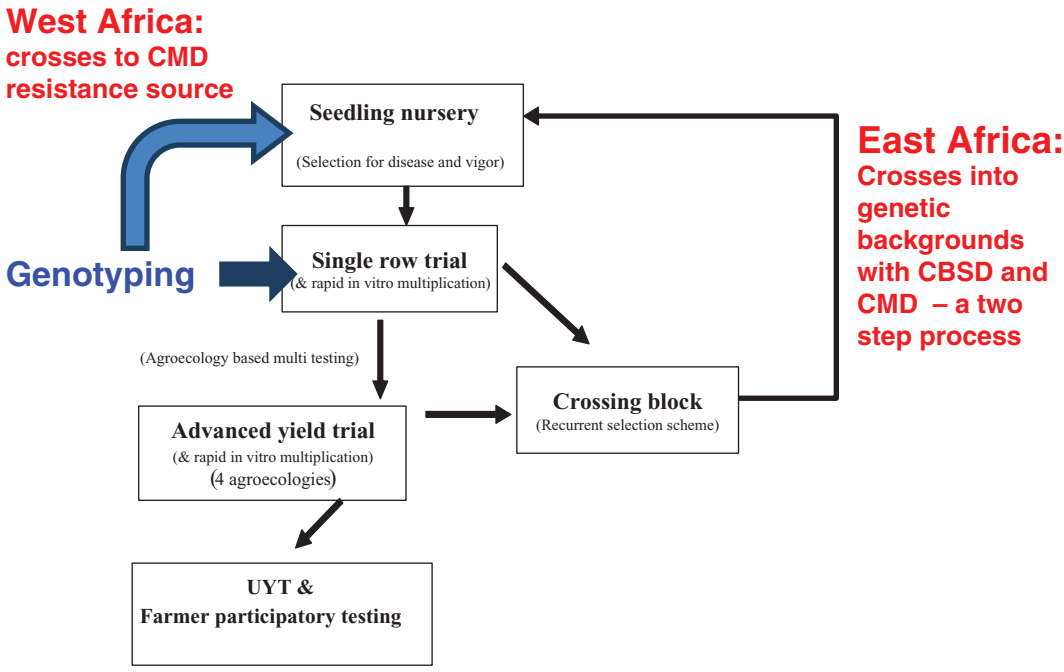


Plate 15.11. A Marker-Assisted Selection-based fast-track evaluation scheme implemented in the cassava breeding community of practice in Africa, leading to release of cultivars in 5-6 years.

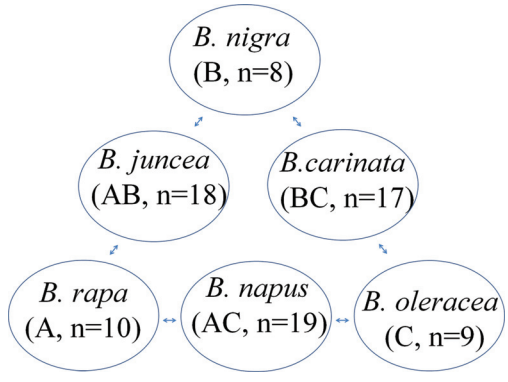


Plate 16.1. The U-triangle in Brassica species (U 1935).