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Regulatory Role of Transcription Factors in Abiotic Stress Responses in Plants

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Abstract

The major abiotic stresses, such as drought, extreme temperatures, and salinity, are responsible for huge losses in agricultural production. Abiotic stresses induce various biochemical and physiological responses in plants, and abscisic acid (ABA), a plant hormone, plays important roles in the responses. Various stress-responsive genes can be expressed under environmental stresses either through an ABA-dependent or ABA-independent pathway, and various stress signals and ABA share common elements. Both ABA-dependent and ABA-independent signal transduction pathways from stress signal perception to gene expression involve different transcription factors. The major transcription factors involved in abiotic stress response are bZIP proteins, MYB-like proteins, MYC-like bHLH proteins, HD-ZIP proteins, and AP2/EREBP proteins. This chapter provides an extensive review of the AP2/EREBP transcription factors and their potential for abiotic stress tolerance in crops.

21.1
Introduction

The major abiotic stresses, such as water deficit (drought or dehydration), extreme temperatures (heat, cold), and salinity, are responsible for a significant reduction in agricultural production. These abiotic stresses ultimately result in desiccation of the cell and osmotic imbalance, inducing various biochemical and physiological responses in plants. The plant hormone abscisic acid (ABA) plays important roles in response to various stress signals, seed maturation, and dormancy [1]. Various stress-responsive genes can be expressed under environmental stresses either through an ABA-dependent or ABA-independent pathway [2]. Moreover, there is an overlap in the expression pattern of stress-responsive genes under cold, drought, high-salt stress, or ABA application, suggesting that various stress signals and ABA share common elements in the signaling pathway, and that these common elements cross-talk with each other, to maintain cellular homeostasis [2,3].

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Various transcription factors are known to regulate the expression of the stress-responsive genes crucial for plant responses to a range of abiotic and biotic stresses. Genes for transcription factors are induced early and transiently by stress and upregulate the expression of many secondary responsive genes, resulting in stress tolerance [4]. Transcription factors are proteins that act as biological switches to regulate gene expression by binding to short, specific DNA sequences called *cis*-elements that are usually found in the promoter region (or introns) immediately upstream of the transcription start site [5]. In general, a transcription factor is composed of at least two discrete domains – a DNA-binding domain and an activation/repression domain, which operate together to regulate many physiological and biochemical processes by modulating the rate of transcription initiation of target genes [6].

Many of these transcription factors are members of large multigene families. Within the *Arabidopsis thaliana* genome, 32 families of genes have been identified as encoding transcription factors that contain three or more members [7]. Each family is characterized by a unique region of highly conserved amino acid sequence, which usually comprises the DNA-binding domain. Plants devote a large portion of their genome capacity to transcription factors, with the *Arabidopsis* genome coding in excess of 1500 transcription factors, which represents over 5% of the total genes [7,8].

Various transcriptional regulatory mechanisms function in the abiotic stress signal pathways [9,10]. Both ABA-dependent and ABA-independent signal transduction pathways from stress signal perception to gene expression involve different transcription factors. Some transcription factors follow an ABA-dependent signal transduction pathway, while others appear ABA-independent. The ABFs (ABRE-binding factors)/AREBs (ABA-responsive element-binding proteins), MYC/MYB, DREB2 (dehydration-responsive element binding) and NAC (NAM, ATAF, and CUC) transcription factors are involved in ABA-dependent and ABA-independent gene expression pathways under dehydration and salt stress, whereas CBF (CRT-binding factor)/DREB1 involved in cold stress-responsive gene expression through thee ABA-independent pathway [4]. These major stress-responsive transcription factor regulates different genes under different stress conditions, and overexpression of these transcription factors upregulates many genes that are involved in stress response and tolerance [4]. Although there is a no general rule governing the activation of the different classes of stress-responsive genes by the various classes of transcriptional factors, they indeed regulate various stress-inducible genes collectively or separately.

In this chapter, the major transcription factors involved in abiotic stress response: basic region leucine zipper (bZIP) proteins, MYB (myeloblastosis)-like proteins, MYC (myelocytomatosis)-like basic helix–loop–helix (bHLH) proteins, HD-ZIP proteins, and the AP2/EREBP domain family are discussed in detail. The regulatory pathway of DREB1A in *Arabidopsis* and various crop transgenics developed with DREB1A are also discussed in detail.

21.2
bZIP Proteins

The bZIP transcription factors, in plants, regulate processes including pathogen defense, light and stress signaling, seed maturation, and flower development.

There are two structural regions present on the α-helix of the bZIP domain [11]: an around 16-amino-acid basic region and a heptad repeat of leucines or other bulky hydrophobic amino acids [12]. The basic region contains nuclear localization signals and an invariant N–x7–R/K motif to contact the DNA, whereas the heptad repeat creates an amphipathic helix. To bind DNA, two subunits form a coiled-coil structure (the so-called zipper) [11]. bZIP transcription factors, also known as AREBs [1] or ABFs [13] bind to an ABA-responsive, *cis*-acting element named ABRE (ABA-responsive element) present in the promoter regions of ABA-responsive genes [14–16]. A conserved sequence, PyACGTGGC, has been reported to function as an ABRE in many ABA-responsive genes [17–19]. Plant bZIP proteins preferentially bind to DNA sequences with an ACGT core. Binding specificity is regulated by flanking nucleotides. Plant bZIPs preferentially bind to the A-box (TACGTA), C-box (GACGTC), and G-box (CACGTG) [20], but there are also examples of non-palindromic binding sites [13,21]. Nucleotides around the ACGT core motif are important for determining the binding specificity of bZIP proteins. However, a single copy of ABRE is not sufficient for ABA-responsive transcription. Furthermore, a coupling element is required to specify the function of ABRE as together these constitute an ABA-responsive complex in the regulation of ABA-responsive gene expression [22]. Most of the known coupling elements have similarity with ABREs and contain an AyGCGT motif [23]. Furthermore, the G-box resembles the ABRE motif and functions in the regulation of plant genes in a variety of environmental conditions, such as red light, UV light, anaerobiosis, and wounding [24]. G-box-binding proteins also contain a bZIP motif [24].

Several genes for bZIP that bind to ABREs *in vitro* or are inducible by ABA have been isolated [14–16]. A cDNA for the ABRE-binding protein EmBP-1 was first shown to encode a bZIP protein containing a basic DNA-binding domain linked to a ZIP domain [17]. bZIP proteins are a large family of transcription factors in plants with 75 members present in *Arabidopsis*; using common domains the AtbZIP family can be subdivided into 10 groups [12]. The functional information available suggests that group A bZIP proteins of *Arabidopsis* are involved in ABA or stress signaling [1,4].

21.3
MYB-Like Proteins

MYB proteins are a superfamily of transcription factors that play regulatory roles in developmental processes and defense responses. The v-MYB gene of avian myeloblastosis virus (AMV) was the first MYB gene to be identified [25]. Three

v-MYB-related genes, c-MYB, A-MYB, and B-MYB, were subsequently found in many vertebrates, and are thought to be involved in the regulation of cell proliferation, differentiation, and apoptosis [26].

The first plant MYB gene *C1*, was isolated from *Zea mays* (encoding for a c-MYB-like transcription factor that is involved in anthocyanin biosynthesis) [27]. The fact that MYB genes exist widely in eukaryotes suggests that these genes might be very ancient during the course of evolutionary. Interestingly, the numbers of MYB genes in plants are remarkably higher than those in fungi or animals [8].

About 52-amino-acid imperfect repeats are present in the MYB domain. Each domain contains one to three repeats and these repeats adopt a helix–turn–helix conformation that intercalates in the major groove of the DNA. The MYB domain of c-MYB, a mammalian transcription factor, contains three imperfect repeats, R1, R2, and R3 [27]. Typically, three regularly spaced tryptophan residues are present in each MYB repeat, participating in a hydrophobic cluster that is presumably involved in the specific recognition of DNA [28].

Plant MYB proteins have been classified into three major groups: R2R3-MYB, with two adjacent repeats; R1R2R3-MYB, with three adjacent repeats; and a heterogeneous group collectively referred to as the MYB-related proteins, which usually, but not always, contain a single MYB repeat [29–31]. In the past decade, the R2R3-MYB genes have been extensively studied. These were reported to be involved in many physiological and biochemical processes. More than 150 plant MYB-like proteins known so far contain either two or only one sequence related helix–turn–helix motif in their DNA-binding domain [32]. Animal c-MYB genes contain three helix–turn–helix motif-encoding repeats (R1R2R3 class genes), which were identified in different plant evolutionary lineages, including mosses, ferns, and monocots [32]. The DNA-binding domain consisting of three MYB repeats existed before the divergence of the animal and plant lineages. R1R2R3-MYB genes may have a conserved function in eukaryotes and R2R3-MYB genes may predominantly regulate plant-specific processes that evolved during plant speciation. The MYB superfamily has the largest number of members of any *Arabidopsis* gene family [7]; 198 genes encoding MYB repeats have been identified in the *A. thaliana* genome, among these 126 are R2R3-MYB, five are R1R2R3-MYB, 64 are MYB-related, and three are atypical MYB genes [33].

21.4 MYC-Like bHLH Proteins

bHLH transcription factors have reportedly being present in three eukaryotic kingdoms. In 1989, Murre *et al.* [34] identified a region that shared a significant number of identical amino acids in DNA-binding proteins from animals and this region has become known as the bHLH domain. In 1989, Ludwig *et al.* [35] identified a regulatory gene *Lc* of anthocyanin biosynthesis in *Zea mays* and showed that the predicted protein shared the bHLH domain. With the identification of the Ino4p protein from yeast [36], it became clear that bHLH proteins constitute a

ubiquitous family of regulators in eukaryotes and that the bHLH domain is an ancient component of transcriptional regulation. Moreover, recent genome sequencing and expressed sequence tag (EST) programs have indicated the existence of many more bHLH genes in various eukaryotic species.

This family is defined by the bHLH signature domain, which consists of 60 amino acids with two functionally distinct regions. Typically, a bHLH domain comprises a stretch of about 18 hydrophilic and basic amino acids at the N-terminal end of the domain, followed by two regions of hydrophobic residues predicted to form two amphipathic α-helices separated by an intervening loop of variable sequence and length [37,38]. The basic region, at the N-terminal end of the domain, is involved in DNA binding and the HLH region, at the C-terminal end, functions as a dimerization domain [34,39]. Outside of the conserved bHLH domain, these proteins exhibit considerable sequence divergence [40]. Studies with mammalian bHLH proteins have shown that the conserved HLH structure is required for dimerization between two bHLH proteins [39,41,42]. Two separate polypeptides lead to the formation of homodimers and/or heterodimers with the interaction of the HLH regions and that the basic region of each polypeptide binds to half of the DNA recognition sequence [43,44]. Some bHLH proteins form homodimers or heterodimer with closely related members of the family, whereas some bHLH proteins form heterodimers with one or several different partners [45]. The bHLH proteins recognizes a six bases core DNA sequence motif called E-box (5-CANNTG-3). There are different types of E-boxes and the most common is the palindromic G-box (5-CACGTG-3). The conserved amino acids present in the basic region of the protein recognize the core DNA motif, whereas other residues in the domain dictate specificity for a given type of E-box [46]. Each bHLH protein has a binding site preference for the central two bases of the CANNTG motif [47]. Two MYC recognition sequences in the 67-bp region of the *rd22* promoter are CACATG. The bacterially expressed rd22BP1 fusion protein evidently recognizes and binds only the first CACATG motif [48]. In addition, flanking nucleotides outside of the hexanucleotide core have been shown to play a role in binding specificity [40,45,49] and there is evidence that a loop residue in the protein plays a role in DNA binding through elements that lie outside of the core recognition sequence [38]. The conserved amino acids His–Glu–Arg (H-E-R) in the basic region of bHLH proteins, at positions 5, 9, and 13, are most important for DNA binding. In non-plant bHLH proteins, His5 and Glu9 residues contact with the outer two nucleotides of the E-box motif, whereas Arg13 is in contact with the two inner nucleotides of the motif [40,50,51]. The DNA backbone is contacted by basic residues at positions 10 and 12, and these are also conserved in the majority of plant proteins. The highly conserved hydrophobic residues in helix 1 and 2 are believed to be necessary for dimerization. In *A. thaliana*, a leucine residue is present at position 23 in every bHLH protein, which emphasizes the likely importance of this residue in dimerization.

The *AtbHLH* genes constitute one of the largest families of transcription factors in *A. thaliana* with significantly more members than are found in most animal species and about an equivalent number to those in vertebrates. Heim *et al.* [52] identified 133 bHLH proteins in *Arabidopsis* and classified them in to 12 groups

(subfamilies) of related sequences. Within each subfamily genes contain similar number of introns with conserved positions. In the same way, the encoded proteins of the subfamily show similar lengths, similar positions for the bHLH domain, and also show similarity in amino acid sequences outside the DNA-binding domain. Toledo-Ortiz *et al.* [53] identified 147 bHLH protein-encoding genes in *Arabidopsis* and classified them in to 21 subfamilies based on bHLH domain sequences.

In *A. thaliana*, three proteins, PvPG1, AtbHLH006/rd22BP1/RAP-1 (both group III), and AtbHLH008/PIF3 (group VII) [54], have been shown to bind a sequence identical or very similar to the B variant of the animal E-box motif, which is also identical to the G-box core motif (CACGTG) – a ubiquitous regulatory DNA element found in plants that is also bound by some bZIP transcription factors [55].

Most of the *Arabidopsis* bHLH proteins contain the H-E-R configuration within the bHLH domain-like group B proteins from animals [52], suggesting that these proteins evolved from group B proteins present in early eukaryotic lineages. The bHLH proteins of groups VIII and X act as negative regulators; because of variations in H-E-R configuration they have lost the DNA-binding ability, but retained the dimerization ability, and group X proteins are now classified as group D [56].

Most group III members with known functions act as transcription factors regulating genes of flavonoid metabolism. Subgroup IIIf are involved in very different processes: flavonoid/anthocyanin biosynthesis (AtbHLH0012/MYC1 and AtbHLH042/TT8) and trichome initiation (AtbHLH001/GL3). Three members of group XII, AtbHLH044/BEE1, AtbHLH058/BEE2, and AtbHLH050/BEE3 (BR enhanced expression), from *A. thaliana* have been linked to multiple pathways regulating plant growth and development [57]. Based on the current characterization of a limited number of plant bHLH proteins, this family of transcription factors has a range of different roles in plant cell and tissue development as well as plant metabolism [52].

21.4.1 Cooperation of MYC and MYB Proteins

Cooperation of MYC and MYB proteins has been reported in plants, but not in animals [58–60]. Genetic analysis of the anthocyanin biosynthetic pathway in *Z. mays* has identified a group of bHLH genes required for production of the purple anthocyanin pigments: R (R-s and R-p), B, Lc, Sn [61], and R-ch Hopi [62]. In *Z. mays*, bHLH proteins and other transcription factors Cl or P, both R2R3-MYB proteins, together regulate pigmentation in tissues. Anthocyanin production was also controlled in a tissue-specific manner by other members of the *R* gene family and MYB proteins in *Z. mays* and other species, In maize, the *C7* and *PI* genes encoding MYB homologs have been reported to require the *RIB* gene product for MYC homologs to *trans*-activate target genes, such as *Bronze1* and *A1* for anthocyanin biosynthesis [58–60,63]. Functional analysis of maize *B* and *C7* genes has demonstrated that the N-terminal domain of the B (MYC homolog) protein interacts with the C1 (MYB homolog) protein [64]. The maize *C7* gene is regulated by ABA and the *viviparous7* gene during seed maturation [65]. The bHLH/MYB

partnership has been shown to be important in the differentiation of *A. thaliana* trichomes through the interaction of a bHLH protein encoded by glabra3 (GL3) and the R2R3-MYB protein glabrous1 (GL1) [66]. Transgenic plants overexpressing AtMYC2 and/or AtMYB2 cDNAs not only had higher sensitivity to ABA, but also enhanced ABA-induced gene expression of *rd22* and *AtADH1* [67]. Microarray analysis of the transgenic plants overexpressing both AtMYC2 and AtMYB2 cDNAs revealed that several ABA-inducible genes also are upregulated in the transgenic plants [67]. These studies suggest interaction of MYC and MYB proteins in ABA-induced gene expression in vegetative tissues under dehydration stress conditions.

21.5 HD-ZIP Proteins

The HD-ZIP family is characterized by a homeodomain (HD) followed by a leucine zipper (ZIP) domain motif. This feature is found only in plant HD proteins [68–70]. HD-ZIP proteins are transcription factors encoded by a class of homeobox genes. It is striking that HD-ZIP proteins have not been described in animals, despite characterization of more than 100 HD proteins from animal systems [71]. HD-ZIP proteins mediate aspects of development that are unique to plants, such as the coupling of development to environmental signals [72].

HD-ZIP proteins are characterized by the presence of two functional domains: a HD responsible for DNA binding [73,74] and a ZIP located immediately C-terminal to the HD involved in protein–protein interaction. The homeobox, a 183-bp DNA sequence element, encodes a 61-amino-acid sequence, known as the HD. The homeobox was first identified in developmental genes of *Drosophila* [75,76]. The spacing of the HD and the putative ZIP in *Arabidopsis* HD-ZIP proteins is identical to the distance between the DNA-binding domain and the ZIP in bZIP proteins [77–81]. Moreover, the presence of characteristic hydrophobic and charged residues within the heptad repeats is analogous to the bZIP class [77–79,82,83]. These similarities suggest that HD-ZIP proteins may, like members of the bZIP class, utilize the ZIP motif as a dimerization domain [69].

Despite sequence similarities, HD-ZIP proteins participate in a variety of processes during plant growth and development [84]. The *Arabidopsis* genome contains 47 HD- ZIP genes, which have been grouped into four different classes (HD-ZIP I–IV) based on gene structure, presence of unique domains, and function [85,86]. In *Arabidopsis*, the HD-ZIP class I comprises 17 members encoding proteins of a similar size (around 35 kDa) including a well-conserved HD domain and a less-conserved ZIP motif. HD-ZIP class I proteins are generally involved in responses related to abiotic stress, ABA, blue light, de-etiolation, and embryogenesis. HD-ZIP I proteins recognize and bind the pseudopalindromic sequence CAAT(A/T)ATTG [87]. The expression of many HD-ZIP class I genes is dependent on water and light conditions; reports have shown that transcript levels of ATHB5, −6, −7, −12, and −16 were significantly influenced by water-deficit stress, osmotic stress, or exogenous treatment with ABA and different light conditions [85,88–96].

21.6
AP2/EREBP Domain Proteins

The AP2/EREBP family of transcription factors was first characterized in *Arabidopsis* apetala 2 (AP2) [97], which is involved in floral morphogenesis, and in tobacco EREBP1 [98], which is involved in ethylene-responsive gene expression, and hence named the AP2/EREBP family. All AP2/EREBP proteins possess typical structural characteristics of transcription factors such as a DNA-binding domain (AP2/EREBP), nuclear localization signals (basic amino acid sequences), and putative transcription activation domains (acidic region, serine-rich region, etc.) [99].

AP2/EREBP-related genes form a large family, which consists of many members found in several plant species. There are 145 distinct genes encoding the AP2/EREBP-type proteins in *Arabidopsis* and these proteins were classified into five groups: the AP2 subfamily, the RAV subfamily, the DREB subfamily, the EREBP subfamily, and one very specific gene *AL079349*, based on the similarity of their AP2/EREBP DNA-binding domains [100]. Analysis by Feng *et al.* [101] has shown that a total of 147 genes encoding proteins that contained at least one AP2-like domain were present in *Arabidopsis* and the AP2/EREBP family was divided into three main clades: the 19-membered AP2, the six-membered RAV, and the 65-membered EREBP and 57-membered DREB subfamily (EREBP and DREB were considered as one clade). The characteristic AP2 domains found in the EREBP, DREB, and RAV subfamilies were about 60–70 amino acids in length, while that of the AP2 subfamily varied significantly from 41 to 74 amino acids [101].

21.7
DREB Subfamily

This subfamily mainly consists of DREB proteins. The deduced amino acid sequences of DREB proteins showed significant sequence similarity with the conserved DNA-binding domain found in the EREBP and AP2 proteins [99]. The DREB proteins specifically recognize and bind to a *cis*-element, known as DRE (dehydration-responsive element) A/GCCGAC or CRT (C-repeat element)-conserved core sequence CCGAC [102]. DRE or CRT *cis*-elements exist widely in promoters of plant genes such as *rd29A*, *rd17*, *kin1*, and so on, which are induced by dehydration, high-salt, and cold stresses [102]. The entire consensus amino acids are conserved in EREBP and DREB subfamilies of proteins, except that amino acids 14 and 19 are valine (Val) and glutamate (Glu) in the DREB subgroup and alanine (Ala) and aspartic acid (Asp) in the EREBP subgroup [99]. Yeast *in vivo* analysis showed that the conserved Val and Glu residues are crucial in the regulation of the binding activity of DREB1A to the DRE *cis*-element [99].

Many DREB/CBF homologs, including *Arabidopsis CBF1, CBF2,* and *CBF3* [103], rice *DREB1A* and *DREB1B* [104], and tomato *CBF1* [105], are induced rapidly by low temperatures. Some of the CBF homologs may also be induced by drought, high salinity, or ABA treatment, as *Arabidopsis CBF4* is involved in drought resistance [106], whereas *DDF1* and *DDF2* are involved in the regulation of gibberellin (GA) biosynthesis and high-salinity tolerance [107]. Other homologs, such as *OsDREB1C* [104] and *HvCBF2* [108], are constitutively expressed. Even freeze-sensitive plants that apparently lack cold-acclimation capability, such as rice, maize, and tomato, have multiple CBF homologs. For example, three CBF homologs have been identified in tomato, but two of them, *LeCBF2* and *LeCBF3,* are not responsive to either cold, drought, high salinity, or ABA treatment [105].

The components of the CBF cold-response pathway are highly conserved in flowering plants and not limited to those that cold acclimate [109]. Homologs of *Arabidopsis CBF* genes have been identified and characterized in more than 20 species, including rapeseed [109,110], barley [111], rice [104], wheat [112], maize [113], tomato [105], *Capsella bursa* [114], pepper [115], soybean [116], oat [117], perennial ryegrass [118], *Eucalyptus* [119], *Populus* [120], and grape [121]. The proteins of the DREB subfamily were further divided into six groups: A1–6, among which A1 and A2 were the two largest groups [100].

Xiong and Fei [118] have further classified 59 DREB1/CBF homologs into separate clades. This classification included 41 DREB1/CBF homologs reported for 14 plant species, and 18 additional CBF homologs from genomic and/or EST sequences of rice, maize, poplar, and loblolly pine by BLAST search. Nearly all the known DREB1/CBF genes (54 out of 59) were classified into this A1 group (CBF family), including six *Arabidopsis* DREB1 proteins. Group A1 (CBF family) is further divided into monocot and eudicot subgroups. In the monocot subgroup, 32 homologs were grouped into three clades, represented by *OsDREB1A/CBF3, OsDREB1B/CBF1,* and *OsDREB1C/CBF2,* respectively. Three to four subclades were recognized in the *CBF2* and *CBF3* clades. The CBF genes in the eudicot subgroup further subdivided in to clades as parallels to the taxonomic classification. The existence of multiple subclades, each of which includes CBF gene members from different species, suggests that the CBF gene was duplicated many times and diverged before or during speciation from the common monocot ancestor. The clustering of CBF homologs in eudicot plants suggests that duplications of CBF homologs in eudicot plants are independent events, and duplication and divergence occurred after speciation [118].

The eight *Arabidopsis* proteins, including DREB2A and DREB2B, were classified in to the A2 group. Four soybean DREB homologs were classified into the A5, A6, and A2 subgroups. Wheat *TaDREB1* was also classified into the A2 subgroup (the *TaDREB1* sequence showing higher similarity with DREB2 than *DREB1* genes of *Arabidopsis*). This further indicates that their functions may be distinct from that of other DREB1/CBF genes.

21.8 CBF/DREB Genes from *Arabidopsis*

Several stress-inducible genes, such as *rd29A* and *cor15A* of *Arabidopsis*, are induced through the ABA-independent pathway. A 9-bp conserved sequence TACCGACAT, the DRE, has been identified in the promoter region of the *rd29A* gene as an essential *cis*-acting element in the ABA-independent response to dehydration, high salinity, and cold [122]. Similar *cis*-acting elements named CRT or low-temperature-responsive element (LTRE), both containing an A/GCCGAC motif that forms the core of the DRE sequence CCGAC, regulate cold-inducible gene expression, including the *COR15A* gene from *Arabidopsis* [3,123], the *BN115* gene from *Brassica napus* [124], and the *WCS120* gene from wheat [125]. Identification of DRE/CRT *cis*-elements led to the isolation of *trans*-acting proteins, which specifically bind to these elements and are similar to ERF/AP2 proteins. These transcription factors are known as CBFs or DREB proteins. Three *CBF/DREB1* genes of *Arabidopsis*, *CBF1/DREB1B*, *CBF2/DREB1C*, and *CBF3/DREB1A*, were induced by cold [126–128], whereas *CBF4/DREB1D* is induced by osmotic stress [100,106] and other two, *DDF1/DREB1F* and *DDF2/DREB1E*, are induced by high-salinity stress [100,107]. The DREB2 proteins also bind to DRE/CRT like CBF/DREB1 proteins; however, DREB2 proteins are involved in drought-responsive gene expression, but not in cold [128], suggesting the existence of cross-talk between the CBF/DREB1 and DREB2 pathways. Six DREB2-related genes in the *Arabidopsis* genome were also reported, but the expression levels of these genes were low under stress conditions [100].

CBF1, *CBF2*, and *CBF3* genes constitute a small gene family organized as a cluster on chromosome IV of *Arabidopsis* in the order *DREB1B/CBF1*, *DREB1A/CBF3*, and *DREB1C/CBF2* [103,126]. *CBF2* (GenBank accession no. AF062924) and *CBF3* (GenBank accession no. AF062925) open reading frames (ORFs) contain 651 nucleotides each, that are 84% identical to each other. *CBF2* and *CBF3* ORFs show a high degree of similarity to *CBF1*, 81% and 84%, respectively. Furthermore, the sequences of *CBF1*, *CBF2*, and *CBF3* do not appear to have any introns interrupting their ORFs [103].

The CBF polypeptides contain a 58-amino-acid motif known as the AP2 domain, which is evolutionarily conserved in plants [103]. It has been noted that all CBF/DREB1 proteins share common signature motifs (PKK/RPAGRxKFxETRHP and DSAWR) that bracket the AP2 domain and those motifs are found in CBF-like proteins that are conserved across species [109]. Apart from the DNA-binding domain, each DREB protein contains a basic region in its N-terminal region that might function as a nuclear localization signal and an acidic C-terminal region that might act as an activation domain for transcription [128].

Moreover, CBF1, CBF2, and CBF3 proteins show potential recognition sites for protein kinase C and casein kinase II. Some of these sites are conserved among the three CBF polypeptides, such as Ser13, Ser56, which is inside of the AP2 domain, and Thr151 [103]. A Ser/Thr-rich region following the DNA-binding domain and a glutamine-rich region in the C-terminal region were found in both DREB2A and

DREB2B proteins [128]. Study of cold response in wheat showed that phosphorylation was essential for the binding of nuclear factors to LTRE/DRE motifs in the *wcs120* promoter [129]. In the CBF proteins, a similar Ser/Thr-rich region was identified as a putative interaction domain, which is modified by other regulatory molecules in a signal transduction pathway [112].

21.9
CBF/DREB Regulation in *Arabidopsis*

21.9.1
Promoter Regions of the CBF/DREB Genes of *Arabidopsis*

The 5′ regulatory sequences of *CBF1*, *CBF2*, and *CBF3* genes have diverged more than the coding regions, but still keep a high level of similarity, which may result in the identical expression patterns shown by these genes. Comparison of the CBF1–3 promoter regions revealed that nucleotide sequences around the ATG initiation codons and TATA-box sequences are conserved [130]. Six conserved sequences have been reported in promoter regions of CBF1–3 genes [130]. Motifs similar to the G-box and ABRE-related sequences (T/CACGTGG/TC), MYB (C/TAACNA/G), and MYC recognition sites (CANNTG) have been reported in *CBF1–3* promoter regions [130]. *CBF1*, *CBF2*, and *CBF3* were not responsive to ABA, indicating that the CANNTG sequence, repeated several times in their upstream regions, was not sufficient to confer ABA responsiveness in the context of CBF promoters [103].

Medina *et al.* [103] also find some more motifs in the 5′ regions of *CBF1–3* genes, the core CANNTG consensus motif, as well as the CACGTC and TACGTG related sequences, which are present in the promoter region of many genes that are regulated by different environmental stresses and ABA. Furthermore, the pentamer CAGCC, which corresponds to the LTRE core sequence CCGAC in reverse orientation, was present in the CBF promoters [103]. The sequence CCGTC, which differs in only one nucleotide from the LTRE motif, was also found in the 5′ region of *CBF1*. Whether these sequences can confer the low-temperature response remains to be seen [103].

Sequence analysis of 5′-flanking regions of *DREB2A* and *DREB2B* genes showed that both genes are interrupted by a single intron at identical positions in their leader sequence. Several conserved sequences were found in the promoter regions of both DREB2 genes [131].

21.9.2
Expression of CBFs is Modulated by Temperature

Zarka *et al.* [132] carried out cold stress experiments with *Arabidopsis* to determine the effect of temperature on the cold-sensing mechanism. Studies by Zarka *et al.* [132] indicated that "the cold-sensing mechanism is not a 'binary' on and off system, it acts like a rheostat to adjust the level of CBF transcript accumulation to

the level of low temperature input." In cold-shock experiments, *CBF1–3* were rapidly induced upon exposure of plants to low temperature; CBF transcript levels reached a maximum at about 3 h and then declined significantly, but remained elevated over those found in warm-grown plants during the course of the 3-week experiment [132].

In *Arabidopsis*, the temperature-change experiments indicated that the CBF genes become inactive within minutes of transferring plants from low to warm temperature; the CBF transcripts had a half-life of only 7.5 min at warm temperatures [132], a value that is among the shortest described for plant genes [133]. The magnitude of the cold shock affected the peak levels of the CBF transcripts; the levels of CBF transcripts attained at 4 °C were greater than those attained at 10 °C. The gradual temperature down-shift experiments indicated that the threshold temperature at which accumulation of CBF transcripts became detectable was 14 °C and as the temperature continued to drop, the levels of CBF transcripts continued to increase [132].

Similarly, the cold-stress experiments with *Arabidopsis* indicated that the cold-sensing mechanism became desensitized upon continued exposure of the plants to low temperatures. Plants that had been cold acclimated at 4 °C for 14 day and were returned to warm temperatures for 1 h followed by abrupt transfer to 4 °C showed no detectable increase in CBF transcript levels. This experiment suggested that the cold-sensing mechanism became desensitized at 4 °C upon extended incubation and it could become resensitized at 4 °C after 24 h at warm temperatures [132]. These experiments also indicated that the desensitization that occurred upon exposure to 4 °C did not eliminate the ability of the plants to sense and respond to further drops in temperature. When plants that had been cold acclimated at 4 °C for 14 days were directly transferred to 0 or −5 °C, an increase in CBF levels occurred [132].

21.9.3
Regulation of the CBF Pathway in *Arabidopsis*

The stress induction of the *Arabidopsis CBF1–3* [126] and *DREB2* genes [128,131] is ABA independent, while the dehydration-induced expression of *CBF4* is controlled by ABA [106]. DREB/CBF gene expression may be controlled by Ca^{2+}-related processes, because both mutations in the Ca^{2+}/H^{+} transporter *CAX1* (calcium exchanger 1) and Ca^{2+}-sensor protein CBL1 have altered patterns of DREB/CBF gene expression [134,135]. These results suggest that *CAX1* ensures the accurate development of the cold acclimation response in *Arabidopsis* by controlling the induction of CBF/DREB and the corresponding target genes by regulating Ca^{2+} homeostasis in response to low temperatures [135]. The promoter regions of CBFs have no evident DRE/CRT elements and thus these genes do not appear to be subject to auto-regulation [126]. The expression of the CBF genes is apparently repressed by either their own gene products or the products of their downstream target genes, ensuring transient and tightly controlled expression of these genes [136,137]. A differential temporal pattern in the expression of CBF genes has been

uncovered in response to low temperature; the expression of *CBF1/DREB1B* and *CBF3/DREB1A* precedes that of *CBF2/DREB1C* [138]. Novillo *et al.* [138] showed that the expression of *CBF1/DREB1B* and *CBF3/DREB1A* was negatively regulated by *CBF2/DREB1C*.

21.9.3.1 Upstream Regulators of the CBF Pathway

Since the CBF transcripts start to accumulate within 15 min of a plant's exposure to cold, it was proposed that a transcription factor(s) already present in the cell at normal growth temperature recognizes the CBF promoters and induces CBF expression upon activation by cold stress [126]. Some factors and components controlling the cold-induced expression of CBFs have recently been characterized by mutational screens [139].

Positive Regulators of CBF Expression

ICE A dominant negative mutation of *ICE1* (inducer of CBF expression 1), the *ice1* mutant in *Arabidopsis*, results in almost complete elimination of *CBF3/DREB1A* transcript accumulation in response to low temperatures. However, *ice1* had little effect on cold-induced accumulation of *CBF2/DREB1C* transcripts [137]. Mutational analysis of the *CBF2/DREB1C* promoter identified two segments, designated ICEr1 and ICEr2, which work in concert to impart cold-regulated *CBF2* expression [132]. These studies indicate that differences exist in the mechanism of activation within the CBF/DREB1 family. *ICE1* encodes a constitutively expressed and nuclear-localized MYC-like bHLH transcriptional activator, which has been shown to bind specifically to the MYC recognition sequences in the *CBF3* promoter [137], but not to a putative MYB recognition sequence. ICE1 protein is inactive under non-stress conditions and upon exposing a plant to cold, modification of either ICE1 (most probably activated by phosphorylation [140]) or an associated protein would allow ICE1 protein to bind to the MYC *cis*-elements of the CBF promoter and induce *CBF3* expression [126]. Overexpression of the *ICE1* gene driven by the constitutive promoter in transgenic plants induced the expression of *CBF3* and its target genes *rd29A* and *COR15A* only at cold temperatures, but not at warm temperatures, suggesting that cold-induced modification of ICE1 protein is necessary [137]. The signaling components that transduce the cold stress signal to *ICE1* remain to be identified. It is also unclear whether *ICE1* also functions in other abiotic stress response pathways.

LOS4 Another gene that has a positive role in CBF expression is *LOS4* (low expression of osmotically responsive genes), which encodes DEAD-box RNA helicase, indicating that it functions in the regulation of RNA metabolism. Expression of CBFs and their downstream target genes as well as cold acclimation are impaired in *los4-1* mutant plants [141]. Interestingly, *los4-1* plants are highly sensitive to chilling when exposed to cold in darkness. This could specifically be due to impaired expression of *CBF2* in *los4-1* plants since *CBF2* alone was

expressed when wild-type *Arabidopsis* plants were exposed to cold during darkness [141].

The *CBF2* ortholog of birch shows higher expression when exposed to cold in darkness than in light [140], implying that the expression of CBF genes is also regulated by light. Light signaling mediated by phytochrome B has been reported to be necessary for cold-induced gene expression through the DRE/CRT element [142]. In addition, transient accumulation of the *CBF2* transcripts has been shown in response to far-red light and this accumulation was found to be phytochrome A dependent [143].

Negative Regulators of CBF Expression

HOS1 *Arabidopsis* plants with *hos1* and *hos2* (high expression of osmotically responsive gene) mutations show enhanced expression of a set of cold-inducible genes under cold stress, indicating that *HOS1* [144,145] and *HOS2* [144] are negative regulators of cold signal transduction. The *hos2* and *hos1* mutations enhance the cold-inducible genes by a different mechanism [144]. *HOS1* is a negative regulator of CBF expression, where *hos1* mutant plants showed enhanced expression of *CBF2, CBF3,* and their downstream target genes, and increased capacity to cold-acclimate in cold treatment. The *hos2* mutants also showed enhanced cold-responsive genes, but failed to show freezing tolerance. Molecular genetic analysis of the *HOS1* locus of *Arabidopsis* showed that early cold-signaling components upstream of the CBF/DREB1 might be regulated by specific ubiquitin-mediated degradation [145]. The *HOS1* gene encodes a protein that contains a RING finger motif, like IAP (inhibitor of apoptosis) proteins of animals. HOS1 may act as a E3 ubiquitin ligase, like IAP proteins of animals, by targeting a positive regulator(s) of CBF/DREB1 expression in the cold signaling pathway [145]. HOS1 resides in the cytoplasm, but appears to relocate to the nucleus upon cold treatment, suggesting that HOS1 may relay the cold signal to the nucleus to regulate the expression of *CBF/DREB1* genes [145]. The *hos1* mutation results in sustained and super-induction of *CBF2* and *CBF3*, and their target regulon genes are specifically induced during cold stress, but salt or ABA induction of these genes was not substantially altered [146].

FIERY Two additional negative regulators of the CBF pathway, *FIERY1* (*FRY1*) and *FIERY2* (*FRY2*), have been characterized [147,148]. Transcript levels of *CBF2* and stress-responsive genes were increased in *Fry1* mutant *Arabidopsis* plants under cold stress, but these plants were impaired in cold acclimation. *FRY1* encodes an inositol polyphosphate 1-phosphatase and involves in reduction of stress responses by controlling the turnover of the second messenger IP3 (inositol 1,4,5-triphosphate) [147]. Like *Fry1* mutants, *Fry2* mutant plants are also not acclimatized to cold stress despite very high expression of CBFs and their target genes [148]. This further indicates that either the downregulation of the CBF genes is essential for cold acclimation or that *fry1* and *fry2* mutations have pleiotropic effects on processes involved in the development of freezing tolerance [147,148].

MYB15 Apart from MYC recognition sequences, *CBF* promoters also have MYB recognition sequences. The *Arabidopsis* MYB15 is involved in cold regulation of *CBF* genes and in the development of freezing tolerance. The MYB15 transcription factor interacts with ICE1 and binds to MYB recognition sequences in the promoters of *CBF* genes. *MYB15* gene overexpression resulted in reduced expression of CBF genes and reduced freezing tolerance, whereas *myb15* mutant plants show increased tolerance to freezing stress. These results suggest that MYB15 controls the expression of *CBFs* and other genes in response to cold stress and is part of a complex network [149].

21.9.3.2 Downstream Regulators of the CBF Pathway

Apart from the CBF/DREB1, pathway there are multiple parallel and converging pathways involved in enhanced freezing tolerance. *Arabidopsis* mutants with enhanced freezing tolerance in the absence of cold acclimation (*eskimo*) [150] and mutants sensitive to freezing (*sfr*) [151] have been characterized to dissect the complex signaling pathway involved in increased freezing tolerance.

SFR6 is also Required for COR Gene Expression The *sfr6* mutant of *Arabidopsis* was identified based on its specific failure to gain freezing tolerance after cold acclimation treatment [151,152]. Transcriptome analysis indicated that the *sfr6* mutant is deficient in CRT/DRE-regulated COR gene expression during cold, osmotic stress, or exogenous ABA [152,153], whereas the cold-inducible expression of CBFs is unaffected. Transcriptome analysis also indicated that the *sfr6* mutation downregulates cold-regulated genes that do not have a DRE/CRT element in their promoters. These results indicate that SFR6 affects either a component in the signaling pathway downstream of CBF transcription or a component in an independent pathway that is simultaneously required for COR gene expression [152,153].

Histone Acetyl Transferase *Arabidopsis* has mutations or altered activities in transcriptional adapter ADA and GCN5, a histone acetyl transferase (HAT), which affected low-temperature regulation of COR gene expression without affecting the expression of CBF/DREB1 genes. These results indicate that *CBF1*-mediated transcription may require the transcriptional adapter ADA and the HAT GCN5 for the regulation of COR regulation [154,155].

21.9.4 CBF3 Integrates the Activation of Multiple Components of the Cold Response

Transgenic *Arabidopsis* plants overexpressing *CBF3* (*DREB1A*) of *Arabidopsis* under the control of the CaMV 35S promoter showed elevated levels of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose [156]. These plants had elevated *P5CS2* transcript levels (2- to 3-fold over the control plant) suggesting that the increase in proline levels resulted, at least in part, from increased expression of the key proline biosynthetic enzyme Δ1-pyrroline-5-carboxylate synthase (P5CS) [156]. The *P5CS2* transcript levels as

well as proline accumulation in cold-acclimated *CBF3*-overexpressing plants were 2- to 3-fold higher than in cold-acclimated control plants. Analysis of the promoter region of *P5CS2* gene revealed two core DRE regulatory elements CCGAC, within 350 nucleotides upstream of the ATG start codon [157], but whether *CBF3* binds to these sequences and activates expression was not determined [156]. The levels of total sugars in cold-acclimated transgenic *Arabidopsis* plants overexpressing *CBF3* were approximately 3-fold higher than those in control plants. Total soluble sugars significantly accounted for as much as 20% of the total dry weight of plant material in the cold-acclimated *CBF3*-overexpressing plants [156]. Two enzymes that have key roles in determining the levels of sucrose in plant cells are sucrose phosphate synthase (SPS) and sucrose synthase (SuSy) [158]. The transcript levels of these two genes were the same in transgenic and control plants. Thus, the effects of *CBF3* on sugar levels do not appear to be mediated by altering transcription of the SPS- or SuSy-encoding genes. These results suggest that *CBF3* integrates the activation of multiple components of cold acclimation response [156].

21.9.4.1 ESK1

Another *Arabidopsis* gene, *eskimo1* (*ESK1*), that affects the levels of proline and sugars, and has a major effect on freezing tolerance, has been identified [150]. While the concentrations of free proline and total sugars were elevated to 30-fold in the *esk1* and 2-fold in the mutant plants, respectively, the expression of the *COR* genes was not affected. Thus, *ESK1* appeared to be a negative regulator of *P5CS* transcription at warm temperatures. It was proposed that one possibility could be that ESK1 may be a transcriptional repressor that binds to the promoter of one or both of the *Arabidopsis P5CS* genes [157] at warm temperature and keeps transcription at a relatively low level. At low temperatures, *CBF3* could either directly bind to the *P5CS* promoter(s) and overcome this repression by *ESK1* or induce the expression of some other protein that inactivates *ESK1*.

Xin and Browse [150] proposed that *ESK1* defines a signaling pathway of cold acclimation that is distinct from that which mediates expression of the *COR* genes and cold acclimation, and is not a simple, linear signaling pathway activating the full set of processes responsible for increasing freezing tolerance. Instead, they proposed a model in which parallel or branched signaling pathways activate "distinct suites" of cold acclimation responses.

21.9.5 Parallel Pathway to CBFs

In addition to enhanced expression of LEA (late embryogenesis abundant)-type genes, multiple abiotic stress tolerance of CBF-overexpressing transgenic plants might also be in part due to accumulation of compatible osmolytes [156] and enhanced oxidative stress tolerance [159,160]. It was not clear how osmolyte biosynthesis and antioxidant defense pathways were activated in CBF-overexpressing plants [161]. Genome-wide expression analysis showed that CBF overexpression also

induced transcription factors, such as AP2 domain proteins (*RAP2.1* and *RAP2.6*), a putative zinc finger protein, and R2R3-MYB73 [162], that may regulate osmolyte biosynthesis and antioxidant defense genes [161].

21.9.5.1 RAV1 and ZAT12 May Follow Parallel Pathways to CBFs

Two transcription factors, RAV1 (AP2) [163] and ZAT12 (zinc finger) [164], had patterns of expression that were similar to those of CBF. Neither RAV1 nor ZAT12 transcript levels were affected in CBF-overexpressing plants; they probably operate in pathways that are parallel to those of the CBFs [162].

21.10 *DREB1A*-Targeted Genes

In *Arabidopsis*, *DREB1A* gene expression was rapid and transient in response to cold treatment, reached a maximum at 2 h, and then decreased, whereas the expression of *DREB1A* target genes was increased slowly and gradually after cold treatment within 10 h [9]. These results support the view that *DREB1A* regulates the expression of the *DREB1A* target genes, such as *rd29A*, *erd10*, *cor15A*, *rd17*, *kin2*, and *RAFL06-16-B22* [102,165]. In *Arabidopsis*, among 41 cold-inducible genes, 32 genes contained either the DRE or DRE-related CCGAC core motifs in their promoter regions, suggesting that DRE is a major *cis*-acting element involved in cold-inducible gene expression [9]. Studies by Sakuma *et al.* [100] and Liu *et al.* [128] indicated that the binding of the DREB proteins to the DRE sequence is highly specific. However, these proteins had different binding specificities to the second or third nucleotides of DRE [128]. Presumably, CBF1 and CBF2 have overlapping, if not identical, roles to those of CBF3 [156]. Medina *et al.* [103] hypothesized that differences in the sequences of the CCGAC core element and/or in the sequences that surround it may result in the recruitment of distinct CBF proteins. A similar situation has been described for the G-box sequence CANNTG and the bZIP proteins [166].

Maruyama *et al.* [167] identified 38 *DREB1A* target genes in DREB1A-over-expressing transgenic plants and the *DREB1A* target genes were classified into two groups. The first group includes proteins that are believed to function in stress tolerance. Examples of such proteins include LEA proteins, antifreeze proteins, hydrophilic proteins, RNA-binding protein, galactinol synthase, and protease inhibitors. The second group contains protein factors that are involved in further regulation of signal transduction and gene expression that probably function in response to stress. The transcription factors STZ/ZAT10 (STZ) and At5g04340 are two specific examples of *DREB1A* downstream target genes, and these two transcription factors may repress the transactivation of genes. The recombinant *DREB1A* protein bound to A/GCCGACNT more efficiently than to A/GCCGACNA/G/C and Maruyama *et al.* [167] identified a consensus DRE A/GCCGACNT sequence in the promoter regions (from –51 to –450) of the direct downstream genes of *DREB1A*. Sakuma *et al.* [168] found that both *DREB2A* and

DREB1A proteins can bind to the DRE sequence, but the DNA-binding specificities of each to the neighboring sequences of the DRE core motif were slightly different; therefore, the downstream genes of each are partially different. Fourteen genes were identified as candidates for direct targets of *DREB2A*, whereas nine genes encoded LEA class proteins [168].

21.11
Overexpression of DREB Genes in Plant Species

The ability of the CBF/DREB1 transcription factors to activate the DRE/CRT class of stress-responsive genes has further been demonstrated by studies on overexpression or enhanced inducible expression of CBF/DREB1 that have resulted in activation of the target genes in several model species as well as crop species.

21.11.1
Overexpression of DREB Genes in Transgenic *Arabidopsis*

Overexpression of *Arabidopsis CBF/DREB1* genes increased tolerance of the transgenic *Arabidopsis* plants to freezing, salt, or drought stress, suggesting that regulation of the CBF/DREB1 class of genes in plants is important for the development of stress tolerance [155]. Strong tolerance to freezing stress was observed in transgenic *Arabidopsis* plants that overexpress *CBF1/DREB1B* under the control of the CaMV 35S promoter [169]. Overexpression of *DREB1A/CBF3* under the control of the CaMV 35S promoter also increased the tolerance to drought, high-salinity, and freezing stresses [102,128,156]. Constitutive overexpression of CBFs strongly activated the expression of several LEA-type genes, enhancing freezing and osmotic stress tolerance of transgenic *Arabidopsis* [102,128,169]. Overexpression of DREB1-type proteins conferred high stress tolerance in transgenic *Arabidopsis* plants, whereas the plants overexpressing DREB2-type proteins failed to show any stress tolerance [106,128]. These results indicate that DREB1-type proteins are constitutively active in plants, but that DREB2-type proteins probably require modification in response to stress for their activation in plants [113]. Sakuma *et al.* [168] showed that DREB2A protein has a negative regulatory domain in its central region (amino acids 136–165) and the deletion of this domain transforms DREB2A into the constitutive active form. The overexpression of *DREB2A CA* (modified to be constitutively active) activated the expression of many stress-inducible genes and improved tolerance to drought in transgenic *Arabidopsis*.

Nevertheless, overexpression of DREB1A protein has also been reported to cause severe growth retardation under normal growth conditions. Use of the stress-inducible *rd29A* promoter instead of the constitutive CaMV 35S promoter for the overexpression of *DREB1A* minimized the negative effects on plant growth [102]. Transgenic *Arabidopsis* plants carrying *rd29A:DREB1A* showed low levels of constitutive expression of *LEA* genes, and enhanced expression under cold,

dehydration, and salt stresses. Both the *rd29A:DREB1A* and *CaMV 35S:DREB1A* transgenic plants showed enhanced tolerance to freezing, drought, and salt stresses,, but tolerance levels of *rd29A:DREB1A* transgenics were significantly higher than those of *CaMV 35S:DREB1A* transgenics [102]. Moreover, the constitutive overexpression of CBFs resulted in severe growth retardation and reduction in seed production, even under a normal environment [128]. Similar responses were observed in transgenic tomato overexpressing *Arabidopsis DREB1B/CBF1*, resulting in the development of a dwarf phenotype, which could be prevented by exogenous application of GA3 [159,160]. These observations suggest that inhibition of gibberellin biosynthesis is a function common to *DREB1/CBF* genes.

A gibberellin-deficient *Arabidopsis* mutant designated *dwarf and delayed flowering 1* (*ddf1*) showed dwarfism and late flowering. The contents of bioactive GA4 and GA1 were in fact decreased in *ddf1*, but the transcription level of the GA20 oxidase gene did not decrease. Genetic and molecular analyses revealed that the *ddf1* phenotypes are caused by increased or ectopic expression of a putative AP2 transcription factor (DDFs). Isolation and characterization of *DDFs* (*DREB1E* and *DREB1F*) revealed that these genes are phylogenetically closer to *DREB1* genes and *DDF1* mRNA is strongly induced by high-salinity stress. Moreover, transgenic plants overexpressing *DDF1* showed increased tolerance to high-salinity stress. These results suggest that *DDF1* is involved in the regulation of gibberellin biosynthesis and stress tolerance. Interestingly, DNA microarray analysis using the Affimetrix GeneChip 8K did not detect these changes in transcript levels of gibberellin-related genes in transgenic *Arabidopsis* overexpressing *CBF1–3* [162]. These results suggest that the inhibition of gibberellin biosynthesis might be caused by other mechanisms like post-transcriptional modification of GA20 oxidase, induction of an inhibitory subunit of GA20 oxidase, or production of unknown enzymes that catabolize C19-gibberellin intermediates [107].

21.11.2
Heterologous Expression of *Arabidopsis* DREB Genes in Transgenic Plants

Overexpression of *Arabidopsis* DREB1/CBF genes in transgenic crop plants improved freezing, drought, and salt tolerance [102,128,159,160,169] (Table 21.1). Constitutive overexpression of *Arabidopsis* CBF genes in *B. napus* was shown to induce expression of *Bn115* and *Bn28*, an ortholog of the CBF-targeted *Arabidopsis* gene *COR6.6*, increasing freezing tolerance in both non-acclimated and cold-acclimated plants [109]. Similar reports on tomato (*Lycopersicon*) plants ectopically expressing *Arabidopsis CBF1/DREB1B* showed enhanced resistance to water-deficit, chilling, and oxidative stresses [159,160]. These transgenics exhibited growth retardation showing a dwarf phenotype, and the fruit and seed numbers and fresh weight of the transgenic tomato plants were apparently less than those of the wild-type plants under normal growth conditions. Exogenous gibberellic acid treatment reversed the growth retardation and enhanced growth of transgenic tomato plants, but did not affect the level of water-deficit resistance/chilling

Table 21.1 Functional analysis of DREB genes in transgenic plants.

DREB gene	Gene source	Transgenic plant	Tolerance to	Reference
DREB1A/CBF3	*A. thaliana*	*A. thaliana*	Freezing, salt, and drought tolerance	[102,128,156]
		B. napus	Freezing and drought tolerance	[109]
		Triticum sp.	Drought tolerance	[170]
		Nicotiana tabacum	Drought and cold tolerance	[171]
		Arachis hypogaea	Drought tolerance	[172]
		Lycopersicon esculentum	No significant tolerance	[105]
		O. sativa	Drought and salt tolerance	[173]
		S. tuberosum	Salt tolerance	[174]
DREB1B/CBF1	*A. thaliana*	*A. thaliana*	Freezing tolerance	[156,169]
		B. napus	Freezing and drought tolerance	[109]
		Fragaria ananassa	Freezing tolerance	[175]
		L. esculentum	Drought, chilling, and salt tolerance	[159,160]
		O. sativa	No significant tolerance	[176]
		Populus sp.	Freezing tolerance	[120]
DREB1C/CBF2	*A. thaliana*	*B. napus*	Freezing and drought tolerance	[109]
DREB1D/CBF4	*A. thaliana*	*A. thaliana*	Freezing and drought tolerance	[106]
DREB1F/DDF1	*A. thaliana*	*A. thaliana*	High salinity tolerance	[107]
AhDREB1	*Atriplex hortensis*	*N. tabacum*	Survived better under salt and drought stresses	[177]
BnCBF5 and *17*	*B. napus*	*B. napus*	Freezing tolerance, increased photosynthetic capacity	[178]
CIG-B	*Prunus avium*	*A. thaliana*	Salt and freezing tolerance	[179]
GhDREB1	*Gossypium hirsutum*	*N. tabacum*	Freezing	[180]
HvDREB1	*Hordeum vulgare*	*A. thaliana*	Salinity	[181]
LeCBF1	*L. esculentum*	*A. thaliana*	Freezing tolerance	[105]
	L. esculentum	*L. esculentum*	No significant tolerance	
LpCBF3	*Lolium perenne*	*A. thaliana*	Enhanced freezing tolerance	[118]
OsDREB1A	*O. sativa*	*A. thaliana*	Drought, salt, and freezing tolerance	[104]
OsDREB1F	*O. sativa*	*O. sativa, A. thaliana*	Drought, salt, and freezing tolerance	[182]

OsDREB1G	*O. sativa*	*O. sativa*	Drought	[183]
TaDREB1	*Triticum aestivum*	*O. sativa*	Dwarf phenotypes observed	[112]
		A. thaliana	No significant tolerance	
ZmDREB1	*Z. mays*	*A. thaliana*	Drought and freezing tolerance	[113]
AtDREB2A-CA	*A. thaliana*	*A. thaliana*	Drought	[184]
AtDREB2C	*A. thaliana*	*A. thaliana*	Thermotolerance	[185]
OsDREB2B	*O. sativa*	*A. thaliana*	Drought, thermotolerance	[186]
PeDREB2	*Populus euphratica*	*N. tabacum*	Salinity	[187]
PgDREB2A	*Pennisetum glaucum*	*N. tabacum*	Hyperionic, hyperosmotic	[188]
ZmDREB2A	*Z. mays*	*A. thaliana*	Drought, thermotolerance	[189]

tolerance in these plants. Moreover, stomata of the transgenic *CBF1* tomato plants closed more rapidly than the wild-type after water-deficit treatment with or without gibberellic acid pretreatment and contained higher levels of proline than wild plants under normal or water-deficit conditions. The level of H_2O_2 in the transgenic plants was lower than that in the wild-type plants under either normal or cold conditions. Subtractive hybridization performed to isolate the responsive genes of heterologous *Arabidopsis CBF1* in transgenic tomato plants revealed *catalase 1* (*CAT1*) activation.

In rice (*Oryza sativa*), the *CBF1/DREB1B* of *Arabidopsis* was introduced under the control of the maize ubiquitin promoter [176]. Cold tolerance in the transgenics was not significantly different from that of the wild-type plants, as determined by ion leakage, chlorophyll fluorescence, and survival rates. However, the cold-responsive genes contain DRE/CRT elements in their regions, and *lip5*, *lip9*, and *OsDhn1* were upregulated in the transgenic plants, suggesting that the cold signal transduction pathway involving *CBF1* is partially conserved in this cold-labile plant [176]. Similarly, overexpression of the *A. thaliana DREB1A* gene under the control of a stress-inducible promoter from the *rd29A* gene in transgenic wheat was reported to delay water-stress symptoms when compared with controls in greenhouse conditions [171]. The transgenic wheat lines further started to show water stress symptoms (loss of turgor and bleaching of the leaves) after 15 days of withdrawal of water, whereas the control plants showed these symptoms sooner than 10 days and severe symptoms (death of all leaf tissue) were evident in the controls after 15 days. In the tobacco model system, overexpression of *DREB1A* improved drought and low-temperature stress tolerance with the stress-inducible *rd29A* gene promoter minimizing the negative effects on the plant growth. Furthermore, overexpression of stress-inducible genes targeted by *DREB1A* were detected [170].

Transgenic rice were developed (*O. sativa* cv. Nakdong) by using the *Agrobacterium*-mediated transformation method [173] using the *ubiquitin 1* promoter, together with its intron (*Ubi1*), to drive the constitutive expression of *DREB1A*

(*CBF3*) of *Arabidopsis*. Transgenic rice showed elevated tolerance to drought and high salinity, and produced relatively low levels of tolerance to low-temperature exposure. These data were in direct contrast to *CBF3* in *Arabidopsis*, which is known to function primarily to enhance freezing tolerance. Microarray and RNA gel-blot analyses showed that 12 target genes were activated in transgenic rice plants in normal conditions and 13 additional genes were induced on exposure to drought stress (more then 1.6-fold increase). Interestingly, these transgenic plants exhibited neither growth inhibition nor visible phenotypic alterations despite constitutive expression of the *CBF3* in contrast to the results previously obtained from *Arabidopsis* where the transgenic plants were stunted [102].

Among the tuber crops, potato (*Solanum tuberosum*) was transformed with the *A. thaliana DREB1A* gene driven by the *rd29A* gene promoter via *Agrobacterium*-mediated transformation [174]. Some of the transgenic potato lines showed significantly higher resistance to salt stress than the controls [174] and this tolerance correlated with the copy number of the *DREB1A* insert with few exceptions [174]. Transgenic groundnut plants that carry the *DREB1A* gene of *A. thaliana* driven by the *rd29A* gene promoter of *A. thaliana* showed higher transpiration efficiency than the wild plants under water-limiting conditions [172].

These studies indicate that genetic engineering of CBFs and potentially other transcription factors with stress-specific promoters in crops appears to be a viable approach for engineering tolerance to multiple stresses, including salt stress. While reports indicate that constitutive overexpression of *Arabidopsis DREB1A* improved drought and low-temperature stress tolerance, regulation of transgene expression via the stress-inducible *rd29A* promoter minimized the negative effects on plant growth in model and crop species. This substantial enhanced tolerance to water stress indicates that a combination of the *rd29A* promoter and *DREB1A* is useful for improvement of various kinds of transgenic plants that are tolerant to environmental stress.

21.11.3
DREB Genes Have Discrepant Expression in Monocots and Dicots

A wheat DREB gene, *TaDREB1* (similar to *Arabidopsis DREB2A*), exerted different effects in different transgenic plants [112]. Overexpression of the *TaDREB1* gene under unstressed conditions caused a dwarf phenotype in transgenic rice, whereas in transgenic *Arabidopsis* this dwarf phenotype was not observed. This discrepancy might be due to the possibility that a gene originated from monocots functioned effectively in transgenic monocots and a gene from dicots was effective only in dicots [112]. Further studies on the transgenic plants will elucidate more details.

21.11.4
CBF/DREB1 Genes of *Arabidopsis* and Rice are Functionally Different

The *CBF1/DREB1B* gene of *Arabidopsis* was introduced into rice under the control of the maize ubiquitin promoter [176] and cold tolerance in the transgenics was not

significantly different from that of the wild-type plants. However, the cold-responsive genes *lip5*, *lip9*, and *OsDhn1* were upregulated in these transgenic plants, suggesting that the cold signal transduction pathway involving *CBF1* is partially conserved in this cold-labile plant [176]. Dubouzet *et al.* [104] have reported that rice CBF/DREB1 genes are functionally similar to *Arabidopsis* CBF/DREB1, although the specificity of their binding to the CRT/DRE element is somewhat different. Interestingly, transgenic *Arabidopsis* expressing *CaMV 35S:OsDREB1A* did not significantly upregulate such *COR* genes as *erd10* and *kin2/cor6.6*. These results together demonstrate that the CBF/DREB1 genes of *Arabidopsis* and rice are functionally different. The fact that rice is much more sensitive to cold than *Arabidopsis* is probably due to the lack of homologs of some *Arabidopsis* CBF/DREB1 target genes in rice. For example, the COR15a protein previously identified as cryoprotective [176,190] is not found in the rice database. Over 36 cold-induced genes in rice have been reported, but most of these genes did not match with the CBF-regulated genes of *Arabidopsis* [191].

21.12 Conclusion

Abiotic stresses such as drought, salinity, and extreme temperatures are major causes of losses in agriculture production. Many genes are involved in plant responses to stresses and transcription factors play a key role in the stress response by regulating other genes, whose products function in providing stress tolerance to plants. The major transcription factors involved in abiotic stress are bZIP, MYB/MYC, HD-ZIP, and AP2/EREBP. Transcription factors play a crucial role in providing tolerance to multiple stresses generally in both an ABA-dependent and ABA-independent manner, and through respective *cis*-elements and DNA-binding domains. Understanding the molecular mechanisms of plant responses to abiotic stresses is very important as it facilitates in exploiting them to improve stress tolerance and productivity. Many studies were conducted on DREB transcription factors by developing transgenics using different promoters and these studies suggested that using a stress-responsive promoter is a better option than using a constitutive promoter.

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