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REVIEW

Regulons involved in osmotic stress-responsive and cold stress-responsive gene expression in plants

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Many plants, including *Arabidopsis thaliana*, increase in freezing tolerance in response to low non-freezing temperatures, a phenomenon which is known as cold acclimation. Molecular studies in plants have shown that several genes with various functions are induced by low temperature (cold stress) and osmotic stress such as drought and high salinity. For several stress-inducible genes, *cis*-acting elements in promoters regions and the corresponding transcription factors that affect the expression of these genes have been analyzed in *Arabidopsis*. The dehydration-responsive element (DRE)/C-repeat (CRT), *cis*-acting element, is involved in osmotic stress- and cold stress-inducible gene expression. Transcription factors that bind to the DRE/CRT were isolated and named DRE-binding protein 1 (DREB1)/CRT-binding factor (CBF) and DREB2. The DREB1A/CBF3, DREB1B/CBF1 and DREB1C/CBF2 regulons are involved in cold stress-responsive gene expression, whereas, the DREB2 regulon is involved in osmotic stress-responsive gene expression. In previous experiments, overexpression of the *DREB1*/*CBF* genes in transgenic *Arabidopsis* plants upregulated several stress-inducible genes and increased tolerance to freezing, drought and high-salinity stresses. Subsequent to their discovery, the *DREB1*/*CBF* genes have been successfully used to improve abiotic stress tolerance in a number of different crop plants. Interestingly, homologous genes of *DREB1*/*CBF* have been found in many other plant species including tomato and rice, which are unable to undergo cold acclimation. Thus, it is apparent that the *DREB1*/*CBF* regulon is ubiquitous within higher plants. Current research endeavors are focusing to identify additional transcription factors that are associated with stress response. The ultimate goal of regulon biotechnology is the control of signal transduction networks, a manipulation which in turn is expected to improve stress tolerance in plants.

Abbreviations – ABA, abscisic acid; ABF, ABRE-binding factor; ABRE, ABA-responsive element; AREB, ABRE-binding protein; bHLH, basic helix-loop-helix; bZIP, basic-domain leucine zipper; CBF, C-repeat-binding factor; CRT, C-repeat; DRE, dehydration-responsive element; DREB, DRE-binding protein; ERD, early responsive to dehydration; ICE1, inducer of CBF expression 1; LEA, late embryogenesis abundant; LUC, luciferase; MYBR, MYB recognition site; MYCR, MYC recognition site; NACR, NAC recognition site; RD, responsive to dehydration; SnRK, SNF1-related protein kinase; ZF-HD, zinc-finger homeodomain.

Introduction

As plants are sessile organisms, they are forced to survive in environments with variable environmental stresses such as cold stress and osmotic stress which includes drought and high salinity. Many plants, including the extensively characterized model plant *Arabidopsis thaliana*, exhibit an increase in freezing tolerance in response to low, non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow 1999). A number of genes have been described that respond to cold and osmotic stress in plants (Shinozaki and Yamaguchi-Shinozaki 1997, Thomashow 1999, Zhang et al. 2004a), and it is thought that their gene products may play important roles for acclimation of plants. Recently, 299 drought-inducible genes, 213 high-salinity stress-inducible genes, and 54 cold-inducible genes were identified using a cDNA microarray containing approximately 7000 independent full-length *Arabidopsis* cDNA clones (Seki et al. 2002, Shinozaki et al. 2003). Functions of their gene products have been predicted from comparisons of sequence homology with known proteins. Genes induced during osmotic and cold stress conditions are thought to function not only in protecting cells from stress by the production of important metabolic proteins (functional proteins) but also in the regulation of genes for signal transduction in the stress response (regulatory proteins). Such examples of functional proteins include water channel proteins, chaperones, proteases, late embryogenesis-abundant (LEA) proteins, and enzymes that are involved with the synthesis of osmoprotectants [compatible solutes: sugars, proline (Pro), etc.]. Examples of stress-related regulatory proteins include transcription factors, protein kinases, and enzymes for phosphoinositide turnover, and enzymes for the synthesis of the plant hormone abscisic acid (ABA). Multiple studies have attempted to augment plant stress tolerance by overexpressing various kinds of functional proteins such as enzymes for the synthesis of osmoprotectants and ion transporters (Chen and Murata 2002, Zhang et al. 2004a). However, it has become evident that the engineering of single enzymes is not sufficient, because multiple stress responses are necessary for plants to endure severe stress conditions.

In plants, it is possible for a single transcription factor to control the expression of many target genes through the specific binding of the transcription factor to *cis*-acting element in the promoters of their respective target genes. This type of a transcription unit is called a "regulon." Analysis of the expression mechanisms of osmotic stress- and cold stress-responsive genes revealed apparent presence of multiple regulons in *Arabidopsis* (Figs 1 and 2). Among them, the dehydration-responsive element-binding protein (DREB1)/C-repeat (CRT)-binding factor (CBF) regulon is involved in both osmotic stress- and cold stress-responsive gene expression, and the other regulons including the DREB2 regulon are involved in osmotic stress-responsive gene expression. It is expected that biotechnological efforts which strive to control gene expression within these regulons will improve the tolerance against multiple stresses in plants.

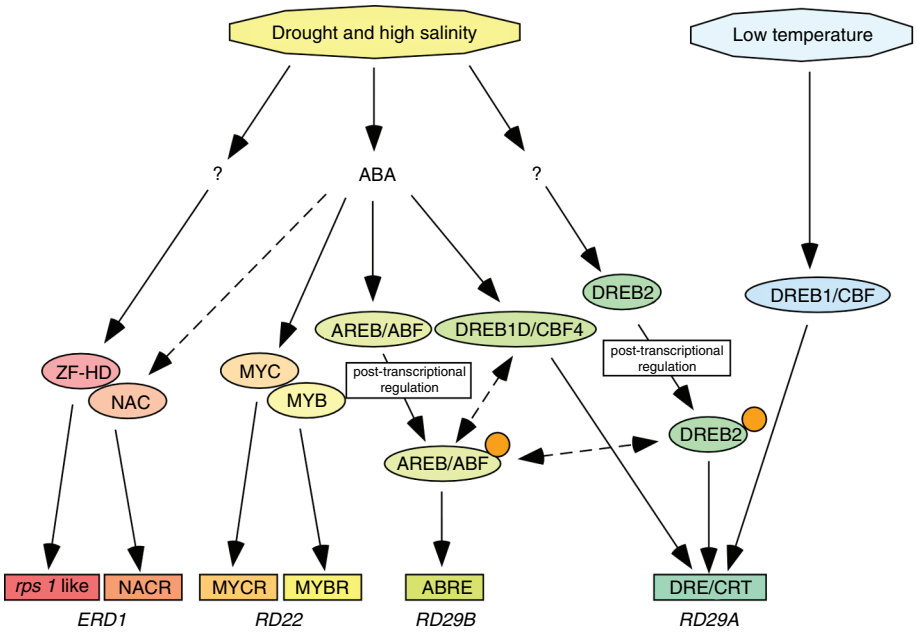

Fig. 1. Regulatory network of gene expression in response to cold stress and osmotic stress such as drought and high salinity: specificity and crosstalk of gene networks. *Cis*-acting elements that are involved in stress-responsive transcription are shown in boxes. Transcription factors that control stress-inducible gene expression are shown in ovals. Small circles indicate the modification of transcription factors in response to stress signals for their activation, such as phosphorylation. Dotted lines indicate possible regulation. Double arrow lines indicate possible crosstalk.

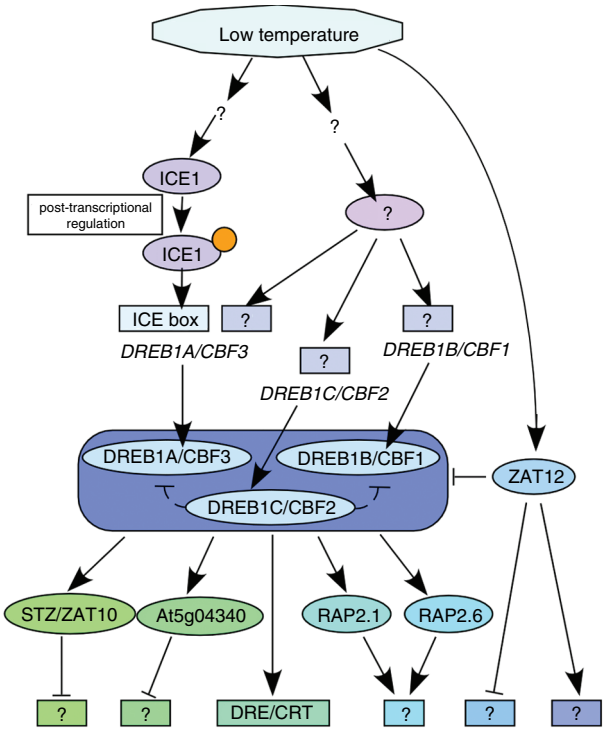

Fig. 2. Regulatory network of gene expression in response to cold stress. *Cis*-acting elements that are involved in stress-responsive transcription are shown in boxes. Transcription factors that control stress-inducible gene expression are shown in ovals. Small circles indicate the modification of transcription factors in response to stress signals for their activation, such as phosphorylation.

DREB regulons involved in osmotic stress- and cold stress-responsive gene expression in *Arabidopsis*

The promoter of an *Arabidopsis* drought-, high-salinity- and cold-inducible gene *responsive to dehydration 29A* (*RD29A*) encoding a LEA-like protein contains two major *cis*-acting elements. Specifically, the ABA-responsive element (ABRE) is involved in ABA-responsive gene expression (see below), and the DRE/CRT is involved in osmotic stress- and cold stress-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki 1994). DRE/CRT (CCGAC) is a *cis*-acting element that functions in ABA-independent gene expression in response to abiotic stress (Figs 1 and 2). Transcription factors belonging to the AP2 (APETALA2)/ethylene-responsive element-binding factor (ERF) family that bind to the DRE/CRT have been isolated and termed DREB1/CBF and DREB2 (Gilmour et al. 1998, Liu et al. 1998, Stockinger et al. 1997). The conserved DNA-binding motif of DREB1/CBF and DREB2 is A/GCCGAC (Sakuma et al. 2002). The DREB1A/CBF3 (DREB1A), DREB1B/CBF1 (DREB1B), and DREB1C/CBF2 (DREB1C) are highly similar in amino acid sequences, and the genes occur tandemly within the *Arabidopsis* genome (Gilmour et al. 1998, Shinwari et al. 1998). These *DREB1/CBF* genes are quickly and transiently induced by cold stress, and their products activate the expression of multiple stress-inducible target genes. The *DREB2* genes are induced by dehydration, leading to the expression of various genes that are involved in drought-stress tolerance (Liu et al. 1998, Nakashima et al. 2000). In the *Arabidopsis* genome, 145 AP2/ERF-related proteins are encoded, and the DREB subfamily is included within the A-1–A-6 subgroups (Sakuma et al. 2002) (Fig. 3). The A-1 subgroup contains six *Arabidopsis* proteins including DREB1A, DREB1B, and DREB1C, and the A-2 subgroup contains eight *Arabidopsis* proteins, including DREB2A and DREB2B.

Improved stress tolerance of transgenic plants overexpressing *DREB1/CBF*

Overexpression of the *DREB1A*, *DREB1B*, and *DREB1C* genes in transgenic *Arabidopsis* plants showed increased tolerance to freezing, drought, and high salt concentrations (Gilmour et al. 2004, Jaglo-Ottosen et al. 1998, Kasuga et al. 1999, Liu et al. 1998). These observations led the researchers to believe that DREB1A, DREB1B, and DREB1C proteins function without post-translational modification of the proteins in the development of stress tolerance. Overexpression of *DREB1A*, *DREB1B*, and *DREB1C* genes results in multiple biochemical changes that are associated with cold acclimation (Gilmour et al. 2000, 2004). Examples of such changes in plants overexpressing *DREB1A* included elevated levels of Pro and total soluble sugars such as sucrose, raffinose, glucose, and fructose. Plants overexpressing *DREB1A* also had elevated delta (1)-(P5CS) pyrroline-5-carboxylate synthase transcript levels. Accumulation of P5CS suggests that the resultant increase in Pro levels is, at least in part, related to the increased expression of the key Pro biosynthetic enzyme P5CS. Collectively, these results lead us to propose that DREB1/CBF integrates the activation of multiple components of the cold acclimation response.

A side effect of the overexpression of *DREB1A*, *DREB1B*, or *DREB1C* in transgenic *Arabidopsis* is dwarfism (Gilmour et al., 2000, 2004, Kasuga et al. 1999, Liu et al. 1998). Similarly, the development of dwarf phenotypes was also found in transgenic tomato overexpressing *Arabidopsis DREB1B*, and it was prevented by exogenous application of gibberellin (GA) (Hsieh et al. 2002a, b). In comparison with wild-type tomato plants, GA-treated transgenic plants still exhibited a

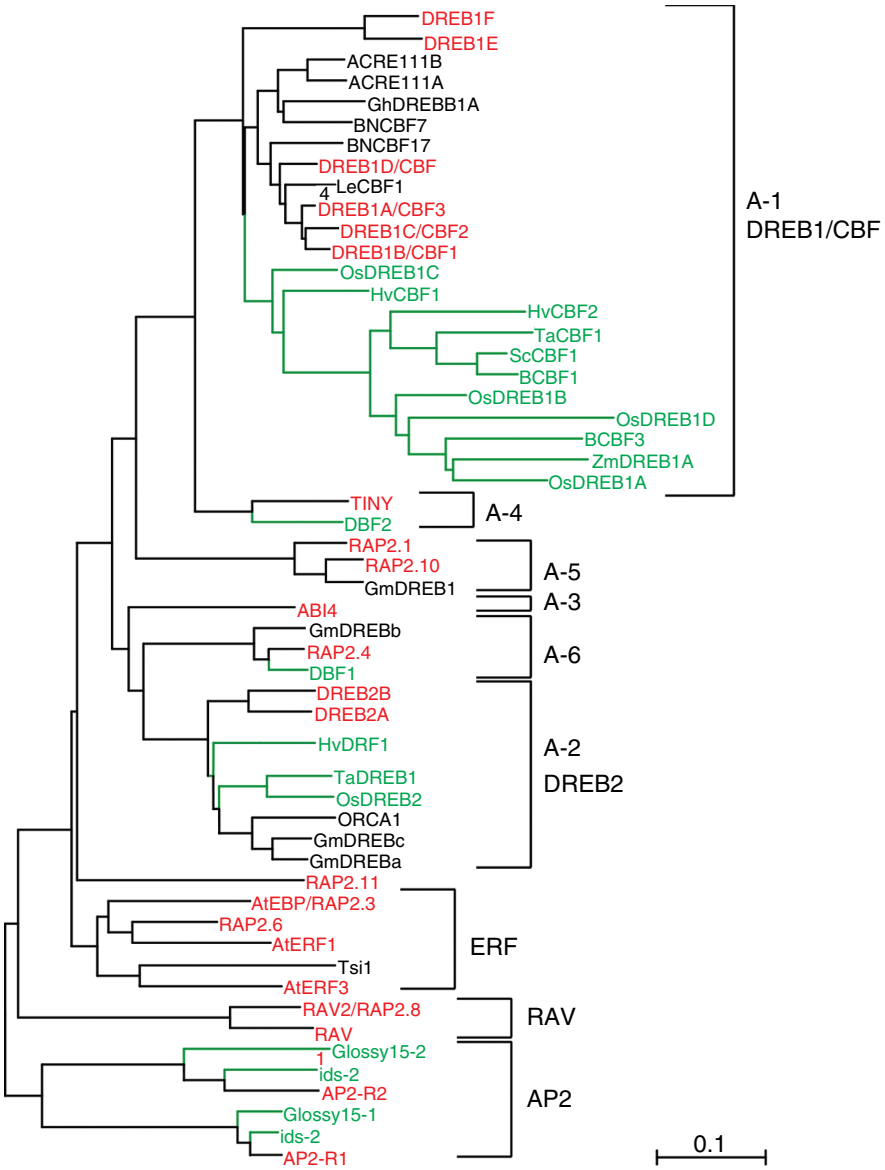

Fig. 3. Phylogenic analysis of the major dehydration-responsive element-binding protein 1 (DREB1)/C-repeat-binding factor (CBF), DREB2, and other proteins having the ethylene-responsive element-binding factor (ERF)/AP2 domains. A phylogenetic tree of the ERF/AP2 domains was constructed by CLASTAX, and the scale indicates branch lengths. A-1–A-6 indicate subgroups proposed by Sakuma et al. (2002). This figure was modified from Dubouzet et al. (2003), Sakuma et al. (2002), and Qin et al. (2004). The plant species and the accession number of each appended protein were DREB1D/CBF4 (*Arabidopsis thaliana*, At5g51990); DREB1E (*Arabidopsis thaliana*, At1g63030); DREB1F (*Arabidopsis thaliana*, At1g12610); GmDREB1 (*Glycine max*, AF514908); GmDREBa (*Glycine max*, AY542886); GmDREBb (*Glycine max*, AY296651); GmDREBc (*Glycine max*, AY244760). Genes from *Arabidopsis thaliana* are shown in red; genes from monocot plants in blue (wheat, rice, rye, and barley); and genes from dicot plants in black (*Brassica napus*, cotton, tomato, and tobacco).

greater degree of dehydration and chilling tolerance. However, it is important to note that microarray analysis did not detect the changes in transcript levels of any known GA-related genes in transgenic *Arabidopsis* overexpressing *DREB1A*, *DREB1B*, or *DREB1C* (Fowler and Thomashow 2002). Recently, DREB1F is reported to be involved in the regulation of GA biosynthesis and stress tolerance (Magome et al. 2004). At the present time, it is not yet clear whether other DREB1/CBF proteins are related to GA synthesis or not.

Many candidates for the DREB1/CBF target genes have been identified using microarray analyses (Fowler and Thomashow 2002, Maruyama et al. 2004, Seki et al. 2001). Maruyama et al. (2004) searched for downstream genes in transgenic plants overexpressing DREB1A using the full-length cDNA microarray and Affymetrix GeneChip array and identified 38 genes as putative DREB1A downstream target genes. Subsequent to their identification, the downstream genes of DREB1A 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 protein, RNA-binding protein, an enzyme required for sugar biosynthesis (galactinol synthase), and protease inhibitors. It is likely that these gene products function to increase the tolerance to drought, high salt, and freezing stresses in transgenic *Arabidopsis*. The second group contains

protein factors that are involved in further regulation of signal transduction and gene expression that probably functions in response to stress. Examples of such proteins from the second group include transcription factors (C_2H_2 zinc finger DNA-binding proteins and AP2/ERF-type DNA-binding protein) and enzymes that are involved in phospholipid metabolism (phospholipase C). Genes encoding transcription factors containing a C_2H_2-type zinc-finger (ZF) motif constitute a large family in higher plants. The transcription factors STZ/ZAT10 (STZ) and At5g04340 are two specific examples of DREB1A downstream target genes (Fig. 2). STZ has been shown to repress the trans-activation of genes through an essential DLN-box/EAR-like repression motif in its C-terminal region. The At5g04340 protein also contains this same repression motif in its C-terminal region as well. Recently, Sakamoto et al. (2004) analyzed transgenic *Arabidopsis* plants overexpressing STZ using a cDNA microarray and found that many genes related to photosynthesis and carbohydrate metabolism were suppressed in these plants; suggesting that STZ is involved in the mechanism of growth retardation of the *35S* : *DREB1A* transgenic plants. Stress-related signaling has been reported to result in the repression of genes associated with plant growth and development. Thus, it is apparent that DREB1A, DREB1B, and DREB1C not only controls the activation of genes involved in stress tolerance but also represses genes that are involved in plant growth and development, such as photosynthesis-related genes. Maruyama et al. (2004) also searched for conserved sequences in the promoter regions of the direct downstream genes and found A/GCCGACNT in their promoter regions from -51 to -450 as a consensus DRE. The recombinant DREB1A protein bound to A/GCCGACNT more efficiently than to A/GCCGACNA/G/C.

Vogel et al. (2005) used the Affymetrix GeneChip containing probe sets for approximately 24 000 *Arabidopsis* genes to define a core set of cold-responsive genes and to determine which genes were targets of DREB1C and six other transcription factors that appeared to be coordinately regulated with DREB1C. A total of 514 genes were placed in the core set of cold-responsive genes, 302 of which were upregulated and 212 that were downregulated. Hierarchical clustering and bioinformatic analysis indicated that the 514 cold-responsive transcripts could be assigned to one of seven distinct expression classes. Furthermore, these analyses identified multiple potential novel *cis*-acting cold-regulatory elements. Eighty-five cold-induced genes and eight cold-repressed genes were assigned to the DREB1C regulon. An additional nine cold-induced genes and 15 cold-repressed genes were assigned to a regulon controlled by ZAT12. Of the 25 core cold-induced genes that were most highly upregulated, 19 genes were induced by DREB1C and two additional genes were regulated by both DREB1C and ZAT12. Thus, the large majority of the most highly induced genes belong to the DREB1/CBF and ZAT12 regulons (Fig. 2). Constitutive expression of ZAT12 in *Arabidopsis* caused a small, but reproducible, increase in freezing tolerance, indicating a role for the ZAT12 regulon in cold acclimation. In addition, ZAT12 downregulated the expression of the *DREB1/CBF* genes, a phenomenon which indicates a role for ZAT12 in a negative regulatory circuit that dampens expression of the DREB1/CBF cold-response pathway (Fig. 2).

Upstream of the DREB1/CBF regulon

The *inducer of CBF expression 1* (*ICE1*) gene was identified through map-based cloning of the *Arabidopsis ice1* mutation, which affected the expression of the *DREB1A* promoter : *luciferase* transgene (Chinnusamy et al. 2003). *ICE1* encodes a MYC-type basic helix-loop-helix (bHLH) transcription factor that regulates the expression of *DREB1A* but not those of the other *DREB1/CBF* genes (Fig. 2). Overexpression of *ICE1* in transgenic plants resulted in improved freezing tolerance, supporting an important role for ICE1 in the cold stress response. Molecular analysis of the *DREB1C* promoter has identified multiple *cis*-acting elements that are involved in cold-inducible gene expression (Y. Imura et al., unpublished data) (Shinwari et al. 1998, Zarka et al. 2003), and a DNA-binding protein which interacts with the promoter region containing these elements has been cloned and shown to be a MYC-type bHLH transcription factor that is different from ICE1 (Y. Imura et al., unpublished data). These results suggest the redundant involvement of MYC-type bHLH transcription factors in the regulation of the *DREB1/CBF* gene expression (Fig. 1). A cold signal is necessary for the activation of the ICE proteins, but the mechanism of this signal still remains to be solved. Analysis of the *cbf2* mutant, in which the *DREB1C* gene was disrupted, indicated that DREB1C is a negative regulator of DREB1A and DREB1B expression and plays a central role in the stress tolerance of *Arabidopsis* (Novillo et al. 2004) (Fig. 2). Collectively, these data suggest that the regulation of *DREB1/CBF* gene expression might be more complex than was previously thought.

The DREB1/CBF regulon in other plants

Many species from tropical regions, such as tomato, rice, and maize, are unable to tolerate freezing and suffer chilling injury when exposed to low temperatures. In contrast, plants from temperate regions, such as wheat, canola, and *Arabidopsis*, are able to survive

both chilling and freezing temperatures. The dynamic ability of these plants to increase in freezing tolerance in response to low temperature is a process that is known as cold acclimation (Thomashow 1999). Recent advances in the understanding of DREB1/CBF suggests that the DREB1/CBF regulon has an important role for cold acclimation. Homologous genes of *DREB1/CBF* have been found in many plant species such as wheat, canola, rye, and *Brassica napus*; all of which are capable of undergoing cold acclimation (Jaglo et al. 2001, Zhang et al. 2004a) (Fig. 3). Interestingly, Zhang et al. (2004b) reported that tomato, a chilling-sensitive plant, encodes three *DREB1/CBF* homologs, *LeCBF1-3*, that are present in a tandem array in the genome. Only the tomato *LeCBF1* gene was found to be cold inducible. Constitutive overexpression of *LeCBF1* in transgenic *Arabidopsis* plants induced expression of DREB1/CBF-targeted genes and increased freezing tolerance. These data clearly indicated that *LeCBF1* encodes a functional homolog of the *Arabidopsis* DREB1/CBF proteins. Overexpression of *Arabidopsis DREB1B* (*CBF1*) in tomato has been shown to increase the "chilling" and drought tolerance of transgenic tomato plants (Hsieh et al. 2002a, b). However, constitutive overexpression of either *LeCBF1* or *Arabidopsis DREB1A* in transgenic tomato plants did not increase "freezing" tolerance (Zhang et al. 2004b). Gene expression studies, including the use of a cDNA microarray representing approximately 8000 tomato genes, only identified four genes that were induced 2.5-fold or more in the *LeCBF1* or *DREB1A* overexpressing plants. Three of the four identified genes were putative members of the tomato DREB1/CBF regulon as they were also upregulated in response to low temperature. From these results, they concluded that an intact DREB1/CBF cold-response pathway is present in tomato. However, the tomato DREB1/CBF regulon differs from that of *Arabidopsis* and appears to be considerably smaller and less diverse in function.

In a similar line of study, Dubouzet et al. (2003) isolated rice homologs for *DREB1/CBF* and *DREB2*, four *OsDREB1*s, and one *OsDREB2* from rice genomic sequences and determined that they function in stress-inducible gene expression. Similar to its *Arabidopsis* homolog, overexpression of *OsDREB1A* in *Arabidopsis* revealed that this gene has a similar function in stress-responsive gene expression and stress tolerance. These data indicate that similar transcription factors function in dicotyledonous and monocotyledonous plants. However, in microarray and RNA blot analyses, some stress-inducible target genes of the DREB1A proteins that only have ACCGAC as DRE were not overexpressed in the *OsDREB1A* transgenic *Arabidopsis* plants. The OsDREB1A protein bound to GCCGAC more preferentially than to ACCGAC, whereas, the DREB1A proteins bound to both GCCGAC and ACCGAC with similar efficiency. Recently, Oh et al. (2005) developed transgenic rice plants that constitutively expressed *Arabidopsis DREB1A*. The overexpression of *DREB1A* in transgenic rice resulted in elevated tolerance to drought and high salinity and produced relatively low levels of tolerance to low-temperature exposure. Similarly, our group also developed transgenic rice plants that constitutively expressed *DREB1A* or *OsDREB1A* genes (Y. Ito et al., unpublished data). In our case, however, these factors in transgenic rice elevated tolerance to drought, high salinity, and low temperature. A novel DREB1/CBF transcription factor named ZmDREB1A was also identified in maize (Qin et al. 2004). It was found that the maize ZmDREB1A was involved in cold-responsive gene expression, and overexpression of the *ZmDREB1A* gene in *Arabidopsis* resulted in increased drought and freezing tolerance.

The DREB2 regulon involved in osmotic stress-responsive gene expression

In contrast to the *DREB1/CBF* genes, overexpression of *DREB2* in transgenic plants does not improve stress tolerance, a phenomenon which suggests that DREB2 proteins require post-translational activation (Liu et al. 1998). The DREB2 protein is expressed under normal growth conditions and is activated in the early stage of the osmotic stress response through post-translational modification (Fig. 1). Domain analysis of DREB2A revealed that a negative regulatory domain exists in the central section of DREB2A, and deletion of this region makes DREB2A a constitutive active form (DREB2A-CA) (Y. Sakuma et al., unpublished data). Transgenic *Arabidopsis* plants overexpressing this constitutive active form of DREB2A (DREB2A-CA) showed growth retardation and improved tolerance to drought stress. Subsequent microarray analysis utilizing RNA isolated from DREB2A-CA overexpressing plants revealed that many dehydration-inducible genes were expressed in the transgenic plants even under non-stressed conditions. However, it is important to note that several unique genes were upregulated exclusively in DREB2A-CA overexpression *Arabidopsis* plants and were not upregulated in the transgenic *Arabidopsis* plants overexpressing DREB1A.

Other regulons involved in osmotic stress-responsive gene expression

ABA plays an important role in the signal transduction of osmotic stress in plants. ABRE (ABRE : ACGTGG/TC)

is a major *cis*-acting element that functions to regulate ABA-responsive gene expression (Fig. 1). In *Arabidopsis*, it was determined that two ABRE motifs are important for the regulation of ABA-responsive expression of the *RD29B* gene encoding a LEA-like protein (Uno et al. 2000). The basic leucine zipper transcription factor ABRE-binding protein (AREB)/ABRE-binding factor (ABF) can bind to ABRE and activate ABA-dependent gene expression (Choi et al. 2000, Uno et al. 2000). Activation of the AREB1/ABF2 (AREB1) and AREB2/ABF4 (AREB2) proteins has been shown to require an ABA-mediated post-translational modification (Uno et al. 2000), which is probably an ABA-dependent phosphorylation event (Fig. 1). It is likely that the ABA-activated protein kinases, including a SNF1-related protein kinase 2, might phosphorylate and activate the AREB/ABF-type proteins (Yoshida et al. 2002). In *Arabidopsis*, overexpression of ABF3 or AREB2 caused ABA hypersensitivity, reduced transpiration rate, and enhanced drought tolerance of the transgenic plants (Kang et al. 2002). The AREB1 is reported to be an essential component of glucose signaling, and its overexpression affects multiple stress tolerance including drought, salt, and heat (Kim et al. 2004).

The induction of the *Arabidopsis* drought-inducible gene *RD22*, which encodes a protein having a homology to an unidentified seed protein, is mediated by ABA. A MYC transcription factor, AtMYC2, and a MYB transcription factor, AtMYB2, have been shown to bind *cis*-elements, MYC-recognition site: CANNTG and MYB-recognition site: C/TAACNA/G in the *RD22* promoter and cooperatively activate *RD22* (Abe et al. 1997) (Fig. 1). These MYC and MYB proteins are synthesized after endogenous levels of ABA accumulate, thereby indicating that their role is in a late stage of the stress responses. Overexpression of both AtMYC2 and AtMYB2 not only caused an ABA-hypersensitive phenotype but this also improved the osmotic stress tolerance of transgenic plants (Abe et al. 2003). Microarray analysis of MYC- and MYB-overexpressing transgenic plants revealed target genes for MYC and MYB. Examples of such target genes included the alcohol dehydrogenase gene and ABA- or jasmonic acid (JA)-inducible genes (Abe et al. 2003). These data indicate that crosstalk between ABA- and JA-responsive gene expression occurs via the AtMYC2 transcription factor.

The early responsive to dehydration 1 (*ERD1*) gene encoding a Clp protease regulatory subunit responds to dehydration and high salinity before the accumulation of ABA. This pattern of gene regulation suggests that an ABA-independent pathway exists in the dehydration stress response of *Arabidopsis* (Nakashima et al. 1997). Analysis of the *ERD1* promoter identified two novel *cis*-acting elements that are involved in induction by dehydration stress (Simpson et al. 2003). Base substitution analysis showed that a 14-bp *rps1*-like region (CACTAAATTGTCAC) and a CATGTG motif are necessary for the induction of the *ERD1* gene in dehydrated plants (Fig. 1). Tran et al. (2004) isolated three cDNA clones encoding proteins that bind to the 63-bp promoter region of *ERD1*, the specific location which contains the CATGTG motif (Fig. 1). These three cDNA clones encode proteins which belong to the NAC transcription factor family including RD26. Microarray analysis of transgenic plants overexpressing the *NAC* genes revealed that several drought-inducible genes were upregulated in the transgenic plants. It is important to note that these plants also exhibited significantly increased drought tolerance. However, *ERD1* was not upregulated in the transgenic plants. Recently a one-hybrid screening approach was used to isolate cDNAs encoding the transcription factor that binds to the *rps1* site 1-like sequence. These cDNAs encode Zinc-finger homeodomain (ZF-HD) transcription factors (L.-S. P. Tran et al., unpublished data). Overexpression of both NAC and ZF-HD proteins activated the expression of *ERD1* under non-stressed normal growth conditions in the transgenic *Arabidopsis* plants.

Crosstalk between the DREB regulons and the other regulons

Many osmotic-stress and cold-inducible genes contain both DRE/CRT and ABRE motifs in their promoter regions. It was originally thought that these *cis*-acting elements would function independently from one another. However, precise analysis of these *cis*-acting elements, which both reside within the *RD29A* promoter region, revealed that DRE/CRT functions cooperatively with ABRE as a coupling element in ABA-responsive gene expression in response to drought stress (Narusaka et al. 2003). This observation indicates that there are interactions between the DREB and the AREB/ABF regulons (Fig. 1).

Recently, an osmotic stress-inducible *DREB1D* gene has been identified (Haake et al. 2002). Genes of the *DREB1/CBF* family are mainly induced by cold stress, but the drought-inducible gene *DREB1D* functions to provide crosstalk between DREB2 and DREB1/CBF regulatory systems. The drought-inducible expression of *DREB1D* is controlled by ABA-dependent pathways, suggesting that DREB1D may function in the slow response to drought that relies upon the accumulation of ABA (Fig. 1). Moreover, ABA induces the *DREB1/CBF*

gene transcription and subsequent induction of cold-regulated genes via the DRE/CRT promoter element (Knight et al. 2004). A maize DRE-binding protein, DBF1, has been shown to function as a transcriptional activator of the *responsive to ABA 17* (*rab17*) promoter by ABA (Kizis and Pages 2002). This also suggests that that the DRE/CRT is involved in an ABA-dependent pathway for the regulation of stress-inducible genes in some plants.

Application of regulon biotechnology to improve stress tolerance in crop plants

As the DREB1/CBF regulon is ubiquitous within the higher plants (Fig. 3), "DREB technology" which aims to control the expression of the DREB1/CBF regulon is expected to improve abiotic stress tolerance in crop plants. At the present time, the *DREB1*/*CBF* genes of *Arabidopsis* have been successfully used to engineer and augment abiotic stress tolerance in a number of different species. However, constitutive overexpression of the *DREB1*/*CBF* genes in plants showed an undesirable dwarf phenotype (Gilmour et al. 2000, Liu et al. 1998). As a means to overcome this problem, stress-inducible promoters that have low background expression under normal growth condition have been used in conjunction with the *DREB1*/*CBF* genes to achieve increased stress tolerance without growth retardation (Kasuga et al. 1999). Constitutive overexpression of *Arabidopsis DREB1A* improved drought and low-temperature stress tolerance in tobacco, and regulation of transgene expression via the stress-inducible *RD29A* promoter minimized the negative effects on plant growth (Kasuga et al. 2004). Similarly, the *Arabidopsis DREB1A* gene was placed under control of the *RD29A* promoter and transferred via biolistic transformation into bread wheat (Pellegrineschi et al. 2004). In comparison with controls, plants expressing the *DREB1A* gene exhibited a 10-day delay in wilting when water was withheld. This substantial increased resistance 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. We are currently collaborating with many research groups, and we have the common goal to improve stress-tolerant crop plants utilizing regulon biotechnology (Nakashima and Yamaguchi-Shinozaki, 2005). It is hoped in the future that the collective efforts and results of these collaborative studies will positively contribute to sustainable food production in developing countries and with help to prevent global-scale environmental damage that is resultant from abiotic stress.

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