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REVIEW

Molecular Studies on Stress-Responsive Gene Expression in *Arabidopsis* and Improvement of Stress Tolerance in Crop Plants by Regulon Biotechnology

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Abstract

Molecular studies have shown that several genes with various functions are induced by environmental stresses such as drought, high-salinity and low temperature in plants. Most of the dehydration responsive genes are induced by the plant hormone abscisic acid (ABA), but others are not. Expression analyses of dehydration-responsive genes have provided at least four independent regulatory systems (regulons) for gene expression in a model plant *Arabidopsis thaliana*. The *cis*-acting elements in the promoters of some genes that have a typical stress-inducible expression profile and the transcription factors that affect the expression of these genes have been analyzed. Transcription factors that bind to a DRE/CRT (dehydration-responsive element / C-repeat) *cis*-acting element were isolated and termed DREB1/CBF (DRE-binding protein 1/ C-repeat binding factor) and DREB2 (DRE-binding protein 2). Overexpression of DREB1/CBF in transgenic *Arabidopsis* plants increased tolerance to freezing, drought and high salt concentrations. The *DREB1/CBF* genes have been successfully used to improve abiotic stress tolerance in a number of different crop plants. Studies on the other transcription factors associated with stress response are in progress. We collaborate with many research groups to improve stress tolerant crop plants utilizing regulon biotechnology. We hope the results of these collaborative studies will contribute to the sustainable food production in developing countries and help to prevent the global-scale environmental damage.

Discipline: Biotechnology
Additional key words: DREB1, environmental stress, transcription factors, transgenic plants

Introduction

As plants are sessile organisms, they are directly exposed to environmental stresses such as drought, high salinity and low temperature. Plants respond to environmental stress, and the transduced signals cause expression of numerous genes associated with stress tolerance. A number of genes have been described that respond to environmental stresses such as drought, high salinity and low temperature in plants[4,15,33,47,48,60].

We isolated more than 60 independent cDNAs for dehydration inducible genes using molecular techniques such as differential screening in a model plant *Arabidopsis thaliana*[33,47,48]. Recently, 299 drought-inducible genes, 54 cold-inducible genes, and 213 high-salinity-stress-inducible genes were identified using a cDNA microarray containing around 7,000 independent *Arabidopsis* full-length cDNA groups[46,48]. Functions of their gene products have been predicted from sequence homology with known proteins. Genes induced during dehydration stress conditions are thought to function not only in protecting cells from dehydration by the production of important metabolic proteins (functional proteins) but also in the regulation of genes for signal transduction in the dehydration stress response (regulatory proteins). The functional proteins contain water channel proteins, chaperons, proteases, LEA (Late Embryogenesis Abundant) proteins, and enzymes for the synthesis of osmoprotectants (compatible solutes; sugars, proline, etc.). The regulatory proteins contain transcription factors, protein kinases, and enzymes for phosphoinositide (PI) turn-

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over, and enzymes for the synthesis of the plant hormone abscisic acid (ABA). So far, various kinds of functional proteins such as enzymes for the synthesis of osmoprotectants were overexpressed in plants to improve the stress tolerance[5,59]. However, it seems that the engineering of one enzyme is not enough as many kinds of stress responses are necessary for plants to survive in severe stress conditions.

In plants, one transcription factor can control the expression of many target genes through the specific binding of the transcription factor to the *cis*-acting element in the promoters of the target genes. Such kind of a transcription unit is called a "regulon". Northern analysis of dehydration-inducible genes revealed that there appear to be at least four independent regulons in *Arabidopsis* (Fig. 1). They are (1) DREB regulon, (2) NAC (NAM, ATAF1, 2, and CUC2) and ZF-HD (zinc-finger homeodomein) regulon, (3) AREB/ABF (ABA-responsive element binding protein / ABA-responsive element binding factor) regulon, and (4) MYC (myelocytomatosis oncogene) and MYB (myeloblastosis oncogene) regulon. The DREB regulon and the NAC and ZF-HD regulon are ABA-independent. The AREB/ABF regulon and the MYC and MYB regulon are ABA-dependent. Regulon biotechnology, by controlling the expression of the regulon system, is expected to improve the tolerance against stresses in plants.

DREB regulon involved in ABA-independent gene expression

1. Isolation of DREB1/CBF regulon and DREB2 regulon

The promoter of an *Arabidopsis* drought-, high-salinity- and cold-inducible gene *RD29A* (*responsive to dehydration 29A*) encoding a LEA-like protein has been

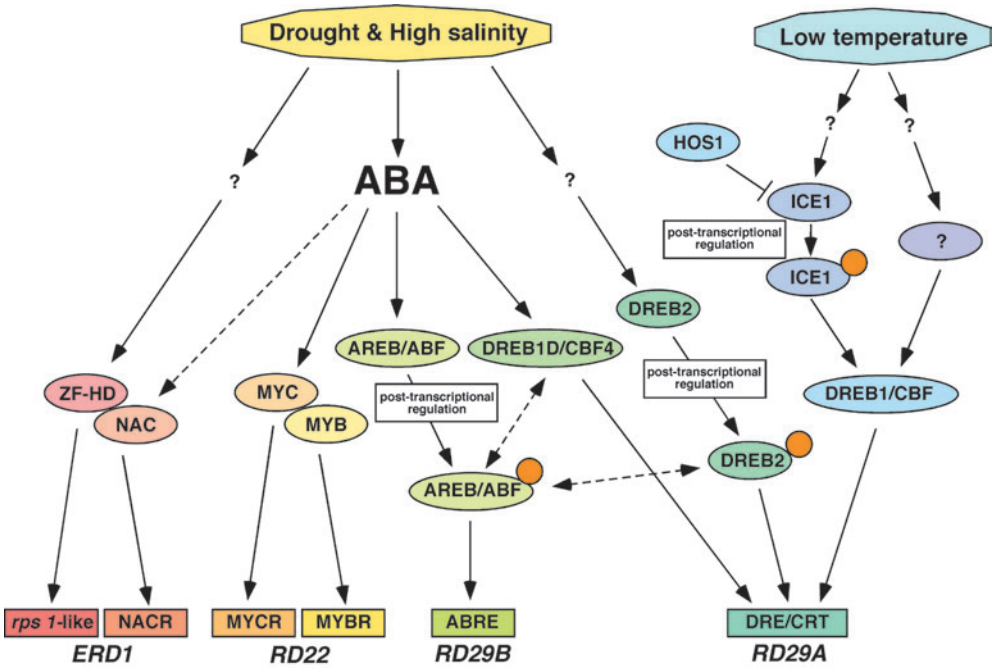

Fig. 1. Regulatory network of gene expression in response to drought, high salinity and cold stresses: specificity and crosstalk of gene networks

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 circles or 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 cross talk. ABF: ABRE-binding factor, ABRE: ABA-responsive element, AREB: ABRE-binding protein, CBF: C-repeat-binding factor, CRT: C-repeat, DRE: dehydration-responsive element, DREB: DRE-binding protein, *ERD*: early responsive to dehydration, ICE: inducer of CBF expression, MYBR: MYB recognition site, MYCR: MYC recognition site, NACR: NAC recognition site, *RD*: responsive to dehydration, ZF-HD: zinc finger homeodomain protein.

found to contain two major *cis*-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE)/C-repeat (CRT), that are involved in stress-inducible gene expression[56]. DRE/CRT (CCGAC) is a *cis*-acting element that functions in ABA-independent gene expression in response to abiotic stress (Fig. 1). Transcription factors belonging to the AP2/ERF (APETALA2 / ethylene-responsive element binding factor) family that bind to DRE/CRT have been isolated and termed DREB1/CBF and DREB2[10,26,50]. The conserved DNA-binding motif of DREB1/CBF and DREB2 is A/GCCGAC[42]. The *DREB1/CBF* genes are quickly and transiently induced by cold stress, and their products activate the expression of target stress-inducible genes. The *DREB2* genes are induced by dehydration, leading to the expression of various genes that are involved in drought-stress tolerance[26].

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

Overexpression of *DREB1A/CBF3* in transgenic *Arabidopsis* plants showed increased tolerance to freezing, drought and high salt concentrations[17,19,26], suggesting that the DREB1A/CBF3 proteins function without modification of the proteins in the development of stress tolerance. Many candidates for the DREB1A/CBF3 target genes have been identified using microarray[9,30,45]. Most of these target genes contain DRE- or DRE-related CCGAC core motif sequences in their promoter regions. We analyzed the expression of these candidate genes using RNA gel blot and identified more than 40 genes as the DREB1A downstream genes. Many of the products of these genes were proteins known to function against stress and were probably responsible for the stress tolerance of the transgenic plants. The downstream genes also included genes for transcription factors involved in further regulation of signal transduction and gene expression in response to stress.

The overexpression of the *DREB1/CBF* gene results in multiple biochemical changes associated with cold acclimation[11]: *DREB1A/CBF3*-expressing plants had elevated levels of proline (Pro) and total soluble sugars, including sucrose, raffinose, glucose, and fructose. Plants overexpressing *DREB1A/CBF3* also had elevated *P5CS* (for delta(1)-pyrroline-5-carboxylate synthase) transcript levels suggesting that the increase in Pro levels resulted, at least in part, from increased expression of the key Pro biosynthetic enzyme P5CS. These results lead us to propose that DREB1A/CBF3 integrates the activation of multiple components of the cold acclimation response.

Dwarfism is observed in transgenic *Arabidopsis* overexpressing *DREB1A/CBF3*, *DREB1B/CBF1*, *DREB1C/CBF2* or *DREB1D/CBF4*[11,12,19,26]. The development of dwarf phenotypes was also found in transgenic tomato overexpressing *Arabidopsis DREB1B/CBF1*, and it was prevented by exogenous application of gibberellin (GA)[14]. These suggest that an inhibition of GA biosynthesis is a function common to the *DREB1/CBF* genes. However, microarray analysis did not detect the changes in transcript levels of GA-related genes in transgenic *Arabidopsis* overexpressing *DREB1A/CBF3*, *DREB1B/CBF1*, or *DREB1C/CBF2*[9]. Recently, DREB1F is reported to be involved in the regulation of GA biosynthesis and stress tolerance[29]. It is not clear yet whether other DREB1/CBF proteins are related to GA synthesis or not.

In contrast to the *DREB1/CBF* genes, overexpression of *DREB2* in transgenic plants does not improve stress tolerance, suggesting that DREB2 proteins require posttranslational activation[26]. The DREB2 protein is expressed under normal growth conditions and is activated in the early stage of the osmotic stress response through posttranslational modification (Fig. 1).

3. Regulation of the expression of DREB1/CBF regulon

The *ICE1 (inducer of CBF expression 1*) gene was identified through the map-based cloning of the *Arabidopsis ice1* mutation, which affected the expression of the *DREB1A*/*CBF3* promoter-*LUC* (*luciferase*) transgene[6]. *ICE1* encodes a MYC-type bHLH (basic helix-loop-helix) transcription factor that regulates the expression of *DREB1A*/*CBF3* but not of other *DREB1*/*CBF* genes (Fig. 1). 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/CBF2* promoter has identified multiple *cis*-acting elements that are involved in cold-inducible gene expression[57] (Imura et al., unpublished data). The DNA-binding protein has been cloned and shown to be a MYC-type bHLH transcription factor that is different from ICE1 (Imura et al., unpublished data). These results suggest the redundant involvement of MYC-type bHLH transcription factors in the up-regulation of the *DREB1/CBF* genes. A cold signal is necessary for the activation of the ICE proteins but the mechanism of this signal remains to be solved. Analysis of the *cbf2* mutant, in which the *DREB1C/CBF2* gene was disrupted, indicated that DREB1C/CBF2 is a negative regulator of *DREB1A/CBF3* and *DREB1B/CBF1* expression and plays a central role in stress tolerance in *Arabidopsis*[35]. These data suggest that the regulation of the expression of the *DREB1/CBF* genes might be more complex than previously thought.

NAC and ZF-HD regulon involved in ABA-independent gene expression

The *ERD1* (*early responsive to dehydration 1*) gene encoding a Clp (caseinolytic protease) protease regulatory subunit responds to dehydration and high salinity before the accumulation of ABA, suggesting the existence of an ABA-independent pathway in the dehydration stress response[31]. Analysis of the *ERD1* promoter identified two novel *cis*-acting elements that are involved in induction by dehydration stress[49]. 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). Recently, we isolated three cDNA clones encoding proteins that bind to the 63-bp promoter region of *ERD1*, which contains the CATGTG motif[52] (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 up-regulated in the transgenic plants, and the plants showed significantly increased drought tolerance. However, *ERD1* was not up-regulated in the transgenic plants. We recently isolated zinc-finger homeodomain (ZF-HD) transcription factors containing a homeodomain that can bind to the *rps1* site 1-like sequence using the yeast one-hybrid system. Overexpression of both NAC and ZF-HD proteins activated the expression of *ERD1* under unstressed normal growth conditions in the transgenic *Arabidopsis* plants.

AREB/ABF regulon involved in ABA-dependent gene expression

ABRE (ABA-responsive elements: ACGTGG/TC) is a major *cis*-acting element in ABA-responsive gene expression (Fig. 1). Two ABRE motifs are important in the ABA-responsive expression of the *Arabidopsis* gene *RD29B* encoding a LEA-like protein[53]. The bZIP (basic leucine zipper) transcription factors ABRE-binding protein (AREB)/ABRE-binding factor (ABF) can bind to ABRE and activate ABA-dependent gene expression[7,53]. Activation of the AREB1 and AREB2 proteins has been shown to require an ABA-mediated modification[53], which is probably ABA-dependent phosphorylation (Fig. 1). Overexpression of ABF3 or AREB2/ABF4 caused ABA hypersensitivity, reduced transpiration rate and enhanced drought tolerance of the transgenic plants[18]. The AREB1/ABF2 is reported to be an essential component of glucose signaling and its overexpression affects multiple stress tolerance including drought, salt and heat[22].

MYC and MYB regulon involved in ABA-dependent gene expression

The induction of the *Arabidopsis* drought-inducible gene *RD22* encoding a protein having a homology to an unidentified seed protein is mediated by ABA, and this gene requires protein biosynthesis for its ABA-dependent expression[1]. A MYC transcription factor, AtMYC2 (*Arabidopsis thaliana* MYC 2), and a MYB transcription factor, AtMYB2 (*Arabidopsis thaliana* MYB 2), have been shown to bind *cis*-elements, MYCR (MYC-recognition site: CANNTG) and MYBR (MYB-recognition site: C/TAACNA/G) in the *RD22* promoter and cooperatively activate *RD22*[1] (Fig. 1). These MYC and MYB proteins are synthesized after the accumulation of endogenous ABA, indicating that their role is in a late stage of the stress responses. Microarray analysis of MYC- and MYB-overexpressing transgenic plants revealed target genes for MYC and MYB, such as the alcohol dehydrogenase gene and ABA- or jasmonic-acid (JA)-inducible genes[2]. Overexpression of both AtMYC2 and AtMYB2 not only caused an ABA-hypersensitive phenotype but also improved the osmotic-stress tolerance of the transgenic plants[2].

Recently, AtMYC2 transcription factors function as members of a MYC-based regulatory system conserved in dicotyledonous plants with a key role in JA-induced defense gene activation[3,28]. These reports highlight the crosstalk between biotic stress signaling and abiotic stress signaling.

Crosstalk between the DREB regulon and the other regulons

Many drought- and cold-inducible genes contain both DRE/CRT and ABRE motifs in their promoters. These *cis*-acting elements are thought to function independently. However, precise analysis of these *cis*-acting elements in the *RD29A* gene expression revealed that DRE/CRT functions cooperatively with ABRE as a coupling element in ABA-responsive gene expression in response to drought stress[34]. This indicates that there are interactions between the DREB regulon and the AREB/ABF regulon (Fig. 1).

Recently, an osmotic-stress inducible *CBF4/DREB1D* gene has been identified[12]. Genes of the *DREB1/CBF* family are mainly induced by cold stress, but the drought-inducible gene *CBF4/DREB1D* functions to provide crosstalk between DREB2 and DREB1/CBF regulatory systems. The drought-inducible expres-

sion of *CBF4/DREB1D* is controlled by ABA-dependent pathways, suggesting that CBF4/DREB1D may function in the slow response to drought that relies on 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[24]. A maize DRE-binding protein, DBF1, has been shown to function as a transcriptional activator of the *rab17 (responsive to abscisic acid 17)* promoter by ABA[23]. This also suggests the existence in some plants of an ABA-dependent pathway for the regulation of stress-inducible genes that involves DRE/CRT.

Gene expression in recovery process from abiotic stress in *Arabidopsis*

Microarray analysis has revealed many genes that respond to rehydration after drought stress, indicating their involvement in the process of recovery from abiotic stress[36]. The products of these genes are thought to function not only in recovery from stress but also in cell growth and elongation. The expression and function of the rehydration-inducible *ERD5* gene encoding a proline dehydrogenase (ProDH) gene has been precisely analyzed. This gene is involved in the degradation of the proline that accumulates during dehydration[32]. Promoter analysis of the *ProDH* gene revealed an important *cis*-acting element, ACTCAT, that is involved in rehydration-inducible gene expression[43]. Many rehydration-inducible gene promoters contain the ACTCAT motif. Recently we showed that the ATB2 subgroup bZIP proteins functions as transcriptional activators in hypoosmolarity-responsive expression of the *ProDH* gene in *Arabidopsis*[44]. The molecular information in the process of recovery from abiotic stress may allow us to improve the resilient plants.

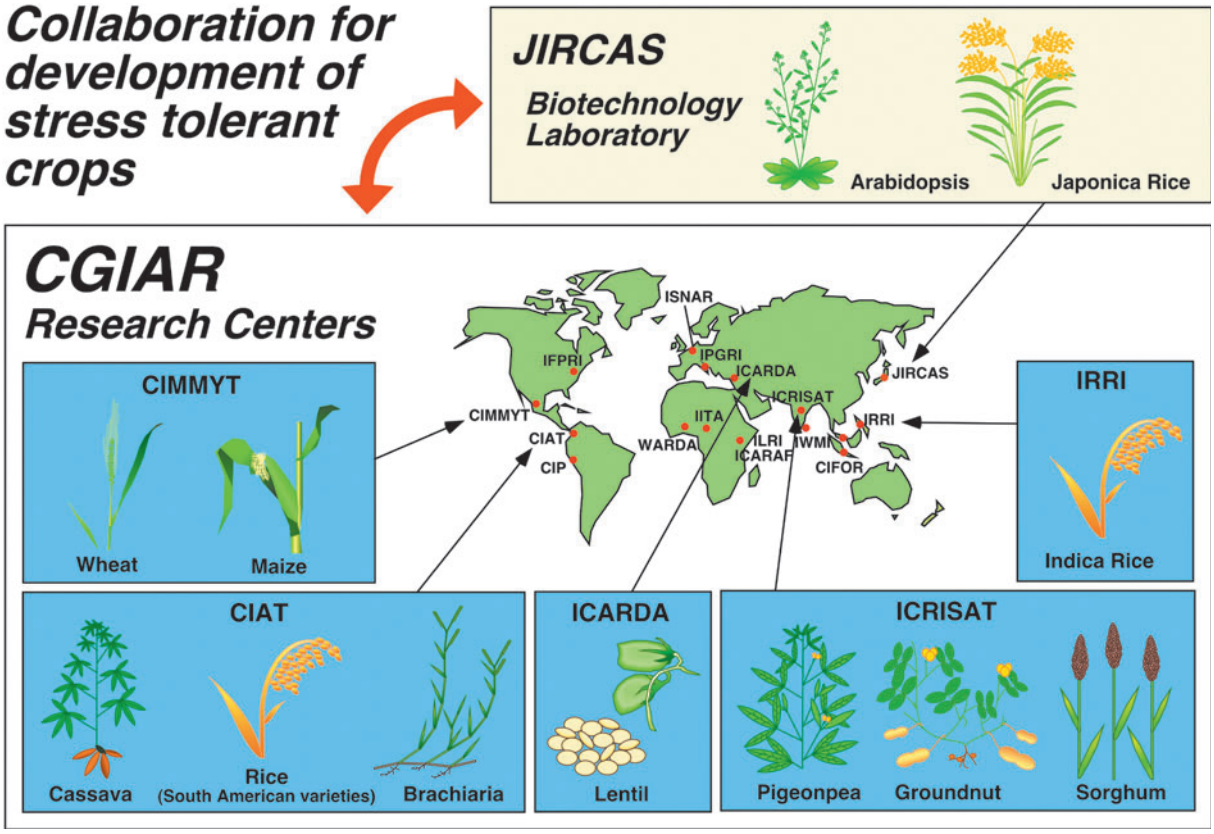

Fig. 2. Collaboration for development of stress tolerant crops

CGIAR: Consultative Group on International Agricultural Research, CIAT: International Center for Tropical Agriculture (Colombia), CIMMYT: International Maize and Wheat Improvement Center (Mexico), ICARDA: International Center for Agricultural Research in the Dry Areas (Syrian Arab Republic), ICRISAT: International Crops Research Institute for the Semi-Arid Tropics (India), IRRI: International Rice Research Institute (The Philippines), JIRCAS: Japan International Research Center for Agricultural Sciences (Japan).

Table 1. Abiotic stress tolerance by regulon biotechnology

Transcription factor	Type	Gene source	Transgenic Species	Promoter	Tolerance	Reference
DREB1A/CBF3	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S, RD29A*	Freezing, salt, and drought	11, 19, 26
			Wheat	*RD29A*	Drought	39
			Tobacco	*35S, RD29A*	Freezing and drought	20
			Brassica napus	*35S*	Freezing and drought	16, 59
DREB1B/CBF1	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S*	Freezing	11, 17
			Tomato	*35S, HVA22*	Drought, chilling, and oxidative stress	13, 14, 25
			Strawberry	*35S*	Freezing	37
			Brassica napus	*35S*	Freezing and drought	16, 59
DREB1C/CBF2	AP2/ERF	*Arabidopsis*	*Brassica napus*	*35S*	Freezing and drought	16, 59
DREB1D/CBF4	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S*	Freezing and drought	12
DREB1F/DDF1	AP2/ERF	*Arabidopsis*	*Arabidopsis*	*35S*	High salinity	29
ZmDREB1	AP2/ERF	Maize	*Arabidopsis*	*35S*	Drought and freezing	40
OsDREB1A	AP2/ERF	Rice	*Arabidopsis*	*35S*	Drought, salt, and freezing	8
Tsi1	AP2/ERF	Tobacco	Tobacco	*35S*	Salt	38
JERF1	AP2/ERF	Tomato	Tobacco	*35S*	Salt	58
ICE1	HLH	*Arabidopsis*	*Arabidopsis*	Superpromoter*	Freezing	6
AtMYC2 & AtMYB2	MYC & MYB	*Arabidopsis*	*Arabidopsis*	*35S*	Osmotic stress	2
CpMYB10	MYB	*Craterostigma plantagineum*	*Arabidopsis*	*35S*	Drought and salt	55
Osmyb4	MYB	Rice	*Arabidopsis*	*35S*	Cold and freezing tolerance	54
AREB1/ABF2	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Drought, salt, heat, and oxidative stress	22
AREB2/ABF4	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Drought, salt, chilling, freezing, heat, and oxidative stress	18, 22
ABF3	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Drought, salt, chilling, freezing, heat, and oxidative stress	18, 22
ABI5	bZIP	*Arabidopsis*	*Arabidopsis*	*35S*	Water stress	27
ABI3 (plus ABA)	VP1	*Arabidopsis*	*Arabidopsis*	*35S*	Freezing	51
SCOF-1	Zn finger	Soybean	*Arabidopsis*	*35S*	Low temperature stress	21
			Tobacco	*35S*	Low temperature stress	21
STZ	Zn finger	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	41
ANAC019/ANAC	NAC	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	52
ANAC055/AtNAC3	NAC	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	52
ANAC072/RD26	NAC	*Arabidopsis*	*Arabidopsis*	*35S*	Drought	52

* Superpromoter consists of three copies of the octopine synthese upstream-activating sequence in front of the manopine synthese promoter.

Application of regulon biotechnology to improve stress tolerance in crop plants

The orthologous genes of *DREB1/CBF* have been found in many crop plants such as canola, broccoli, tomato, alfalfa, wheat, barley, corn, and rice[59]. These indicate that the DREB1/CBF regulon system is ubiquitous in the plant kingdom, and the "DREB technology" with controlling the expression of the DREB1/CBF regulon system is expected to improve the tolerance against stresses in crop plants. So far the *DREB1/CBF* genes of *Arabidopsis* have been successfully used to engineer abiotic stress tolerance in a number of different species (Table 1). For example, constitutive overexpression of the *Arabidopsis DREB1/CBF* genes in canola results in increased freezing tolerance[16] and drought tolerance[59].

We have isolated rice orthologs for DREB1/CBF and DREB2, four OsDREB1s and one OsDREB2, in the rice genome sequence and they function in stress-inducible gene expression[8]. Overexpression of OsDREB1A in *Arabidopsis* revealed that this gene has a similar function to that of its *Arabidopsis* homolog in stress-responsive gene expression and stress tolerance. This indicates that similar transcription factors function in dicotyledons and monocotyledons. A novel DREB1/CBF transcription factor ZmDREB1A was also identified in *Zea mays*[40]. The ZmDREB1A was involved in cold-responsive gene expression and overexpression of the *ZmDREB1A* gene in *Arabidopsis* which resulted in increased drought and freezing tolerance.

However, constitutive overexpression of the *DREB1/CBF* genes in plants showed dwarf phenotype[11,26]. To overcome these problems, 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 the growth retardation[19,25]. Constitutive overexpression of *Arabidopsis DREB1A/CBF3* improved drought- and low-temperature stress tolerance in tobacco[20]. The stress-inducible *RD29A* promoter minimized the negative effects on the plant growth in tobacco. Furthermore, we detected overexpression of stress-inducible target genes of DREB1A/CBF3 in tobacco. The *Arabidopsis DREB1A/CBF3* gene was placed under control of the *RD29A* promoter and transferred via biolistic transformation into bread wheat[39]. Plants expressing the *DREB1A/CBF3* gene demonstrated substantial resistance to water stress in comparison through checks under experimental greenhouse conditions, manifested by a 10-day delay in wilting when water was withheld. These results indicate 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.

Now we collaborate with many research groups to improve stress tolerant crop plants utilizing regulon biotechnology (Fig. 2). We hope the results of these collaborative studies will contribute to the sustainable food production in developing countries and help to prevent the global-scale environmental damage.

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