

Development of Drought-Resistant and Water Stress-Tolerant Crops through Biotechnology

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Abstract

Plant productivity is markedly affected by drought stress. We reported that a *cis*-acting promoter element, dehydration-responsive element (DRE), plays an important role in regulating gene expression in response to drought stress in *Arabidopsis*. The DRE is also involved in low-temperature- and salt-responsive gene expression. The transcription factor DREB1A specifically interacts with the DRE and induces the expression of stress tolerance genes. Overexpression of the cDNA encoding DREB1A in transgenic *Arabidopsis* plants activated the expression of many of these stress tolerance genes under normal growing conditions and resulted in improved tolerance to drought, salt loading, and freezing. We prepared a cDNA microarray using full-length *Arabidopsis* cDNAs to identify the target stress tolerance genes of DREB1A. Twelve stress-inducible genes were identified as the target genes of DREB1A, and six of them were new. However, the use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter to drive the expression of DREB1A resulted in severe growth retardation under normal growing conditions. In contrast, the expression of DREB1A from the stress-inducible *rd29A* promoter gave rise to minimal effects on plant growth while providing an even greater tolerance to stress conditions than did the expression of the gene from the 35S CaMV promoter. As the DRE-related regulatory element is not limited to *Arabidopsis*, the DREB1A cDNA and the *rd29A* promoter may be useful to improve the stress tolerance of agriculturally important crops by gene transfer.

Introduction

Plant growth is markedly affected by environmental abiotic stresses, such as drought, high salinity and low temperature. Plants respond and adapt to these stresses in order to survive against abiotic stress. Among these abiotic stresses, drought or water deficit is the most severe limiting factor of plant growth and crop production. Drought stress induces various biochemical and physiological responses in plants. Recently, a number of genes have been described that respond to drought at the transcriptional level^{3, 6, 24, 30}. The products of these genes are considered to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response (Fig.1)^{25, 26}.

Genetic engineering is considered to be useful for improving the stress tolerance of plants. Recently, several different approaches have been attempted to improve the stress tolerance of plants by gene transfer⁹. The genes selected for transformation were those involved in encoding enzymes required for the biosynthesis of various osmoprotectants^{4, 12, 29}. Other genes that have been selected for transformation include those that

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encoded enzymes for modifying membrane lipids, LEA protein, and detoxification enzyme^{8, 14, 16, 31}). In all these experiments, a single gene for a protective protein or an enzyme was overexpressed under the control of the 35S cauliflower mosaic virus (CaMV) constitutive promoter in transgenic plants, although a number of genes have been shown to function in environmental stress tolerance and response. The genes encoding protein factors that are involved in the regulation of gene expression and signal transduction and function in stress response seem to be useful to improve the tolerance of plant to stresses by gene transfer as they can regulate many stress-inducible genes involved in stress tolerance.

Drought is one of the most severe environmental stresses and it affects almost all the plant functions. Abscisic acid (ABA) is produced under water deficit conditions and plays important roles in tolerance against drought. Most of the drought-inducible genes that have been studied to date are also induced by ABA^{25, 26}. Several reports have described genes that are induced by dehydration but are not responsive to exogenous ABA treatments^{25, 26}. These findings suggest the existence of ABA-independent as well as ABA-dependent signal-transduction cascades between the initial signal of drought stress and the expression of specific genes^{25, 26}. To understand the molecular mechanisms of gene expression in response to drought stress, *cis*- and *trans*-acting elements that function in ABA-independent and ABA-responsive gene expression by drought stress have been precisely analyzed^{25, 26}. In this article, we summarize recent progress of our research on *cis*- and *trans*-acting factors involved in ABA-independent gene expression in drought stress response. We also report stress tolerance of transgenic plants that overexpress a single gene for a stress-inducible transcription factor, using *Arabidopsis* as a model.

Variety of functions of drought-inducible genes

Various genes respond to drought-stress in various species, and functions of their gene products have

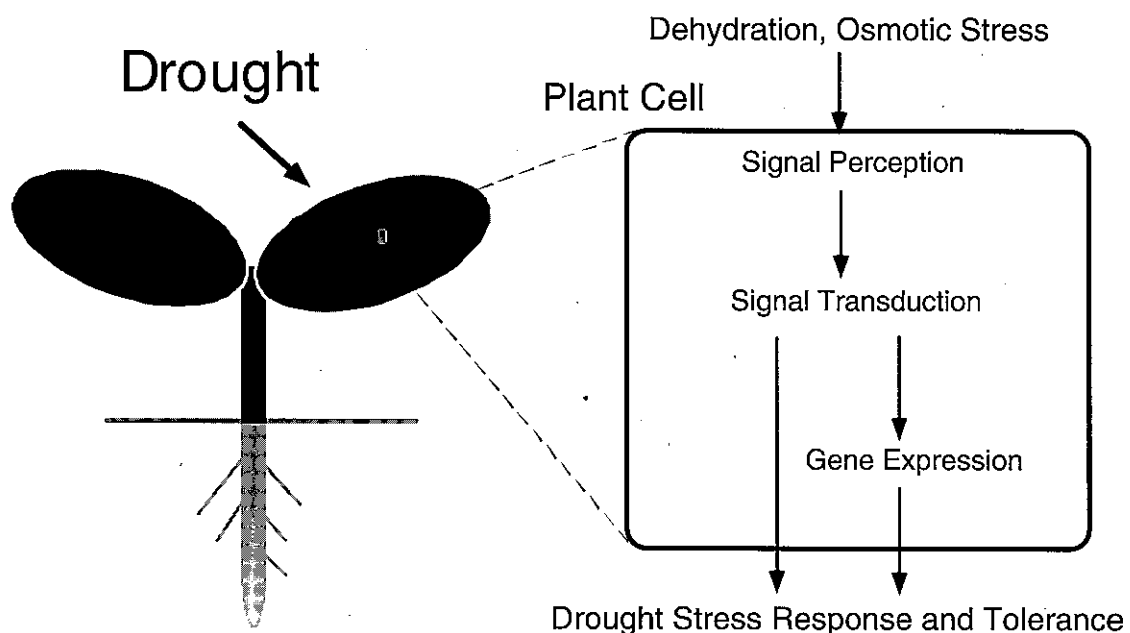


Fig. 1 Schematic representation of molecular responses to drought stress in plant cells. Molecular and cellular responses to drought stress include perception of dehydration signal, signal transduction to cytoplasm and nucleus, gene expression, and responses and tolerance to drought stress.

been predicted from sequence homology with known proteins. Many drought-inducible genes are also induced by salt stress and low temperature, which suggests the existence of similar mechanisms of stress responses. Genes induced during drought-stress conditions are considered to function not only in protecting cells from water deficit by the production of important metabolic proteins but also in the regulation of genes for signal transduction in the drought stress response^{3, 6, 24, 25, 26, 30}. Thus, these gene products are classified into two groups (Fig. 2). The first group includes proteins that probably function in stress tolerance; such as chaperones, LEA proteins, osmotin, antifreeze proteins, mRNA binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes and various proteases. LEA proteins, chaperones and mRNA binding proteins have been analyzed biochemically and shown to be involved in protecting macromolecules like enzymes, lipids and mRNAs from dehydration. Proline, glycine betaine and sugars function as osmolytes and protect cells from dehydration. Key enzymes of several osmolytes have been cloned and analyzed biochemically. Water channel proteins, sugar transporters and proline transporters are considered to function in the transport of water, sugars and proline through plasma membranes and tonoplast to adjust osmotic pressure under stress conditions. Detoxification enzymes, such as glutathione S-transferase,

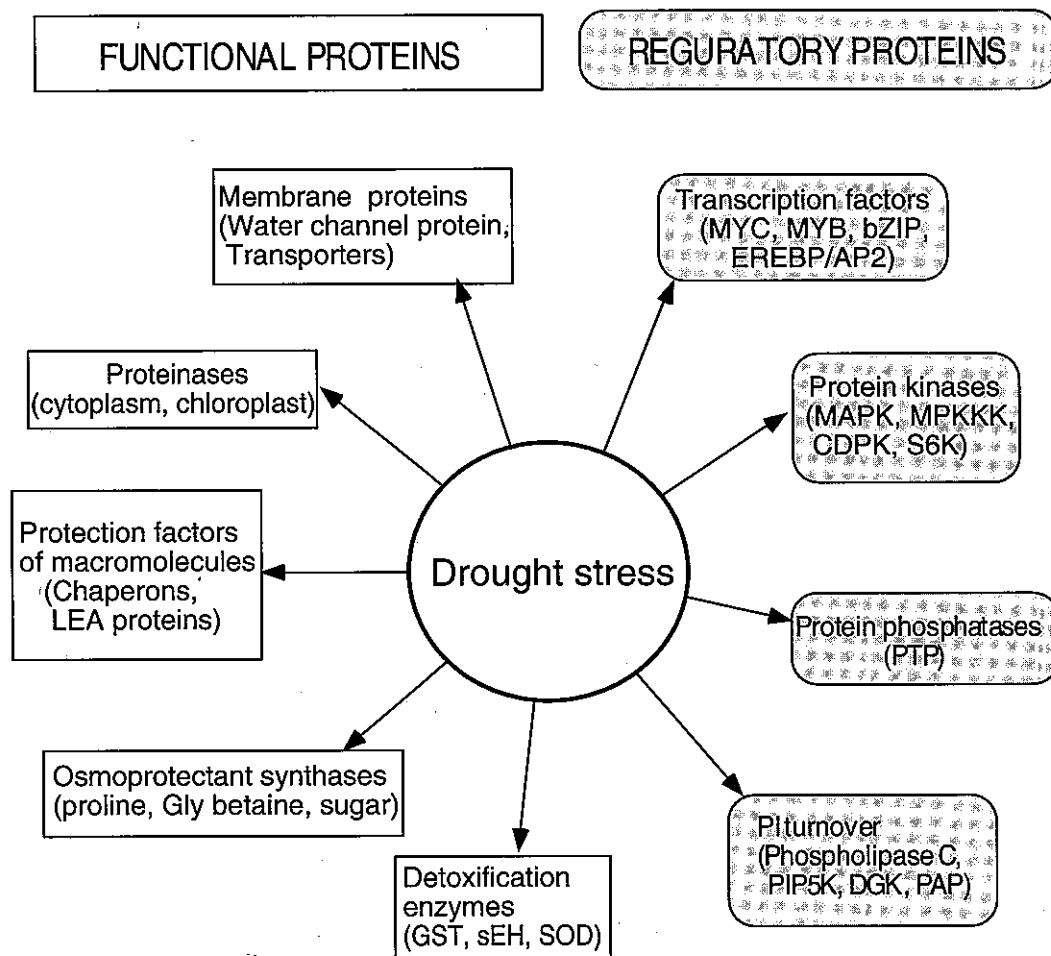


Fig. 2 Drought-stress inducible genes and their possible functions in stress tolerance and response.

Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (functional proteins), and the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (regulatory proteins).

superoxide dismutase, and a soluble epoxide hydrolase are involved in the protection of cells from active oxygen. Proteases including thiol proteases, Clp protease, and ubiquitin are considered to be required for protein turnover and recycling of amino acids.

The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response: protein kinases, transcription factors and enzymes in phospholipid metabolism^{3, 6, 24, 25, 26, 30}. Genes for a variety of transcription factors that contain typical DNA binding motifs, such as bZIP, MYB, MYC, ERF/AP2 and Zinc fingers, have been demonstrated to be stress-inducible^{3, 26}. These transcription factors function in further regulation of various functional genes under stress conditions. Various protein kinases, such as MAP kinases, calcium dependent protein kinases (CDPK), SNF1 related protein kinase and ribosomal S6 kinase were demonstrated to be induced or upregulated by dehydration^{18, 26}. Stress-inducible genes for protein phosphatases have been reported³². These protein kinases and phosphatases may be involved in the modification of functional proteins and regulatory proteins involved in stress signal transduction pathways. Phospholipids, such as inositol 1,4,5-triphosphate, diacylglycerol and phosphatidic acid are considered to be involved in stress signaling process in plants²⁶. Enzymes involved in phospholipid metabolism of which genes are stress inducible may play important roles in stress signaling as well. Existence of a variety of drought-inducible genes suggests complex responses of plants to drought stress. Their gene products are involved in drought stress tolerance and stress responses.

Expression of dehydration-induced genes in response to environmental stresses and ABA

The expression patterns of genes induced by drought were analyzed by RNA gel-blot analysis. Results indicated broad variations in the timing of induction of these genes under drought conditions. All the drought-inducible genes are induced by high salinity stress. Most of the drought-inducible genes also respond to cold stress but some of them do not, and vice versa. Many genes respond to ABA whereas some others do not^{3, 6, 24, 25, 26, 30}. ABA-deficient mutants were used to analyze drought-inducible genes that respond to ABA. Several genes were induced by exogenous ABA treatment, but were also induced by cold or drought in ABA-deficient (*aba*) or ABA-insensitive (*abi*) *Arabidopsis* mutants. These observations indicate that these genes do not require an accumulation of endogenous ABA under cold or drought conditions, but do respond to ABA. There are ABA-independent as well as ABA-dependent regulatory systems of gene expression under drought stress. Analysis of the expression of ABA-inducible genes showed that several genes require protein biosynthesis for their induction by ABA, suggesting that at least two independent pathways exist between the production of endogenous ABA and gene expression under stress conditions.

As shown in Fig. 3, it is now hypothesized that at least four independent signal transduction pathways function in the activation of stress-inducible genes under dehydration conditions: two are ABA-dependent (pathways I and II) and two are ABA-independent (pathways III and IV)^{25, 26}. One of the ABA-dependent pathways requires protein biosynthesis (pathway I). *Cis*- and *trans*-acting factors involved in ABA-induced gene expression have been extensively analyzed in one of the ABA-dependent pathways (pathway II). One of the ABA-independent pathways overlaps with that of the cold response (pathway IV). There are several drought-inducible genes that do not respond to either cold or ABA treatment, which suggests that there is the fourth pathway in the dehydration stress response (pathway III). Recently, based on genetic analysis of *Arabidopsis* mutants with the *rd29A* promoter::luciferase transgene, the existence of drought-, salt- and cold-specific signaling pathways in stress response was suggested, and crosstalks between these signaling pathways were also observed.

Identification of a *cis*-acting element, DRE, involved in drought-responsive expression

Numbers of genes are induced by drought, salt and cold in *aba* (ABA-deficient) or *abi* (ABA-insensitive) *Arabidopsis* mutants, suggesting that these genes do not require ABA for their expression under cold or drought conditions. Among these genes, the expression of a drought-inducible gene for *rd29A/lti78/cor78* was extensively analyzed³⁹. At least two separate regulatory systems function in gene expression during drought and cold stress; one is ABA-independent (Fig. 3, pathway IV) and the other is ABA-dependent (pathway II).

To analyze the *cis*-acting elements involved in the ABA-independent gene expression of *rd29A*, we constructed chimeric genes with the *rd29A* promoter fused to the β glucuronidase (*GUS*) reporter gene and transformed *Arabidopsis* and tobacco plants with these constructs. The *GUS* reporter gene driven by the *rd29A* promoter was induced at significant levels in transgenic plants by dehydration, low temperature, or high-salt or by treatment with ABA³⁹. The deletion, the gain-of-function and the base substitution analysis of the promoter region of *rd29A* gene revealed that a 9-bp conserved sequence, TACCGACAT (DRE, Dehydration Responsive Element), is essential for the regulation of the expression of *rd29A* under drought conditions. Moreover, DRE has been demonstrated to function as a *cis*-acting element involved in the induction of *rd29A* by either low temperature or high-salt stress³⁹. Therefore, DRE seems to be a *cis*-acting element involved in

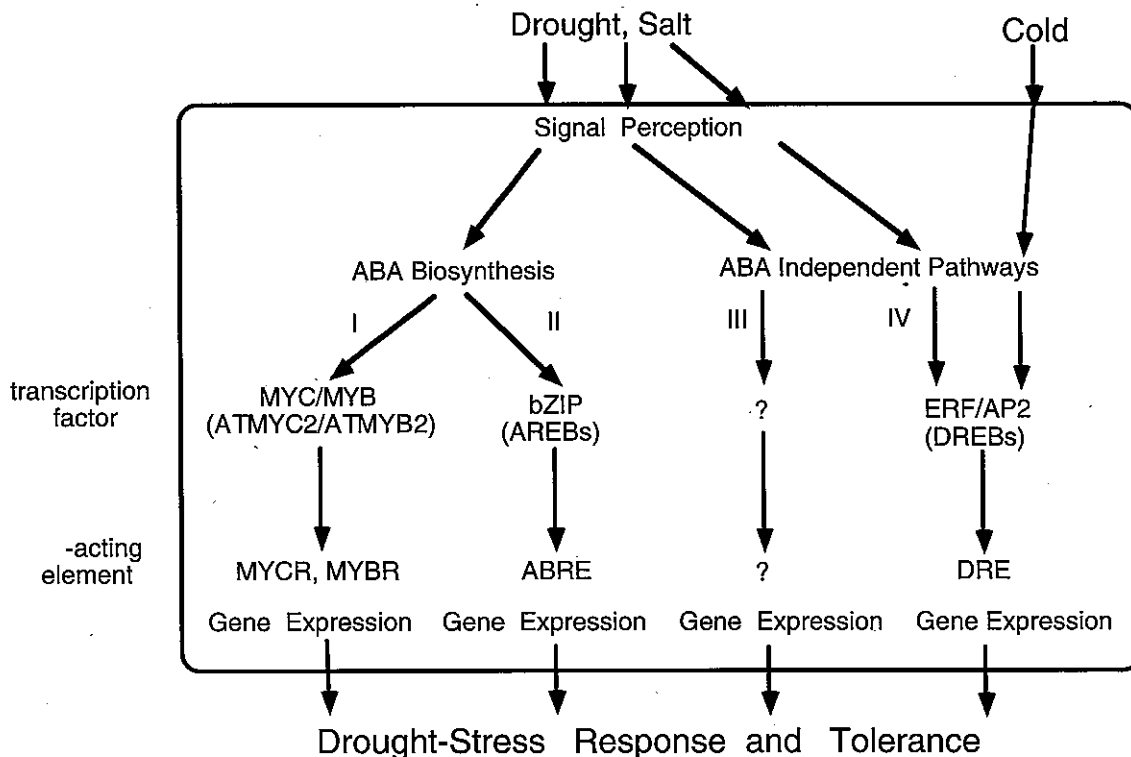


Fig. 3 Signal transduction pathways between initial dehydration stress signal and gene expression.

There are at least four signal transduction pathways: two are ABA-dependent (I and II) and two are ABA-independent (III and IV). Protein synthesis is necessary for one of the ABA-dependent signal pathways (I). ABRE is involved in one of the ABA-dependent pathway (II). In one of the ABA-independent pathways, DRE is involved in the regulation of genes not only by drought and salt but also by cold stress(IV). Another ABA-independent pathway is controlled by drought and salt, but not by cold (III).

gene induction by dehydration, high-salt, or low temperature, but does not function as an ABA-responsive element in the induction of *rd29A*.

Important roles of the DRE binding proteins during drought and cold stresses

Two cDNA clones that encode DRE binding proteins, DREB1A and DREB2A, were isolated by using the yeast one-hybrid screening technique¹⁹. The deduced amino acid sequences of DREB1A and DREB2A showed significant sequence similarity with the conserved DNA binding domains found in the ERF and APETALA2 proteins that function in ethylene-responsive expression and floral morphogenesis, respectively^{20, 21}. Each DREB protein contained 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. These data suggest that each DREB cDNA encodes a DNA binding protein that might function as a transcriptional activator in plants.

The ability of the DREB1A and DREB2A proteins expressed in *Escherichia coli* to bind the wild-type or mutated DRE sequences was examined using the gel retardation method¹⁹. The results indicate that the binding of these two proteins to the DRE sequence is highly specific. To determine whether the DREB1A and DREB2A proteins are capable of transactivating DRE-dependent transcription in plant cells, we performed transactivation experiments using protoplasts prepared from *Arabidopsis* leaves. Coexpression of the DREB1A or DREB2A proteins transactivated the expression of the *GUS* reporter gene driven by the DRE sequence in *Arabidopsis* leaf protoplasts. These results suggest that the DREB1A and DREB2A proteins function as transcription activators involved in the cold- and dehydration-responsive expression, respectively, of the *rd29A*

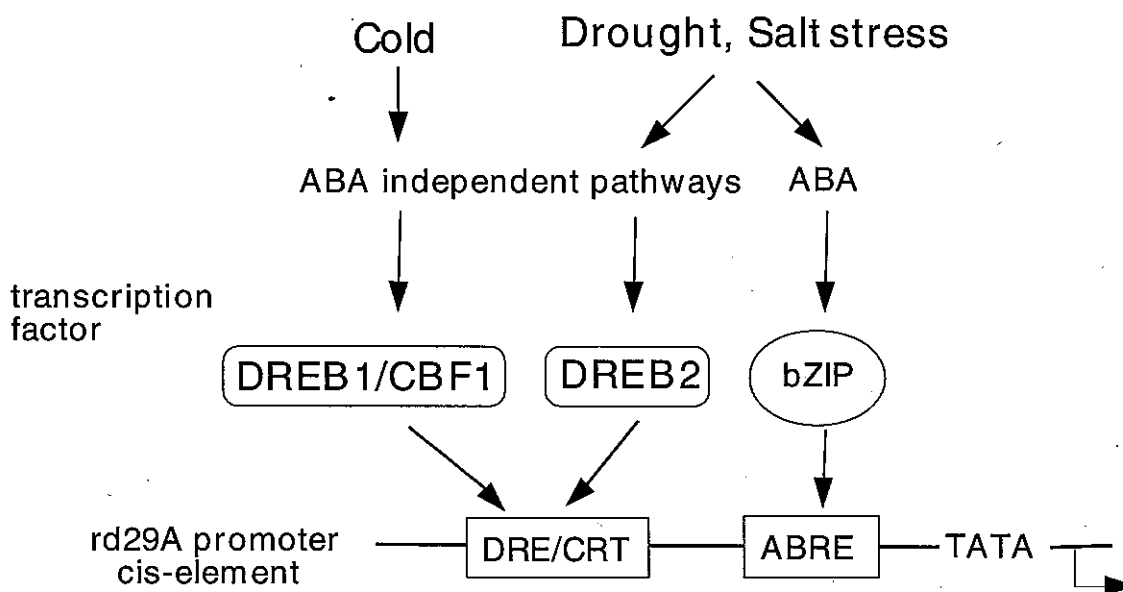


Fig. 4 A model of the induction of the *rd29A* gene and *cis*- and *trans*-acting elements involved in stress-responsive gene expression.

Two *cis*-acting elements, DRE and ABRE, are involved in the ABA-independent and ABA-responsive induction of *rd29A*, respectively. Two types of DRE binding proteins, DREB1/CBF1 and DREB2, separate two different signal transduction pathways in response to cold and drought factors, respectively. Genes for ABRE binding proteins encode bZIP transcription factors.

gene (Fig. 4)¹⁵.

We isolated cDNA clones encoding two DREB1A homologs (named DREB1B and DREB1C)¹⁵. The DREB1B clone was identical with CBF1²⁸. We also isolated cDNA clones encoding a DREB2A homolog and named it DREB2B. Expression of the *DREB1A* gene and its two homologs was induced by low-temperature stress²⁷, whereas expression of the *DREB2A* gene and its single homolog was induced by dehydration¹⁹. These results indicate that two independent families of DREB proteins, DREB1 and DREB2, function as *trans*-acting factors in two separate signal transduction pathways under low temperature and dehydration conditions, respectively (Fig. 4)¹⁵.

Analysis of the roles of DREB1A and DREB2A by using transgenic plants

We generated transgenic plants in which the DREB1A or DREB2A cDNAs were introduced to overproduce the DREB proteins to analyze the effects of overproduction of the DREB1A and DREB2A proteins on the expression of the target *rd29A* gene. *Arabidopsis* plants were transformed with vectors carrying fusions of the enhanced CaMV 35S promoter and the DREB1A (35S:DREB1A) or DREB2A (35S:DREB2A) cDNAs in the sense orientation^{15,17}. All of the transgenic plants carrying the 35S:DREB1A transgene (the 35S:DREB1A plants) showed growth-retardation phenotypes under normal growth conditions. The 35S:DREB1A plants showed variations in phenotypic changes in growth retardation that may have been due to the different levels of expression of the DREB1A transgenes for the position effect¹⁵.

To determine whether overproduction of the DREB1A protein caused the expression of the target gene in unstressed plants, we compared the expression of the *rd29A* gene in control plants carrying the pBI121 vector. Transcription of the *rd29A* gene was low in the unstressed wild-type plants but high in the unstressed 35S:DREB1A plants¹⁵. The level of the *rd29A* transcripts under the unstressed control condition was found to depend on the level of the DREB1A transcripts¹⁵. To determine whether overproduction of the DREB1A protein caused the expression of other target genes, we evaluated the expression of its target stress-inducible genes. In the 35S:DREB1A plants the *kin1*, *cor6.6/kin2*, *cor15a*, *cor47/rd17* and *erd10* genes were expressed strongly under unstressed control conditions, as was the *rd29A* gene^{11,13,26}.

Recently we have prepared a cDNA microarray using 1300 full-length *Arabidopsis* cDNAs to identify further target stress-inducible genes of DREB1A²⁹. mRNA prepared from the 35S:DREB1A plants and wild-type control plants were used for the preparation of Cy3-labeled and Cy5-labeled cDNA probes, respectively. These cDNA probes identified genes with expression levels more than two times greater in the 35S:DREB1A transgenic plants than in wild-type control plants as DREB1A target genes. In total, 12 DREB1A target genes were identified by cDNA microarray analysis. Among them, six were shown to be DREB1A target genes: *rd29A*, *kin1*, *cor6.6/kin2*, *cor15a*, *cor47/rd17* and *erd10*. Also, among the six novel DREB1A target genes that had not been identified as DREB1A target genes previously, we found cDNAs showing sequence identity with putative cold acclimation protein, DC1.2 homolog, enolase and cysteine proteinase inhibitor, and *erd4* cDNA. All these gene products presumably function in stress tolerance in plant cells.

In contrast, the transgenic plants carrying the 35S:DREB2A transgene (the 35S:DREB2A plants) showed little phenotypic change. In the 35S:DREB2A transgenic plants, the *rd29A* mRNA did not accumulate significantly, although the *DREB2A* mRNA accumulated even under unstressed conditions¹⁵. Expression of the DREB2A protein is not sufficient for the induction of the target stress-inducible gene. Modification, such as phosphorylation of the DREB2A protein, seems to be necessary for its function in response to dehydration (Fig. 4). However, DREB1 proteins can function without modification.

Drought, salt and freezing stress tolerance in transgenic plants

The tolerance to freezing and dehydration of the transgenic plants was analyzed using the 35S:DREB1A plants grown in pots at 2 °C for 3 weeks. When plants were exposed to a temperature of -6 °C for 2 days, returned to 22 °C, and grown for 5 days, all of the wild-type plants died, whereas the 35S:DREB1A plants survived at a high frequency^{11,15}. Freezing tolerance was correlated with the level of expression of the stress-inducible genes under unstressed control conditions (Fig. 5; between 80 and 30%, survival)^{11,15}.

To determine whether the introduction of the DREB1A gene enhances tolerance to dehydration stress, we did not water the plants for 2 weeks. Although all of the wild-type plants died within 2 weeks, between 70 and 20% of the 35S:DREB1A plants survived and continued to grow after rewatering. Drought tolerance was also dependent on the level of expression of the target genes in the 35S:DREB1A plants under unstressed conditions (Fig. 4)^{11,15}.

Overexpression of the DREB1A cDNA, driven by the constitutive 35S CaMV promoter in transgenic plants, activated strong expression of the target stress-inducible genes under unstressed conditions, which, in turn, increased the tolerance of freezing, salt and drought stresses^{11,15}. Jaglo-Ottosen *et al.* reported that CBF1 overexpression also enhances freezing tolerance⁹. However, the overexpression of stress-inducible genes controlled by the DREB1A protein caused severe growth retardation under normal growth conditions^{11,15}.

To resolve the problem of growth retardation, we used the stress-inducible *rd29A* promoter to generate overexpression of DREB1A in transgenic plants (*rd29A*:DREB1A plants)¹¹. Because the *rd29A* promoter was stress-inducible and contained binding sites for the DREB1A protein, it did not generate the expression of the DREB1A transgene at high levels under unstressed conditions; instead, it rapidly amplified the expression of the DREB1A transgene only under dehydration, salt, and low-temperature stress. The *rd29A*:DREB1A plants showed a strong stress tolerance even though their growth retardation under normal growing conditions was not significant. Moreover, the growth and the productivity of these plants were almost the same as those of the wild-type plants under normal growing conditions¹¹.

On the contrary, the *rd29A*:DREB1A transgenic plants were more tolerant to the stresses than the 35S:DREB1A plants that exhibited growth retardation under normal growing conditions (Fig. 5)¹¹. As the *rd29A* gene is one of the target genes of the DREB1A protein, the *rd29A* promoter is more suitable for the tissue specific expression of the DREB1A gene in plants than the 35S CaMV promoter. In the *rd29A*:DREB1A plants, the target gene products seem to be strongly accumulated in the same tissues that express the products under stress conditions. These results indicate that the combination of the DREB1A cDNA with the *rd29A* promoter would be quite useful for improving drought, salt and freezing-stress tolerance in transgenic plants.

In previous studies, we showed that DRE also functions in gene expression in response to stress in tobacco plants^{33, 34}, which suggests the existence of similar regulatory systems in tobacco and other crop plants. DRE-related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes^{10, 22}. These observations suggest that both the DREB1A cDNA and the *rd29A* promoter can be used to improve the dehydration, salt and freezing tolerance of crops by gene transfer.

To apply this system to tobacco, the DREB1A cDNA was overexpressed in transgenic tobacco. Overexpression of DREB1A improved drought, salt, and cold stress tolerance in tobacco as well as in *Arabidopsis*. These results indicate that the *Arabidopsis* DREB1A gene can be used to improve the stress tolerance of other dicotyledonous crops. In addition, we have also isolated DREB homologues in rice. OsDREB1A, 1B and OsDREB2A share a significant similarity with *Arabidopsis* DREB genes in structure, stress responsiveness and basic function in the stress signal transduction pathway in rice. We consider that the DREB/DRE regulatory system also can be used to improve the stress tolerance of monocotyledonous crops

by gene transfer. Currently we are collaborating with several institutes, which are working on transformation of agriculturally important crops to obtain various kinds of stress-tolerant crops such as wheat, maize and legumes.

Conclusion

Molecular mechanisms of drought stress response and tolerance have been extensively studied for the past ten years. Many genes that are regulated by drought stress have been reported in a variety of plants. Analyses of stress-inducible gene expression have revealed the presence of multiple signal-transduction pathways between the perception of drought-stress signal and gene expression. It was suggested that at least four different transcription factors function in the regulation of the dehydration-inducible genes; two are ABA-responsive and two are ABA-independent. This explains the complex stress response observed after exposure of the plants to drought stress. Genetic analysis of *Arabidopsis* mutants with the *rd29A* promoter::Luciferase transgene also suggests complex signaling pathways in drought-, salt- and cold-stress responses⁷. Some genes

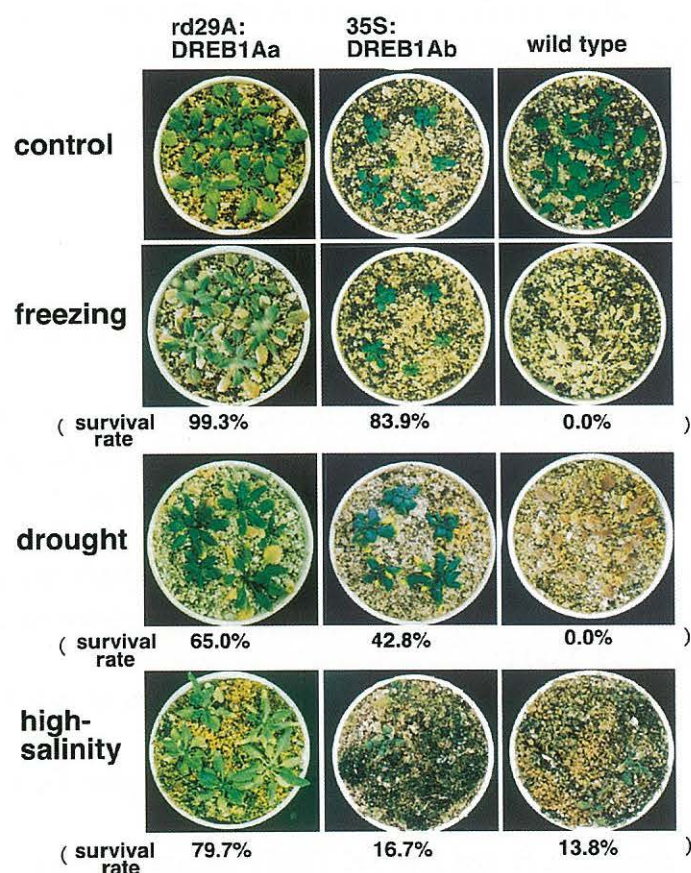


Fig. 5 Freezing, drought and salt stress tolerance of the 35S:DREB1Ab, and *rd29A*:DREB1Aa transgenic plants.

The stress treatments were conducted as described in the text.

control = 3-week-old plants growing under normal conditions; freezing = plants exposed to a temperature of -6 °C for 2 days and returned to 22 °C for 5 days; drought = water withheld for 2 weeks; high-salinity = plants soaked in 600 mM NaCl solution for 2 h and transferred to pots under normal growing conditions for 3 weeks.

are rapidly induced by drought stress in 10 min whereas others are slowly induced in a few hours after the accumulation of endogenous ABA. Several genes for various transcription factors are induced by drought stress and ABA at transcriptional levels, which might be involved in the regulation of slowly expressed genes of which products function in stress tolerance and adaptation. In addition, many genes for the factors involved in the signal-transduction cascades, such as protein kinases and enzymes involved in PI turnover, are upregulated by drought stress signal²⁶. These signaling factors might be involved in the amplification of the stress signals and adaptation of plant cells to drought-stress conditions. Molecules that function as osmosensors and ABA-receptors have not been identified. Based on the knowledge of osmosensors in yeast and bacteria, cloning of homologues of the two-component histidine kinase as an osmosensor is in progress in higher plants. Molecular analyses of these factors should provide a better understanding of the signal-transduction cascades during drought stress. Transgenic plants that modify the expression of these genes will give more information on the function of their gene products.

Sequencing of *Arabidopsis* entire genome was completed by the end of 2000, which means that the structures of all the 26,000 genes of *Arabidopsis* were determined^{1,2}. All the stress-inducible genes will be identified by systematic analysis of gene expression. In the next decade, we consider that it will be important to develop novel methods to analyze complex networks of stress responses of higher plants. Reverse genetic approach as well as classical genetics will become more important to understand not only the functions of stress-inducible genes but also complex signaling process in environmental stress responses. Efficient gene disruption methods as well as transgenic approaches using antisense or sense constructs also contribute to the precise understanding of molecular mechanisms of stress response.

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