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Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor

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Received 2 July 1998; accepted 4 January 1999

Plant productivity is greatly affected by environmental stresses such as drought, salt loading, and freezing. We reported previously that a *cis*-acting promoter element, the dehydration response element (DRE), plays an important role in regulating gene expression in response to these stresses. The transcription factor DREB1A specifically interacts with the DRE and induces expression of stress tolerance genes. We show here that overexpression of the cDNA encoding DREB1A in transgenic 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. However, use of the strong constitutive 35S cauliflower mosaic virus (CaMV) promoter to drive expression of *DREB1A* also resulted in severe growth retardation under normal growing conditions. In contrast, 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 expression of the gene from the CaMV promoter.

Keywords: drought tolerance, freezing tolerance, transgenic plants, multigene expression, stress-inducible promoter

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Drought, salt loading, and freezing are stresses that cause adverse effects on the growth of plants and the productivity of crops. The physiologic response to these stresses arises out of changes in cellular gene expression. Expression of a number of genes has been demonstrated to be induced by these stresses[1,2]. The products of these genes can be classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response[3]. The first group includes proteins that likely function by protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes[3–5]. The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism[3].

Recently, several different gene transfer approaches have been employed to improve the stress tolerance of plants[6]. The transferred genes included those encoding enzymes required for the biosynthesis of various osmoprotectants[7–9] or those encoding enzymes for modifying membrane lipids[10,11], LEA protein[12], and detoxification enzyme[13]. Each of these experiments involved transfer of a gene encoding a single specific stress-protective protein, expressed from the 35S cauliflower mosaic virus (CaMV) promoter. To investigate the possibility of simultaneously enhancing tolerance toward multiple stresses (i.e., drought, salt loading, and freezing) by gene transfer, we transferred a gene encoding a stress-inducible transcription factor that regulates many genes involved in stress tolerance in *Arabidopsis thaliana*.

Analyses of the expression of dehydration-inducible genes in *Arabidopsis* have indicated that at least four independent signal pathways function in the induction of stress-inducible genes in response to dehydrastion[3]. Two are abscisic acid (ABA)-dependent and two are ABA-independent. One of the two ABA-independent pathways overlaps with that of the cold-response. Several stress-induced genes, such as *rd29A*, are induced through this separate ABA-independent pathway[14–18]. A *cis*-acting element has been identified in the promoter region of the *rd29A* gene and is responsible for dehydration- and cold-induced expression[19]. This sequence (TACCGACAT), termed the dehydration-responsive element (DRE), is essential for the regulation of dehydration-responsive gene expression[19] and is found in the promoter regions of other dehydration- and cold-stress inducible genes[18,20]. The cDNAs encoding the DRE-binding proteins, DREB1A and DREB2A, have been isolated by yeast one-hybrid screening[21]. Both proteins specifically bind and activate transcription of genes containing the DRE sequence in *Arabidopsis*.

Expression of the *DREB1A* cDNA under control of the 35S CaMV promoter in transgenic plants gave rise to strong constitutive expression of the stress-inducible genes, and increased tolerance to freezing, salt, and drought stresses[21]. A cDNA encoding CRT/DRE binding protein (CBP1) was isolated from *Arabidopsis* and when overexpressed also enhanced freezing tolerance[22,23]. However, the overexpression of these genes resulted in severe growth retardation under normal growth conditions. Here, we use the stress-inducible *rd29A* promoter to drive expression of *DREB1A*, with the aim of minimizing the negative effects on plant growth experienced with use of the 35S CaMV promoter. We observed improved stress tolerance of the transgenic plants and much improved growth under nonstressed conditions.

Results

Preparation of transgenic *Arabidopsis*. *Arabidopsis* (Columbia ecotype) plants were transformed with vectors expressing the DREB1A cDNA from either a modified 35S CaMV promoter[24] or the stress-inducible *rd29A* promoter. Eighteen antibiotic-resistant *Arabidopsis*

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transformants carrying the 35S:DREB1A transgene (35S:DREB1A plants) and 43 transformants carrying the *rd29A*:*DREB1A* transgene (*rd29A*:DREB1A plants) were generated using a vacuum infiltration method[25].

Analyses of the 35S:DREB1A plants. Growth of the 35S:DREB1A transformants was compared with wild-type control plants after 35 (Fig. 1A) and 53 days (Fig. 1B). The transgenic plants demonstrated varying degrees of growth retardation, which we postulated to be due to variation in expression of the *DREB1A* transgene[21]. Three of the 18 transformants showed severe growth inhibition, and were classified as 35S:DREB1Aa plants. Transformants exhibiting lesser degrees of growth retardation were classified as either 35S:DREB1Ab (moderate phenotypic changes) or 35S:DREB1Ac (mild phenotypic changes) plants. The growth retardation affected seed numbers under normal growth (control) conditions. We observed a significant reduction in the number of seeds produced by the 35S:DREB1Aa and 35S:DREB1Ab plants compared with wild-type plants. The number of seeds harvested from the 35S:DREB1Aa plants was less than 1% of the wild-type controls. In contrast, the 35S:DREB1Ac plants produced a number of seeds similar to wild-type plants.

The expression of *DREB1A* and its target genes was analyzed in 35S:DREB1Ab and 35S:DREB1Ac plants and compared with control plants transformed with the pBI121 vector (Fig. 2). In the 35S:DREB1Ab plants, the *kin1, cor6.6/kin2, cor15a, cor47/rd17,* and *erd10* stress-response genes[3,26] were strongly expressed under control conditions, as was the *rd29A* gene. The expression of these genes in the 35S:DREB1Ac plants was only slightly higher than in wild-type plants but was noticeably lower than in 35S:DREB1Ab plants under unstressed conditions. In contrast, we detected no difference in the expression of the stress-inducible *AtP5CS, erd1, rd22,* and *rd29B* genes[14,27–29] between the 35S:DREB1A and wild-type plants. These results indicate that overexpression of the DREB1A protein leads to specific induction of its target stress-response genes, but not of nontarget stress-response control genes under conditions in the 35S:DREB1A plants, and that the level of stress-responsive gene expression correlates with levels of *DREB1A* mRNA.

Analyses of the *rd29A*:DREB1A plants. *rd29A*:DREB1A plants were then examined under growth conditions. The *DREB1A* cDNA was driven by the strong stress-inducible *rd29A* promoter. Nearly all of the *rd29A*:DREB1A plants exhibited slight growth retardation under control conditions (Fig. 3). However, unlike the 35S:DREB1A plants, there were no significant differences in growth retardation between the transgenic *rd29A*:DREB1A plant lines. Moreover, seed numbers for the *rd29A*:DREB1A transformants were similar to wild-type plants grown under control conditions.

The expression pattern of the *DREB1A* and *rd29A* genes was similar between the various *rd29A*:DREB1A transformant plants. Expression of these genes in transgenic plant *rd29A*:DREB1Aa is shown in Figure 4. The *DREB1A* transgene was expressed at levels above the endogenous gene in wild-type plants even under control conditions, yet was strongly induced by dehydration, salt, cold stress, and treatment with ABA in the *rd29A*:DREB1Aa plants. Under control conditions, expression of the *DREB1A* gene in the *rd29A*:DREB1Aa plants was much less than in the 35S:DREB1Aa plants and similar to that in the 35S:DREB1Ac plants (Fig. 4). In contrast, we detected similar strong expression of the *DREB1A* gene in both the *rd29A*:DREB1Aa and 35S:DREB1Aa plants under conditions of stress (dehydration, salt, low temperature, and treatment with ABA). We observed similar patterns of expression of the *rd29A* gene in the *rd29A*:DREB1Aa plants. The *rd29A* gene was expressed at low levels under control conditions and was strongly induced by each of the stress treatments. A similar pattern of expression was observed for other stress-inducible, DREB1A-responsive genes in the *rd29A*:DREB1A transgenic plants (data not shown).

Freezing, drought, and salt stress tolerance of the transgenic plants. The tolerance of the *rd29A*:DREB1A plants to freezing, dehy-

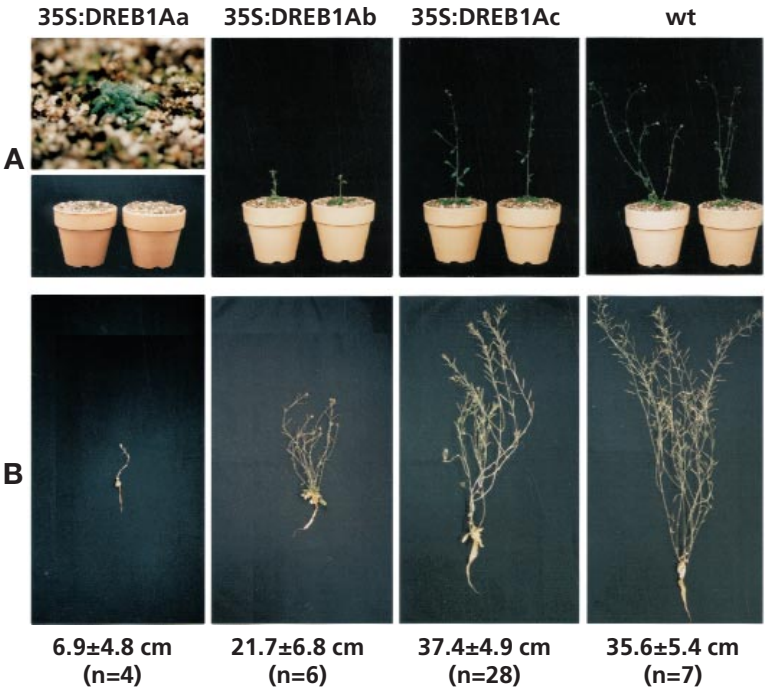

Figure 1. Phenotypes of the 35S:DREB1Aa, 35S:DREB1Ab, and 35S:DREB1Ac plants in relation to wild-type plants (pBI121). (A) Plants grown for 35 days. (B) Plants grown for 53 days. The average height of each plant line grown for 53 days is indicated under each picture.

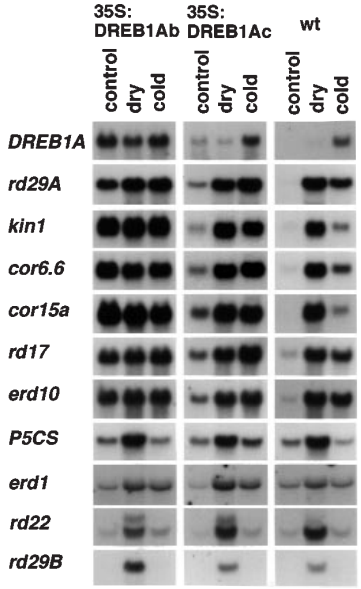

Figure 2. Expression of *DREB1A* target gene mRNAs in 35S:DREB1A transgenic plants and in the wild-type controls. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arbidopsis* plants that had been dehydrated for 5 h (dry), treated at 4°C for 5 h (cold), or left untreated (control). The DNA fragments of the full-length *DREB1A, KIN1, COR6.6, cor15a, RD17, ERD10, AtP5CS, ERD1,* and *RD22* cDNAs and the 3′-terminal specific DNA fragment of *rd29A* and *rd29B* were used as probes.

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dration, and salt stress was compared with that of the 35S:DREB1Ab and 35S:DREB1Ac plants grown in pots or on agar plates at 22°C for 3 weeks (Fig. 5A). When plants grown in pots were exposed to temperatures of -6°C for 2 days, then returned to 22°C and grown for 5 days, less than 10% of the wild-type plants survived, whereas 77.9% and 96.2% of the 35S:DREB1Ab and *rd29A*:DREB1Aa plants survived, respectively (Table 1A). The surviving transgenic plants continued to grow and subsequently flowered. Freezing tolerance of the 35S:DREB1A plants correlated with the level of expression of the stress-inducible DREB1A-responsive genes under control conditions: fewer of the 35S:DREB1Ac plants survived than 35S:DREB1Ab plants (48.8% vs. 77.9% survival, respectively; Table 1A). In contrast, the freezing tolerance of the *rd29A*:DREB1Aa plants was much stronger than that of the 35S:DREB1Ab plants, even though the expression of the stress-inducible DREB1A-responsive genes was weak under control conditions (Fig. 5A and B).

To test whether overexpression of the *DREB1A* gene enhanced tolerance to dehydration, the wild-type and transgenic plants grown in pots were not watered for 2 weeks (Fig. 5A). Nearly all the wild-type plants died within this 2-week period, whereas 69.2% of the 35S:DREB1Ab transgenic plants survived this level of drought stress and continued to grow when watering resumed (Table 1B). The toler-

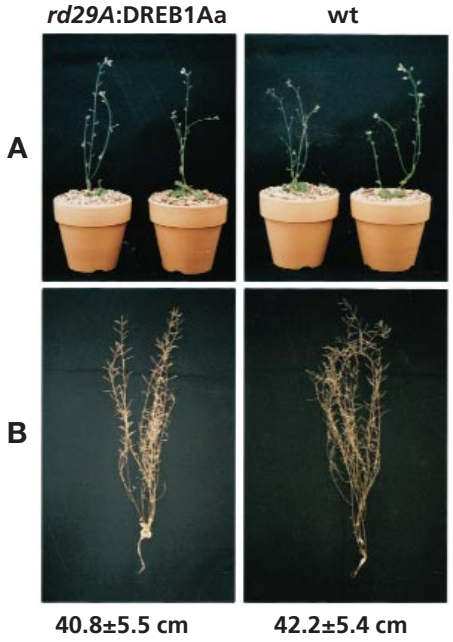

Figure 3. Phenotypes of the *rd29A*:DREB1Aa and wild-type control plants (transformed with the vector pBI121) under control conditions. (A) Plants grown for 30 days. (B) Plants grown for 63 days. The average height of each plant line grown for 63 days is indicated under each picture.

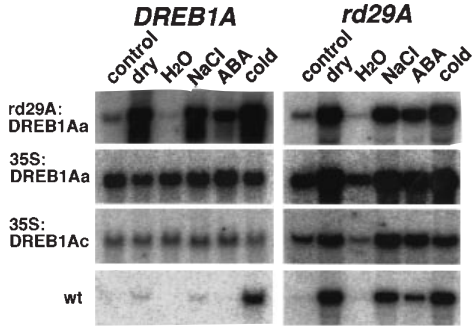

Figure 4. Expression of the *DREB1A* and *rd29A* genes in 35S:DREB1A and *rd29A*:DREB1A transgenic plants. Each lane was loaded with 20 µg of total RNA prepared from transgenic *Arbidopsis* plants that had been dehydrated (dry), grown hydroponically in 250 mM NaCl (NaCl), in water (H_2O), or in 100 µM ABA (ABA), or transferred to agar plates at 4°C (cold), or untreated (control). The stress treatments were continued for 5 h.

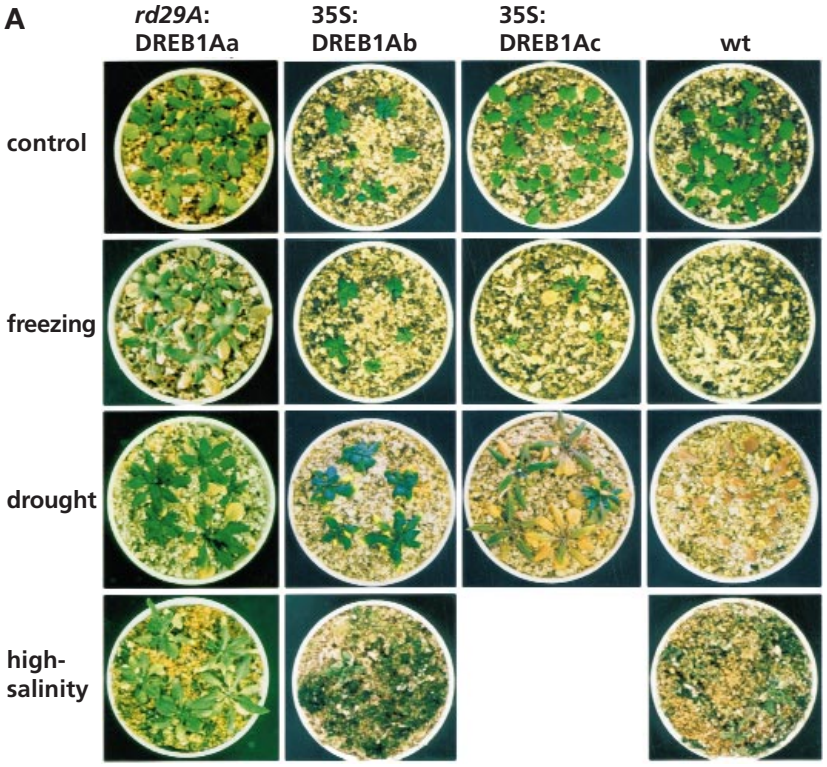

Figure 5. (A) Freezing, drought, and salt stress tolerance of the 35S:DREB1Ab, 35S:DREB1Ac, and *rd29A*:DREB1Aa transgenic plants. The stress treatments were performed as described in the text. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C 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 control conditions for 3 weeks. (B) Expression of the *DREB1A* and *rd29A* genes in the 35S:DREB1A and *rd29A*:DREB1A transgenic plants subjected to freezing, drought, and salt stress. Control: 3-week-old plants growing under normal conditions; freezing: plants exposed to a temperature of -6°C for 2 days; drought: water withheld for 5 days; control: plants grown on GM agar plates for 2 weeks; high-salinity: plants that were grown on agar plates for 2 weeks, soaked in 600 mM NaCl solution for 2 h.

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Table 1. Survival rates of transgenic plants.

A. Freezing tolerance

	Survival	Total	%
rd29A:DREB1A	177	184	96.2
35S:DREB1Ab	67	86	77.9*
35S:DREB1Ac	40	82	48.8*
wt	9	95	9.5*

B. Drought tolerance

	Survival	Total	%
rd29A:DREB1A	92	120	76.7
35S:DREB1Ab	45	65	69.2
35S:DREB1Ac	23	58	39.7*
wt	1	55	1.8*

C. High-salinity stress tolerance

	Survival	Total	%
rd29A:DREB1A	125	159	78.6
35S:DREB1Ab	10	34	29.4*
wt	7	39	17.9*

Number of plants surviving exposure to (A) freezing, (B) drought, and (C) high-salinity. Experiments were repeated five times. Five to fifty plants were tested in each experiment. Statistical significance, compared with the value of *rd29A*:DREB1A, was determined by χ^2 test (*p<0.005).

ance of the 35S:DREB1A plants to drought stress depended on the level of the expression of DREB1A-responsive genes under control conditions: 39.7%, 69.2%, and 76.7% survival was observed for the 35S:DREB1Ac, 35S:DREB1Ab, and *rd29A*:DREB1A plants, respectively (Table 1B). To examine the tolerance of the transgenic plants to salt stress, plants grown on agar plates were removed from the plates, soaked in 600 mM NaCl solution for 2 h, and then grown in pots for 3 weeks (Fig. 5A). Only 17.9% of the wild-type plants survived this treatment compared with 29.4% of the 35S:DREB1Ab plants (Table 1C). In contrast, the *rd29A*:DREB1A plants were highly tolerant of the salt stress (78.6% survival; Table 1C).

The expression of *DREB1A* and DREB1A-responsive genes was examined in the transgenic plants after freezing, drought, and high-salinity stress treatments (Fig. 5B). The *DREB1A* and *rd29A* genes were weakly expressed even under control conditions in the *rd29A*:DREB1Aa plants. Upon exposure to stress conditions, expression of these genes was elevated to similar levels in *rd29A*:DREB1Aa to that in 35S:DREB1Ab plants (Fig. 5B). These results indicate that strong expression of the DREB1A-responsive genes under stress conditions correlated with tolerance to freezing, drought, and high-salinity stress.

Discussion

Many genes have been demonstrated to respond to drought, high salt levels, and cold stress, and the proteins encoded by these genes are thought to function in protecting cells from these stresses. In the present study, we were able to produce transgenic plants that were highly drought-, salt-, and freezing-tolerant by overexpressing a single gene for a stress-inducible transcription factor, DREB1A. DREB1A binds to the *cis*-acting DRE and regulates the expression of many stress-inducible genes under drought, salt, and cold stress in *Arabidopsis*[21]. The expression of the transferred *DREB1A* gene and its stress-inducible target genes were correlated in the transgenic plants under control conditions. We detected the overexpression of six stress-inducible DREB1A-responsive genes in both 35S:DREB1A and *rd29A*:DREB1A transgenic plants. The genes encoding proteins involved in stress tolerance seem to be overproduced even under control conditions in the 35S:DREB1A transgenic plants, but are rapidly overproduced only in response to stress treatments in the *rd29A*:DREB1A plants. These target genes (*rd29A, kin1, cor6.6/kin2, cor47/rd17, cor15a,* and *erd10*) contain the DRE or related motifs[18–20,22], and are normally induced by dehydration, salt, and cold stresses. In contrast, the *AtP5CS, erd1, rd22,* and *rd29B* genes, which do not contain the DRE or related sequences[27–30] and are not targets of DREB1A, were not overexpressed in *DREB1A* transgenic plants. However, we believe that additional stress-inducible genes may be overexpressed in these transgenic plants under control conditions.

LEA proteins appear during the maturation of embryos and desiccation of seeds, and are also induced by drought, salt, and cold stresses in the vegetative tissues of various plants[31,32]. These proteins are quite hydrophilic and are believed to function by directly protecting plant cells from these stresses. One stress-inducible DREB1A-responsive gene, *rd29A,* encodes a protein similar to the LEA proteins[14]. The *cor47/rd17* and *erd10* genes both encode group 2 LEA proteins[31]. The *kin1* and *cor6.6/kin2* genes encode proteins that are structurally similar to the alanine-rich antifreeze proteins produced by some fish[20]. These similarities in structure and expression suggest that the products of these genes may have similar functions in plants. The *cor15a* gene encodes a protein that is targeted to the stromal compartment of the chloroplasts[33], and enhances the freezing tolerance of *Arabidopsis* leaf protoplasts[34].

Cold acclimation increases the freezing tolerance of plants[2]. Various genes are induced during cold acclimation, such as *rd29A/cor78/lti78, kin1, cor6.6/kin2, cor15a, cor47/rd17,* and *erd10.* The DRE is involved in the induction of these genes in response to low temperatures[1]. Overexpression of *DREB1A* induced expression of these genes under control conditions and increased the freezing tolerance of transgenic plants in a fashion reminiscent of what occurs during cold-acclimation of wild-type plants. Overexpression of CBF1, a DREB1A homolog, both enhanced freezing-stress tolerance and increased the expression of *cor15a, cor6.6,* and *cor47* (ref. 23*).* These observations demonstrate that the target genes of both the DREB1A and CBF1 encode proteins that function in protecting cells from freezing stress. Overexpression of DREB1A also enhanced drought and salt tolerance in the transgenic plants, demonstrating that the proteins encoded by the target stress-inducible genes also function in protecting cells from drought and salt stress.

The 35S:DREB1A plants exhibited varying degrees of growth retardation under control conditions. The extent of both growth retardation under control conditions and stress tolerance correlated with the level of constitutive expression of the DREB1A transcript and its target genes. These results indicate that stress tolerance of the 35S:DREB1A plants comes at the expense of growth and productivity.

To overcome the problem of growth retardation, we used the stress-inducible *rd29A* promoter to overexpress DREB1A. The *rd29A* promoter is stress-inducible and contains binding sites for the DREB1A protein. This promoter allowed low levels of DREB1A expression during unstressed conditions, yet permitted rapid high-level expression of the DREB1A transgene during exposure to dehydration, salt, and low-temperature stress. Thus, strong stress tolerance was accomplished without significant growth retardation under control conditions. Indeed, the growth and seed production of these plants was similar to wild-type plants under normal growing conditions. *rd29A*:DREB1A plants are more tolerant to stress than the 35S:DREB1A plants. As the *rd29A* gene is one of the target genes of the DREB1A protein, the *rd29A* promoter is highly suited to the tissue-specific expression of DREB1A. In *rd29A*:DREB1A plants, the target gene products appear to accumulate in the same tissues that express the stress-inducible genes during stress conditions. These results indicate that transgenic expression of the DREB1A cDNA from the *rd29A* promoter should be quite useful for improving drought, salt, and

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freezing-stress tolerance in plants. Previously, we showed that the DRE also functions in stress response in tobacco plants[14,19], 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[35,36]. These observations suggest that both the DREB1A cDNA and the *rd29A* promoter can be used to improve the dehydration, salt, and freezing tolerance of agriculturally important crops by gene transfer.

Experimental protocol

Overexpression of *DREB1A* in transgenic plants. The 35S:DREB1A plasmid was constructed as described previously[20]. To construct *rd29A*:DREB1A, a *Bam*HI fragment of the *DREB1A* cDNA was cloned into the *Bam*HI site of the pBI29APNot. pBI29APNot was constructed by ligation of the *Hind*III fragment of the *rd29A* promoter (-861 to +63) into the *Hind*III site of pBI101 (Clontech, Palo Alto, CA). The *Hind*III fragment of the *rd29A* promoter was amplified by PCR with the primers, 5´-AAGCTTGCCATAGATGCAATTAATC-3´ and 5´-AGCTTTTGGAAAGATTTTTTTCTTTCCAA-3´. The resulting plasmid was digested with *Sma*I and *Sac*I to delete the β-glucuronidase coding region, and ligated to a *Sma*I-*Not*I-*Sac*I polylinker (Takara, Tokyo, Japan). The constructs were introduced into *Agrobacterium tumefaciens* C58 as described previously[19]. The *Arabidopsis* plants selected for transformation were grown under continuous illumination in 9 cm pots at approximately 2500 lux and 22°C for 6 weeks. Plants were transformed by the vacuum infiltration method[22].

Phenotypes of the transgenic plants. The transgenic 35S:DREB1A and *rd29A*:DREB1A plants, which were grown on germination medium (GM) agar plates containing kanamycin (30 mg/L) for 7 days, were transferred to 9 cm pots filled with a 1:1 mixture of perlite and vermiculite, then were watered with 1000-fold diluted Hyponex (Osaka, Japan). They were photographed after the numbers of days indicated in Figures 2 and 4.

RNA gel blot analysis. *Arabidopsis* was grown on GM agar plates for 3 weeks and exposed to dehydration, high-salt, cold-stress, and ABA treatments, as described previously[19]. The plants were subjected to stress treatments for 5 h and then frozen in liquid nitrogen for further analyses. Isolation of total RNA and northern blot hybridization were performed as described previously[19]. The DNA fragments of the full-length *DREB1A, RD17, ERD10, AtP5CS, ERD1,* and *RD22* cDNAs and the 3´-terminal specific DNA fragments of *rd29A* and *rd29B* were used as probes. Probes for the *kin1, cor6.6,* and *cor15a* genes were obtained by PCR from cDNAs prepared from ABA-treated *Arabidopsis* plants.

Freezing, drought, and high-salt stress tolerance of the transgenic plants. Plants were grown in 9 cm pots filled with a 1:1 mixture of perlite and vermiculite. They were grown under continuous illumination of approximately 2500 lux at 22°C. Separate samples of the 3-week-old plants were exposed to freezing and drought stresses. Freezing stress was conducted by exposing the plants to -6°C temperatures for 2 days, then returning to 22°C for 5 days. Drought stress was conducted by withholding water for 2 weeks. High-salt stress was created by soaking plants that were grown on agar plates and gently pulled out of the growing medium in 600 mM NaCl solution for 2 h. The plants were then transferred to pots under normal growing conditions for 3 weeks. The numbers of plants that survived and continued to grow were counted. The statistical significance of the values was determined using chi-squared test.

Acknowledgments

The authors thank Satomi Yoshida, Atsuko Konishi, and Ekuko Ohgawara for their excellent technical assistance. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences, which awarded a grant to K.Y.-S. This work was also supported in part by the Special Coordination Fund of the Science and Technology Agency, by the Hunan Frontier Science Program, and by a grant in aid from Japan's Ministry of Education, Science, and Culture to K.S.

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