

# Functional Analysis of an *Arabidopsis* Transcription Factor, DREB2A, Involved in Drought-Responsive Gene Expression

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Transcription factors DREB1A/CBF3 and DREB2A specifically interact with *cis*-acting dehydration-responsive element/C-repeat (DRE/CRT) involved in cold and drought stress-responsive gene expression in *Arabidopsis thaliana*. Intact DREB2A expression does not activate downstream genes under normal growth conditions, suggesting that DREB2A requires posttranslational modification for activation, but the activation mechanism has not been clarified. DREB2A domain analysis using *Arabidopsis* protoplasts identified a transcriptional activation domain between residues 254 and 335, and deletion of a region between residues 136 and 165 transforms DREB2A to a constitutive active form. Overexpression of constitutive active DREB2A resulted in significant drought stress tolerance but only slight freezing tolerance in transgenic *Arabidopsis* plants. Microarray and RNA gel blot analyses revealed that DREB2A regulates expression of many water stress-inducible genes. However, some genes downstream of DREB2A are not downstream of DREB1A, which also recognizes DRE/CRT but functions in cold stress-responsive gene expression. Synthetic green fluorescent protein gave a strong signal in the nucleus under unstressed control conditions when fused to constitutive active DREB2A but only a weak signal when fused to full-length DREB2A. The region between DREB2A residues 136 and 165 plays a role in the stability of this protein in the nucleus, which is important for protein activation.

## INTRODUCTION

Plant growth and productivity are affected by various abiotic stresses, such as drought, high salinity, and low temperature. Plants respond and adapt to these stresses at the physiological and biochemical levels. Moreover, many genes that function in stress tolerance are induced under stress conditions (Thomashow, 1999; Bray et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2003). Most of these stress-inducible genes are controlled by abscisic acid (ABA), but some are not, indicating the involvement of both ABA-dependent and ABA-independent regulatory systems in stress-responsive gene expression (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Several stress-inducible genes, such as *RD29A* and *COR15A*, are induced through the ABA-independent pathway. A 9-bp conserved se-

quence, TACCGACAT, named the dehydration-responsive element (DRE), is an essential *cis*-acting element for regulation of *RD29A* induction in the ABA-independent response to dehydration, high salinity, and cold (Yamaguchi-Shinozaki and Shinozaki, 1994). Similar *cis*-acting elements, named C-repeat (CRT) and low-temperature-responsive element, both containing an A/GCCGAC motif that forms the core of the DRE sequence, regulate cold-inducible gene expression (Baker et al., 1994; Jiang et al., 1996; Thomashow, 1999).

Three cDNAs encoding DRE binding proteins, *CBF1*, *DREB1A*, and *DREB2A*, have been isolated by the yeast one-hybrid screening method (Stockinger et al., 1997; Liu et al., 1998). The proteins showed significant sequence similarity in the conserved DNA binding domain found in the ERF/AP2 proteins. They specifically bind to the DRE/CRT sequence and activate the transcription of genes driven by the DRE/CRT sequence in *Arabidopsis thaliana*. We isolated two cDNA clones homologous to *DREB1A* (*DREB1B* and *DREB1C*) and one cDNA homologous to *DREB2A* (*DREB2B*; Liu et al., 1998; Shinwari et al., 1998). Gilmour et al. (1998) also reported cloning of two *CBF1* homologues, *CBF2* and *CBF3*, from *Arabidopsis*. *CBF1* is identical to *DREB1B*, and its two homologues, *CBF2* and *CBF3*, are identical to *DREB1C* and *DREB1A*, respectively. Expression of the *DREB1/CBF* genes is induced by cold stress but not by drought and high-salinity stress. By contrast, expression of the *DREB2*

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genes is induced by drought or high-salt stresses (Liu et al., 1998). Both DREB1/CBF and DREB2 proteins bind to DRE/CRT, but DREB1/CBF are thought to function in cold-responsive gene expression, whereas DREB2 proteins are involved in drought-responsive gene expression. More recently, Sakuma et al. (2002) reported three novel *DREB1/CBF*-related genes that were not expressed at high levels under various stress conditions. However, one of them, *CBF4/DREB1D*, is induced by osmotic stress (Haake et al., 2002; Sakuma et al., 2002), and the other two, *DDF1/DREB1F* and *DDF2/DREB1E*, are induced by high-salinity stress (Sakuma et al., 2002; Magome et al., 2004), suggesting the existence of crosstalk between the CBF/DREB1 and DREB2 pathways.

Overexpression of *DREB1/CBF* driven by the 35S promoter of *Cauliflower mosaic virus (CaMV)* caused growth retardation under normal growth conditions and increased tolerance to dehydration, high salinity, and freezing in transgenic *Arabidopsis* (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999), suggesting that the DREB1A/CBF proteins function without modification in cold stress-responsive gene expression. More than 40 genes downstream of DREB1/CBF have been identified through the use of both cDNA and GeneChip microarrays (Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004). Many of their protein products are probably responsible for the stress tolerance of transgenic plants. The downstream genes also include genes for transcription factors, such as C2H2 zinc-finger-type and AP2/ERF-type transcription factors, suggesting the existence of further regulation of gene expression downstream of the DRE/DREB1 (CRT/CBF) regulon (Fowler and Thomashow, 2002; Maruyama et al., 2004). Conserved sequences in the promoter regions of the genes directly downstream of *DREB1A* were analyzed, and A/GCCGACNT was found in their promoter regions between -51 and -450 as a consensus DRE (Maruyama et al., 2004). DREB homologues have been reported from various species of both monocots and dicots, such as *Brassica napus*, tomato (*Solanum lycopersicum*), barley (*Hordeum vulgare*), maize (*Zea mays*), rice (*Oryza sativa*), rye (*Secale cereale*), and wheat (*Triticum aestivum*) (Jaglo et al., 2001; Choi et al., 2002; Gao et al., 2002; Xue, 2002; Dubouzet et al., 2003; Qin et al., 2004). These genes have also been shown to function in cold stress tolerance in those plants.

The DREB2 protein also has a conserved ERF/AP2 DNA binding domain and recognizes the DRE sequence. A database search of the *Arabidopsis* whole-genome sequence found at least six DREB2 homologues encoded in the *Arabidopsis* genome other than DREB2A and DREB2B. *DREB2A* and *DREB2B* are induced strongly by drought and high salinity, but the others are not. In addition, expression levels of the other six *DREB2* homologues under stress conditions are very low. Therefore, among the eight DREB2-type proteins, DREB2A and DREB2B are thought to be major transcription factors that function under drought and high-salinity stress conditions (Nakashima et al., 2000; Sakuma et al., 2002). However, overexpression of DREB2A in transgenic plants neither caused growth retardation nor improved stress tolerance, suggesting that the DREB2A protein requires posttranslational modification, such as phosphorylation, for its activation (Liu et al., 1998), but the activation mechanism has not been elucidated yet.

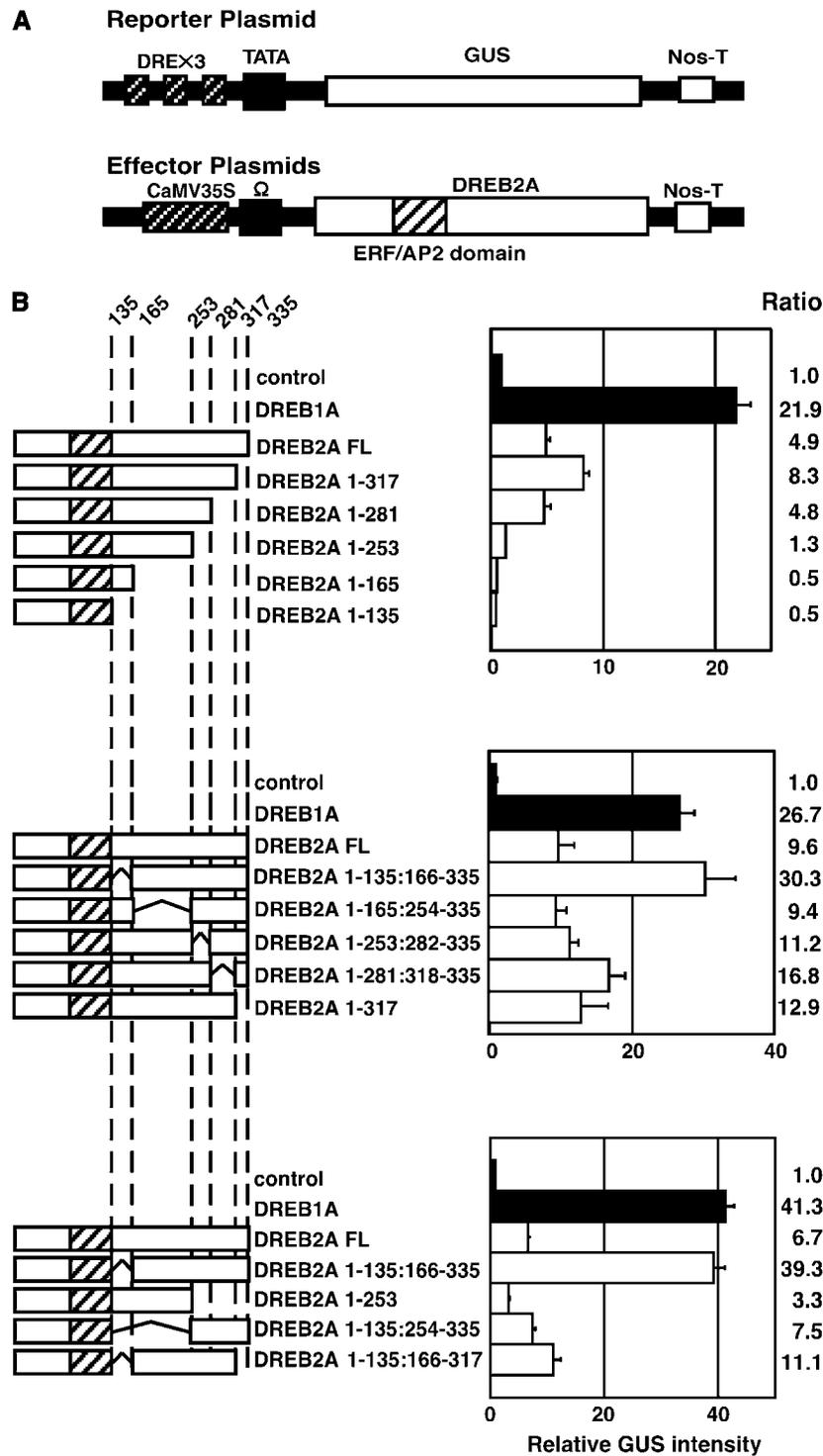
In this study, we performed domain analysis of the DREB2A protein and found that its C-terminal region functions as a transcriptional activation domain and that its middle region functions as a negative regulatory domain in the regulation of DREB2A activity. We succeeded in producing a constitutive active form of DREB2A by deletion of this negative regulatory domain. We analyzed genes downstream of DREB2A using transgenic plants overexpressing the constitutive active form of DREB2A and identified many drought-inducible genes lying downstream of DREB2A. We also analyzed the stress tolerance of the transgenic plants and found improvement of drought stress tolerance. These results indicate that DREB2A plays important roles in stress response and tolerance to drought in *Arabidopsis*.

## RESULTS

### Transcriptional Activation Activity of Deletion Mutants of DREB2A

Because the C-terminal region of DREB2A is rich in acidic amino acids, the transcriptional activation domain of DREB2A has been predicted to exist in this region (Liu et al., 1998). To identify the transcriptional activation domain of DREB2A, we performed transactivation experiments using protoplasts prepared from *Arabidopsis* T87 suspension-cultured cells. According to the results of the Chou-Fasman analysis for prediction of secondary structure (Chou and Fasman, 1978), the C-terminal half of the DREB2A protein, from just behind the DNA binding domain to the C-terminal end of the protein (amino acid residues 136 to 335), is divided into five parts. We constructed a series of effector plasmids without each part of DREB2A and a reporter plasmid having a  $\beta$ -glucuronidase (*GUS*) reporter gene driven by three copies of a DRE sequence (Figure 1A). These plasmids were cotransfected into *Arabidopsis* protoplasts, and then transactivation of the reporter gene was analyzed. As shown in Figure 1B, coexpression of the full-length DREB2A (DREB2A FL) cDNA resulted in ~5 to 10 times higher transactivation of the reporter gene than without DREB2A (control). Deletion of the C-terminal region between amino acids 254 and the C-terminal end of DREB2A decreased transactivation of the reporter gene to the basal level (Figure 1B, DREB2A 1-253). On the other hand, an internal deletion mutant lacking the region between amino acids 136 and 253 showed a similar activation level as that of the DREB2A FL (Figure 1B, DREB2A 1-135:254-335). These results indicate that the C-terminal region of DREB2A between amino acids 254 and the C-terminal end is required for transcriptional activation.

Interestingly, deletion of the region between amino acids 136 and 165 of DREB2A resulted in a significant increase of its activity (Figure 1B, DREB2A 1-135:166-335). Coexpression of this effector plasmid transactivated the *GUS* reporter gene ~30 times higher than the basal level and three times higher than that of the DREB2A FL. This level of activation was equivalent to that of the DREB1A effector construct (Figure 1B). We could not detect this kind of high activation using forms of DREB2A with the other regions deleted; therefore, we named this construct the constitutive active form of DREB2A. These results suggest the



**Figure 1.** Domain Analysis of the C-Terminal Region of the DREB2A Protein in Protoplasts Prepared from *Arabidopsis* T87 Cells.

**(A)** Schematic diagram of the reporter and effector constructs used in cotransfection experiments. The reporter construct contained 75-bp fragments of the *RD29A* promoter tandemly repeated three times (DRE $\times$ 3), -61 *RD29A* minimal TATA promoter, and *GUS* reporter gene. The effector constructs contain the *CaMV 35S* promoter and the tobacco mosaic virus  $\Omega$  sequence (Gallie et al., 1987) fused to the DREB2A cDNA with or without C-terminal deletion. Nos-T indicates the polyadenylation signal of the gene for nopaline synthetase.

**(B)** Transactivation of the *RD29A* promoter-*GUS* fusion gene by DREB1A, DREB2A FL, or C-terminal-region deletion mutants of DREB2A. Deleted regions of the DREB2A mutants are indicated as numbers of amino acid residues. Striped boxes indicate the AP2/ERF DNA binding domain. To normalize for transfection efficiency, the *CaMV 35S* promoter-luciferase plasmid was cotransfected in each experiment. Bars indicate the standard error of six replicates. Ratios indicate the degree of expression compared with the value obtained with the empty effector plasmid.

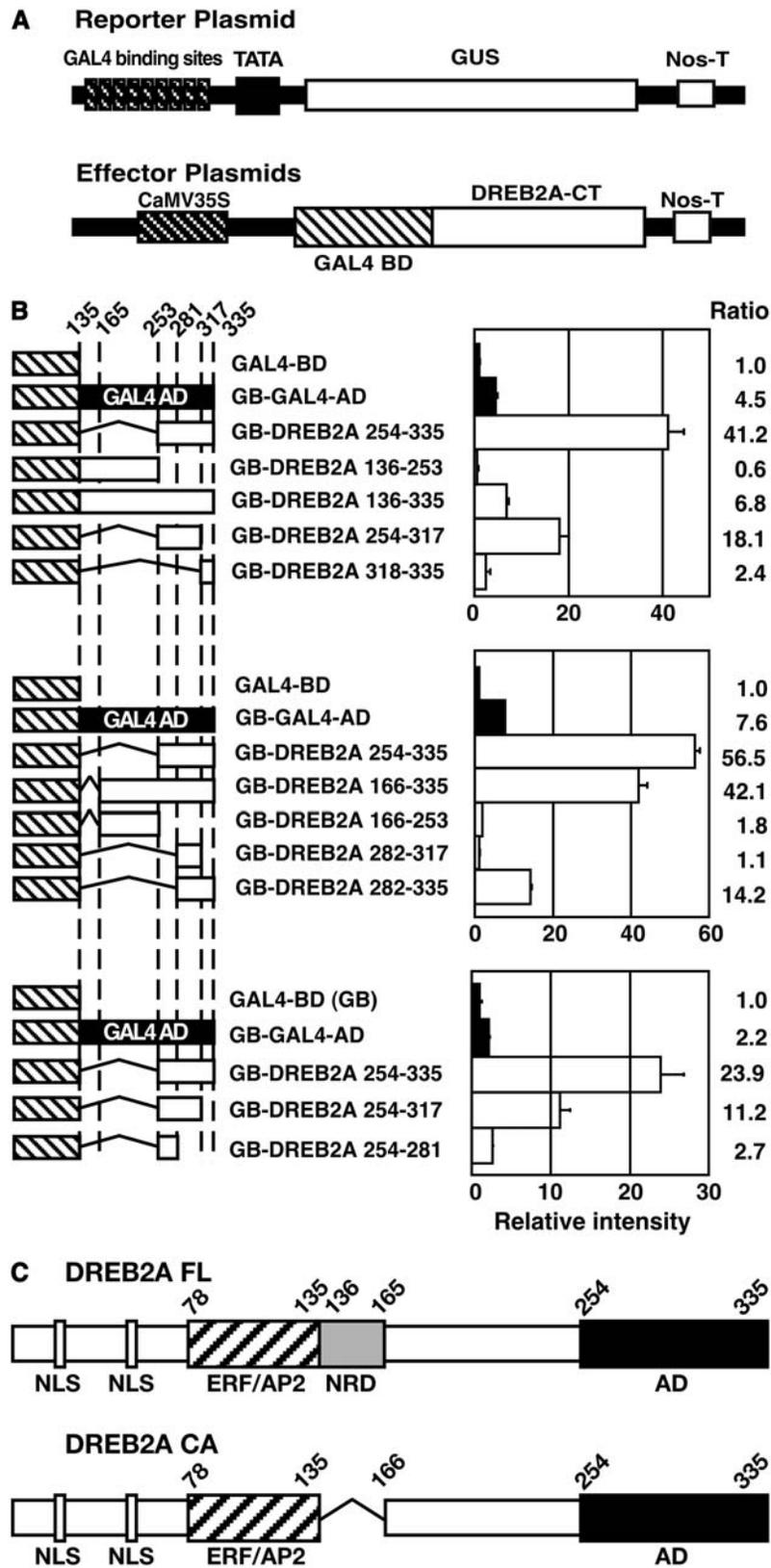


Figure 2. Transcriptional Activation with the C-Terminal Region of DREB2A Fused to the GAL4 Binding Domain.

presence of a negative regulatory domain in the region between amino acids 136 and 165.

To further analyze the activation domain of DREB2A, we prepared fusion genes that consist of the DNA binding domain of the yeast transcriptional activator *GAL4* (Ma et al., 1988) and various *DREB2A* fragments. The effector plasmids were co-transfected into protoplasts of *Arabidopsis* T87 cells with a reporter plasmid that contains nine copies of a *GAL4* binding site fused to the *CaMV 35S* minimal promoter and the *GUS* reporter gene (Figure 2A). As shown in Figure 2B, effector constructs of GB-DREB2A 136-253 and GB-DREB2A 166-253 that lack the region between amino acid 254 and the C-terminal end of DREB2A did not activate the expression of the reporter gene. The effector construct GB-DREB2A 254-335 containing the region from amino acid 254 to the C-terminal end of DREB2A showed the highest activation levels of the reporter gene among all effector constructs. When the fragment of GB-DREB2A 254-335 was extended toward the N terminus, no further increase of reporter activity was observed (Figure 2B, GB-DREB2A 136-335 and GB-DREB2A 166-335).

The region between amino acids 254 and 335 was divided into three parts and analyzed to identify the minimum activation domain of DREB2A. Each part could not or could only weakly stimulate the expression of the reporter gene when it was fused to the *GAL4* binding domain (Figure 2B, GB-DREB2A 254-281, GB-DREB2A 282-317, and GB-DREB2A 318-335), and a deletion of at least one part significantly decreased the activity of the *GUS* reporter compared with GB-DREB2A 254-335 (Figure 2B, GB-DREB2A 254-317 and GB-DREB2A 282-335). Although the effector construct GB-DREB2A 166-335 induced reporter gene expression at almost the same level as did GB-DREB2A 254-335, transactivation by GB-DREB2A 136-335, which contains the region between amino acids 136 and 165, was approximately one-sixth that of GB-DREB2A 254-335. These results indicate that the region between amino acids 254 and 335 functions as an activation domain of DREB2A and that the region between amino acids 136 and 165 negatively controls the transcriptional activation ability of DREB2A.

Figure 2C shows schematic diagrams of the DREB2A FL and the constitutive active form of DREB2A (DREB2A CA). DREB2A is predicted to have nuclear localization signals in its N-terminal region. The ERF/AP2 DNA binding domain and an activation domain exist in the central region and the C-terminal region, respectively. DREB2A also contains a negative regulatory domain adjoining the DNA binding domain. DREB2A CA can be generated by deletion of this negative regulatory domain.

### Overexpression of DREB2A CA in *Arabidopsis*

To analyze the function of DREB2A, we overexpressed *DREB2A CA*, *DREB2A* 1-135:166-335, which gave the highest activity in the transient transactivation experiments (Figure 1B), in *Arabidopsis* plants. This constitutive active form of *DREB2A* was overexpressed under the control of the enhanced *CaMV 35S* promoter (Mitsuhara et al., 1996). The tobacco mosaic virus  $\Omega$  sequence (Gallie et al., 1987) was inserted upstream of the mutant *DREB2A* fragment to increase the translational level. Fifty-five transgenic *Arabidopsis* plants were generated using the vacuum infiltration method (Bechtold et al., 1993). Expression levels of the transgene in these transgenic T2 plants were analyzed by RNA gel blot analysis, and we selected three lines, *35S:DREB2A CA-a*, *35S:DREB2A CA-b*, and *35S:DREB2A CA-c*, that showed strong, moderate, and weak transgene expression, respectively, for further analysis (Figure 3).

We observed the growth of the *35S:DREB2A CA* plants under normal growing conditions and compared them with control plants containing the pBI121 vector (wild type) and *35S:DREB2A FL* plants overexpressing the *DREB2A FL* cDNA (Figures 3A to 3C). All the *35S:DREB2A CA* plants showed significant growth retardation compared with the control plants, and the level of retardation was correlated with the levels of expression of the transgene and of a DREB2A target gene, *RD29A* (Figure 3E). By contrast, only very weak growth retardation was observed in *35S:DREB2A FL* plants overexpressing *DREB2A FL*. The *35S:DREB2A CA* plants had rounded, slightly darker leaves with short petioles. These phenotypes appeared in the *35S:DREB2A CA-a* plants more notably than in the *35S:DREB2A-c* plants (Figure 3D).

### Microarray and RNA Gel Blot Analyses of Transgenic *Arabidopsis* Plants Overproducing DREB2A CA

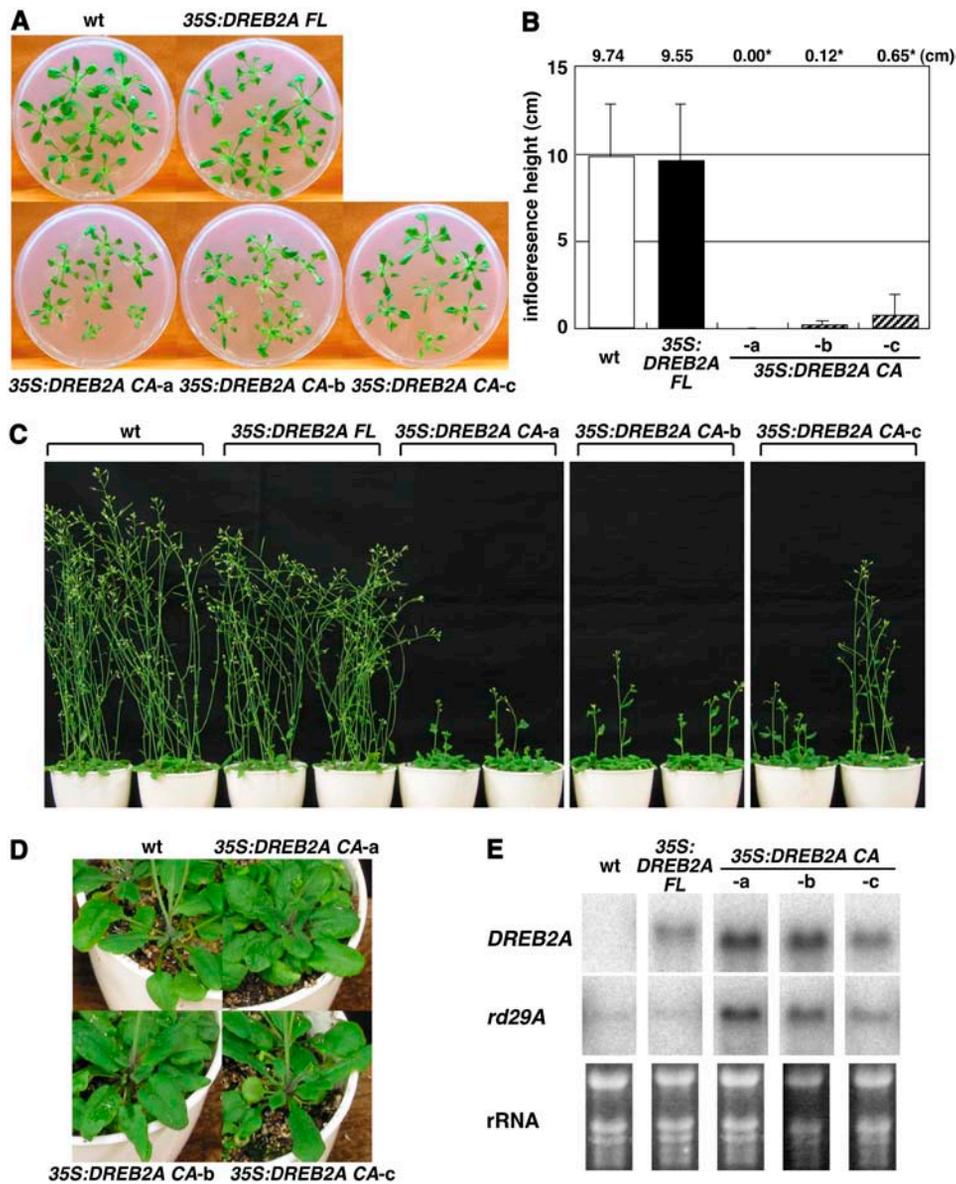
To determine the genes downstream of DREB2A in *Arabidopsis*, we performed array analysis using a full-length cDNA microarray containing ~7000 *Arabidopsis* full-length cDNAs (Seki et al., 2002). We used three independent transgenic *Arabidopsis* plants overexpressing DREB2A CA: *35S:DREB2A CA-a*, *35S:DREB2A CA-b*, and *35S:DREB2A CA-c*. Cy3- and Cy5-labeled cDNA probes were prepared from mRNAs isolated from the *35S:DREB2A CA* and control plants, respectively, grown under unstressed control conditions. These probes were hybridized with the cDNA microarray, and the expression profiles of the ~7000 genes were analyzed. To assess the reproducibility, we

**Figure 2.** (continued).

**(A)** Schematic diagrams of the reporter and effector constructs. The *GUS* reporter plasmid contains nine copies of the *GAL4* binding site fused to the *CaMV 35S* minimal TATA box. The effector plasmids encode the *GAL4* DNA binding domain (BD; amino acids 1 to 147) alone or the *GAL4* binding domain fused to either the *GAL4* activation region (amino acids 768 to 881) or C-terminal regions of DREB2A (white bar).

**(B)** Transactivation of the *GAL4* binding sites-*GUS* fusion gene by the fusion proteins of the *GAL4* DNA binding domain with the *GAL4* activation domain (AD) or C-terminal regions of the DREB2A protein. Striped boxes indicate the *GAL4* DNA binding domain. To normalize for transfection efficiency, the *CaMV 35S* promoter-luciferase plasmid was cotransfected in each experiment. Bars indicate the standard error of six replicates. Ratios indicate the degree of expression compared with the value obtained from the plasmid that encodes only the *GAL4* DNA binding domain.

**(C)** Schematic diagrams of the DREB2A proteins. Numerals indicate the position of each domain in amino acid numbers from the N terminus. NLS, nuclear localization signal; ERF/AP2, ERF/AP2 DNA binding domain; NRD, negative regulatory domain; AD, activation domain.



**Figure 3.** Effects of Overexpressing *DREB2A CA* in Transgenic Plants under Unstressed Conditions.

**(A)** The 25-d-old seedlings carrying *DREB2A CA* driven by the 35S promoter (35S:*DREB2A CA*-a, -b, and -c), the *DREB2A FL* construct driven by the 35S promoter (35S:*DREB2A FL*), or pBI121 (wt).

**(B)** Inflorescence heights of 30-d-old transgenic plants. Average inflorescence heights and standard deviations were calculated using 12 plants. Asterisks indicate that these plants have significantly shorter inflorescences than wild-type plants (Student's *t* test,  $P < 0.001$ ).

**(C)** Photographs of 35-d-old transgenic plants

**(D)** Close-up of the wild-type and 35S:*DREB2A CA* plants shown in **(C)**.

**(E)** RNA gel blot analysis of *DREB2A* and *RD29A* in wild-type and transgenic plants. Each lane was loaded with 5  $\mu$ g of total RNA prepared from 3-week-old transgenic *Arabidopsis* plants. Ethidium bromide-stained rRNA image is shown as a loading control.

repeated each experiment three times and further analyzed the genes showing a signal intensity  $>2000$  in at least one experiment. Table 1 lists the genes whose expression was increased more than five times in the 35S:*DREB2A CA*-a plants (Maruyama et al., 2004). We regarded genes with an expression ratio  $>5$  in the 35S:*DREB2A CA*-a plants as candidates for *DREB2A* down-

stream genes. Seventeen genes were identified as candidates by cDNA microarray analysis. Expression ratios of most identified genes were correlated with levels of mRNA accumulation of *DREB2A CA* among the three transgenic lines (see Supplemental Table 1 online; European Bioinformatics Institute ArrayExpress database; accession number E-MEXP-317).

**Table 1.** Microarray Analysis of 35S:DREB2A CA Plants

Gene Name	AGI Locus Identifier	Ratio <sup>a</sup>	DRE <sup>b</sup>	ABRE <sup>b</sup>	Description <sup>c</sup>
Genes whose expression in 35S:DREB2A CA-a plants was increased more than five times compared with expression in wild-type plants					
<i>RD29A/COR78/LTI140</i> <sup>d,e</sup>	At5g52310	15.5	-131 to -126, -168 to -163, -225 to -220, -275 to -270	-65 to -60	Hydrophilic protein
<i>AtGalS3</i> <sup>d</sup>	At1g09350	12.8	-391 to -396, -787 to -792, -815 to -820	-275 to 270	Galactinol synthase
<i>RD29B/LTI65</i> <sup>e</sup>	At5g52300	12.4	-172 to -167	-45 to -40, -73 to -68, -92 to -97, -178 to -173, -719 to -724	Hydrophilic protein
<i>At1g52690</i> <sup>e</sup>	At1g52690	11.9	-47 to -42	-200 to -195, -238 to -233, -235 to -240, -635 to -630, -632 to -637	Late embryogenesis-abundant protein, putative
<i>At4g33720</i>	At4g33720	10.2		-26 to -21, -210 to -215	SCP-like extracellular protein
<i>COR15A</i> <sup>d,e</sup>	At2g42540	10.5	-183 to -178, -360 to -355, -417 to -422	-70 to -65, -131 to -126, -128 to -133, -304 to -309	Late embryogenesis-abundant protein, putative
<i>MT2A/AtMT-1/AtMT-K/MT2</i> <sup>e</sup>	At3g09390	9.9	-182 to -177		Metallothionein protein, putative
<i>RD17/COR47</i> <sup>d,e</sup>	At1g20440	9.8	-161 to -156, -966 to -961, -995 to -990	-345 to -350, -919 to 914	Dehydrin
<i>At1g32860</i>	At1g32860	9.4	-71 to -66, -615 to -610	-587 to -592	Glycoside hydrolase family 17 protein
<i>At1g69870</i> <sup>e</sup>	At1g69870	8.9	-342 to 337	-102 to -97, -200 to -195	Proton-dependent oligopeptide transport family protein
<i>PDC2</i>	At5g54960	7.0	-303 to -308, -372 to -377	-944 to -939	Pyruvate decarboxylase
<i>LEA14/LSR3</i> <sup>d,e</sup>	At1g01470	6.0	-66 to -61, -384 to -389, -414 to -409	-103 to -108, -147 to -152, -646 to -651	Late embryogenesis-abundant protein, putative
<i>At2g23120</i> <sup>d,e</sup>	At2g23120	6.0	-127 to -122	-75 to -70, -100 to -105	Unknown protein
<i>At5g54170</i>	At5g54170	5.6			Unknown protein
<i>At5g62350</i> <sup>d</sup>	At5g62350	5.5	-40 to -35, -192 to -187, -560 to -555		Plant invertase/pectin methylesterase inhibitor
<i>At3g53990</i> <sup>e</sup>	At3g53990	5.3	-196 to -201, -983 to -988		Universal stress protein family
<i>ATGRP7</i> <sup>d</sup>	At2g21660	5.2	-87 to -82	-307 to 312	RNA recognition motif-containing RNA binding protein
Genes whose expression by RNA gel blot analysis in DREB2A CA-a plants was increased less than five times compared with that in wild-type plants					
<i>KIN1</i> <sup>d,e</sup>	At5g15960	3.8	-119 to -114	-77 to -72, -147 to -152, -361 to -366	Late embryogenesis-abundant protein
<i>KIN2/COR6.6</i> <sup>d,e</sup>	At5g15970	3.7	-131 to -126	-72 to -67, -69 to -74, -89 to -85, -86 to -91, -159 to -164, -380 to -385	Late embryogenesis-abundant protein
<i>At1g22985</i> <sup>e</sup>	At1g22985	3.4	-42 to -47, -123 to -118		AP2 domain-containing DNA binding protein, putative
<i>COR15B</i> <sup>d,e</sup>	At2g42530	2.8	-194 to -189, -748 to -753	-70 to -65, -150 to -155	Late embryogenesis-abundant protein
<i>COR414-TM1</i> <sup>d</sup>	At1g29395	1.9	-327 to -332	-39 to -44, -45 to -50, -86 to -81, -187 to -182	Thylakoid membrane protein
<i>At2g02100</i> <sup>d</sup>	At2g02100	1.3	-181 to -186		Plant defensin-fusion protein, putative (PDF2.2)

<sup>a</sup> Median ratio of three repeats. Ratio was defined as follows: fluorescence intensity of each cDNA of 35S:DREB2A CA-a/fluorescence intensity of each cDNA of vector control line.

<sup>b</sup> DRE sequences (A/GCCGAC) or ABRE sequences (ACGTGG/T) observed in 1000 nucleotides existing upstream of the 5'-termini of the longest cDNA clones isolated are listed. Numbers indicate the numbers of nucleotides beginning at the 5'-termini of the longest cDNA clones isolated. A minus sign means that the nucleotides exist upstream of the 5'-terminus of the putative transcription site.

<sup>c</sup> Descriptions from Munich Information Center for Protein Sequences database.

<sup>d</sup> DREB1A target genes (Maruyama et al., 2004).

<sup>e</sup> Direct downstream genes of DREB2A.

In our previous study, the DREB2A protein specifically bound to six nucleotides (A/GCCGAC) of the DRE core motif (Sakuma et al., 2002). We searched for this core motif in the promoter regions of the 17 identified genes. The 1000-bp promoter regions upstream of putative transcriptional initiation sites that are the 5'-ends of the full-length cDNA clones were used for this search (Seki et al., 2002). We found the DRE motif(s) in the promoter region of 15 genes. Because many stress-inducible genes that have the DRE motif(s) contain the abscisic acid-responsive element (ABRE) core motif (ACGTGG/T) in their promoter regions, we also searched for ABRE in the promoter regions of the 17 genes. Thirteen contained the ABRE motif(s), and 12 contained both DRE and ABRE motifs in their 1000-bp promoter regions. As the DREB1A protein was also reported to bind specifically to the DRE core motif (Sakuma et al., 2002), we compared these 17 genes with the genes downstream of DREB1A identified by Maruyama et al. (2004). We found only eight genes in common.

For further analysis of the genes that are upregulated by overexpression of DREB2A CA, we performed RNA gel blot analysis. The total RNAs isolated from the wild-type, 35S:DREB1A-b, 35S:DREB2A CA-a, 35S:DREB2A CA-b, and 35S:DREB2A CA-c plants with or without stress treatments of dehydration for 5 h and 4°C for 5 h were used for RNA gel blot analysis (Figure 4). The 35S:DREB1A-b plants corresponded to 35S:DREB1Ab described by Kasuga et al. (1999), and this line showed moderate phenotypic change among the DREB1A overexpressors. Figure 4A shows expression of the DREB1A and DREB2A transgenes in the transgenic plants. DREB1A was overexpressed in the 35S:DREB1A-b transgenic plants, and the active form of DREB2A was overproduced in the 35S:DREB2A CA transgenic plants. We subjected genes whose expression ratios were >5 and the genes *KIN1*, *KIN2*, *At1g22985*, *COR15B*, *COR414-TM1*, and *At2g02100* to RNA gel blot analysis. These latter genes, except for *At1g22985*, have DRE in their promoter regions and showed expression of >5 in transgenic plants overexpressing DREB1A (Maruyama et al., 2004), whereas the expression ratios in the 35S:DREB2A CA-a plants were <5 (Table 1). Although the expression ratio of *At1g22985* in the 35S:DREB2A CA-a plants was 3.4, we confirmed the expression of this gene using RNA gel blot analysis, as it encodes an interesting protein, a transcription factor that contains the ERF/AP2 DNA binding domain, suggesting the existence of further regulation of gene expression downstream of DREB2A. RNA gel blot analysis showed that accumulation of mRNAs of 11 genes under unstressed control conditions was increased in the 35S:DREB2A CA plants compared with that in the wild-type plants but not in the 35S:DREB1A-b plants (Figure 4B, lanes 1, 5, 8, 11, and 14). Most of these genes responded to drought and/or cold stress in the wild-type plants; in particular, accumulation of *RD29B*, *At1g52690*, and *At1g69870* transcripts increased in response to drought stress treatment (Figure 4B, lanes 1 to 3). Figure 4C shows genes that were induced equivalently in the 35S:DREB1A-b plants and the 35S:DREB2A CA plants. Although *At2g02100* and *COR414-TM1* were overexpressed in the 35S:DREB1A-b plants and have DREs in their promoter regions, their expression was not increased in the 35S:DREB2A CA plants (Figure 4D, lanes 5, 8, 11, and 14). Expression of *COR15A*, *KIN1*, *KIN2*, and *COR15B* was increased in both the 35S:DREB1A and the 35S:DREB2A CA plants, but their expres-

sion levels were much higher in the 35S:DREB1A-b plants than in the 35S:DREB2A CA plants (Figure 4D, lanes 5, 8, 11, and 14).

### Promoter Analysis of DREB-Upregulated Genes

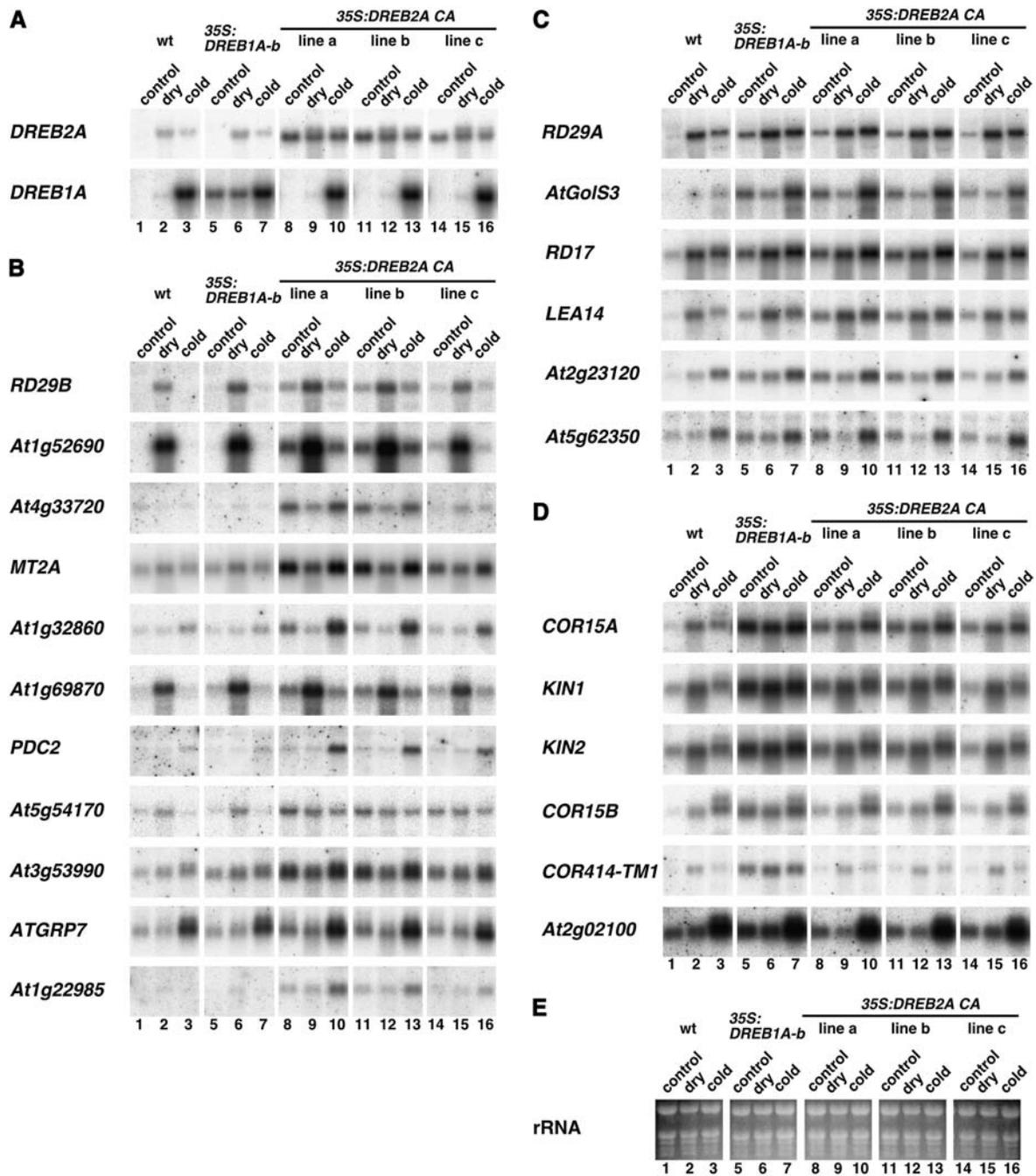
To understand why several genes downstream of DREB2A are different from those downstream of DREB1A, we compared the DRE sequences in the promoter regions of the DREB1A- and DREB2A-upregulated genes. The genes listed in Figures 4B and 4D were selected as DREB2A- and DREB1A-upregulated genes, respectively. We searched for the DRE core motif (A/GCCGAC) in the promoter regions of the genes, using the 500-bp promoter regions upstream of putative transcriptional initiation sites at the 5'-end of the full-length cDNA clones for this search (Seki et al., 2002). We found 8 and 11 DRE sequences in the promoter regions of the DREB1A-upregulated and DREB2A-upregulated genes, respectively, and analyzed them with 20 neighboring nucleotides (10 on each side) using the sequence logo programs (Schneider and Stephens, 1990; Figures 5A and 5B).

Recently, we reported that the DREB1A protein has the highest affinity to the A/GCCGACNT sequence, which we determined by detailed analysis of the promoter region of the DREB1A downstream genes (Maruyama et al., 2004). We found A/GCCGACNT in the promoter regions of 75% of the DREB1A-upregulated genes. However, only four DRE sequences (36%) were A/GCCGACNT in the promoter regions of the DREB2A-upregulated genes. Although the last T of A/GCCGACNT is critical for the binding affinity of the DREB1A protein to DRE, the binding affinity of the DREB2A protein may not be affected by a base substitution at the corresponding position of DRE (Figure 5A; Maruyama et al., 2004). To confirm this hypothesis, we performed a gel mobility shift assay with DNA fragments containing the ACCGACAT or ACCGACAA/C/G sequences as competitors (Figures 5C and 5D). Addition of the unlabeled DNA fragment containing ACCGACAT reduced the extent of binding of DREB1A more effectively than that of the unlabeled DNA fragment containing ACCGACAA/C/G (Figure 5D; Maruyama et al., 2004). On the other hand, we could not detect a difference in the binding affinity of DREB2A between the DNA fragments containing ACCGACAT and those containing ACCGACAA/C/G.

Among the 11 DRE sequences found in the promoter sequences of the DREB2A-upregulated genes, 1 contained GCCGAC and 10 contained ACCGAC as the DRE core motifs (Figure 5B). This low frequency of G at this nucleotide position (position 11 in Figure 5B) suggests low binding affinity of the DREB2A protein to the GCCGAC sequence. To analyze binding characteristics of DREB2A to ACCGAC and GCCGAC, we performed a gel mobility shift assay using 75-bp DNA fragments containing A/C/G/TCCGAC (Figures 5C and 5E). The addition of unlabeled ACCGACAT or GCCGACAT competitors inhibited the binding of the DREB1A protein equivalently. By contrast, the addition of an unlabeled ACCGACAT fragment was more effective at binding the DREB2A protein than the addition of an unlabeled DNA fragment containing C/G/TCCGACAT.

### Drought and Freezing Stress Tolerance of 35S:DREB2A CA Transgenic Arabidopsis Plants

We compared the drought and freezing stress tolerance of the 35S:DREB2A CA plants with that of the 35S:DREB1A-b and



**Figure 4.** Expression of the *DREB1A*- and *DREB2A*-Upregulated Genes in the Plants Carrying pBI121, *35S:DREB2A CA*, or *35S:DREB1A* Constructs.

RNAs were prepared from transgenic *Arabidopsis* plants that had been dehydrated for 5 h (dry), treated at 4°C for 5 h (cold), or untreated (control). The full-length cDNA was used as probe for each gene except for *DREB2A*, *RD29A*, and *RD29B*; the 3'-terminal-specific DNA fragments were used as probes for these three genes.

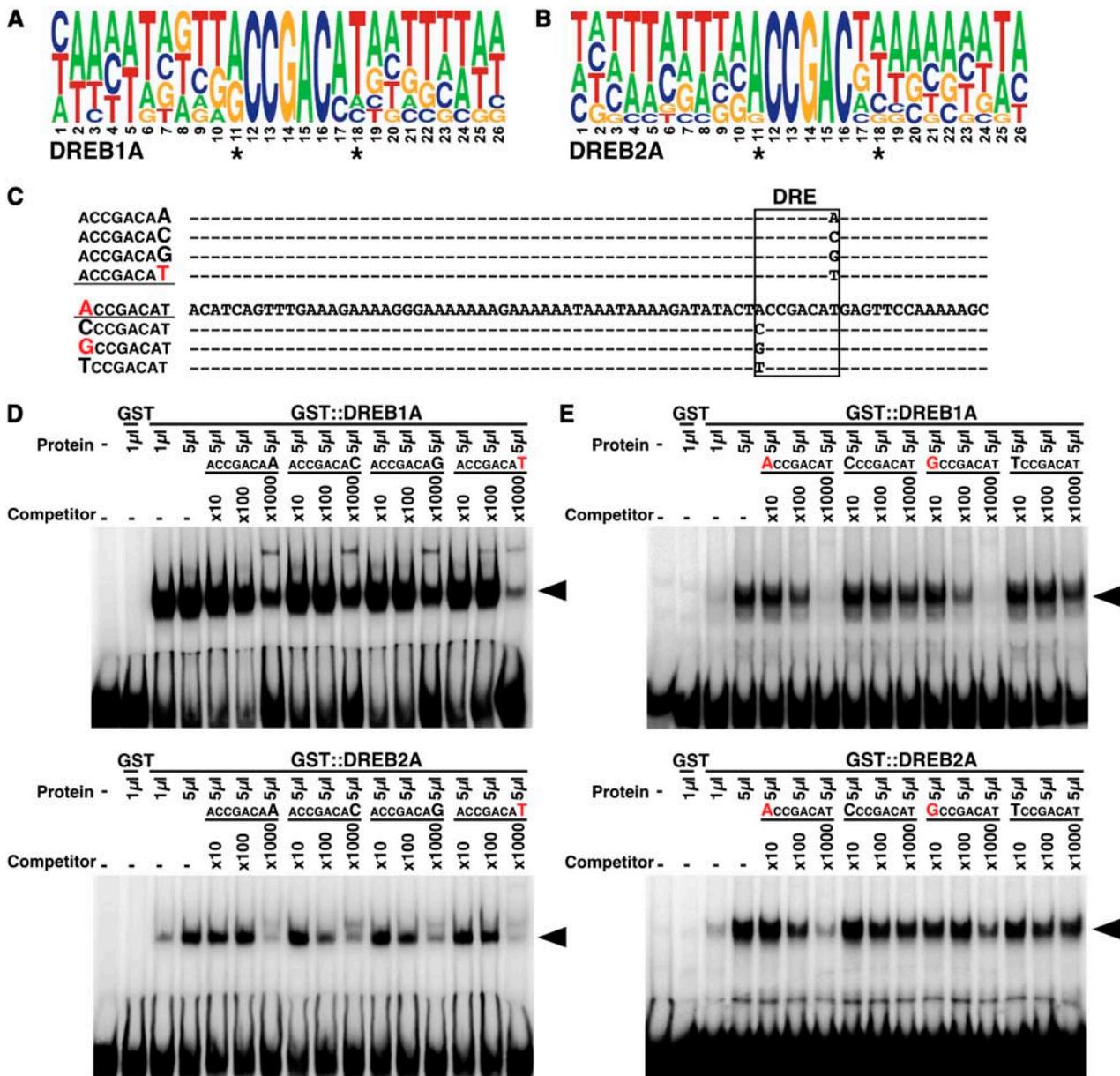
(A) Expression of the transgenes.

(B) Genes that showed higher expression in *35S:DREB2A* plants.

(C) Genes that showed equivalent expression in *35S:DREB1A* plants and *35S:DREB2A CA* plants.

(D) Genes that showed higher expression in *35S:DREB1A* plants.

(E) Ethidium bromide-stained rRNA image is shown as a loading control.



**Figure 5.** Promoter Analysis of the DREB1A- and DREB2A-Upregulated Genes.

(A) and (B) Sequence logo for the DRE core sequence (A/GCCGAC) with 20 adjacent nucleotides found in the promoter regions of DREB1A-upregulated genes (*At2g02100*, *COR414-TM1*, *COR15A*, *COR15B*, *KIN1*, and *KIN2*) and DREB2A-upregulated genes (*RD29B*, *At1g52690*, *MT2A*, *At1g32860*, *At1g69870*, *PDC2*, *At3g53990*, *ATGRP7*, and *At1g22985*). The asterisks indicate nucleotide positions analyzed by gel mobility shift assay.

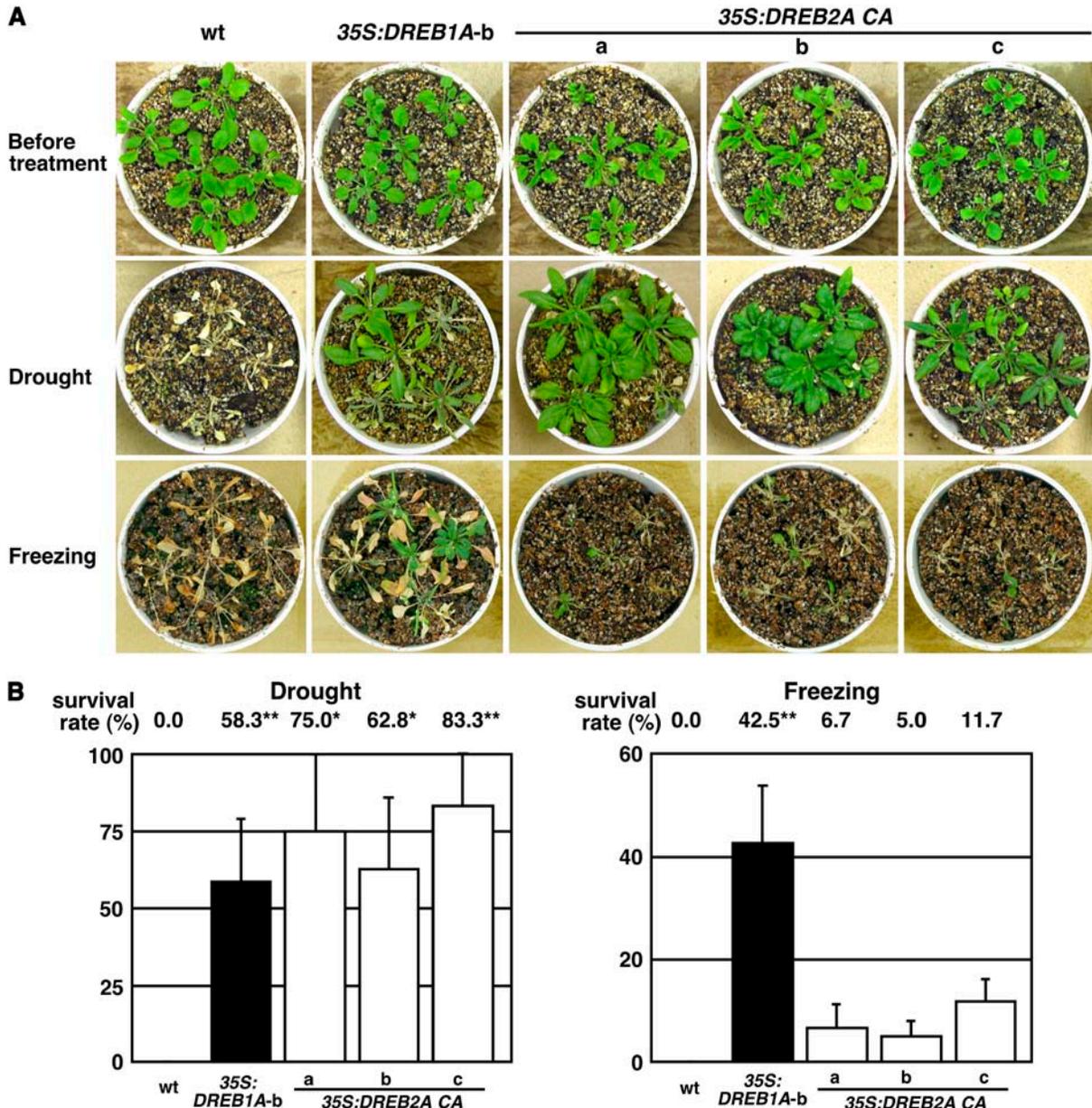
(C) Probe and competitor sequences used in the gel mobility shift assay. Sequences of the 75-bp fragment of the wild-type *RD29A* promoter (ACCGACAT, underlined) and the mutants that were used as competitors. The red letters indicate nucleotides mentioned in the text. The wild-type fragment was used also as a probe.

(D) and (E) Competitive DNA binding assay of recombinant DREB1A and DREB2A proteins using A/C/G/TCCGACAT or ACCGACAA/C/G/T as competitors. Glutathione S-transferase (GST) or recombinant protein solutions were preincubated with or without unlabeled competitors for 5 min at 25°C. <sup>32</sup>P-labeled probe was then added, and the mixture was incubated for 30 min at 25°C. As competitors, 10×, 100×, or 1000× excess amounts of the unlabeled fragments were used. The retarded bands are indicated by arrowheads.

wild-type plants (Figure 6). The plants were grown on germination medium agar plates for 3 weeks, then transferred to soil and grown for 1 week at 22°C. For drought stress treatment, water was withheld for 2 weeks. They were then watered and grown under control conditions for 3 d. This treatment killed all wild-type plants, but ~60% of the *35S:DREB1A-b* plants survived. Like

*35S:DREB1A-b*, the *35S:DREB2A CA* plants exhibited tolerance to drought, with survival rates of 62.8 to 83.3%.

For the freezing stress treatment, the plants were exposed to a temperature of -6°C for 30 h and returned to 22°C for 5 d. All the wild-type plants died in this treatment, whereas ~40% of the *35S:DREB1A-b* plants survived. By contrast, only 5.0 to 11.7%

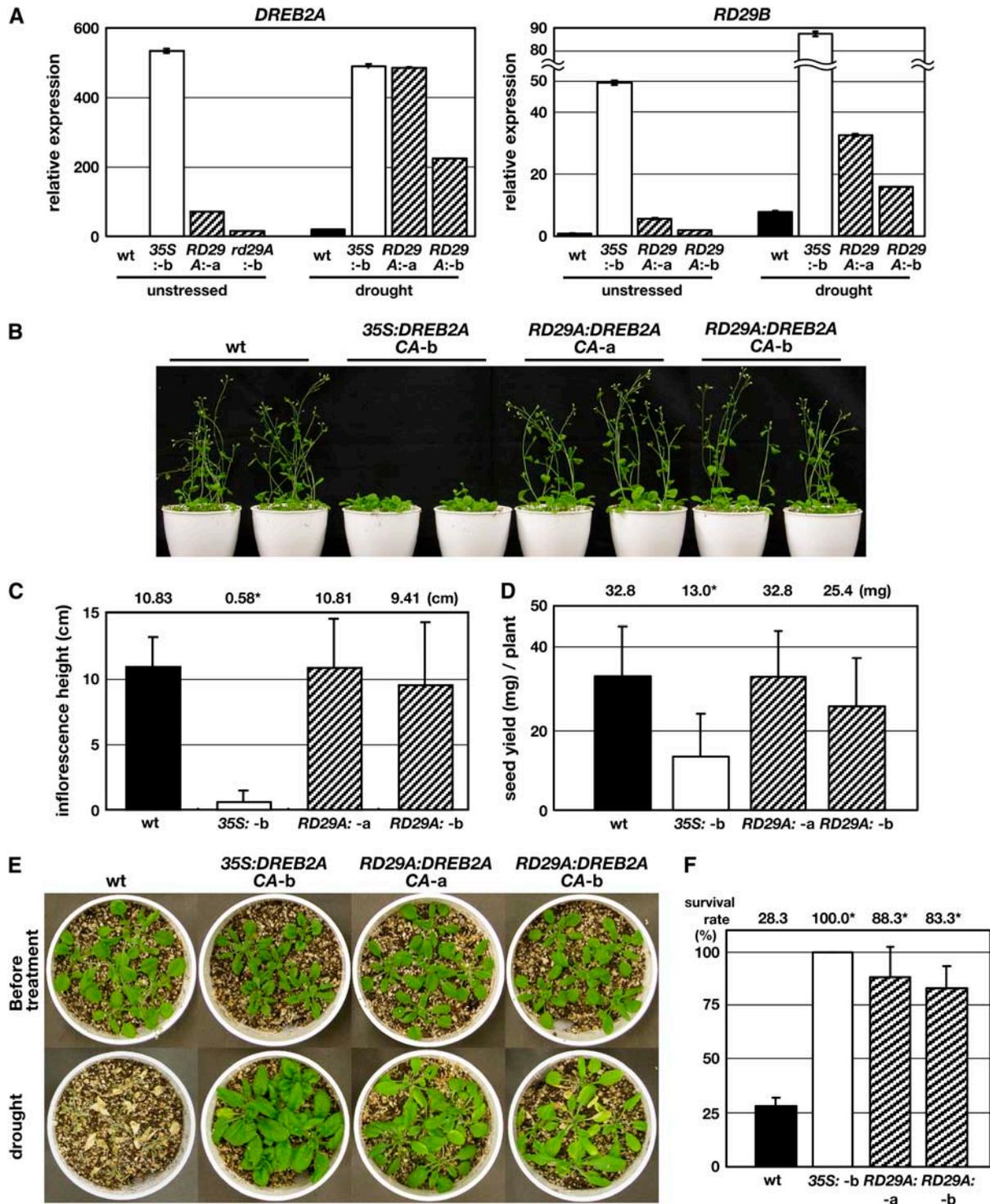


**Figure 6.** Drought and Freezing Tolerance of the *35S:DREB2A CA* and *35S:DREB1A* Plants.

The stress treatments were conducted as described in the text. Drought: water withheld from plants for 2 weeks; freezing: 4-week-old plants exposed to -6°C for 30 h and returned to 22°C for 5 d.

(A) Photographs of plants before and after stress treatments.

(B) Survival rates of plants exposed to drought and freezing stress. Average survival rates and standard errors were calculated using results of three replicated experiments. Twenty plants (five plants/pot) were tested in each experiment. In all experiments, the plants with asterisks had significantly higher survival rates than wild-type plants ( $\chi^2$  test, \*P < 0.05, \*\*P < 0.01).



**Figure 7.** Phenotypes and Drought Stress Tolerance of the *RD29A:DREB2A* CA Transgenic Plants.

**(A)** Gene expression profiles of *DREB2A* and *RD29B* in the wild-type plants, *35S:DREB2A* CA-*b* plants (*35S:-b*), and *RD29A:DREB2A* CA plants (*RD29A:-a* and *RD29A:-b*). RNAs were isolated from unstressed control plants or plants that were treated for 30 min under drought condition. Accumulation of the *DREB2A* and *RD29B* mRNAs was measured by quantitative RT-PCR. Data represent means and standard errors of three replications.

**(B)** Photographs of vector control plants and transgenic plants overexpressing *DREB2A* CA. The plants were grown on germination medium agar plates for 3 weeks, then transferred to soil and grown for 2 weeks.

of the *35S:DREB2A CA* plants survived (Figures 6A and 6B). These results indicate that the genes downstream of DREB2A play an important role in drought stress tolerance but are not sufficient to withstand freezing stress.

#### Use of Stress-Inducible Promoters to Overexpress DREB2A CA Minimized the Negative Effects on Plant Growth

We have reported that overexpression of DREB1A driven by the stress-inducible *RD29A* promoter is able to improve plant stress tolerance significantly with minimum growth retardation (Kasuga et al., 1999, 2004). Because overexpression of DREB2A CA with the *CaMV 35S* promoter showed a negative effect on plant growth, we tried to use this technique to overexpress DREB2A CA. We generated 20 transgenic plants overexpressing DREB2A CA cDNA driven by the *RD29A* promoter (*RD29A:DREB2A CA*). Accumulation of DREB2A mRNA and of mRNAs of its downstream gene *RD29B* in these transgenic plants was analyzed by quantitative RT-PCR. We selected lines *RD29A:DREB2A CA-a* and *RD29A:DREB2A CA-b* for further analysis.

We compared these two lines with wild-type and *35S:DREB2A CA-b* plants. Expression of both DREB2A and *RD29B* was enhanced in the *35S:DREB2A CA-b* plants even under unstressed condition. By contrast, expression levels of these genes in the *RD29A:DREB2A CA-a* and *RD29A:DREB2A CA-b* plants under unstressed conditions were relatively low compared with those in *35S:DREB2A CA-b*. After 30 min of dehydration, expression of DREB2A and *RD29B* in the *RD29A:DREB2A CA-a* plants was increased by 24 and 4 times, respectively, compared with that in the wild-type plants (Figure 7A).

Growth of the *RD29A:DREB2A CA* plants was compared with that of the wild-type and *35S:DREB2A CA-b* plants. Figure 7B shows 5-week-old plants of the wild type, *35S:DREB2A CA-b*, *RD29A:DREB2A CA-a*, and *RD29A:DREB2A CA-b*. Unlike the *35S:DREB2A CA-b* plants, there were no significant differences in growth between the vector control and *RD29A:DREB2A CA* plants. We then analyzed seed yields of these plants (Figure 7C). The seed yield of *35S:DREB2A CA-b* was reduced to 40% of the wild type. By contrast, the seed yield of the *RD29A:DREB2A CA-a* plants was similar to that of wild-type plants grown under control conditions, and that of *RD29A:DREB2A CA-b* plants was 77% of the wild type. The tolerance of the *RD29A:DREB2A CA* plants to drought was compared with that of the *35S:DREB2A CA-b* and wild-type plants (Figures 7D and 7E). Plants grown in pots were not watered for 2 weeks. Only 21.3% of the wild-type

plants survived. However, the survival rates of *RD29A:DREB2A CA-a* and *-b* plants were much higher, at 88.3 and 83.3%, respectively (Figures 7E and 7F).

#### Subcellular Accumulation Patterns of DREB2A FL and DREB2A CA Fused to Synthetic Green Fluorescent Protein

To study subcellular accumulation patterns of the DREB2A FL protein and the DREB2A CA protein, we constructed chimeric genes with the synthetic green fluorescent protein (*sGFP*) gene (Niwa et al., 1999) fused to DREB2A FL cDNA (*35S:GFP-DREB2A FL*) or DREB2A CA cDNA (*35S:GFP-DREB2A CA*) (Figure 8A). The fusion genes were introduced into *Arabidopsis* plants and were overexpressed under the control of the *CaMV 35S* promoter. As a control, the *sGFP* gene was also overexpressed in transgenic *Arabidopsis* plants. Two lines of transgenic plants were analyzed for each *sGFP-DREB2A* construct. The accumulation of mRNA of each fusion gene was almost the same among the four lines of transgenic plants (Figure 8B). However, expression of the *RD29A* gene in the *35S:GFP-DREB2A FL* plants was remarkably lower than that in the *35S:GFP-DREB2A CA* plants (Figure 8C). Moreover, in the *35S:GFP-DREB2A FL* plants, only very weak green fluorescent signals were observed in the nuclei (Figures 8D and 8E). By contrast, strong green fluorescent signals were clearly observed in the nuclei of the *35S:GFP-DREB2A CA* plants (Figures 8D and 8E). These results suggest that the DREB2A CA protein is more stable than the DREB2A FL protein in the nucleus of the transgenic plant.

#### DISCUSSION

DREB2A and DREB1A were simultaneously isolated as transcription factors that recognize a *cis*-acting element, DRE/CRT, through the use of a yeast one-hybrid screening method (Liu et al., 1998). Although the function of DREB1A in cold stress-responsive gene expression has been analyzed extensively, that of DREB2A has not been elucidated yet (Shinozaki and Yamaguchi-Shinozaki, 2003). DREB2A seems to function mainly in ABA-independent water stress-inducible gene expression, since expression of DREB2A is strongly induced by drought and high-salinity stresses but not by ABA treatment (Liu et al., 1998). However, we could not detect any phenotypic changes in a T-DNA insertion mutant of DREB2A under water stress conditions (data not shown). The DREB2A family consists of eight members, and at least DREB2B is strongly induced by drought

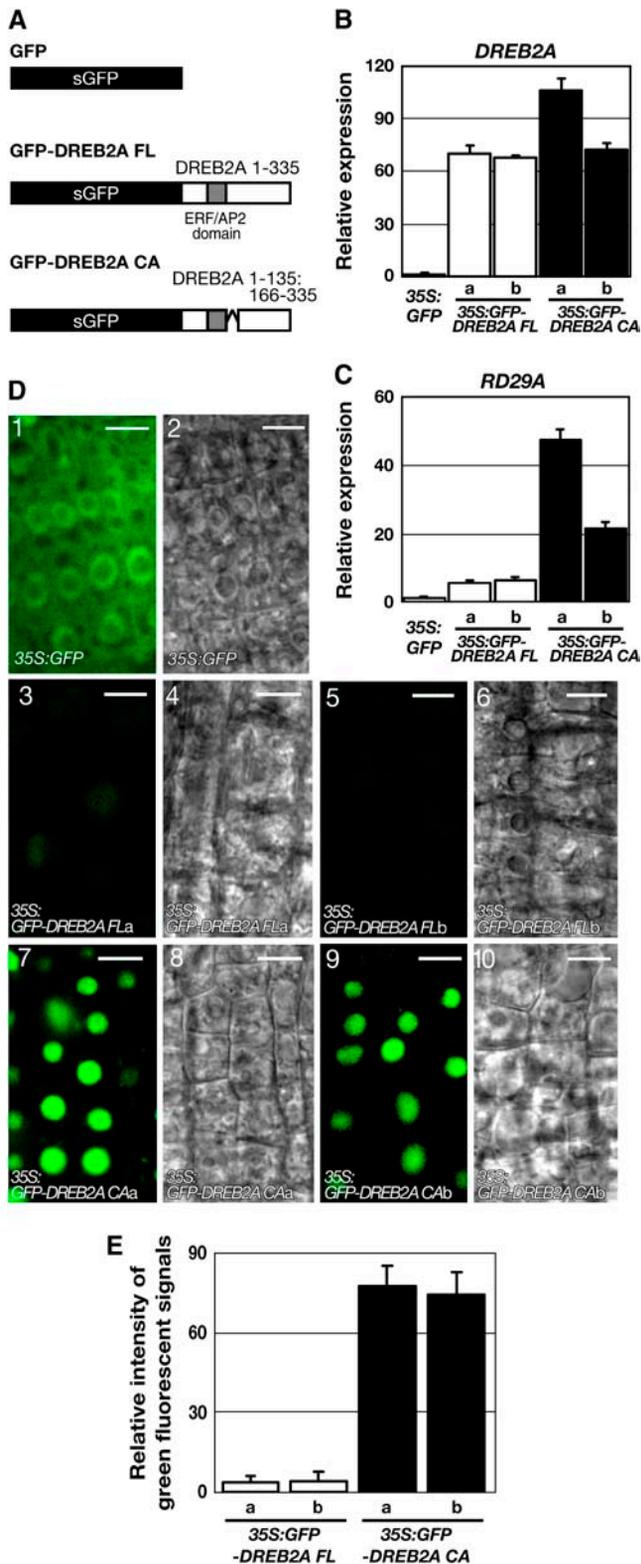
#### Figure 7. (continued).

(C) Inflorescence heights of 5-week-old transgenic plants. Average inflorescence heights and standard deviations were calculated using 12 plants. Asterisk indicates that these plants had significantly shorter inflorescences than wild-type plants (Student's *t* test,  $P < 0.001$ ).

(D) Seeds were harvested from 3-month-old vector control plants, *35S:DREB2A CA-b* plants, or *RD29A:DREB2A CA* plants, and air-dry seed was weighed. The average yield of each line was calculated from yields of 20 plants. Error bars show standard deviation. Asterisk indicates that these plants had significantly lower seed yields than wild-type plants (Student's *t* test,  $P < 0.001$ ).

(E) Photographs of plants before and after stress treatment. Stress treatment was performed as described in Figure 6.

(F) Survival rates of plants exposed to drought stress. Average survival rates and standard errors were calculated using results of three replicated experiments. Twenty plants (five plants/pot) were tested in each experiment. Asterisk indicates that in all experiments, these plants had significantly higher survival rates than wild-type plants ( $\chi^2$  test,  $P < 0.001$ ).



**Figure 8.** Subcellular Accumulation Patterns of DREB2A FL and DREB2A CA Fused to sGFP.

(A) Structure of the chimeric genes with sGFP fused to DREB2A FL (35S:GFP-DREB2A FL) or DREB2A CA (35S:GFP-DREB2A CA).

and high-salinity stresses like DREB2A (Sakuma et al., 2002). Thus, the function of the DREB2A family genes may be redundant under water stress conditions, and a loss-of-function approach may not be effective. In this study, we have made important progress in the understanding of the function of DREB2A in water stress-responsive gene expression using a gain-of-function approach with DREB2A CA.

We showed that an acidic region (amino acids 254 to 335) in the C terminus of DREB2A plays an important role as a transcriptional activation domain of the DREB2A protein in transactivation experiments using protoplasts (Figure 2). An acidic region in the C terminus of DREB1A was also reported to function as an activation domain (Liu et al., 1998). Although both DREB1A and DREB2A contain an acidic activation domain, overexpression of these proteins showed different phenotypic changes in transgenic plants (Liu et al., 1998). Overexpression of DREB1A not only induced strong expression of the downstream genes under unstressed conditions but also caused dwarfed phenotypes in the plants. These transgenic plants were tolerant to freezing and drought. By contrast, overexpression of DREB2A FL cDNA did not induce expression of the downstream genes under unstressed conditions and showed few phenotypic changes in transgenic plants (Figure 3; Liu et al., 1998). Therefore, the translated DREB2A protein seems to be an inactive form under unstressed conditions, and modification under stress seems to be necessary for its activation. We showed that the deletion of the 136 to 165 amino acid region significantly increased DREB2A activity in *Arabidopsis* protoplasts (Figure 1), and the resultant protein is active in transgenic plants even under unstressed control conditions (Figure 3). Therefore, this region may function negatively in the activation of the DREB2A protein under unstressed control conditions.

Using microarray and RNA gel blot analyses of the 35S: DREB2A CA plants, we identified 21 genes that were upregulated by overexpression of DREB2A CA (Table 1, Figure 4). However, it may be difficult to determine the downstream genes of DREB2A accurately using only array and RNA gel blot analyses, as we used the strong constitutive *CaMV* 35S promoter to overexpress DREB2A CA. Therefore, we analyzed expression of these 21 upregulated genes under stress conditions and searched for the DRE core motif in their promoter region. Among these 21

(B) and (C) Expression of DREB2A and RD29A in the transgenic *Arabidopsis* plants carrying 35S:GFP, 35S:GFP-DREB2A FL, or 35S:GFP-DREB2A CA. Data represent means and standard errors of three replications.

(D) Confocal microscope images of sGFP fluorescence (1, 3, 5, 7, and 9) and Nomarski microscope images (2, 4, 6, 8, and 10) of young roots from transgenic *Arabidopsis* plants carrying 35S:GFP (1 and 2), 35S:GFP-DREB2A FL (3 to 6), or 35S:GFP-DREB2A CA (7 to 10). Bars = 10  $\mu$ m.

(E) Relative intensities of green fluorescent signals in the nuclei of transgenic *Arabidopsis* plants carrying 35S:GFP-DREB2A FL or 35S:GFP-DREB2A CA. Average signal intensity was calculated from signals of >40 nuclei observed in three fields of view per line. Error bars represent standard deviation. The 35S:GFP-DREB2A CA plants gave significantly stronger green fluorescent signals than 35S:DREB2A FL plants (Student's *t* test,  $P < 0.001$ ).

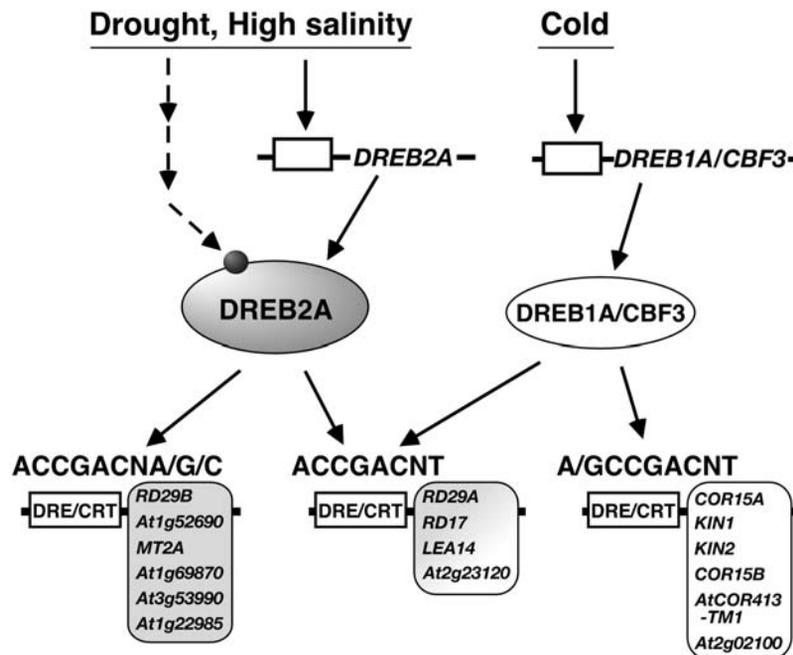
upregulated genes in the *35S:DREB2A CA* plants, 12 genes (*RD29B*, *At1g52690*, *At1g69870*, and *At3g53990* [Figure 4B]; *RD29A*, *RD17*, *LEA14*, and *At2g23120* [Figure 4C]; and *COR15A*, *KIN1*, *KIN2*, and *COR15B* [Figure 4D]) were upregulated under drought stress, and their promoter regions carry the DRE core motif(s) (Table 1). The other two genes, *MT2A* and *At1g22985* (Figure 4B), were upregulated weakly under drought stress, and we found the DRE core motif(s) in their promoter regions (Table 1).

These results suggest that these 14 genes are candidates for direct targets of DREB2A. Nine of these 14 genes encode LEA class proteins, which are thought to protect macromolecules, such as enzymes and lipids, from dehydration (Table 1; Shinozaki and Yamaguchi-Shinozaki, 1999). Overproduction of these proteins probably improves drought stress tolerance in the transgenic plants.

Both DREB1A and DREB2A have been reported to recognize a core sequence of DRE, G/ACCGAC (Liu et al., 1998; Sakuma et al., 2002). However, we revealed that some DREB2A downstream genes are not DREB1A downstream genes, and some DREB1A downstream genes are not DREB2A downstream genes (Figures 4B and 4D). Moreover, although some genes downstream of DREB1A, such as *COR15A*, *COR15B*, *KIN1*, and *KIN2*, are recognized by DREB2A also, their expression levels in the *35S:DREB2A CA* plants were significantly lower than those in the *35S:DREB1A* plants (Figure 4D). These differences in species or expression level of the downstream genes between the two DREB proteins may be a reason for the lower freezing tolerance of the *35S:DREB2A CA* plants than that of the *35S:DREB1A* plants. *COR15A*, *COR15B*, *KIN1*, *KIN2*, and two genes that are

overexpressed only in the *35S:DREB1A-b* plants, *At2g02100* and *COR414-TM1*, may play important roles in improving freezing stress tolerance of DREB1A overexpressors. Recently, we have elucidated that the DREB1A protein has the highest affinity to the A/GCCGACNT sequence by detailed analysis of the promoter region of the DREB1A downstream genes (Maruyama et al., 2004). To understand why some of the genes downstream of DREB1A and DREB2A differ, we performed promoter analysis of the DREB1A- and DREB2A-upregulated genes and gel mobility shift assay with DNA fragments containing the DRE core sequence. We found that the DREB2A protein could recognize not only A/GCCGACNT, but also A/GCCGACNA/G/C, and prefers ACCGAC to GCCGAC. These different binding specificities between DREB1A and DREB2A may explain why these proteins control some different downstream genes.

Chimeric genes with *sGFP* fused to *DREB2A FL* or *DREB2A CA* were expressed in the transgenic *Arabidopsis* plants. Although only weak green fluorescence was observed in the nucleus of the *35S:GFP-DREB2A FL* plants, strong signals were observed in the nucleus of the *35S:GFP-DREB2A CA* plants (Figure 8). Both full-length and active-form DREB2A proteins contain nuclear localization signals in their N-terminal regions (Figure 2C, NLS); the DREB2A FL protein is probably also targeted at the nucleus. We suspected that the DREB2A FL protein is unstable in the transgenic plants compared with its constitutive active form. DREB2A CA does not contain the negative regulatory domain between amino acids 136 and 165 of DREB2A. Using the PESTfind program (<https://emb1.bcc.univie.ac.at/content/section/6/45/>), we found a PEST sequence (RSDASEVTSTSSQSEVCTVETPGCV) in this region. The PEST



**Figure 9.** Model of the Induction of Genes Regulated by DREB1A and DREB2A under Drought, High-Salinity, and Cold Stress Conditions.

The genes downstream of the DREB proteins are categorized into three groups. The middle group contains downstream genes shared by DREB1A and DREB2A. The other groups consist of DREB1A- and DREB2A-specific downstream genes.

sequence exists in rapid-turnover proteins and regulates the activity of proteins that require a rapid change of activity by controlling their accumulation (Rechsteiner and Rogers, 1996). The DREB2A FL protein containing the PEST sequence may be degraded rapidly by the ubiquitin-proteasome system, whereas DREB2A CA may have a long lifetime in the nucleus.

Figure 9 shows a model of the induction of genes regulated by DREB1A and DREB2A under drought, high-salinity, and cold stress conditions. The *DREB1A* gene is induced by cold stress, and *DREB2A* is induced by drought and high-salinity stresses. Modification of the DREB2A protein may be necessary for its activation under drought and high-salinity stress conditions. The genes downstream of the DREB proteins are categorized into three groups. The first group consists of downstream genes shared by DREB1A and DREB2A; most of these have ACCGACNT in their promoter regions. The second group consists of DREB1A-specific downstream genes; these genes have A/GCCGACNT in their promoters. The third group consists of DREB2A-specific downstream genes; we found ACCGACNA/G/C frequently in their promoter regions. These different downstream genes between the DREB1A and DREB2A proteins result in different stress tolerance to cold and drought in plants.

In conclusion, the DREB2A protein has a negative regulatory domain in its central region, and the deletion of this domain transforms DREB2A into the constitutive active form. Overexpression of DREB2A CA activated the expression of many stress-inducible genes and improved tolerance to drought in transgenic *Arabidopsis*. We found that both DREB2A and DREB1A can bind to the DRE sequence, but the DNA binding specificities of each to the neighboring sequences of the DRE core motif were slightly different; therefore, the downstream genes of each are partially different. Green fluorescent signals of the sGFP fused to DREB2A CA were remarkably higher than those of the sGFP fused to the DREB2A FL in the nucleus of the transgenic plants under unstressed control conditions. Thus, these results suggest that stability of the DREB2A protein is important for its activation, and the activated DREB2A regulates drought stress-responsive gene expression, which enhances drought stress tolerance in plants.

## METHODS

### Plant Materials

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 3 weeks, as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). Stress treatments for RNA gel blot analysis and stress tolerance tests were performed as described previously (Liu et al., 1998). *Arabidopsis* T87 suspension-cultured cells were maintained as described previously (Takahashi et al., 2001).

### Transient Expression Experiments

Effector and reporter plasmids used in the transient transactivation experiment examining C-terminal deletion mutants of DREB2A were constructed as described previously (Liu et al., 1998). Effector plasmids that encode the GAL4 DNA binding domain fused to the C-terminal region of DREB2A and a reporter plasmid that contains the GAL4 binding sequence were constructed as described previously (Urao et al., 1996).

Insert fragments used for construction of effector plasmids were amplified by PCR using the primer pairs shown in Supplemental Table 2 online.

Isolation of *Arabidopsis* T87 cell protoplasts and polyethylene glycol-mediated DNA transfection were performed as described previously (Sato et al., 2004).

### Plant Transformation

Plasmids used for the transformation of *Arabidopsis* were constructed with a mutant DREB2A fragment that lacks amino acid residues 136 to 165. The fragment was cloned into a multicloning site of the pBE2113Not vector (Liu et al., 1998) or the pBIRD29AP-Not vector (Kasuga et al., 1999). The constructed plasmid was introduced into *Agrobacterium tumefaciens* C58 cells. Plants were transformed as described previously (Liu et al., 1998).

### Microarray Analysis

Total RNA was isolated with Trizol reagent (Invitrogen) from 3-week-old plants having pBI121 or overexpressing DREB2A CA. Three independent transgenic lines were used for each experiment. mRNAs were prepared using the PolyATract mRNA isolation system III (Promega). Preparation of fluorescent probes, microarray hybridization, and scanning have been described previously (Seki et al., 2002). The reproducibility of the microarray analysis was assayed by triplicate replication of each experiment. Raw data were analyzed by GeneSpring version 5.1 software (Silicon Genetics) and normalized using the Lowess normalization method. Expression ratios and *t* test *P* values of the cross-gene error model were also calculated by GeneSpring. The genes showing a signal intensity >2000 in at least one experiment were considered for the analysis. We studied genes that showed expression ratios >5 with *P* values <0.05 in the experiments for 35S:DREB2A CA-a. The median, not average, expression ratio among three replications was used to select genes to be subjected to RNA gel blot analysis (see Supplemental Table 1 online).

### RNA Analysis

Total RNA was extracted with Trizol reagent. RNA gel blot analysis was performed as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994). For real-time quantitative RT-PCR, cDNA was synthesized from total RNA using Revertra Ace (Toyobo) with random hexamer primers according to the manufacturer's instructions. Real-time PCR was performed on a Light Cycler (Roche Diagnostics) using an SYBR Premix Ex Taq kit (Takara) according to the manufacturer's instructions. The primer pairs used in the real-time PCR are shown in Supplemental Table 3 online. Amounts of template cDNA that were used in each PCR reaction were corrected by the results of quantification of 18S rRNA. Three determinations were performed for each sample.

### Gel Mobility Shift Assay

Recombinant GST fusion proteins were prepared as described previously (Liu et al., 1998). A 429-bp (119 to 547) fragment of the DREB1A cDNA and a 500-bp (167 to 666) fragment of the DREB2A cDNA were cloned into the *EcoRI-Sall* sites of the pGEX-4T-1 vector (Pharmacia). The recombinant plasmid was transformed to *Escherichia coli* BL21. Production and purification of the GST fusion proteins were performed as described previously (Urao et al., 1993). The 75-bp probe fragments containing DRE from the *RD29A* promoter with or without base substitution were prepared by PCR and cloned into the *Bam*HI site of the pBluescript II SK- vector (Sakuma et al., 2002). The gel mobility shift assay was performed as described previously (Sakuma et al., 2002).

### Construction of 35S:GFP-DREB2A and 35S:GFP-DREB2A CA Fusion Genes and Observation of Subcellular Localization of Green Fluorescent Signals in Transgenic Plants

The 35S:GFP-DREB2A and 35S:GFP-DREB2A CA plasmids were constructed by cloning of cDNAs encoding the DREB2A FL protein or the internally deleted DREB2A 1-135:166-335 protein into a pGreenII0029 E12-35S- $\Omega$  NsGFP vector. That vector was constructed as follows: an E12-35S- $\Omega$ -multicloning site-Nos terminator cassette was subcloned from pBE2113Not (Liu et al., 1998) into pGreenII0026 (<http://www.pgreen.ac.uk>), and then sGFP cDNA amplified by PCR with the primer pair 5'-GGGACTAGTATGGTGAGCAAGGGCGAG-3' and 5'-GGGTCTAGATGCTTGTACAGCTCGTCCAT-3' was inserted into the *Xba*I site. The construct was introduced into *Arabidopsis* plants as described above in the section about plant transformation. The GFP fluorescence in the young roots of 3-week-old transgenic plants was analyzed with the confocal laser scanning microscope LSM5 PASCAL (Carl Zeiss). All photographs were taken at the same magnification. The relative intensity of the green fluorescent signal in the nucleus was measured using ImageJ software (National Institutes of Health, <http://rsb.info.nih.gov/ij/>).

### Accession Numbers

*Arabidopsis* Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *DREB2A* (At5g05410), *DREB1A* (At4g25480), *RD29A* (At5g52310), *RD29B* (At5g52300), *MT2A* (At3g09390), *PDC2* (At5g54960), *ATGRP7* (At2g21660), *AtGolS3* (At1g09350), *RD17* (At1g20440), *LEA14* (At1g01470), *COR15A* (At2g42540), *COR15B* (At2g42530), *KIN1* (At5g15960), *KIN2* (At5g15970), and *COR414-TM1* (At1g29395). The complete set of microarray data has been deposited in the European Bioinformatics Institute ArrayExpress database under accession number E-MEXP-317 ([www.ebi.ac.uk/arrayexpress/](http://www.ebi.ac.uk/arrayexpress/)).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Table 1.** Results of Microarray Analysis for Genes Subjected to RNA Gel Blot Analysis.

**Supplemental Table 2.** Primer Pairs Used for Effector Plasmid Construction.

**Supplemental Table 3.** Primer Pairs Used for Real-Time PCR.

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**Functional Analysis of an *Arabidopsis* Transcription Factor, DREB2A, Involved in Drought-Responsive Gene Expression**

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