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

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Transcription factors DREB1A/CFG3 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, 2003). Several 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, 2003). Several stress-inducible genes, such as RD29A and COR15A, are induced through the ABA-independent pathway. A 9-bp conserved sequence, 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 gene...
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 β-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×3), –61 RD29A minimal TATA promoter, and GUS reporter gene. The effector constructs contain the CaMV 35S promoter and the tobacco mosaic virus 1 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 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 Ω sequence (Galie 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, 3SS:DREB2A CA-a, 3SS:DREB2A CA-b, and 3SS:DREB2A CA-c, that showed strong, moderate, and weak transgene expression, respectively, for further analysis (Figure 3).

We observed the growth of the 3SS:DREB2A CA plants under normal growing conditions and compared them with control plants containing the pBI121 vector (wild type) and 3SS:DREB2A FL plants overexpressing the DREB2A FL cDNA (Figures 3A to 3C). All the 3SS: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 3SS:DREB2A FL plants overexpressing DREB2A FL. The 3SS:DREB2A CA plants had rounded, slightly darker leaves with short petioles. These phenotypes appeared in the 3SS:DREB2A CA-a plants more notably than in the 3SS: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: 3SS:DREB2A CA-a, 3SS:DREB2A CA-b, and 3SS:DREB2A CA-c. Cy3- and Cy5-labeled cDNA probes were prepared from mRNAs isolated from the 3SS: 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...
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 downstream 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).

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 µg of total RNA prepared from 3-week-old transgenic Arabidopsis plants. Ethidium bromide–stained rRNA image is shown as a loading control.
Table 1. Microarray Analysis of 3SS:DREB2A CA Plants

<table>
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<tr>
<th>Gene Name</th>
<th>AGI Locus Identifier</th>
<th>Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DRE&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ABRE&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Description&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes whose expression in 3SS:DREB2A CA-a plants was increased more than five times compared with expression in wild-type plants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td><strong>RD29A/AtCOR78/AtLTI140</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>At5g52310</td>
<td>15.5</td>
<td>−131 to −126, −168 to −163, −225 to −220, −275 to −270</td>
<td>−65 to −60</td>
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<td>−275 to 270</td>
<td>Galactinol synthase</td>
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<td>At1g52690</td>
<td>11.9</td>
<td>−47 to −42</td>
<td>−200 to −195, −238 to −233, −235 to −240, −635 to −630, −632 to −637</td>
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<td>At4g33720</td>
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<td>−345 to −350, −919 to 914</td>
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<td>Pyruvate decarboxylase</td>
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<td>−100 to −105</td>
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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

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<th>Gene Name</th>
<th>AGI Locus Identifier</th>
<th>Median Ratio&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Description&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>3.7</td>
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<td>−72 to −67, −69 to −74, −89 to −85, −86 to −91, −159 to −164, −380 to −385</td>
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<td>−39 to −44, −45 to −50, −86 to −81, −187 to −182</td>
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<sup>a</sup>Median ratio of three repeats. Ratio was defined as follows: fluorescence intensity of each cDNA of 3SS: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 overexpressers. 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 recognizes the DRE sequence.

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 ACCAGACAT or ACCGACAA/C/G sequences as competitors (Figures 5C and 5D). Addition of the unlabeled DNA fragment containing ACCAGACAT 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 DREB2A to GCCGAC and ACCGAC, which reduces the extent of binding of DREB1A more effectively than that of the unlabeled DNA fragment containing ACCGACAA/C/G. 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.

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 (ACCAGACA, 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. 32P-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% survival was observed in the 35S:DREB2A CA plants.

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 un-stressed 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:sGFP-DREB2A FL) or DREB2A CA cDNA (35S:sGFP-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:sGFP-DREB2A FL plants was remarkably lower than that in the 35S:sGFP-DREB2A CA plants (Figure 8C). Moreover, in the 35S:sGFP-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:sGFP-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 elucidated 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...
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 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

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).

(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 μm.

(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:GFP-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 ACCGACACA/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 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 (Sakuma et al., 2002).

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 (Satoh 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 PolyATtract 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-genec 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-Sal 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 BamHI 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-DREB2CA Fusion Genes and Observation of Subcellular Localization of Green Fluorescent Signals in Transgenic Plants

The 35S:GFP-DREB2A and 35S:GFP-DREB2CA 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 E2-35S-1-NaGFP vector. That vector was constructed as follows: an E2-35S::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′-GGGACTAGTATGGTGACCAAGGGAG3′ and 5′-GGGTTCTAGATGCCTGACGCTCCAT-3′ was inserted into the XbaI 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 LMS5 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 (At5g5410), DREB1A (At4g25480), RD29A (At5g25310), RD29B (At5g25300), MT2A (At5g09390), PDC2 (At5g54960), ATGRP7 (At2g1660), AtGoI52 (At1g09350), RO17 (At1g02440), LEA14 (At1g01470), COR15A (At2g42540), COR15B (At2g42530), KIN1 (At5g15960), KIN2 (At5g15970), and COR414-TM1 (At1g23935). 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/).

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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