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An *Arabidopsis* Gene Family Encoding DRE/CRT Binding Proteins Involved in Low-Temperature-Responsive Gene Expression

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In higher plants, a *cis*-acting element, DRE/CRT, is involved in gene expression responsive to drought and low-temperature stress. To understand signal transduction pathways from the cold stress signal to gene expression, we characterized a gene family for DRE/CRT-binding proteins DREB1A and CBF1 in *Arabidopsis thaliana*. DREB1A and CBF1 were shown to be involved in low-temperature-responsive gene expression. We screened an *Arabidopsis* genomic DNA library with the cDNA fragment of DREB1A as a probe and isolated *DREB1A* and 2 related genes, *DREB1B* (= CBF1) and *DREB1C*. These were arrayed in the order B, A, C in an 8.7 kb region of *Arabidopsis* chromosome 4. Northern blot analysis using gene-specific probes showed that the 3 *DREB1* genes are induced mainly by cold stress but not by osmotic stress in leaves, roots, and stems. Several conserved sequences were found in the promoter regions of all 3 genes. The β-glucuronidase (GUS) reporter gene driven by the *DREB1* promoters was induced at transcriptional level by low temperature in transgenic *Arabidopsis* plants. © 1998 Academic Press

Plants are inevitably confronted during their life cycle with numerous environmentally determined stresses that can be detrimental to their survival. In order to meet such challenges, plants not only possess structural physical barriers against environmental stress but also have evolved inducible mechanisms that allow them to respond to environmental stimuli. A number of genes that respond to desiccation and low temperature at the transcriptional level have been described recently, and their gene products are thought to be involved in stress response and tolerance (1-4).

It is important to know how plants sense low temperature and water deficit and process this information to alter gene expression. Promoter analyses have been performed with many stress-inducible genes by drought, low temperature, and high salinity in a variety of plants. ABA-independent as well as ABA-dependent regulatory mechanisms have been shown to be involved in transcription of those stress-inducible genes. Identification of a *cis*-acting element involved in responsiveness to drought, low temperature, and high salt stress in *Arabidopsis thaliana* is an important advance in this regard. A 9-bp conserved sequence, TACCGACAT, termed the dehydration-responsive element (DRE), is essential for the induction of the *rd29A* gene (also known as *cor78* and *lti78*) of *Arabidopsis* not only by osmotic stress caused by drought and high salinity but also by low-temperature stress (5). However, DRE is not involved in ABA-responsive gene expression. DRE-related motifs have been reported in promoters of multiple genes regulated by osmotic and low-temperature stress, including *kin1*, *cor6.6*/*kin2*, and *rd17*/*cor47* in *Arabidopsis* (6, 7). A similar motif (C-repeat; CRT) was also reported in the promoter region of cold- and dehydration-inducible *cor15A* (8). The CCGAC core sequence was shown to be important for cold-responsive gene expression in the 5′ promoter region of the cold-inducible *Brassica napus* gene *BN115* (9).

We isolated 2 types of cDNAs encoding DRE binding proteins (DREB1A and DREB2A) in *Arabidopsis* using yeast one-hybrid screening with cDNA libraries prepared from dehydrated or cold-treated *Arabidopsis* plants (10). The deduced amino acid sequences of DREB1A and DREB2A showed no significant sequence similarity except in the conserved DNA-binding domain found in the EREBP and AP2 proteins. Both

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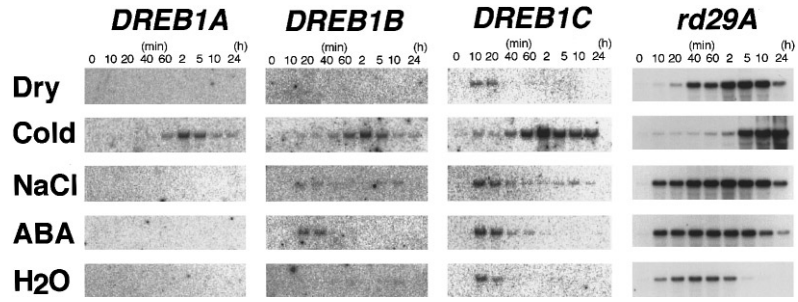


FIG. 1. Expression of *DREB1* gene family in response to low-temperature stress. Each lane was loaded with 20 μg of total RNA from 3-week-old *Arabidopsis* plants that had been dehydrated (Dry), transferred to 4°C (Cold), transferred for hydroponic growth in 250 mM NaCl (NaCl), transferred to 100 μM ABA (ABA), or transferred to water (H_2O) for hydroponic growth as described in Materials and Methods. The number in each lane is the number of minutes (0, 10, 20, 40, 60) or hours (2, 5, 10, 24) after the initiation of treatment before the isolation of RNA. RNA was analyzed by RNA gel blotting with gene-specific probes of *DREB1A*, *DREB1B*, *DREB1C*, or *rd29A*.

DREB1A and DREB2A specifically bound to the DRE sequence in vitro and activated the transcription of the β-glucuronidase (GUS) reporter gene driven by the DRE sequence in *Arabidopsis* protoplasts. Expression of the *DREB1A* gene was induced by cold stress, and expression of the *DREB2A* gene was induced by dehydration. Two independent DREB proteins, DREB1A and DREB2A, function as transcriptional activators in 2 separate signal transduction pathways under low temperature and dehydration conditions, respectively (10). Overexpression of the DREB1A cDNA in transgenic *Arabidopsis* plants induced strong expression of its target genes under unstressed control conditions, but overexpression of the DREB2A cDNA induced weak expression of the target genes. The transgenic plants that overexpress the DREB1A cDNA revealed tolerance not only to freezing but also to dehydration (10).

We also isolated 2 cDNA clones homologous to DREB1A (DREB1B and DREB1C) and 1 cDNA homologous to DREB2A (DREB2B) from *Arabidopsis* plants (10). The *Arabidopsis* cDNA CBF1, which encodes a transcriptional activator containing an EREBP-AP2-domain that binds to CRT/DRE, has also been characterized (11). The DREB1B cDNA is identical to CBF1. Jaglo-Ottosen et al. (1998) also reported that CBF1 overexpression in transgenic *Arabidopsis* plants induced strong expression of the target genes under unstressed conditions and enhanced freezing tolerance (12). These data indicate that the DREB1/CBF1 genes are transcriptionally induced by low temperature and then their gene products induce the expression of multiple target genes, achieving tolerance to freezing and drought in transgenic plants. By contrast, *DREB2A* was induced by drought and high-salinity stress, but its product, DREB2A, could not strongly induce the target genes involved in stress tolerance. This suggests that post-translational modification is needed for the activation of the DREB2A protein.

To understand signal transduction pathways from perception of the cold-stress signal to gene expression, it is important to analyze the structure and expression of the *DREB1A-C* genes and their promoters. In this study, we isolated genomic clones corresponding to the

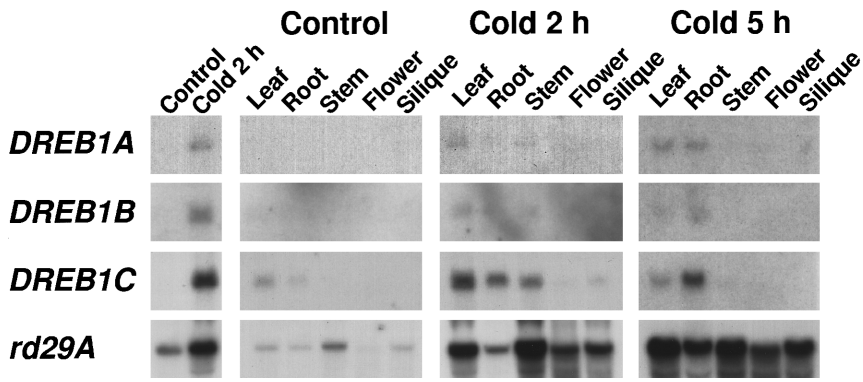


FIG. 2. Tissue-specific Northern blot analysis of the *DREB1* gene family. To detect *DREB1* mRNAs, 40 μg of tissues (Leaf, Root, Stem, Flower and Silique) that had been transferred to 4°C (Cold) for 2 or 5 h or untreated (Control) were used as described in Materials and Methods. RNA was extracted from 6 to 7-week-old plants grown in pots for all tissues except leaves, or from plants grown on agar plates for leaves. The same procedure was followed as for Figure 1.

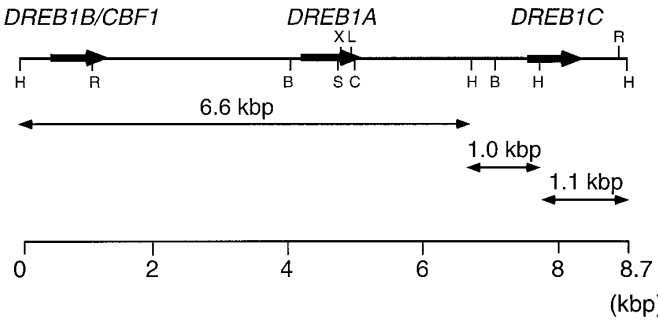


FIG. 3. Restriction maps of the *DREB1* clones. The upper restriction map of the *DREB1* genes was derived from the 3 lower restriction maps of the *Hin*d III clones of these fragments. These sites were connected through PCR. Restrictions are shown as H (*Hin*d III), R (*Eco* RV), B (*Xba* I), S (*Sac* I), C (*Hin*c II), X (*Xho* I), and L (*Sal* I). Arrows indicate the direction of transcription of each gene.

DREB1A-C cDNAs by screening an *Arabidopsis* genomic library with the DREB1A cDNA as a probe, and found 3 *DREB1* genes located close to each other on the *Arabidopsis* genome. The putative promoters of these genes were fused to the *GUS* reporter gene for the analysis of regulatory elements involved in cold-responsive transcription in transgenic *Arabidopsis* plants.

MATERIALS AND METHODS

Plant materials. Plants (*Arabidopsis thaliana* ecotype Columbia) were grown aseptically on germination medium (GM) agar (13) containing 0.8% Bacto-agar (Difco, Detroit, MI, USA) for 2-4 weeks under continuous light (3000 lux). Rosette plants were used in stress-treatment experiments.

Stress and hormone treatments and RNA isolation. Arabidopsis rosette plants grown for 3-4 weeks on GM agar plates were subjected to various environmental stresses, such as dehydration, high salt, low temperature, or exogenous ABA. Plants were dehydrated in Petri dishes at 60% relative humidity and 22°C under dim light (100 lux); or transferred to distilled water, 250 mM NaCl, or 100 μM ABA (mixed isomers) under dim light; or grown in an incubator at 4°C under dim light. In each case, the plants were stressed for varying lengths of time, washed gently, and then immediately frozen in liquid nitrogen. RNAs were isolated from plants as previously described (14).

Hybridization analysis of RNA. Gene-specific DNA fragments of the DREB1A, DREB1B, and DREB1C cDNA clones were used for Northern blot hybridization analysis. The following DNA fragments containing the 3′ non-coding regions of the 3 cDNAs were used as gene-specific probes: an *Sal* I-*Sma* I fragment (position 729-933) for DREB1A, an *Sph* I-*Bam*HI fragment (position 721-937) for DREB1B, and an *Hae* III-*Bam*HI fragment (position 694-944) for the DREB1C cDNA. DNA fragments were labeled by the random primer method with [α-^{32}P]-dCTP (Amersham, Aylesbury, UK) by using a random primed DNA labeling kit (Boehringer Mannheim). The labeled fragments were hybridized with RNA-blotted filters as previously described (15).

Isolation of clones containing the DREB1 genes from Arabidopsis genomic library. An *Arabidopsis* genomic library (Clontech, Palo Alto, CA, USA) was screened by the plaque hybridization method (16). A DREB1A cDNA fragment (1.4 kb in size) was used as a probe (10). Positive clones were plaque-purified and their DNAs were isolated by phenol extraction followed by CsCl gradient centrifugation (17).

Subcloning and sequencing DNA fragments containing the DREB1 genes. DNA fragments containing the *DREB1* genes were subcloned into the pBluescriptII SK$^-$ (pSK$^-$) vector (Stratagene, La Jolla, CA, USA). We used a 6.6 kb *Hin*d III fragment for *DREB1A* and *DREB1B*, and 1.0 kb and 1.1 kb *Hin*d III fragments for *DREB1C* (Fig. 3). An exo/mung deletion kit (Takara, Kyoto, Japan) was used to make a deletion series to allow the DNA sequence of the 6.6-kb fragment to be determined. Plasmid DNA templates for sequencing were prepared with an automatic plasmid isolation system (Model PI-100, Kurabo, Osaka, Japan) and sequenced by using a chemical robot (Catalyst 800, Perkin Elmer, San Jose, CA, USA) and a semi-automatic DNA sequencer (Model 373A, Perkin Elmer). Nucleotide and amino acid sequences were analyzed with the Genetyx software system (Software Development Co., Tokyo, Japan). We connected the 3 *Hin*d III fragments by sequencing PCR products that bridged the *Hin*d III sites.

Mapping of the DREB1 genes. The *Arabidopsis* CIC library constructed in yeast artificial chromosomes (YACs; 18) was screened by using the DREB1A cDNA fragment as a probe as described previously (19). After hybridization with the radiolabeled probe at 65°C overnight, the filters were washed in 0.1 × SSC and 0.1% SDS at 65°C. A Bio Image Analyzer (Fuji Film Co., Tokyo, Japan) was used to obtain images of hybridized RNA bands.

Primer extension analysis. The primer extension experiment was performed as previously described (20) using the [γ-^{32}P]ATP-labeled oligonucleotide, which corresponds to the complementary sequence of the upstream parts of the *DREB1* genes: 5′-AAAGACAGAGATCTTTTAGT-3′ (complementary to positions +78 to +97; Fig. 5A) for *DREB1A;* 5′-AAGACAGATATACTATCTTT-3′ (complementary to positions +75 to +94; Fig. 5B) for *DREB1B;* and 5′-GACAGAGATCTTCTACTTAC-3′ (complementary to positions +80 to +99; Fig. 5C) for *DREB1C*. The mRNA for the primer extension was extracted from *Arabidopsis* rosette plants incubated for 2 h by the guanidine thiocyanate/CsCl method, and was purified by using an oligo-dT column.

Transgenic plants. DNA fragments 1123, 611, and 877 bp long (*DREB1A, DREB1B* and *DREB1C*, respectively) containing the 984-,

FIG. 4. Map position of *DREB1A-C,* located on chromosome 4.

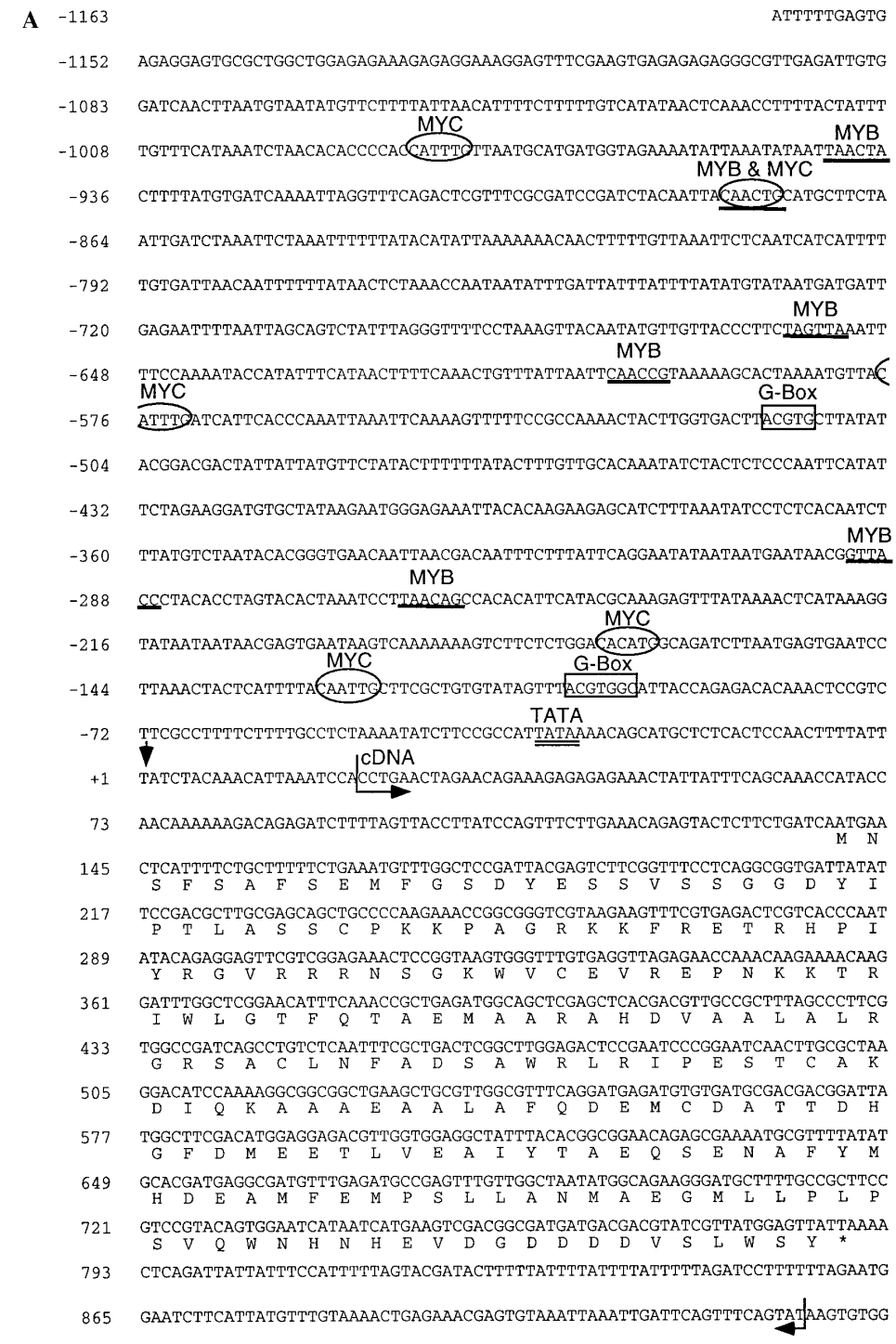


FIG. 5. Nucleotide sequences of *DREB1A* (A), *DREB1B* (B), and *DREB1C* (C). Nucleotide sequences of the non-coding strand including the coding regions and their 5′ and 3′ flanking regions are shown. Numbers refer to nucleotides relative to the 5′ end of their mRNAs. Putative TATA boxes are double underlined. Sequences homologous to the G-box are boxed. Sequences homologous to MYC are circled, and those homologous to MYB are underlined. The deduced amino acid sequences are shown below the nucleotide sequences in single letter codes. The 5′ and 3′ ends of the *DREB1A-C* cDNAs are shown with arrows.

433-, and 725-bp regions from the site of initiation of transcription were ligated into the *Bam*HI-*Hin*d III site of the promoterless *GUS* expression vector pBI101 (Clontech, Palo Alto, CA, USA). The vectors containing the *DREB1A, DREB1B,* or *DREB1C* promoter—*GUS* fusion constructs were then transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* via triparental mating with an *E. coli* strain that contained a mobilization plasmid, pRK2013. The pBI 101 vectors containing the *DREB1A, B,* or *C* promoter—*GUS* fusion

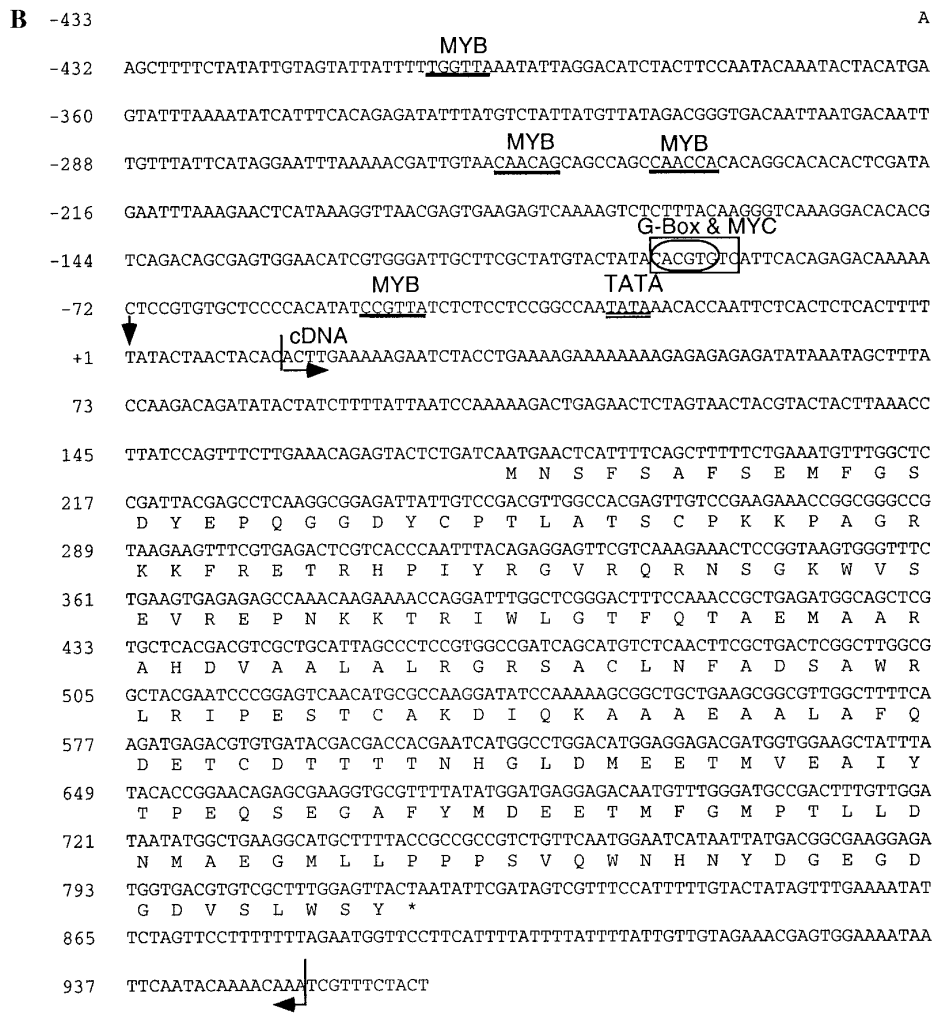


FIG. 5—*Continued*

constructs were transferred into *A. tumefaciens* C58 as described previously (5). *Arabidopsis* plants were transformed by the vacuum infiltration method as described previously (21). Transgenic *Arabidopsis* plants were grown at 22°C under a 16 h light—8 h dark cycle.

Northern blot analysis of transgenic Arabidopsis plants. Transgenic *Arabidopsis* rosette plants grown on GM agar plates were subjected to various environmental stresses, such as dehydration, low temperature, high salt, or exogenous ABA for 2 or 5 h to detect their maximum expression (5), and were then frozen in liquid nitrogen. RNA samples were extracted from the frozen plants as described previously (15). DNA fragments used as probes were those containing the coding region of the GUS reporter gene or fragment containing the full length of their respective DREB1 cDNAs.

RESULTS

Accumulation of DREB1 mRNA in Response to Low Temperature

Expression of the *DREB1A*, *DREB1B*, and *DREB1C* genes under various stress conditions was analyzed by Northern blot analysis and compared with that of the *rd29A* gene. The 3 *DREB1* genes were induced mainly by low-temperature stress, whereas the target *rd29A* gene was induced by low-temperature, drought, and salt stresses (Fig. 1). Induction of the genes was detected within 40 min and reached a maximum at 2 h after low-temperature treatment at 4°C. In contrast, the *rd29A* gene was induced by low-temperature stress within 2 h and was strongly expressed at 5 h. The 3 *DREB1* genes were induced during cold stress before the *rd29A* gene. Expression of the *DREB1C* gene was quickly and transiently induced within 10 min by dehydration, high-salt, and ABA treatments and decreased by 40 min. This transient expression of *DREB1C* was detected even in the control plants transferred to water (Fig. 1: H_2O). These results suggest that the *DREB1C* gene may be expressed in response to touch or wounding stress as well as to low-temperature stress.

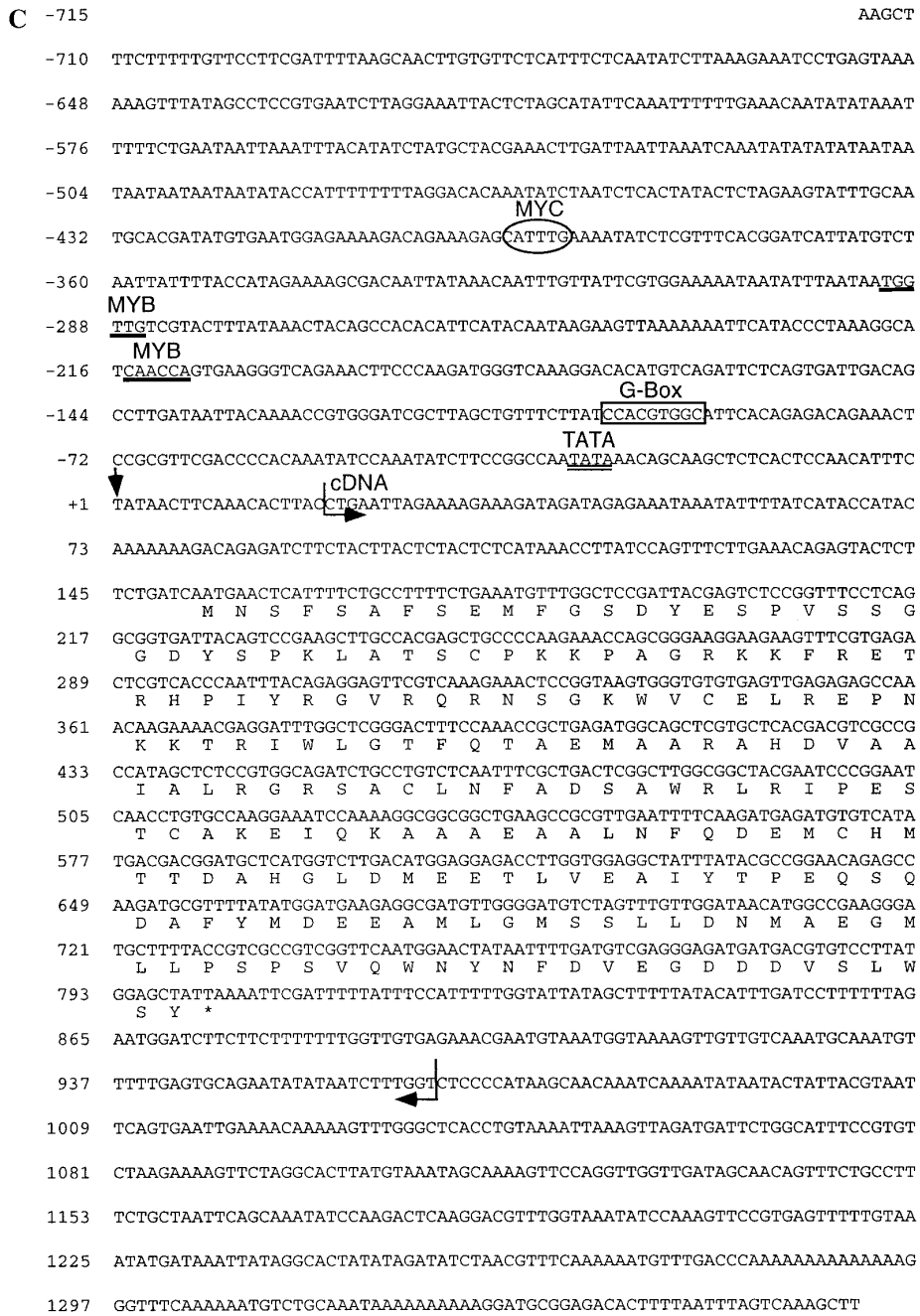
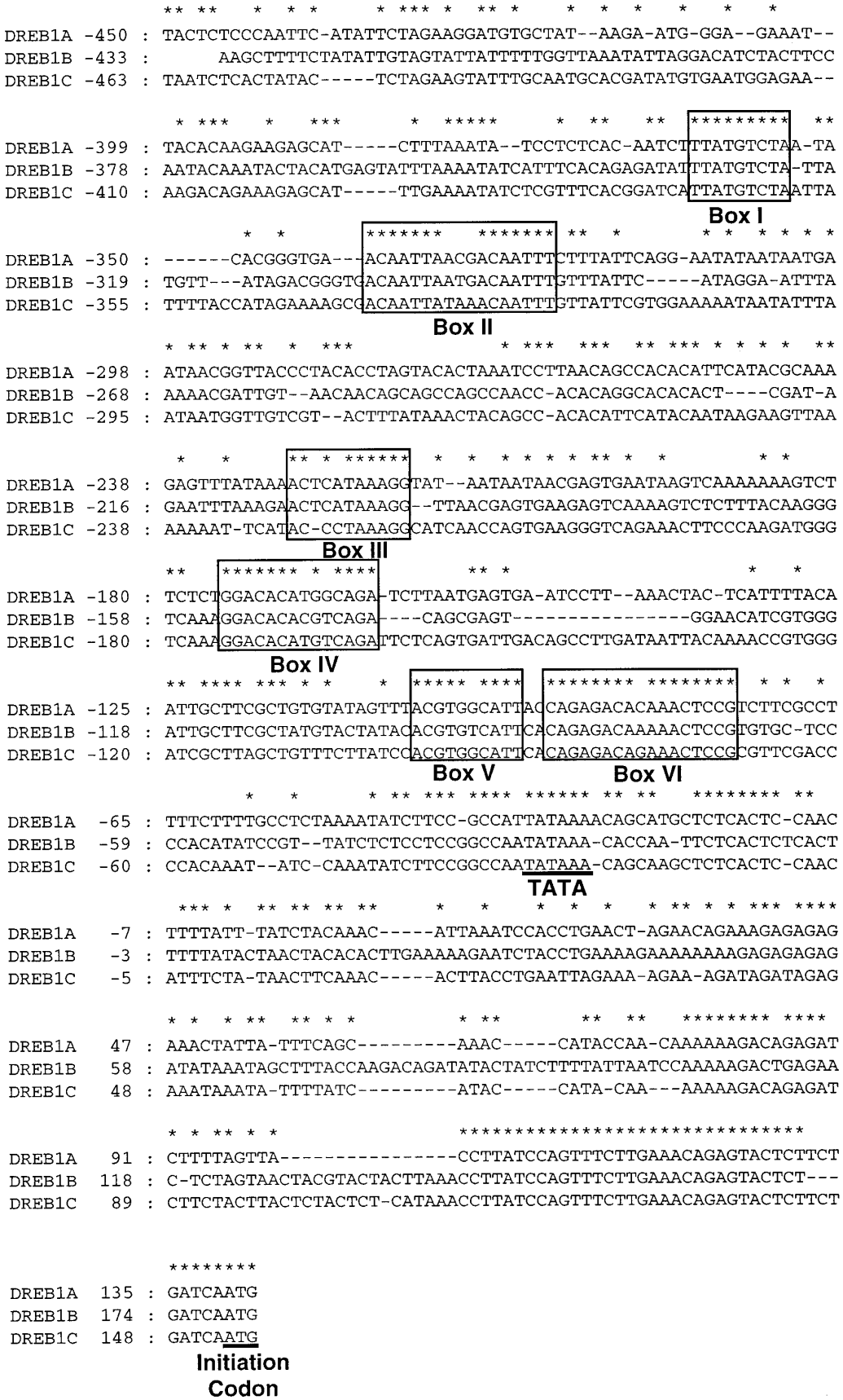


FIG. 5—*Continued*

Figure 2 shows tissue-specific expression of 3 *DREB1* genes in whole plants as well as in specific tissues treated by cold stress for 2 or 5 h. The 3 *DREB1* genes are expressed mainly in leaves, roots, and stems. Strong expression of the *rd29A* gene was observed in all tissues of plants treated at 4°C.

FIG. 6. Comparison of the nucleotide sequences of the promoter regions of the 3 *DREB1* genes. Asterisks denote sequence identity among all 3 sequences. Boxes show conserved sequences among the 3 genes (Box I, Box II, Box III, Box IV, Box V, and Box VI). The TATA box and the ATG initiation codon are underlined.


DREB1A -450 : TACTCTCCCAATTC-ATATTCTAGAAGGATGTGCTAT--AAGA-ATG-GGA--GAAAT--
DREB1B -433 : AAGCTTTTCTATATTGTAGTATTATTTTTGGTTAAATATTAGGACATCTACTTCC
DREB1C -463 : TAATCTCACTATAC-----TCTAGAAGTATTTGCAATGCACGATATGTGAATGGAGAA--
DREB1A -399 : TACACAAGAAGAGCAT-----CTTTAAATA--TCCTCTCAC-AATCTTTATGTCTAA-TA
DREB1B -378 : AATACAAATACTACATGAGTATTTAAAATATCATTTCACAGAGATATTTATGTCTA-TTA
DREB1C -410 : AAGACAGAAAGAGCAT-----TTGAAAATATCTCGTTTCACGGATCATTATGTCTAATTA
Box I
DREB1A -350 : ------CACGGGTGA---ACAATTAACGACAATTTCTTTATTCAGG-AATATAATAATGA
DREB1B -319 : TGTT---ATAGACGGGTGACAATTAATGACAATTTGTTTATTC-----ATAGGA-ATTTA
DREB1C -355 : TTTTACCATAGAAAAGCGACAATTATAAACAATTTGTTATTCGTGGAAAAATAATATTTA
Box II
DREB1A -298 : ATAACGGTTACCCTACACCTAGTACACTAAATCCTTAACAGCCACACATTCATACGCAAA
DREB1B -268 : AAAACGATTGT--AACAACAGCAGCCAGCCAACC-ACACAGGCACACACT----CGAT-A
DREB1C -295 : ATAATGGTTGTCGT--ACTTTATAAACTACAGCC-ACACATTCATACAATAAGAAGTTAA
DREB1A -238 : GAGTTTATAAAACTCATAAAGGTAT--AATAATAACGAGTGAATAAGTCAAAAAAAGTCT
DREB1B -216 : GAATTTAAAGAACTCATAAAGG--TTAACGAGTGAAGAGTCAAAAGTCTCTTTACAAGGG
DREB1C -238 : AAAAAT-TCATAC-CCTAAAGGCATCAACCAGTGAAGGGTCAGAAACTTCCCAAGATGGG
Box III
DREB1A -180 : TCTCTGGACACATGGCAGA-TCTTAATGAGTGA-ATCCTT--AAACTAC-TCATTTTACA
DREB1B -158 : TCAAAGGACACACGTCAGA----CAGCGAGT----------GGAACATCGTGGG
DREB1C -180 : TCAAAGGACACATGTCAGATTCTCAGTGATTGACAGCCTTGATAATTACAAAACCGTGGG
Box IV
DREB1A -125 : ATTGCTTCGCTGTGTATAGTTTACGTGGCATTACCAGAGACACAAACTCCGTCTTCGCCT
DREB1B -118 : ATTGCTTCGCTATGTACTATACACGTGTCATTCACAGAGACAAAAACTCCGTGTGC-TCC
DREB1C -120 : ATCGCTTAGCTGTTTCTTATCCACGTGGCATTCACAGAGACAGAAACTCCGCGTTCGACC
Box V
Box VI
DREB1A -65 : TTTCTTTTGCCTCTAAAATATCTTCC-GCCATTATAAAACAGCATGCTCTCACTC-CAAC
DREB1B -59 : CCACATATCCGT--TATCTCTCCTCCGGCCAATATAAA-CACCAA-TTCTCACTCTCACT
DREB1C -60 : CCACAAAT--ATC-CAAATATCTTCCGGCCAATATAAA-CAGCAAGCTCTCACTC-CAAC
TATA
DREB1A -7 : TTTTATT-TATCTACAAAC-----ATTAAATCCACCTGAACT-AGAACAGAAAGAGAGAG
DREB1B -3 : TTTTATACTAACTACACACTTGAAAAAGAATCTACCTGAAAAGAAAAAAAAGAGAGAGAG
DREB1C -5 : ATTTCTA-TAACTTCAAAC-----ACTTACCTGAATTAGAAA-AGAA-AGATAGATAGAG
DREB1A 47 : AAACTATTA-TTTCAGC---------AAAC-----CATACCAA-CAAAAAAGACAGAGAT
DREB1B 58 : ATATAAATAGCTTTACCAAGACAGATATACTATCTTTTATTAATCCAAAAAGACTGAGAA
DREB1C 48 : AAATAAATA-TTTTATC---------ATAC-----CATA-CAA---AAAAAGACAGAGAT
DREB1A 91 : CTTTTAGTTA----------CCTTATCCAGTTTCTTGAAACAGAGTACTCTTCT
DREB1B 118 : C-TCTAGTAACTACGTACTACTTAAACCTTATCCAGTTTCTTGAAACAGAGTACTCT---
DREB1C 89 : CTTCTACTTACTCTACTCT-CATAAACCTTATCCAGTTTCTTGAAACAGAGTACTCTTCT
DREB1A 135 : GATCAATG
DREB1B 174 : GATCAATG
DREB1C 148 : GATCAATG
Initiation Codon

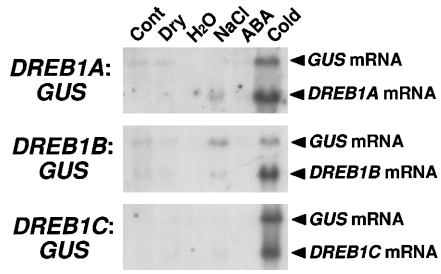


FIG. 7. Analysis of the effects of various stress treatments on the induction of the *DREB1* promoter—*GUS* fusion genes in transgenic *Arabidopsis* plants. Northern blotting was used to measure the amount of *GUS* mRNA or endogenous *DREB1* mRNAs in transgenic *Arabidopsis* plants that had been dehydrated (Dry), transferred to 4°C (Cold), transferred from agar plates for hydroponic growth in 250 mM NaCl (NaCl), transferred to 100 μM ABA (ABA), transferred to water (H_2O) for hydroponic growth, or untreated (Cont). All treatments were done for 2 or 5 hours. Northern blotting was done as described in Materials and Methods. DNA fragments for the coding regions of *GUS* or the *DREB1A, DREB1B,* or *DREB1C* cDNAs were used as probes.

Isolation and Structural Organization of 3 DREB1 Genes

We screened an *Arabidopsis* genomic library using the DREB1A cDNA fragment as a probe, and isolated 3 *Hin*d III DNA fragments (6.6, 1.1, and 1.0 kb) that contained the *DREB1* genes. Nucleotide sequences of these 3 genomic *Hind* III fragments were determined. We connected the sequences of these fragments based on PCR analysis. The 3 genes are tandemly arrayed in a locus covering an 8.7-kb region of the *Arabidopsis* genome. *DREB1A* and *DREB1B* are located on the 6.6-kb *Hin*d III fragment; *DREB1C* is located on 2 *Hin*d III fragments of 1.0- and 1.1-kb length (Fig. 3).

We determined the chromosomal position of the *DREB1* genes. The DREB1A cDNA fragment was used to screen an *Arabidopsis* CIC library constructed in YACs. We identified 3 YACs (CIC1E5, CIC1G9, and CIC10G2) hybridized with the DREB1A cDNA probe. These 3 YACs constitute a contig containing a restriction fragment length polymorphism (RFLP) marker, m600, on chromosome 4. These results show that *DREB1A* is located near m600 (Fig. 4). This map position is consistent with a report that a BAC clone, M7J2, carrying the DREB1 gene family, was mapped near m600 on chromosome 4 (http://muntjac.mips.biochem.mpg.de/arabi/index.html).

Structure of 3 DREB1 Genes

The nucleotide sequences of the genomic DNA fragments were determined (*DREB1A,* Fig. 5A; *DREB1B,* Fig. 5B; *DREB1C,* Fig. 5C). These sequences contain no introns in their coding regions (10). The 5′ ends of their transcripts were determined by using the primer extension method (data not shown), and are indicated in the nucleotide sequences in Figure 5 by position +1. Typical TATA box sequences were located about −30 nucleotides upstream from the 5′ ends. We searched the upstream regions of the 3 *DREB1* genes for putative *cis*-acting motifs. We found motifs similar to the G-box and ABRE-related sequences (T/CACGTGG/TC; 20, 22). In addition, we found motifs related to the MYB (C/TAACNA/G; 23-24) and MYC recognition sites (CANNTG; 25). Comparison of the DREB1 promoter regions revealed that nucleotide sequences around the ATG initiation codons and TATA box sequences are conserved (Fig. 6). We found 6 conserved sequences (TTATGTCTA: Box I; ACAATT-ANNNACAATTT: Box II; ACTCA/CTAAAGG: Box III; GGACACAT/CGG/TCAGA: Box IV; ACGTGG/TCATT: Box V; and CAGAGACANAAACTCCG: Box VI) in the 3 promoter regions.

Expression of the DREB Promoter—GUS Fusion Genes in Transgenic Arabidopsis

DNA fragments of the *DREB1A, DREB1B,* and *DREB1C* genes of length 1123, 611, and 877 bp contained promoter regions of length 984, 433, and 725 bp, respectively. These DNA fragments were ligated into the *Bam*HI-*Hin*d III site of the promoterless GUS expression vector pBI 101. We examined the effects of environmental stresses, such as dehydration, high salt, the application of ABA, and low temperature, on the expression of the *DREB1* promoter—*GUS* fusion genes in transgenic *Arabidopsis* (Fig. 7). Northern blot analysis was used to analyze the level of induction of the *GUS* gene driven by the *DREB1* promoters. We analyzed 6 independent transgenic *Arabidopsis* plants. The *GUS* gene driven by the *DREB1C* promoter responded strongly to low-temperature stress (Fig. 7). The expression level of the *GUS* gene was the same as that of the endogenous *DREB1C* gene. The *GUS* genes driven by the *DREB1A* and *DREB1B* promoters were also induced by low-temperature stress, but the level of their expression was lower than that of *DREB1C.* The *DREB1B* promoter—*GUS* gene responded to high salinity and low-temperature; this may be due to the use of the shortest promoter fragment, 433 bp, in the DREB1B construct. These results suggest that all 3 *DREB1* promoters contain *cis*-acting elements involved in low-temperature-responsive gene expression, but that the *DREB1C* and *DREB1A* promoters have only enough elements to show a high level of induction by low-temperature stress.

DISCUSSION

Molecular responses to low-temperature stress are associated with the induction of various genes mediated by a *cis*-acting element, DRE/CRT (2). Proteins encoded by the DREB1A and CBF1 cDNAs bind to DRE/CRT and activate the transcription of their target

genes, which in turn increase tolerance to freezing (10-12). Previously, we isolated 2 cDNA clones homologous to the DREB1A cDNA (DREB1B and DREB1C; 10). We analyzed the specific expression of the 3 *DREB1* genes (Fig. 1). All 3 genes were strongly induced by low-temperature stress. The timing of the induction was similar among the 3 genes and preceded that of the *rd29A* gene. These observations suggest that these 3 *DREB1* genes form a small gene family and have similar functions in the transcription of target genes by low-temperature stress.

Expression of these 3 *DREB1* genes is induced mainly by low-temperature stress in leaves, roots, and stems (Fig. 2). By contrast, the *rd29A* gene is induced by low-temperature stress also in flowers and siliques. These results suggest the existence of *DREB1*-related genes other than *DREB1A-C* whose products are involved in low-temperature-responsive expression in flowers and siliques. Alternatively, the expression of the *rd29A* gene in flowers and siliques may depend on a different *cis*-acing element. On the other hand, *DREB1C* is transiently induced within 10 min by any of the treatments we tested (Fig. 1). This may be due to touch stimuli or wounding stress. *DREB1C* was expressed most strongly among the 3 genes. We also detected the expression of *DREB1C* in unstressed control leaves and roots (Fig. 2). This induction of gene expression may be due to touch or wounding during the preparation of specific tissues.

The *Arabidopsis* genome contains at least 3 *DREB1* genes tandemly arrayed in a locus of 8.7-kb length (Fig. 3). Sequence analysis shows that they are very closely related and have no introns (Figs. 5 and 6). These observation suggest that duplication of these *DREB1* genes produced a small multigene family during the species' evolution. The presence of similar multigene families is often observed in stress-inducible genes of plants, such as drought-responsive genes of rice and *Arabidopsis* (8, 16, 20) and pathogenesis-related genes of tobacco (26).

Analysis of transgenic *Arabidopsis* plants containing fusion genes with the 5′ regions of the *DREB1* genes and the *GUS* reporter gene showed that all 3 *DREB1* promoters show cold-responsive gene expression (Fig. 7). Several conserved motifs were found in the 5′ regions of the 3 *DREB1* genes (Figs. 5 and 6). Moreover, conserved DNA sequences (named Box I-VI) were found in the promoter regions of these genes (Fig. 6). Box V contains the G-box motif found in promoter regions of several genes that are induced by environmental stimuli (27). In common with a number of environmentally controlled genes, promoters of the several low-temperature-regulated genes also contain the G-box element (28). This element is the core motif of a number of *cis*-acting regulatory sequences, including the ABA-responsive element (ABRE; 20, 29, 30). However, the *DREB1* genes were not induced by ABA treatment, which suggests that the G-box motif in Box V does not function as an ABA-responsive element. To date, no motifs that specifically function in cold-stress-responsive expression but not in drought response have been identified. We are currently evaluating promoter constructions containing these conserved sequence motifs to identify *cis*-acting elements involved in low-temperature-specific expression of these genes.

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