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The Arabidopsis *CBF* Gene Family Is Composed of Three Genes Encoding AP2 Domain-Containing Proteins Whose Expression Is Regulated by Low Temperature but Not by Abscisic Acid or Dehydration[1]

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We have identified two genes from Arabidopsis that show high similarity with *CBF1*, a gene encoding an AP2 domain-containing transcriptional activator that binds to the low-temperature-responsive element CCGAC and induces the expression of some cold-regulated genes, increasing plant freezing tolerance. These two genes, which we have named *CBF2* and *CBF3*, also encode proteins containing AP2 DNA-binding motifs. Furthermore, like CBF1, CBF2 and CBF3 proteins also include putative nuclear-localization signals and potential acidic activation domains. The *CBF2* and *CBF3* genes are linked to *CBF1*, constituting a cluster on the bottom arm of chromosome IV. The high level of similarity among the three *CBF* genes, their tandem organization, and the fact that they have the same transcriptional orientation all suggest a common origin. *CBF1*, *CBF2*, and *CBF3* show identical expression patterns, being induced very rapidly by low-temperature treatment. However, in contrast to most of the cold-induced plant genes characterized, they are not responsive to abscisic acid or dehydration. Taken together, all of these data suggest that *CBF2* and *CBF3* may function as transcriptional activators, controlling the level of low-temperature gene expression and promoting freezing tolerance through an abscisic acid-independent pathway.

Many plant species from temperate regions can increase their freezing tolerance in response to low, nonfreezing temperatures (Levitt, 1980; Sakai and Larcher, 1987). This process, called cold acclimation, involves several biochemical and physiological changes that seem to be regulated through changes in gene expression (Thomashow, 1994). Genetic analyses revealed that multiple genes are involved in cold acclimation (Thomashow, 1990), and a wide number of genes whose transcript levels accumulate in response to low temperatures have been isolated and characterized (Thomashow, 1994; Hughes and Dunn, 1996; Capel et al., 1997; Gana et al., 1997; Hong et al., 1997; Capel et al., 1998; Kiyosue et al., 1998; Urao et al., 1998). However, the precise role that these genes play in the process of cold acclimation remains to be determined.

During the past few years a major goal in the study of gene expression induced by low temperature has been to determine the specific *cis*-acting regulatory sequences. Yamaguchi-Shinozaki and Shinozaki (1994) first identified two 9-bp DNA elements in the promoter of the Arabidopsis *RD29A* gene that activated gene expression in response to low temperature and drought when fused to a reporter gene. The two elements contained the low-temperature DRE core sequence CCGAC, also named C-repeat by Thomashow and colleagues (Baker et al., 1994). This sequence, hereafter referred to as LTRE, has been found to be essential for the low-temperature responsiveness of other cold-induced plant genes, including the Arabidopsis gene *COR15A* (Baker et al., 1994), the *Brassica napus* gene *BN115* (Jiang et al., 1996), and the wheat gene *WCS120* (Ouellet et al., 1998). Although many of the changes in gene expression that occur during the process of cold acclimation are mediated by ABA (Bray, 1993; Welin et al., 1994), the results of Yamaguchi-Shinozaki and Shinozaki (1994) revealed that the LTRE is not responsive to ABA, suggesting that it imparts cold- and dehydration-regulated gene expression through an ABA-independent pathway.

A step toward increased understanding of the molecular mechanisms that control cold acclimation and, therefore, how low temperatures regulate gene expression was the isolation and characterization of a cDNA from Arabidopsis encoding a C-repeat/DRE/LTRE-binding protein, CBF1 (C-repeat/DRE-Binding Factor; Stockinger et al., 1997). *CBF1* was described as a single- or low-copy-number gene, the expression levels of which did not change appreciably in plants exposed to low temperatures or water stress. The deduced CBF1 amino acid sequence indicated that the protein had in its N-terminal region a potential nuclear localization sequence followed by an AP2 DNA-binding motif and an acidic C-terminal half that might act as an activator domain (Stockinger et al., 1997). Furthermore, expression analyses in yeast demonstrated that CBF1 could function as a transcriptional activator, since it promoted the transcription of a reporter gene containing the LTRE as an activator sequence. Recently, Jagglo-Ottosen et al. (1998)

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Abbreviations: DRE, drought-responsive element; LTRE, low-temperature-responsive element; ORF, open reading frame.

showed that overexpression of *CBF1* in Arabidopsis transgenic plants induced the expression of some cold-regulated genes and increased freezing tolerance of non-cold-acclimated, transformed plants. They concluded that CBF1 is a positive regulator of the cold-acclimation process, controlling gene expression and promoting freezing tolerance.

An interesting question raised from the results mentioned above is whether Arabidopsis contains homologs of *CBF1* or other genes encoding LTRE-binding proteins. Here we describe the identification and characterization of two Arabidopsis genes homologous to *CBF1*. Both genes, which we have called *CBF2* and *CBF3*, are organized in tandem with *CBF1* on chromosome 4 of Arabidopsis and constitute a small gene family. Like *CBF1*, both genes encode proteins containing putative nuclear localization signals, AP2 DNA-binding motifs, and potential acidic activation domains. We also show that expression of *CBF1*, *CBF2*, and *CBF3* is induced very early and in a transient manner during the process of cold acclimation but is not induced by ABA or dehydration treatments. Based on these results, we suggest a potential role for *CBF2* and *CBF3* in cold acclimation and freezing tolerance.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis Heyhn. ecotype Columbia (Col) was purchased from Lehle Seeds (Tucson, AZ). Four-week-old plants were used for all of the experiments. Plants were grown at 22°C under long-day photoperiods (16 h of cool-white fluorescent light, photon flux of 70 μmol m^{-2} s^{-1}) in pots containing a mixture of perlite, vermiculite, and sphagnum (1:1:1), and irrigated with water and mineral nutrient solution (Haughn and Somerville, 1986) once a week. Low-temperature treatments were performed at 4°C for different periods under the same light and photoperiodic conditions. For ABA treatments, plants were sprayed with 100 μM ABA and leaves were harvested 3 h later. The ABA stock solution (1 mM) was prepared in DMSO. Control plants were sprayed with water containing the same final concentration of the ABA solvent. Water stress was induced by transferring the plants to Petri dishes and allowing them to lose 50% of their fresh weight. After the treatments, leaves were immediately frozen in liquid nitrogen and stored at −80°C until their use.

Molecular Biology Methods

The Arabidopsis P1 genomic clone M7J2, corresponding to the bottom arm of chromosome IV, was sequenced in-frame as part of the European Arabidopsis Genome Sequencing Project. A shotgun library approach was used to determine the DNA sequence of the insert (Povinelli and Gibbs, 1993; Anderson et al., 1994). The DNA sequence was obtained by using the IR *Taq* DNA-sequencing kit (Boehringer Mannheim) and an automated DNA sequencer (Li-Cor, Lincoln, NE). Genomic DNA extractions were carried out according to the method of Dellaporta et al. (1983). Total RNA was isolated from different plant organs according to the method of Nagy et al. (1988). Restriction digestions, cloning, and DNA- and RNA-blot hybridizations were performed following standard protocols (Sambrook et al., 1989).

For DNA- and RNA-blot hybridizations, DNA probes were radioactively labeled with [α-^{32}P]dCTP using the Megaprime kit (Amersham). A 692-bp *Rsa*I fragment from *CBF2* and a 1124-bp *Hin*dIII fragment from *CBF3* containing corresponding coding regions were cloned into pBluescript and used as probes to simultaneously detect all members of the *CBF* gene family. Alternatively, DNA fragments partially encompassing the 3′-noncoding regions were used as gene-specific probes. The *CBF1*-specific probe consisted of a 181-bp PCR-amplified fragment containing 25 nucleotides of coding sequence and 156 nucleotides of 3′-noncoding region that was obtained by using the primers 5′-GTGAAGCAAAGAAGTAGAAAACG-3′ and 5′-GTGACGTGTCGCTTTGGAGTTAC-3′ (Stockinger et al., 1997).

The specific probe for the *CBF2* gene consisted of a 199-bp PCR-amplified fragment produced by using the primers 5′-GCATTTAAGAATAGCCCACAC-3′ and 5′-CGACGGCGATGATGACGACGT-3′. The fragment had 40 nucleotides of coding sequence and 159 nucleotides of 3′-noncoding region. The *CBF3*-specific probe consisted of a 227-bp PCR-amplified fragment covering 38 nucleotides of coding sequence and 189 nucleotides of 3′-noncoding region. The fragment was obtained by using the primers 5′-TATTTTGATTTGTTGCTTATGG-3′ and 5′-TCGAGGGAGATGATGACGTGTCC-3′. In addition, a 700-bp PCR-amplified fragment from the Arabidopsis *KIN1* gene was also used as a probe (Kurkela and Frank, 1990). This fragment was produced by using the primers 5′-CCCGGATCCGGCACCACCACTCCCTTTAG-3′and 5′-GGGGAATTCGAATATAAGTTTGGCTCGTC-3. Finally, as an RNA-loading control, a probe consisting of a 700-bp *Eco*RI-*Hin*dIII fragment corresponding to the *RBP4*-coding region (Kim et al., 1990) was used.

Databases were searched for sequence similarities using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (Altschul et al., 1997). A comparison of the nucleotide and amino acid sequences was performed with the software package PC/Gene 6.5 (Intelligenetics, Mountain View, CA). Putative phosphorylation sites were identified by comparing the amino acid sequences against all phosphorylation sites stored in the PROSITE pattern database (Bairoch et al., 1997).

RESULTS

Identification and Characterization of *CBF2* and *CBF3*, Two Genes Homologous to *CBF1*

The sequence of the Arabidopsis genomic clone M7J2 included one ORF, which, when compared with the databases, resulted in an identical coding sequence to the *CBF1* cDNA described by Stockinger et al. (1997). This clearly indicated that the *CBF1* gene does not have introns interrupting its ORF. Two new ORFs that showed a high degree

of similarity to *CBF1*, 81% and 84%, were also identified. These ORFs, which we named *CBF2* (accession no. AF062924) and *CBF3* (accession no. AF062925), contained 651 nucleotides each that were 84% identical to each other. Furthermore, when compared with *CBF1*, *CBF2* and *CBF3* did not appear to have any introns interrupting their ORFs. *CBF2* and *CBF3* were located 3 and 7 kb downstream of *CBF1*, respectively (Fig. 1A), suggesting that *CBF1*, *CBF2,* and *CBF3* constitute a small gene family organized as a cluster on chromosome IV of Arabidopsis. A search for additional *CBF* genes in the Arabidopsis genome was performed by DNA-blot hybridization experiments under low-stringency conditions. The probes used were DNA fragments containing *CBF2*- or *CBF3*-coding sequences (Fig. 1B). The results obtained revealed that both probes hybridized with the same restriction fragments in each digestion, the only difference being their relative intensities. Moreover, the number and molecular size of the fragments recognized by both probes were in agreement with the *CBF* gene organization shown in Figure 1A, indicating that no more *CBF*-related genes are present in the genome of Arabidopsis.

Analysis of the 5′ regions of *CBF1*, *CBF2,* and *CBF3* (Fig. 2) showed that these regions also have a moderate level of similarity. In addition, they showed the presence of sequences with similarity to known regulatory sequences identified in other plant genes. Thus, the core CANNTG consensus motif, as well as the CACGTC- and TACGTG-related sequences, which are present in the promoter region of many genes that are regulated by different environmental stresses and ABA (Guiltinan et al., 1990; Williams et al., 1992; Busk and Pages, 1998), were found in the promoters of the *CBF* genes. Furthermore, the pentamer CAGCC, which corresponds to the LTRE core sequence (CCGAC) in reverse orientation, was present in the *CBF* promoters. The sequence CCGTC, which differs in only one nucleotide from the LTRE motif, was also found in the 5′ region of *CBF1* (Fig. 2).

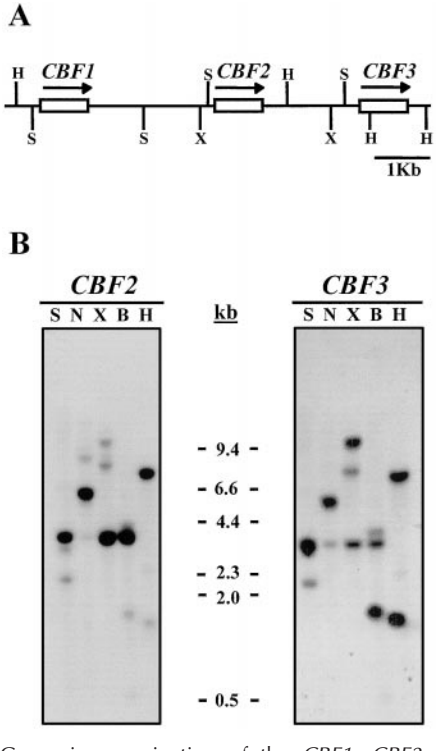

Figure 1. Genomic organization of the *CBF1, CBF2,* and *CBF3* genes. A, Physical map of *CBF* genes on chromosome IV of Arabidopsis. The ORFs are shown with open bars. The restriction sites are *Hin*dIII (H), *Xba*I (X), and *Sca*I (S). The direction of transcriptions is indicated by arrows. B, DNA-blot hybridizations of Arabidopsis genomic DNA (4 μg) digested with *Sca*I (S), *Nde*I (N), *Xba*I (X), *Bgl*II (B), and *Hin*dIII (H). The probes used were the 692-bp *Rsa*I fragment from *CBF2* and the 1124-bp *Hin*dIII fragment from *CBF3*, as described in "Materials and Methods." The positions of molecular size markers are in the center.

The coding regions of *CBF2* and *CBF3* encoded two polypeptides of 216 amino acids each, with a predicted molecular mass of 24 kD. The pIs for both polypeptides were low: 5.0 for CBF2 and 4.9 for CBF3. The amino acid alignment of CBF2 and CBF3 revealed that 76% of the residues were identical and 85% were similar (Fig. 3A). When compared with CBF1, CBF2 and CBF3 also showed a very high degree of similarity (84% and 86%, respectively; Fig. 3A). Like CBF1, CBF2 and CBF3 included in their N-terminal regions basic residues that potentially represent nuclear localization signals (Raikhel, 1992) and putative AP2 DNA-binding domains (Weigel, 1995; Ohme-Takagi and Shinshi, 1995). Comparison of the AP2 domains from the CBF proteins and the Arabidopsis DNA-binding protein AtEBP (Buttner and Singh, 1997) revealed high similarity (Fig. 3B). Furthermore, CBF2 and CBF3 also contained acidic C-terminal fragments (pIs of 3.8 and 3.6, respectively) that might act as transcriptional activation domains (Hahn, 1993). In addition, they showed potential recognition sites for protein kinase C and casein kinase II (Kennelly and Krebs, 1991). Some of these sites were conserved among the three CBF polypeptides as Ser-13, Ser-56, which is inside of the AP2 domain, and Thr-151 (Fig. 3A).

The Expression of *CBF1*, *CBF2*, and *CBF3* Genes Is Specifically Induced by Low Temperature in Different Organs of Arabidopsis

Although *CBF1* was first described as not regulated by low temperature (Stockinger et al., 1997), the fact that its overexpression in transgenic plants induces the accumulation of some cold-regulated transcripts (Jagglo-Ottosen et al., 1998) suggested that its expression could be induced by low temperature. In addition, the sequences found in the 5′ regions of all of the *CBF* genes that show similarity to regulatory elements (see above) supported this assumption.

To analyze whether the expression of *CBF* genes is induced by low temperature, total RNA was prepared from leaves of 4-week-old Arabidopsis plants exposed to 4°C for different periods. Considering the high similarity among the coding sequences of the three *CBF* genes analyzed, *CBF1*-, *CBF2*-, and *CBF3*-specific transcripts were identified by RNA-blot hybridizations with specific probes prepared from DNA fragments corresponding to their 3′-untranslated regions (see "Materials and Methods"). DNA-blot hybridizations showed that each probe hybridized with

Figure 2. Alignment of the 5′ upstream sequences of *CBF1*, *CBF2*, and *CBF3*. Six-hundred-eighty bases of the 5′-untranslated region of *CBF1* were aligned with 720 and 702 bases of the corresponding regions of *CBF2* and *CBF3*, respectively. Asterisks indicate nucleotides identical to the *CBF1* sequence. Hyphens indicate gaps inserted in the sequences for better alignment. The initiation ATGs and putative TATA boxes (TATAAA) are double underlined. CANNTG motifs and related sequences CACGTC and TACGTG are shown in gray boxes. Black boxes highlight the CAGCC pentamers. The CCGTC sequence is single underlined.

only a single restriction fragment in each digestion, demonstrating that it specifically recognized the corresponding gene (not shown). As shown in Figure 4, *CBF1*, *CBF2*, and *CBF3* transcripts accumulated with very similar kinetics in response to low temperatures. An increase of *CBF* mRNA levels was already detectable after 30 min of exposure to low temperature, reaching maximal levels of accumulation after 1 h. This increase was transient, since transcript levels decreased thereafter.

To date, most characterized cold-regulated genes are also responsive to ABA and water stress (Thomashow, 1994; Hughess and Dunn, 1996; Rouse et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Capel et al., 1997; Hong et al., 1997; Kirch et al., 1997). To determine whether the accumulation of *CBF* mRNAs was specifically regulated by low temperature or was also responsive to these treatments, Arabidopsis plants were either treated with 100 μM ABA or dehydrated until losing 50% of their initial fresh weight. Total RNAs isolated from these plants and from controls were used to perform RNA-blot hybridizations with the *CBF*-specific probes previously described. Figure 4 shows that *CBF* transcripts did not accumulate in response to ABA or dehydration, indicating that the expression of *CBF* genes is specifically regulated by low temperature. As positive controls for treatments and RNA loading, hybridizations with *KIN1* and *RBP4* probes were carried out, respectively (Fig. 4). The *KIN1* probe recognizes a gene from Arabidopsis, the expression of which is induced by low temperature, ABA, and dehydration treatments (Kurkela and Franck, 1990), whereas the *RBP4* probe recognizes an Arabidopsis gene that is expressed constitutively (Kim et al., 1990).

The accumulation of *CBF* mRNAs in different organs of Arabidopsis in response to low temperature was studied in total RNA isolated from leaves, stems, and roots of plants exposed to 4°C for 1 h. As controls, RNAs from the same organs of plants grown at 20°C were also isolated. RNA-blot hybridizations were performed with the *CBF*-specific probes used in previous experiments. The results obtained revealed that, in response to low temperature, *CBF* transcripts accumulated to similar levels in all organs analyzed (Fig. 5). To monitor RNA loading, hybridizations with the *RBP4* probe (see above) were carried out with the same membranes.

DISCUSSION

A significant step toward understanding the molecular mechanisms that control the process of cold acclimation was the recent isolation of a cDNA corresponding to Arabidopsis CBF1, the first identified C-repeat/DRE/LTRE-binding factor (Stockinger et al., 1997). Here we present the identification and characterization of two novel Arabidopsis genes, *CBF2* and *CBF3*, both of which are highly similar to *CBF1.* Furthermore, we demonstrate that the expression of all three *CBF* genes is regulated by low temperature.

Genomic sequence analyses indicated that *CBF1*, *CBF2*, and *CBF3* do not show introns among their coding sequences and are organized in tandem on chromosome 4 of Arabidopsis. The high similarity existing among *CBF1*, *CBF2*, and *CBF3*, together with their close linkage and the fact that they have the same transcriptional orientation, clearly suggests a common origin, probably by two consecutive duplications of an ancestral gene and subsequent divergence through mutations. It is interesting that the same origin has been proposed for the members of five families of low-temperature-responsive genes from Arabidopsis, in which the homologous genes are arranged in tandem in the genome (Nordin et al., 1991; Gilmour et al., 1992; Kurkela and Borg-Franck, 1992; Horvath et al., 1993; Wilhelm and Thomashow, 1993; Yamaguchi-Shinozaki and Shinozaki, 1993; Welin et al., 1994; Capel et al., 1997). However, the reason that low-temperature-regulated genes are so frequently organized in tandem remains unknown.

The 5′ regulatory sequences of *CBF1*, *CBF2*, and *CBF3* have diverged more than the coding regions but still keep a high level of similarity, which may result in the identical expression patterns shown by these genes. The fact that

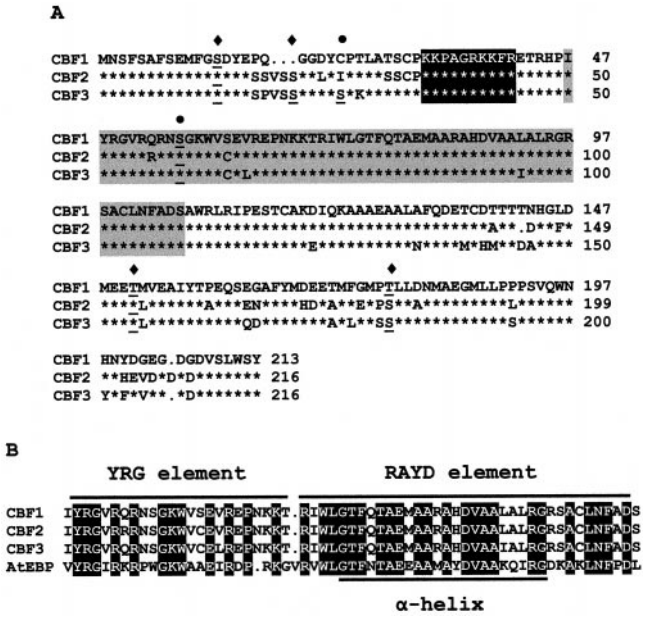

Figure 3. Amino acid sequences of CBF1, CBF2, and CBF3 proteins. A, Sequence alignment. Amino acid residues identical to the CBF1 sequence at a given position are indicated by asterisks. Points represent gaps inserted in the sequences for better alignment. Black box corresponds to the potential nuclear localization signals. Gray boxes highlight the AP2 domains. Underlined amino acids indicate potential recognition sites for protein kinase C (●) and casein kinase II (◆). B, Comparison of the AP2 domains from CBF1, CBF2, and CBF3 proteins and the Arabidopsis ethylene-responsive element-binding protein AtEBP (Bütner and Singh, 1997). Identical amino acids are highlighted in black boxes. Points represent gaps inserted in the sequences for better alignment. Residues belonging to the conserved AP2 domain elements YRG and RAYD (Okamuro et al., 1997) are indicated. Amino acids in the RAYD conserved element predicted to form an amphipatic α-helix that might promote DNA binding (Okamuro et al., 1997) are underlined.

CBF1, *CBF2*, and *CBF3* are not responsive to ABA seems to indicate that the CANNTG sequence, repeated several times in their upstream regions, is not sufficient to confer ABA responsiveness in the context of *CBF* promoters. The consensus LTRE core sequence, CCGAC, which has been reported to be essential for the low-temperature responsiveness of several genes (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki 1994; Jiang et al., 1996; Ouellet et al., 1998), is not found in the *CBF* promoters. Only the variant CCGTC, which differs in just one nucleotide from the LTRE, is present in the 5′ region of *CBF1*. Whether this sequence can confer the low-temperature response remains to be seen. However, it is interesting to emphasize that the pentamer CAGCC, which is the LTRE core sequence in reverse orientation, is present in the promoters of all *CBFs*.

CBF2 and CBF3 polypeptides contain a 60-amino acid motif, the AP2 domain, that is evolutionarily conserved in plants (Okamuro et al., 1997) and has been described as a DNA-binding domain (Ohme-Takagi and Shinshi, 1995; Weigel, 1995). CBF2 and CBF3, like CBF1, also have potential nuclear localization sequences in their N-terminal regions and acidic C-terminal fragments. Moreover, CBF1,

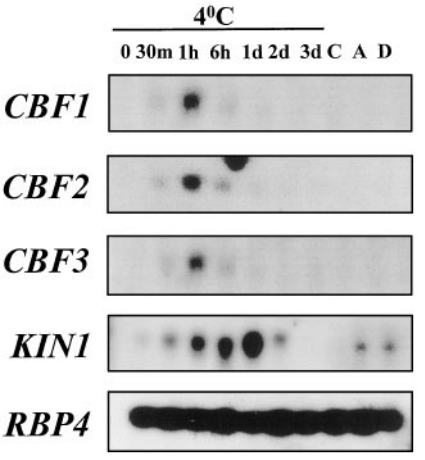

Figure 4. *CBF1*, *CBF2*, and *CBF3* transcripts accumulate in response to low temperature but not in response to ABA or water stress. RNA-blot hybridizations were performed with total RNA (10 μg per lane) isolated from leaves exposed to 4°C for the indicated time, sprayed with 100 μM ABA (A), sprayed with the ABA solvent (C), or dehydrated until losing 50% of their fresh weight (D). The probes used were the 3′ fragments of *CBF1*, *CBF2*, and *CBF3* and the fragments of the *KIN1* and *RBP4* genes described in "Materials and Methods."

CBF2, and CBF3 show potential recognition sites for protein kinase C and casein kinase II. Some of these sites are conserved among the three proteins, and one of them, Ser-56, is located in the AP2 domain. Recently, Vazquez-Tello et al. (1998) proposed that the expression of *WCS120*, a low-temperature-inducible gene from wheat that contains two LTREs in its promoter region, may be regulated by nuclear factors whose binding activity is modulated by phosphorylation/dephosphorylation mechanisms. We speculate that the potential phosphorylation sites found in the CBF proteins may play an important role in their functions.

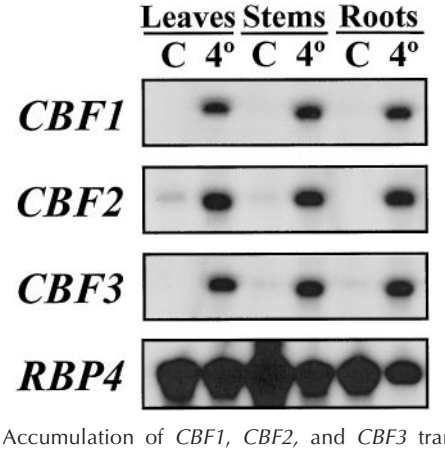

Figure 5. Accumulation of *CBF1*, *CBF2*, and *CBF3* transcripts in different organs of Arabidopsis in response to low temperature. RNA-blot hybridizations were carried out with total RNA (15 μg per lane) isolated from leaves, stems, and roots of plants grown at 22°C (C) or exposed to 4°C for 1 h (4°). The specific probes for the *CBF* genes and the probe used for *RBP4* are described in "Materials and Methods."

Expression analyses revealed that *CBF2* and *CBF3* are positively regulated by low temperature. Furthermore, in contrast to previous data (Stockinger et al., 1997), *CBF1* transcripts also accumulated in response to low temperature in our experimental conditions. The cold-inducible expression of *CBF* genes does not show marked differences. It is not organ specific, since CBF mRNAs accumulate to similar levels in different organs of Arabidopsis, and is transiently regulated. The accumulation of *CBF* transcripts increases rapidly after plants are transferred to low-temperature conditions, reaching the highest levels after 1 h of exposure and decreasing thereafter. This expression pattern suggests that *CBF* genes should be involved in responses that are transiently produced when plants are exposed to low temperatures, and fits with the notion that their induction should be an early amplification event in the low-temperature-induced signaling cascade, preceding and prompting the accumulation of transcripts corresponding to *CBF*-regulated genes. On the other hand, the expression of *CBF* genes is not regulated by ABA treatment or water stress, two conditions that have been shown to increase plant freezing tolerance (Cloutier and Siminovitch, 1982; Chen and Gusta, 1983; Mäntylä et al., 1995) and induce the expression of most cold-inducible genes (Thomashow, 1994; Hughes and Dunn, 1996; Rouse et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Capel et al., 1997; Hong et al., 1997; Kirch et al., 1997).

CBF1 has been described as a transcriptional activator that binds to the LTRE sequence, inducing the expression of some low-temperature genes and increasing freezing tolerance (Stockinger et al., 1997; Jagglo-Ottosen et al., 1998). Our data indicate that *CBF2* and *CBF3* have a high degree of homology with *CBF1* (>84%) and show an identical expression pattern. This strongly suggests that they may fulfill a similar function as *CBF1*, controlling the level of low-temperature-regulated gene expression and promoting freezing tolerance. The fact that *CBF* genes are not responsive to ABA indicates that they should be involved in regulating the expression of low-temperature-inducible genes through an ABA-independent pathway. Moreover, taking into consideration that LTRE is able to confer response to dehydration (Yamaguchi-Shinozaki and Shinozaki, 1994), the fact that the expression of *CBF* genes is not induced by this stress suggests that LTRE-binding proteins other than *CBFs* should mediate dehydration-regulated gene expression through LTREs.

Recently, the isolation and characterization of five Arabidopsis cDNAs encoding LTRE-binding proteins have been reported (Liu et al., 1998). Three of these proteins, designated DREB1A, DREB1B, and DREB1C, correspond to CBF2, CBF1, and CBF3, respectively. The other two proteins, named DREB2A and DREB2B, also contain AP2 DNA-binding domains, basic residues in their N-terminal regions that might function as nuclear localization signals, and acidic C-terminal regions that might act as transcriptional activation domains. Transactivation experiments using Arabidopsis protoplasts from leaves revealed that CBF2 (DREB1A) and DREB2A proteins can function as transcriptional activators. Furthermore, overexpression of *CBF2* (*DREB1A*) cDNA resulted in transgenic plants showing strong expression of the target genes under unstressed conditions, an increase in tolerance to freezing and dehydration, and a dwarfed phenotype. In contrast, transgenic plants overexpressing *DREB2A* cDNA revealed few phenotypic changes and showed weak expression of the target genes under unstressed conditions. It is interesting that the expression of the *DREB2A* and *DREB2B* genes seems to be strongly induced in leaves by dehydration and high-salt stress, but not by low temperature, as with the *CBF* (*DREB1*) genes. All of these results confirm that, like CBF1, CBF2 and CBF3 are *trans*-acting factors that regulate low-temperature-induced gene expression promoting freezing tolerance. The results also show that DREB2 is an independent family of LTRE-binding proteins that function in a separate signal transduction pathway under dehydration conditions.

Considering the large number of genes whose expression is induced in response to low temperature, we hypothesized that differences in the sequences of the CCGAC core element and/or in the sequences that surround it may result in the recruitment of distinct CBF proteins. A similar situation has been described for the G-box sequence CANNTG and the bZIP proteins (Williams et al., 1992). The availability of all *CBF* genes makes it possible to perform in vivo and in vitro experiments to test this hypothesis.

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