CBF2/DREB1C is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in *Arabidopsis*

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CBF/DREB1 **(C-repeat-binding factor/dehydration responsive element-binding factor 1) genes encode a small family of transcriptional activators that have been described as playing an important role in freezing tolerance and cold acclimation in** ***Arabidopsis*****. To specify this role, we used a reverse genetic approach and identified a mutant,** ***cbf2*****, in which the** ***CBF2/DREB1C*** **gene was disrupted. Here, we show that** ***cbf2*** **plants have higher capacity to tolerate freezing than WT ones before and after cold acclimation and are more tolerant to dehydration and salt stress. All these phenotypes correlate with a stronger and more sustained expression of CBF/DREB1-regulated genes, which results from an increased expression of** ***CBF1/DREB1B*** **and** ***CBF3/DREB1A*** **in the mutant. In addition, we show that the expression of** ***CBF1/DREB1B*** **and** ***CBF3/DREB1A*** **in response to low temperature precedes that of** ***CBF2/DREB1C*****. These results indicate that CBF2/DREB1C negatively regulates** ***CBF1/DREB1B*** **and** ***CBF3/DREB1A*****, ensuring that their expression is transient and tightly controlled, which, in turn, guarantees the proper induction of downstream genes and the accurate development of** ***Arabidopsis*** **tolerance to freezing and related stresses.**

Freezing temperatures greatly limit the geographical distribution and growing season of plants and cause negative effects on crop quality and productivity. As a consequence, appreciable effort has been conducted to determine the adaptive mechanisms plants have evolved to survive this adverse environmental condition. Many plants, including *Arabidopsis*, increase their freezing tolerance in response to low, nonfreezing temperatures. This process, called cold acclimation (1), is complex and involves a number of biochemical and physiological changes, ranging from alterations in lipid composition to accumulation of sugars (2). Different studies have suggested that low-temperature-regulated gene expression is critical in plants for cold acclimation (2). Low-temperature-responsive genes encode a diverse number of proteins, including enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants, molecular chaperones, antifreeze proteins, among others, with a believed function in freezing tolerance (2).

During the past few years, substantial progress has been made toward understanding how low temperatures regulate gene expression. In particular, a family of transcription factors in *Arabidopsis* known either as C-repeat-binding factor (CBF)1, CBF2, and CBF3 (3, 4) or dehydration-responsive element-binding factor (DREB)1B, DREB1C, and DREB1A (5), respectively, has been identified. These factors belong to the Apetala 2/ethylene-responsive element-binding protein (AP2/EREBP) family of DNA-binding proteins (6) and bind to the cold- and dehydration-responsive DNA regulatory element (DRE) (7), also termed C-repeat (CRT) (8). CRT/DRE elements contain the conserved CCGAC core sequence, which is sufficient to induce gene transcription under cold stress (7, 8) and is present in the promoters of many cold-inducible genes (2). Interestingly, the *CBF/DREB1* genes do not contain the CCGAC sequence in their promoters but are also induced by low temperature. This induction is transient and precedes that of cold-inducible genes with the CRT/DRE cis-element (4, 5, 9). Ectopic overexpression of *CBF1/DREB1B* and *CBF3/DREB1A* in *Arabidopsis* results in the constitutive expression of downstream cold-inducible genes, even at warm temperatures and in increased freezing tolerance (5, 10–12), suggesting that *CBF/DREB1* genes may play an important role in cold acclimation. In addition, overexpression of *CBF3/DREB1A* also enhances drought and salt tolerance (5, 11). To our knowledge, overexpression of *CBF2/DREB1C* has not been reported.

Unfortunately, mutant plants in the *CBF/DREB1* genes have not been so far identified, which has prevented the analysis of their actual contribution to the cold-acclimation response. In fact, despite the extensive investigations carried out, our understanding of *CBF/DREB1* gene function(s) remains elusive, and a clear role of their requirement for stress tolerance has not still emerged. For example, whether all three *CBF/DREB1* genes are required for freezing tolerance and cold acclimation and how the expression of *CBF/DREB1* genes is regulated in response to low temperatures are essential questions that are still unanswered. To dissect the precise role of these genes and shed some light on these issues, we screened a transferred DNA (T-DNA) mutagenized population of *Arabidopsis* for plants containing T-DNA insertions in the *CBF/DREB1* genes. Here, we report on the isolation and characterization for the first time of a mutant plant in which a *CBF/DREB1* gene, namely *CBF2/DREB1C*, is disrupted. The results obtained indicate that CBF2/DREB1C plays a critical role in the development of *Arabidopsis* tolerance to freezing and other related stresses by controlling the precise expression of *CBF1/DREB1B* and *CBF3/DREB1A* and, hence, that of the downstream genes. On the basis of these results, a model for the function of CBF2/DREB1C in cold acclimation and the regulation of *CBF/DREB1* gene expression in response to low temperature is proposed.

Materials and Methods

Plant Materials, Growth Conditions, and Treatments. Seeds from *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia, were purchased from Lehle Seeds (Round Rock, TX). Plants were grown in pots containing a mixture of organic substrate and vermiculite (3:1, vol/vol) and irrigated with mineral nutrient solution (13) once a week. Plants for dehydration and salt tolerance assays were grown under sterile conditions in Petri dishes containing GM medium (Murashige and Skoog medium (14) supplemented with 1% sucrose) solidified with 0.8% (wt/vol) agar. In all cases, plants were developed at 20°C under a long-day photoperiod (16

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Abbreviations: CRT, C-repeat; CBF, CRT-binding factor; DREB, dehydration-responsive element-binding factor; FW, fresh weight; T-DNA, transferred DNA; DRE, dehydration-responsive DNA-regulatory element.

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h of cool-white fluorescent light, photon flux of 70 μM m^{-2}·s^{-1}). All treatments were performed on 3-week-old plants.

Low-temperature treatments were performed by transferring plants to a growth chamber set to 4°C for different periods of time under the light and photoperiodic conditions described above. Freezing assays were carried out in a temperature programmable freezer. Nonacclimated or cold-acclimated (7 days at 4°C) plants were exposed to 4°C for 30 min in darkness and subsequently temperature was lowered by 2°C per h. The final desired freezing temperature was maintained for 6 h, and then the temperature was increased again to 4°C at the same rate. After thawing at 4°C for 4 h in the dark, plants were returned to their original growth conditions (see above). Tolerance to freezing was determined as the capacity of plants to resume growth after 7 days of recovery under control conditions. Dehydration was induced by removing plants from the medium, placing them on a dry filter paper, and allowing them to develop for 2 days without watering. The rate of dehydration was estimated as the percentage of initial fresh weight (FW) that remains after treatment. Salt stress was accomplished by transferring plants to new Petri dishes containing the agar medium plus 100 mM NaCl. Tolerance was estimated by determining the root elongation and the FW of the plants after 7 days of treatment. After low-temperature treatment, plants used for RNA-blot hybridizations were immediately frozen in liquid N_2 and stored at −80°C until their use.

Identification of the *cbf2* Mutant. The *cbf2* mutant was identified by PCR screening of ≈30,000 *Arabidopsis* T-DNA insertion lines (J.M.A. and J.R.E., unpublished data), by using specific oligonucleotides for the *CBF2/DREB1C* gene (5′-TCCGGTTTCCTCAGGCGGTGATTACA-3′ and 5′-TAAGGACACGTCATCATCTCCCTGAC-3′) and the T-DNA (5′-GCTCATGATCAGATTGTCGTTTCCCGCCTT-3′ and 5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′). DNA sequencing showed that the T-DNA insertion was 179 bp upstream of the start codon of *CBF2/DREB1C*.

Cosegregation Analysis. Cosegregation of the T-DNA insertion with the mutant (stress-tolerant) phenotypes was determined by crossing homozygous *cbf2* plants to WT *Arabidopsis* of the same ecotype (Columbia). For cosegregation analysis, the genotype of segregating F_2 plants was analyzed by PCR with *CBF2/DREB1C* and T-DNA-specific primers. More than 95% of homozygous *cbf2* plants showed tolerant phenotypes, and 96% of plants that were homozygous WT displayed WT phenotypes. Several homozygous WT and mutant F_2 plants were self-crossed and their descendents analyzed. Ninety percent of the progeny from the *cbf2* homozygous parents were phenotypically mutant, and 91% of the progeny from WT parents were phenotypically WT, demonstrating that the mutant phenotypes are genetically linked to the *cbf2* locus.

Genetic Complementation Analysis. A 1,810-bp DNA fragment containing the *CBF2/DREB1C* gene and 1,138 bp of its native promoter was obtained by PCR. The fragment was then cloned upstream of the nopaline synthase terminator in pCAMBIA1381 binary vector. The resulting plasmid was introduced into *Agrobacterium* C58C1 and used for transformation of *cbf2* mutant. T_1 plants were selected by using hygromycin.

Molecular Biology Methods. Total RNA was isolated as described (15). Restriction digestions, cloning, and RNA-blot hybridizations were performed by following standard protocols (16). Specific probes for the *CBF/DREB1* genes have been described (9). The probe for *LTI78* was a 1.0-kb genomic fragment produced by using the primers 5′-CGGGATTTGACGGAGAACCA-3′ and 5′-ACCATAATACATCAAAGACG-3′. The probe for *KIN-1* was a 700-bp genomic fragment obtained by using the primers 5′-GGCACCACACTCCCTTTAGC-3′ and 5′-GAATATAAGTTTGGCTCGTC-3′. The *COR47* and *COR15A* probes were 400-bp and 1.0-kb fragments, respectively, obtained from the corresponding cDNAs (17, 18). The *RCI1A* probe consisted of a 200-bp DNA fragment corresponding to the 3′ untranslated region (19). The *RCI2A* probe consisted of a 300-bp DNA fragment from the 3′ noncoding region (20). The probe for *DREB2A* was a 300-bp genomic fragment produced by using the primers: 5′-GATGTGGATCAGAGTCACTT-3′ and 5′-CAACAGTCGTTGTGGGATTAAGG-3′. The *L18* probe was a 791-bp cDNA fragment (21). Equal RNA loading in the experiments was monitored by rRNA staining. RNA samples for each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice.

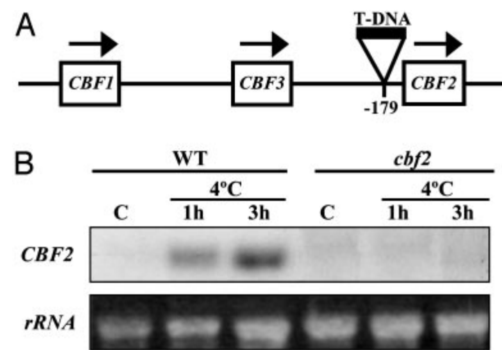

Fig. 1. Structure of *CBF/DREB1* cluster and T-DNA insertion in the *CBF2/DREB1C* gene. (*A*) Schematic representation of the *CBF/DREB1* cluster with the T-DNA insertion. The site of insertion in the *cbf2* mutant, 179 bp upstream of the *CBF2/DREB1C* start codon, is represented. Arrows indicate the direction of transcription in the *CBF/DREB1* genes. The *CBF* locus is not drawn to scale. (*B*) RNA-blot hybridization by using a specific probe for *CBF2/DREB1C* and total RNA (20 μg) prepared from 3-week-old rosette leaves of WT and *cbf2* mutant plants grown under control conditions (C) or exposed to 4°C for 1 h and 3 h. Equal amounts of RNA were present in each sample as confirmed by ethidium bromide staining of rRNAs.

Results

Isolation and Characterization of the *cbf2* Mutant. A T-DNA mutagenized population of *Arabidopsis* was screened by PCR for plants containing an insertion in the *CBF/DREB1* genes. From a population of ≈30,000 individual T-DNA insertion lines, we identified a single mutant plant (*cbf2*) bearing a disruption mutation in *CBF2/DREB1C*. Sequence analysis revealed that the insertion was within the putative TATA box (9), 179 bases upstream of the start codon (Fig. 1*A*). The progeny of heterozygous plants showed a segregation of the *cbf2* phenotypes (described below) of ≈1:3 between mutant and WT (data not shown), indicating that *cbf2* is a recessive mutation in a single nuclear gene. RNA-blot analysis confirmed that the *CBF2/DREB1C* mRNA did not accumulate in the mutant after cold stress treatment (Fig. 1*B*), suggesting that the *cbf2* is a null or severely hypomorphic allele. Compared to the WT under control growth conditions, *cbf2* mutant plants did not exhibit any obvious morphological or developmental abnormality.

The *cbf2* Mutation Enhances Constitutive Freezing Tolerance and Cold Acclimation. To understand the precise role of *CBF2/DREB1C* in cold acclimation, we examined the freezing tolerance of the *cbf2* mutant. Plants, with or without cold acclimation for 7 days at 4°C, were exposed for 6 h to different freezing temperatures. Plant survival was scored after 7 days of recovery under control growth conditions. Unexpectedly, both nonacclimated and cold-acclimated mutant plants were significantly more tolerant to freezing than the corresponding WT plants (Fig. 2). The enhancement of freezing tolerance in *cbf2* plants compared with WT was very similar before

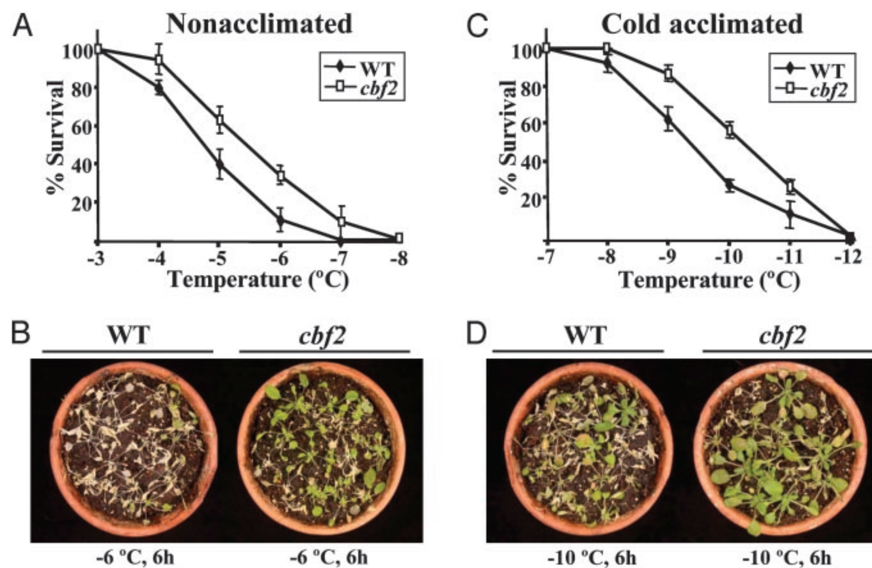

Fig. 2. Freezing tolerance of *cbf2* mutant plants. Three-week-old WT and *cbf2* plants grown under long-day photoperiods at 20°C were exposed to different freezing temperatures for 6 h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7 days of recovery under unstressed conditions. (*A*) Tolerance of nonacclimated plants. (*B*) Representative nonacclimated WT and *cbf2* plants 7 days after being exposed to −6°C for 6 h. (*C*) Tolerance of cold-acclimated (7 days at 4°C) plants. (*D*) Representative cold-acclimated WT and *cbf2* plants 7 days after being exposed to −10°C for 6 h. In *A* and *C*, data are expressed as means of three independent experiments with 50 plants each. Bars indicate SE.

and after cold acclimation. In fact, a comparison of LT_{50} (temperature that causes 50% lethality) values between nonacclimated mutant and WT plants showed that *cbf2* plants had LT_{50} values of −5.7°C, whereas WT plants had a LT_{50} of −4.8°C (Fig. 2*A*). In the case of cold-acclimated plants, the LT_{50} values of *cbf2* and WT plants were −10.4°C and −9.4°C, respectively (Fig. 2*C*). The increased freezing tolerance manifested by the mutant with respect to the WT was very apparent in both nonacclimated (Fig. 2*B*) and cold-acclimated (Fig. 2*D*) plants. Therefore, the *cbf2* mutation does not impair cold acclimation. Rather, it actually enhances both the constitutive freezing tolerance and the freezing tolerance of *Arabidopsis* after cold acclimation. These results indicate that CBF2/DREB1C acts as a negative regulator of freezing tolerance in *Arabidopsis*.

***cbf2* Mutant Plants Show Increased Tolerance to Dehydration and Salt Stress.** To characterize the capacity of the *cbf2* mutant to respond to other types of stresses, we examined its tolerance to dehydration and high salt. Dehydration was induced by maintaining plants on a dry filter paper for 2 days without watering. The rate of dehydration was determined as the percentage of initial FW remaining after treatment. Mutant and WT plants did not show significant differences in their initial FW values (data not shown). After dehydration, *cbf2* plants maintained an average of 22% of their initial FW, whereas WT plants maintained only 13.5% (Fig. 3*A*). No differences in stomatal closure were found in any case (data not shown). Correspondingly, *cbf2* plants had a much less severe dehydration phenotype than that observed in WT plants (Fig. 3*B*). This indicates that the mutation at the *CBF2/DREB1C* gene significantly increases the tolerance of *Arabidopsis* to dehydration. The tolerance to salt stress was estimated by determining the root elongation in *cbf2* and WT plants after growing for 7 days in a medium containing 100 mM NaCl. WT and *cbf2* mutant plants showed identical root elongation under control conditions. The FW of the plants after the stress treatment also provided to be an estimate of their salt tolerance. Mutant plants subjected to salt stress displayed 60% and 66% of the root elongation and FW shown by unstressed WT plants. In turn, WT plants only showed 48% and 40% of the root elongation and FW that they displayed under control conditions (Fig. 3*C*). These significant differences among *cbf2* and WT plants were clearly apparent at the phenotypic level (Fig. 3*D*) and demonstrate that the *cbf2* mutant also has an increased tolerance to salt stress. Taken together, these data reveal that, in addition to freezing tolerance, CBF2/DREB1C acts as a negative regulator of dehydration and salt tolerance in *Arabidopsis*. Importantly, the freezing, dehydration, and salt tolerance phenotypes shown by *cbf2* were found to be genetically linked to the T-DNA insertion, as demonstrated by cosegregation analysis (see *Materials and Methods*).

The *cbf2* Mutation Enhances Transcription of *CBF1/DREB1B*, *CBF3/DREB1A*, and Downstream Genes. In an attempt to reconcile the unexpected results described above with earlier predictions on the role of CBF2/DREB1C, we examined the impact of the *cbf2* mutation on the transcript levels of several cold-inducible genes. The *LTI78*, *KIN1*, *COR47*, and *COR15A* genes have been described as CBF/DREB1 target genes and to be part of the CBF/DREB1 regulon (22, 23). Interestingly, mRNAs for these cold-inducible genes were found to be constitutively expressed at low levels in the *cbf2* mutant under unstressed control conditions (Fig. 4*A*). After cold treatment, the levels of these mRNAs were further induced and more sustained than in the WT (Fig. 4*A*). Moreover, the effects of the *cbf2* mutation on the expression of CBF/DREB1 target genes seem to be specific because the expression of *RCI1A*, *RCI2A*, and *DREB2A* cold-inducible genes (19, 20, 24), which do not contain CRT/DRE elements in their promoters (ref. 25; GenBank accession nos. AL391145 and AB010692), was unaffected in control and cold-treated *cbf2* plants (Fig. 4*A*). We conclude from these results that CBF2/DREB1C negatively regulates the transcription of downstream CBF/DREB1 target genes and is essential for the accurate expression of these genes in response to low temperature.

Overexpression of *CBF1/DREB1B* and *CBF3/DREB1A* in transgenic *Arabidopsis* has been reported to induce constitutive expression of the CRT/DRE-containing genes, which in turn would promote freezing, dehydration and salt tolerance (5, 10–12). Furthermore, a positive correlation was found between the expression levels of *CBF1/DREB1B* and *CBF3/DREB1A*, the level of expression of the CBF/DREB1-target genes, and the

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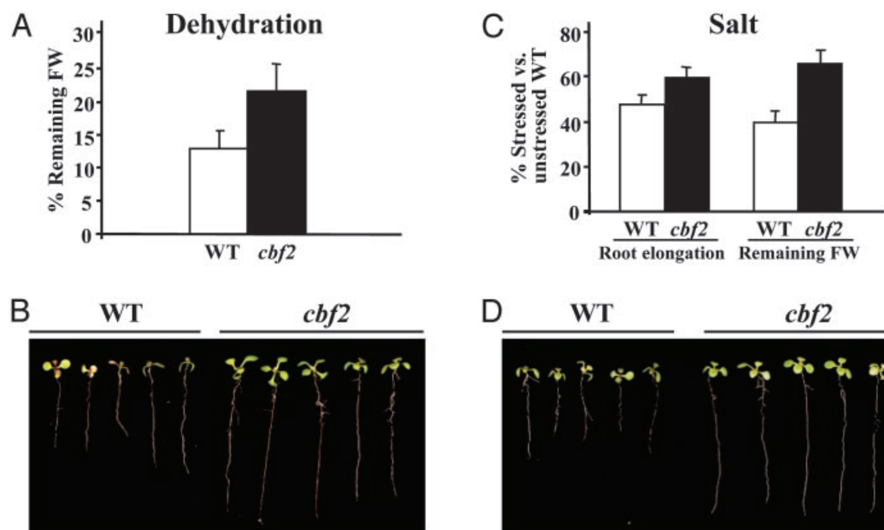

Fig. 3. Tolerance to dehydration and salt stress of *cbf2* mutant plants. (*A*) Dehydration tolerance of 3-week-old WT and *cbf2* plants. Tolerance was estimated as the percentage of initial FW that remains after transferring plants to a dry filter paper and allowing them to develop for 2 days without watering. (*B*) Representative WT and *cbf2* plants after dehydration treatment. (*C*) Salt tolerance of 3-week-old WT and *cbf2* plants. Tolerance was estimated by determining the root elongation and FW of plants transferred to a medium containing 100 mM NaCl for 7 days. These values are represented as a percentage of root elongation and FW of WT unstressed plants. (*D*) Representative WT and *cbf2* plants after salt treatment. In *A* and *C*, data are expressed as means of three independent experiments with 20 plants each. Bars indicate SE. Values obtained from WT and *cbf2* were in all cases significantly different ($P < 0.05$) as determined by Student's *t* test.

level of tolerance to stress conditions (5, 10–12). One hypothesis to explain the intriguing physiological and molecular phenotypes shown by the *cbf2* mutant is that the level of expression of *CBF1/DREB1B* and/or *CBF3/DREB1A* under control conditions and/or in response to low temperature may be higher than in the WT. RNA-blot analysis revealed that under control conditions, the transcript levels of both *CBF1/DREB1B* and *CBF3/DREB1A* were higher in *cbf2* plants than in the WT. After cold treatment, the level of expression of *CBF1/DREB1B* and *CBF3/DREB1A* was more sustained in the *cbf2* mutant compared to the WT (Fig. 4*B*). These data indicate that CBF2/DREB1C acts to negatively regulate the expression of *CBF1/DREB1B* and *CBF3/DREB1A*. Nevertheless, because the three *CBF/DREB1* genes are arranged in tandem, the possibility exists that the T-DNA insertion in the *cbf2* mutant could induce directly the expression of *CBF1/DREB1B* and *CBF3/DREB1A*. Complementation of the mutant with the *CBF2/DREB1C* gene driven by its native promoter reestablished the WT expression patterns of *CBF1/DREB1B* and *CBF3/DREB1A* and those of their target genes (Fig. 4*C*). This complementation confirms that *CBF2/DREB1C* is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A*. As expected from these results, the expression of *CBF2/DREB1C* in *cbf2* mutant also reestablished the WT phenotypes to freezing, dehydration, and salt tolerance (data not shown).

Contrary to what was described in *CBF3/DREB1A*-overexpressing plants (10–12), *cbf2* mutants did not display a growth-retardation phenotype. The degree to which overexpressing plants were stunted in growth was shown to positively correlate with the level of *CBF3/DREB1A* expression and, therefore, with the level of expression of CBF/DREB1 target genes (11, 12). It seems likely that the lack of effect on growth and development observed in *cbf2* plants is due to their low levels of *CBF3/DREB1A* expression compared with the overexpressing plants.

The Expression of *CBF1/DREB1B* and *CBF3/DREB1A* in Response to Cold Precedes That of *CBF2/DREB1C*. The three *CBF/DREB1* genes are generally assumed to be induced at the same time in response to low temperature. Nevertheless, because the induction is transient and the results described above demonstrate that CBF2/DREB1C represses *CBF1/DREB1B* and *CBF3/DREB1A*, this might not be the case. To test this possibility, we performed a detailed analysis of the induction of each individual *CBF/DREB1* gene by exposing *Arabidopsis* plants to 4°C for various periods of time. RNA-blot hybridizations revealed that *CBF1/DREB1B* and *CBF3/DREB1A* transcripts have very similar patterns of accumulation. The transcripts started to accumulate after 15 min of cold treatment, and they increased over the next 90 min. Subsequently, transcript levels decreased rapidly (Fig. 5). *CBF2/DREB1C* transcripts accumulated at a slower rate, reaching maximal levels after 2.5 h of cold exposure and then gradually declining (Fig. 5). From these results, we conclude that the three *CBF/DREB1* genes are not induced at the same time in response to low temperature, *CBF1/DREB1B* and *CBF3/DREB1A* preceding *CBF2/DREB1C*.

Discussion

Low temperature triggers the expression of the CBF/DREB1 family of transcription factors (4, 5, 9). It has been suggested that these factors have an important role in cold acclimation from the observation that ectopic expression of *CBF1/DREB1B* and *CBF3/DREB1A* induces the transcription of genes containing the CRT/DRE promoter element and enhances freezing tolerance of nonacclimated *Arabidopsis* transgenic plants (5, 10–12). Nevertheless, the individual contribution of each *CBF/DREB1* gene to that adaptive response has not been examined by loss-of-function analysis and important points on their function(s) remain uncertain. In this study, we have identified and characterized *cbf2*, a mutant with a T-DNA disrupting the *CBF2/DREB1C* gene. Our results show that *cbf2* mutants have higher capacity to tolerate freezing than WT plants before and after cold acclimation and are more tolerant to dehydration and salt stress. Interestingly, these unexpected phenotypes correlate with an increased expression of *CBF1/DREB1B*, *CBF3/DREB1A*, and, hence, downstream-regulated genes. The CBF/DREB1 regulon has been described as including genes that act in concert to improve freezing tolerance (10, 26). Furthermore,

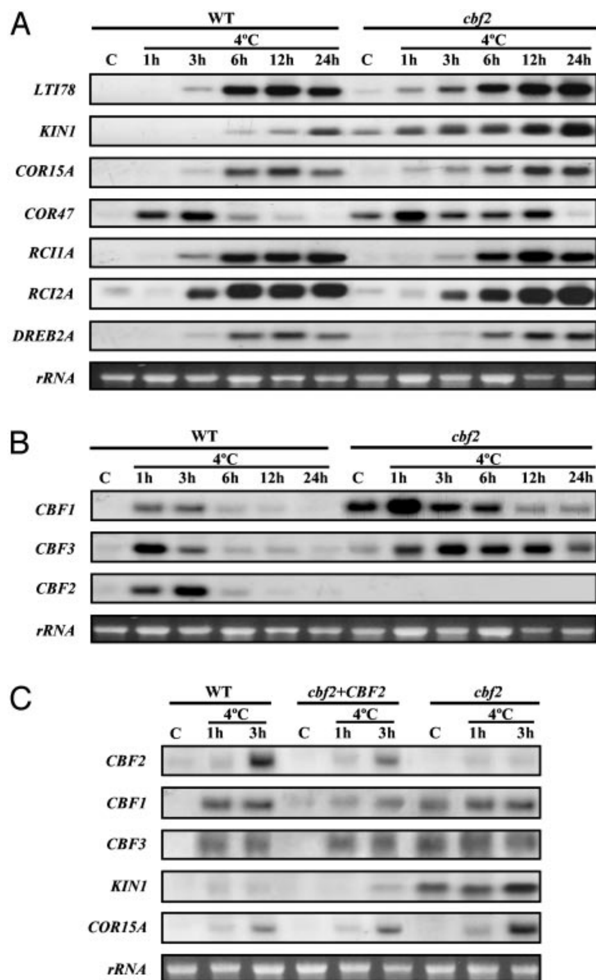

Fig. 4. Transcript levels of cold-induced genes in the *cbf2* mutant and in the complemented *cbf2* mutant. RNA-blot hybridizations were performed with total RNA (20 μg) isolated from 3-week-old rosette leaves of WT, *cbf2*, and complemented *cbf2* (*cbf2*+*CBF2*) plants grown under control conditions (C) or exposed to 4°C for the indicated times. In all cases, gene-specific probes were used for the hybridizations. (*A*) Transcript levels of *LTI78*, *KIN1*, *COR15A*, *COR47*, *RCI1A*, *RCI2A*, and *DREB2A* cold-inducible genes in WT and *cbf2*. *LTI78*, *KIN1*, *COR15A*, and *COR47* contain the CRT/DRE element in their promoters, whereas *RCI1A*, *RCI2A*, and *DREB2A* do not. (*B*) Transcript levels of *CBF*/*DREB1* genes in WT and *cbf2*. (*C*) Transcript levels of *CBF*/*DREB1*, *KIN1*, and *COR15A* genes in WT, *cbf2*, and *cbf2*+*CBF2*. Equal amounts of RNA were present in each sample as confirmed by ethidium bromide staining of rRNAs.

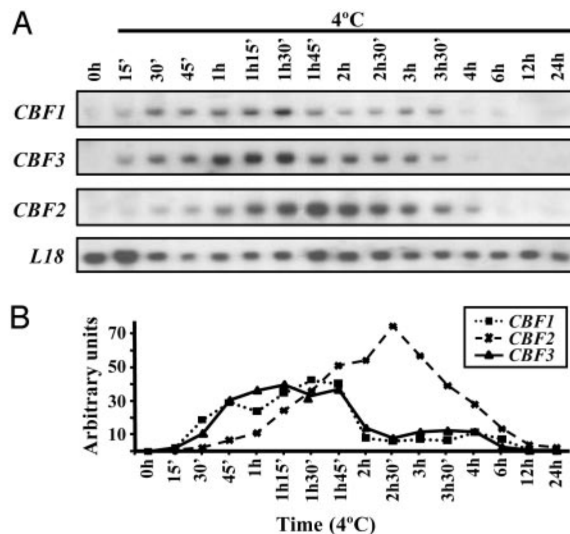

Fig. 5. Transcript levels of *CBF*/*DREB1* genes in response to low temperature. (*A*) RNA-blot hybridizations were performed with total RNA (20 μg) isolated from 3-week-old rosette leaves of Columbia plants grown under control conditions (C) or exposed to 4°C for the indicated times. Specific probes were used for the hybridizations. Hybridization with a probe representing *L18*, a ribosomal gene, was used to normalize signals from the *CBF*/*DREB1* genes. (*B*) Quantitative representation of the relative expression of *CBF*/*DREB1* genes (*CBF*/*L18*) in response to low temperature.

high expression levels of CBF/DREB1 target genes have been shown to result in improved tolerance to dehydration and salt stress (5, 11). Therefore, the increased tolerance to freezing, dehydration, and salt stress exhibited by *cbf2* mutant plants is likely a consequence of their high levels of CBF/DREB1-regulated gene expression. These data indicate that CBF2/DREB1C negatively regulates *CBF1*/*DREB1B* and *CBF3*/*DREB1A* expression and plays a critical role not only in cold acclimation but also in the proper development of constitutive tolerance to freezing and related stresses in *Arabidopsis*.

The mechanism(s) whereby the expression of *CBF*/*DREB1* genes is regulated by low temperature is unknown at present. Analysis of transgenic *Arabidopsis*-expressing reporter genes fused to *CBF*/*DREB1* promoters has revealed that the induction of these genes in response to cold is regulated at the transcriptional level (ref. 4; F.N. and J.S., unpublished data). On the other hand, available evidence has suggested that *CBF*/*DREB1* genes are not subjected to autoregulation, because the CRT/DRE core motif, CCGAC, is not present in their promoters (4, 9) and overexpression of *CBF1*/*DREB1B* does not result in accumulation of *CBF3*/*DREB1A* transcripts (4). Thus, it was proposed (4) that induction of *CBF*/*DREB1* genes in response to low temperature could involve the modification of either a CBF/DREB1 activator, named ICE (inducer of CBF expression), that would be inactive at warm temperatures or an associated protein that would allow the activator to induce the expression of these genes. Recently, the identification of an *Arabidopsis* MYC-like basic helix–loop–helix transcriptional activator, called ICE1, that binds the *CBF3*/*DREB1A* promoter has been reported (27). *ICE1* is expressed constitutively, and its overexpression enhances the expression of the CBF/DREB1 regulon in the cold and improves the freezing tolerance of the *Arabidopsis* transgenic plants (27). The expression of the *CBF*/*DREB1* genes has also been suggested to be feedback inhibited by their gene products or by products of their downstream target genes (24). Our results provide direct evidence that *CBF*/*DREB1* gene expression can be controlled, at least in part, by the CBF/DREB1 factors themselves. These results are consistent with the role of CBF2/DREB1C as a negative regulator of the expression of *CBF1*/*DREB1B* and *CBF3*/*DREB1A*, and, therefore, also of their downstream-target genes. In fact, the *cbf2* mutation leads to an accumulation of *CBF1*/*DREB1B* and *CBF3*/*DREB1A* transcripts that results in the induction of target genes and subsequent increase in freezing, dehydration, and salt tolerance.

Because *CBF*/*DREB1* transcripts are not usually detected in RNA-blot hybridizations under control conditions, it is generally presumed that *CBF*/*DREB1* genes are not transcribed at normal growth temperatures. Therefore, if CBF2/DREB1C is required to repress the expression of *CBF1*/*DREB1B* and *CBF3*/*DREB1A*, it could be expected that these genes were expressed at those temperatures. A detailed analysis of the quantitative data available from different microarray experiments (26–28) reveals that, under nonstressed conditions, the transcript levels of *CBF2*/*DREB1C* are 5- to 8-fold higher than those of *CBF1*/*DREB1B* and *CBF3*/*DRE*B1A, which is consistent with the

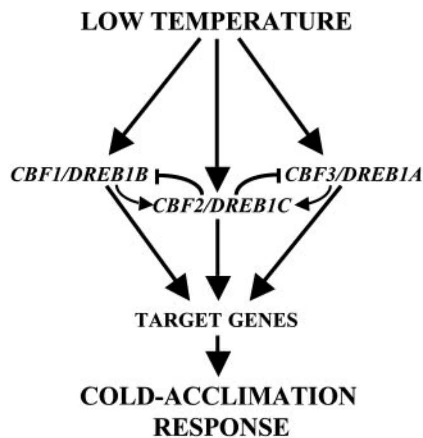

Fig. 6. Proposed model for the function of CBF2/DREB1C in cold acclimation and the regulation of *CBF/DREB1* gene expression in response to low temperature. Arrowheads and end lines indicate positive and negative regulation, respectively.

function that we propose for CBF2/DREB1C. Another general assumption concerning the expression of *CBF/DREB1* genes is that they have a similar pattern of induction in response to low temperature. Our results demonstrate that this assumption is not the case, the expression of *CBF1/DREB1B* and *CBF3/DREB1A* preceding that of *CBF2/DREB1C*. This asynchronism, together with the negative regulation of *CBF1/DREB1B* and *CBF3/DREB1A* by CBF2/DREB1C, may be important to ensure that *CBF/DREB1* gene expression is transient and tightly controlled. Based on the results described here, a hypothetical model for CBF2/DREB1C function during cold acclimation is presented in Fig. 6. Under control conditions, steady-state levels of CBF2/DREB1C would be repressing *CBF1/DREB1B* and *CBF3/DREB1A* expression. When plants are exposed to low temperature, the activation of different, perhaps specific, regulators, such as ICE1 (27), would induce very rapidly the expression of these genes escaping from CBF2/DREB1C repression. Later on, probably once CBF1/DREB1B and CBF3/DREB1A products attain certain levels, *CBF2/DREB1C* is induced, which in turn would provoke the suppression of *CBF1/DREB1B* and *CBF3/DREB1A* transcription. We propose that CBF1/DREB1B and CBF3/DREB1A proteins, likely in the presence of low temperature, could contribute to activate *CBF2/DREB1C* expression. Finally, CBF1/DREB1B and CBF3/DREB1A would activate the transcription of downstream target genes and, subsequently, the development of the cold-acclimation response (5, 10–12). The possibility remains that, in addition to repressing *CBF1/DREB1B* and *CBF3/DREB1A*, CBF2/DREB1C might also activate cold-induced gene expression. Unfortunately, such a function cannot be revealed by studying the *cbf2* mutation alone, given that *CBF1/DREB1B* and *CBF3/DREB1A* likely have a redundant role in this regard and thus mask the effect of the mutation on cold-induced gene expression. Needless to say, this model does not rule out the existence of additional regulatory mechanisms involved in controlling the precise expression of *CBF/DREB1* genes (24, 29–31).

As mentioned before, freezing temperature is a common stress condition that adversely affects plant growth and agricultural production. Even modest increases (1–2°C) in the freezing tolerance of crop species would have a dramatic impact on agricultural productivity and profitability (32). Determining the molecular mechanisms that plants have evolved to survive freezing would not only increase our fundamental knowledge of how plants adapt to changes in the environment but would also contribute to the development of new strategies to improve the tolerance of crop species to this adverse situation. Here, we demonstrate that CBF2/DREB1C plays a crucial role in the development of *Arabidopsis* tolerance to freezing and other related stresses by ensuring the accurate expression of *CBF1/DREB1B* and *CBF3/DREB1A* and, hence, that of the CBF/DREB1-target genes. Although the mechanisms by which CBF2/DREB1C regulates *CBF1/DREB1B* and *CBF3/DREB1A* expression are currently unknown and need to be investigated, these findings provide a new perspective on the genetic control of freezing tolerance and further our understanding of the molecular basis of plant responses to adverse environmental conditions.

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1. Levitt, J. (1980) in *Responses of Plants to Environmental Stresses* (Academic, New York), 2nd. Ed.
2. Thomashow, M. F. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 571–599.
3. Stockinger, E. J., Gilmour, S. J. & Thomashow, M. F. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1035–1040.
4. Gilmour, S. J., Zarka, D. G., Stockinger, E. J., Salazar, M. P., Houghton, J. M. & Thomashow, M. F. (1998) *Plant J.* **16,** 433–442.
5. Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. & Shinozaki, K. (1998) *Plant Cell* **10,** 1391–1406.
6. Riechmann, J. L. & Meyerowitz, E. M. (1998) *Biol. Chem.* **379,** 633–646.
7. Yamaguchi-Shinozaki, K. & Shinozaki, K. (1994) *Plant Cell* **6,** 251–264.
8. Baker, S. S., Wilhelm, K. S. & Thomashow, M. F. (1994) *Plant Mol. Biol.* **24,** 701–713.
9. Medina, J., Bargues, J., Perol, M., Pérez-Alonso, J. & Salinas, J. (1999) *Plant Physiol.* **119,** 463–469.
10. Jaglo-Ottosen, K. R., Gilmour, S. J., Zarka, D. G., Schabenberger, O. & Thomashow, M. F. (1998) *Science* **280,** 104–106.
11. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. & Shinozaki, K. (1999) *Nat. Biotechnol.* **17,** 287–291.
12. Gilmour, S. J., Sebolt, A. M., Salazar, M. P., Everard, J. D. & Thomashow, M. F. (2000) *Plant Physiol.* **124,** 1854–1865.
13. Haughn, G. & Somerville, C. (1986) *Mol. General Genet.* **204,** 430–434.
14. Murashige, Y. & Skoog, F. (1962) *Plant Physiol.* **15,** 473–497.
15. Logeman, J., Schell, J. & Willmitzer, L. (1987) *Anal. Biochem.* **163,** 26–20.
16. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) in *Cloning: A Laboratory Manual,* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
17. Gilmour, S. J., Artus, N. N. & Thomashow, M. F. (1992) *Plant Mol. Biol.* **18,** 13–21.
18. Gilmour, S. J., Lin, C. & Thomashow, M. F. (1996) *Plant Physiol.* **111,** 293–299.
19. Jarillo, J. A., Capel, J., Leyva, A., Martínez-Zapater, J. M. & Salinas, J. (1994) *Plant Mol. Biol.* **25,** 693–704.
20. Capel, J., Jarillo, J. A., Salinas, J. & Martínez-Zapater, J. M. (1997) *Plant Physiol.* **115,** 569–576.
21. Baima, S., Sessa, G., Ruberti, I. & Morelli, G. (1995) *Gene* **153,** 171–174.
22. Thomashow, M. F. (2001) *Plant Physiol.* **125,** 89–93.
23. Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y. & Shinozaki, K. (2001) *Plant Cell* **13,** 61–72.
24. Guo, Y., Xion, L., Ishitani, M. & Zhu, J. K. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 7786–7791.
25. Medina, J., Catalá, R. & Salinas, J. (2001) *Plant Physiol.* **125,** 1655–1656.
26. Fowler, S. & Thomashow, M. F. (2002) *Plant Cell* **14,** 1675–1690.
27. Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.-H., Hong, X., Agarwal, M. & Zhu, J. K. (2003) *Genes Dev.* **17,** 1043–1054.
28. Chen, W., Provart, N. J., Glazebrook, J., Katagiri, F., Chang, H. S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S. A., *et al*. (2002) *Plant Cell* **14,** 559–574.
29. Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B. & Zhu, J. K. (2001) *Genes Dev.* **15**, 912–924.
30. Lee, H., Guo, Y., Ohta, M., Xiong, L., Stevenson, B. & Zhu, J. K. (2002) *EMBO J.* **21,** 2692–2702.
31. Gong, Z., Lee, H., Xiong, L., Jagendorf, A., Stevenson, B. & Zhu, J. K. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 11507–11512.
32. Steponkus, P. L., Uemura, M., Joseph, R. A., Gilmour, S. J. & Thomashow, M. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14570–14575.