the manufacturer. The coding sequence for dCtBP, containing codons 8 to 383, was inserted into the pGEX-5X-3 expression plasmid (Pharmacia) and transformed into strain BL21::DE3 pLysS of *Escherichia coli*. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 hours. Cells were then sonicated in buffer A [20 mM tris-HCl (pH 7.9), 0.2 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40], and total lysates containing the GST and GST-dCtBP proteins were immobilized onto glutathione-agarose beads (Sigma) in buffer A. Binding assays were done as described (*29*). ^{35}S-labeled proteins were fractionated on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

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31. A GST-dCtBP fusion protein containing amino acid residues 8 to 383 was injected into a rat (Pocono Rabbit Farm, PA). The preimmune serum did not detectably cross-react with fixed embryos. The GST-dCtBP antiserum specifically stained nuclei in mixed-stage embryos. Reduced staining was detected in l(3)03463 homozygous embryos. Embryos were fixed and preincubated in bovine serum albumin as described (*15*). The rat serum was diluted 1:1000, and dCtBP was visualized using a 1:200 dilution of tetramethyl rhodamine isothiocyanate–conjugated antibodies to rat immunoglobulin (Jackson Labs).
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Arabidopsis CBF1 Overexpression Induces *COR* Genes and Enhances Freezing Tolerance

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Many plants, including *Arabidopsis*, show increased resistance to freezing after they have been exposed to low nonfreezing temperatures. This response, termed cold acclimation, is associated with the induction of *COR* (cold-regulated) genes mediated by the C-repeat/drought-responsive element (CRT/DRE) DNA regulatory element. Increased expression of *Arabidopsis* CBF1, a transcriptional activator that binds to the CRT/DRE sequence, induced *COR* gene expression and increased the freezing tolerance of nonacclimated *Arabidopsis* plants. We conclude that CBF1 is a likely regulator of the cold acclimation response, controlling the level of *COR* gene expression, which in turn promotes tolerance to freezing.

Studies of the molecular basis of plant tolerance to freezing have focused primarily on the cold acclimation response, the process by which plants increase their tolerance to freezing in response to low nonfreezing temperatures (*1*). Cold acclimation is associated with biochemical and physiological changes and alterations in gene expression (*1*, *2*). Studies of genes stimulated by low temperature have revealed that many, including the *Arabidopsis COR* genes, encode hydrophilic polypeptides that potentially promote tolerance to freezing (*1–3*). Indeed, constitutive expression of *COR15a* (which encodes the chloroplast-targeted polypeptide COR15am) in transgenic *Arabidopsis* plants improves the freezing tolerance of chloroplasts frozen in situ and of protoplasts frozen in vitro (*4*). Unlike cold acclimation, however, *COR15a* expression has no discernible effect on the survival of frozen plants (*2*, *5*).

Genetic analyses indicate that multiple genes are involved in cold acclimation in plants (*6*). Several *COR* genes are coordinately stimulated along with *COR15a* in response to low temperature (*2*, *7*), which suggests that *COR15a* might act in concert with other *COR* genes to enhance tolerance to freezing in plants. If so, expression of the entire battery of *COR* genes would have a greater effect on freezing tolerance than *COR15a* expression alone. To test this hypothesis, we attempted to induce expression of the *COR* gene "regulon" with the *Arabidopsis* transcriptional activator CBF1 (CRT/DRE binding factor 1) (*8*), a putative *COR* gene regulator. CBF1 binds to the cis-acting CRT (C-repeat)/DRE (drought-responsive element) sequence (*9*, *10*), a DNA regulatory element that stimulates transcription in response to both low temperature and water deficit (*9*). The element is present in the promoters of multiple *COR* genes including *COR15a*, *COR78* (also known as RD29A and LTI78), and *COR6.6* (*10–12*). Expression of *CBF1* in yeast (*Saccharomyces cerevisiae*) activates expression of reporter genes that have the CRT/DRE as an upstream activator sequence (*8*).

We created transgenic *Arabidopsis* plants that overexpress *CBF1* by placing a cDNA encoding CBF1 under the control of the strong cauliflower mosaic virus (CaMV) 35S RNA promoter and transforming the chimeric gene into *Arabidopsis* ecotype RLD plants (*13*). Initial screening gave rise to two transgenic lines, A6 and B16, that accumulated *CBF1* transcripts at high concentrations. Southern blot analysis indicated that the A6 plants had a single DNA insert and the B16 plants had multiple inserts. Examination of fourth generation homozygous A6 and B16 plants indicated that amounts of *CBF1* transcript were higher in nonacclimated A6 and B16 plants than they were in nonacclimated RLD plants (Fig. 1A). Quantities of *CBF1* transcript were greater in the A6 plants than in the B16 plants (Fig. 1A).

CBF1 overexpression induced *COR* gene expression without a low-temperature stimulus (Fig. 1A). Specifically, greater than normal amounts of *COR6.6*, *COR15a*, *COR47*, and *COR78* transcripts were detected in nonacclimated A6 and B16 plants. In nonacclimated A6 plants, *COR* transcript concentrations approximated those found in cold-acclimated RLD plants. In nonacclimated B16 plants, they were less than in cold-acclimated RLD plants. Immunoblot analysis indicated that the amounts of the COR15am (Fig. 1B) and COR6.6 polypeptides were also elevated in the A6 and B16 plants, with a higher level of expression in A6 plants. We were unable to identify the CBF1 protein in either RLD or transgenic plants (*5*). Overexpression of *CBF1* did not affect transcript concentrations of *eIF4A*

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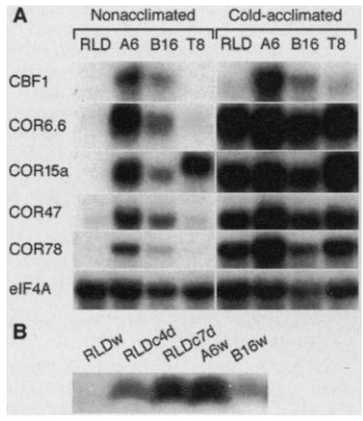

Fig. 1. Expression of *CBF1* and *COR* genes in RLD and transgenic *Arabidopsis* plants. (**A**) *CBF1* and *COR* transcripts. Leaves from nonacclimated and 3-day cold-acclimated plants (*20*) were harvested and total RNA was prepared and analyzed for *CBF1* and *COR* transcripts by RNA blot analysis with ^{32}P-radiolabeled probes (*21*). The autoradiograms for *CBF1* resulted from 3-day film exposure and those for *COR6.6* and *COR15a* were from a 3-hour exposure (the ^{32}P-radiolabeled probes were of similar specific activity). (**B**) COR15am proteins. Total soluble protein (100 μg) was prepared from leaves of the nonacclimated RLD (RLDw), 4-day cold-acclimated RLD (RLDc4d), 7-day cold-acclimated RLD (RLDc7d), and nonacclimated A6 and B16 plants; the amounts of COR15am were determined by immunoblot analysis with antiserum raised against the COR15am polypeptide (*22*). No reacting bands were observed with preimmune serum.

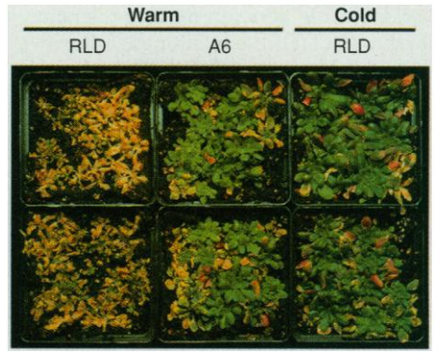

Fig. 3. Freezing survival of RLD and A6 *Arabidopsis* plants. Nonacclimated (Warm) RLD and A6 plants and 5-day cold-acclimated (Cold) RLD plants were frozen at −5°C for 2 days and then returned to a growth chamber at 22°C (*24*). A photograph of the plants after 7 days of regrowth is shown.

(eukaryotic initiation factor 4A) (*14*), a constitutively expressed gene that is not responsive to low temperature (Fig. 1A), and had no obvious effects on plant growth and development.

Two additional transgenic lines, K16 and 1-11, that overexpress *CBF1* have recently been identified. Northern blot analysis of nonacclimated T2 generation plants indicated that, in both of these lines, *COR* gene expression is also higher than that in nonacclimated RLD plants.

CBF1 overexpression increased the tolerance of plants to freezing (Fig. 2), as determined by the electrolyte leakage test (*15*).

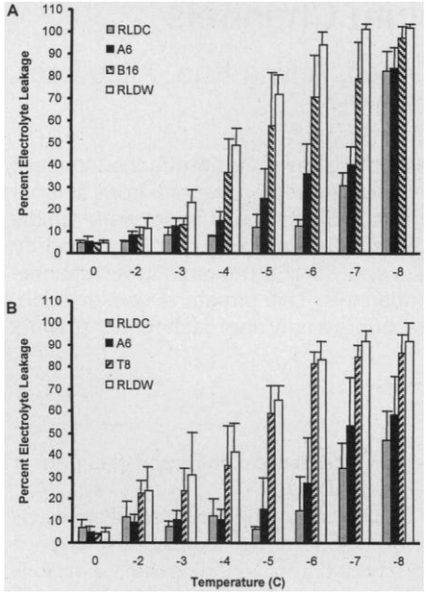

Fig. 2. Freezing tolerance of leaves from RLD and transgenic *Arabidopsis* plants. Leaves from nonacclimated RLD (RLDw) plants, 10-day cold-acclimated RLD (RLDc) plants, and nonacclimated A6, B16, and T8 plants were frozen at the indicated temperatures and the extent of cellular damage was estimated by measuring electrolyte leakage (*23*). Error bars indicate standard deviations.

Detached leaves were frozen to various subzero temperatures and, after thawing, cellular damage (due to freeze-induced membrane lesions) was estimated by measuring ion leakage from the tissues. Leaves from nonacclimated A6 and B16 plants were more tolerant to freezing than those from nonacclimated RLD plants (Fig. 2). The freezing tolerance of leaves from nonacclimated A6 plants exceeded that of leaves from nonacclimated B16 plants (Fig. 2A), which had lower levels of *CBF1* and *COR* gene expression (Fig. 1A). T8 transgenic plants (*4*), which constitutively express only *COR15a* (under control of the CaMV 35S RNA promoter) (Fig. 1A), were less freezing tolerant than A6 plants (Fig. 2B).

A comparison of EL_{50} values (the freezing temperature that results in release of 50% of tissue electrolytes) of leaves from RLD, A6, B16, and T8 plants is presented in Table 1. Data from multiple experiments indicate that the freezing tolerance of leaves from nonacclimated A6 and B16 plants was greater than that of leaves from nonacclimated RLD and T8 plants and that leaves from nonacclimated A6 plants were more freezing tolerant than leaves from nonacclimated B16 plants.

The enhancement of freezing tolerance in A6 plants was apparent in whole plant survival tests (Fig. 3). Nonacclimated A6 plants displayed variable, but greater, freezing tolerance than nonacclimated RLD plants (Fig. 3). No difference in plant survival was detected between nonacclimated B16 and RLD plants and nonacclimated T8 and RLD plants.

Our results demonstrate that constitutive overexpression of the *Arabidopsis* transcriptional activator CBF1 induces expression of *Arabidopsis COR* genes and increases the freezing tolerance of nonacclimated plants. These results are consistent with CBF1 having a role in regulating *COR* gene expression and further link the *COR* genes to plant cold acclimation. The increase in freezing tolerance brought about by expressing the battery of CRT/DRE-regulated *COR* genes was greater than that brought about by overexpressing *COR15a* alone, which implicates

Table 1. Comparison of EL_{50} values of leaves from RLD and transgenic *Arabidopsis* plants. EL_{50} values were calculated and compared by analysis of variance (*25*). EL_{50} ± SE (*n*) are presented on the diagonal line for leaves from nonacclimated RLD (RLDw), cold-acclimated (7 to 10 days) RLD (RLDc), and nonacclimated A6, B16, and T8 plants. *P* values for comparisons of EL_{50} values are indicated in the intersecting cells.

	EL_{50} values				
	RLDw	RLDc	A6	B16	T8
RLDw	−3.9 ± 0.21 (8)	$P < 0.0001$	$P < 0.0001$	$P = 0.0014$	$P = 0.7406$
RLDc		−7.6 ± 0.30 (4)	$P = 0.3261$	$P < 0.0001$	$P < 0.0001$
A6			−7.2 ± 0.25 (6)	$P < 0.0001$	$P < 0.0001$
B16				−5.2 ± 0.27 (5)	$P = 0.0044$
T8					−3.8 ± 0.35 (3)

additional *COR* genes in freezing tolerance. Whether CRT/DRE-containing *COR* genes are involved in bringing about the full array of biochemical and physiological changes that occur with cold acclimation (*1*, *2*) remains to be determined.

Freezing temperatures greatly limit the geographical distribution of native and cultivated plants and often cause severe losses in agricultural productivity (*16*). Traditional plant breeding approaches have met with limited success in improving the freezing tolerance of agronomic plants (*6*). The freezing tolerance of the best wheat varieties today is essentially the same as the most freezing-tolerant varieties developed in the early part of this century. Biotechnology, however, may offer new strategies. Here we show that the freezing tolerance of nonacclimated *Arabidopsis* plants is enhanced by increasing the expression of the *Arabidopsis* regulatory gene *CBF1*. The CRT/DRE DNA regulatory element we have targeted here is not limited to *Arabidopsis* (*17*) and thus may provide a way to improve the freezing tolerance of crop plants.

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13. Standard procedures were used for plasmid manipulations (*18*). The CBF1-containing Ase I–Bgl II fragment from pACT-Bgl+ (*8*) was gel purified, Bam HI linkers were ligated to both ends, and the fragment was inserted into the Bam HI site in pCIB710 [S. Rothstein *et al.*, *Gene* **53**, 153 (1987)], which contains the CaMV 35*S* RNA promoter and terminator. The chimeric plasmid was linearized at the Kpn I site and inserted into the Kpn I site of the binary vector pCIB10g (Ciba-Geigy, Research Triangle Park, NC). The plasmid was transformed into *Agrobacterium tumefaciens* strain C58C1(pMP90) by electroporation. *Arabidopsis* plants were transformed by the vacuum infiltration procedure [N. Bechtold, J. Ellis, G. Pelletier, *C. R. Acad. Sci. Ser. III Life Sci.* **316**, 1194 (1993)] as modified [A. van Hoof and P. J. Green, *Plant J.* **10**, 415 (1996)].
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20. *Arabidopsis thaliana* ecotype RLD plants were grown in pots under continuous light (~100 μE m^{-2} s^{-1}) at 22°C for 18 to 25 days as described (*19*). In some cases, plants were then cold acclimated at 2.5°C under continuous light (~50 μE m^{-2} s^{-1}) for various amounts of time.
21. Total RNA was isolated from plant leaves and subjected to RNA blot analysis by high-stringency hybridization and wash conditions as described (*8*, *19*). DNA probes were gel purified and radiolabeled with ^{32}P by random priming according to standard procedures (*18*).
22. Total soluble protein was isolated from plant leaves, fractionated by tricine SDS–polyacrylamide gel electrophoresis, and transferred to 0.2-μm nitrocellulose as described (*4*). COR15am protein was detected with antiserum raised to purified COR15am and protein A–conjugated alkaline phosphatase (Sigma) (*4*).
23. Electrolyte leakage tests were conducted as described (*15*, *19*) with the following modifications. Two to four detached leaves from nonacclimated or cold-acclimated plants were placed in a test tube and submerged for 1 hour in a −2°C bath containing water and ethylene glycol in a completely randomized design, after which ice crystals were added to nucleate freezing. After an additional hour of incubation at −2°C, the samples were cooled in decrements of 1°C each hour. Samples (five replicates for each data point) were thawed overnight on ice and incubated in 3 ml of distilled water with shaking at room temperature for 3 hours. Electrolyte leakage from leaves was measured with a conductivity meter. The solution was then removed, the leaves were frozen at −80°C (for at least 1 hour), and the solution was returned to each tube and incubated for 3 hours to obtain a value for 100% electrolyte leakage.
24. Pots (9 cm) containing about 40 nonacclimated *Arabidopsis* plants (20 days old) and 4-day cold-acclimated plants (25 days old) (*20*) were placed in a completely randomized design in a −5°C cold chamber in the dark. After 1 hour, ice chips were added to each pot to nucleate freezing. Plants were removed after 2 days and returned to a growth chamber at 22°C.
25. Model curves fitting up to third-order linear polynomial trends were determined for each electrolyte leakage experiment. To ensure unbiased predictions of electrolyte leakage, trends significantly improving the model fit at the 0.2 probability level were retained. EL_{50} values were calculated from the fitted models. An unbalanced one-way analysis of variance, adjusted for the different number of EL_{50} values for each plant type, was determined by using SAS PROC GLM [SAS Institute, *SAS/STAT User's Guide, Version 6* (SAS Institute, Cary, NC, 1989)].

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Structural Conservation in Prokaryotic and Eukaryotic Potassium Channels

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Toxins from scorpion venom interact with potassium channels. Resin-attached, mutant K^+ channels from *Streptomyces lividans* were used to screen venom from *Leiurus quinquestriatus hebraeus*, and the toxins that interacted with the channel were rapidly identified by mass spectrometry. One of the toxins, agitoxin2, was further studied by mutagenesis and radioligand binding. The results show that a prokaryotic K^+ channel has the same pore structure as eukaryotic K^+ channels. This structural conservation, through application of techniques presented here, offers a new approach for K^+ channel pharmacology.

Scorpion toxins inhibit ion conduction through potassium channels by occluding the pore at the extracellular opening. A single toxin protein binds very specifically to a single K^+ channel to cause inhibition. The toxins are 35 to 40 amino acids in length and have a characteristic fold that is held rigidly by three disulfide bridges (*1*). They are active site inhibitors, because when they bind to the channel they interact energetically with K^+ ions in the pore (*2–4*). The interaction between these inhibitors and the pore of K^+ channels has been exploited to gain insights into the structure and function of K^+ channels.

Studies employing site-directed mutagenesis of the Shaker K^+ channel have mapped the scorpion toxin binding site to regions corresponding to the extracellular entryway of the K^+ channel from *Streptomyces lividans* (the KcsA channel) (*4–9*). Although the amino acids of the K^+ channel selectivity filter are highly conserved, the residues lining the entryway are quite variable. As if to mirror the amino acid variation at the binding site, the toxins are also highly variable in