RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance

Zhizhong Gong*, Hojoung Lee*, Liming Xiong*, André Jagendorf†, Becky Stevenson*, and Jian-Kang Zhu*‡

*Department of Plant Sciences, University of Arizona, Tucson, AZ 85721; and †Department of Plant Biology, Cornell University, Ithaca, NY 14853

Contributed by André Jagendorf, July 5, 2002

Susceptibility to chilling injury prevents the cultivation of many important crops and limits the extended storage of horticultural commodities. Although freezing tolerance is acquired through cold-induced gene expression changes mediated in part by the CBF family of transcriptional activators, whether plant chilling resistance or sensitivity involves the *CBF* genes is not known. We report here that an *Arabidopsis thaliana* mutant impaired in the cold-regulated expression of *CBF* genes and their downstream target genes is sensitive to chilling stress. Expression of *CBF3* under a strong constitutive promoter restores chilling resistance to the mutant plants. The mutated gene was cloned and found to encode a nuclear localized RNA helicase. Our results identify a regulator of *CBF* genes, and demonstrate the importance of gene regulation and the CBF transcriptional activators in plant chilling resistance.

Low temperature is an important environmental factor that greatly influences the growth, development, survival, and distribution of plants (1). Most plants from temperate regions can cold-acclimate, i.e., they show increased tolerance to freezing temperatures after an exposure to low, nonfreezing temperatures (2). At least part of the basis of cold acclimation is that exposure to low temperatures induces the expression of many plant genes (2, 3). Some of the cold-induced gene products such as COR15A have been shown to mitigate membrane damage caused by freezing stress (4, 5).

The dehydration-responsive element (DRE)/C-repeat (CRT) cis-element is present in the promoters of many of the cold-responsive genes such as *RD29A* (also known as *COR78* or *LTI78*) and *COR15A* (6–8). In *Arabidopsis thaliana*, the CBF family of transcriptional activators, also known as DREB1s, bind to the DRE/CRT element and activate the expression of *COR/RD* genes (8, 9). Cold induces rapid and transient expression of *CBF1*, *CBF2*, and *CBF3*, which in turn activate the downstream *COR* genes (10). Ectopic expression of *CBF1* or *CBF3* in transgenic *Arabidopsis* plants leads to constitutive expression of *COR* genes and enhanced freezing tolerance without cold-acclimation treatment (9, 11). These studies demonstrate a critical role of the CBF regulon in the acquisition of freezing tolerance. An important challenge ahead is to identify regulators of the *CBF* genes.

Recently, the *Arabidopsis* HOS1 protein was shown to be a negative regulator of *CBF* genes (12). *CBFs* and their downstream *COR* genes show enhanced cold induction in *hos1* mutant plants (12, 13). The *Arabidopsis esk1* mutants are constitutively freezing-tolerant, but are not affected in the expression of genes with the DRE/CRT cis-element, suggesting that *ESK1* may be involved in a CBF-independent cold-response pathway (14). The *sfr6* mutation reduces cold and abscisic acid (ABA) induction of the CRT/DRE genes but has no effect on the expression of *CBF* genes (15). Therefore, no positive regulator of *CBF*s has been identified to date.

Many important crops and fruits that originated from the tropics or subtropics, such as rice, maize, tomato, banana, and orange, are injured or killed by exposure to low, nonfreezing temperatures in the range of 0–12°C (16, 17). Chilling injury also limits the extended storage of many vegetables and fruits (16). Plant responses to chilling are multigenic and the molecular mechanisms of chilling sensitivity or resistance are not well understood (18). Chilling sensitivity has been attributed to membrane lipid phase transitions (17, 19–21), impaired rRNA modification in plastids (22), and metabolic imbalance (1). Genetic enhancement of the degree of fatty acid unsaturation of membrane lipids was shown to improve chilling resistance in some plants (23, 24). In naturally chilling resistant plants such as *Arabidopsis*, chilling temperature-induced gene expression is critical for acquired freezing tolerance. Whether the gene induction is also important for chilling resistance is not known. If gene regulation is important, does the CBF regulon play a role in this process, as it does in freezing tolerance?

During a genetic screen for *Arabidopsis* mutants with deregulated expression of the *RD29A-LUC* reporter gene (25), we isolated a mutant, *los4-1*, which shows a reduced *RD29A-LUC* expression in response to cold, but not to ABA or high salt. Northern blot analysis indicated that the mutation also decreases the expression of the endogenous *RD29A* and other *COR/RD* genes under cold stress. The *CBF* genes show reduced or delayed cold induction in *los4-1* mutant plants. Unexpectedly, *los4-1* mutant plants are very sensitive to chilling temperatures, particularly in the dark. We show here that constitutive expression of the *CBF3* gene reverses the chilling sensitivity of *los4-1* mutant plants. The *LOS4* gene was isolated by map-based cloning, and found to encode a DEAD-box RNA helicase protein that is localized in both the nucleus and cytoplasm. Our study not only identifies a positive regulator of *CBF* genes, but also reveals a critical role of gene regulation and the CBF regulon in plant chilling resistance.

Materials and Methods

Plant Growth, Mutant Isolation. Plant growth conditions were same as described (25). *A. thaliana* (ecotype C24) expressing the chimeric *RD29A-LUC* gene (referred to as wild-type in this study) were mutagenized with ethyl methanesulfonate, and mutants defective in cold regulation were screened by luminescence imaging by using a CCD camera as described (25). Mutants were backcrossed to the wild type for four times to eliminate other mutations from the genetic background.

Northern Blot Analysis. Two-week-old wild-type and mutant plants grown on standard 0.6% agar medium containing Murashige and Skoog salts supplemented with 3% sucrose were treated with either low temperature, ABA, or NaCl. Total RNA was extracted from whole seedlings (≈0.3 g) and RNA analysis was performed as described (12). Gene-specific DNA probes used in this analysis were as described (12, 25). For the Δ9-desaturase

Abbreviations: DRE, dehydration-responsive element; CRT, C-repeat; SSLP, simple sequence-length polymorphism; ABA, abscisic acid; GFP, green fluorescent protein.

‡To whom reprint requests should be addressed. E-mail: jkzhu@ag.arizona.edu.

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gene (AAF82163) and the *LOS4* gene, full-length cDNAs were used as probes.

Chilling and Freezing Treatments. For chilling tests, plants grown in soil (Metro-Mix 350, Scott-Sierra Horticultural Products Co., Marysville, OH) in growth chambers with 16 h light at 22°C, 8 h dark at 18°C, and 70% relative humidity for 2 weeks were transferred to 4°C in dark or light (20 μmol·m^{-2}·s^{-1}). For freezing tests, 2-week-old plants grown in soil treated at 4°C in light (20 μmol·m^{-2}·s^{-1}) for 4 days were transferred to a temperature-programmable freezer (Percival Scientific Co., Boone, IA). Ice chips were sprayed onto the plants to initiate uniform freezing. The plants were kept at −1°C for 16 h and then gradually decreased to −8°C during 7 h. The plants were further kept at −8°C for 6 h, then removed to 4°C for 24 h. Pictures were taken on the 3rd day after the plants were transferred from 4°C to room temperature.

Determination of Sucrose and Proline Contents. Samples were taken at 10 o'clock in the morning. Sucrose contents were determined as described (26). Proline contents were measured as described (27).

Positional Cloning and Complementation of *los4-1* Mutants. For genetic mapping, the homozygous *los4-1* mutant in C24 background was crossed to wild type of the Columbia ecotype. The F_2 population produced by selfing F_1 individuals was screened for *los4-1* chilling-sensitive mutants by a treatment at 4°C for 2 weeks. Simple sequence-length polymorphism (SSLP) markers were developed and used for mapping. DNA samples for the SSLP mapping were prepared from single leaves of a total of 563 mutant F_2 plants. By using SSLP markers, we first mapped *los4* to chromosome 3 between markers nga162 and nga112. Markers GAPAB-1, T32N15-1, F18B3-1, CDC2BG-1, F4F15-1, and F22O6-1 were then used to narrow down the *los4* mutation within four BAC clones: F3C22, F8J2, T4D2, and F4P12. Further mapping with newly developed SSLP markers on 1,126 chromosomes pinpointed *los4* mutation between the end (63.5 kb) of BAC clone F8J2 (total, 99.8 kb) and the beginning (18.7 kb) of BAC clone F4P12 (total, 144.6 kb). To identify the *los4* mutation, we sequenced all predicted genes in this region in both *los4-1* and wild-type plants.

For *los4* complementation, a PCR product amplified from BAC clone T4D2 by using a forward primer, 5′-ATGTCGACCATGATCAGTAATATCTTTGCATCCTC-3′ and a reverse primer, 5′-TCAAGAAAACAGACGACATAG-3′, was cloned into the binary vector pCAMBIA1200. The clone insert was sequenced to verify that no PCR or cloning errors occurred. The construct was introduced into homozygous *los4-1* mutant plants by *Agrobacterium*-mediated transformation. Transformants were selected on MS medium containing 25 mg/liter hygromycin and transferred to soil for growth. After 1 week, the plants in soil were treated at 4°C for 2 weeks in the dark to test for chilling resistance.

***LOS4* cDNA Cloning.** *Arabidopsis* poly(A) RNAs were reverse transcribed with a 21-mer oligo(dT) primer and were used as templates for PCR amplification of *LOS4* cDNA. The forward primer, 5′-CATCTAGAATGGCGGATACGGTAGAGAAAGTTCCCACC-3′ (underline indicates *Xba*I site for later use), and reverse primer, 5′-ACGGTACCTCACTCGTCCAGCAGGCCAGCTTCCTTTAATGC-3′ (underline indicates *Kpn*I site for later use), were designed according to the corresponding genomic sequence of T4D2.40. The reverse transcription–PCR products were cloned into pBluescript SK(−) digested with *Eco*RV, and the resultant clones verified by sequencing.

CBF3 Overexpression in *los4-1* Mutants. For *CBF3* overexpression, the *CBF3* ORF was amplified from *Arabidopsis* (ecotype Columbia) genomic DNA by PCR with a forward primer: 5′-TCTAGATGAACTCATTTTCTGCTTTTTCTG-3′ (*Xba*I site is underlined) and a reverse primer 5′-GGTACCTTTTAATAACTCCATAACGATACGTC-3′ (*Kpn*I site is underlined). The PCR product was first cloned into pBluescript SK(−) and confirmed by sequencing. Then the *CBF3* ORF was released by digesting with *Xba*I and *Kpn*I, and subcloned into pBIB vector under control of the superpromoter, which consists of three copies of the octopine synthase upstream-activating sequence in front of the manopine synthase promoter (28). *Agrobacterium tumefaciens* strain GV3101 containing this binary construct was used to transform *los4-1* mutant plants. Transformants were screened on MS medium containing 25 mg/liter hygromycin.

Analysis of LOS4-GFP Fusion Protein. For the LOS4-GFP fusion construct, PCR was done by using a forward primer 5′-AGGAATTCATGGCGGATACGGTAGAGAAAGTTCCCACC-3′ (*Eco*RI site is underlined), and a reverse primer, 5′-GTGGATCCCACTCGTCCAGCAGGCCAGCTTCCTTTAATGC-3′ (*Bam*HI site is underlined) to amplify the full ORF of *LOS4*. The PCR product was digested with *Eco*RI and *Bam*HI, and ligated to a pEGAD vector, previously double-digested with the same two enzymes, resulting in the fusion of LOS4 in-frame with GFP at the C terminus to make a GFP-LOS4 fusion protein (29). *A. tumefaciens* strain GV3101 containing this construct was used to transform *Arabidopsis* (ecotype Columbia). Thirty lines of glufosinate herbicide-resistant transgenic plants were selected, and analyzed for GFP expression in their T_2 progeny. The staining of nuclei and GFP analysis were done as described (12).

Results

Isolation of the *los4-1* Mutant. Transgenic *A. thaliana* plants (ecotype C24) containing the *RD29A-LUC* transgene (referred to herein as wild type) emit luminescence in response to low temperature, osmotic stress, or ABA treatment (25). Mutants with altered luminescence responses to one or combinations of treatments were selected from ethyl methanesulfonate mutagenized *RD29A-LUC* plants by luciferase imaging (25). Preliminary analysis identified several mutants exhibiting a decreased response only to cold, but not to ABA or osmotic (high-salt) stress. One of these mutants, designated as *los4-1*, was chosen for detailed characterization. Fig. 1*A* shows a comparison of luminescence images of *los4-1* and wild-type seedlings under cold (without light), ABA, and high-salt treatments. The luminescence images of *los4-1* seedlings seem less bright under cold but not under ABA or NaCl treatment. Quantitation of the luminescence intensities indicates that *los4-1* plants have a cold response that is about 40% lower than that of the wild type at 24 h (Fig. 1*B*). The peak luminescence intensities of *los4-1* plants are slightly higher than those of wild-type plants under ABA treatment. Under NaCl treatment, the luminescence intensities are not significantly different between *los4-1* and the wild type (Fig. 1*B*). Because we later discovered that *los4-1* plants show a severe-chilling-sensitive phenotype in the dark but not in the light, the luminescence responses of mutant and wild-type plants treated in the cold and light were also compared, and a similar difference was found as in the dark (data not shown).

Homozygous *los4-1* mutant plants were backcrossed to the wild type. The resulting F_1 plants all exhibited a wild-type phenotype in luminescence responses and chilling sensitivity. The F_2 progeny from the selfed F_1 segregated ≈3:1 for wild type/mutant based on the chilling-sensitive phenotype described later (data not shown). These results indicate that the *los4-1* mutant is caused by a recessive mutation in a single nuclear gene.

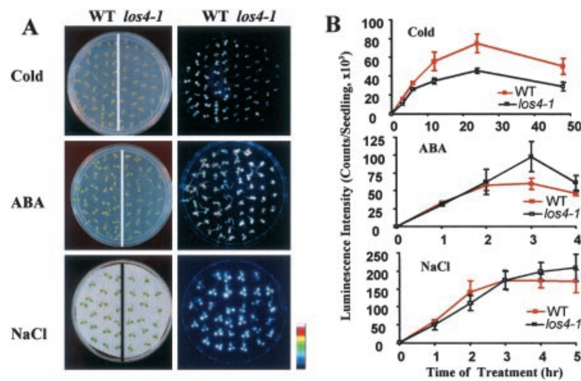

Fig. 1. The *los4-1* mutation reduces *RD29A-LUC* expression in response to cold but not to ABA or hyperosmotic stress. (*A*) *RD29A-LUC* expression was indicated by luminescence images and quantitatively measured as luminescence intensity. Wild-type (*Left*) and *los4-1* (*Right*) seedlings, and their luminescence images taken after treatment at 4°C, or with 100 μM ABA for 3 h or 300 mM NaCl for 5 h. The color scale at right shows the luminescence intensity from dark blue (lowest) to white (highest). (*B*) Time courses of luminescence intensities of wild-type and *los4-1* plants under cold, ABA, or NaCl treatment. Data represent means and standard errors ($n = 20$). Black square, wild type; open square, *los4-1*.

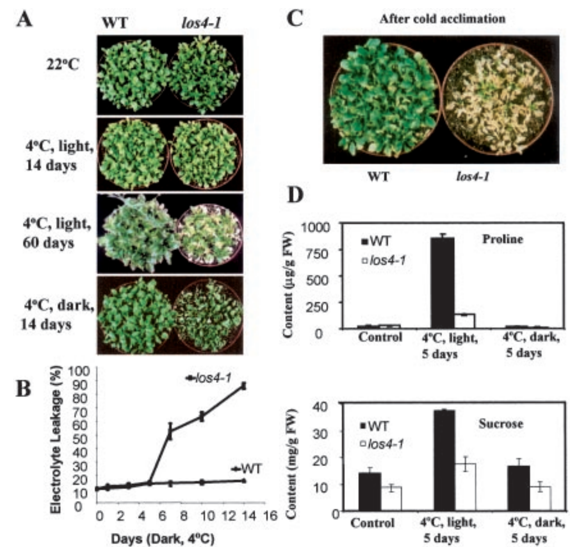

Fig. 3. Chilling and freezing sensitivity of *los4-1* mutant plants. (*A*) Wild-type and *los4-1* mutant plants grown at 22°C, without chilling treatment, or treated at 4°C for 2 weeks or 2 months in light, or 2 weeks in dark. (*B*) Electrolyte leakage. Electrolyte leakage assays were done by using wild-type and *los4-1*mutant leaves from 2-week-old plants grown in soil and treated at 4°C in the dark. (*C*) Wild-type and *los4-1* mutant plants grown at 22°C for 2 weeks were cold-acclimated at 4°C for 4 days in light and then treated at −8°C for 6 h. (*D*) Sucrose and proline contents in *los4-1* mutant and wild-type plants treated at 4°C in light or dark for 5 days.

The *los4-1* Mutation Reduces Cold Induction of *CBFs* and Their Downstream Genes. RNA blot analysis was performed to determine whether *los4-1* affects the expression of endogenous *RD29A* and other cold-responsive genes. Under cold treatment in the light, the transcript levels of *RD29A*, *KIN1*, and *COR15A* were lower in *los4-1* plants than in the wild type at the 48-h time point when the expression was the highest (Fig. 2). *los4-1* plants also showed lower levels of expression of these genes at the 12-h time point. Intriguingly, at the 24- h time point the expression levels of these genes were very low in the wild-type and lower than in *los4-1* plants (Fig. 2), which may have to do with circadian regulation of these genes (30). Under cold treatment in the dark, the expression levels of these genes also seemed lower in *los4-1* plants, except for the 48- h time point (Fig. 2). A cold-induced desaturase (AAF82163) gene showed a reduced expression in *los4-1* caused by the cold treatment either in the light or dark (Fig. 2)

Because most of the cold-responsive genes above are known to be activated by the CBF transcription factors (11), the expression levels of *CBF* genes were compared between *los4-1* and the wild type. Under cold treatment in the light, *CBF1* had a peak level of induction at 6 h in the wild type, but a reduced peak level in *los4-1* at 24 h (Fig. 2). *CBF3* also had a peak level of induction at 6 h in the wild type, and it was not substantially induced in *los4-1* at any time point (Fig. 2). Under cold treatment in the dark, *CBF1* and *CBF3* were not detected in either the wild type or *los4-1*. Under cold and light, *CBF2* had a peak level of induction at 3 h in the wild type and a reduced peak level at 24 h in *los4-1* (Fig. 2). Under cold and dark, *CBF2* had a strong peak level of induction at 3 h in the wild type but a much weaker induction in *los4-1*, which peaked at 6 h. These results show that the *los4-1* mutation has a complicated effect on cold-induced gene expression but it generally lowers the levels of induction.

***los4-1* Mutant Plants Are Chilling-Sensitive.** No apparent phenotypic difference existed between the *los4-1* mutant and wild-type plants grown at warm temperatures (Fig. 3*A*). The *los4-1* and wild-type plants grown at warm temperatures for 2 weeks were transferred to 4°C either in the light or dark. After cold treatment in the dark for 2 weeks, *los4-1* mutant plants were severely damaged and their leaves died completely (Fig. 3*A*). In contrast, cold treatment in the dark for 2 weeks caused no apparent damage to wild-type plants. Cold treatment in the light for 2 weeks did not cause damage to the wild-type or *los4-1* mutant plants (Fig. 3*A*). However, prolonged cold treatment (e.g., 2 months) in the light caused substantial damage to the leaves of *los4-1*, but not as much to wild-type plants (Fig. 3*A*).

To quantify the extent of chilling injury of *los4-1* plants in the

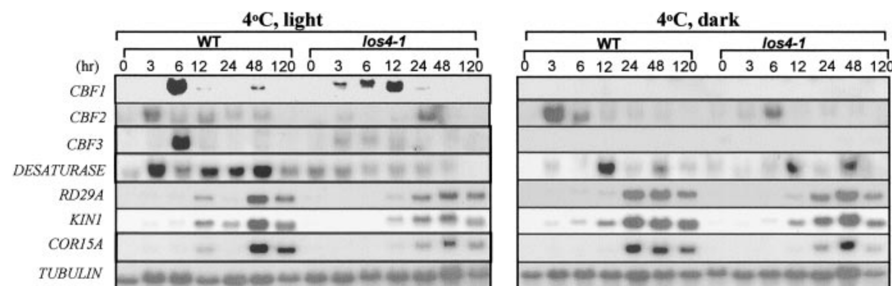

Fig. 2. Transcript levels of cold-regulated genes in *los4-1* and wild-type plants. Plants were treated at 4°C for the indicated times. The *TUBULIN* gene was used as a loading control. WT, wild type.

dark, electrolyte leakage was assayed in leaves excised from soil-grown plants treated at 4°C in the dark for different times. Wild-type leaves showed little increase in electrolyte leakage during a 2-week cold treatment in the dark. However, *los4-1* leaves showed a dramatic increase in electrolyte leakage after 7 days of cold treatment in the dark (Fig. 3*B*). After 2 weeks, approximately 90% of cellular electrolytes in *los4-1* leaves leaked out, indicating that the mutant leaves were killed.

***los4-1* Mutant Plants Are Defective in Cold Acclimation.** Because of the defect in cold-responsive gene expression in *los4-1* mutant plants, we were interested in testing whether the mutant has a reduced capacity to cold-acclimate. Because the mutant does not show a chilling sensitivity in the light when treated for a relatively short duration, we performed cold-acclimation treatment at 4°C in the light for 4 days. Without cold acclimation, no difference occurred in freezing tolerance between *los4-1* and wild-type plants grown at warm temperatures for 3 weeks (data not shown). After 4 days of cold acclimation in the light, the wild-type plants acquired a high degree of freezing tolerance and could survive a 6-h treatment at −8°C (Fig. 3*B*). However, *los4-1* mutant plants were either completely killed or severely damaged by the same freezing treatment (Fig. 3*B*). The results demonstrate that the *los4-1* mutation impairs cold acclimation.

Reduced Accumulation of Proline and Sucrose in *los4-1* Mutant Plants. *Arabidopsis* plants ectopically expressing *CBF3* show enhanced accumulation of proline (Pro) and total soluble sugars after cold acclimation, and these osmolytes may contribute to freezing tolerance (31). We determined the sucrose and proline contents after cold acclimation in wild-type and *los4-1* plants. Treatment at 4°C under light for 5 days induced significant proline accumulation in the wild type (Fig. 3*C*). In contrast, the same treatment only led to a slight increase in proline level in *los4-1* mutant plants (Fig. 3*C*). In the dark, the cold treatment did not induce proline accumulation in either *los4-1* or the wild type (Fig. 3*C*).

Induction of sucrose accumulation at 4°C under light was lower in *los4-1* than in the wild type (Fig. 3*C*). Cold treatment in the dark failed to up-regulate sucrose content significantly in either *los4-1* or the wild type. These results are consistent with the proposed roles of proline and sucrose in freezing tolerance, although they do not provide an explanation of the severe chilling sensitivity of *los4-1* plants in the dark.

Ectopic Expression of *CBF3* in *los4-1* Suppresses the Chilling- and Freezing-Sensitive Phenotypes. We hypothesized that the defect in the regulation of *CBF* genes in *los4-1* may be responsible for the chilling and freezing sensitivity of the mutant plants. To test this hypothesis, *CBF3* was expressed under a strong constitutive super promoter (28) in *los4-1* mutant plants (Fig. 4). Fourteen independent *CBF3* overexpression lines were tested and survived 4°C treatment in the dark for 2 weeks. Two independent transgenic lines were selected for detailed characterization. The transcripts of *CBF3* were detected by RNA blot analysis in both lines under normal growth conditions when none of the endogenous *CBF* genes was expressed. The ectopic expression of *CBF3* in one of these lines is shown in Fig. 4*B*. *CBF3* expression resulted in constitutive *RD29A-LUC* expression without cold treatment (Fig. 4*A*). Chilling resistance cosegregated with the *CBF3* transgene in the T_2 progeny of the transformants (data not shown). One homozygous T_3 population is illustrated in Fig. 4*C*, which shows that all *los4-1* plants transformed with the constitutive *CBF3* transgene became resistant to chilling treatment in the dark for 2 weeks. In addition, when tested at 4°C in the light for more than 2 months, the CBF3-expressing *los4-1* plants were not damaged (data not shown). A homozygous T_3 population was examined for freezing tolerance after cold acclimation at 4°C

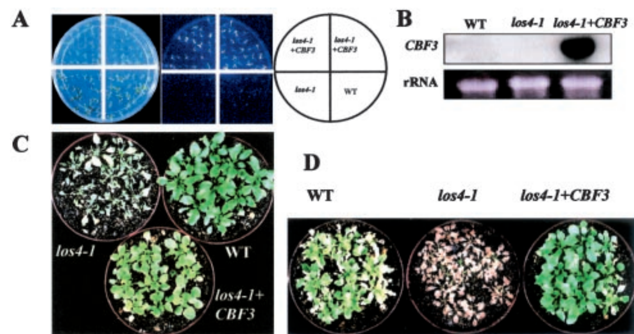

Fig. 4. Overexpression of the *CBF3* gene reverses the chilling-sensitive phenotype of *los4-1* mutant plants. (*A*) *RD29A-LUC* luminescence images of two *CBF3* transgenic lines (line 1 on the right, heterozygous; line 2 on the left, homozygous), compared with *los4-1* mutant and wild-type plants. The plants were grown at 22°C and not treated with any stress. (*B*) Expression of *CBF3* in one transgenic line (line 2) without cold treatment. (*C*) *los4-1* mutant plants transformed with the *CBF3* gene were chilling-resistant. Transgenic line 2, wild-type and *los4-1* mutant plants were treated at 4°C in dark for 14 days. (*D*). Freezing tolerance test of transgenic line 2 plants. After cold acclimation at 4°C for 4 days in light, wild-type (WT), *los4-1* mutant, and *los4-1* overexpressing CBF3 plants were treated at −8°C for 6 h.

in the light for 1 week, and the transformants were found to perform even better than the wild-type control plants (Fig. 4*D*). These results show that constitutive expression of *CBF3* reversed the chilling- and freezing-sensitive phenotypes of *los4-1* mutant plants.

Positional Cloning of the *LOS4* Gene. We isolated the *LOS4* locus by using a map-based cloning strategy. A segregating F_2 family was obtained from a cross between plants homozygous for *los4-1* mutation in the C24 background and wild-type plants of Columbia ecotype. *los4-1* mutant plants were selected from the F_2 population after a cold treatment in the dark for 2 weeks. An initial survey of 45 of these mutant plants with polymorphic markers placed *LOS4* to chromosome III, between the SSLP markers nga162 and nga112. Subsequent mapping with more mutant plants by using markers GAPAB, T32N15, F18B3, CDC2BG, F4F15, and F22O6 narrowed down *LOS4* to a contig of four BAC clones: F3C22, F8J2, T4D2, and F4P12 (Fig. 5*A*). New SSLP markers were developed on the basis of the sequence of these BAC clones. By surveying 1,126 recombinant chromosomes, we found two recombination events at about 89.8 kb on the BAC clone F3C22 (total, 100 kb), one recombination at about 63.5 kb on BAC clone F8J2 (total, 99.8 kb), no recombination at about 78.9 kb on BAC clone T4D2 (total, 92.6 kb), and two recombinations at 18.7 kb on BAC clone F4P12 (total, 144.6 kb) (Fig. 5*A*). We sequenced candidate ORFs between the nearest flanking markers on F8J2 and F4P12, and compared the sequences from *los4-1* with those from wild-type plants. In the hypothetical T4D2.40 gene, a G-to-A mutation was discovered in the *los4-1* mutant.

To confirm that the *los4-1* mutant phenotypes are caused by this mutation in the T4D2.40 gene, we conducted a complementation test by using the wild-type T4D2.40 gene amplified by PCR from BAC clone T4D2. Twenty *los4-1* mutant plants transformed with the T4D2.40 gene were obtained. When tested under cold treatment in the dark for 2 weeks, all of the transformants showed a chilling-resistant, wild-type phenotype. Homozygous T_3 progeny from two independent transformants were further tested and found to exhibit the wild-type level of chilling resistance; one of the two lines is shown in Fig. 5*B*. The T_3 seedlings were also examined for *RD29A-LUC* expression under cold stress, and the expression level was found to be the

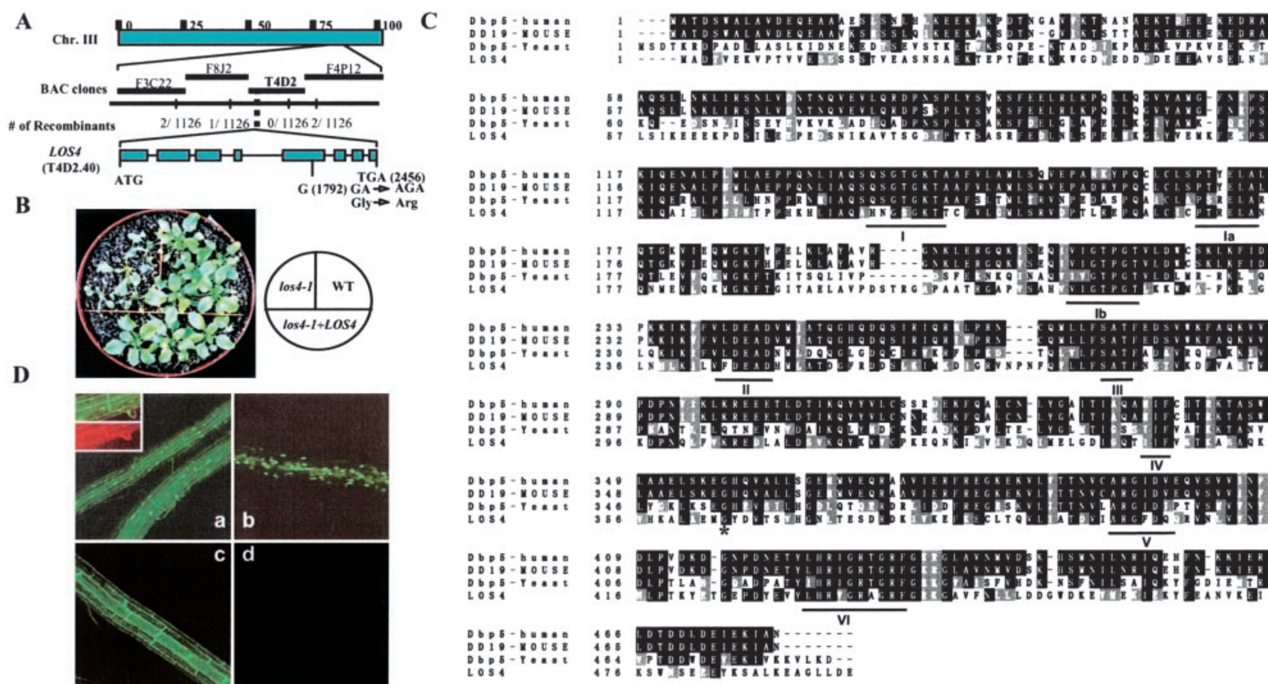

Fig. 5. Positional cloning and characterization of the *LOS4* gene. (*A*) Genetic mapping of *LOS4*. Genetic mapping delimited *LOS4* between the bottom of BAC clone F8J2 and the top of BAC clone F4P12. The *los4* mutation was identified by sequencing and comparing all predicted genes in this region from *los4-1* mutant and wild-type plants. Structure of *LOS4* and the position of the *los4* mutation are indicated. Positions are relative to the initiation codon. Filled boxes indicate the ORF, and lines between boxes indicate introns. (*B*) Complementation of *los4-1* mutant by the wild-type *LOS4* gene. *los4-1* mutant, wild-type, and *los4-1* mutant transformed with the wild-type *LOS4* gene (indicated as *los4-1*+*LOS4*) were treated at 4°C in dark for 14 days. The picture was taken 3 days after the plants were removed from 4°C to 22°C. (*C*) Alignment of LOS4 amino acid sequence with other RNA helicases. Amino acid residues in black indicate identical matches, and those in gray indicate conserved substitutions. The specific motifs conserved in DEAD-box RNA helicases are underlined. The mutation in *los4-1* mutant is marked by a star. GenBank accession numbers for the RNA helicases are as follows: Dbp5-Human, AJ237946; DD19-Mouse, Q61655; Dbp5-Yeast, NP 014689. (*Da*) A GFP-LOS4 fusion protein is localized in both nuclei and cytoplasm in the cells in transgenic *Arabidopsis*. Cells were stained with propidium iodide to indicate the nuclei (inset in red). (*Db*) A transgenic plant (line N7) expressing a GFP fused with a transcriptional factor as a positive control for nuclear localization (29). (*Dc*) Line Q1 expressing GFP fused with an acidic ribosomal protein as positive control for cytoplasmic localization (29). (*Dd*) A plant without GFP is shown as negative control.

same as that in wild-type plants (data not shown). These results prove that T4D2.40 is the *LOS4* gene.

***LOS4* Encodes a Putative RNA Helicase.** LOS4 cDNA was obtained by reverse transcription–PCR. A comparison between the cDNA and genomic sequences suggests that the *LOS4* gene contains 8 exons and 7 introns. *LOS4* is predicted to encode a protein of 496 amino acid residues with an estimated molecular mass of approximately 55.3 kDa. Database searches revealed that LOS4 belongs to the family of DEAD-box RNA helicases (Fig. 5*C*). LOS4 has all of the eight characteristic motifs of the DEAD-box protein family, including the DEAD (Asp-Glu-Ala-Asp) tetrapeptide in the motif II that is diagnostic of this family of helicases (32). The *los4-1* point mutation (GCA to AGT) occurs in the 5th exon, and changes the conserved Gly-364 to Arg. *LOS4* is a single copy gene in the *Arabidopsis* genome.

To determine the subcellular localization of the LOS4 protein, *LOS4* cDNA was fused in-frame at its N terminus with the green fluorescence protein (GFP) marker. GFP-LOS4 fusion protein was expressed under the CaMV 35S promoter in transgenic *Arabidopsis* plants. The subcellular localization of GFP-LOS4 was determined by green fluorescence imaging under a confocal microscope. The GFP-LOS4 protein was detected in both nuclei and cytoplasm in the cells (Fig. 5*D*). As positive controls, plants expressing a known nuclear localized GFP fusion protein (GFP fused to a transcriptional factor) and a known cytoplasmic localized GFP fusion protein (GFP fused with an acidic ribosomal protein) (29) are shown in Fig. 5*D*. Plants not transformed with any GFP fusion construct did not emit any green fluorescence and are shown as a negative control (Fig. 5*D*).

Discussion

Genetic screening by using the *RD29A-LUC* system has identified a *Arabidopsis* mutant, *los4-1*, which is altered in cold-responsive gene regulation and chilling and freezing tolerance. Our results show that LOS4 plays an important role in the positive regulation of the expression of *CBF* genes. To our knowledge, this is the first time that a positive regulator of *CBF* genes has been identified. In nonplant systems, RNA helicases are known to be involved in every step of RNA metabolism, including nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay, and organellar gene expression (32). However, the functions of RNA helicases are poorly understood in plants (33, 34). Among all sequenced genomes including those of human, fly, worm, and yeast, *Arabidopsis* has the largest number of DEAD-box RNA helicase genes, estimated to be more than 50 (34, 35). Three RNA helicase genes were recently cloned from *Arabidopsis* mutants; one is thought to be required for posttranscriptional gene silencing in *Arabidopsis* (36), the other two yielded no clear clues to their biochemical function (37, 38). How *LOS4* positively regulates the expression of *CBF* genes in *Arabidopsis* is unclear at the present time. Because *LOS4* gene encodes a putative RNA helicase, its function must be involved in RNA metabolism. In cyanobacteria, a cold-induced DEAD-box RNA helicase has been suggested to unwind cold-stabilized secondary structure in

the 5′-untranslated region of RNA during cold stress (39). LOS4 may directly control the stability or other aspects of *CBF* transcripts under cold treatment. It is also possible that LOS4 may affect *CBF* transcript accumulation indirectly by regulating the metabolism of transcripts of activators or repressors of *CBF* genes.

The *los4* mutation impairs both cold signaling (e.g., *CBF* regulation) and chilling resistance of plants, implicating a genetic connection between the two important aspects of low-temperature responses. The expression of *CBF* genes under chilling treatment was either blocked or delayed in *los4-1* mutant plants. Ectopic expression of *CBF3* could suppress the chilling sensitivity of *los4-1* mutant plants. Together, these results demonstrate that the *CBF* regulon is critical for chilling resistance, in addition to its well known role in freezing tolerance. Ectopic or overexpression of *CBF* genes may lead to improved chilling resistance in some naturally chilling sensitive crops.

Light alleviates the chilling sensitivity of *los4-1* mutant plants. Cold treatment in the light-induced proline and sucrose accumulation in *los4-1* mutant plants, although the induced levels were still lower than in the wild type. The moderate accumulation of proline and sucrose may help protect *los4-1* cells from chilling injury. It seems that light as a signal helps activate certain chilling tolerance mechanisms including the expression of some *CBF* genes and the accumulation of proline and sucrose. In addition, photosynthesis-generated energy supply might also be beneficial for chilling resistance.

Chilling temperatures reduce the fluidity of cellular membranes (17, 19–21). The CBF transcriptional activators may control the expression of fatty acid desaturase genes important for the adjustment of cellular membrane fluidity under chilling stress. This control is suggested by the defect of *los4-1* mutant in the cold induction of a fatty acid desaturase gene (Fig. 2). However, in *Arabidopsis*, a failure in membrane fatty acid unsaturation retards plant growth under chilling temperatures but does not confer severe chilling sensitivity (21, 40). So the severe-chilling-sensitive phenotypes of *los4-1* mutant plants cannot be attributed only to the lower expression of fatty acid desaturase gene(s). Our results suggest that the defects in cold-induced expression of *COR* genes and desaturase genes combined may be the main culprit for chilling sensitivity in the *los4-1* mutant, and perhaps in some naturally chilling-sensitive plants as well. Recently, genetic studies showed that dehydrins in cowpea seeds are associated with chilling tolerance during seed germination (41). Many of the vegetatively expressed *COR* genes controlled by *CBFs* are probably functionally related to the seed dehydrins and may thus be important for chilling tolerance in vegetative tissues. Although CBFs are known to regulate multiple molecular and physiological processes associated with cold acclimation (31), the full spectrum of CBF-regulated cellular processes remain to be defined. The precise causes of chilling sensitivity or injury are also poorly understood. Future identification of the entire spectrum of CBF-regulated processes will help reveal some of the unknown causes of chilling sensitivity in plants.

We thank Dr. Julio Salinas for the desaturase gene probe and Dr. David W. Ehrhardt for pEGAD vector. This work was supported by U.S. Department of Agriculture National Research Initiative Grant 2000-00664 and National Science Foundation Grant IBN-9808398 (to J.-K.Z.).

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