

ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*

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Cold temperatures trigger the expression of the CBF family of transcription factors, which in turn activate many downstream genes that confer chilling and freezing tolerance to plants. We report here the identification of *ICE1* (*inducer of CBF expression 1*), an upstream transcription factor that regulates the transcription of *CBF* genes in the cold. An *Arabidopsis ice1* mutant was isolated in a screen for mutations that impair cold-induced transcription of a *CBF3* promoter-luciferase reporter gene. The *ice1* mutation blocks the expression of *CBF3* and decreases the expression of many genes downstream of *CBFs*, which leads to a significant reduction in plant chilling and freezing tolerance. *ICE1* encodes a MYC-like bHLH transcriptional activator. *ICE1* binds specifically to the MYC recognition sequences in the *CBF3* promoter. *ICE1* is expressed constitutively, and its overexpression in wild-type plants enhances the expression of the *CBF* regulon in the cold and improves freezing tolerance of the transgenic plants.

[*Keywords:* Cold signaling; freezing tolerance; bHLH protein; CBF regulation; transcriptome]
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Cold is an environmental factor that limits the geographical distribution and growing season of many plant species, and it often adversely affects crop quality and productivity (Thomashow 1999). Most temperate plants can acquire tolerance to freezing temperatures by a prior exposure to low nonfreezing temperatures, a process known as cold acclimation (Guy 1990; Hughes and Dunn 1996; Browse and Xin 2001). Plants of tropical and subtropical origins are sensitive to chilling temperatures (0°C–10°C) and are incapable of cold acclimation. Many studies have suggested that cold-regulated gene expression is critical in plants for both chilling tolerance (Gong et al. 2002; Hsieh et al. 2002) and cold acclimation (Knight et al. 1999; Thomashow 1999; Tahtiharju and Palva 2001). Cold-responsive genes encode a diverse array of proteins such as enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants; molecular chaperones, antifreeze proteins, and others with a presumed function in tolerance to the dehydration caused by freezing (Guy 1990; Thomashow 1999; Mohapatra et al. 1989).

Many of the cold and dehydration responsive genes have in their promoters one or several copies of the *DRE/*

CRT cis-element, which has the core sequence, CCGAC (Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997). A family of transcription factors known as *CBFs* or *DREB1s* binds to this element and activates transcription of the downstream cold and dehydration-responsive genes (Stockinger et al. 1997; Liu et al. 1998). Interestingly, the *CBF/DREB1* genes are themselves induced by low temperatures. This induction is transient and precedes that of the downstream genes with the *DRE/CRT cis*-element (Thomashow 1999). Therefore, there is a transcriptional cascade leading to the expression of the *DRE/CRT* class of genes under cold stress. Ectopic expression of *CBFs/DREB1s* in plants turns on downstream cold-responsive genes even at warm temperatures and confers improved freezing tolerance (Jaglo-Ottosen et al. 1998; Liu et al. 1998).

Since *CBF* transcripts begin accumulating within 15 min of plants' exposure to cold, Gilmour et al. (1998) proposed that there is a transcription factor already present in the cell at normal growth temperature that recognizes the *CBF* promoters and induces *CBF* expression upon exposure to cold stress. Gilmour et al. (1998) named the unknown activator(s) "ICE" (*inducer of CBF expression*) protein(s) and hypothesized that upon exposing a plant to cold, modification of either ICE or an associated protein would allow ICE to bind to *CBF* promoters and activate *CBF* transcription.

Genetic analysis in *Arabidopsis* plants expressing the

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firefly luciferase reporter gene driven by the *CRT/DRE* element-containing *RD29A* promoter (Ishitani et al. 1997) has identified several mutants with deregulated cold-responsive gene expression. The *hos1* (*high expression of osmotically responsive genes*) mutant shows an enhanced cold-induction of *CBFs* and their downstream cold responsive genes (Ishitani et al. 1998). *HOS1* encodes a RING finger protein that is present in the cytoplasm at normal growth temperatures, but accumulates in the nucleus upon cold treatment. Since many RING-finger proteins are known to serve as ubiquitin E3 ligases, *HOS1* has been proposed to function by targeting certain positive regulator(s) of *CBFs* for ubiquitination and degradation (Lee et al. 2001). The transcription of *CBF* genes is also under feedback repression by its own gene product or its downstream target gene products. This was revealed by studies on the *los1* mutant that is defective in the translational elongation factor 2 gene (Guo et al. 2002). The *los1* mutation blocks cold induction of genes with the *CRT/DRE* element, but causes superinduction of the *CBF* genes. It was shown that protein synthesis in *los1* mutant plants is disrupted specifically in the cold. Therefore, cold-induced *CBF* transcripts cannot be translated to activate downstream genes, and feedback repression cannot occur, leading to superinduction of *CBF* transcripts (Guo et al. 2002).

Another *Arabidopsis* mutation, *los2*, also impairs cold induction of *CRT/DRE* element-containing genes (Lee et al. 2002). *LOS2* encodes a bifunctional enolase that can bind to the promoter of *ZAT10*, a zinc finger transcriptional repressor. *ZAT10* expression is rapidly and transiently induced by cold in the wild type, and this induc-

tion is stronger and more sustained in the *los2* mutant. Therefore, *LOS2* may control the expression of delayed cold response genes via transcriptional repression of *ZAT10* (Lee et al. 2002). The *Arabidopsis LOS4* locus is involved in the accumulation of *CBF* transcripts under cold treatment (Gong et al. 2002). *los4-1* mutant plants are sensitive to chilling stress, and the chilling sensitivity can be rescued by ectopic expression of *CBF3* (Gong et al. 2002). *LOS4* encodes a DEAD-box RNA helicase, suggesting that RNA metabolism may be involved in cold responses.

We carried out a genetic screen (Chinnusamy et al. 2002) to identify cold signaling components upstream of the *CBF* proteins. A cold-responsive bioluminescent *Arabidopsis* plant was engineered by expressing the firefly luciferase (*LUC*) coding sequence under the control of the *CBF3* promoter. Homozygous *CBF3-LUC* plants were chemically mutagenized and mutants with altered cold-induced *CBF3-LUC* expression were isolated by luminescence imaging. Here we report on the *ice1* mutant, which is impaired in the cold-induction of *CBF3-LUC* and is defective in cold acclimation. *ICE1* encodes a MYC-like bHLH transcriptional activator that binds to the *CBF3* promoter. Thus, *ICE1* plays a key role in regulating cold-responsive gene expression and cold tolerance in *Arabidopsis*.

Results

Identification of the *ICE1* locus

As noted above, *Arabidopsis* plants containing the *CBF3-LUC* transgene emitted bioluminescence in re-

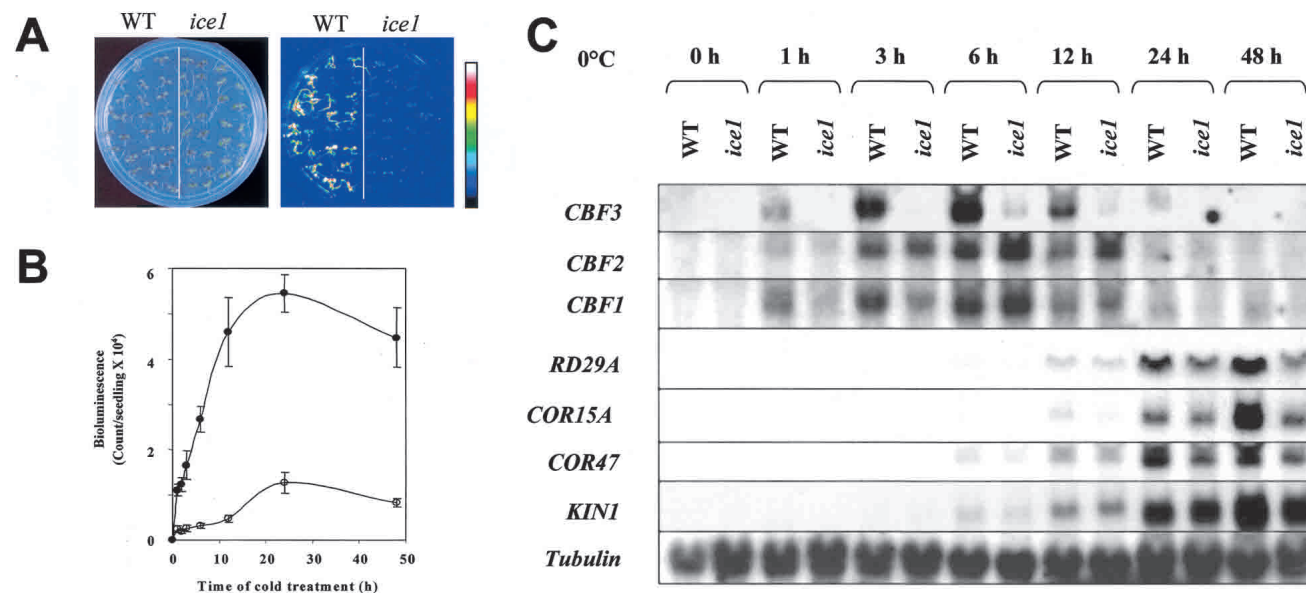


Figure 1. The *ice1* mutation blocks the cold-induction of *CBF3* and affects the expression of other cold-responsive genes. (A) Morphology (left) and *CBF3-LUC* luminescence images (right) of wild-type and *ice1* seedlings. Luminescence images of the plants were collected after 12 h of cold (0°C) treatment. (B) Quantitation of the luminescence intensities of wild-type (solid circles) and *ice1* (open circles) seedlings in response to different durations of cold treatment. (C) Transcript levels of *CBFs* and their downstream target genes in wild-type and *ice1* plants in response to cold treatment. Seedlings were either not treated (0 h) or treated with cold (0°C) for the indicated durations (h). The tubulin gene was used as a loading control. WT, wild type.

sponse to cold stress (Fig. 1A,B). The homozygous *CBF3-LUC* plants (herein referred to as wild type) were mutagenized by ethylmethane sulfonate, and the resulting M2 population was screened for mutants with aberrant bioluminescence responses under cold stress using a low light imaging system (Chinnusamy et al. 2002). Several mutants showing abnormal cold regulation of *CBF3-LUC* expression were recovered. One of these mutant lines, designated as *ice1*, is virtually blocked in *CBF3-LUC* expression in the cold (Fig. 1A,B). In response to treatment at 0°C, wild-type plants showed strong luminescence, while the *ice1* mutant showed very little induction of luminescence throughout the duration of cold treatment (Fig. 1A,B). After 12 h of cold treatment, *ice1* plants showed nearly 10 times less luminescence than that of wild-type plants, and are obviously defective in the cold regulation of *CBF3-LUC* expression (Fig. 1B).

The *ice1* mutant plant was crossed with *CBF3-LUC* wild-type plants and the resulting F1 plants were examined for *CBF3-LUC* expression after 12 h of cold treatment at 0°C. As determined by luminescence imaging, all F1 plants showed reduced cold-induced *CBF3-LUC* expression similar to that of *ice1*. An F2 population from the selfed F1 segregated in an ~3:1 ratio between mutant

and wild type (data not shown). These results show that *ice1* is a dominant mutation in a single nuclear gene.

ice1 mutant plants are defective in cold-regulated gene expression

RNA blot analysis was carried out to analyze the effect of *ice1* mutation on the transcript levels of endogenous *CBFs* and their target cold stress-responsive genes. Consistent with the imaging results, cold induction of the endogenous *CBF3* gene was greatly impaired (almost abolished) in *ice1* mutant plants (Fig. 1C). Wild-type plants showed *CBF3* induction after 1 h of cold stress and the expression peaked at 6 h. In contrast, *CBF3* induction was almost abolished in *ice1* plants (Fig. 1C). While the *CBF1* induction level in the *ice1* mutant was lower than that of wild type at 1 and 3 h of cold stress, its induction level at 6 and 12 h was similar to that in the wild type. The *CBF2* induction level was slightly lower in *ice1* at 1 h of cold treatment, whereas at 6 and 12 h, the induction level was higher in the mutant (Fig. 1C). We also examined the cold induction of the downstream target genes of *CBFs*. The expression levels of *RD29A*, *COR15A*, and *COR47A* under cold stress were lower in *ice1* than in the

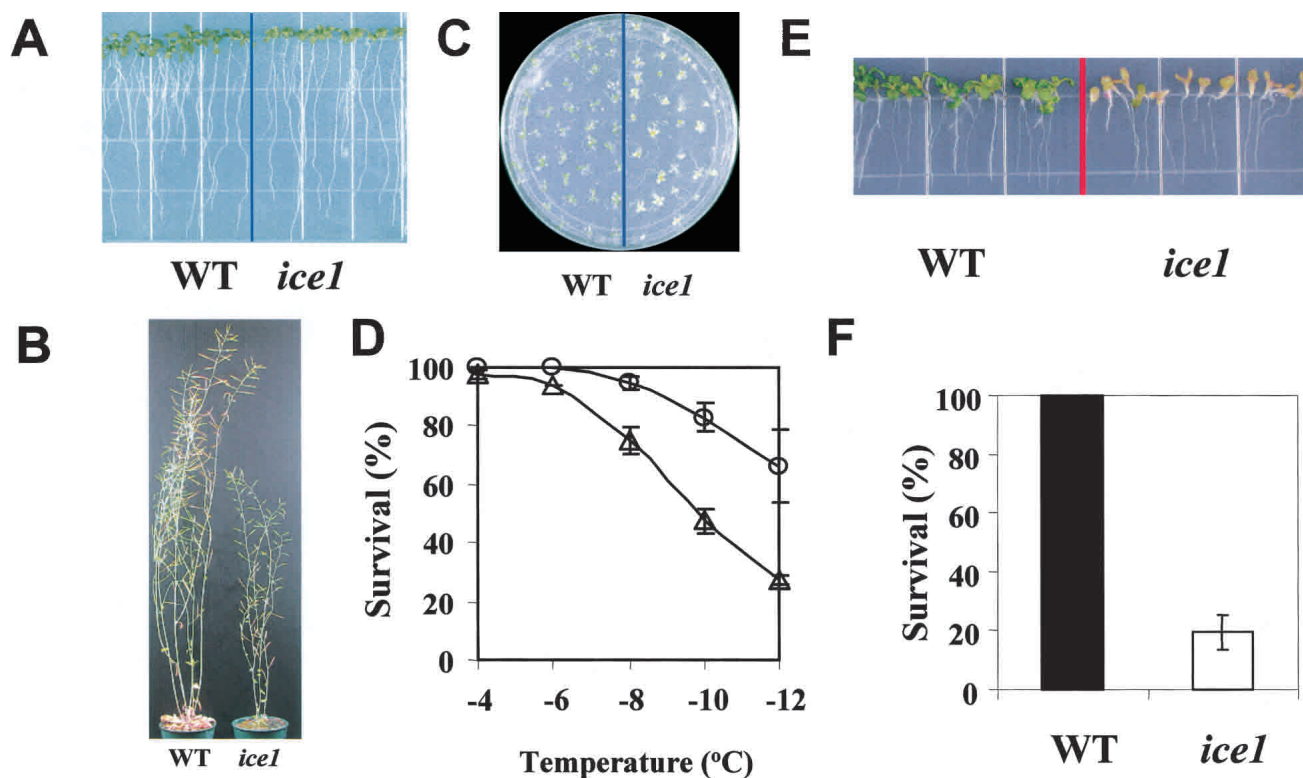


Figure 2. Morphology and freezing and chilling sensitivity of *ice1* mutant plants. (A) Wild-type and *ice1* seedlings in nutrient medium on agar under normal growth conditions. (B) Wild-type and *ice1* plants in soil under normal growth conditions. (C) *ice1* plants are defective in cold acclimation. Ten-day-old seedlings grown at 22°C were incubated for 4 d in light at 4°C before freezing treatment at -12°C. The picture was taken 3 d after the freezing treatment. (D) Comparison of survival rates after freezing treatments at the indicated temperatures. Open circles and open triangles represent wild-type and *ice1* plants, respectively. (E) *ice1* plants are sensitive to prolonged chilling treatment. After germination at 22°C, the plants were grown at 4°C for 6 wk. (F) Comparison of survival rates after 6 wk of chilling stress.

wild type, while the induction of *KIN1* was lower in *ice1* only after 48 h of cold stress (Fig. 1C).

Consistent with these RNA blot results, microarray analysis using Affymetrix near full genome genechips showed that out of 306 genes induced threefold or more in the wild type by a 6-h cold treatment, 217 are either not induced in the *ice1* mutant or their induction is 50% or less of that in the wild type (Supplementary Tables 1A, 2). Thirty-two of these encode putative transcription factors, suggesting that ICE1 may control many cold-responsive regulons. For 87 of the 306 cold-induced genes, their induction levels in the wild type and *ice1* differ by less than twofold (Supplementary Table 1B). Interestingly, two genes show higher levels of cold induction in the *ice1* mutant (Supplementary Table 1C).

The *ice1* mutation impairs chilling and freezing tolerance

At normal growth temperatures, *ice1* and wild-type seedlings were similar in size (Fig. 2A). Although adult *ice1* plants were smaller, they were not very different from the wild type in flowering time and fertility (Fig. 2B). Ten-day-old seedlings of *ice1* and wild type grown on separate halves of the same agar plates were cold acclimated at 4°C for 4 d and then subjected to a freezing tolerance assay. The *ice1* mutant was less freezing-tolerant than the wild type at all freezing temperatures (Fig. 2C,D). Freezing at -10°C for 2 h killed about 50% of *ice1* mutant plants, whereas <20% of wild-type plants were killed at this temperature (Fig. 2D). When newly germinated (at 22°C) *ice1* and wild-type seedlings were transferred to 4°C (with $30 \pm 2 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light), chilling injury became apparent in the mutant after 4 wk of cold treatment (Fig. 2E). After 6 wk of chilling stress, 100% of wild-type but only 20% of *ice1* mutant plants survived (Fig. 2F).

Positional cloning of ICE1

To map the *ice1* mutation, a homozygous *ice1* mutant in the *CBF3-LUC* Columbia background was crossed to wild-

type plants of the Ler ecotype. F1 plants from the cross were selfed to produce F2 seeds. Since the *ice1* mutation is dominant, we selected from the segregating F2 population seedlings with the wild-type phenotype (based on plant size and morphology) for mapping. A total of 662 wild-type plants were selected and used for mapping with simple sequence length polymorphism and cleaved amplified polymorphic sequence markers (see Materials and Methods section for details), which initially placed *ICE1* on the middle of chromosome 3, then narrowed its location to a 58-kb region on the MLJ15 and MDJ14 BAC clones. Candidate genes in this region were amplified from homozygous *ice1* mutant plants and sequenced. The sequences were compared with the published sequence of *Arabidopsis* ecotype Columbia and a single G-to-A mutation in the hypothetical MLJ15.14 gene was found.

To confirm that MLJ15.14 is the *ICE1* gene, the MLJ15.14 gene including 2583 bp upstream of the initiation codon and 615 bp downstream of the stop codon was cloned from *ice1* mutant plants. This fragment was inserted into a binary vector and introduced into *CBF3-LUC* Columbia wild-type plants by *Agrobacterium*-mediated transformation. Transgenic plants were selected based on their hygromycin resistance, and cold-induced bioluminescence in the T2 lines was compared with that of the wild type. The MLJ15.14 gene from *ice1* suppressed cold-induced luminescence from the wild-type plants (Fig. 3A,B) and reduced the plant height to that of *ice1* mutant (data not shown), thus confirming that MLJ15.14 is *ICE1*.

ICE1 encodes a constitutively expressed and nuclear-localized MYC-like bHLH transcription factor

The open reading frame of *ICE1* was determined by sequencing cDNAs obtained by reverse transcriptase PCR (RT-PCR). *ICE1* is predicted to encode a protein of 494 amino acids, with an estimated molecular mass of 53.5 kD. Database searches revealed that *ICE1* contains a MYC-like bHLH domain at its C-terminal half (Fig. 4A,B). Over the entire length of the protein, *ICE1* shows

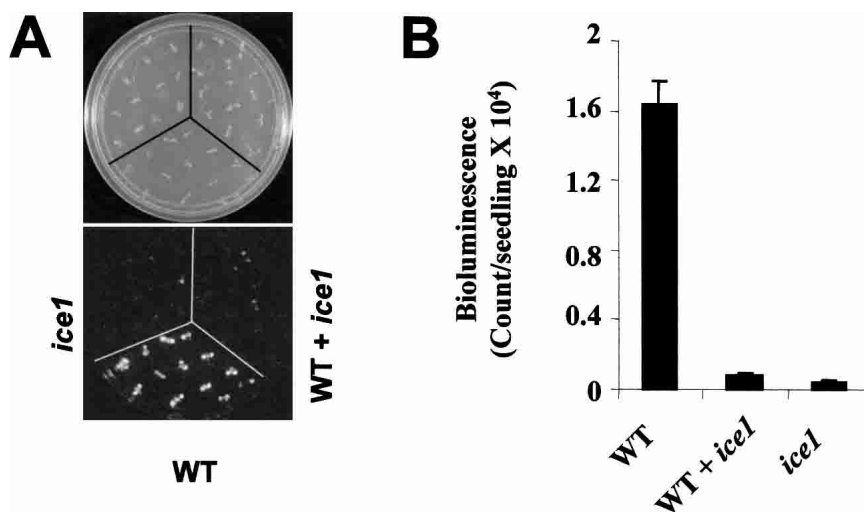


Figure 3. Confirmation of *ICE1* gene cloning by expressing the dominant *ice1* mutant allele in wild-type plants. (A) Expression in wild type of a genomic fragment containing the *ice1* mutation recapitulates the *ice1* mutant phenotype. Seven-day-old seedlings of the wild type, *ice1*, and wild type transformed with the mutant *ice1* gene grown on MS agar medium were subjected to luminescence imaging after 12 h of cold (0°C) stress. (B) Quantitation of *CBF3-LUC* bioluminescence levels in wild type (WT), *ice1* and wild type transformed with the mutant *ice1* gene after 12 h of cold (0°C) stress.

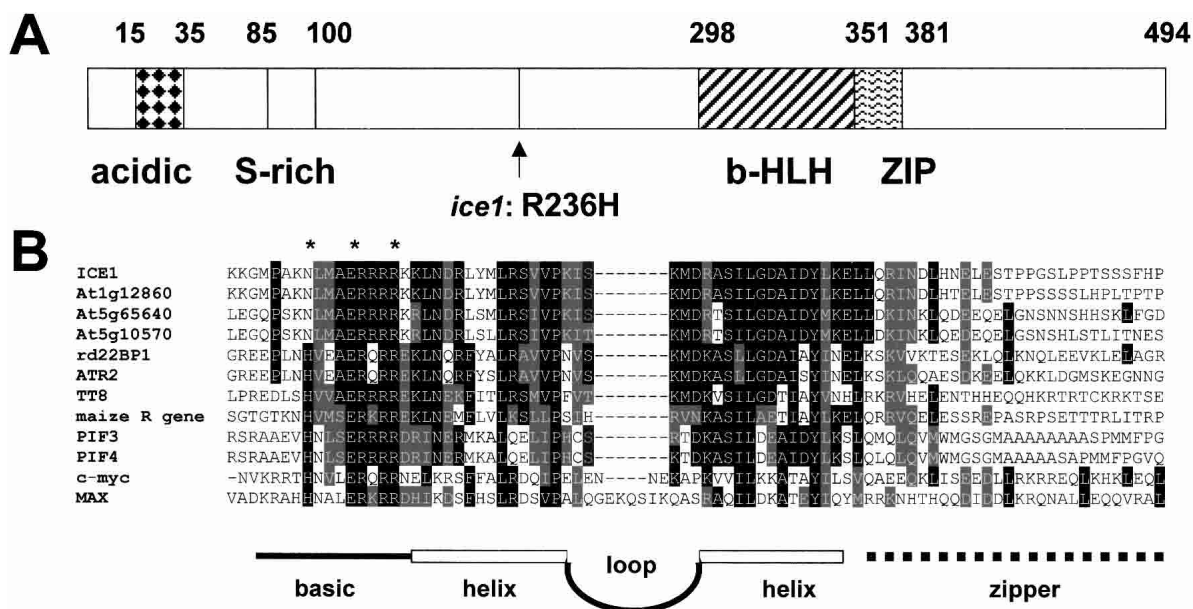


Figure 4. *ICE1* encodes a bHLH protein. (A) Overall domain structure of ICE1 protein. A putative acidic domain (acidic), serine-rich region (S-rich), bHLH domain, and possible zipper region (ZIP) are indicated. The arrow indicates the amino acid residue changed in the *ice1* mutant. (B) Sequence alignment of the bHLH domains and ZIP regions of ICE1 and other plant and animal bHLH proteins. Identical and similar residues are shown in black and gray, respectively. The basic region is indicated by a bold line and the helix–loop–helix domain is indicated by open boxes connected with a loop. The zipper region is indicated as a broken line. DDJB/EMBL/GenBank accession numbers, with amino acid numbers in parentheses, are: ICE1, AY195621 (300–398); At1g12860, NM_101157 (638–731); At5g65640, NM_125962.1 (171–269); At5g10570, NM_121095.2 (144–242); rd22BP, AB000875 (446–544); ATR2, NM_124046.1 (409–507); maize R gene, M26227 (410–508); TT8, AJ277509 (357–455); PIF3, AF100166 (254–352); PIF4, AJ440755 (255–353); MAX, P52161 (21–107); c-myc, 1001205A (354–435). Asterisks indicate amino acid residues of MAX that are known to interact with nucleotides (Grandori et al. 2000).

amino acid sequence similarity to an unknown protein of *Arabidopsis* (At1g12860). The *ice1* mutation changes Arg 236, conserved in these two *Arabidopsis* proteins, to His. The bHLH domain of ICE1 shows high amino acid similarity to that of known MYC-related bHLH transcription factors (Fig. 4B). All MYC binding promoter elements contain the CA nucleotides that are contacted by a conserved glutamic acid in the bHLH zipper domain (Grandori et al. 2000). This glutamic acid residue (Glu 312) is also conserved in the basic DNA binding domain of ICE1 (Fig. 4B). The bHLH family of transcription factors is characterized by an acidic domain near the N terminus and a conserved bHLH DNA binding and dimerization domain near the C terminus (Purugganan and Wessler 1994). All these features are present in ICE1 protein (Fig. 4A).

To analyze the expression pattern of *ICE1* in different tissues, T2 lines of transgenic *Arabidopsis* plants expressing an *ICE1* promoter-*GUS* transgene were analyzed. *GUS* expression was detected in roots, leaves, stem, and floral parts. Semiquantitative RT-PCR analysis also showed that *ICE1* was expressed constitutively and the expression was stronger in leaves and stems than in other tissues (Fig. 5A,B). RNA blot analysis showed that the *ICE1* transcript was slightly up-regulated by cold, NaCl, and ABA, but not by dehydration (Fig. 5C).

To examine the subcellular localization of the ICE1

protein, ICE1 was fused in-frame to the C-terminal side of the green fluorescent protein (GFP) and expressed under control of the CaMV 35S promoter. Confocal imaging of GFP fluorescence in T2 transgenic plants showed that the GFP-ICE1 fusion protein is present in the nucleus under either warm (Fig. 5D) or cold temperatures (data not shown).

ICE1 binds to MYC recognition sites in the *CBF3* promoter

ICE1 has a bHLH domain and its amino acid sequence in the basic region is highly conserved with other bHLH proteins (Fig. 4B), and therefore may recognize promoter elements similar to the DNA binding sites for known bHLH proteins. These proteins recognize DNA with the consensus sequence CANNTG (Meshi and Iwabuchi 1995). In the promoter region of *CBF3*, there are five potential MYC-recognition elements within a 1-kb region upstream of the transcription initiation site (Shinwari et al. 1998). These possible MYC-recognition sites, designated MYC-1 through MYC-5, fall into four groups because MYC-3 and MYC-5 share the same consensus sequence, CATTG (Fig. 6A). Thus, MYC-3 was used to represent both MYC-3 and MYC-5. To determine whether ICE1 binds to these MYC-recognition sites in

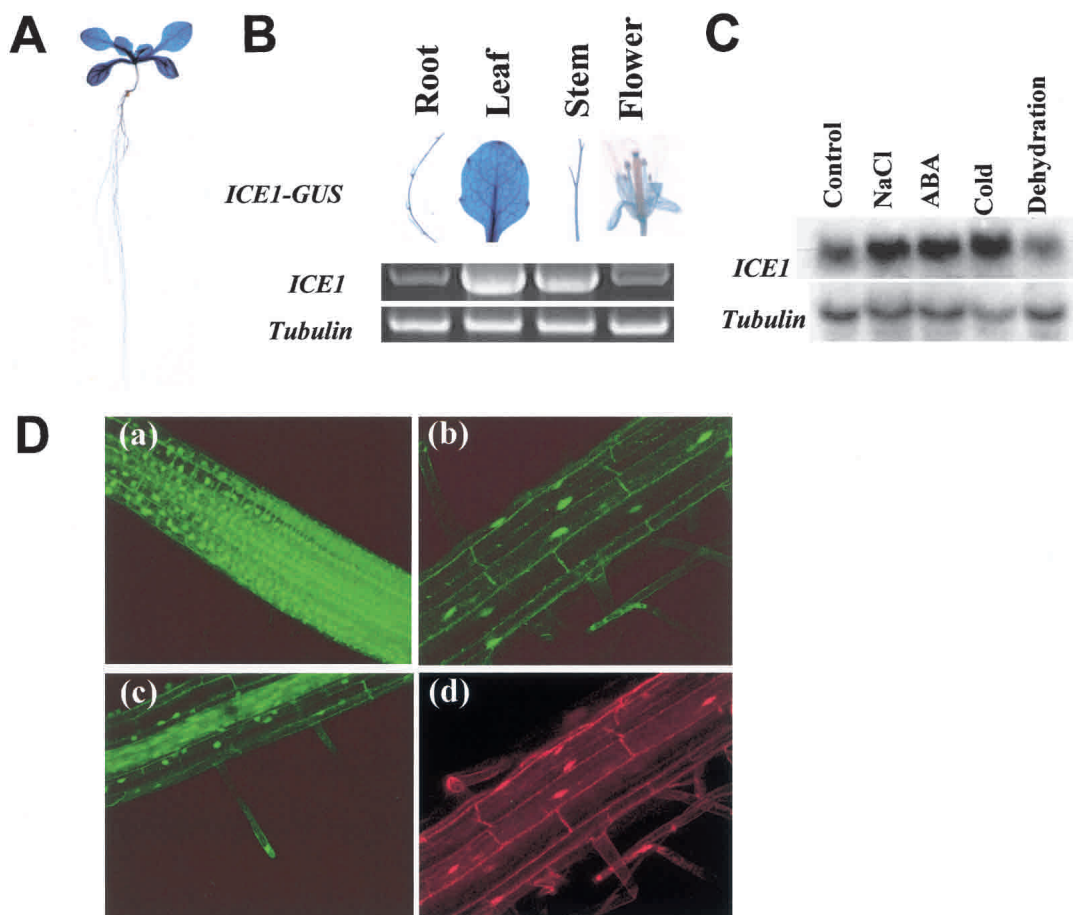


Figure 5. Expression of the *ICE1* gene and subcellular localization of the ICE1 protein. (A) *ICE1* promoter-driven *GUS* expression pattern in a wild-type seedling. (B) *ICE1* promoter-*GUS* expression in different plant tissues, and the corresponding *ICE1* transcript levels as determined by RT-PCR analysis. The tubulin gene was used as an internal control in the RT-PCR. (C) RNA blot analysis of *ICE1* expression in wild-type seedlings under various abiotic stresses. Plants with the following treatments are shown: control, MS salt only; NaCl, 300 mM NaCl for 5 h; ABA, 100 μ M abscisic acid for 3 h; Cold, 0°C for 2 h; Dehydration, air drying for 30 min. (D) Localization of GFP-*ICE1* fusion protein in the nucleus. Panels a–c show confocal images of root cells in GFP-*ICE1* transgenic plants, and panel d shows the location of nuclei as indicated by propidium stain.

the *CBF3* promoter, we expressed and purified His-*ICE1* fusion protein from *E. coli*. Four DNA fragments encompassing each possible MYC-recognition site were used for interaction with His-*ICE1* in an electrophoresis mobility shift assay (EMSA).

Several complexes were observed when *ICE1* was incubated with any of the four DNA fragments (MYC-1 through MYC-4), indicating that *ICE1* is able to bind to these sequences (Fig. 6B). The MYC-2 fragment formed one major complex with *ICE1*, while the other DNA fragments formed several complexes with *ICE1*. These complexes were abolished by the addition of increasing amounts of cold competitors with the same sequences, but not by P1 or P2, which contains a putative MYB-recognition site and a nonrelated sequence, respectively (Fig. 6B). This specificity of competition strengthens the hypothesis that the interaction between DNA and *ICE1* requires the MYC-recognition sequences. When the MYC-2 fragment was used as a probe, the complex was

most efficiently competed off by the cold MYC-2 competitor, suggesting that *ICE1* has a higher affinity for the MYC-2 site than for the other sites (Fig. 6C). The complex formed by *ICE1* and the MYC-2 fragment was less affected by a mutated competitor than by the wild-type competitor (Fig. 6D). Together, these results show that *ICE1* interacts specifically with the MYC-recognition sites in the *CBF3* promoter. The *ice1* mutation does not appear to affect *ICE1* interaction with the *CBF3* promoter, because the Arg 236 to His mutant form of *ICE1* was also able to bind to the MYC-2 probe (Fig. 6E).

ICE1 is a transcriptional activator that positively regulates *CBF* expression

Transient expression assays were carried out to determine whether *ICE1* acts as a transcriptional activator or repressor. An effector plasmid was constructed by fusing *ICE1* with the DNA binding domain of the yeast GAL4

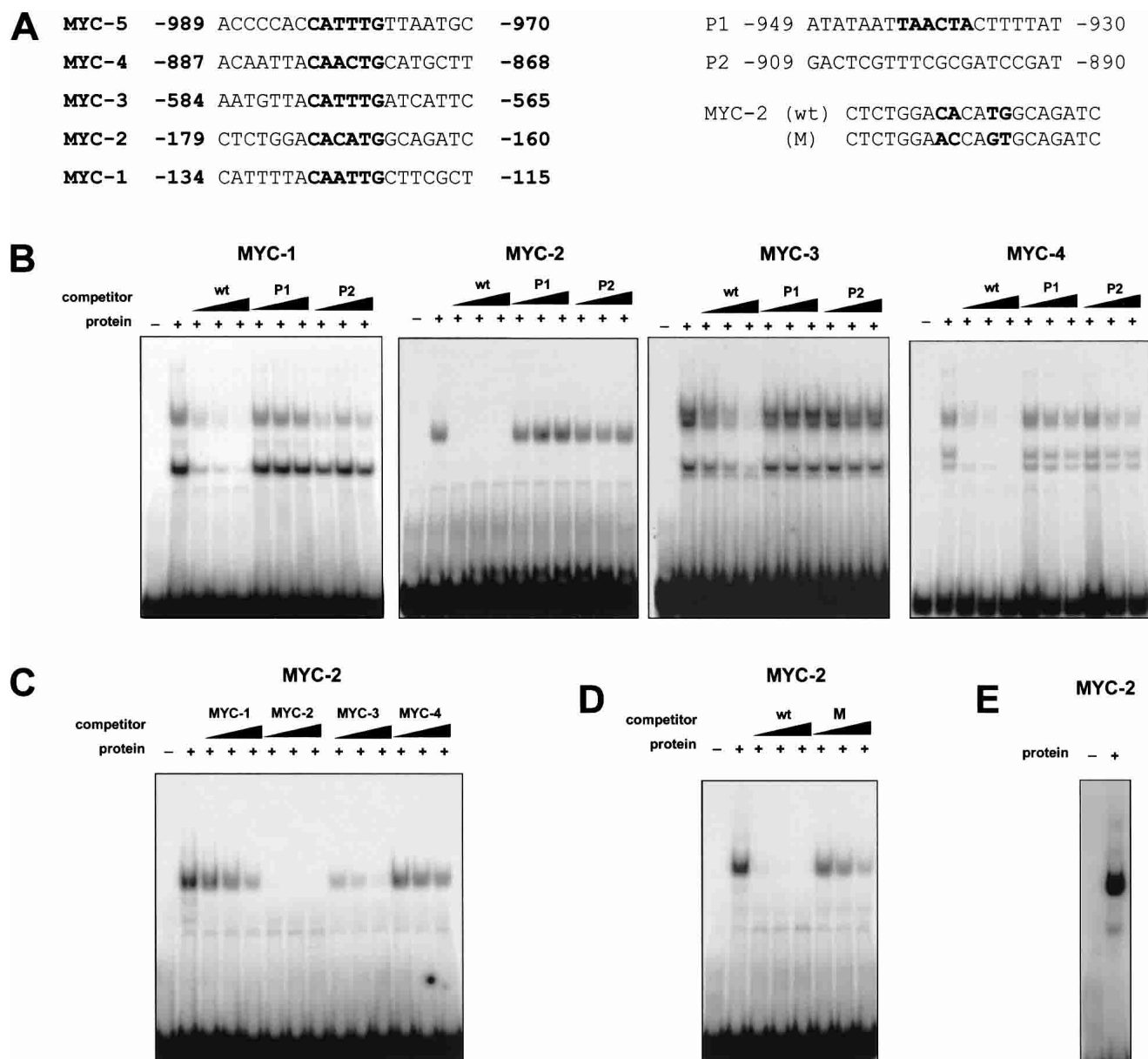


Figure 6. ICE1 protein binds to the MYC-recognition elements in the *CBF3* promoter. (A) Sequences and positions of oligonucleotides within the *CBF3* promoter used in the EMSA. Letters in bold indicate sequences of MYC-recognition motifs in MYC-1 through MYC-5. Bold letters in the P1 oligonucleotide are a putative MYB-recognition motif. (B) Interaction between ICE1 protein and 32 P-labeled MYC-1 through MYC-4 DNA fragments. (C) ICE1 binds to the MYC-2 DNA fragment more strongly than to the other DNA fragments. (D) Consensus nucleotide residues in the MYC-recognition motif are important for the interaction between ICE1 and the MYC-2 DNA fragment. (E) *ice1* mutant protein also binds to the MYC-2 DNA fragment. The labeled oligonucleotides used in each experiment are indicated at the top of each panel. Triangles indicate increasing amounts of unlabeled oligonucleotides for competition in B, C and D, which correspond to 50-, 100-, and 250-fold excess of each probe.

transcriptional activator (*GAL4-ICE1*; Fig. 7A). When the wild-type *GAL4-ICE1* and a *GAL4*-responsive reporter gene, *GAL4-LUC*, were delivered into *Arabidopsis* leaves by particle bombardment, the luciferase activity increased 20-fold relative to the control with or without an effector plasmid containing only the *GAL4* DNA binding domain (Fig. 7B). The Arg 236 to His mutant form of *GAL4-ICE1* also activated the *GAL4*-responsive transcription (Fig. 7B). These results suggest that ICE1 is

a transcriptional activator, and that the *ice1* mutation does not affect the function of the transcriptional activation domain.

A null allele of *ice1* created by T-DNA insertion does not show any phenotypes of the dominant *ice1* mutant (data not shown), suggesting that there is functional redundancy in the *ICE1* gene family. We overexpressed *ICE1* in wild-type *Arabidopsis* plants by using the strong constitutive super promoter. None of the overexpression

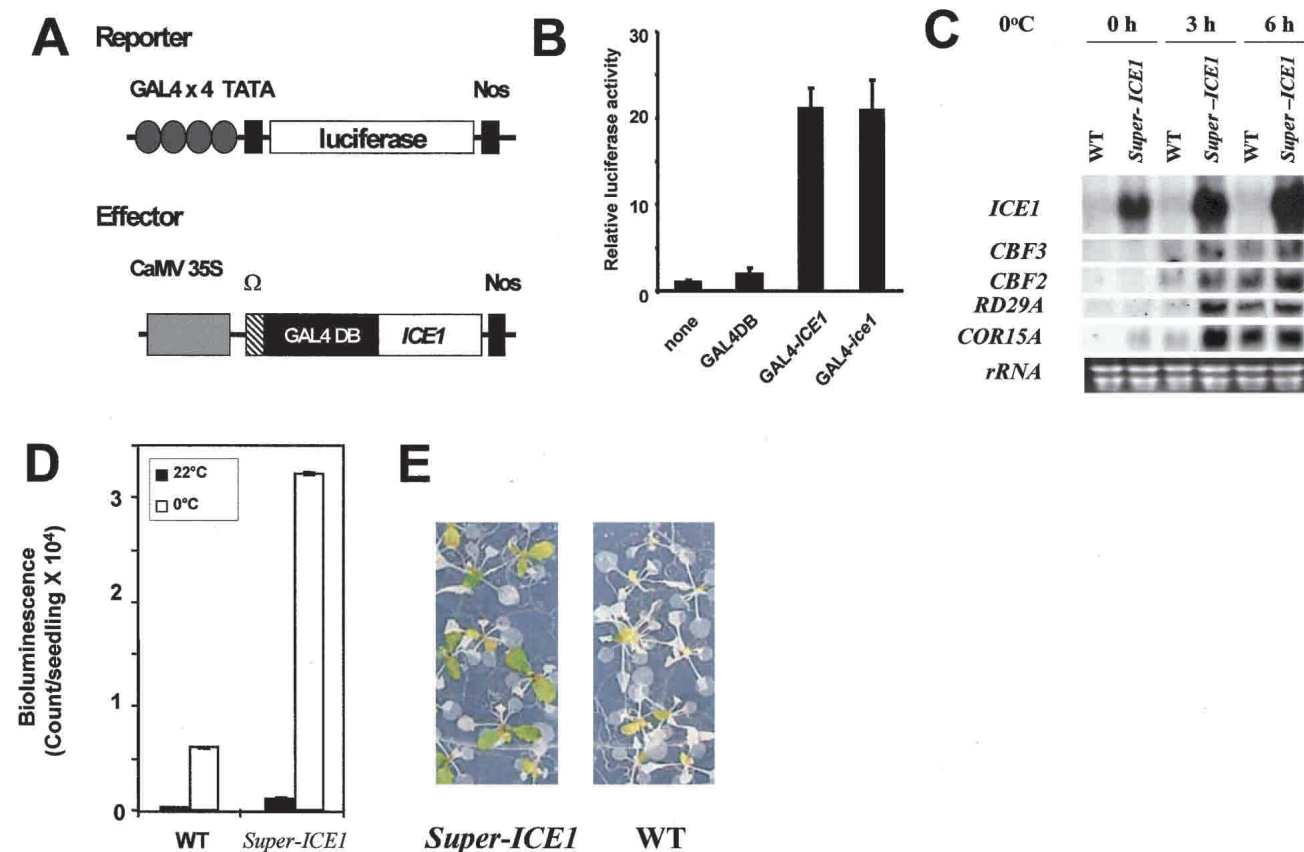


Figure 7. ICE1 is a transcriptional activator and its overexpression enhances the *CBF* regulon in the cold and improves freezing tolerance. (A) Schematic representation of the reporter and effector plasmids used in the transient expression assay. A *GAL4*-responsive reporter gene was used in this experiment. Nos, the terminator signal of the nopaline synthase gene; Ω, the translational enhancer of tobacco mosaic virus; *GAL4 DB*, the DNA-binding domain of the yeast transcription factor *GAL4*. (B) Relative luciferase activities after transfection with *GAL4-LUC* and *35S-GAL4-ICE1* or *35S-GAL4-ice1*. To normalize values obtained after each transfection, a gene for luciferase from *Renilla* was used as an internal control. Luciferase activity is expressed in arbitrary units relative to the activity of *Renilla* luciferase [as described in Ohta et al. (2001)]. The values are averages of three bombardments, and error bars indicate standard deviations. (C) RNA blot analysis of *ICE1* and cold-responsive gene expression in wild-type and *ICE1* overexpressing transgenic (*Super-ICE1*) plants. Seedlings were either not treated (0 h) or treated with low temperature (0°C) for 3 or 6 h. Ethidium bromide stained rRNA bands are shown as loading control. (D) *CBF3-LUC* expression (indicated as luminescence intensity) in wild-type and *ICE1* overexpressing transgenic (*Super-ICE1*) plants. (E) Improved survival of *ICE1* overexpressing transgenic (*Super-ICE1*) plants after a freezing treatment.

lines showed any *ice1* mutant phenotypes. RNA blot analysis showed that *ICE1*-overexpression did not activate *CBF3* expression at warm temperatures. However, *ICE1*-overexpression enhanced the expression of the endogenous *CBF3* gene as well as the *CBF3-LUC* reporter gene in the cold (Fig. 7C,D). Cold-induction of *CBF2*, *RD29A*, and *COR15A* was also enhanced in the *Super-ICE1* transgenic plants (Fig. 7C). When the *Super-ICE1* transgenic plants and wild-type control plants in the same agar plates were cold acclimated at 4°C for 5 d and then subjected to freezing treatment at -8°C for 4 h, the *ICE1* overexpression transgenic seedlings showed a higher survival rate (75.9% ± 6.5%) than that of control plants (37.2% ± 12.6%; Fig. 7E). The *ICE1* overexpression transgenic plants did not exhibit obvious growth or developmental abnormalities (data not shown). These results suggest that ICE1 is a positive regulator of *CBF3*,

and that the dominant nature of *ice1* is likely caused by a dominant negative effect of the mutation.

Discussion

Cold temperatures trigger the transcription of the *CBF* family of transcription factors, which in turn activate the transcription of genes containing the *DRE/CRT* promoter element (Thomashow 1999). The *CBF* target genes presumably include some transcription factors (Fowler and Thomashow 2002). Therefore, cold signaling for freezing tolerance requires a cascade of transcriptional regulations. In the present study, we have identified ICE1, a very upstream transcription factor of this cascade. Our results show that ICE1 is a positive regulator of *CBF3* and has a critical role in cold acclimation. *ICE1* encodes a MYC-like bHLH transcription factor. Five pu-

tative MYC recognition sequences are present in the *CBF3* promoter, while *CBF1* and *CBF2* promoters each contain one such element (Shinwari et al. 1998). This is consistent with the fact that *CBF3* is more strongly affected by the *ice1* mutation than are *CBF1* or *CBF2*. DNA binding assays showed that ICE1 can specifically bind to the MYC recognition sequences on the *CBF3* promoter but not to a putative MYB recognition sequence (Fig. 6). The *ice1* mutation abolishes *CBF3* expression, and reduces the expression of CBF-target genes in the cold. Consistent with its role in cold-responsive gene regulation, ICE1 is important for chilling and freezing tolerance of *Arabidopsis* plants.

The *ice1* mutation also affects the cold-induction of *CBF1* and *CBF2*; their expression is slightly reduced early in the cold, but at later time points the expression is not reduced. Instead, the expression of *CBF2* is actually enhanced in the *ice1* mutant after 6 and 12 h of cold treatment. The expression of *CBF* genes is known to be repressed by their gene products or the products of their downstream target genes (Guo et al. 2002). The correlation between the reduced *CBF3* expression and enhanced *CBF2* induction suggests that CBF3 may repress *CBF2* expression. When the *CBF2* gene is disrupted, *CBF1* and *CBF3* show more sustained induction in the cold (J. Salinas, pers. comm.), suggesting that CBF2 may repress the expression of *CBF1* and *CBF3*. The potential negative regulation of each other among the CBF transcription factor genes may be important for ensuring that their expression is transient and tightly controlled.

The three *CBF* genes are generally presumed to be functionally redundant. Their individual contribution has not been examined by loss-of-function analysis. Even though the *ice1* mutation only blocks the expression of *CBF3*, the downstream genes such as *RD29A*, *COR15A*, and *COR47* are substantially affected. This suggests that CBF3 plays a critical role in the cold regulation of these genes. In comparison, the cold regulation of *KIN1* is less affected by the *ice1* mutation. Therefore, it is possible that the three *CBF* genes may each have their own set of preferred target genes.

ICE1 is expressed constitutively in all tissues (Fig. 5A,B), and is only slightly up-regulated by cold (Fig. 5C). Consistent with what has been speculated for "ICE" proteins (Gilmour et al. 1998), cold-induced modification of the ICE1 protein or of a transcriptional cofactor appears to be necessary for ICE1 to activate the expression of *CBFs*. This is supported by our evidence because *ICE1* is expressed constitutively and localized in the nucleus, but the *CBF* expression requires cold treatment; and transgenic lines constitutively overexpressing *ICE1* do not show *CBF3* expression at warm temperatures, but have a higher level of *CBF3* expression at cold temperatures. The ability of transcription factors to activate gene transcription may be regulated by protein phosphorylation and dephosphorylation in the cytoplasm or in the nucleus (reviewed by Liu et al. 1999). The *ice1* mutation is very near potential serine phosphorylation residues (Ser 243 and Ser 245), and thus might affect the phosphorylation/dephosphorylation of ICE1.

It is known that MYC-related bHLH transcription factors require MYB cotranscription factors and/or WD-repeat containing factors for transcriptional activation of target genes (Spelt et al. 2000; Walker et al. 1999). The promoters of *CBFs* contain MYC as well as potential MYB recognition sequences (Shinwari et al. 1998), suggesting that a MYB-related transcription factor may also be involved in the cold induction of *CBFs*. The *ice1* mutation, which substitutes Arg 236 with His, may interfere with hetero-oligomer formation between ICE1 and an ICE1-like protein or a MYB-related cofactor. Alternatively, the putative dominant negative effect of *ice1* could be a consequence of *ice1* interference with potential ICE1 homo-oligomer formation, protein stability, nuclear localization, or cold-induced posttranslational modification of ICE1.

Materials and methods

Plant materials and mutant isolation

The *CBF3* promoter, a region from 1126 to 100 bp upstream of the initiation codon, was obtained by PCR using the following primer pair: 5'-TCATGGATCCACCATTGTTAATGCATGATGG-3' and 5'-GCTCAAGCTTCTCTGTTCTAGTTCAGG3'. This promoter was placed in front of the firefly luciferase (*LUC*) coding sequence in a plant transformation vector (Ishitani et al. 1997). *Arabidopsis thaliana* ecotype Columbia (with the *glabrous1* mutation) was transformed with *Agrobacterium tumefaciens* containing this *CBF3-LUC* construct by the floral dipping method. Plants homozygous for the *CBF3-LUC* transgene were selected from the second generation after transformation. One such plant with a single copy of the *CBF3-LUC* transgene was chosen for subsequent experiments (hereafter referred to as wild type). This wild-type plant did not show any bioluminescence when grown under normal growth conditions, but emitted bioluminescence when cold stress was imposed. The *CBF3-LUC* plant seeds were mutagenized with ethyl methanesulfonate (EMS). Seedlings of the M2 generation were used to screen for mutants defective in cold-regulated *CBF3-LUC* expression by luminescence imaging. Seven-day-old seedlings grown on 0.6% agar plates containing 3% sucrose and 1× Murashige and Skoog (MS) salts (JRH Biosciences) were screened for deregulated luciferase expression in response to low temperature treatment at 0°C for 12 h, using a low-light video imaging system (Princeton Instruments). Luminescence intensities of individual seedlings were quantified with the WinView software provided by the camera manufacturer (Princeton Instruments; Chinnusamy et al. 2002).

Chilling and freezing tolerance assays

Chilling sensitivity of *ice1* and wild-type plants were tested by exposing the seedlings immediately after radicle emergence. After 2 d of stratification at 4°C, mutant and wild-type seeds were germinated at 22°C on MS nutrient medium with 3% sucrose and 1.2% agar. Chilling stress was imposed by incubating the seedlings at 4°C ± 1°C with 30 ± 2 μmole quanta · m⁻² · s⁻¹ light. Freezing tolerance was assayed as described (Xin and Browse 1998). Briefly, wild-type and *ice1* seeds were sown on agar (0.9%) plates with Gamborg basal salts and 1.5% sucrose. After 2 d of stratification at 4°C, the plates were kept at 22°C under 50 ± 2 μmole quanta m⁻² · s⁻¹ continuous light. Ten-day-old seed-

lings were cold acclimated at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 30 ± 2 $\mu\text{mole quanta. m}^{-2} \cdot \text{s}^{-1}$ light for 4 d. These plants on petri dishes were placed on ice in a freezing chamber (Percival Scientific) set to $-1^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$ for 16 h. Ice chips were sprinkled on these plants before the chamber was programmed to cool at 1°C h^{-1} . Petri dishes of plants were removed after being frozen at desired temperatures for 2 h unless indicated otherwise, thawed at 4°C for 12 h in the dark, and then transferred to 22°C under 50 ± 2 $\mu\text{mole quanta. m}^{-2} \cdot \text{s}^{-1}$ continuous light. Survival of the seedlings was scored visually after 2 d.

Gene expression analysis

For RNA analysis, 10-day-old seedlings of wild-type and *ice1* plants grown on separate halves of the same MS agar plates were used. Total RNA extracted from control and stressed plants was analyzed by RNA blotting as described by Liu and Zhu (1997). The *RD29A* gene-specific probe was from the 3' noncoding region (Liu and Zhu 1997). *COR15A* and *COR47* cDNAs (Gilmour et al. 1992; Lin and Thomashow 1992) were kindly provided by M.F. Thomashow (Michigan State University). The *CBF2* and *CBF3* gene-specific probes were generated by PCR with the following primer pairs: *CBF2*-forward primer, 5'-TTC GATTTTTATTTCCATTTTTGG-3'; *CBF2*-reverse primer, 5'-CCAAACGTCCCTGAGTCTTGAT-3'; *CBF3*-forward primer, 5'-TAAAACTCAGATTATTATTTCCATTT-3'; *CBF3*-reverse primer, 5'-GAGGAGCCACGTAGAGGGCC-3'. The probe for *KIN1* (Kurkela and Franck 1990) was a 0.4-kb *EcoRI* fragment of the *Arabidopsis* EST clone YAP368T7. The β -tubulin gene was used as a loading control and was amplified by PCR with the following primer pairs: forward primer (5'-CGTGGATCA CAGCAATACAGAGCC-3') and reverse primer (5'-CCTCCT GCACTTCCACTTCGTCTTC-3').

For Affymetrix GeneChip array analysis, 20 μg of total RNA from the wild-type and *ice1* seedlings with or without cold treatment (6 h under light) were extracted using the RNeasy Plant Mini Kit (Qiagen) and used to make biotin-labeled cRNA targets. The Affymetrix *Arabidopsis* ATH1 genome array GeneChips, which contain >22,500 probe sets representing ~24,000 genes, were used and hybridization, washing, and staining were carried out as directed in the manufacturer's manual. Microarray data were extracted from scanned GeneChip images and analyzed using Microarray Suite version 5.0.1 (Affymetrix).

Mapping and cloning of the ICE1 locus

Genetic analysis of F_1 and F_2 progenies of the *ice1* cross with wild type showed that *ice1* is a dominant mutation. Hence, to clone *ICE1*, a homozygous *ice1* plant was crossed with the *Arabidopsis* Landsberg erecta (Ler) ecotype and the F_2 progeny from self-pollinated F_1 were used to select mapping samples with the wild-type phenotype. Genomic DNA extracted from these seedlings was used for PCR-based mapping with simple sequence polymorphism markers or cleaved amplified polymorphic sequence markers. New SLP mapping markers on F16J4, MTC11, MLJ15, MDJ14, K17E12, and T32N15 BAC clones were developed based on insertion/deletions identified from the Ce-reon *Arabidopsis* polymorphism and Ler sequence collection (<http://www.arabidopsis.org>). Genomic DNA corresponding to candidate genes was amplified by PCR from *ice1* mutant and wild-type plants and sequenced to identify the *ice1* mutation.

For *ice1* mutant complementation, the MLJ15.14 gene, including 2583 bp upstream of the initiation codon and 615 bp downstream of the stop codon, was PCR-amplified by LA Taq polymerase (Takara) using *ice1* mutant genomic DNA as template. The PCR primers used were as follows: forward primer:

5'-AGGGATCCGGACCACCGTCAATAACATCGTAAAGT AG-3'; reverse primer: 5'-CGAATTCTAACCGCCATTAAC TATGTCTCTCTCTATCTC-3'. The resulting 5035-bp fragment was T-A cloned into the pCR2.1 TOPO vector (Invitrogen) and then subcloned into pCAMBIA1200 between the *BamHI* and *EcoRI* sites. This and all other constructs described here were completely sequenced to ensure that they did not contain PCR or cloning errors. The binary construct was then introduced into *Agrobacterium* strain GV3101 and transformed into *CBF3-LUC* Columbia wild-type plants. Hygromycin-resistant transgenic plants were selected and their T_2 progenies were tested for *CBF3-LUC* expression in response to cold stress.

Analysis of ICE1 expression

The promoter region (2589 bp upstream from the initiation codon) of the *ICE1* gene was PCR-amplified with the following primer pair: forward primer, 5'-AGGGATCCGGACCACCGT CAATAACATCGTAAAGTAG-3'; reverse primer, 5'-CGAAT TCGCCAAAGTTGACACCTTTACCCCAAAG-3'. The resulting fragment was digested with *BamHI* and *EcoRI* and inserted into the pCAMBIA1391 binary vector. This *ICE1* promoter-*GUS* construct was introduced into *Agrobacterium* strain GV3101 and transformed into wild-type *Arabidopsis*. T_2 transgenic lines resistant to hygromycin were analyzed for *ICE1*-promoter driven *GUS* expression. For *GUS* staining, T_2 seedlings grown on MS agar plates were incubated with X-Gluc. for 12 h at 37°C and then washed 5 times with 70% (v/v) ethanol at 70°C to remove chlorophyll. *ICE1* expression was also examined by quantitative RT-PCR analysis of RNA prepared from wild-type roots, leaves, stems, and flowers. The *ICE1* cDNA was amplified by RT-PCR using the following primers: forward primer: 5'-GCGATGGGTCTTGACGGAAACAATGGTG-3' and reverse primer 5'-TCAGATCATACCAGCATACCCTGCTGTATCG-3'. The tubulin gene was used as an internal control in the RT-PCR analysis. Tubulin cDNA was amplified using the following primers: forward primer: 5'-GTCAAGAG GTTCTCAGCAGTA-3' and reverse primer 5'-TCACCTTCT TGATCCGCAGTT-3'.

Overexpression of ICE1

The *ICE1* cDNA was amplified from *Arabidopsis* (ecotype Columbia) RNA by RT-PCR using the following primers: a forward primer: 5'-GCTCTAGAGCGATGGGTCTTGACGGAA ACAATGGTG-3' and a reverse primer 5'-GGGGTACCTC AGATCATACCAGCATACCCTGCTGTATCG-3'. The PCR product was digested with *XbaI* and *KpnI*, and cloned into the 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 (Li et al. 2001). *Agrobacterium tumefaciens* strain GV3101 containing this binary construct was used to transform *Arabidopsis* plants. Transformants were selected on MS medium containing hygromycin (30 mg/L).

Expression and localization of GFP-ICE1 fusion protein

The full-length *ICE1* cDNA was obtained from wild-type plants by RT-PCR using the following primers: forward primer, 5'-AGGAATTCGCGATGGGTCTTGACGGAAACAATGGTG-3'; reverse primer, 5'-CTGGATCCTCAGATCATACCAG CATACCCTGCTGTATCG-3'. The resulting PCR fragment was digested with *EcoRI* and *BamHI* and cloned into the binary vector pEGAD downstream from the CaMV 35S promoter. This

GFP-ICE1 construct was introduced into *Agrobacterium* strain GV3101 and transformed into wild-type *Arabidopsis*. T2 transgenic lines resistant to Basta (glufosinate) were selected and analyzed for GFP expression. To visualize the nucleus, root tissues were stained with propidium iodide (1 µg/mL). Green fluorescence (GFP expression) and red fluorescence (propidium iodide staining) analyses of transgenic plants were performed with a confocal laser-scanning microscope.

DNA binding assay

The wild-type and mutant *ICE1* cDNAs were amplified by RT-PCR and inserted into *NdeI* and *BamHI* sites in the expression vector pET14b (Novagen). Wild-type and mutant His-ICE1 fusion proteins were prepared from *E. coli* cells (BL21 DE3) according to the instruction manual of His-Bind Buffer Kit (Novagen). The electrophoresis mobility shift assay (EMSA) was carried out as described (Hao et al. 1998). The following double-stranded oligonucleotides listed in Figure 6A (MYC-1, MYC-2, MYC-3, MYC-4, and MYC-5) were used as probes and competitors in EMSAs. Nucleotide sequences P1 (-949 to -930) and P2 (-909 to -890) were also used as competitors. P1 contains a putative MYB-recognition site. P2 does not contain any typical *cis*-elements. DNA probes were end-labeled with [γ -³²P]dCTP using the Klenow fragment and purified through a Sephadex G-50 column. The labeled probes (ca 0.02 pmole) were incubated for 20 min at room temperature with 2.3 µg of purified His-ICE1 fusion protein in 1× binding buffer (Hao et al. 1998) supplemented with 20 pmole poly(dI-dC). The resulting DNA-protein complexes were resolved by electrophoresis on a 6% polyacrylamide gel in 0.5× TBE buffer and visualized by autoradiography. For competition experiments, unlabeled competitors were incubated with the His-ICE1 fusion protein on ice for 30 min prior to the addition of labeled probes.

Transient expression assay

The wild-type (*ICE1*) and mutant (*ice1*) cDNAs were amplified by RT-PCR, digested with *SmaI* and inserted into *SmaI* and *SalI* sites of the plant expression vector 35S-GAL4 DB (Ohta et al. 2000). The plasmid DNA of the resulting effector, *GAL4-ICE1*, and a *GAL4* responsive reporter, *GAL4-LUC* (Ohta et al. 2000) were delivered into *Arabidopsis* leaves using particle bombardment (Ohta et al. 2001).

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