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A R2R3 Type MYB Transcription Factor Is Involved in the Cold Regulation of CBF Genes and in Acquired Freezing Tolerance*

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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 freezing tolerance to plants. It has been shown previously that the cold regulation of *CBF3* involves an upstream bHLH-type transcription factor, ICE1. ICE1 binds to the Myc recognition sequences in the *CBF3* promoter. Apart from Myc recognition sequences, *CBF* promoters also have Myb recognition sequences. We report here that the *Arabidopsis* MYB15 is involved in cold-regulation of *CBF* genes and in the development of freezing tolerance. The *MYB15* gene transcript is up-regulated by cold stress. The MYB15 protein interacts with ICE1 and binds to Myb recognition sequences in the promoters of *CBF* genes. Overexpression of *MYB15* results in reduced expression of *CBF* genes whereas its loss-of-function leads to increased expression of *CBF* genes in the cold. The *myb15* mutant plants show increased tolerance to freezing stress whereas its overexpression reduces freezing tolerance. Our results suggest that MYB15 is part of a complex network of transcription factors controlling the expression of *CBFs* and other genes in response to cold stress.

Cold temperatures have a huge impact on the survivability and distribution of living organisms. Plants, being sessile, have evolved efficient mechanisms to sense and adapt to low temperature stress. Plant responses to adverse low temperature are manifested at physiological, molecular and biochemical levels. Many temperate plants have the potential to increase their freezing tolerance after a prior exposure to nonfreezing temperatures, a process known as cold acclimation (1–3). At the molecular level, a specific set of proteins is induced in response to low temperature, which helps plants cope with chilling and freezing stress (4–8). Proteins induced during cold acclimation include enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids, and antioxidants, molecular chaperones, antifreeze proteins, and many others with a presumed function in tolerance to cellular dehydration caused by apoplastic freezing (1, 4, 9).

Promoters of many of the cold-responsive genes have the *DRE/CRT/LTRE* (dehydration responsive element/C-repeat/low temperature responsive element) sequence, a cis element necessary and sufficient for gene transcription under cold stress (10–12). The CBF/DREB family of transcription factors binds to this sequence and activates cold-responsive genes (11, 13). The *CBF* transcription factor genes are also induced by cold, and their induction is regulated by components upstream in the cold response pathways (14–17). In addition, it has been shown that a loss-of-function mutation in *CBF2* results in increased expression of *CBF1* and *CBF3*, implying that CBF2 negatively regulates the expression of *CBF1* and *CBF3* (18).

In addition to the CBF pathway, recent studies have revealed the presence of parallel pathways associated with cold acclimation (19–21). Some important components mediating cold tolerance through CBF-independent pathways include homeodomain and MYB-type transcription factors (22, 23). Support for the existence of CBF-independent pathways has also come from the analysis of the *eskimo1* mutants of Arabidopsis (24), which are constitutively freezing tolerant, without any apparent effect on the CBF regulon. Apart from large changes in gene transcript levels, extensive reconfiguration of the metabolome also takes place in response to cold temperatures (25, 26).

A critical component in the activation of *CBF3* and a number of other cold-responsive transcription factor genes in Arabidopsis is ICE1 (14). ICE1 is a constitutively expressed transcription factor of the bHLH[2] family that can bind to the Myc recognition elements in the *CBF3* promoter. A dominant mutation in *ICE1* blocks the cold induction of *CBF3* and many other transcription factors, and reduces the expression of their downstream genes (14, 27). Apart from Myc recognition sequences, many putative Myb binding sequences are present in the promoters of *CBF* genes (28) indicating that MYB-like transcription factors may also play a role in controlling *CBF* gene expression. Furthermore, some reports suggest that the interplay of MYC-like bHLH transcription factors and MYB co-transcription factors and/or WD repeat containing factors is required for transcriptional activation of target genes (29, 30).

In the present study, we have identified a MYB-like transcription factor involved in the cold regulation of *CBF* genes. This transcription factor, referred to as MYB15, interacts physically with ICE1. MYB15 binds to sequences in the promoters of *CBF1*, *2*, and *3* genes. Transgenic plants overexpressing *MYB15*

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[2] The abbreviations used are: bHLH, basic helix-loop-helix; GST, glutathione *S*-transferase; GFP, green fluorescent protein; Luc, luciferase; RNAi, RNA interference; EMSA, electrophoretic mobility shift assay; WT, wild type.

show reduced levels of *CBF3*, *CBF2* and *CBF1* transcripts in the cold. *MYB15* loss-of-function mutant plants show increased levels of *CBF3* as well as *CBF1* and *CBF2*. Overexpression of *MYB15* results in decreased tolerance to freezing stress, whereas its knock-out mutant exhibits increased freezing tolerance. These results suggest that MYB15 is involved in the cold-regulation of *CBF* genes and in cold stress tolerance.

EXPERIMENTAL PROCEDURES

Gene Expression Analysis—For RNA analysis, 10-day-old seedlings of wild-type and *ice1* mutant plants grown on separate halves of the same Murashige-Skoog (MS) nutrient agar plate were used. Total RNA extracted from control and stressed plants was analyzed by RNA blotting as described by Liu and Zhu (31). RNA isolated from the transgenic plants overexpressing MYB15, the RNAi line of MYB15 and the *myb15* T-DNA mutant was extracted and transferred to nylon membranes. The membrane was probed with *MYB15* cDNA corresponding to the full-length open reading frame, or gene-specific probes of *CBF3*, *CBF2*, and *CBF1*. β-Tubulin or actin gene was used as a loading control. For checking the *MYB15* expression analysis in various tissues RNA was extracted from roots, leaves, stems, and flowers. 2 μg of total RNA was used to make cDNA using Superscript II cDNA synthesis kit (Invitrogen). The first strand cDNA template was used to amplify *MYB15* gene using forward primer 5′-GGAATTCCATATGACGAGCTCGAACAGTACTAG-3′ and reverse primer 5′-CGCGGATCCCTAGCCAATACATCGAACCAGAAG-3′. β-Tubulin gene was amplified as an internal loading control using the following primers: forward primer: 5′-GTCAAGAGGTTCTCAGCAGTA-3′ and reverse primer 5′-TCACCTTCTTGATCCGCAGTT-3′.

Yeast 2-Hybrid Interaction Studies—*MYB15* was amplified with primers 5′-GATGGGAAGAGCTCCATGCTG-3′ and 5′-CCGCTCGAGCTAGCCAATACATCGAACCAG-3′ and cloned in the SmaI and XhoI sites of the *pACT2* vector (prey vector). The C-terminal region (corresponding to 266–494 amino acids) of ICE1 was amplified from pMal-ICE1 DNA (ICE1 cloned in MBP fusion vector) as a template with 5′-TGAGACTGGGATTGAGGTTTCTG-3′ and 5′-CAAGCTTGCCTGCAGGTCGAC-3′ primers and cloned in the SmaI and SalI sites of pAS2 vector (bait vector). For mapping, the interacting domain deletions of the C-terminal portion of ICE1 were PCR-amplified with gene-specific primers and cloned in NcoI and BamHI sites of the pAS2 vector. Prey and different bait plasmids were co-transformed in the Y190 strain of yeast, and colonies were selected on SC-Trp-Leu medium (32). Resultant colonies were assayed for β-Gal activity.

Expression and Purification of Fusion Protein in Escherichia coli—Full-length *MYB15* open reading frame (cloned in pGEMT-easy) was amplified with the gene-specific primer 5′-CGGGATCCATGGGAAGAGCTCCATGCTGTG-3′ and SP6 primer. The amplicon was cloned in the BamHI and SalI sites of the pMAL vector (NEB, Beverley, MA) and pGEX 4T-1 vector (Amersham Biosciences). Full-length *AtMYB79* cDNA was amplified with 5′-CGGGATCCGAATGGTGGAAGAAGTTTGGAGAAA-3′ and 5′-CCGCTCGAGTTAACAAAATGGAATCACCAAGTT-3′ and cloned in BamHI and XhoI sites of pGex 4T-1 vector. The MBP-MYB15 fusion protein was purified according to the manufacturer's instructions. GST-fused MYB15 and AtMYB79 constructs were transformed into *E. coli* BL21(codon plus) cells (Stratagene, La Jolla, CA). Single colonies were grown overnight at 37 °C, transferred to fresh 20× volume of Luria-Bertani media and further cultured for 1 h. Recombinant protein expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. The cells were harvested by centrifugation (5,000 × *g*, 10 min, 4 °C), and the pellets were resuspended in prechilled lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 100 μg/ml lysozyme) and incubated on ice for 15 min. Dithiothreitol (50 mM), phenylmethanesulfonyl fluoride (1 mM), and *N*-lauroyl sarcosine (1%) were added before 1-min sonication. The sonicate was clarified by centrifugation at 30,000 × *g* for 15 min at 4 °C. Triton X-100 (1.5%) was added in the supernatant and mixed, followed by the addition of glutathione-agarose beads (Sigma). After overnight incubation at 4 °C, the beads were pelleted and washed extensively with prechilled PBS. GST-fused proteins were eluted with 100 mM glutathione (Sigma), 50 mM Tris, pH 8.8.

DNA Binding—For binding with *CBF* promoters, different fragments were PCR-amplified from the *CBF* promoters (details of the regions are shown in Fig. 3) with KOD polymerase (Novagen, San Diego, CA). Amplified fragments were eluted from agarose gel with use of the QiaQuick gel purification kit (Qiagen, Valencia, CA). Eluted fragments were end-labeled with [γ-P^{32}]ATP and T_4 polynucleotide kinase. A total of 500 pg of the labeled probe was incubated with 500 ng of purified MBP-MYB15 fusion protein at room temperature for 30 min. For competition, purified protein was incubated with 100 ng of unlabeled fragments for 30 min at room temperature prior to their incubation with the labeled probe. The DNA-protein complex was resolved on 5% polyacrylamide gel in 0.5× TBE and visualized by autoradiography.

Transient Expression Assays—*MYB15* cDNA was cloned in SmaI and SalI sites of the plant expression vector [^{35}S]GAL4-DB (33). The plasmid DNA of the resulting effector *GAL4-ICE1* (14) and a GAL4 responsive reporter, *GAL4-LUC* (33), were delivered into Arabidopsis protoplasts by PEG-mediated DNA uptake (34).

In Vitro Pull-down Assay—*In vitro* pull-down assays were carried out to confirm the physical interaction of MYB15 and ICE1. Full-length *MYB15* cloned in pGEM-T easy and *AtMYB79* (cloned in EcoRI and XhoI sites of pBCSK; Stratagene) were used for *in vitro* transcription and translation. Full-length *ICE1* and *ABI2* open reading frames were cloned in EcoRI/SalI and NcoI/EcoRI sites of pCITE4a. A total of 2 μg each of the linearized plasmid was *in vitro* transcribed with use of the Megascript T7 RNA polymerase kit (Ambion, Austin, TX), and 10 μg of the purified transcript of *MYB15* and *AtMYB79* was *in vitro* translated with use of the Flexi Rabbit Reticulocyte system (Promega, Madison, WI) in the presence of [^{35}S]methionine. S-tag-ICE1, and S-tag-ABI2 transcripts were translated in the absence of [^{35}S]methionine, and their proteins were purified with use of the S-tag purification kit (Novagen) according to the manufacturer's instructions. S-Tag-ICE1 and S-tag-ABI2 bound on the S-Tag slurry were used to pull down ^{35}S-labeled MYB15. In a separate experiment, ^{35}S-labeled ICE was produced and used for pull-down assays with either GST-

MYB15 or GST-MYB79 proteins. Pull-down assays were performed as described (32).

Expression and Localization of MYB15—For construction of the *MYB15* promoter-GUS fusion, a 2.0-kb fragment upstream of the start codon of *MYB15* cDNA was PCR-amplified with 5′-CCCAAGCTTATACCATATCAAATCTGAGAAAG-3′ and 5′-CGCGGATCCATTTGTGATTGCTGATAAAAGAAG-3′ primers from the Arabidopsis (Col-0 ecotype) genomic DNA and cloned in HindIII and BamHI sites of pCAMBIA1391Z. The resultant plasmid was mobilized in *Agrobacterium* strain and transformed in Col-0 Arabidopsis plants by floral infiltration (35). The transgenic plants were selected on MS medium containing 30 mg/liter of hygromycin. Transgenic seedlings were histochemically stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 21 days as described in Jefferson *et al.* (36) and visualized under an Olympus FZX12 dissecting microscope. For construction of the GFP fusion, full-length *MYB15* cDNA was PCR-amplified with 5′-CCGGAATTCATGGGAAGAGCTCCATGCTGTGAG-3′ and 5′-CGCGGATCCCTAGCCAATACATCGAACCAGAAG-3′ primers and cloned in EcoRI and BamHI sites of pEGAD vector containing a bialaphos acetyltransferase selectable marker gene (37). For confocal microscopy, MYB15-GFP transgenic seedlings selected on MS medium supplemented with 50 mg/liter phosphinothricin were mounted on glass slides, and images were visualized under a Zeiss 510 Meta confocal microscope with a 488-nm excitation laser and a 522/DF35 emission filter.

Transgenic Plants and T-DNA Knock-outs—MYB15 was amplified with 5′-GCTCTAGAATGGGAAGAGCTCCATGCTGTGA-3′ and 5′-GGGGTACCCTAGCCAATACATCGAACCAGA-3′ and cloned in XbaI/KpnI sites of pRT105 vector. The cassette containing the 35 S promoter-MYB15-nos terminator was excised from the resulting plasmid and cloned in the PstI site of the pCAMBIA3300 vector. The final construct was mobilized into the GV3101 *Agrobacterium* strain. Transformation of Arabidopsis plants (CBF3-LUC background) was carried out by *Agrobacterium*-mediated floral infiltration. The T1 transgenic plants were selected by spraying 30 mg/liter basta 3 times, at 3-day intervals, 2 weeks after imbibition. Seeds from each T1 plant (T2) were individually collected and used in the initial analysis. Selected T2 plants were further propagated, and homozygous lines of overexpression plants were used for analysis. For the construction of the RNAi construct, 348 bp of *MYB15* was amplified with 5′-GGACTAGTCGGCGCGCCGATATCGATGAAAGCTTCT-3′ and 5′-GGTACCATTTAAATCTAGAGCCCGGCTAAGAGATCT-3′ primers. The resulting PCR product was cloned in AscI and SwaI sites of the pFGC5948 vector. The construct was introduced into Arabidopsis (CBF3-LUC background) and transformants were selected on MS medium supplemented with 30 mg/liter hygromycin.

Seeds of T-DNA mutant of MYB15 available in ABRC (SALK_151976) were used to find the homozygous T-DNA insertion line. After confirmation of homozygous T-DNA insertion, gene knock-out was confirmed by RT-PCR of MYB15 with gene-specific primers. RNA was extracted from the homozygous line and analyzed for CBF expression.

Freezing Tolerance Assays—For the freezing tolerance assay, seeds of the MYB15 overexpression line and wild-type plants (CBF3-LUC) were sown in pot media. Ion leakage test after freezing was carried out essentially as described by Ishitani *et al.* (15). Briefly, for each treatment, one excised leaf was placed in a test tube containing 100 μl of deionized H_2O, and the tube was placed in a circulating freezing bath (VWR Scientific, San Francisco, CA) set at 0 °C. For each temperature treatment three replicates were taken. The temperature of the bath was programmed to decrease to −10 °C at 2 °C per hour. When the designated temperature was reached, tubes were removed and placed immediately on ice to allow gradual thawing. The leaflets then were transferred carefully to another tube containing 25 ml of deionized water and shaken overnight, followed by measurement of conductivity. The tubes with the leaves were then autoclaved. After cooling down to room temperature, conductivities of the solutions were measured again. The percentage of electrolyte leakage was calculated as the percentage of the conductivity before autoclaving over that after autoclaving. The ion leakage experiment was repeated twice with three replicates in each experiment. Representative results from one of the experiments are presented here.

Whole plant freezing was assayed as described (24). Briefly, wild-type and *myb15* seeds were sown in separate halves of the same agar (0.9%) plate with Gamborg basal salts and 1.5% sucrose. Three plates were used for each point of freezing temperatures. After 2 days of stratification at 4 °C, the plates were kept at 22 °C under 50 ± 2 μmol quanta $m^{-2}{\cdot}s^{-1}$ continuous light. Ten-day-old seedlings were cold-acclimated at 4 °C± 1 °C and 30 ± 2 μmol quanta $m^{-2}{\cdot}s^{-1}$ light for 4 days. Plants in Petri dishes were placed on ice in a freezing chamber (Percival Scientific) set to −1 °C ± 0.1 °C for 16 h. Ice chips were sprinkled on the plants before the chamber was programmed to cool at 1 °C/h. The Petri dishes were removed after being frozen at the desired temperatures for 2 h, thawed at 4 °C for 12 h in the dark, and then transferred to 22 °C under 50 ± 2 μmol quanta $m^{-2}{\cdot}s^{-1}$ continuous light. Survival of the seedlings was scored visually after 2 days.

RESULTS

MYB15 Expression in Wild Type and ice1 Mutant Plants—To find candidate MYB transcription factors that may function together with ICE1 in cold response pathways, we examined DNA microarray data from a comparison of the transcriptomes of wild type and *ice1* mutant plants treated with cold for 6 h (14). We found that the expression of *AtMyb15* (designated as *MYB15* herein) was higher in *ice1* mutant than that in the wild type. RNA blot analysis showed that *MYB15* expression is up-regulated by cold stress in both the wild type and *ice1* mutant plants (Fig. 1*A*). Consistent with the microarray data, *MYB15* expression level is higher in *ice1* after 6 h of cold treatment. The expression level is also higher after 3 h of cold treatment, but the level becomes lower in *ice1* after 12 h of cold treatment (Fig. 1*A*).

Semi-quantitative RT-PCR as well as in silico examination using Genevestigator indicated that *MYB15* is expressed constitutively at low levels in all plant tissues (not shown). Transgenic plants expressing the GUS reporter gene under the control of the *MYB15* promoter were analyzed to determine the tissue distribution of *MYB15*. GUS activity was detected in

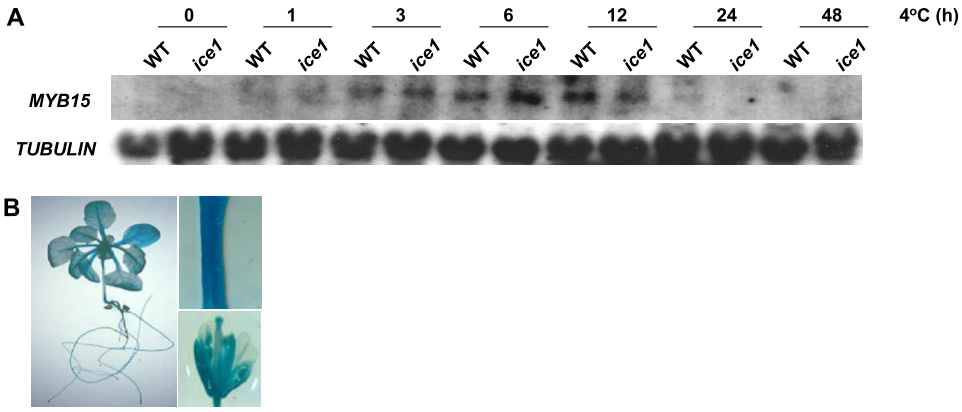

FIGURE 1. **MYB15 gene expression pattern and regulation.** *A*, *MYB15* transcript in wild-type (*WT*, CBF3-LUC background) and *ice1* mutant plants under normal and cold stress conditions. The tubulin gene was used as a loading control. *B*, histochemical localization of GUS activity in seedling, stem, and flower of transgenic Arabidopsis expressing a *MYB15* promoter-GUS fusion construct.

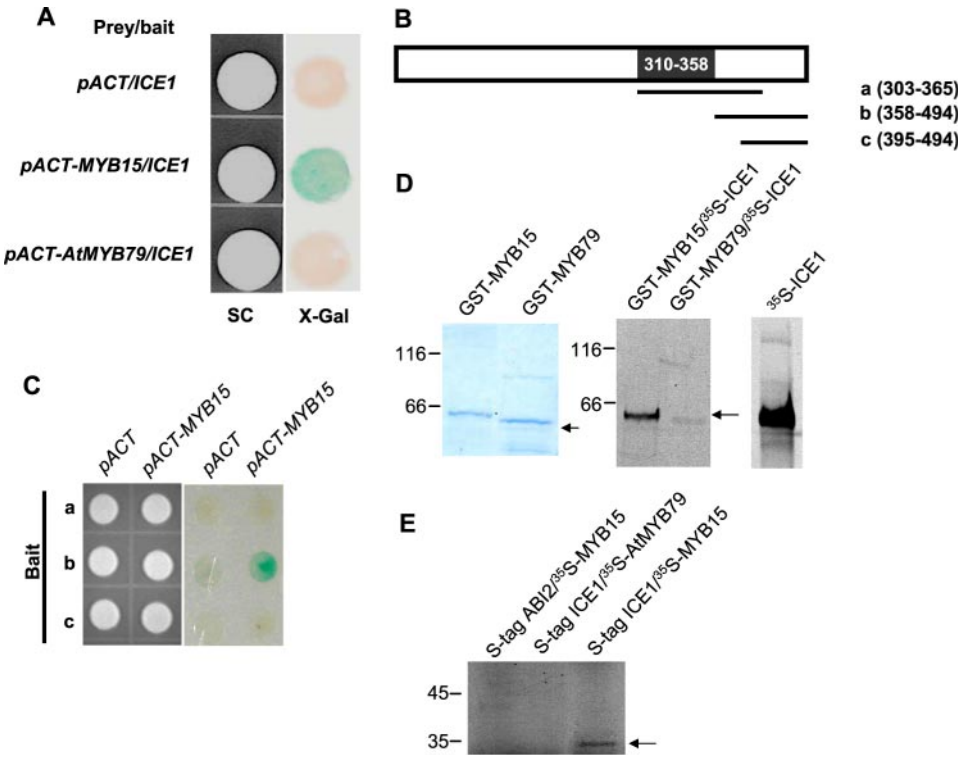

FIGURE 2. **MYB15 interacts with ICE1.** *A*, different prey and bait combinations used for the yeast 2-hybrid assay are indicated. *B*, different regions (*a*–*c*) of ICE1 used as bait to map its interacting domain. The *boxed area* in the line diagram represents the bHLH region of ICE1 protein. *C*, interaction of the depicted regions of ICE1 with MYB15 as prey. *D*, *in vitro* pull-down assay with GST-tagged proteins. The combinations used are indicated at the *top*, and the molecular mass markers (kDa) are indicated at the *left* of the panel. Coomassie Blue-stained gel of the GST-tagged proteins is shown in the *left panel*, and the autoradiogram is shown in the *right panel*. [^{35}S]ICE1 used for the pull-down assay is shown in the *far right*. *E*, *in vitro* pull-down assay with S-tagged proteins. The combinations used are indicated at the *top*, and the molecular mass markers are indicated at the *left* of the panel.

roots, leaves, stems, and floral parts (Fig. 1*B*), further indicating that *MYB15* is ubiquitously expressed.

MYB15 Interacts with ICE1—Yeast 2-hybrid analysis was used to determine whether MYB15 might interact with ICE1. Different portions of ICE1 protein were used as bait, and full-length MYB15 was used as prey to determine their interaction. Because the full-length ICE1 protein itself gave strong activation of the β-Gal reporter gene we used portions of ICE1 as bait. MYB15 did not interact with N-terminal portion (1–266 amino acids) of ICE1 (data not shown), but interacted strongly with the C-terminal portion (266–494 amino acids) of ICE1 (Fig. 2*A*). The interaction of MYB15 and ICE1 was specific, because the prey vector or AtMYB79 failed to activate the reporter gene (Fig. 2*A*). The C-terminal portion of ICE1 was further narrowed down by deletions (Fig. 2*B*) and then used as baits to determine the MYB15 interacting domain in ICE1. The region corresponding to amino acid residues 358–494 of ICE1 was found to interact with MYB15 (Fig. 2*C*).

We used protein pull-down assays to confirm the interaction between ICE1 and MYB15. GST-MYB15 was able to pull down ^{35}S-labeled ICE1 (Fig. 2*D*). Similarly S-tagged ICE1 was able to pull down ^{35}S-labeled MYB15 (Fig. 2*E*). Their interaction was specific, because neither GST-MYB79 nor S-Tag-ABI2 was able to pull-down either ICE1 or MYB15 proteins, respectively. These results suggest that MYB15 interacts specifically with ICE1.

MYB15 Binds to Myb Recognition Sites in the Promoters of CBF Genes—Electrophoretic mobility shift assays (EMSA) were carried out to determine whether MYB15 could bind to elements in *CBF* promoters. Different portions of the *CBF* promoters were PCR-amplified and used for EMSA. One major complex was observed with fragments II (−750/−500) and III (−500/−300) of the *CBF1* promoter, whereas other regions of the *CBF1* promoter had no binding with MYB15 (Fig. 3*A*). When *CBF2* promoter fragments were used, binding was observed with fragments I (−1000/−750) and II (−500/−270), whereas no binding was observed with fragment III (−270/−20). MYB15 was able to bind to all 4 fragments of the *CBF3* promoter. These complexes were abolished by the addition of cold competitors with the same sequences.

Transcription factors belonging to the Myb family have binding specificity to either type I Myb recognition sequences (-CNGTT(A/G)-) or type II (-G(G/T)T(A/T)GTT(A/G)-) and type IIG (-G(G/T)T(A/T)GGT(A/G)-; Ref. 38) Myb recognition sequences. MYB15 preferentially binds to type II and type IIG and binds to a much lesser extent to type I Myb recognition

sequence (38). The *CBF* promoter regions used in this study was found to have many sequences closely related to Type II and Type IIG Myb recognition sites. A detailed presentation of the sequences present in the regions of these promoters and the binding of MYB15 is shown in Table 1. The results indicate that MYB15 can bind to the *CBF* promoters, and the binding is possibly mediated by the Myb recognition sequences.

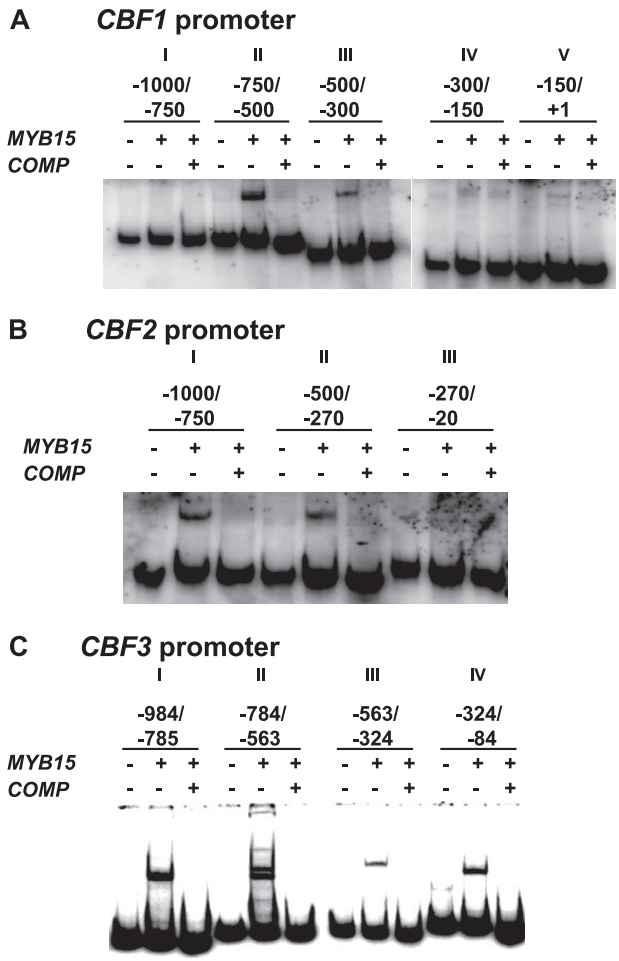

FIGURE 3. **Evaluation of MYB15 protein binding to *CBF* promoter elements by EMSA.** *A*, *CBF1* promoter; *B*, *CBF2* promoter; *C*, *CBF3* promoter. Five fragments for *CBF1*(I, −1000 to −750 bp; II, −750 to −500; III, −500 to −300; IV, −300 to −150 and V, −150 to +1), three fragments for *CBF2* (I, −1000 to −750; II, −500 to −270 and III, −270 to −20) and four fragments for *CBF3* (I, −984 to −785; II, −784 to −563; III, −563 to −324 and IV, −324 to −84) were PCR-amplified and used for EMSA studies.

MYB15 Is Nuclear-localized and Is a Transcriptional Activator in Transient Assays—To examine the subcellular localization of the MYB15 protein, full-length *MYB15* cDNA was fused in-frame to the C terminus of the green fluorescent protein (GFP) coding sequence. GFP-MYB15 fusion driven by a CaMV 35 S promoter was expressed in transgenic Arabidopsis plants. Confocal imaging of GFP fluorescence in the transgenic plants showed that the GFP-MYB15 fusion protein is present in the nucleus (Fig. 4*A*), suggesting that MYB15 is nuclear-localized under nonstressed conditions. Cold stress did not change the nuclear localization of MYB15 (data not shown).

Transient expression assays were carried out to determine whether MYB15 might be a transcriptional activator or repressor. An effector plasmid was constructed by fusing MYB15 with the DNA binding domain of the yeast GAL4 transcriptional activator under the control of a CaMV 35 S promoter (*GAL4-MYB15*; Fig. 4*B*). When the GAL4-MYB15 and a GAL4-responsive reporter gene, *GAL4-LUC*, were delivered into Arabidopsis protoplasts by PEG-mediated DNA uptake, the luciferase activity increased 10-fold relative to the control with or without an effector plasmid containing only the GAL4 DNA binding domain (Fig. 4*B*). These results indicate that MYB15 might be a transcriptional activator.

MYB15 Overexpression Reduces the Expression of CBF Genes under Cold Stress—*MYB15* was overexpressed under the control of the *CaMV* 35 S promoter in wild-type Arabidopsis plants harboring the CBF3-LUC reporter gene. CBF3-LUC luminescence intensities were analyzed in homozygous seedlings of the *MYB15* overexpression lines. As in the wild type, no detectable luminescence was observed in *MYB15* overexpression lines without cold treatment. After cold treatment, CBF3-LUC was expressed at a high level in wild-type plants (Fig. 5, *A* and *B*). However, *MYB15* overexpression lines did not show high CBF3-LUC expression even after the cold treatment (Fig. 5, *A* and *B*).

Northern analysis confirmed that *MYB15* was ectopically expressed to high levels in the overexpression lines (Fig. 5*C*). Under conditions of cold stress, *MYB15* transcript levels increased to even higher levels in the overexpression lines. We also examined the expression of *MYB13* and *MYB14*, two genes most closely related to *MYB15*. These two genes were not up-regulated substantially by cold stress (Fig. 5*C*). Their transcript levels appeared to be reduced in MYB15 overexpression lines (Fig. 5*C*).

TABLE 1

Presence of type II and type IIG Myb recognition sequences in the promoters of *CBF1*, *-2*, and *-3*

The fragments used for mobility shift assay are indicated in the left columns, and the binding is indicated in the column to the right of the sequences.

	CBF1		CBF2		CBF3	
Frag. I	-TTGTTG-(Type II) -TTGTTG-(Type II)	−[a]	-TTGTTA-(Type II) -TTGTTA-(Type II)	++[b]	-TTGTTA-(Type II) -TTGTTA-(Type II)	+++[c]
Frag. II	-TGGTTA-(Type II)	+++	-TTGTTA-(Type II) -TGGTTG-(Type II)	+++	-TTGTTA-(Type II) -TAGTTA-(Type II)	+++
Frag. III	-TGGTTA-(Type II)	+++			-TTGTTG-(Type II) -TTGGTG-(Type IIG)	+[d]
Frag. IV		−			-CGGTTA-(Type II)	+++
Frag. V	-CCGTAA-(Type I)	−				

[a] −, no binding.
[b] ++, medium binding.
[c] +++, strong binding.
[d] +, weak binding.

We further analyzed one of the overexpression line (no. 10) for expression of *CBFs* and their downstream genes. Under control conditions, transcript of none of the *CBF* genes was detected in either the wild type or *MYB15* overexpression line.

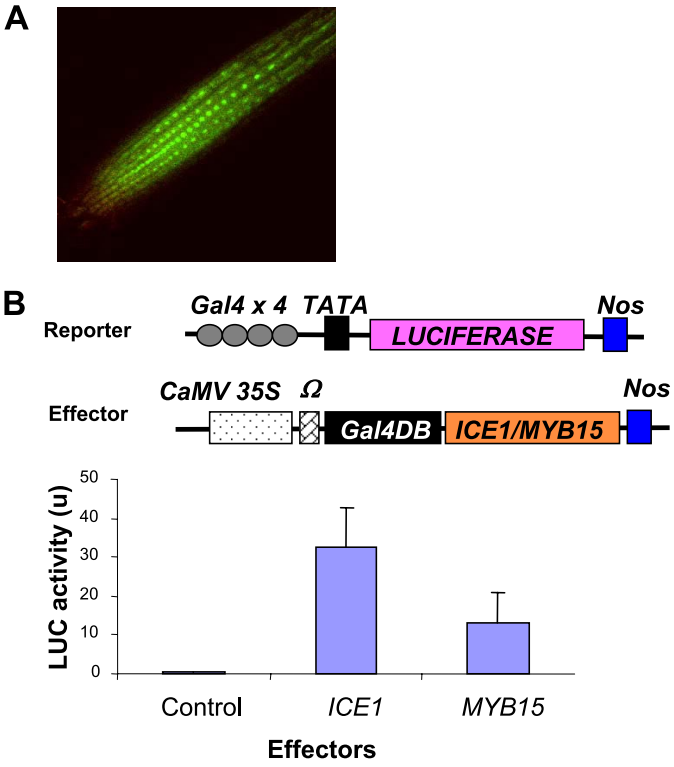

FIGURE 4. **MYB15 is a nuclear-localized transcriptional activator.** *A*, localization of GFP-MYB15 protein in the nuclei of root cells of GFP-MYB15 transgenic plants. *B*, relative luciferase reporter activities after transfection with Gal4-Luc and 35 S-Gal4DB-MYB15 or 35 S-Gal4DB-ICE1. To normalize 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 (42).

Under 3 h of cold stress, more *CBF3* as well as CBF1 and CBF2 transcript was present in the wild-type plants as compared with the *MYB15* overexpression line (Fig. 5*D*). At the other time points, there was less difference in the expression of *CBF* genes between the wild type and *MYB15* overexpression line. No substantial difference in *COR15A* and *RD29A*, two downstream genes of CBFs, was seen between the different genotypes (Fig. 5*D*).

MYB15 Knock-down or Knock-out Causes Increased Expression of CBF Genes under Cold Stress—We generated *MYB15* RNAi lines in the *CBF3-LUC* background to study the effect of *MYB15* loss-of-function. In the RNAi lines, *CBF3-LUC* expression was induced by cold treatment to much higher levels compared with the wild-type (Fig. 6, *A* and *B*). *MYB15* gene knockdown in the RNAi lines was confirmed by RT-PCR analysis (Fig. 6*C*). We tested the expression of endogenous *CBFs* and their downstream genes in one of the *MYB15* RNAi lines (no. 9). At 3 h of cold stress there was little difference in expression of *CBF1* and *CBF2* genes in wild type and RNAi line no. 9 (Fig. 6*D*). However, *CBF3* exhibited higher expression in the RNAi line no. 9, than WT at this time point. At 6 and 12 h of cold stress *CBF1*, *CBF2*, and *CBF3* genes all had higher transcript levels in the RNAi line as compared with the wild type. However, the downstream genes *COR15A* and *RD29A* had no substantial differences in their expression between wild type and the RNAi line (Fig. 6*D*).

When the SALK T-DNA lines became available, we identified a homozygous T-DNA mutant in *MYB15* (Fig. 7*A*). RT-PCR analysis showed that the homozygous line (no. 28) had a complete loss of *MYB15* transcript whereas both the wild type and a heterozygous line (no. 30) showed *MYB15* expression (Fig. 7*B*). The homozygous T-DNA mutant was analyzed for the expression of the *CBFs* and their downstream genes. Both the mutant and WT plants showed strong expression of *CBF1*,

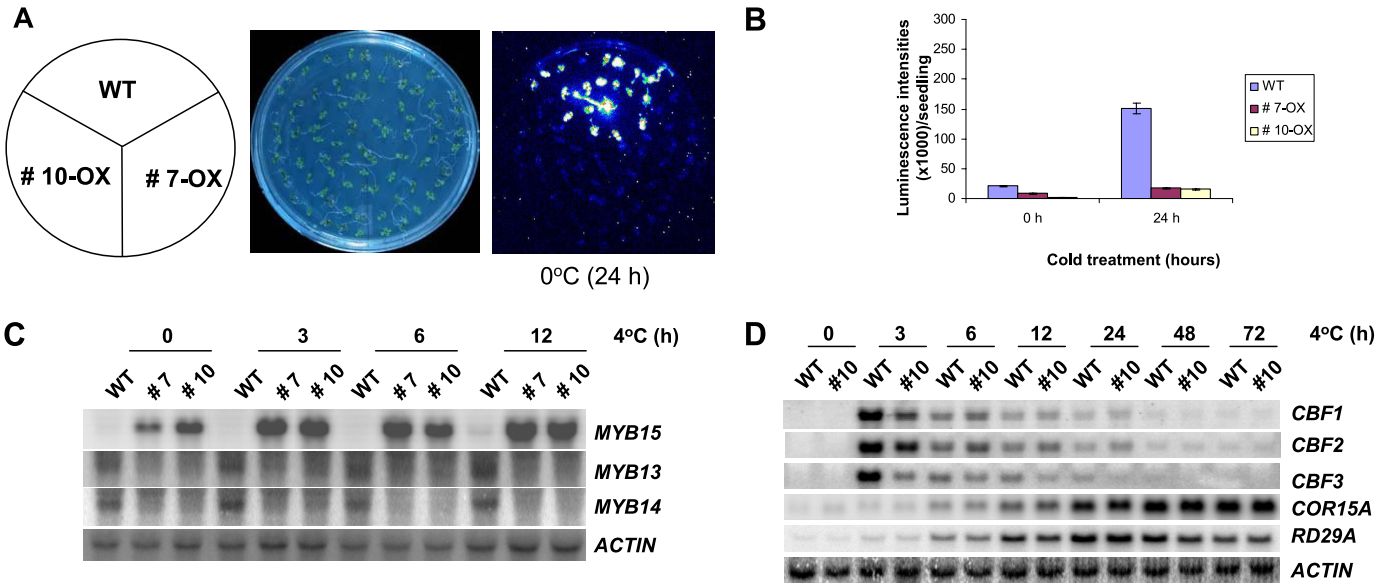

FIGURE 5. **Ectopic expression of *MYB15* leads to decreased *CBF* expression under cold stress.** *A*, images of seedlings and luminescence of the wild type and the *MYB15* overexpression lines (7 and 10). Images were taken after 24-h cold treatment. *B*, quantitation of luminescence intensities from the wild type and the *MYB15* overexpression lines (7 and 10) in response to cold stress. *C*, steady state levels of *MYB15* and related Myb transcription factors *MYB13* and *MYB14* transcripts in *MYB15* overexpression lines 7 and 10. Actin was used as a loading control. *D*, steady state levels of *CBF1*, *2*, *3* and downstream genes *COR15A* and *RD29A* transcripts in *MYB15* overexpression line 10.

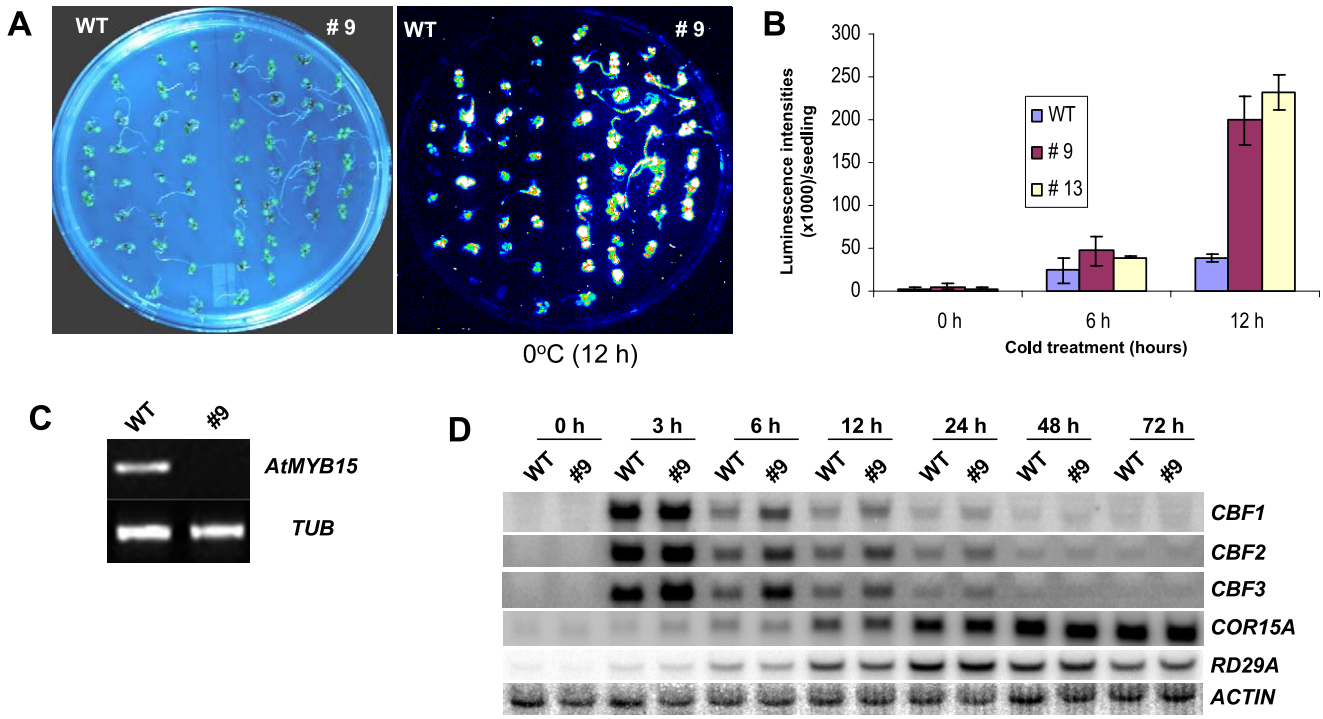

FIGURE 6. **MYB15 knock-down by RNAi leads to increased *CBF* expression.** *A*, luminescence of the wild type and *MYB15* RNAi lines 9 and 13. *B*, quantitation of luminescence intensities from the wild-type and the *MYB15* RNAi lines 9 and 13 during cold stress. *C*, RT-PCR of WT and RNAi line (9). Tubulin (*TUB*) was used an internal control. *D*, steady state levels of *CBF1*, *-2*, *-3*, and downstream genes *COR15A* and *RD29A* transcripts in *MYB15* RNAi line 9. Actin was used as a loading control.

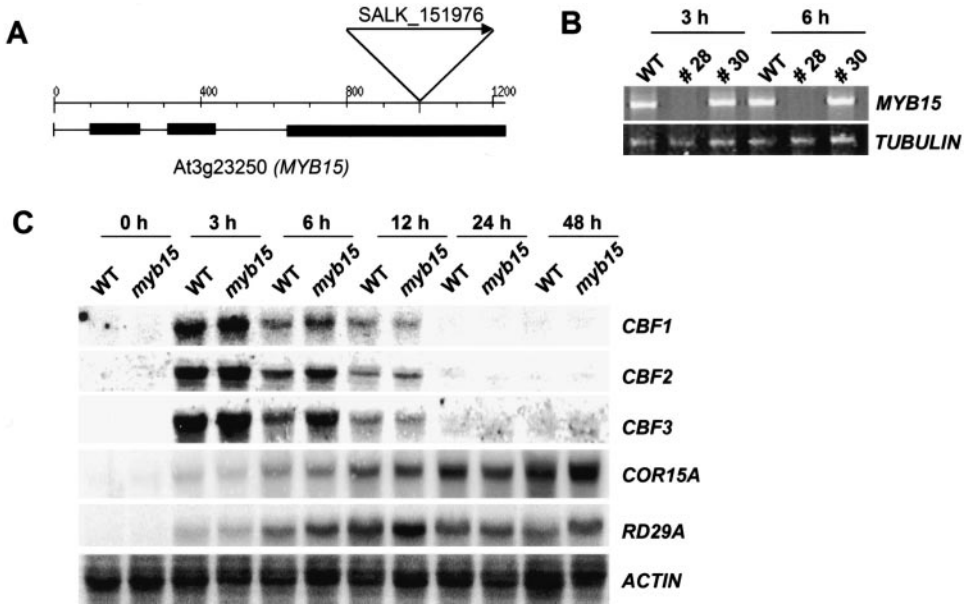

FIGURE 7. **Analysis of the *myb15* T-DNA line.** *A*, graphical representation of the T-DNA insertion in *MYB15*. *B*, RT-PCR of homozygous (28), heterozygous (30) and WT plants to confirm gene knock-out. *C*, expression levels of *CBFs*, *COR15A* and *RD29A* genes in the homozygous T-DNA line. The duration of the cold treatment are indicated at the *top*. Actin was used as a loading control.

-2, and *-3* after 3 h of cold treatment. However, these genes appeared to have higher levels of expression in the mutant as compared with the wild-type seedlings (Fig. 7*C*). The higher levels of expression of the *CBF* genes in the mutant were more evident at 6 h of cold stress (Fig. 7*C*). However, after 12 h of cold treatment, the expression of the *CBF* genes was similar between the mutant and wild-type plants (Fig. 7*C*). *COR15A* had higher expression in the mutant as compared with wild type after 48 h of cold stress. Similarly, *RD29A* had higher expression in the *myb15* mutant as compared with the wild type at the 12 and 48 h time points during cold stress. These results indicate that *MYB15* may play a negative role in controlling the expression of the *CBF* genes *in vivo*. We checked the expression of *MYB13* and *MYB14*, and found that their expression was not altered in the *myb15* T-DNA mutant (data not shown).

MYB15 Negatively Regulates Plant Freezing Tolerance—To evaluate the effect of over and underexpression of *MYB15* on plant freezing tolerance, an electrolyte leakage test was conducted. Overexpression of *MYB15* resulted in higher levels of electrolyte leakage, indicating a decreased freezing tolerance (Figs. 8, *A* and *B*). The LT_{50} values for the wild-type and MYB15 overexpression plants were −6.8 °C and −6.0 °C, respectively, under nonacclimation conditions. After acclimation, the LT_{50} for the wild-type plants increased to −9.8 °C but was only −7.9 °C for the MYB15 overexpression plants. In contrast, the *myb15* T-DNA mutant was more tolerant to freezing stress under both acclimation and nonacclimation conditions (Fig. 8,

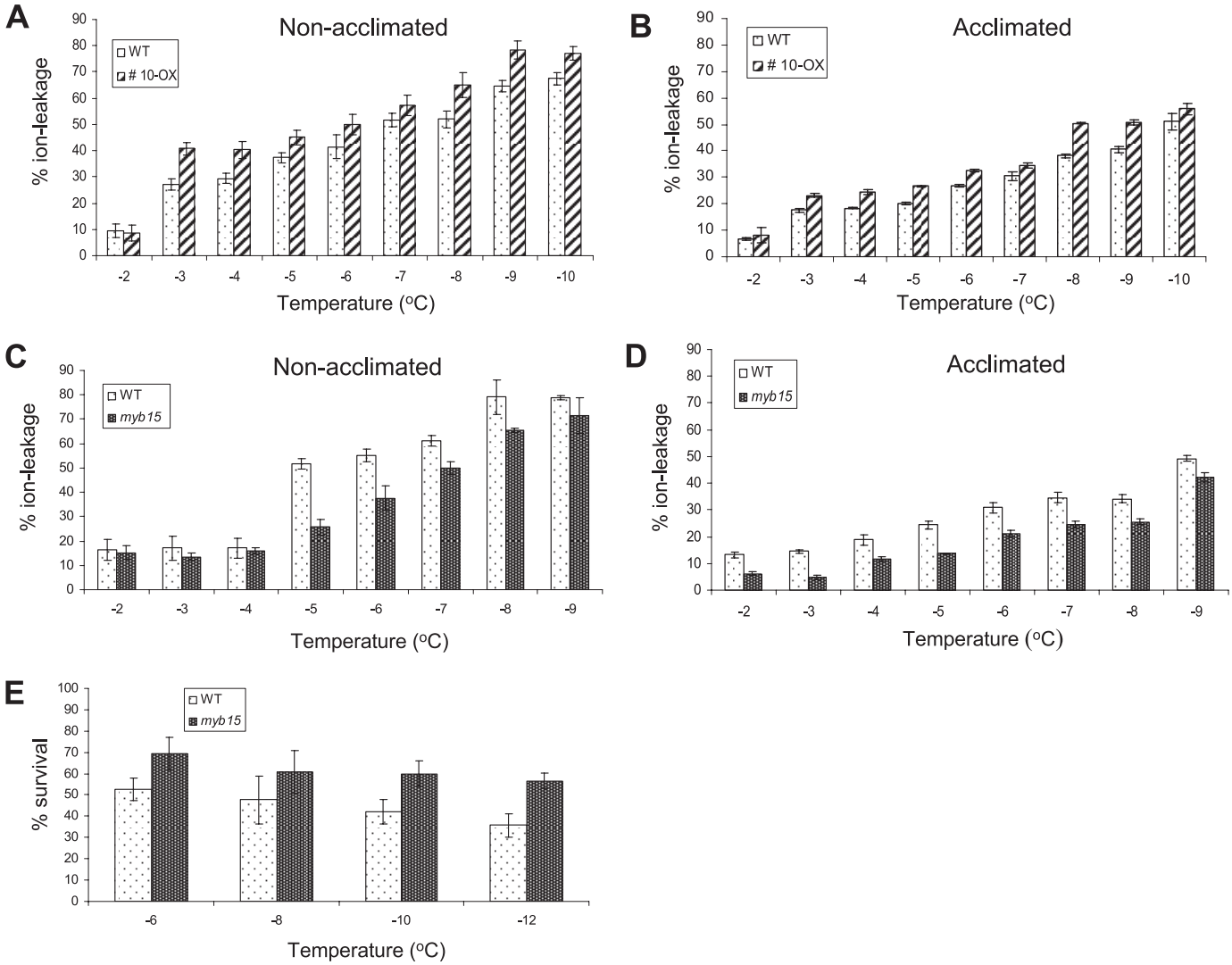

FIGURE 8. **Effect of MYB15 overexpression and knock-out on plant freezing tolerance.** *A*—*D*, ion-leakage test. Error bar represents S.E. ($n = 3$). *A*, WT and overexpression line 10 without acclimation. *B*, WT and overexpression line 10 after acclimation for 48 h at 4 °C. *C*, WT and T-DNA knock-out line without acclimation, *D*, WT and T-DNA knock-out line after acclimation for 48 h at 4 °C. *E*, survival rate of *myb15* and WT seedlings after freezing at different temperatures. *Error bars* represent S.E. in the mean percentage of survival ($n = 3$).

C and *D*). The LT_{50} values for the nonacclimated wild-type and mutant plants were −4.9 °C and −7 °C, respectively.

The *myb15* mutant was also subjected to a whole seedling freezing assay to evaluate the role of MYB15 in plant survival after freezing treatment. The *myb15* mutant exhibited increased survival as compared with wild-type seedlings under different freezing temperatures (Fig. 8*E*). For example, nearly 60% of the mutant seedlings but only about 40% of wild-type seedlings survived a −12 °C freezing treatment. Together, these results show that *MYB15* overexpression results in decreased freezing tolerance, whereas its knock-out results in increased freezing tolerance.

DISCUSSION

Cold acclimation involves a cascade of transcriptional events. Certain constitutively expressed transcription factors are presumably activated in response to cold, and these would turn on the transcription of cold-induced transcription factors such as the CBFs. CBF proteins can then activate the expression of downstream cold-responsive genes that encode proteins with protective effects. ICE1 has been identified as a constitutive transcription factor upstream of CBF3 (14). In the present study, another transcription factor, MYB15, was found to play a role in the regulation of *CBF* genes under cold stress. MYB15 physically interacts with ICE1. Combinatorial interactions between transcription factors have been shown to be important for the regulation of downstream genes (29, 30, 39). Although the functional consequence of MYB15-ICE1 interaction on the expression of the *CBF* genes is unclear at the present time, the observation of such an interaction, together with other functional data, strongly support a role for MYB15 in cold-regulated gene expression.

The interaction between R2R3MYB proteins and bHLH proteins is known to occur through a conserved signature sequence in MYB proteins and N-terminal domain in subgroup III of bHLH proteins (40). Neither ICE1 nor MYB15 have these conventional signature sequences, therefore the interaction between them is possibly mediated by novel interaction domains.

MYB15 is expressed at a low level in all tissues and is localized in the nucleus. Under cold stress, *MYB15* expression is up-regulated. Interestingly, the kinetics of this up-regulation is altered in the *ice1* mutant. ICE1 may directly (through binding to *MYB15* promoter) or indirectly (*i.e.* through its downstream genes) attenuate *MYB15* expression in response to cold. MYB15 belongs to the R2R3-Myb family of transcription factors and can bind to the promoter fragments of all three *CBF* genes, consistent with the presence of type II Myb recognition sequences in these promoters. The role of MYB15 in planta is supported by data showing that *MYB15* over- and underexpression alters the expression of *CBF* genes and affects freezing tolerance.

Results from the transient assays showed that MYB15 has transcriptional activation activity. However, data from MYB15 overexpression lines and T-DNA mutant plants indicated that MYB15 is a negative regulator of *CBF* expression. The effect of MYB15 on *CBF1* and *2* is similar as on *CBF3*. The *CBF* genes show increased expression in the T-DNA knock-out mutant plants and decreased expression in the overexpression lines. MYB15 interaction with ICE1 and binding to *CBF* promoter elements suggest a likely direct effect of MYB15 on *CBF* gene expression, although we cannot rule out an additional indirect effect. Interpretation of these genetic data could be complicated by the complex network of transcriptional regulation of genes. For example, *in vitro* studies showed that CBF2 is a positive regulator of downstream cold-responsive genes and its overexpression in transgenic plants leads to increased expression of downstream cold-responsive genes and enhanced freezing tolerance (13, 41, 42). However, in *cbf2* knock-out mutant plants, downstream cold-responsive genes and freezing tolerance are also increased (18). This paradoxical result may be explained by a possible negative regulation of CBF2 on *CBF1* and *3* expression, and the overcompensation of *CBF1* and *3* expression in *cbf2* mutant then leads to the observed phenotypes. An examination of Myb genes closely related to *MYB15* revealed that *MYB13* and *14* expression was reduced in *MYB15* overexpression plants, but their expression was not affected by *myb15* knock-out. Besides MYB15 and ICE1, there are probably other Myb and Myc family of transcription factors involved in the regulation of *CBF* genes. These proteins may interact with themselves and/or with each other both physically and genetically and thus form a complex web of transcription factors. To have a more complete understanding of MYB15 function in cold responsive gene regulation, more of these transcription factors will need to be identified and characterized in the future. It is possible that MYB15 may function as both an activator and repressor depending on the target promoter sequences and interacting proteins, as have been documented for some transcription factors in animals (43–47).

There is a further complication in understanding the role of MYB15 in cold-regulated gene expression. Although the *CBF* genes are negatively affected by MYB15 levels, the CBF downstream genes *COR15A* and *RD29A* are largely unaffected in the MYB15 overexpression or underexpression (RNAi or T-DNA knock-out) plants. Recent studies have suggested that the CBFs are not the sole transcription factors involved in the regulation of these downstream genes (22, 23). Besides negatively regulating *CBFs*, MYB15 may at the same time positively regulate other transcriptional activators of the downstream genes. It is also possible that MYB15 may negatively regulate certain transcriptional repressors of the downstream genes. These possibilities point again to a complex web of transcriptional regulation of cold responsive gene expression. Notwithstanding the precise function of MYB15 in this transcriptional network, our results show that MYB15 plays a role in freezing tolerance and in the regulation of *CBF* genes under cold stress.

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