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 eskiomo1 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 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...
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′-GGAATTCATGACAAGCTCGACATGCATAG-3′ and reverse primer 5′-CGCGGGATCCCTAGCCAATCGATGCGAGCTG-3′ and cloned in SmaI and XhoI sites of the pGEX 4T-1 vector. For mapping, the interacting domain deletions of MYB15, the RNAi line of MYB15 and the 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′-GGAATTCATGACAAGCTCGACATGCATAG-3′ and reverse primer 5′-CGCGGGATCCCTAGCCAATCGATGCGAGCTG-3′. β-Tubulin gene was amplified as an internal loading control using the following primers: forward primer: 5′-GTCAAGAGGTTCTCTAGACGATA-3′ and reverse primer 5′-TCACCTTCTTCTAGATCCGAGTT-3′.

**Yeast 2-Hybrid Interaction Studies**—MYB15 was amplified with primers 5′-GATGGGAAGAGCTCATGTCGAGC-3′ and 5′-CGGCTCGAGCTTGGCCAATACATCGAACCAG-3′ and cloned in the SmaI and Xhol 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 C-terminal region (corresponding to 266–494 amino acids) of the SmaI and XhoI sites of the pBCSK; 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-32P]ATP and T4 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 Sall sites of the plant expression vector [35S]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 Xhol sites of pBCKS; Stratagene) were used for in vitro transcription and translation. Full-length ICE1 and ABI2 open reading frames were cloned in EcoRI/Sall and Ncol/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 [35S]methionine. S-tag-ICE1, and S-tag-ABI2 transcripts were translated in the absence of [35S]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 the S3-labeled MYB15. In a separate experiment, 35S-labeled ICE was produced and used for pull-down assays with either GST-
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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'-CGGGATCC-3' and 5'-GGGATCC-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'-CGGGATCC-3' and 5'-GGGATCC-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 of 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'-GGTATCGCTTATCCATATCAATCTGCTGTGA-3' and 5'-GGGTACCCATGGCAAGAGCTCCATGCTGTGA-3' primers and cloned in EcoRI/HindIII sites of pRT105 vector. The cDNA was PCR-amplified with gene-specific primers. RNA was extracted from the 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 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 H2O, and the tube was placed in a circulating freezing bath (WVR 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⁻²s⁻¹ continuous light. Ten-day-old seedlings were cold-acclimated at 4 °C ± 1 °C and 30 ± 2 μmol quanta m⁻²s⁻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⁻²s⁻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. 1A). 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. 1A).

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...
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roots, leaves, stems, and floral parts (Fig. 1B), 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. 2A). The interaction of MYB15 and ICE1 was specific, because the prey vector or AtMYB79 failed to activate the reporter gene (Fig. 2A). The C-terminal portion of ICE1 was further narrowed down by deletions (Fig. 2B) 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. 2C).

We used protein pull-down assays to confirm the interaction between ICE1 and MYB15. GST-MYB15 was able to pull down 35S-labeled ICE1 (Fig. 2D). Similarly S-tagged ICE1 was able to pull down 35S-labeled MYB15 (Fig. 2E). 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 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)GGT(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 sequences.
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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.

**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.

| Frag. | CBF1       | CBF2       | CBF3       |
|-------|------------|------------|------------|
| I     | -TGGTTG-(Type II) | -TGGTTA-(Type II) | -TGGTTA-(Type II) |
| II    | -TGGTTA-(Type II) | +       | -TGGTTA-(Type II) |
| III   | +       | +       | +       |
| IV    | +       | +       | +       |
| V     | -CGGTTA-(Type IIG) | -CGGTTA-(Type II) |

\(a\) = no binding.
\(b\) = weak binding.
\(c\) = medium binding.
\(d\) = strong binding.

MYB15 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. 4A), 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 GAL4 DNA binding domain of the yeast GAL4 transcriptional activator under the control of a CaMV 35 S promoter (GAL4-MYB15; Fig. 4B). 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. 4B). 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. 5C). 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. 5C). Their transcript levels appeared to be reduced in MYB15 overexpression lines (Fig. 5C).

**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 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. 5C). 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. 5C). Their transcript levels appeared to be reduced in MYB15 overexpression lines (Fig. 5C).
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.

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. 5D). 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. 5D).

**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 knock-down in the RNAi lines was confirmed by RT-PCR analysis (Fig. 6C). 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. 6D). 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. 6D).

When the SALK T-DNA lines became available, we identified a homozygous T-DNA mutant in MYB15 (Fig. 7A). 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. 7B). 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,
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 LT50 values for the wild-type and MYB15 overexpression plants were −6.8 °C and −6.0 °C, respectively, under nonacclimation conditions. After acclimation, the LT50 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, A and B). The LT50 values for the wild-type and MYB15 overexpression plants were −6.8 °C and −6.0 °C, respectively, under nonacclimation conditions. After acclimation, the LT50 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, A and B).
The LT₅₀ 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 compared with wild-type seedlings under different freezing temperatures (Fig. 8E). 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 icel 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 plants 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 I4 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 other 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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