A Novel MYBS3-Dependent Pathway Confers Cold Tolerance in Rice[1][W][OA]

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Rice (*Oryza sativa*) seedlings are particularly sensitive to chilling in early spring in temperate and subtropical zones and in high-elevation areas. Improvement of chilling tolerance in rice may significantly increase rice production. MYBS3 is a single DNA-binding repeat MYB transcription factor previously shown to mediate sugar signaling in rice. In this study, we observed that MYBS3 also plays a critical role in cold adaptation in rice. Gain- and loss-of-function analyses indicated that MYBS3 was sufficient and necessary for enhancing cold tolerance in rice. Transgenic rice constitutively overexpressing MYBS3 tolerated 4°C for at least 1 week and exhibited no yield penalty in normal field conditions. Transcription profiling of transgenic rice overexpressing or underexpressing MYBS3 led to the identification of many genes in the MYBS3-mediated cold signaling pathway. Several genes activated by MYBS3 as well as inducible by cold have previously been implicated in various abiotic stress responses and/or tolerance in rice and other plant species. Surprisingly, MYBS3 repressed the well-known DREB1/CBF-dependent cold signaling pathway in rice, and the repression appears to act at the transcriptional level. *DREB1* responded quickly and transiently while *MYBS3* responded slowly to cold stress, which suggests that distinct pathways act sequentially and complementarily for adapting short- and long-term cold stress in rice. Our studies thus reveal a hitherto undiscovered novel pathway that controls cold adaptation in rice.

Rice (*Oryza sativa*) is one of the most important food crops in the world, and increases in rice yield could significantly ease the pressure on world food production. Rice is also a powerful model for functional genomics study for dissecting genetic networks of stress responses in cereal crops. Low temperatures are one of the major environmental stresses that adversely affect rice productivity in temperate and subtropical zones and in high-elevation areas. Rice seedlings are particularly sensitive to chilling in early spring in these areas, leading to slow seedling development, yellowing, withering, reduced tillering, and stunted growth (Andaya and Mackill, 2003). Rice cannot be grown in approximately 7,000,000 hectares of land in south and southeast Asia due to cold stress (Sthapit and Witcombe, 1998); in temperate regions such as California, cold is an important stress that results in delayed heading and yield reduction due to spikelet sterility (Peterson et al., 1974). Thus, improvement of chilling tolerance may significantly increase rice production.

Plants respond and adapt to cold stress at the molecular and cellular levels as well as induce an array of biochemical and physiological alterations that enable them to survive (Bohnert et al., 1995; Browse and Xin, 2001). Under cold stress, the expression of many genes is induced in various plant species (Hughes and Dunn, 1996; Thomashow, 1999), and the products of these genes function not only in adaptations promoting stress tolerance, such as biosynthesis of osmotica (Chen and Murata, 2002; Taji et al., 2002), generation of antioxidants (Prasad et al., 1994), and increased membrane fluidity (Murata and Los, 1997; Orvar et al., 2000), but also in the regulation of gene expression and signaling transduction in stress responses, such as transcription factors and proteins involved in RNA processing and nuclear export (Yamaguchi-Shinozaki and Shinozaki, 2006; Chinnusamy et al., 2007). Deciphering the mechanisms by which

plants perceive and transmit cold signals to cellular machinery to activate adaptive responses is of critical importance for developing breeding strategies to enhance cold stress tolerance in crops.

In Arabidopsis (*Arabidopsis thaliana*) and rice, the CBF/DREB1-dependent cold response pathway has been shown to play a predominant role in freezing tolerance through the process of cold acclimation (Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 2006; Chinnusamy et al., 2007). The DREB1/CBF family, including DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2, is able to bind to and activate the cis-acting elements DRE (for dehydration-responsive element; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997) and CRT (for C-repeat; Baker et al., 1994) on promoters of several cold-responsive (*COR*) genes (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Medina et al., 1999).

Rice *DREB1A* and *DREB1B* are induced by cold stress, and constitutive overexpression of these genes leads to induction of stress-responsive genes, increased tolerance to high salt and cold, and growth retardation under normal conditions in transgenic Arabidopsis and rice (Dubouzet et al., 2003; Ito et al., 2006), indicating the evolutionary conservation of the DREB1/CBF cold-responsive pathway in monocots and dicots. However, in comparison with Arabidopsis and other cereals like wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) that cold acclimate (Wen et al., 2002), rice does not undergo an acclimation process and is more sensitive to low-temperature exposures. Microarray analysis demonstrated the existence of 22 cold-regulated genes in rice, which have not been reported in Arabidopsis (Rabbani et al., 2003). These studies also indicate that plant species vary in their abilities to adapt to cold stress.

Other rice proteins have also been shown to be involved in cold tolerance. For example, a zinc-finger protein, iSAP1, confers cold, dehydration, and salt tolerance in transgenic tobacco (*Nicotiana tabacum*; Mukhopadhyay et al., 2004); the rice MYB4 transcription factor confers chilling and freezing tolerances by enhancing COR gene expression and Pro accumulation in Arabidopsis (Vannini et al., 2004) and improves cold and drought tolerances by accumulating osmolyte in transgenic apple (*Malus pumila*; Pasquali et al., 2008). Overexpression of the rice cold-, drought-, and salt-inducible MYB3R-2 (an R1R2R3 MYB gene) enhances cold, drought, and salt tolerance by regulating some stress-responsive genes involved in the CBF-dependent or CBF-independent pathways in Arabidopsis (Dai et al., 2007; Ma et al., 2009).

The expression of *DREB1* is subject to regulation by several factors. For example, it is affected by members in the same *DREB1* family. The Arabidopsis *cbf2* mutant, in which CBF2/DREB1C is disrupted, shows higher freezing, dehydration, and salt tolerance than the wild-type plant, indicating that DREB1C/CBF2 acts as a repressor of CBF1/DREB1B and CBF3/DREB1A expression (Novillo et al., 2004). The expression of DREB1/CBF is activated by Inducer of CBF Expression1 (a MYC-like basic helix-loop-helix-type transcription factor; Chinnusamy et al., 2003), CAX1 (a Ca^{2+}/H^+ transporter; Catala et al., 2003), CBL1 (a Ca^{2+} sensor; Albrecht et al., 2003), and LOS4 (a DEAD box RNA helicase; Gong et al., 2002) and repressed by FRY2 (a transcription factor; Xiong et al., 2002), HOS1 (a putative RING finger E3 ligase; Lee et al., 2001), and ZAT12 (a C_2H_2 zinc finger transcription factor; Vogel et al., 2005) during cold acclimation in Arabidopsis. The mechanism by which these factors affect the expression of CBF/DREB1 is not clear.

Previously, three MYB transcription factors, MYBS1, MYBS2, and MYBS3, each with a single DNA-binding domain (1R MYB), were identified in rice and shown to bind specifically to the TA box (TATCCA) in the sugar response complex (SRC) of the α-amylase gene (α*Amy3*) promoter (Lu et al., 2002). MYBS1 and MYBS2 transactivate, while MYBS3 represses, the sugar starvation-inducible α*Amy3* SRC activity in rice (Lu et al., 2002). The rice *MYBS3* homolog in Arabidopsis (Arabidopsis Genome Initiative code no. At5g47390) is activated by abscisic acid (ABA), $CdCl_2$, and NaCl (Yanhui et al., 2006). Recently, we found that the expression of *MYBS3* was induced by cold, which prompted us to study its functions in rice in more detail. In this report, by both gain- and loss-of-function analyses, we show that *MYBS3* is essential for cold stress tolerance in rice. Transcription profiling of transgenic rice overexpressing or underexpressing *MYBS3* led to the identification of genes that are activated or repressed by *MYBS3* and play diverse functions. The DREB1-dependent cold response signaling pathway is among those repressed by MYBS3 in rice. Our studies suggest that the DREB1- and MYBS3-dependent pathways may complement each other and act sequentially to adapt to immediate and persistent cold stress in rice.

RESULTS

Expression of *MYBS3* Is Ubiquitous and Activated by Cold Stress

Expression of *MYBS3* was found to be ubiquitous in all tissues in seedlings and mature plants and in cultured suspension cells of rice (Fig. 1A). The regulation of *MYBS3* expression by various stresses was investigated by subjecting rice seedlings to ABA (20 μM), drought (air dry), cold (4°C), salt (200 mM NaCl), and heat (45°C) treatments. The accumulation of *MYBS3* mRNA was induced by cold in roots and by cold and salt in shoots (Fig. 1B) but was reduced by ABA in shoots (Fig. 1C). The expression pattern of *MYBS3* and *DREB1A* under cold stress was further compared. The amount of *MYBS3* mRNA was detectable at 28°C and increased 5-fold at 4°C after 72 h; in contrast, the accumulation of *DREB1A* mRNA was barely detectable at
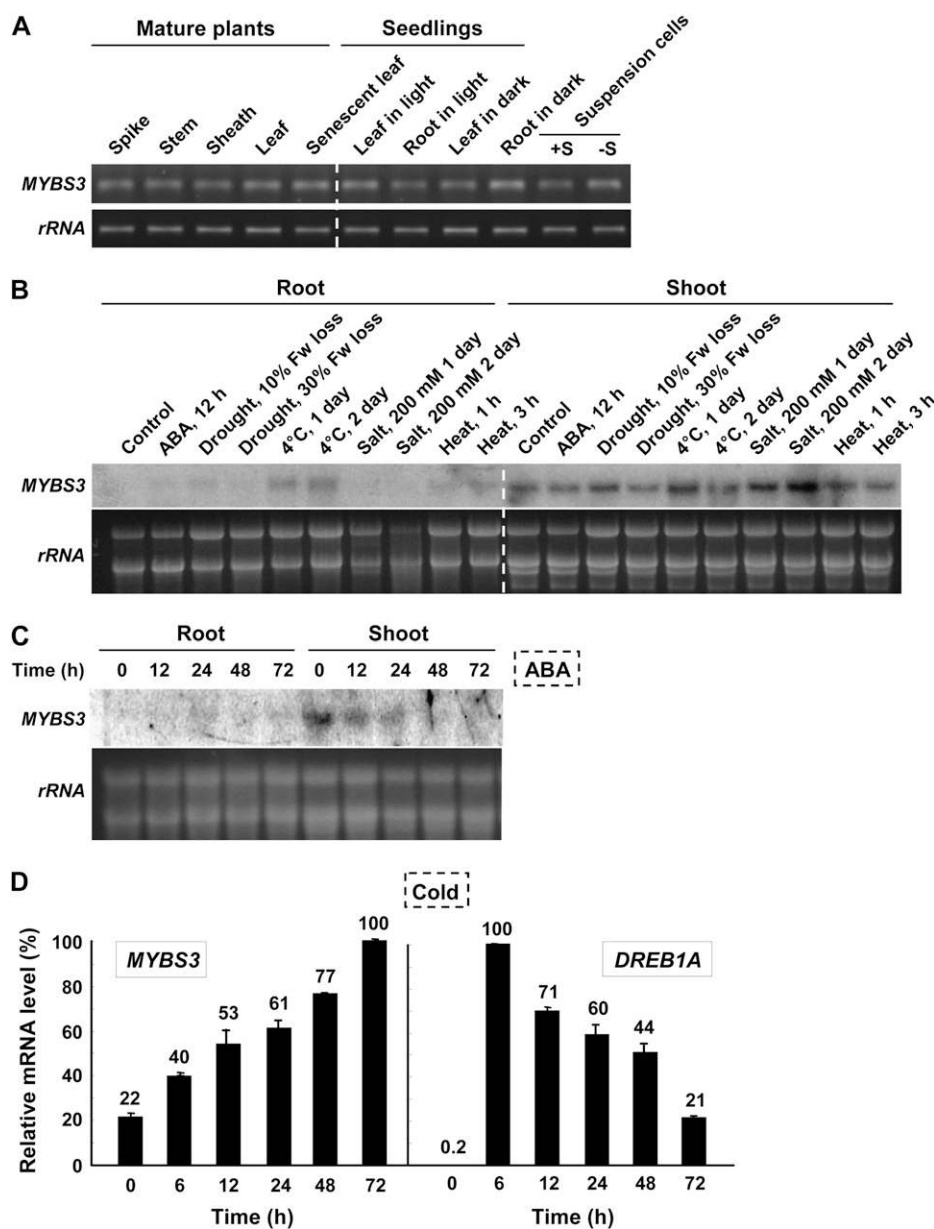
Figure 1. *MYBS3* is constitutively expressed in all rice tissues and also responds to cold. A, Rice tissues were collected from 3-month-old plants grown in the field, 7-d-old seedlings cultured hydroponically, and suspension cells cultured with or without Suc (S) for 2 d. Total RNAs were isolated from rice tissues, and RT-PCR analysis for accumulation of *MYBS3* mRNA was performed. The 18S ribosomal RNA was used as an internal control. B, Ten-day-old rice seedlings were treated with various stresses as indicated. C, Ten-day-old rice seedlings were treated with 20 μM ABA for up to 72 h. For B and C, total RNAs were isolated from seedlings and subjected to northern-blot analyses. The ethidium bromide-stained 18S rRNA was used as an RNA loading control. D, Ten-day-old rice seedlings were shifted from 28°C to 4°C and incubated for 72 h. Total RNAs were isolated from seedlings and subjected to quantitative real-time RT-PCR analysis. The highest mRNA level was assigned a value of 100, and mRNA levels of other samples were calculated relative to this value. The error bars indicate the SE of three replicates.

28°C, increased drastically after shifting to 4°C, and peaked at 6 h, but it declined to one-fifth after 72 h (Fig. 1D).

To determine whether *MYBS3* is regulated by cold at the transcriptional level, the 2.5-kb *MYBS3* promoter was fused to the reporter gene *GFP* encoding a green fluorescence protein and introduced into the rice genome. The *Ubiquitin* (*Ubi*) promoter fused to *GFP* was used as a control. Transgenic rice seedlings were grown at 4°C. Under the control of the *MYBS3* promoter, the accumulation of *GFP* mRNA was 2.5 times higher at 12 h and stayed high up to 24 h (Fig. 2A, top). In contrast, under the control of the *Ubi* promoter, the accumulation of *GFP* mRNA decreased by nearly 50% at 6 h and then stayed at similar levels up to 24 h (Fig.

2A, bottom). This result indicates that the *MYBS3* promoter is activated by cold.

A previous study has shown that MYBS3 is a transcriptional repressor of $\alpha Amy3$ SRC in rice suspension cells (Lu et al., 2002). To determine whether MYBS3 is localized in the nucleus, the *Ubi* promoter was fused to the *MYBS3-GFP* fusion DNA. The *Ubi::MYBS3-GFP* and *Ubi::GFP* constructs were introduced into the rice genome. Protoplasts were isolated from transformed calli, incubated at 4°C or 28°C, and examined. Accumulation of MYBS3-GFP was detected mainly in the nucleus, whereas GFP alone was distributed throughout the cell except the vacuole, at both 4°C and 28°C (Fig. 2B), suggesting that MYBS3 is constitutively localized in the nucleus.
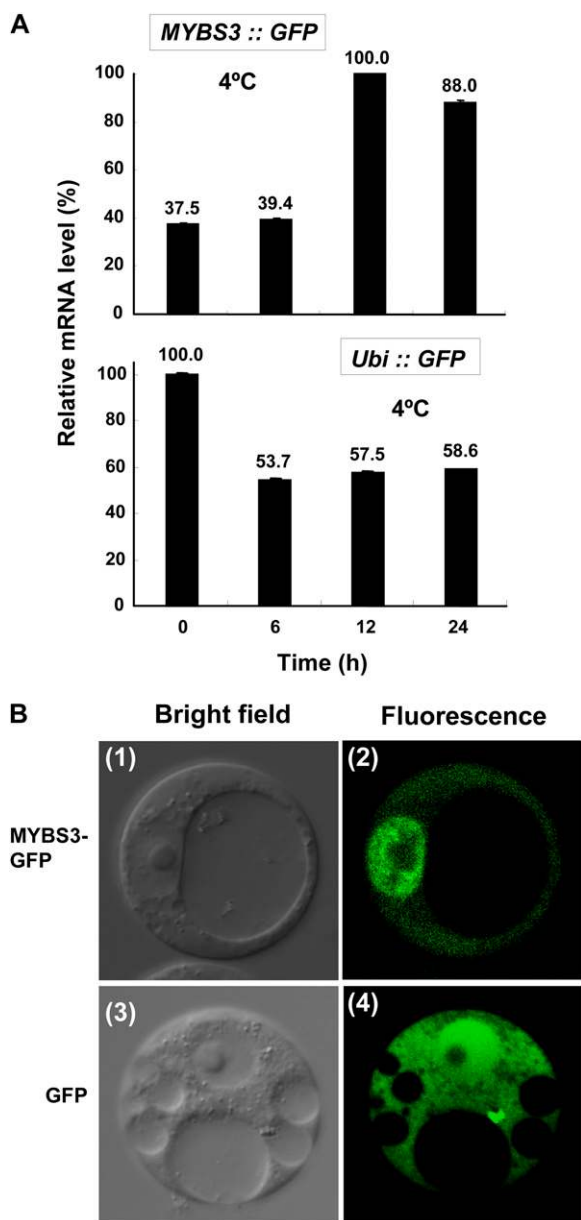
A

B

Figure 2. The *MYBS3* promoter is up-regulated by cold stress, and the MYBS3 transcription factor is constitutively localized in the nucleus. A, Rice transformed with the *MYBS3::GFP* construct or the *Ubi::GFP* construct. Transgenic rice seedlings were cultivated hydroponically at 28°C for 10 d and then transferred to 4°C for up to 24 h. Total RNA was isolated from leaves and subjected to quantitative real-time RT-PCR analysis. The highest mRNA level was assigned a value of 100, and mRNA levels of other samples were calculated relative to this value. The error bars indicate the SE of three replicates. B, Protoplasts were isolated from transformed rice calli expressing GFP alone or the MYBS3-GFP fusion protein. Panels 1 and 2 show protoplasts expressing the MYBS3-GFP fusion protein; GFP was detected mainly in the nucleus. Panels 3 and 4 show protoplasts expressing GFP alone; GFP was detected in the nucleus and cytoplasm. Panels 1 and 3 are bright-field images, and panels 2 and 4 are fluorescent field images.

MYBS3 Is Sufficient and Necessary for Cold Tolerance in Rice

Since MYBS3 was induced by cold, its role in cold tolerance in rice was explored by gain- and loss-of-function approaches. Constructs *Ubi::MYBS3* and *Ubi::MYBS3*(RNAi) (for RNA interference; Supplemental Fig. S1) were introduced into the rice genome, and several transgenic lines were obtained. Compared with the untransformed wild-type rice, the accumulation of *MYBS3* mRNA was higher in *MYBS3* overexpression [*MYBS3*(Ox)] lines S3(Ox)-110-1 and S3(Ox)-112-7 and lower in *MYBS3* underexpression [*MYBS3*(Ri)] lines S3(Ri)-42-10 and S3(Ri)-52-7 (Fig. 3A). Each of these lines contained only one copy of inserted DNA.

To test the cold tolerance of transgenic rice, seedlings were shifted from 28°C to 4°C. *MYBS3*(Ox) lines and wild-type plants remained normal, while *MYBS3*(Ri) lines started to show leaf rolling at 4°C after 8 h (Fig. 3B) and both wild-type and *MYBS3*(Ri) lines showed leaf rolling and wilting at 4°C after 24 h in hydroponic culture (Fig. 3C; Supplemental Fig. S2) or 1 week in soil (Fig. 4). Seedlings seemed to be more cold sensitive in hydroponic culture, probably due to weaker growth in hydroponic culture than in soil. Line S3(Ox)-110-1, which accumulated three times more *MYBS3* mRNA than S3(Ox)-112-7 (Fig. 3A), conferred higher cold tolerance than line S3(Ox)-112-7 (Fig. 4C). Quantitative analysis also indicated that *MYBS3*(Ox) lines were more cold tolerant than wild-type plants and *MYBS3*(Ri) lines, and wild-type plants were more cold tolerant than *MYBS3*(Ri) lines (Table I). These observations suggest that *MYBS3* is sufficient and necessary for cold tolerance in rice, and the degree of cold tolerance correlates with the *MYBS3* expression level.

The morphology of transgenic rice was similar to the wild-type plants, except under greenhouse growth conditions, where plants of the *MYBS3*(Ox) lines were 20% shorter, had 30% lower tiller numbers, and headed 1 week later than the wild-type and *MYBS3*(Ri) lines (Supplemental Fig. S3). However, in field conditions, most agronomic traits and yield of *MYBS3*(Ox) lines were similar to those of the wild type (Table II).

MYBS3 Regulates the Expression of Genes with Diverse Functions

To identify downstream genes regulated by *MYBS3* under cold stress, seedlings of S3(Ox)-110-1, S3(Ri)-52-7, and the wild type were grown at 4°C and 28°C for 24 h. Total RNAs were isolated for microarray analysis using the Affymetrix Rice GeneChip array containing 55,515 probe sets. Relative change was calculated by comparing the data for a *MYBS3*(Ox) line or a *MYBS3*(Ri) line against those for wild-type plants grown at 4°C and 28°C, generating six comparisons. Only relative changes of 3-fold or more were taken to be
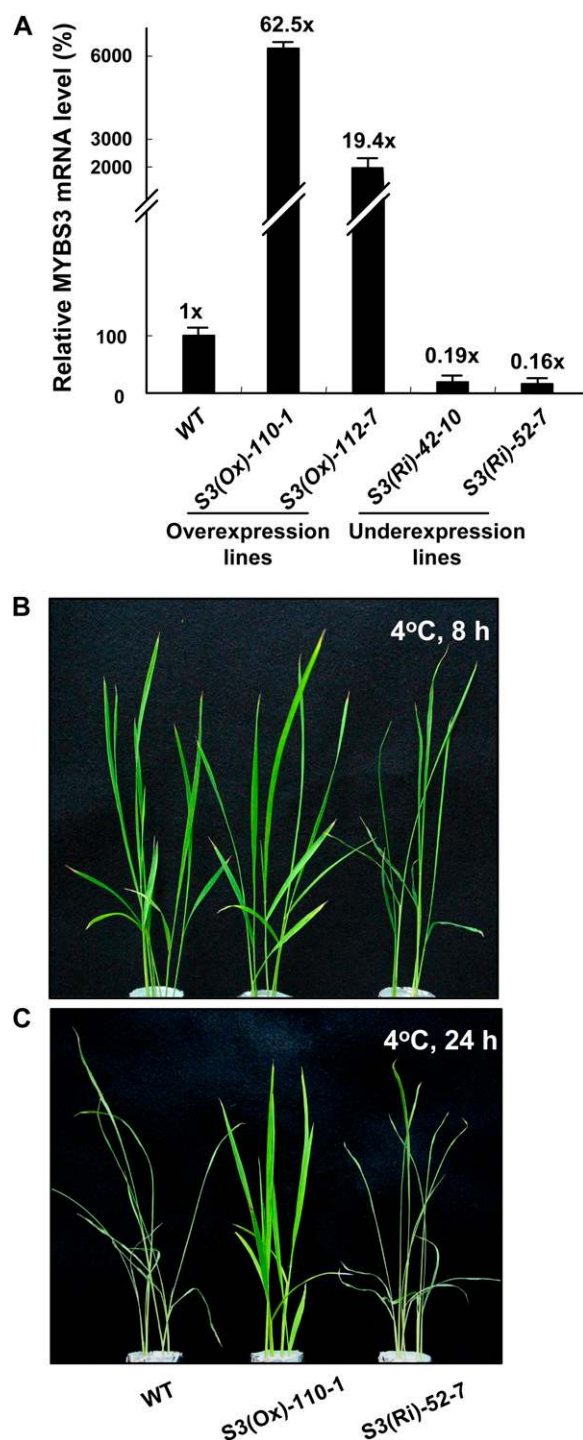
Figure 3. *MYBS3* is sufficient and necessary for cold tolerance in rice. A, RNAs isolated from leaves of 10-d-old rice seedlings of *MYBS3* overexpression [S3(Ox)] lines and MYBS3 underexpression [S3(Ri)] lines were subjected to quantitative real-time RT-PCR analysis. The mRNA level of the wild type (WT) was assigned a value of 100, and mRNA levels of other samples were calculated relative to this value. Values followed by x indicate relative induction. The error bars indicate the SE of three replicates. B and C, Ten-day-old seedlings of the wild type and lines S3(Ox)-110-1 and S3(Ri)-52-7 were incubated at 4°C for 8 h (B) or 24 h (C).

significantly different. Based on a Venn diagram analysis, 89 genes were up-regulated in the *MYBS3*(Ox) line (compared with the wild type) at either 4°C or 28°C, and 1,466 genes were up-regulated in the wild type at 4°C (compared with 28°C; Supplemental Fig. S4A, left). Among these genes, 17 genes were up-regulated by overexpression of *MYBS3* as well as up-regulated by cold in the wild type (Supplemental Table S1). On the other hand, 291 genes were down-regulated in the *MYBS3*(Ox) line (compared with the wild type) at either 4°C or 28°C, and 871 genes were down-regulated in the wild type at 4°C (compared with 28°C; Supplemental Fig. S4A, right). Among these genes, 53 genes were down-regulated by overexpression of *MYBS3* as well as down-regulated by cold in the wild type (Supplemental Table S1).

Another analysis revealed that 389 genes were up-regulated in the *MYBS3*(Ri) line (compared with the wild type) at either 4°C or 28°C (Supplemental Fig. S4B, left). Among these genes, 17 genes were up-regulated by underexpression of *MYBS3* as well as up-regulated by cold in the wild type (Supplemental Table S2). On the other hand, 124 genes were down-regulated in the *MYBS3*(Ri) line (compared with the wild type) at either 4°C or 28°C (Supplemental Fig. S4B, right). Among these genes, 37 genes were down-regulated by overexpression of *MYBS3* as well as by cold in the wild type (Supplemental Table S2).

The cold- and *MYBS3*-regulated genes seem to be involved in diverse functions, and many of them have also been shown to be regulated by drought and salt stresses (Supplemental Tables S1 and S2). Among the 17 genes up-regulated by overexpression of *MYBS3* as well as up-regulated by cold in the wild type, five genes that have also been shown to be up-regulated by drought (Supplemental Table S1) and cold (Jain et al., 2007), such as genes encoding Glu decarboxylase, WRKY77, multidrug resistance protein 4, and trehalose-6-phosphate phosphatase (TPP1 and TPP2), were selected for further quantitative real-time reverse transcription (RT)-PCR analysis. The accumulation of mRNA of all five genes was significantly increased in the wild type and further increased in the *MYBS3*(Ox) line but reduced in the *MYBS3*(Ri) line at 4°C (Fig. 5; Supplemental Table S3), indicating that these genes are downstream of the *MYBS3*-mediated cold signaling pathway.

MYBS3 Suppresses the *DREB1*-Dependent Pathway under Prolonged Cold Stress

We noticed that in the microarray analysis, the *DREB1* family, including *DREB1A*, *DREB1B*, and *DREB1C*, and another two *DREB1*-like genes (ERF#025 and ERF#104) were up-regulated in the wild type at 4°C, but the induction was surprisingly reduced or abolished in the *MYBS3*(Ox) line at 4°C (Supplemental Fig. S5). To investigate how MYBS3 regulates *DREB1* gene expression, the accumulation of mRNAs of three *DREB1* genes was further analyzed with the

Figure 4. Overexpression of MYBS3 confers cold tolerance in rice for up to 1 week. Seedlings of *MYBS3*(Ox) and *MYBS3*(Ri) lines were grown in soil for 10 d. A, Seedlings kept at 28°C for 1 week. B, Seedlings shifted to 4°C for 1 week. C, Enlarged images from B.
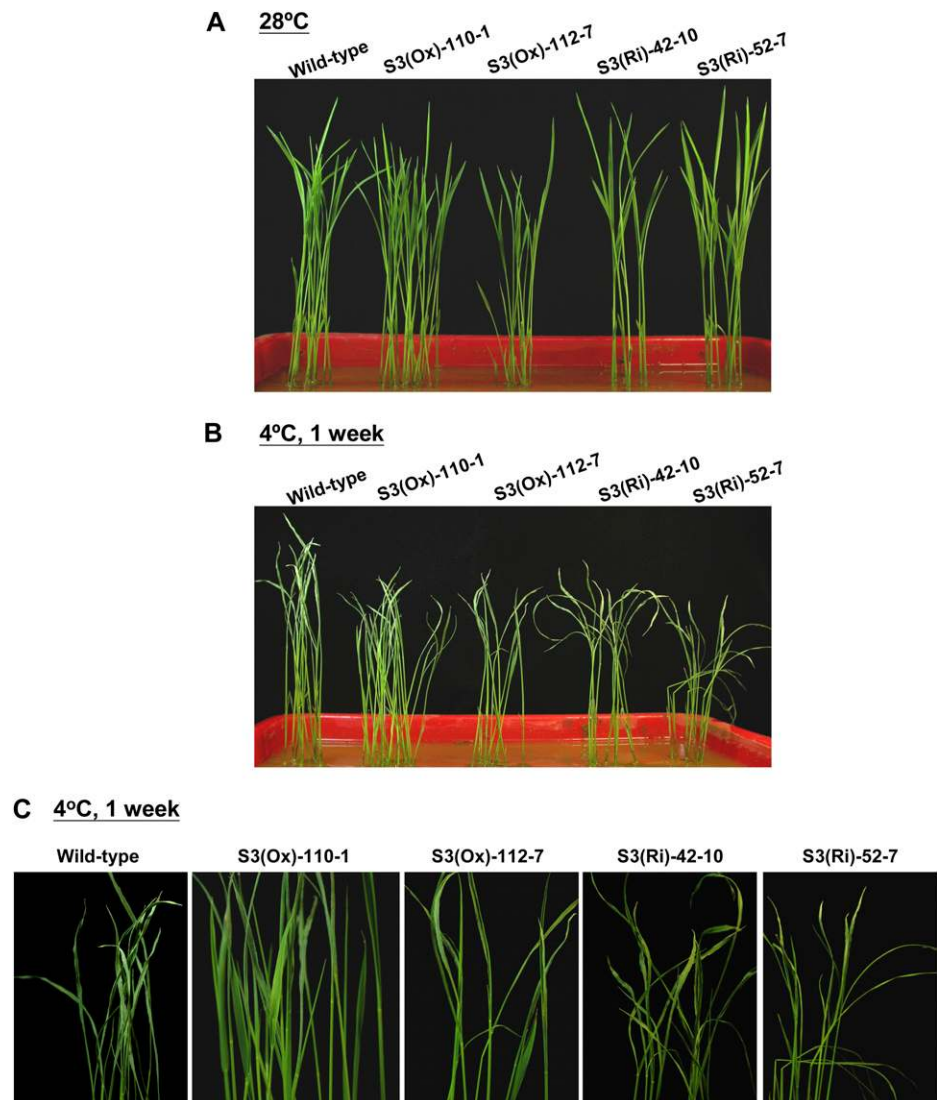
quantitative real-time RT-PCR analysis. As shown in Figure 6, compared with the wild type, accumulation of *MYBS3* mRNA increased significantly at 28°C and was further induced 2-fold at 4°C in the *MYBS3*(Ox) line. The accumulation of *MYBS3* mRNA was reduced in the *MYBS3*(Ri) line at both 4°C and 28°C. In contrast, the cold-induced *DREB1A*, *DREB1B*, and *DREB1C* expression was significantly suppressed in the *MYBS3*(Ox) line at 4°C. Furthermore, the cold inducibility of *αAmy3/RAmy3D* and a cytochrome P450 gene, both members of the cold-inducible *DREB1A* regulon (Ito et al., 2006), was also significantly reduced in the *MYBS3*(Ox) line at 4°C. The accumulation of *DREB1*, *αAmy3/RAmy3D*, and cytochrome P450 mRNAs was significantly higher in the *MYBS3*(Ri) line than in the *MYBS3*(Ox) line at 4°C, although the levels did not reach that in the wild type at 4°C.

Our previous study has shown that MYBS3 represses *αAmy3* SRC through the TA box (Lu et al., 2002). Examination of promoter regions within 1 kb

upstream of the translation start codon (ATG) revealed the presence of the TA box and/or its variants in *DREB1* genes (Fig. 7). To determine whether MYBS3 represses *DREB1* promoters, a rice embryo transient expression assay was performed. Rice embryos were cotransfected with the effector construct containing

Table I. *MYBS3(Ox) lines are more cold tolerant*

Number of plants survived is after exposure to 4°C for 24 h. Experiments were repeated four times. Five to eight plants per line were tested in each experiment.

Line	No. of Plants Survived	Total No. of Plants Tested	Survival Rate
			%
Wild type	3	30	10.0 ± 0.0
S3(Ox)-110–1	18	21	85.7 ± 10.5
S3(Ox)-112-7	26	32	81.3 ± 11.1
S3(Ri)-42-10	0	20	0.0 ± 0.0
S3(Ri)-52-7	0	21	0.0 ± 0.0

the *Ubi* promoter fused to *MYBS3* cDNA and the reporter construct containing *DREB1A* (1,054 bp), *DREB1B* (747 bp), or *αAmy3* SRC (105 bp) promoter sequence fused to luciferase (*Luc*) cDNA. Both *DREB1* promoters were significantly induced at 4°C, but only the *DREB1B* promoter was repressed by overexpression of *MYBS3* at 4°C (Fig. 8). The *αAmy3* SRC was repressed by overexpression of *MYBS3* at both 4°C and 28°C, consistent with the role of MYBS3 as a repressor of *αAmy3* SRC (Lu et al., 2002). These results indicate that MYBS3 could repress *DREB1B* promoter and *αAmy3* SRC at 4°C.

DISCUSSION

A Novel MYBS3-Mediated Cold Signaling Pathway

In this study, both gain- and loss-of-function analyses demonstrated that the MYBS3-mediated pathway is essential for cold stress tolerance in rice. We showed that *DREB1A* responds early and transiently, which is consistent with previous reports in Arabidopsis and rice (Liu et al., 1998; Shinwari et al., 1998; Dubouzet et al., 2003; Vogel et al., 2005), whereas *MYBS3* responds relatively slowly, to cold stress in rice (Fig. 9). The DREB1-mediated process is most likely crucial in responding to short-term cold stress (cold shock), and the MYBS3-mediated system is more important for long-term adaptation to persistent cold stress.

Transcriptome profiling analyses suggest that multiple cold response pathways exist in Arabidopsis and rice (Fowler and Thomashow, 2002; Vogel et al., 2005; Cheng et al., 2007; Chinnusamy et al., 2007). However, the MYBS3-mediated cold signaling pathway has never been observed previously. MYBS3 acts as a transcriptional repressor of *αAmy3* SRC in the sugar signaling pathway in rice (Lu et al., 2002) and is constitutively localized in the nucleus in cultured rice suspension cells (Fig. 2B). These studies indicate that MYBS3 may play multiple regulatory roles in plant growth in addition to cold response in rice. Consequently, gene expression in the *MYBS3*(Ox) or *MYBS3*

Table II. *Comparison of agronomic traits of a MYBS3(Ox) line with the wild type grown in the field*

Twenty plants each of the wild type and line S3(Ox)-110-1 per replicate, with a total of three replicates, were grown during February to July 2008.

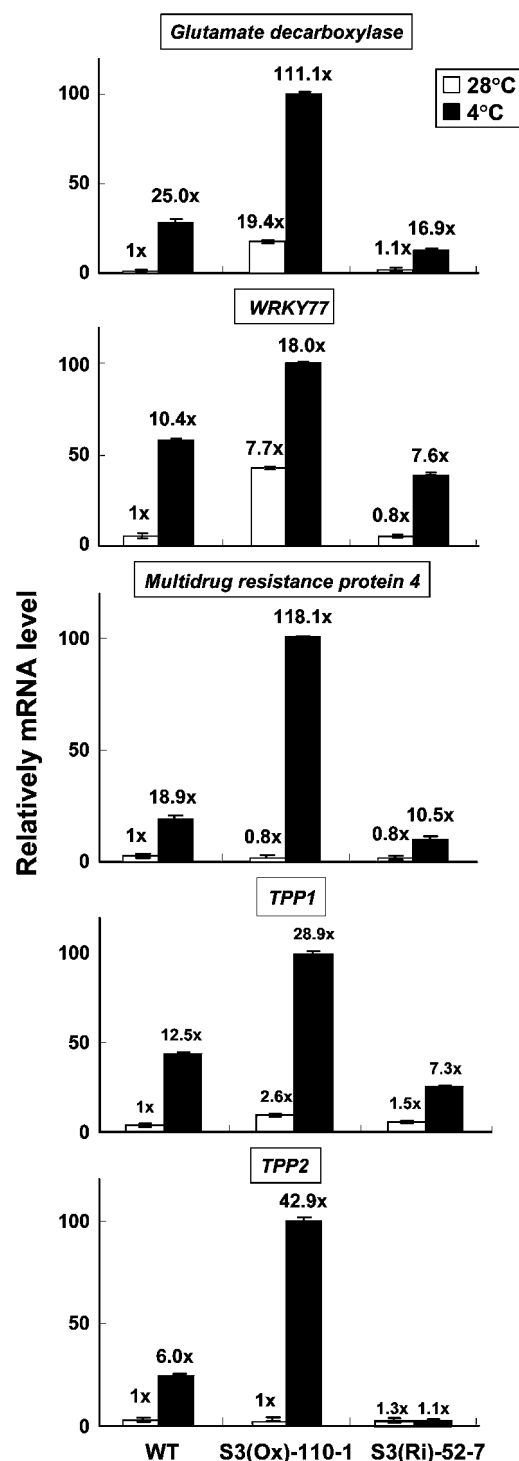| Trait	Wild Type	S3(Ox)-110-1
Plant height (cm)	101.6 ± 3.7	95.5 ± 5.4
Tiller number	12.3 ± 2.5	19.5 ± 6.8
Panicle number per plant	12.9 ± 2.9	19.0 ± 6.1
Panicle length (cm)	18.9 ± 1.3	18.4 ± 0.1
Grain number per panicle	118.0 ± 14.5	103.5 ± 8.8
Fertility (%)	95.7 ± 1.2	93.8 ± 1.7
Grain yield (g per plant)	41.7 ± 11.0	45.4 ± 15.9
One thousand grain weight (g)	26.3 ± 0.5	24.3 ± 0.4

Figure 5. Cold-activated genes that are activated in the *MYBS3*(Ox) line and repressed in the *MYBS3*(Ri) line. Ten-day-old seedlings of the wild type (WT) and lines S3(Ox)-110-1 and S3(Ri)-52-7 were incubated at 4°C for 24 h. Total mRNAs were isolated, and the accumulation of mRNA of the indicated genes was analyzed by quantitative real-time RT-PCR analysis. The mRNA level in the wild type at 28°C was assigned a value of 1, and mRNA levels of other samples were calculated relative to this value. Values followed by x indicate relative induction. Error bars indicate the SE of three replicates. Accession numbers of the indicated genes are provided in "Materials and Methods."
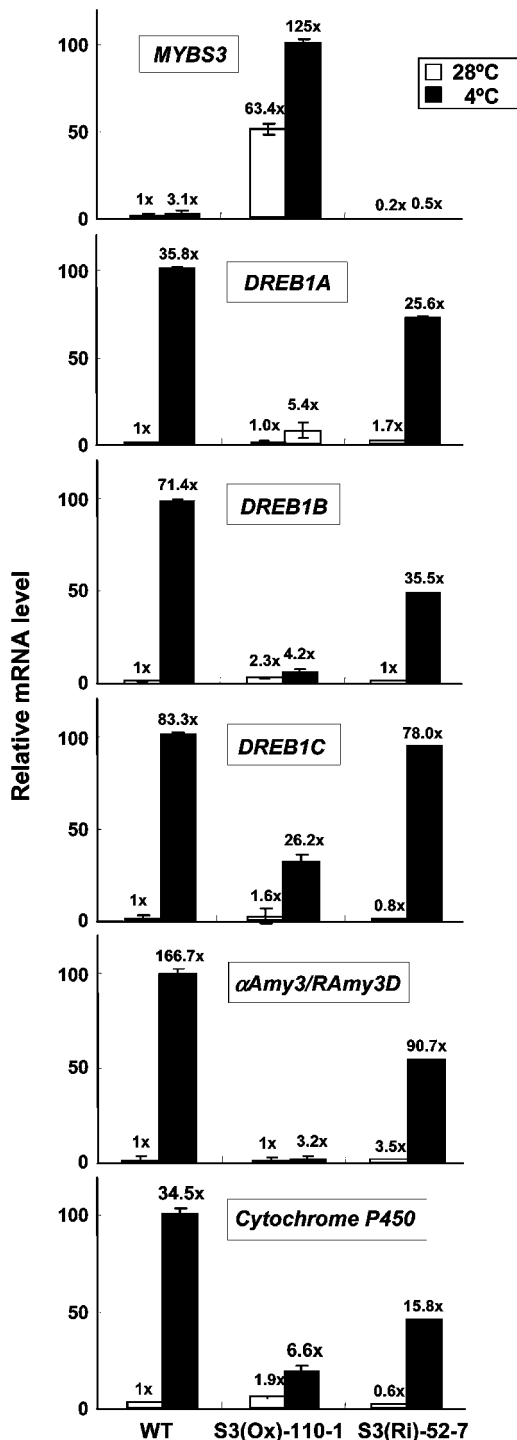
Figure 6. *MYBS3* suppresses the expression of the *DREB1* family and their downstream genes under cold stress. Ten-day-old seedlings of the wild type (WT) and lines S3(Ox)-110-1 and S3(Ri)-52-7 were incubated at 4°C for 24 h. Total mRNAs were isolated, and the accumulation of mRNA of the indicated genes was determined by quantitative real-time RT-PCR analysis. The mRNA level in the wild type at 28°C was assigned a value of 1, and mRNA levels of other samples were calculated relative to this value. Values followed by x indicate relative induction. Error bars indicate the sε of three replicates. Accession numbers of the indicated genes are provided in "Materials and Methods."

(Ri) line altered at 28°C may not, whereas that altered at 4°C may, be involved in cold response.

The MYBS3-regulated genes encompass a wide range of functions. In the microarray analysis, among the 17 genes up-regulated by at least 3-fold by over-expression of *MYBS3* as well as by cold in the wild type (Supplemental Fig. S4A), several of them have previously been implicated in stress responses and/or tolerance in plants (Supplemental Table S1), such as Glu decarboxylase, which catalyzes the conversion of Glu to γ-aminobutyrate and is activated in response to heat in Arabidopsis roots (Bouche et al., 2004) and to anoxia in rice roots (Aurisano et al., 1995); WRKY77, which activates the ABA-inducible *HVA22* promoter in cereal grains (Xie et al., 2005), and several WRKYs have been shown to confer biotic and abiotic stress tolerance in Arabidopsis (Ross et al., 2007; Lai et al., 2008; Zhou et al., 2008); and multidrug resistance protein 4, whose expression is activated by arsenate and arsenite stresses in rice seedlings (Chakrabarty et al., 2009) and whose homologous genes confer salt tolerance (Lee et al., 2004) and oxidative stress tolerance against pathogens (Sun et al., 2006) in various plant species.

*TPP*s are a group of genes worth noting. Trehalose is a disaccharide sugar widely distributed in bacteria, fungi, plants, and invertebrate animals; it is produced from Glc by trehalose-6-phosphate synthase (TPS) and TPP and serves as sugar storage, metabolic regulator, and protectant against abiotic stresses (Strom and Kaasen, 1993; Elbein et al., 2003). Trehalose has been shown to stabilize dehydrated enzymes, proteins, and lipid membranes as well as to protect biological structures from damage during desiccation (Elbein et al., 2003). *TPP1* and *TPP2* are two major TPP genes expressed in rice seedlings (Shima et al., 2007). Their expression is induced by cold and other abiotic stresses (Pramanik and Imai, 2005; Shima et al., 2007; Ge et al., 2008). Trehalose accumulates rapidly and transiently, which follows the transient induction of TPP activity, in rice tissues during chilling stress (Pramanik and Imai, 2005). Overexpression of *TPS* and *TPP* enhances the accumulation of trehalose and tolerance to cold stress in transgenic tobacco and rice (Garg et al., 2002; Jang et al., 2003; Ge et al., 2008; Iordachescu and Imai, 2008). However, the regulatory mechanism of *TPP*s by cold or other stresses is unclear.

The accumulation of these MYBS3-activated genes was significantly increased in the *MYBS3*(Ox) line and decreased in the *MYBS3*(Ri) line at 4°C (Fig. 5). This study suggests that MYBS3 may confer stress tolerance to transgenic rice through the activation of these genes whose products are involved either in the regulation of gene expression for cold adaptation or for protection of cells from chilling injury.

Complexity in Cold Regulation

The temporal expression patterns and magnitudes of activation of *DREB1A* and *MYBS3* expression by
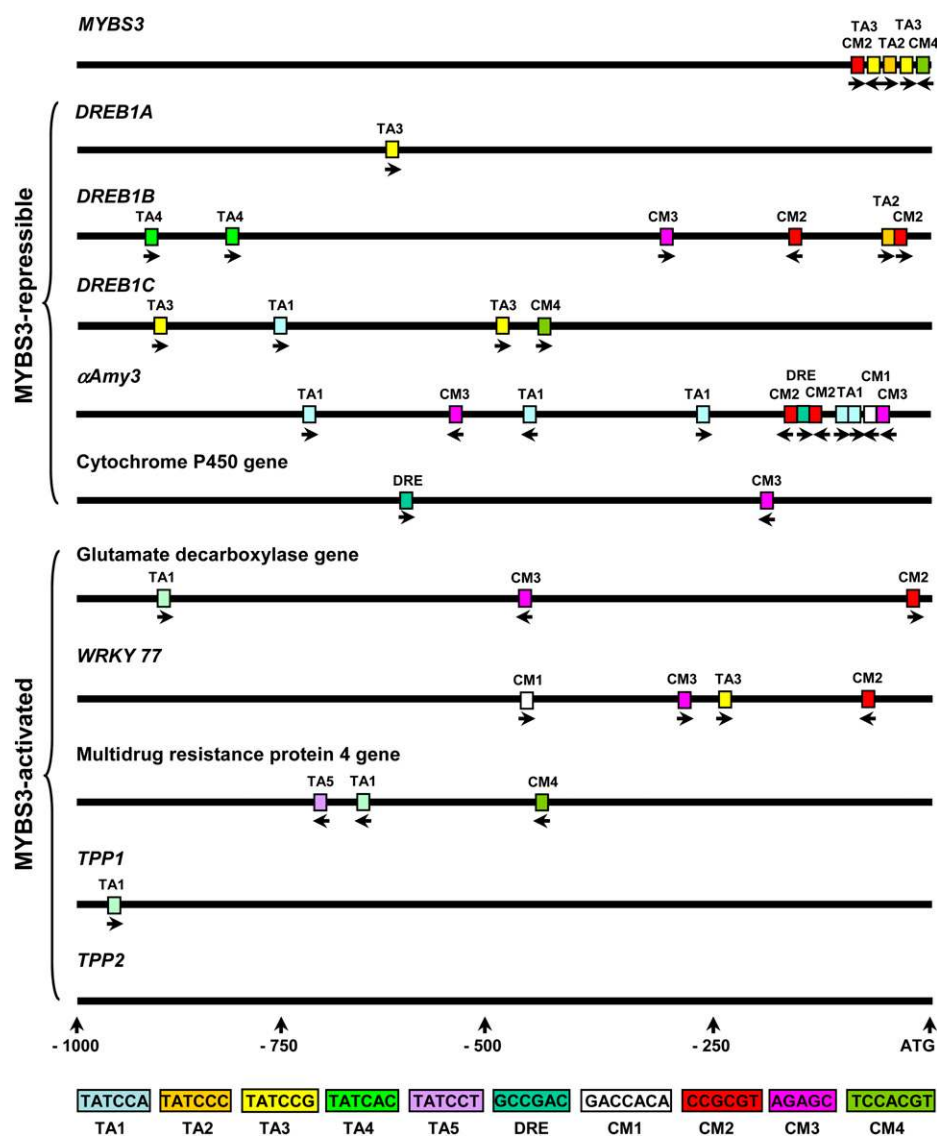
Figure 7. Putative cis-acting elements present in promoters of cold-inducible and MYBS3-activated or -repressible genes. Positions of the TA box, DRE, and CMs within 1 kb upstream of the translation start codon ATG are indicated by color-coded boxes, and their nucleotide sequences are shown at the bottom of the graph. Horizontal arrows indicate the orientation of the TA box, DRE, and CMs in the promoter region. Vertical arrows indicate the position upstream of ATG.

cold are quite different (Figs. 1 and 8). Several factors have been found to regulate the expression of *DREB1/CBF* as mentioned in the introduction, but detailed information about the cold signaling pathways upstream of *DREB1/CBF* is rather limited. Recently, a calmodulin-binding transcription factor was found to bind to the conserved motif 2 (CM2) present in promoters (within 200 bp upstream of ATG), and function as a positive regulator, of the rapidly cold-inducible *CBF2* and *ZAT12* transcription factors in Arabidopsis (Doherty et al., 2009). CM2 is present in one copy in the *MYBS3* promoter (-117 to -112 upstream of ATG; Fig. 7; Supplemental Table S4). For cold up-regulated but *MYBS3* down-regulated genes, CM2 is present in two copies each in *DREB1B* (-134 to -129 and -80 to -75) and *αAmy3* (-158 to -153 and -149 to -144) promoters; for cold up-regulated and *MYBS3* up-regulated genes, CM2 is present in the Glu decarboxylase (-54 to -49) and *WRKY77* (-96 to -91) promoters (Fig. 7;

Supplemental Table S4). Some other CMs shared by the Arabidopsis *CBF2* and *ZAT12* promoters (Doherty et al., 2009) could also be found in *DREB1B*, *DREB1C*, *αAmy3*, and cytochrome P450 promoters (Fig. 7; Supplemental Table S4), but the functions of these cis-acting elements and the identifies of their interacting transcription factors in cold signaling have not been determined (Doherty et al., 2009).

CM1 to CM7 have been found in the Arabidopsis *CBF2* promoter (within 200 bp upstream of ATG; Doherty et al., 2009); however, only CM4 is present in the 1-kb promoter region of *DREB1C* (the rice *CBF2* homolog; Fig. 7), suggesting that the mechanism of cold regulation in the *DREB1/CBF* family might have diverged throughout evolution. No CM is present in the 1-kb promoter region of *DREB1A*, indicating that unidentified cis-acting element(s) could be responsible for cold induction of *DREB1A*. It appears that combinations of various cis-acting elements and interacting
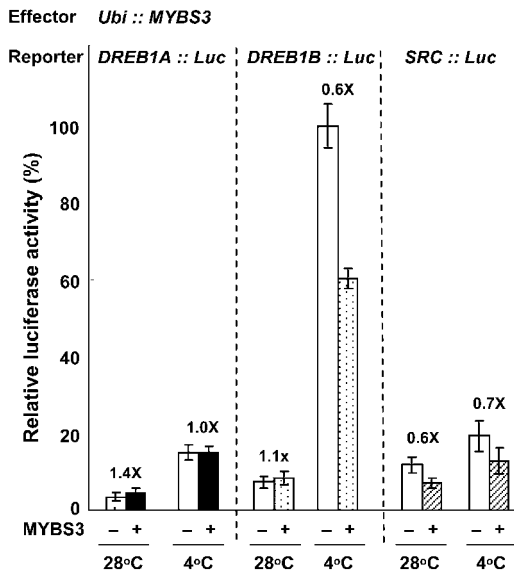
Figure 8. The *DREB1B* promoter and *αAmy3* SRC are repressed by MYBS3. The rice embryo transient expression assay was performed as described (Lu et al., 2007). Construct *Ubi::MYBS3* served as an effector, and *DREB1A::Luc*, *DREB1B::Luc*, and *αAmy3* SRC::*Luc* served as reporters. Effector and reporter constructs were cotransfected into rice embryos and incubated at 28°C or 4°C for 24 h, and the *Luc* activity was determined. Values followed by x indicate fold induction or repression of *Luc* by MYBS3 overexpression. The error bars indicate the SE of three replicates.

transcription factors constitute the quantitative and temporal regulation of the DREB1- and MYBS3-dependent cold signaling cascades.

It is also noticed that the DREB1A target sequence DRE (Ito et al., 2006) is present in *αAmy3* (−153 to −148) and cytochrome P450 (−605 to −600) promoters, and interestingly, it overlaps with the two CM2s in the *αAmy3* promoter (Fig. 7). None of the 1-kb promoter regions of *MYBS3*-activated genes further characterized in this study contains DRE (Fig. 7).

How MYBS3 represses the expression of the *DREB1* regulon is unclear. The TA box has been shown to function in both sense and antisense orientations (Lu et al., 1998). Promoters of the cold-inducible but *MYBS3*-repressible genes shown in Figure 6, except cytochrome P450, contain the TA box or its variants (Yu, 1999; Wang et al., 2007) in the sense or antisense orientation (Fig. 7; Supplemental Table S5), which could be the target of repression by MYBS3. However, in the transient expression assay, the 747-bp *DREB1B* promoter and 105-bp *αAmy3* SRC, but not the 1,054-bp *DREB1A* promoter, were repressed by overexpression of *MYBS3* at 4°C (Fig. 8). One explanation is that the TA3 box (−625 to −620) in the 1,054-bp *DREB1A* promoter did not function as well as the TA2 box (−85 to −80) in the 747-bp *DREB1B* promoter and the TA1 box (the canonical TA box; two copies between −116 to −105) in the 105-bp *αAmy3* SRC in the rice embryo transient expression assay.

How MYBS3 activates the expression of downstream genes in the cold signaling pathway (Fig. 5), by serving as a transcriptional activator or repressing a transcriptional repressor, is unclear. However, except for *TPP2*, other MYBS3 up-regulated genes also contain the TA box or its variants (Fig. 7; Supplemental Table S5). Both MYBS1 and MYBS3 bind specifically to the TA box; however, MYBS1 activates and MYBS3 represses *αAmy3* SRC under sugar starvation (Lu et al., 2002). MYBs with a single DNA-binding domain (1R MYB) have been proposed to bind DNA as a dimer, and MYBS1 does whereas MYBS3 does not form a homodimer (Lu et al., 2002). Whether MYBS3 could be converted into an activator, by interacting with another 1R MYB and forming a heterodimer or with other transcription factor(s), remains for further study.

Taken together, the above studies suggest the complexity of cold regulation in plants, which involves multiple cis-acting elements and transcription factors. Additionally, the regulation of the MYBS3-dependent pathway differs from that of the DREB1- or reactive oxygen species-mediated pathway in response to cold stress (Ito et al., 2006; Cheng et al., 2007), which suggests that MYBS3 defines a new signaling pathway mediating cold adaptation in rice. It appears that distinct regulatory pathways function in fine-tuning the qualitative and quantitative gene expression for short- and long-term cold adaptation in rice (Fig. 9).

MYBS3 as a Tool for Improving Cold Stress Tolerance in Crops

Compared with microbial TPP, the rice TPP has been shown to be rather unstable, which leads to low-level
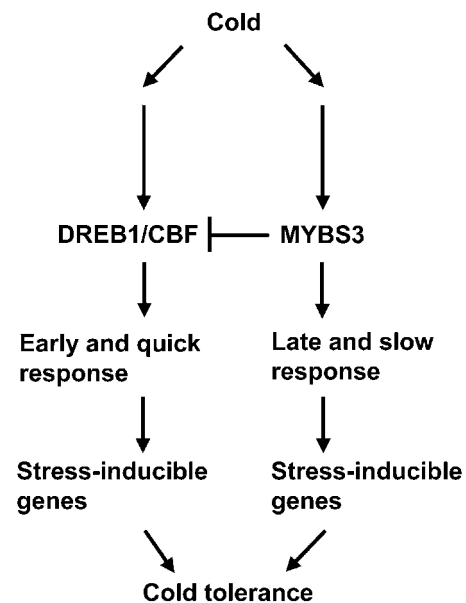

Figure 9. Proposed role of *MYBS3* in cold stress tolerance in rice. Details of the model are described in the text.

accumulation of trehalose in rice under normal growth conditions (Shima et al., 2007). Although in wild-type plants the expression of two rice *TPP*s is induced by cold, it peaks around 24 h and declines afterward at 4°C to 6°C (Pramanik and Imai, 2005; Ge et al., 2008). The significant activation of *TPP* expression in the *MYBS3*(Ox) line (Fig. 5) may increase the accumulation of trehalose to levels high enough to confer cold tolerance in rice.

In the *MYBS3*(Ri) line, the expression of three *DREB1* genes was 50% to 94% that of the wild type at 4°C (Fig. 6), probably due to weaker growth and reduced cellular activities of plants under cold stress, as mentioned above that MYBS3 may play multiple regulatory roles in plant growth in addition to cold response in rice. However, it suggests that high-level *DREB1* expression is insufficient to sustain cold tolerance if the level of *MYBS3* expression is too low to efficiently activate the TPP-mediated cold response pathway. Consequently, the sequential expression of *DREB1* and *MYBS3* provides two complementary mechanisms for conferring cold tolerance in rice, with the DREB1-mediated process mediating the immediate cold shock response and the MYBS3-mediated system adjusting the long-term cold adaptation in rice. The antithetical regulation of *αAmy3* in rice seedlings by two different pathways is physiologically meaningful: the transient activation of *αAmy3* expression by *DREB1* allows hydrolysis of reserved starch to answer the immediate need for a carbon source and energy to combat the cold shock, while the subsequent suppression of *αAmy3* expression by *MYBS3* allows rice to conserve carbohydrates until regrowth is allowed at elevated temperatures. It would be interesting to test whether stacking of these two systems, by overexpression of both *DREB1* and *MYBS3*, could further enhance the cold tolerance in rice.

Overexpression of proteins or enzymes associated with stress responses has been a common practice in improving stress tolerance of crop plants. However, constitutive overexpression of these proteins frequently leads to impaired plant growth or yield penalty. For example, although transgenic Arabidopsis and rice constitutively overexpressing CBF/DREB1 and a NAC6 transcription factor are highly tolerant to freezing, the growth rates of these transgenic plants are severely retarded under normal growth conditions (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000; Ito et al., 2006; Nakashima et al., 2007). Using stress-inducible promoters for the expression of these transcription factors minimizes their negative effects on plant growth (Kasuga et al., 1999; Nakashima et al., 2007). In our study, we showed that transgenic seedlings were able to withstand 4°C for at least 1 week after shifting from 28°C (Fig. 4), which could significantly protect seedlings from chilling injury in rice fields in areas that are easily prone to transient temperature drops in early spring. Although the growth of *MYBS3*(Ox) lines was affected to a certain extent in the greenhouse (Supplemental Fig.

S3), the growth and yield of line S3(Ox)-110-1 was normal in the field (Table II). In conclusion, this study not only leads to a better understanding of the mechanism of cold regulation in rice but also presents MYBS3 as an ideal alternative for the improvement of cold tolerance in rice and possibly other crop plants.

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa* 'Tainung 67') was used in this study. Induction of rice calli was performed as described (Yu et al., 1991). For hydroponic culture of rice seedlings, seeds were sterilized with 3% NaOCl for 30 min, washed extensively with distilled water, and germinated in petri dishes with wetted filter papers at 37°C in the dark. After 48 h of incubation, germinated seeds were cultivated in a half-strength Kimura B solution containing the following macronutrients (in mM): $(NH_4)_2SO_4$ (0.18), KNO_3 (0.09), $MgSO_4$ (0.27), KH_2PO_4 (0.09), and $Ca(NO_3)_2$ (0.18) and micronutrients (in μM): iron citrate (0.03), H_3BO_3 (2.5), $MnSO_4 \cdot H_2O$ (0.2), $ZnSO_4 \cdot 7H_2O$ (0.2), $CuSO_4 \cdot 5H_2O$ (0.05), and H_2MoO_4 (0.05). The pH of the solution was adjusted to 4.7 to 4.8 using 0.5 N HCl. The culture solution was replaced with fresh solution every 2 d. Seedlings were grown under a 14-h-light/10-h-dark cycle for 10 d in a 28°C chamber before treatments.

Primers

All primers used for the cloning of cDNAs or plasmid constructions and quantitative RT-PCR are listed in Supplemental Table S6.

Plasmid Construction

The Gateway gene cloning system (Invitrogen) was used to construct the *MYBS3-GFP* fusion gene. Briefly, the full-length cDNA of *MYBS3* was inserted between the *attL1* and *attL2* sites in pENTR/D-TOPO, generating the entry vector pENT-MYBS3. The *CaMV35S* promoter upstream of *GFP* in pCAMBIA1302 (http://www.cambia.org.au/daisy/cambia/585.html) was replaced with the maize (*Zea mays*) *Ubi* promoter, and the *ccdB* DNA fragment flanked by *attR1* and *attR2* sites was inserted between the *Ubi* promoter and *GFP*, generating the destination vector pDEST-GFP. *MYBS3* in pENT-MYBS3 was then inserted upstream of *GFP* in pDEST-GFP through the Gateway λ recombination system, generating p1302-MYBS3-GFP. The 2.5-kb *MYBS3* promoter fragment (upstream of ATG) was PCR synthesized and used for replacement of the *Ubi* promoter in pDEST-GFP, generating the *Ubi::MYBS3-GFP* construct.

For generating constructs used for the embryo transient expression assay, the 1,054-bp *DREB1A* and 747-bp *DREB1B* promoters (upstream of ATG) were PCR synthesized and fused upstream of *Luc* cDNA in pLuc (Lu et al., 1998). Plasmid p3Luc.18 contains *αAmy3* SRC (−186 to −82 upstream of the transcription start site) fused to the *CaMV35S* minimal promoter-*Adh* intron-*Luc* fusion gene (Lu et al., 1998).

For generating constructs used for rice transformation, plasmid pBS-MYBS3 (Lu et al., 2002) containing the *Ubi* promoter fused upstream of *MYBS3* cDNA was linearized with *Eco*RI and inserted into the binary vector pSMY1H (Ho et al., 2000), generating pSMY-MYBS3 (Supplemental Fig. S1). To make the *MYBS3* RNAi construct, a 227-bp sequence derived from the 3′ untranslated region of *MYBS3* cDNA was synthesized by PCR and fused in antisense and sense orientations flanking the 750-bp GFP cDNA. This *MYBS3* RNAi fragment was used to replace the MYBS3 cDNA in pUbi-MYBS3, generating pUbi-MYBS3(Ri). pUbi-MYBS3(Ri) was linearized with *Eco*RI and inserted into the binary vector pSMY1H, generating pSMY-MYBS3(Ri) (Supplemental Fig. S1).

Rice Transformation

Plasmids p1302-MYBS3-GFP, pSMY-MYBS3, and pSMY-MYBS3(Ri) as constructed above were introduced into *Agrobacterium tumefaciens* strain EHA101, and rice transformation was performed as described elsewhere (Ho et al., 2000).

RNA Extraction and Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from leaves of rice seedlings with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). Four micrograms of RNA was used for cDNA preparation with reverse transcriptase (Applied Biosystems), and cDNA was then diluted to 10 ng μL^{-1}. Five microliters of cDNA was mixed with primers and the 2× Power SYBR Green PCR Master Mix reagent and applied to an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The quantitative variation between different samples was evaluated by the delta-delta threshold cycle method, and the amplification of 18S ribosomal RNA was used as an internal control to normalize all data.

Subcellular Localization of the MYBS3-GFP Fusion Protein

Protoplasts were isolated from transformed calli as described (Lu et al., 1998). GFP expression was detected with a LSM510 confocal laser scanning microscope (Carl Zeiss) using a 40× objective lens and the confocal microscopy software release 2.8 (Carl Zeiss).

Stress Treatments

Ten-day-old seedlings cultured in the half-strength Kimura B solution at 28°C and with a 16-h-light/8-h-dark cycle in a growth chamber were used for all stress treatments. Stress treatments were as follows: ABA, seedlings were transferred to a culture solution containing 20 μM ABA; drought, seedlings were air dried until 10% or 30% of fresh weight was lost; cold, seedlings were transferred to 4°C; salt, seedlings were transferred to a culture solution containing 200 mM NaCl; heat, seedlings were transferred to 45°C.

Microarray Analysis

Total RNA was extracted from leaves of rice seedlings using the Qiagen RNeasy Plant Mini Kit (Qiagen) according to the Qiagen manual. RNA quality was examined by the Agilent 2100 bioanalyzer (Affymetrix), and biotinylated target RNA was prepared from total RNA. Samples were hybridized to the Affymetrix Rice GeneChip as described in the GeneChip Expression Analysis Technical Manual. Two biological replicates were performed for cold-treated samples per time point.

The hybridization signals were scanned with an Affymetrix GeneChip scanner 3000 7G, and the cell intensity (CEL) files were obtained from Affymetrix GCOS version 1.4 software. CEL files were loaded into GeneSpring GX 9.0 (Agilent Technologies). Filtering tools in the GeneSpring software were used to identify genes significantly up-regulated and down-regulated between different chips. All genes up-regulated or down-regulated by overexpression or underexpression of *MYBS3* or by cold are listed in Supplemental Tables S7 to S12.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *DREB1A* (Os09g35030); *DREB1B* (Os09g35010); *DREB1C* (Os06g03670); *αAmy3/RAmy3D* (Os08g36910); cytochrome P450 gene (Os02g47470); Glu decarboxylase gene (Os03g13300); *WRKY77* (Os01g40260); multidrug resistance protein 4 gene (Os01g50100); *TPP1* (Os02g44230); *TPP2* (Os10g40550).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Constructs for overexpression and underexpression of *MYBS3* in transgenic rice.

Supplemental Figure S2. MYBS3 is sufficient and necessary for cold tolerance in rice.

Supplemental Figure S3. Plant growth was slightly delayed and reduced in transgenic rice overexpressing *MYBS3* in the greenhouse.

Supplemental Figure S4. Comparison of genes regulated by MYBS3 and cold.

Supplemental Figure S5. MYBS3 suppresses the expression of the *DREB1* and *DREB1*-like family.

Supplemental Table S1. Genes regulated by overexpression of *MYBS3* in transgenic rice as well as by cold in the wild type in the microarray analysis.

Supplemental Table S2. Genes regulated by underexpression of *MYBS3* in transgenic rice as well as by cold in the wild type in the microarray analysis.

Supplemental Table S3. Genes that are up-regulated in *MYBS3*(Ox) and down-regulated in *MYBS3*(Ri) in the microarray analysis.

Supplemental Table S4. The positions of CM motifs in the promoter regions of cold-inducible and *MYBS3* down-regulated or *MYBS3* up-regulated genes.

Supplemental Table S5. The positions of the TA box or its variants in the promoter regions of *MYBS3* down-regulated or *MYBS3* up-regulated genes.

Supplemental Table S6. Primers used in this study.

Supplemental Table S7. Genes up-regulated by overexpression of *MYBS3*.

Supplemental Table S8. Genes down-regulated by overexpression of *MYBS3*.

Supplemental Table S9. Genes up-regulated by underexpression of *MYBS3*.

Supplemental Table S10. Genes down-regulated by underexpression of *MYBS3*.

Supplemental Table S11. Genes up-regulated by cold in the wild type.

Supplemental Table S12. Genes down-regulated by cold in the wild type.

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