A Novel MYBS3-dependent Pathway Confers Cold Tolerance in Rice

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ABSTRACT

Rice 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 (1R) MYB transcription factor previously shown to mediate sugar signaling in rice. In the present 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 over-expressing MYBS3 tolerated 4°C for at least 1 week, and exhibited no yield penalty in normal field conditions. Transcription profiling of transgenic rice over- or under-expressing MYBS3 led to 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 response and/or tolerance in rice and other plant species. Surprisingly, MYBS3 repressed the well-known DREB1/CFB-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 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 which controls cold adaptation in rice.
INTRODUCTION

Rice 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 can not be grown in approximately 7,000,000 hectares of land in south and south-east Asia due to cold stress (Sthapit and Witcombe, 1998); in temperate regions such as California (USA), 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, e.g., 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, e.g., 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 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/CFB family, including DREB1A/CFB3, DREB1B/CFB1, and DREB1C/CFB2, are able to bind to and activate the cis-acting elements DRE (dehydration-responsive element) (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997) or CRT (C-repeat) (Baker et al., 1994) on promoters of several cold-responsive genes (CORs) (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 over-expression 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/CFB cold-responsive pathway in monocots and dicots. However, in comparison to Arabidopsis and other cereals like wheat and barley that cold acclimate (Wen et al., 2002), rice does not undergo 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 (Mukhopadhyay et al., 2004); the rice MYB4 transcription factor confers chilling and freezing tolerances by enhancing the COR gene expression and proline accumulation in Arabidopsis (Vannini et al., 2004), and improves cold and drought tolerances by accumulating osmolyte in transgenic apples (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 subjected 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 Expression 1, ICE1 (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\(_2\)H\(_2\) 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 α-amylase gene (αAmy3) promoter (Lu et al., 2002). MYBS1 and MYBS2 transactivate, while MYBS3 represses, the sugar starvation-inducible αAmy3 SRC activity in rice (Lu et al., 2002). The rice MYBS3 homologue in Arabidopsis (AGI code: At5g47390) is activated by ABA, CdCl₂ 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 the present report, by both gain- and loss-of-function analysis, we show that MYBS3 is essential for cold stress tolerance in rice. Transcription profiling of transgenic rice over- or under-expressing MYBS3 led to 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 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 28°C, increased drastically after shifting to 4°C and peaked at 6 h, but 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. Ubi promoter fused to GFP was used as a control. Transgenic rice seedlings were grown at 4°C. Under the control of MYBS3 promoter, the accumulation of GFP mRNA was 2.5 times higher at 12 h and stayed high up to 24 h (Fig. 2A, upper panel). In contrast, under the control of 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, lower panel). This result indicates that the MYBS3 promoter is activated by cold.
Previous study has shown that MYBS3 is a transcriptional repressor of αAmy3 SRC in rice suspension cells (Lu et al., 2002). To determine whether MYBS3 is localized in 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.

**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) (RNA interference) (Fig. S1) were introduced into the rice genome, and several transgenic lines were obtained. Compared to the untransformed wild type (WT) 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 WT remained normal while MYBS3(Ri) lines started to show leaf rolling at 4°C after 8 h (Fig. 3B), and both WT and MYBS3(Ri) lines showed leaf rolling and wilting at 4°C after 24 h in hydroponic culture (Fig. 3C and 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 WT and $MYBS3$(Ri) lines, and WTs were more cold tolerant than $MYBS3$(Ri) lines (Table 1). 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 WT, 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 WT and $MYBS3$(Ri) lines (Fig. S3). However, in field conditions, most agronomic traits and yield of $MYBS3$(Ox) lines were similar to those of the WT (Table 2).

$MYBS3$ 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 WT were grown at 4 and 28 °C for 24 h. Total RNAs were isolated for microarray analysis using the Affymetrix rice gene chip array containing 55,515 probe sets. Relative change was calculated by comparing the data for $MYBS3$(Ox) line or $MYBS3$(Ri) line against those for WT grown at 4°C and 28°C, generating six comparisons. Only relative changes of 3-fold or more were taken to be significantly different. Based on a Venn diagram analysis, 89 genes were up-regulated in the $MYBS3$(Ox) line (compared with WT) at either 4 or 28 °C, and 1466 genes were up-regulated in WT at 4°C (compared with 28 °C) (Fig. S4A, left panel). Among these genes, 17 genes were up-regulated by over-expression of $MYBS3$ as well as up-regulated by cold in WT (Table S1). On the other
hand, 291 genes were down-regulated in the \textit{MYBS3}(Ox) line (compared with WT) at either 4 or 28 °C, and 871 genes were down-regulated in WT at 4°C (compared with 28 °C) (Fig. S4A, right panel). Among these genes, 53 genes were down-regulated by over-expression of \textit{MYBS3} as well as down-regulated by cold in WT (Table S1).

Another analysis revealed that 389 genes were up-regulated in the \textit{MYBS3}(Ri) line (compared with WT) at either 4 or 28°C (Fig. S4B, left panel). Among these genes, 17 genes were up-regulated by under-expression of \textit{MYBS3} as well as up-regulated by cold in WT (Table S2). On the other hand, 124 genes were down-regulated in the \textit{MYBS3}(Ri) line (compared with WT) at either 4 or 28°C (Fig. S4B, right panel). Among these genes, 37 genes were down-regulated by over-expression of \textit{MYBS3} as well as by cold in WT (Table S2).

The cold- and \textit{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 (Tables S1 and S2). Among the 17 genes up-regulated by over-expression of \textit{MYBS3} as well as up-regulated by cold in WT, five genes that have also been shown to be up-regulated by drought (Table S1) and cold (Jain et al., 2007), such as genes encoding glutamate decarboxylase, WRKY77, multidrug resistance protein 4, and trehalose-6-phosphate phosphatase (TPP1 and TPP2), were selected for further quantitative real-time RT-PCR analysis. The accumulation of mRNA of all five genes was significantly increased in WT and further increased in the \textit{MYBS3}(Ox) line but reduced in the \textit{MYBS3}(Ri) line at 4°C (Fig. 5 and Table S3), indicating that these genes are downstream of the \textit{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 WT at 4°C, but the induction was surprisingly reduced or abolished in the MYBS3(Ox) line at 4°C (Fig. S5). To investigate how MYBS3 regulates DREB1 gene expression, the accumulation of mRNAs of three DREB1 genes was further analyzed with the quantitative real-time RT-PCR analysis. As shown in Fig. 6, compared with WT, 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 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/Rαmy3D and a cytochrome P450 gene, both members of the cold-inducible DREB1A regulon (Ito et al., 2006), were also significantly reduced in the MYBS3(Ox) line at 4°C. The accumulation of DREB1, αAmy3/Rαmy3D and cytochrome P450 mRNAs were significantly higher in the MYBS3(Ri) line than in the MYBS3(Ox) line at 4°C, although levels did not reach to that in WT 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 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 Ubi promoter fused to MYBS3 cDNA and the reporter construct containing DREB1A (1054 bp), DREB1B (747 bp) or αAmy3 SRC (105 bp) promoter
sequence fused to luciferase cDNA (Luc). Both DREB1 promoters were significantly induced at 4°C, but only the DREB1B promoter was repressed by over-expression of MYBS3 at 4°C (Fig. 8). The αAmy3 SRC was repressed by over-expression of MYBS3 at both 4 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 the present 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 MYBS3(Ox) or MYBS3(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 for at least 3-fold by over-expression of MYBS3 as
well as by cold in WT (Fig. S4A), several of them have previously been implicated in stress responses and/or tolerance in plants (Table S1), such as glutamate decarboxylase, which catalyzes the conversion of glutamate to γ-aminobutyrate (GABA) 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); multidrug resistance protein 4, whose expression is activated by arsenate and arsenite stresses in rice seedlings (Chakrabarty et al., 2009) and its homologous genes confer salt tolerance (Lee et al., 2004) and oxidative stress tolerance against pathogens (Sun et al., 2006) in various plant species.

TPPs are a group of genes worthwhile noting. Trehalose is a disaccharide sugar widely distributed in bacteria, fungi, plants and invertebrate animals, and is produced from glucose 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). Over-expression of **TPS** and **TPP** enhances 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 TPPs by cold or other stresses is unclear.
The accumulation of these MYBS3-activated genes were significantly increased in the MYBS3(Ox) line and decreased in the MYBS3(Ri) line at 4°C (Fig. 5). The present 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 \textit{DREB1A} and \textit{MYBS3} expression by cold are quite different (Fig. 1 and Fig. 8). Several factors have been found to regulate the expression of \textit{DREB1/CBF} as mentioned in the introduction, but the detailed information about the cold signaling pathways upstream of \textit{DREB1/CBF} is rather limited. Recently, a calmodulin binding transcription factor (CAMTA) 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 \textit{CBF2} and \textit{ZAT12} transcription factors in Arabidopsis (Doherty et al., 2009). CM2 is present in one copy in the \textit{MYBS3} promoter (-117 to -112 upstream of ATG) (Fig. 7 and Table S4). For cold up-regulated but \textit{MYBS3} down-regulated genes, CM2 is present in two copies each in \textit{DREB1B} (-134 to -129 and -80 to -75) and \textit{\alpha Amy3} (-158 to -153 and -149 to -144) promoters; for cold up-regulated and \textit{MYBS3} up-regulated genes, CM2 is present in the glutamate decarboxylase (-54 to -49) and \textit{WRKY77} promoters (-96 to -91) (Fig. 7 and Table S4). Some of other CMs shared by the Arabidopsis \textit{CBF2} and \textit{ZAT12} promoters (Doherty et al., 2009) could also be found in \textit{DREB1B}, \textit{DREB1C}, \textit{\alpha Amy3} and cytochrome P450 promoters (Fig. 7 and Table S4), but the function of these \textit{cis}-acting elements and the identify of their interacting transcription factors in cold signaling have not been determined (Doherty et al., 2009).
CMs 1-7 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 on the *DREB1/CBF* family might have diverged throughout evolution. No CM is present in the 1-kb promoter region of *DREB1A*, indicating unidentified cis-acting element(s) could be responsible for cold induction of *DREB1A*. It appears that combinations of various cis-acting elements and interacting 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 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 Fig. 6, except cytochrome P450, contain TA box or its variants (Yu, 1999; Wang et al., 2007) in sense or antisense orientation (Fig. 7 and 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 1054-bp *DREB1A* promoter, were repressed by over-expression of MYBS3 at 4°C (Fig. 8). One explanation is that the TA3 box (-625 to -620) in the 1054-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) (2 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 TPP2, other MYBS3 up-regulated genes also contain TA box or its variants (Fig. 7 and 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 one single DNA binding domain (1R MYB) have been proposed to bind DNA as a dimer, and MYBS1 does whereas MYBS3 doesn’t form a homodimer (Lu et al., 2002). Whether MYBS3 could be converted into an activator, by interacting with other 1R MYB and forming a heterodimer or with other transcription factor(s), remains for further study.

Taken together, 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 ROS-mediated pathways 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 expressions 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 accumulation of trehalose in rice under normal growth conditions (Shima et al., 2007). Although in WT plant, the expression of two rice TPPs is induced by cold, it peaks around 24 h and declined afterward at 4-6°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 were 50-94% of the WT 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 rice two complementary mechanisms for conferring cold tolerance in rice, with the DREB1-mediated process mediates the immediate cold shock response and the MYBS3-mediated system adjusts 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 for providing immediate need of carbon source and energy to combat the cold shock, while the subsequent suppression of *αAmy3* expression by *MYBS3* allows rice to conserve carbohydrates until re-growth is allowed at elevated temperatures. It would be interesting to test whether stacking of these two systems, by over-expression 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, though 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, however, 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 minimize their negative effects on plant growth (Kasuga et al., 1999; Nakashima et al., 2007). In the present 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 where are easily prone to transient temperature drops in early spring. Although the growth of MYBS3(Ox) lines was affected to certain extent in the greenhouse (Fig. S3), the growth and yield of line S3(Ox)-110-1 was normal in field (Table 2). In conclusion, the present 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

The rice cultivar *Oryza sativa* L. cv. 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 incubation, germinated seeds were cultivated in a half-strength Kimura B solution containing the following macronutrients (in mM): (NH4)2SO4 (0.18), KNO3 (0.09), MgSO4 (0.27), KH2PO4 (0.09), and Ca(NO3)2 (0.18), and micronutrients (in μM): Fe-citrate (0.03), H3BO3 (2.5), MnSO4·H2O (0.2), ZnSO4·7H2O (0.2), CuSO4·5H2O (0.05), and H2MoO4 (0.05). The pH of the solution was adjusted to 4.7-4.8 using 0.5 N HCl. The culture solution was replaced with fresh solution every 2 days. Seedlings were grown under a 14-h light/10-h dark cycle for 10 days 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 Table S6 online.

Plasmid construction

The GATEWAY gene cloning system (Invitrogen, Carlsbad, CA) 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 ubiquitin
(Ubi) promoter, and the ccdB DNA fragment flanked by attR1 and attR2 sties 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 lambda 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 1054-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 EcoRI and inserted into the binary vector pSMY1H (Ho et al., 2000), generating pSMY-MYBS3 (Fig. S1). To make the MYBS3 RNA interference construct, a 227-bp sequence derived from the 3’UTR of MYBS3 cDNA was synthesized by PCR, 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 EcoRI and inserted into the binary vector pSMY1H, generating pSMY-MYBS3(Ri) (Fig. S1).
Rice transformation

Plasmids p1302-MYBS3-GFP, pSMY-MYBS3, 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, Madison, WI). Four μg of RNA was used for cDNA preparation with reverse transcriptase (Applied Biosystems, Foster City, CA), and cDNA was then diluted to 10 ng/μl. Five μl of cDNA was mixed with primers and the 2X 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 CT method, and the amplification of 18S ribosomal RNA was used as an internal control to normalize all data.

Subcellular localization of MYBS3-GFP fusion protein

Protoplasts were isolated from transformed calli as described (Lu et al., 1998). GFP expression was detected under a LSM510 confocal laser scanning microscope (Carl Zeiss) using a 40X 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 16-h light and 8-h dark cycle in a growth chamber were used for all stress treatments. Stress treatments are 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 Kits (Qiagen, Valencia, CA) according to the Qiagen manual. RNA quality was examined by the Agilent 2100 bioanalyzer (Affymetrix, Palo Alto, CA), and biotinylated target RNA was prepared from total RNA. Samples were hybridized to the Affymetrix Rice GeneChip (Affymetrix, Santa Clara, CA) 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 software Affymetrix GCOS version 1.4 (Affymetrix). CEL files were loaded into GeneSpring GX 9.0 (Agilent Technologies, Palo Alto, CA). 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 over-expression or under-expression of *MYBS3* or by cold are listed in Tables S7 – S12.

**Accession number**

*DREB1A* (Os09g35030); *DREB1B* (Os09g35010); *DREB1C* (Os06g03670); *aAmy3/RAmy3D* (Os08g36910); Cytochrome P450 gene (Os02g47470); Glutamate decarboxylase gene (Os03g13300); *WRKY77* (Os01g40260); Multidrug resistance protein 4 gene (Os01g50100); Trehalose-6-phosphate phosphatase 1 gene (Os02g44230); Trehalose-6-phosphate phosphatase 2 gene (Os10g40550).
Supplemental Data

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

**Supplemental Table S1.** Genes regulated by over-expression of *MYBS3* in transgenic rice as well as by cold in WT in the microarray analysis.

**Supplemental Table S2.** Genes regulated by under-expression of *MYBS3* in transgenic rice as well as by cold in WT 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 position of CM motifs in the promoter region of cold-inducible and *MYBS3* down-regulated or *MYBS3* up-regulated genes.

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

**Supplemental Table S6.** Primers used in this study.

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

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

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

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

**Supplemental Table S11.** Genes up-regulated by cold in WT.

**Supplemental Table S12.** Genes down-regulated by cold in WT.

**Supplemental Figure S1.** Constructs for over-expression and under-expression 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 over-expressing *MYBS3* in 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.

ACKNOWLEDGEMENT

We thank Drs. Harry Iain Wilson and Kuo-Wei Lee for critical review of this manuscript. This work was supported by grants from Academia Sinica and the National Science Council (NSC-94-2321-B-001-023, NSC-95-2321-B-001-015 and NSC96-2321-B-001-007) of the Republic of China.
Table 1. *MYBS3* (Ox) lines are more cold tolerant.

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of plants survived</th>
<th>Total no. of plants tested</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3</td>
<td>30</td>
<td>10.0 ± 0.0</td>
</tr>
<tr>
<td>S3(Ox)-110-1</td>
<td>18</td>
<td>21</td>
<td>85.7 ± 10.5</td>
</tr>
<tr>
<td>S3(Ox)-112-7</td>
<td>26</td>
<td>32</td>
<td>81.3 ± 11.1</td>
</tr>
<tr>
<td>S3(Ri)-42-10</td>
<td>0</td>
<td>20</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>S3(Ri)-52-7</td>
<td>0</td>
<td>21</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Number of plants survived after exposure to 4°C for 24 h. Experiments were repeated 4 times. Five to eight plants per line were tested in each experiment. ±, SD.
Table 2. Comparison of agronomic traits of a *MYBS3(Ox)* line with the wild type grown in field.

<table>
<thead>
<tr>
<th>Trait</th>
<th>WT</th>
<th>S3(Ox)-110-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td>101.6 ± 3.7</td>
<td>95.5 ± 5.4</td>
</tr>
<tr>
<td>Tiller number</td>
<td>12.3 ± 2.5</td>
<td>19.5 ± 6.8</td>
</tr>
<tr>
<td>Panicle number / plant</td>
<td>12.9 ± 2.9</td>
<td>19.0 ± 6.1</td>
</tr>
<tr>
<td>Panicle length (cm)</td>
<td>18.9 ± 1.3</td>
<td>18.4 ± 0.1</td>
</tr>
<tr>
<td>Grain number / panicle</td>
<td>118.0 ± 14.5</td>
<td>103.5 ± 8.8</td>
</tr>
<tr>
<td>Fertility (%)</td>
<td>95.7 ± 1.2</td>
<td>93.8 ± 1.7</td>
</tr>
<tr>
<td>Grain yield (g / plant)</td>
<td>41.7 ± 11.0</td>
<td>45.4 ± 15.9</td>
</tr>
<tr>
<td>1000 Grains weight (g)</td>
<td>26.3 ± 0.5</td>
<td>24.3 ± 0.4</td>
</tr>
</tbody>
</table>

Twenty plants each of WT and line S3(Ox)-110-1 per replicate, and with total of 3 replicates, were grown during February to July, 2008. ±, SD.
LITERATURES CITED


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Peterson ML, Lin SS, Jones D, Rutger JN (1974) Cool night temperatures cause sterility in rice,


and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol 137: 176-189


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 field, 7-day-old seedlings cultured hydroponically, and suspension cells cultured with or without sucrose for 2 days. 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. 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.
Figure 2. MYBS3 promoter is up-regulated by cold stress and MYBS3 transcription factor is constitutively localized in nucleus.

A, Rice transformed with MYBS3::GFP construct or Ubi::GFP construct. Transgenic rice seedlings were cultivated hydroponically at 28°C for 10 days 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 MYBS3-GFP fusion protein. Panels (1) and (2), protoplasts expressing MYBS3-GFP fusion protein, and GFP was detected mainly in the nucleus. Panels (3) and (4), protoplasts expressing GFP alone, and GFP was detected in the nucleus and cytoplasm. Panels (1) and (3), bright field images. Panels (2) and (4), fluorescent field images.
Figure 3. MYBS3 is sufficient and necessary for cold tolerance in rice.

A, RNAs isolated from leaves of 10-day-old rice seedlings of MYBS3 over-expression (S3Ox) lines and MYBS3 under-expression (S3Ri) 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. X indicates relative induction. The error bars indicate the SE of three replicates. B and C, Ten-day-old seedlings of wild type (WT) and lines S3(Ox)-110-1 and S3(Ri)-52-7 were incubated at 4°C for 8 h (B) or 24 h (C).
Figure 4. Over-expression 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 days. A, Seedlings kept at 28°C for 1 week. B, Seedlings shifted to 4°C for 1 week. C, Enlarged photos of B.
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 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 indicated genes was analyzed by a quantitative real time RT-PCR analysis. The mRNA level in the WT at 4°C was assigned a value of 1, and mRNA levels of other samples were calculated relative to this value. Error bars indicate the SE of three replicates. Accession numbers of 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 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 indicated genes was determined by a quantitative real time RT-PCR analysis. The mRNA level in the WT at 28°C was assigned a value of 1, and mRNA levels of other samples were calculated relative to this value. Error bars indicate the SE of three replicates. Accession numbers of indicated genes are provided in Materials and Methods.
Figure 7. Putative cis-acting elements present in promoters of cold-inducible and MYBS3 activated or repressible genes. Positions of TA box, DRE, and CM 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 TA box, DRE, and CM in the promoter region. Vertical arrows indicate the position upstream of ATG.
Figure 8. *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*, or *αAmy3 SRC::Luc* as a reporter. Effector and reporter constructs were co-transfected into rice embryos, incubated at 28°C or 4°C for 24 h, and the luciferase activity was determined. X indicates fold induction or repression of luciferase by MYBS3 over-expression. The error bars indicate the SE of 3 replicates.
Figure 9. Proposed role of MYBS3 in cold stress tolerance in rice. Details of the model are described in the text.