Overexpression of an R1R2R3 MYB Gene, OsMYB3R-2, Increases Tolerance to Freezing, Drought, and Salt Stress in Transgenic Arabidopsis$^{1[C][W][OA]}$

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We used a cDNA microarray approach to monitor the expression profile of rice (Oryza sativa) under cold stress and identified 328 cold-regulated genes. Thirteen such genes encoding MYB, homeodomain, and zinc finger proteins with unknown functions showed a significant change in expression under 72-h cold stress. Among them, OsMYB3R-2 was selected for further study. Unlike most plant R2R3 MYB transcription factors, OsMYB3R-2 has three imperfect repeats in the DNA-binding domain, the same as in animal c-MYB proteins. Expression of OsMYB3R-2 was induced by cold, drought, and salt stress. The Arabidopsis (Arabidopsis thaliana) transgenic plants overexpressing OsMYB3R-2 showed increased tolerance to cold, drought, and salt stress, and the seed germination of transgenic plants was more tolerant to abscisic acid or NaCl than that of wild type. The expression of some cold-related genes, such as dehydration-responsive element-binding protein 2A, COR15a, and RC12A, was increased to a higher level in OsMYB3R-2-overexpressing plants than in wild type. These results suggest that OsMYB3R-2 acts as a master switch in stress tolerance.

Plants are exposed to environmental conditions that frequently impose constraints on growth and development. Among them, low temperature stress is one of the serious environmental stresses affecting plant growth and agricultural production. On exposure of plants to low temperature, a series of genes are induced, the products of which may either directly protect against stress or further control the expression of other target genes (Yamaguchi-Shinozaki and Shinozaki, 2006). In Arabidopsis (Arabidopsis thaliana), a major transcriptional regulatory system that controls abscisic acid (ABA)-independent gene expression in response to low temperatures has been identified (Stockinger et al., 1997; Liu et al., 1998). The system is based on the C-repeat (Baker et al., 1994)/dehydration-responsive element (Yamaguchi-Shinozaki and Shinozaki, 1994) that interacts with C-repeat-binding factors (CBFs). Under cold stress, CBF/dehydration-responsive element-binding protein 1 (DREB1) genes are rapidly and transiently induced and subsequently activate the expression of target genes (Gilmour et al., 1998). Several studies have reported that ectopic overexpression of some CBFs resulted in both activation of target genes and enhanced freezing, salt, or dehydration tolerance of transgenic plants (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Haake et al., 2002).

The CBF pathway is a central component of cold response, but CBF-independent pathways might also be necessary for the cold stress response (Zhu et al., 2004). Direct evidence exists for the activities of some cold-regulated transcription factors (TFs) not participating in the CBF cold-response pathway (Fowler and Thomashow, 2002), which suggests that TFs play a crucial role in controlling downstream gene expression as well as the regulation of cross talk between different signaling pathways. The key to understanding plant cold response lies in the identification of new components involved in these processes and the elucidation of the signaling pathways.

Rice (Oryza sativa) is a model monocot system and one of the most important food crops in Asia (Khush, 1997; Tyagi et al., 1999; Tyagi and Mohanty, 2000; Cantrell and Reeves, 2002). Unlike Arabidopsis and other crops such as wheat (Triticum aestivum), barley (Hordeum vulgare), and rye (Secale cereale), rice is
adversely affected by cold, drought, and salt stress. Cold stress especially limits rice production. Minimizing the loss caused by low temperatures will not only help improve net product but will also extend rice cultivation in marginal lands not able to be cultivated (Khush, 1999; Tyagi and Mohanty, 2000). Rice exposed to cold stress showed marked changes in gene expression, biomembrane lipid composition, and small molecule accumulation (Iba, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). However, much less is known about the regulation mechanism of the rice response to cold stress. Therefore, identifying uncharacterized cold-related genes and defining their functions will enrich the understanding of stress-signaling networks in rice and be important for improving rice tolerance to cold stress.

Here, we report on the isolation and functional characterization of a nuclear-localized R1R2R3 MYB TF designated OsMYB3R-2 (\textit{O. sativa} R1R2R3 MYB-2) in rice. The protein, like animal c-Myb proteins, contains three imperfect repeat sequences in the N-terminal DNA-binding domain (Jin and Martin, 1999). Overexpression of OsMYB3R-2 in Arabidopsis leads to increased tolerance to freezing, drought, and salt stress.

RESULTS
Isolation of Cold-Responsive MYB TFs from Cold-Tolerant Rice

Yuedongdao, a rice variety possessing characteristics of cold tolerance is a crossed progeny of cultivated rice and Dongxiang wild rice (\textit{Oryza rufipogon} Griff.), which is a population of common wild rice with increased cold stress tolerance from Dongxiang in the Jiangxi province of China. The Dongxiang wild rice rhizome can survive at a freezing temperature to $-12.8^\circ$C (Liu et al., 2003a). Our physiological analyses...
showed Yuedongdao and Dongxiang wild rice seedlings survived under 2°C cold treatment for 72 h, whereas cultivated rice (cold-sensitive rice varieties) did not survive (X. Dai, H. Liu, Y. Xu, and K. Chong, unpublished data). Expression profiles of Yuedongdao under cold stress with 2°C for 72 h were monitored by cDNA microarray (BioStar Genechip), which contains approximately 10,000 rice clones (Liu et al., 2003b). The probes were prepared from RNAs isolated from Yuedongdao seedlings under cold treatment for 72 h and nontreated controls. For hybridization, two replicates of a 2-d swap experiment were performed with RNAs extracted independently from a different batch of plants. Results of the two replicates were highly correlated ($r = 0.86$). We considered genes with an expression ratio (treatment to control) 2-fold greater or less than that of control genes (|$\log_2$ ratio| $\geq 1$) as cold-inducible or cold-repressive genes. A total of 328 genes showing reproducible 2-fold up- or down-regulation were selected. Among them, 157 genes were cold inducible and 171 cold repressive (Table I; Supplemen tal Table S1).

We performed semiquantitative reverse transcription (RT)-PCR to confirm the differentially expressed genes identified by microarray analysis. Twenty-six genes representing different expression profiles were analyzed, of which 25 exhibited expression patterns similar to that from microarray analysis (Fig. 1A); only one, R0005G01, showed no significant difference in gene expression between the treatment and control with RT-PCR amplification. Moreover, all randomly scattered expressed sequence tags that represent the same gene showed a similar differentially expressed pattern in the microarray analysis (Supplemental Table I).
Table S1). Thus, our cDNA microarray hybridizations were stable and reliable.

Among the 328 cold-responsive genes, we identified three genes encoding cold-inducible TFs, including two MYB TFs and one homeodomain TF. One, an expressed sequence tag R0481B08 (accession no. BAD81765) encoding a putative R1R2R3 MYB TF was selected for further functional studies. In our microarray hybridization, R0481B08 showed a completely opposite hybridization signal in two dye-exchange replicates (Fig. 1B). The transcript level was increased 2.6-fold when the cold-treated sample was labeled with Cy5 and the untreated sample was labeled with Cy3, whereas in the dye-exchange hybridization, the transcript level was decreased 2.3-fold (Supplemental Table S1). The expression of R0481B08 in the microarray analysis was confirmed by semi-quantitative RT-PCR (Fig. 1A). Moreover, real-time PCR used to further examine its expression in microarray hybridizations showed a similar amount of change as the microarray data (Fig. 1D), which strongly supports the validity of the cold-regulated expression pattern from the microarray analysis (Fig. 1, C and D).

Structural Features, Phylogenetic Tree, and Subcellular Localization of OsMYB3R-2

To investigate the function of R0481B08, we amplified its full-length cDNA by RT-PCR from rice seedlings treated for 72 h at 2°C. The full-length cDNA contains an open reading frame of 587 amino acids with a calculated molecular mass of 63.9 kD. Homological analysis showed that the gene shared the greatest sequence similarity with the MYB TFs from Arabidopsis, rice, Fopulus, and tobacco (Nicotiana tabacum) within the MYB domain. The MYB domain is composed of three imperfect repeat sequences (R1, R2, and R3) of 50 to 53 amino acids in the mammalian MYB protein c-MYB and related proteins A-MYB and B-MYB (R1R2R3-MYB; Carr and Mott, 1991), whereas most of the plant MYB proteins identified thus far contain only two repeats (R2R3-Myb; Jin and Martin, 1999; Stracke et al., 2001). Interestingly, it contains three repeat sequences (Fig. 2A), like animal c-MYB proteins, so this gene is a R1R2R3-type MYB TF and was designated OsMYB3R-2 (O. sativa R1R2R3 MYB-2). Other members of this group of MYB genes from plants (MYB3R-1, MYB3R-2, T48510, AF214117, T48253 from Arabidopsis; NtmybA1, NtmybA2, and NtmybB from tobacco) have been reported recently (Braun and Grotewold, 1999; Kranz et al., 2000), but the biological functions of the genes from Arabidopsis are unknown.

We constructed a phylogenetic tree based on the amino acid sequences of MYB domains of animal and plant Myb proteins (Fig. 2B). The OsMYB3R-2 protein is more similar to AF214117, T48253, MYB3R-1, and NtmybA2 proteins than to plant R2R3-Myb proteins. The OsMYB3R-2 protein showed 66.7% to 74.8% identity with human C-Myb and B-Myb but only 37.6% to 44.1% identity with plant R2R3-Myb proteins. However, OsMYB3R-2 protein did not group with any animal R1R2R3-type Myb proteins (A-Myb, B-Myb, and C-Myb) but, rather, formed a separate branch. OsMYB3R-2 and MYB3R-1 are more closely related to each other than to AJ430051, a putative R1R2R3-type MYB from rice.

Figure 3. Real-time PCR analysis for the expression of OsMYB3R-2 in rice. OSISAP1 was used as a positive control. A and B, Time course of OsMYB3R-2 and OSISAP1 expression during cold treatment. C, OsMYB3R-2 expression response to cold, salt, and drought stress. D, OsMYB3R-2 expression in various tissues. Actin was used as an internal control. Data represent means and S.E of three replicates.
To examine its subcellular localization, OsMYB3R-2 was fused in frame to the 5' terminus of the green fluorescent protein (GFP) reporter gene under the control of the cauliflower mosaic virus 35S promoter (CaMV 35S). The recombinant constructs of the OsMYB3R-2-GFP fusion gene and GFP alone were introduced into onion (Allium cepa) epidermal cells by particle bombardment. As shown in Figure 2C, the OsMYB3R-2-GFP fusion protein accumulated mainly in the nucleus, whereas GFP alone was present throughout the whole cell. Thus, OsMYB3R-2 is a nuclear-localized protein, which is consistent with its predicted function as a TF (Fig. 2C).

**Expression Pattern of OsMYB3R-2 in Response to Cold, Salt, and Drought Stress**

We performed real-time RT-PCR to examine the expression pattern of OsMYB3R-2 under different stress conditions. Under cold stress, the transcripts of OsMYB3R-2 began to increase after 5 h cold treatment and gradually accumulated up to 72 h of treatment (Fig. 3A), which was consistent with our microarray results. In the case of salt and dehydration stress, transcript levels of OsMYB3R were also induced after 5 h treatment as compared with that of non-treated controls (Fig. 3C). To validate this experiment, we used the OSISAP1, a gene encoding zinc-finger protein from rice, as a positive control. OSISAP1 is induced under cold, salt, and drought stress. Under cold treatment, the transcript level of OSISAP1 was increased to a very high level during a 12-h cold treatment and declined thereafter (Mukhopadhyay et al., 2004). We confirmed that the expression of OSISAP1 was induced by cold, desiccation, and salt stress (Fig. 3, B and C), which was consistent with previous studies (Mukhopadhyay et al., 2004). The expression pattern of OsMYB3R-2 under cold stimulation was different from that of OSISAP1, although both were induced by cold stress in rice.

In addition, we examined tissue-specific expression of OsMYB3R-2 in rice using real-time RT-PCR. The OsMYB3R transcripts were detected in all organs tested, but the highest level was in young stems and the lowest in spikes (Fig. 3D).

Taken together, these results suggest that OsMYB3R-2 is induced under cold, salt, and drought stimulation, which suggests that it functions during these stresses.

**Overexpression of OsMYB3R-2 Increases Tolerance to Freezing, Drought, and Salt**

To investigate the function of OsMYB3R-2 in plants, we overexpressed OsMYB3R-2 in transgenic Arabidopsis under control of a CaMV 35S promoter. Transformed lines of Arabidopsis were confirmed by hygromycin selection and Southern blotting. Southern blot was performed by using the DNA digested with HindIII or EcoRI and β-glucuronidase (GUS) gene as a probe. Three transgenic lines were randomly selected and showed different hybridized patterns to the GUS probe (Fig. 4A). In the wild type, however, no signals were detected under the same conditions. Therefore, the three transgenic lines could be independent. Furthermore, RNA gel-blot analysis showed that OsMYB3R-2 was expressed at the higher levels in transgenic Arabidopsis than in the wild type (Fig. 4B).

To examine the possible phenotypes of transgenic lines, T3 progeny of the OsMYB3R-2-overexpressed lines and the wild-type plants were grown in the greenhouse under identical conditions. Compared with wild-type plants, transgenic plants showed a little retarded growth under normal conditions (Fig. 4C).

To investigate the effect of OsMYB3R-2 overexpression on freezing tolerance, T3 transgenic and wild-type seedlings were exposed to −8°C for 10 h. After...
6 d recovery at normal conditions, survival was 26.8% for the wild-type and 84.5% for transgenic lines (Fig. 5, A and B). Phenotypically, most transgenic seedlings were green and could regrow as compared with the wild type, whereas most wild-type seedlings became white and did not regrow after removed to normal conditions. The survival percentage under different low temperatures also showed dramatic difference between the transgenic plants and wild-type plants (Fig. 5, C and D). At −10°C, the proportion of survived wild-type plants decreased to 5.6%, whereas more than 42.8% of transgenic plants survived. Thus, OsMYB3R-2-overexpression plants show high tolerance to freezing stress.

To determine the effect of OsMYB3R-2 overexpression on drought tolerance, 14-d-old plants grown on soil were not watered for 2 weeks and then watered and grown under normal conditions for 7 d (Fig. 6A). After watering was restarted, transgenic plants showed a stronger growth recovery phenotype than wild-type plants. Only 26.7% of the wild-type plants survived this treatment. In contrast, more than 85% of OsMYB3R-2-overexpressed plants survived (Table II), which suggests that the overexpression of OsMYB3R-2 in transgenic Arabidopsis results in greater tolerance to drought stress than in the wild type. The drought-tolerance phenotype of transgenic plants overexpressing OsMYB3R-2 was consistent with slower water loss in detached rosette leaves as compared with the wild type (Fig. 6B).

To test the effect of OsMYB3R-2 overexpression on salt tolerance, transgenic and wild-type seedlings were grown as described in “Materials and Methods.” Seedlings of both genetic backgrounds grew normally in NaCl up to 150 mM, but the transgenic seedlings formed longer roots than the wild type when grown vertically under NaCl treatment (Fig. 6, C and D). When NaCl concentration was increased to 200 mM, the growth of the wild type was completely inhibited and the seedlings showed absence of greening, whereas

**Table II. Survival rates of transgenic plants under drought stress conditions**

| OsMYB3R-2-Overexpressed Lines | Survivala | Totalb | Survivalc |
|-------------------------------|-----------|--------|-----------|
| Wild-type                     | 16        | 60     | 26.7      |
| L5                            | 51        | 60     | 85        |
| L11                           | 60        | 60     | 100       |
| L14                           | 58        | 60     | 96.7      |

aNumber of surviving plants. bTotal plants used in drought assay. cPercentage of surviving plants.
transgenic seedlings were still green and continued to grow (Fig. 6C).

**Germination of OsMYB3R-2-Overexpressed Seeds Is Insensitive to ABA and NaCl**

We tested the effect of ABA and NaCl on germination of OsMYB3R-2-overexpressed seeds. There was no difference in seed germination between the wild-type and transgenic plants under normal conditions (Fig. 7A). In the presence of exogenous ABA, the germination of both wild-type and OsMYB3R-2-overexpressed seeds was inhibited significantly, but transgenic seeds inhibited to a lesser extent (Fig. 7B). For example, at 0.5 μM ABA, approximately 80% of OsMYB3R-2-overexpressed seeds germinated compared with only 30% seeds of the wild type. Under 1.0 μM ABA treatment, most seeds of the wild type did not germinate. In contrast, about one-half the seeds of the transgenic plants germinated and developed green cotyledons and true leaves (Fig. 7, C and D). At ABA levels higher than 2.0 μM, the germination of both wild-type and transgenic seeds was inhibited completely.

We also observed that the germination of transgenic seeds was more tolerant to NaCl than that of wild type under different NaCl treatments (Fig. 7, E and F). At 50 mM NaCl, nearly 79% to 90% of transgenic seeds germinated at day 2 compared with only 35% seed germination for the wild type (Fig. 7E). At 75 mM NaCl, germination of both wild-type and OsMYB3R-2-overexpressed seeds was completely inhibited at day 2 after imbibition. At day 3, although seeds of both plants began to germinate, the germination in the transgenic plants was significantly higher than that of the wild type (Fig. 7F). Nevertheless, both wild-type and transgenic seeds were not observed to germinate at day 5 after imbibition when NaCl concentrations were at 100 mM (data not shown). Thus, overexpression of OsMYB3R-2 increased tolerance to stress in transgenic Arabidopsis plants.

![Figure 6. Effect of OsMYB3R-2 expression on drought and salt tolerance in transgenic Arabidopsis plants. A, Drought tolerance of 35S::OsMYB3R-2. Top, Wild-type and transgenic plants without drought stress treatment. Bottom, Wild-type and transgenic plants were grown for 2 weeks with normal watering, withheld from water for 2 weeks, and then Rewatered for 7 d before photographs were taken. B, Water loss in wild-type and transgenic plants. Detached leaves from 25-d-old plants grown on soil were incubated on a bench, and the fresh weight (FW) was measured at the time intervals indicated. Water loss was calculated from the decrease in FW compared with time zero. Error bars indicate SD. C, Salt tolerance of 35S::OsMYB3R-2. Wild-type and transgenic plants were germinated on MS agar plates, then transferred to a new MS agar plate supplemented with different concentrations of NaCl for 7 d. D, Dose response of transgenic and WT seedlings to NaCl. Data shown are root length. Error bars indicate SD.](image-url)
OsMYB3R-2 in Arabidopsis increased tolerance to NaCl and ABA during seed germination.

OsMYB3R-2 Activates the Expression of Cold-Responsive Genes

To elucidate the molecular mechanism of OsMYB3R-2 in the cold response, we monitored the expression of cold-responsive genes identified in the regulated pathways by real-time PCR analysis. Under 4°C cold treatment for 6 h, the tested marker genes, including RD29A, CBF1, CBF2, CBF3, KIN1, and COR47, showed slight induction in both wild-type and transgenic plants under cold-stress conditions, consistent with previous studies (Kurkela and Franck, 1990; Gilmour et al., 1992, 1998; Yamaguchi-Shinozaki and Shinozaki, 1993; Stockinger et al., 1997). However, under normal conditions (22°C), the expression of DREB2A, COR15a, and RC12A in OsMYB3R-2-overexpressed transgenic plants was substantially higher than that in wild-type plants, whereas no significant induction in expression of RD29A, CBF1, CBF2, CBF3, KIN1, and COR47 in both transgenic and wild-type plants (Fig. 8). COR15a and DREB2A are involved in stress signaling by CBF/DREB1 pathways (Artus et al., 1996; Liu et al., 1998), but RC12A by CBF/DREB1-independent pathways (Medina et al., 2005). Thus, overexpression of OsMYB3R-2 increases expression of DREB2A, COR15a, and RC12A, which are involved in plant tolerance by different pathways.

DISCUSSION

OsMYB3R-2 Encodes a Cold-Responsive R1R2R3 MYB TF

In plants, the transcripts of genes encoding several families of TFs, such as AP2/EREBP, bZIP/HD-ZIP, and several classes of zinc finger domains, are induced after exposure to various abiotic stresses (Shinozaki and Yamaguchi-Shinozaki, 2000; Seki et al., 2001). These TFs function in various pathways to confer stress tolerance in plants (Ingram and Bartels, 1996; Thomashow, 1999; Hasegawa et al., 2000; Zhu, 2002). MYB TFs are involved in numerous processes (Jin and Martin, 1999; Ito et al., 2001; Stracke et al., 2001). So far, only two R2R3-MYB TFs, HOS10 in Arabidopsis and Osmyb4 in rice, may play essential roles in cold stress tolerance.
by a possible CBF-independent pathway (Vannini et al., 2004; Zhu et al., 2005), but the role of R1R2R3 MYB involved in cold stress is poorly understood. In this study, we identified a cold-inducible R1R2R3 MYB TF, OsMYB3R-2, from cold-insensitive rice, a progeny of cultivated rice and common wild rice with cold-tolerance characteristics (Fig. 1).

R1R2R3-MYB genes seem to constitute a small gene family in plants. In Arabidopsis, five R1R2R3-type Myb genes have been described (Braun and Grotewold, 1999; Kranz et al., 2000), but little about their functions is known. Three R1R2R3-type genes from tobacco, NtmybA1, A2, and NtmybB, have been identified to be involved in M-specific activator (MSA)-mediated G2/M-phase-specific transcription through binding to the MSA element and modulating its activity (Ito et al., 2001). Apparently, unlike most plant R2R3-type MYB proteins, OsMYB3R-2 proteins are closer to the plant R1R2R3 MYB and the animal A-, B-, and C-MYB. The presence of the R1R2R3 MYB motif in the OsMYB3R-2 protein, as well as its nuclear localization, demonstrates that OsMYB3R-2 is an R1R2R3-type MYB TF (Fig. 2). R1R2R3-Myb genes occur in different plant evolutionary lineages, including mosses, ferns, and monocots (Kranz et al., 2000). Thus, in contrast to plant R2R3-Myb, the R1R2R3-type plant MYB proteins could have had a conserved function in eukaryotes. From this evidence, we suggest that OsMYB3R-2 plays a conserved role during stress tolerance in rice.

Usually transcriptional factors are induced rapidly during the early phase of the response to cold, drought, and salt stress, reach maximal induction at several hours, and then decrease in expression level (Thomashow, 2001; Yamaguchi-Shinozaki and Shinozaki, 2006). For example, CBF1 and CBF3 showed peak induction at 6 h in wild-type plants and CBF2 showed peak induction at 3 h during stress treatment (Gong et al., 2002). From the nature of early induction, CBF1/CBF2/CBF3 act early in the signal transduction pathway of the stress response. Expression-pattern analysis shows that the activation pattern of OsMYB3R-2 under cold stress differs from that of other stress-inducible TFs previously reported (Dubouzet et al., 2003; Vannini et al., 2004). The transcript level of genes such as OsDREBIA and OSISAPI, required early after stress, increase to a very high level within 1 h after cold treatment (Dubouzet et al., 2003; Mukhopadhyay et al., 2004), continue to increase until 5 or 3 h, remain at elevated levels until 10 or 12 h, and decline thereafter. However, the transcript level of OsMYB3R-2 increases after cold treatment for 5 h and gradually accumulates within 72 h (Fig. 3A). Furthermore, OsMYB3R-2 is induced by drought and salt stress (Fig. 3C), which is dissimilar to Osmyb4, another MYB TF involved in cold stress in rice that was induced only by cold stress for 4 h (Vannini et al., 2004). Thus, OsMYB3R-2 can be classified as a novel R1R2R3-type MYB TF, and this is the first report, to our knowledge, showing that an R1R2R3-type MYB is involved in cold, drought, and salt stress.
Overexpressed OsMYB3R-2 Increases Tolerance to Stress in Arabidopsis

Certain stress-induced proteins have been shown to impart stress tolerance. Overexpression of genes such as CBF/DREB1, OSISAPl, and HVA1 could confer stress tolerance in transgenic plants (Browse and Xin, 2001; Dubouzet et al., 2003; Mukhopadhyay et al., 2004), although their functions remain to be defined. These examples provide a target for improving stress tolerance of crop plants and give an opportunity to understand the function of previously uncharacterized genes. The expression of OsMYB3R-2 in rice is induced with exposure to cold, drought, and salt stress (Fig. 3). In Arabidopsis, the overexpression of OsMYB3R-2 led to increased tolerance to cold, dehydration, and salt stress (Figs. 5–7). Our data suggest that the overexpressed OsMYB3R-2 protein results in enhanced transduction of stress-response signals. Furthermore, the elevated stress tolerance of 35S::OsMYB3R-2 plants coincides with up-regulated stress-responsive genes, including DREB2A, COR15a, and RC12A. COR15a and DREB2A belong to the DRE/CRT class of stress-responsive genes. COR15a from Arabidopsis is induced after cold stress, and its overexpression in transgenic Arabidopsis leads to increased freezing tolerance (Artus et al., 1996; Steponkus et al., 1998). DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREBs are involved in high salinity and drought-responsive gene expression (Liu et al., 1998). Thus, the enhanced stress tolerance in OsMYB3R-2 transgenic plants might depend in part on changes in the expression of those genes. However, several tested CBF class or CBF/DREB inducible marker genes, such as CBF1, CBF2, CBF3, RD29A, COR47, and KIN1, did not show increased expression in the 35S::OsMYB3R-2 plants under normal conditions (Fig. 8), which suggests that some other stress pathways may be involved in OsMYB3R-2-mediated stress tolerance. The high transcription levels of RC12A in 35S::OsMYB3R-2 plants support this deduction. RC12A protein is not a member of the CBF/DREB1 regulon and involvement of CBF/DREB1-independent pathways in modulating stress signaling (Medina et al., 2005). Hydrophilic RC12A protein may contribute to increased stress tolerance in transgenic plants (Thomashow, 1998; Hasegawa et al., 2000). So the mechanism of OsMYB3R-2 may be to increase the expression of some hydrophilic proteins to enhance stress tolerance.

Several lines of evidence indicate that other signal pathways in addition to those mediated by CBF TFs are involved in cold stress (Fowler and Thomashow, 2002; Kreps et al., 2002). Cross talk between those signal transduction pathways is poorly understood. The high transcript level of DREB2A, COR15a, and RC12A in 35S::OsMYB3R-2 plants suggests that OsMYB3R-2 acts as a master switch in stress tolerance and is involved in the complex network controlling stress-responsive genes. Usually, enhanced drought tolerance accompanies hypersensitivity to ABA treatments during seed germination and early seedling development (Hu et al., 2006; Ko et al., 2006). In contrast, in our system, enhanced tolerance to stress accompanies decreased sensitivity of germination to ABA in overexpressed OsMYB3R-2 transgenic plants. In fact, the phenotype exists in other genes previously reported, such as AtHD2c, CaXTH3, and AtTP51 (Avonce et al., 2004; Cho et al., 2006; Srihda and Wu, 2006), although the precise mechanism is still unknown. It is possible that there are various ABA signal transduction pathways involved in both processes of tolerance and germination in Arabidopsis.

OsMYB3R-2 differs from HOS10 and Osmyb4. Importantly, the activated genes in 35S::OsMYB3R-2 Arabidopsis also differ from those in Osmyb4 transgenic Arabidopsis. These results suggest that OsMYB3R-2 is a novel member of the R1R2R3-type MYB family in rice and is involved in stress response.

This study has characterized an R1R2R3-type MYB protein localized at the nucleus in rice and induced by cold, drought, and salt stress. The enhanced stress tolerance of 35S::OsMYB3R-2 Arabidopsis plants reveals that OsMYB3R-2 could mediate signal transduction, regulating some stress-responsive genes involved in CBF-dependent or -independent pathways. Although the detailed mechanism of OsMYB3R-2 involvement in stress is not yet clear, the characterization of OsMYB3R-2 function will provide new insights into stress pathways. This report provides beneficial information for molecular breeding leading to improved stress tolerance of agricultural crops.

MATERIALS AND METHODS

Plant Materials

We used rice (Oryza sativa) L. cv Yuedongdao, which is insensitive to cold stress. Arabidopsis (Arabidopsis thaliana) ecotype Columbia was used in gene transformation.

Rice seeds were surface sterilized for 5 min with ethanol (75% v/v) and 10 min with commercially diluted (1:3 v/v) NaOCl, followed by several rinses with sterile water. Germination was carried out for 72 h on sterile Murashige and Skoog (MS) medium in the dark at 28°C, then grown under 28°C-day/25°C-night temperatures, 12-h-light/12-h-dark cycle, and 50% humidity. After 2 weeks of germination, seedlings underwent several treatments: cold, 2°C for 0.5, 1, 2, 5, 10, 24, 36, 48, 64, and 72 h; drought, transferred to Whatman 3MM paper in a sterile petri dish for 5 h; and high salinity, 250 mM NaCl for 5 h. After all the treatments, seedlings were harvested, frozen in liquid nitrogen, and stored at −70°C for further analysis. Control plants were harvested at the same time as the treated plants.

Microarray Analysis

Rice seedlings were exposed to cold for 72 h; total RNAs from treated plants and nontreated plants were used for preparation of Cy5- and Cy3-labeled cDNA probes. PI005 cDNA microarray (Biostar Genechip) were hybridized with Cy5- and Cy3-labeled probe pairs of cold-treated and nontreated plants. Labeling, hybridization, and washing were performed as described for the CyScribe Post-Labeling kit. Hybridized slides were scanned with use of a GenePix 4000B scanner (Axon Instruments) at 532 and 635 nm to capture the emission of Cy5 and Cy3, respectively. The intensity of each spot at the two wavelengths was transformed into a ratio value with use of the GenePix 4.0 software. Overall intensity of the hybridized slide was normalized by use of GenePix 4.0 software. With the removal of the spots automatically flagged Bad or Not Found by the software, the spots whose
DNA Gel-Blot Analysis

DNA gel blot analysis was performed as described by Wang et al. (2004). Genomic DNA isolated from 3-week-old Arabidopsis seedlings was digested with EcoRI or HindIII, fractionated electrophoretically on 0.8% (w/v) agarose gel, and blotted onto a nylon membrane (Amersham Pharmacia Biotech). The membrane was prehybridized at 65°C for 2 h and hybridized in the same solution containing α-32P-ATP and CTP-labeled GUS for 20 h at 65°C. After hybridization, the membrane was washed once with 2× SSC plus 0.1% SDS at 65°C for 20 min, then twice with 1× SSC plus 0.1% SDS at 37°C for 30 min. The membrane was exposed to x-ray film (Eastman-Kodak) at –70°C for 3 to 7 d.

RNA Gel-Blot Analysis

Total RNA was extracted from 3-week-old seedlings of Arabidopsis with use of Trizol reagent (Invitrogen). Total RNA of 20 μg was electrophoresed on 1.2% agarose gel containing 0.4 m formaldehyde and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech). After hybridization with α-32P-labeled probe, blots were washed with 0.2× SSC containing 0.1% SDS at 65°C for 10 min. The ethidium bromide-stained ribosomal RNA was used as loading control.

Freezing, Drought, and Salt Stress Treatment in Transgenic Arabidopsis

Arabidopsis was grown in 10-cm pots filled with a 1:1 mixture of perlite and vermiculite under a long-day photoperiod (16-h light/8-h dark) at 22°C. Freezing stress involved transferring the 4-week-old or 3-week-old plants into a chamber, decreasing the temperature to −2°C, −4°C, −6°C, −8°C, and −10°C for 10 h respectively, then returning the temperature to 22°C. Plants were analyzed after recovery for 6 d in normal growth conditions.

For the drought stress evaluation, seedlings were grown in pots (10-cm diameter) filled with vermiculite for 2 weeks with constant watering before water was withheld. After 2 weeks without water, all the pots were watered simultaneously, and the plant regrowth was scored 7 d later. Plants were considered dead if all the leaves were brown and there was no regrowth 7 d after rewatering.

For water-loss assay, 10 fully expanded leaves from wild-type and OsMYB3R-2 plants that had developed approximately 14 leaves were detached and weighed at different times to determine the rate of water loss. Each experiment was carried out at least three times.

For the salt tolerance assay, transgenic and wild-type seeds were planted on MS agar plates for germination. Two days after germination, seedlings from each line were carefully transferred to a new MS agar plate supplemented with different concentrations of NaCl. After 7-d growth in treatment media, plants with absent green or dead cotyledons were scored. The root length of the seedlings was measured. We repeated freezing, drought, and salt tolerance experiments three times.

Localization of OsMYB3R-2-GFP Fusion Proteins

The localization assay was performed as described by Wang et al. (2004). The whole coding sequence of OsMYB3R-2 was amplified with two primers (5′-GAC CAG GGC TGT CCT GCT GCC CCT GGT GCT GCT-3′ and 5′-AGC CTT CCT CCT GCT GCT-3′, underlined) and (5′-GGT GGT TAC ATC CAA ATT GGT TG-3′, KpnI site underlined). The PCR product was subcloned into the pBII221 vector to generate pbII221-OsMYB3R-2-GFP containing an OsMYB3R-2-GFP fusion construct under the control of CaMV 35S. The construct was confirmed by sequencing and used for transient transformation of onion (Allium cepa) epidermis via a gene gun (Bio-Rad). Transformed onion cells were observed under a confocal microscope (Nikon).

Transformation of OsMYB3R-2 in Arabidopsis

The digestion product OsMYB3R-2 from pT-OsMYB3R-2 was directionally cloned into the KpnI-BamHI sites of an SN1301 vector to create SN1301-OsMYB3R-2, which carried a GUS marker. OsMYB3R-2 was driven by a CaMV 35S promoter in the construct. The construct was electroporated into the Agrobacterium tumefaciens C58. Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998).

DNA Gel-Blot Analysis

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Germination Assay

The sensitivity of seed germination to ABA and NaCl was assayed on MS agar plates saturated with ABA and NaCl solution (Xiong et al., 2001). Seeds from wild-type and transgenic plants were placed on MS agar plates saturated with distilled water or different concentrations of ABA or NaCl and incubated at 4°C for 48 h before being placed at room temperature under cool-white light for germination. Seeds were considered germinated when radicles completely penetrated the seed coat. Germination was scored daily up to 10 d after seeds were placed at room temperature.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number BAD81765.

Supplemental Data

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

Supplemental Table S1. List of up- or down-regulated genes in Yuedongdao rice under cold stress.
Supplemental Table S2. Primers used in RT-PCR to verify the gene expression in microarray.

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