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***OsMYB3R-2* enhances chilling tolerance in rice**

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Research area: Environmental Stress and Adaptation

Enhanced tolerance to chilling stress in *OsMYB3R-2* transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes

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Abstract

MYB transcription factors play central roles in plant response to abiotic stresses. How stress affects development is poorly understood. Here, we show that *OsMYB3R-2* functions in both stress and developmental processes in rice. Transgenic plants overexpressing *OsMYB3R-2* exhibited enhanced cold tolerance. Cold treatment greatly induced the expression of *OsMYB3R-2*, which encodes an active transcription factor. We showed that OsMYB3R-2 specifically bound to mitosis-specific activators (MSA) *cis*-element (T/C)C(T/C)AACGG(T/C)(T/C)A, a conserved sequence that was found in promoters of cyclin genes such as *OsCycB1*;1 and *OsKNOLLE2*. In addition, overexpression of *OsMYB3R-2* in rice led to higher transcript levels of several G2/M phase-specific genes, including *OsCycB1*;1, *OsCycB2*;1*, OsCycB2*;2, *OsCDC20.*1, than those in *OsMYB3R-2* antisense lines or wild type plants in response to cold treatments. Flow cytometry analysis revealed an increased cell mitotic index in overexpressed transgenic lines of *OsMYB3R-2* after cold treatment. Furthermore, resistance to cold stress in the transgenic plants overexpressing *OsCycB1*;1 was also enhanced. The level of cellular free proline was increased in the overexpressed rice lines of *OsMYB3R-2* and *OsCycB1*;1 transgenic plants compared with wild type under the cold treatment. These results suggest that OsMYB3R-2 target *OsCycB1*;1 and regulate the progress of the cell cycle during chilling stress. *OsCPT1*, which may be involved in the dehydration responsive-element binding factor 1A (DREB1A) pathway, showed the same transcription pattern in response to cold as did *OsCycB1*;1 in transgenic rice. Therefore, a cold resistance mechanism in rice could be mediated by

regulating cell cycle, which is controlled by key genes including *OsMYB3R-2*.

Introduction

Rice, a plant normally grown in tropical and temperate climate zones, is often threatened by cold stress and is especially sensitive to chilling stress at the seedling and reproductive stages (Mukhopadhyay et al., 2004). Staged cold can result in poor germination, stunted seedlings, yellowing or withering and reduced tillering. Unpredictable cold snaps at the reproductive stage delays heading and results in pollen sterility, which was thought to be one of the key factors responsible for the reduction in rice grain yield of rice (Kaneda C, 1974; Mackill DJ, 1997; Andaya and Tai, 2006; Suzuki et al., 2008). Transgenic approaches can be used to improve rice cold tolerance and screening for genes involved in cold tolerance is an important initial step for crop improvement strategy using engineering (Hsieh et al., 2002; Dubouzet et al., 2003; Choi et al., 2004; Choi et al., 2005; Ohnishi et al., 2005; Ito et al., 2006).

Transcription factors including Myb family, which have been widely studied in both animals and plants, play essential roles in regulating gene expression in response to environmental and developmental changes. According to the number of tandem repeats of the SANT 'SWI3, ADA2, N-CoR, and TFIIIB' DNA-binding domains (Rosinski and Atchley, 1998; Jin and Martin, 1999; Stracke et al., 2001), MYB proteins can be divided into three subfamilies: MYB-like proteins (MYB1R factors), R2R3-type MYB factors, and R1R2R3 MYB (MYB3R) factors with one, two or three repeats, respectively (Rosinski and Atchley, 1998).

The *Arabidopsis* MYB family consists of 198 genes and it is one of the largest families among all transcription factor families (Kranz et al., 2000; Yanhui et al., 2006; Pasquali et al., 2008). In *Arabidopsis*, large-scale insertional mutagenesis (Meissner et al., 1999; Stracke et al., 2001) and expression profiling analysis (Kranz et al., 2000; Yanhui et al., 2006) have been used to analyze comprehensive functions and to explore the roles of R2R3 MYB proteins in cell cycle control, secondary metabolism, cellular morphogenesis, meristem formation, hormonal signaling, and stress responses (Salomoni et al., 1997; Meissner et al., 1999). Several R2R3 MYB genes involved in the responses to environmental stimuli such as drought, salt and cold have been studied (Yanhui et al., 2006). *AtMYB2*, together with *AtMYC2*, are transcriptional activators of *RESPONSIVE TO DEHYDRATION22* (*RD22*). Expression of *RD22* is induced by drought and ABA (abscisic acid) (Urao et al., 1993; Ito et al., 1997). *AtMYB102* is a component of the regulatory complex that directs signaling pathways for respond to wounding, osmotic stress, and abscisic acid (ABA) (Denekamp and Smeekens, 2003). *AtMYB60*, a guard-cell-specific transcription factor, regulates stomatal movement in response to drought stress (Cominelli et al., 2005). *HOS10*, encoding an R2R3-type MYB transcription factor, is essential for acclimation to cold stress and may affect tolerance against dehydration via controlling ABA biosynthesis (Zhu et al., 2005). *MYB15* controls the expression of C-repeat binding factors (CBFs) and other genes responding to cold stress (Agarwal et al., 2006). Overexpression of *AtMYB44* can enhance tolerance to drought and salt stresses by reducing the expression of genes encoding PP2Cs, including ABI1, ABI2, AtPP2CA, HAB1, and

HAB2, which negatively regulate ABA signaling (Jung et al., 2008).

In rice, numerous transcription factors have been found to play important roles in response to cold stress. Overexpression of Zinc finger genes such as *OsiSAP8*, *OsCOIN,* and *OsISAP1* confers cold stress tolerance at a seedling stage (Mukhopadhyay et al., 2004; Liu et al., 2007; Kanneganti and Gupta, 2008). Overexpression of *OsbHLH1*, *OsDREB1*/*CBF*, *ROs-bZIP*, *SNAC2* and *OsNAC6* also enhanced transgenic seedling resistance to chilling stress (Wang et al., 2003; Ohnishi et al., 2005; Ito et al., 2006; Cheng et al., 2007; Nakashima et al., 2007; Hu et al., 2008). Overexpression of *OsMYB4* significantly increased tolerance to freezing stress in transgenic *Arabidopsis* (Vannini et al., 2004; Pasquali et al., 2008).

Signaling components and metabolic regulators have also been shown to be involved in stress response. *OsTPP1*, a gene encoding a trehalose-6-phosphate phosphatase, confers cold stress tolerance in rice and activated stress-responsive genes (Pramanik and Imai, 2005; Shima et al., 2007; Ge et al., 2008). *OsLti6* genes (*OsLti6a* and *OsLti6b*) encoding hydrophobic proteins homologous to *Arabidopsis* RCI2 enhanced tolerance to chilling stress in rice seedlings (Morsy et al., 2005). Stress responses in eukaryotic organisms can be mediated by the mitogen-activated protein kinase (MAPK) cascades. Overexpression of *OsMAPK5* conferred tolerance to drought, salt, and cold stresses in rice seedlings (Xiong and Yang, 2003). Stress-responsive *CIPK* genes encoding calcineurin B-like protein-interacting protein kinases such as *OsCIPK03* and *OsCIPK12* also play important roles in improving the tolerance to

chilling stress in rice (Xiang et al., 2007). *OsCDPK7* and *OsCDPK13* encoding Ca^{2+}-dependent protein kinases are positive regulators which enhance cold- and salt-stress tolerance (Saijo et al., 2000; Saijo et al., 2001; Wan et al., 2007; Wang et al., 2008).

The cell cycle is an intrinsic part of plant growth and development. However, less is known about how the cell cycle may be affected upon cold stress and how this may impact the plant's survival. Evidence suggests that cell cycle activities are involved in the stress response mediated by transcription factors (Morano et al., 1999; Luft et al., 2001; Nakai and Ishikawa, 2001; Santilli et al., 2005). MYB proteins, such as NtmybA1, NtmybA2 and NtmybB, may bind specifically bind to the core motif AACGG of DNA sequences, M phase-specific activator (MSA) *cis*-element, in tobacco *in vitro* (Ito et al., 2001; Araki et al., 2004; Suzuki et al., 2006; Haga et al., 2007). NtmybA1 and NtmybA2 mediate the transcription of a G2/M phase-specific gene in tobacco cells, whereas NtmybB functions as a transcription repressor. MYB3R1 and MYB3R4, homologs of NtmybA1 and NtmybA2, respectively, positively regulate cytokinesis mainly through transcriptional activation of the *KN* gene in *Arabidopsis* (Haga et al., 2007).

Our previous studies have indicated that *OsMYB3R-2* transgenic *Arabidopsis* plants are more resistant to freezing, drought, and salt stresses (Dai et al., 2007), leading us to speculate whether the regulation of the cell cycle is involved in the *OsMYB3R-2*-modulated stress response. Here we show that *OsMYB3R-2* is involved

in regulating the responses to cold stress in rice. We demonstrate that OsMYB3R-2 functions as a MYB3R transcription factor targeting to *OsCycB1*;1, which is involved in the G2/M phase transition at low temperature. The transcript level of *OsCPT1*, a putative member of the dehydration responsive element binding factor 1 (DREB1)/CBF pathway, is also enhanced by *OsMYB3R-2*, accompanied by an increased proline level. Our data indicates that in rice, *OsMYB3R-2* may play an important role in the cold-stress signaling pathway modulated by the cell cycle and a putative DREB/CBF pathway.

Results

Molecular characteristics and phenotypes of *OsMYB3R-2* transgenic rice

Transgenic lines of *OsMYB3R-2* in rice were confirmed by hygromycin selection and GUS staining. The results of Northern blot and real-time PCR showed that expression of *OsMYB3R-2* was increased in the four independent overexpressing lines, but decreased in the four antisense lines (Fig. 1A, B). Southern blot analysis with a specific *GUS* probe showed diverse expression patterns in the four overexpressed lines，as well as in the four antisense lines (Fig. 1C, D).

To examine the expression patterns of *OsMYB3R-2 in vivo*, transgenic rice lines were generated with a GUS expression construct driven by a putative *OsMYB3R-2* promoter of 1285-bp in length. GUS staining assay of T_1 rice plants showed strong signals in nearly all tissues examined, including roots, internodes, nodes, leaf blades,

lamina joints, sheaths, glumes of flower organs, and young embryos of immature seeds (Fig. 1E), suggesting that *OsMYB3R-2* is a constitutively expressed gene.

Transgenic rice seeds of either the *OsMYB3R-2* overexpressors or the antisense lines did not differ from those of wild type in germination (Fig. 2). However the overexpressing plants showed growth retardation in comparison to wild type plants under normal conditions (Fig. 2B, C). The length of root cell resulted in shorter roots in the overexpression transgenic lines (Figure S3). When wild-type plants reached the tetraphyllous stage, more than 80% of the *OsMYB3R-2* overexpression plants were still at the trefoil stage (Fig. 2B). Growth retardation was observed in transgenic plants up to the heading stage.

Overexpression of *OsMYB3R-2* enhanced tolerance to chilling stress in rice

To test the possible effect of *OsMYB3R-2* expression on tolerance to chilling, the T_2 transgenic and wild-type seedlings at trefoil stage were exposed to reduced temperature (2□) for various durations, followed by incubation at a normal growth condition in a greenhouse for 2 weeks. Few than 20% of the wild-type seedlings survived a cold treatment of 72 hrs, but none of them were able to resume growth when transferred to normal growth conditions. However, more than 50% of the *OsMYB3R-2* overexpressing seedlings could survive and grow normally (Fig. 3B, C). The survival ratio of antisense seedlings was less than that of the wild type. A time course of the treatment showed drastic differences as the process was extended (Fig.

3D). The wild type and the transgenic seedlings showed no growth difference after chilling treatment for up to 48 h. In contrast, when the time of treatment was extended up to 60 h，more than 80% of the *OsMYB3R-2*-overexpressing plants grew normally, as compared with 55% of the wild type and 45% of the antisense seedlings. Finally after 84 h, neither wild type nor antisense plants survived, but in contrast, 20% of the overexpression lines were still healthy. Therefore, *OsMYB3R-2* plays an important role in regulating tolerance against chilling stress in rice.

To investigate the functions of *OsMYB3R-2* in DREB/CBF stress pathways, we tested the expression patterns of more than 10 genes by RT-PCR in wild-type and transgenic rice plants. One of the genes, *OsCPT1* was activated by *OsMYB3R-2* under cold stress, a deduced target gene of the DREB pathway with the DRE/CRT *cis*-elements (Fig. 4A). A DRE/CRT *cis*-element, CCGACCT, appeared in the upstream sequence (602-596 bp) of *OsCPT1* promoter. The transcription levels of rice DREB genes including *OsDREB1A*, *OsDREB2A* and *OsCBF* didn't show any changes in *OsMYB3R-2* overexpressing transgenic lines under the cold conditions compared with wild type. For the expression patterns of other cold-regulated (*COR*) genes such as *OsCORTM1*, *OsMAT1*, which are the rice homologs of target genes of *Arabidopsis* DREBs (Dubouzet et al., 2003; Chen et al., 2008) (Figure S1 and Table S1), there were no differences between the transgenic lines and wild type. These data suggest that *OsMYB3R-2* may regulate the plant response to cold stress through the deduced *OsCPT1*-involved DREB/CBF pathway in rice.

Under a normal growth conditions (25°C), the levels of cellular free proline did not differ between wild-type and transgenic rice at a range of 112-118 µg fresh-weight (FW) of material (Fig. 4B). In contrast, after cold treatment (2°C), the levels of free proline in *OsMYB3R-2* overexpressing transgenic rice increased substantially with more than 300 µg/g FW as compared with 188 µg/g FW in the wild-type plants. These results were similar to the alterations observed in other transgenic plants overexpreesing the resistant genes such as *OsDREB1, OsCOIN, OsCIPK03* and *OsCIPK12*, which showed resistance to cold stress in rice (Ito et al., 2006; Liu et al., 2007; Xiang et al., 2007). Thus, cellular free proline level is involved in enhanced resistance to cold regulated by *OsMYB3R-2* via a putative DREB/CBF-CPT pathway in rice.

OsMYB3R-2 protein showed transcription activation

A yeast GAL4 system was used to investigate the transcription activation of OsMYB3R-2 (Fig. 5A). OsMYB3R-2 mutants deleted in various domains were tested. The N and C termini of OsMYB3R-2 were truncated and the products termed No. 2 to 14, respectively, with the full-length termed No. 1. Fig. 5 shows a yeast growth analysis on screened medium with SD/-Trp/-Ade, SD/-Trp/-His, or SD/-Trp/-His/-Ade, as well as in the galactosidase assay. Stronger blue signals corresponding to good growth of yeast on both media appeared in Nos. 1, 2, 3, 9, 11 and 13 compared with the control empty vector and the remaining constructs. A common region among constructs was the region of 350-500 amino acids at the C terminus. These results

suggest that the OsMYB3R-2 protein has transcriptional activation activity, and the core region with the activity was from 350-450 at the N terminus.

OsMYB3R-2 targeted type-B cyclin gene *OsCycB1*;1

Bioinformatic analysis showed MSA-like sequences were found in the promoters of cyclin genes in rice (La et al., 2006). Two MSA-like sequences of *OsCycB1*;1 appeared between -200 to -400 bp upstream of the transcription start site. A fully conserved central core pentamer, AACGG, was found in the MSA-like elements. There is a 3-bp less conserved sequence at each side of the core motif. The MSA consensus sequence (T/C)C(T/C)AACGG(T/C)(T/C)A is shown in Fig. 6A (Ferreira et al., 1994; Day et al., 1996; Ito et al., 1997; Ito et al., 1998). It matches the consensus sequences of c-Myb and v-Myb binding sites (Howe and Watson, 1991; Grotewold et al., 1994; Ito et al., 1998), which suggests that the Myb transcription factors may bind the MSA motif.

To test whether OsMYB3R-2 interacts the MSA motif in the promoter of *OsCycB1*;1, electrophoretic mobility shift assay (EMSA) was carried out. EMSA results showed that OsMYB3R-2 can specifically bind the *OsCycB1*;1 promoter of a 378-bp fragment in rice (Fig. 6B, C). EMSA analysis of two MSA elements (RT1 and RT2) from the *OsCycB1*;1 promoter (Fig. 6B, D) showed that the mobility of OsMYB3R-2 specifically shifted with the MSA elements from type-B cyclin genes on the membrane map (Fig. 6E).

Assay of the mutated RT1 sequence of the *OsCycB1*;1 promoter showed that any mutation could weaken the DNA binding ability of OsMYB3R-2 protein (Fig. 6E), whereas the DNA binding ability was abolished by base substitution of RT1mut5. Therefore, we concluded that the CCCAACGG sequence in the *OsCycB1*;1 promoter was recognized by OsMYB3R-2 protein.

We further examined the expression patterns of genes related to the cell cycle (Table S2). Compared to the expression levels of type-A and -D cyclins, the expression levels of type-B cyclin genes *OsCycB1*;1, *OsCycB2*;1, *OsCycB2*;2 and *OsCDC20*.1 were suppressed by cold treatment in both the wild-type and the antisense plants. In the *OsMYB3R-2*-overexpressed lines under the cold treatment, expression patterns of those type-B cyclin genes were the same as those at room temperature (25□) (Fig. 7A).

To test whether the cold tolerance phenotype could be reproduced by overexpressing *OsCycB*s, transgenic rice overexpressing lines of *OsCycB1*;1 as well as RNAi lines were tested for cold stress. The results showed that the wild-type seedling plants with less than 58% could survive after the treatment for 72 hrs, whereas more than 67% of overexpressed *OsCycB1*;1 seedlings could survive and grow normally (Fig. 7B). In contrast, less than 33% of *OsCycB1*;1-RNAi seedlings could resume growth under normal growth conditions. Our data showed that the overexpressing lines of *OsCycB1*;1 enhanced the tolerance to chilling stress compared with wild type. This suggests that *OsCycB1* is likely to be one of the downstream genes regulated by

OsMYB3R-2 under chilling stress.

To investigate whether there is any relationship between proline level and resistance to cold stress in *OsCycB1*;1-overexpressing transgenic rice plants, level of the cellular free proline was monitored. The results showed that under a normal growth condition (25°C), the levels of cellular free proline were similar in both wild-type and *OsCycB1*;1 transgenic rice at a range of 120-124 μg/g fresh-weight (FW) materials (Fig. 7C). In contrast, after cold treatment (2°C), the level of free proline in *OsCycB1*;1-overexpressing transgenic rice increased substantially with 243 μg/g FW as compared with 197 μg/g FW in wild type and 185 μg/g FW in *OsCycB1*;1-RNAi plants. These results of the changed pattern for cellular free proline were similar to those in *OsMYB3R-2* transgenic plants. Taken together, the data suggested that *OsCycB1*;1 was direct regulated by *OsMYB3R-2,* which was involved in the tolerance to cold in rice.

Cell cycle progression in transgenic rice lines

Based on the results of the expression levels of cyclins, we monitored the mitotic index of *OsMYB3R-2*-overexpressed lines under cold conditions. Mitotic index is defined as the ratio between the number of cells in mitosis and the total number of cells, which is used to be a measure for the proliferation status of a cell population. Flow cytometry revealed that DNA content of the *OsMYB3R-2*-overexpressed lines increased at 4^{o}C as compared with the wild type under a normal (28□) or a cold condition (4□) (Fig. 8A-D). Thus, the overexpressing lines possessed more cells in

the G2/M phase, especially under the cold conditions. Under the normal conditions, the overexpressing lines showed a higher mitotic index than the wild-type and the antisense lines. Under cold conditions, in contrast, the mitotic index in the overexpressing lines was markedly higher than that of the wild type; and the index of the antisense lines was notably lower than that of the wild type under cold stress (Fig. 8E). The decreased percentage of the mitotic index under cold stress compared with normal conditions (28□) was 24.3%, 11.6%-14.5% and 33.5%-38.6% in the wild type, the overexpressing lines and the antisense lines, respectively. The changes in the mitotic index correlated to expression pattern of *OsMYB3R-2*. Therefore, we conclude that *OsMYB3R-2*-overexpressing lines possess more cells at the G2/M cell-cycle phase, which promoted an increased mitosis.

Discussion

OsMYB3R-2 is a positive regulator for a subset of G2/M phase-specific genes in rice

MYB3R genes constitute a small gene family of transcription factors in plants that play regulatory roles in the cell cycle and in response to environmental stresses (Ito et al., 2001; Araki et al., 2004; Dai et al., 2007; Haga et al., 2007). *MYB3R* genes exert their functions through binding to MSA elements which mediate the G2/M phase of the cell cycle (Ito et al., 2001; Araki et al., 2004). In *Arabidopsis*, 5 R1R2R3-type Myb genes have been described (Braun and Grotewold, 1999; Kranz et al., 2000; Haga et al.,

2007). *MYB3R1* and *MYB3R4* are two genes homologous to NtmybA and NtmybA2 (Haga et al., 2007), which positively regulate cytokinesis through activating the transcription of several G2/M phase-specific genes in *Arabidopsis*. The defects of multinucleate cells and cell-wall stubs in the *myb3r1 myb3r4* double mutant are caused by the selective reduction of transcript levels of several type-B2 cyclin genes, including *CYCB2*, *CDC20.1* and *KNOLLE* (*KN*) (Haga et al., 2007).

OsMYB3R-2 protein functions as an R1R2R3-type MYB transcription factor. It has three tandem SANT (SWI3, ADA2, N-COR and TFIIIB) DNA-binding domains, is nuclearly localized, and binds to specific *cis*-elements (Dai et al., 2007). OsMYB3R-2 can recognize the consensus sequence (T/C)C(T/C)AACGG(T/C)(T/C)A with the core motif CCAGG (Fig. 6). Although the promoters of *OsCycB1*;1 and *OsKNOLLE2* contain the core motif, only *OsCycB1*;1 (a type-B cyclin gene) can be activated to a high transcriptional level under cold conditions (Fig. 6 and Fig. 7). Therefore, our evidence supports that OsMYB3R-2 functions as a positive regulator to modulate the G2/M phase of the cell cycle via *OsCycB1*;1 in rice.

OsMYB3R-2 coordinates the cell cycle and a deduced DREB/CBF pathway to increase cold tolerance in rice

OsMYB3R-2 functions as a transcription factor with a specific DNA binding characteristic (Fig. 6). The increased mitosis index in transgenic rice of overexpressing *OsMYB3R-2* indicates that *OsMYB3R-2* probably regulates the process of the cell cycle (Fig. 8), showing a similar function to that of its homologs

such as *NtmybA1* and *NtmybA2*, *MYB3R1* and *MYB3R4* (Ito et al., 2001; Araki et al., 2004; Suzuki et al., 2006; Haga et al., 2007). Under cold conditions, we found that some genes of cyclin B-type including *OsCycB1*;1, one target gene of OsMYB3R-2, were activated to high levels of transcription in the *OsMYB3R-2* overexpressing transgenic lines (Fig. 6 and Fig. 7). These results suggested that OsMYB3R-2 play an essential role in maintaining a high progression of the cell cycle under cold stress.

In *Arabidopsis*, the transcript levels of *DREB2A*, *COR15a*, and *RCI2A* are induced in 35S::*OsMYB3R-2* plants (Dai et al., 2007). There is a correlation between the enhanced tolerance to environmental stress in 35S::*OsMYB3R-2* plants and the up-regulation of stress-responsive genes. Our data suggest that OsMYB3R-2 may play an important role in the complex gene network controlling the stress signaling pathways (Dai et al., 2007). *OsDREB* genes, encoding transcription activators, function in response to drought, high salt, and cold stress in rice (Dubouzet et al., 2003; Chen et al., 2008). The expression of *DREB2A* is enhanced in *OsMYB3R-2*-overexpressed plants (Dai et al., 2007). Contrastively , in *OsMYB3R-2* transgenic rice, the transcripts of rice DREB genes and their putative target genes did not show any changes distinctly compared with that in wild type except for *OsCPT1* (Figure S1 and Table S1), The expression of *OsCPT1* , the homolog of *At2g02100*, was activated by *OsMYB3R-2* in overexpressed transgenic lines under cold stress (Fig. 4). In *Arabidopsis*, *At2g02100* which encodes a putative protease inhibitor II is a target gene of DREB1A/CBF3 with unknown function (Dubouzet et al., 2003; Chen et al., 2008). Furthermore, a DRE/CRT *cis*-element (CCGACCT) was involved upstream

of the *OsCPT1* promoter 602-596 bp from the transcription start site in rice, indicating that *OsCPT1* might be involved in the DREB/CBF pathway. Therefore, overexpression of *OsMYB3R-2* activates the expression of *OsCPT1* in the process of response to cold of rice plants, which is putatively targeted by DREB genes.

Two pieces of the core motif (CCAGG) of MSA and a consensus MYB binding site (TAACTG) (Urao et al., 1993) were found in the promoters of *DREB2A* and *RCI2A* in *Arabidopsis*. And a core motif (CCAGG) of MSA and a consensus MYB binding site (TAACTG) were also appeared in the promoter of *COR15A*. There is a report that MYB proteins can specially bind to the specific DNA sequences including their consensus MYB binding sites (Xue, 2005). Therefore, it suggested that OsMYB3R-2 can bind to multiple elements and activate a diverse set of genes.

Tolerance to cold stress is controlled by complex mechanisms involving many changes, including membrane lipid composition, accumulation of compatible solutes, and expression of cold-regulated (*COR*) genes. A downstream change is the up-regulation of cellular proline levels with overexpression of genes showing resistance to cold stress (Thomashow, 1999; Korenjak et al., 2004). We found a remarkable increase in cellular free proline levels with *OsMYB3R-2* overexpression or *OsCycB1*;1 overexpression after cold treatment (Fig. 4B and Fig. 7C). This pattern is similar to that of other genes that enhance resistance to cold stress such as *OsDREB1*, *OsCOIN*, *OsCIPK03* and *OsCIPK12* (Ito et al., 2006; Liu et al., 2007; Xiang et al., 2007). Therefore, the up-regulated level of cellular free proline may be one of the common characteristics of genes conferring resistance to cold stress.

Our experimental observations of rice seedlings with either cold-sensitive traits or resistant traits showed no morphological difference in growth at the early stages under cold conditions. When plants were returned to normal conditions, 25-28□, the leaves rapidly withered in both kinds of plants. However, newly differentiated leaves were formed in *OsMYB3R-2*-overexpressing cold-resistant plants. Cold-resistant plants may have higher competence in maintaining cell division under cold stress than cold-sensitive plants, which is supported by our observations on the cell-cycle (Fig. 8).

Taken together, we conclude that OsMYB3R-2 is a MYB3R transcription factor and *OsCycB1*;1 is one of its target genes regulated under cold conditions. The enhanced cold stress tolerance of 35S::*OsMYB3R-2* and 35S::*OsCycB1*;1 rice plants reveals that OsMYB3R-2 can mediate cold stress signal transduction and regulate some stress-responsive genes involved in cell cycle or a deduced DREB/CBF pathways. Although it is still not clear what the sensing mechanism of *OsMYB3R-2* to chilling stress in rice is, the functions of *OsMYB3R-2* in the cell cycle and cold tolerance will provide new insights into cold stress pathways. The information gained will be beneficial for directing molecular breeding strategies to generate rice varieties with enhanced tolerance to cold stress.

Materials and methods

Plant materials and growth conditions

The plant material used was *Oryza sativa* L. ssp. japonica cv *Zhonghua 10*. Rice seeds were germinated in a small plastic boxes filled with a mixture of flower nutrimental soil and vermiculite (8:1) for at least 14 days. The rice seedlings at trifoliate stage were transferred to grow in big plastic buckets at 30□ during the day and 20□ during the night in a greenhouse.

Plasmid construction and plant transformation

Total RNA of rice seedlings was isolated by use of TRIzol Reagent (Invitrogen). The cDNA of rice was synthesized using of AMV Reverse Transcriptase (Promega). Full-length *OsMYB3R-2* was amplified by RT-PCR with pyrobest DNA polymerase (TaKaRa), ligated into pGEM®-T Easy vector (Promega) and sequenced (Dai et al., 2007). The digestion product of *OsMYB3R-2* from pT Easy-*OsMYB3R-2* was directionally cloned into the *Kpn*I-*Bam*HI sites of a pUN1301 vector to create the pUN1301-*OsMYB3R-2* construct, which carried a GUS marker, with the forward primer 5′GGATCCATGGGGGCCATGGCGATGGTG3′and the reverse primer 5′GGTACCGGTTACATCAAATTGGTTGT3′. A pUN1301-antisense-*OsMYB3R-2* construct was created with the forward primer 5′GGTACCATGGGGGCCATGGCGATGGTG3′ and the reverse primer 5′GGATCCGGTTACATCCAAATTGGTTGT3′. *OsMYB3R-2* was driven by a ubiquitin promoter in the construct. The pUN1301-*OsMYB3R-2* construct was electroporated into the *Agrobacterium tumefaciens* EHA105. Rice embryonic calli were induced on scutella from germinated seeds and transfected with *A. tumefaciens* EHA105

containing the desired binary vector as described previously (Ge et al., 2004). *OsMYB3R-2* transgenic plants were screened in half-strength Murashige and Skoog (MS) medium containing 75 mg L^{-1} hygromycin (Sigma). Transgenic plants of the T_0 generation from calli with hygromycin-resistant plants were transplanted into soil and grown in a greenhouse.

Construction of 35S::*OsCycB1*;1 (ACCESSION No. : AY647458) vector and RNAi plasmid were done according to methods of Jiang et al (2007) and Wang et al (2004). Gene transformation used the method described above.

Construction of *OsMYB3R-2* promoter::*GUS* vector and transformation

The *OsMYB3R-2* promoter of 1285 bp was amplified by PCR from the rice genome and inserted into the upstream of *GUS* at the *Kpn*I-*Bam*HI sites of the pGUS1301 vector (Ge et al., 2004). The *OsMYB3R-2* promoter-pGUS1301 was constructed with the primer pair 5' GGGTACCCCAACTCGTATTGCTCCTCTT 3' and 5' CGGATCCA-CGGCACAAGCACATCCTCA 3'. The construct of the *OsMYB3R-2* promoter-pGUS1301 was electroporated into *A. tumefaciens* EHA105 and transfected into rice embryonic calli as described previously (Ge et al., 2004). GUS staining was used to investigate the *OsMYB3R-2* expression in the T_1 generation of *OsMYB3R-2* promoter::*GUS* transgenic rice.

RT-PCR and real-time PCR

Total RNA was isolated from rice seedlings was isolated by use of TRIzol Reagent (Invitrogen). The cDNA of rice was synthesized using of AMV Reverse Transcriptase (Promega) in a 25-μl reaction containing 2 μg total RNA. Reverse transcription reactions were carried out at 42□ for 60 min followed by chilling on ice for 5 min. An amount of 2 μl of 5-fold diluted cDNA was used as a PCR template in a 20-μl RT reaction mixture. All PCR products were loaded onto 0.8% agarose gel to visualize the amplified cDNAs. RT-PCR reactions were repeated 3 times. RT-PCR of *OsCPT1* involved the forward primer 5´CGGTGGCAGTAGGAAAGTAG3´ and the reverse primer 5´CATGAACAACAGACAAAGGAGA3´ with 28 cycles. *Tubulin* with the forward primer 5'TCAGATGCCCAGTGACAGGA3' and the reverse primer 5'TTGGTGATCTCGGCAACAGA3' was used as a control for 25 cycles. Real-time PCR was used to investigate the level of *OsMYB3R-2* in antisense lines with the primers: 5´AGGTCCACCAATCATTCTCC3´ and 5´GTAAATTCACAAAGTGCAGCG 3´, which were designed in the 3´UTR region to identify the knock-down efficiency of the endogenous *OsMYB3R-2* gene. The methods for real-time PCR were previously described in detail (Dai et al., 2007).

Southern and Northern blot assays

Genomic DNA was isolated from 2-week-old rice seedlings and digested with *Eco*RI or *Hin*dIII. DNA of 20 μg was used for Southern blot analysis as described previously (Ge et al., 2004). The fractioned DNA underwent electrophoresis on 0.7% (w/v) agarose gel and has then blotted onto a nylon membrane (Amersham Pharmacia

Biotech). The membrane was prehybridized at 65□ for 4 h and hybridized in the same solution containing α-^{32}P-dCTP-labeled *GUS* for 20 h at 65□. The *GUS* of 680 bp was amplified by PCR with the forward primer 5'CAACTGGACAAGGCACTAGC3' and the reverse primer 5'AGCGTCGCAGAACATTACAT3'. The membrane was washed with washing buffer (2×SSC plus 0.1% SDS) at 65□ for 20 min after hybridization and then washed twice with 1×SSC plus 0.1% SDS at 65□ for 15 min. The membrane was stored at -70□ for 3-7 days and then exposed to X-ray film (Eastman-Kodak).

Northern blotting was performed as described previously (Ge et al., 2004). Total RNA was extracted from 2-week-old rice seedlings with use of TRIzol Reagent (Invitrogen). Each lane was loaded with 20 μg of total RNA isolated from 2-week-old seedlings. Ethidium bromide-stained rRNA was used as an RNA-loading control. Total RNA was loaded in each lane on 1% agarose gel containing 0.4 M formaldehyde and transferred to Hybond-N$^+$ membrane (Amersham Pharmacia Biotech). A probe of *OsMYB3R-2* cDNA labeled with α-^{32}P-dCTP was prepared by PCR for hybridization. After hybridization for 20 h at 65□, the membrane was washed and exposed to X-ray film. The loading control was ribosomal RNA stained with ethidium bromide.

Treatment of rice seedlings with chilling stress (2°C)

To analyze the response to cold stress, T_2-generation transgenic plants with positive *GUS* staining were used. To ensure that the seedlings were at the same morphological stage as wild-type (WT) and *OsMYB3R-2* transgenic lines, the

transgenic seeds with *OsMYB3R-2* overexpressing were sowed 36 h earlier than those of WT and antisense lines under the same conditions of 12-h light/12-h dark (30□/26□). The seeds of WT and antisense lines were sowed at the same time because they showed no difference in germination and plant growth. All seeds were germinated in a mixture of nutrimental soil and vermiculite (8:1). Two-week-old seedlings at the early period of the tetraphyllous leaf stage were subjected to treatment at 2°C in the Low-temperature Biochemical Incubator (BTI100, LEAD TECH, USA). At 0, 24, 48, 60, 72 and 84 h, cold-treated seedlings were moved to a greenhouse for 2 weeks. Surviving seedlings were photographed and analyzed to investigate the response of *OsMYB3R-2* transgenic plants to chilling stress.

To analyze the response of *OsCycB1*;1 to cold stress, T_1-generation transgenic plants with positive *GUS* staining were used. Two-week-old seedlings at the early period of the tetraphyllous leaf stage were treated at 3°C in the low-temperature cultivation room (7 meters' length and 3.5 meters' width), cold-treated seedlings were moved to a greenhouse for 1 week. Surviving seedlings were analyzed to investigate the response of *OsMYB3R-2* transgenic plants to chilling stress. The method of cold treatment was similar to that described in detail as shown above.

Transactivation assay based on the yeast GAL4 system

The cDNA fragments of *OsMYB3R-2* were generated by PCR amplification, cloned into *Nde*I and *Pst*I sites and fused in-frame to the GAL4 DNA binding domain in the

pGBKT7 vector. ATG bases were added after the *Nde*I site to all the forward primers, except for the forward primer for PCR amplification of full-length *OsMYB3R-2*. A transactivation assay was performed as described (Choi et al., 2004). The constructs of *OsMYB3R-2*-pGBKT7 were transformed into AH109 cells by the lithium acetate-mediated method (Gietz et al., 1992), and transformants were selected on synthetic medium plates (SD medium) lacking tryptophan at 28°C for 2 days. Yeast transformants from SD medium lacking tryptophan were then transferred and streaked onto solid SD agar medium lacking tryptophan/histidine (SD/-Trp/-His), tryptophan/adenine (SD/-Trp/-Ade) or tryptophan/histidine/adenine (SD/-Trp/-His/-Ade) to score the growth response after 3 days. For the β-galactosidase assay, the transformants were blotted on Whatman filter paper, and the cells imprinted on the filter were lysed by freezing in liquid nitrogen, then thawed at room temperature. The filter was then, incubated in 2.5 ml Z buffer containing 0.8 mg 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), which consisted of 16.1 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$, 5.5 g L^{-1} $NaH_2PO_4 \cdot H_2O$, 0.7 g L^{-1} KCl, 0.246 g L^{-1} $MgSO_4 \cdot 7H_2O$ (pH 7.0) at 30°C, and monitored for color reaction.

Determination of cellular proline levels

Fresh material (0.5 g) of 2-week-old seedlings was taken from wild-type and *OsMYB3R-2* transgenic rice (T_2 generation) or *OsCycB1*;1 transgenic rice (T_1 generation) at the same stage with or without cold treatment (4□) for 24 h. The method of obtaining rice seedlings at the same morphological stage was described in

detail in “Treatment of rice seedlings with chilling stress”. The samples were homogenized in 2 ml of 3% aqueous sulfosalicylic acid and centrifuged. The content of cellular free proline was measured using of a spectrophotometer at λ=520 nm as described previously (Wang et al., 2006).

Electrophoretic mobility shift assay (EMSA)

The coding sequence of *OsMYB3R-2* with R1R2R3 tandem copies of DNA binding domains was amplified by PCR and cloned into pGEX-4T-1 vector with a glutathione S-transferase (GST) gene and sequenced. A forward primer, 5´CGCGGATCCATGGGTTGGGGCGCGGTGG3´, and a reverse primer, 5´CCGGAATTCTCAATCAATTGGGTGCTTGTCTG3´, were used in PCR. The OsMYB3R-2-GST fusion protein was induced in the strain BL21 (DE3) in *E. coli* and purified as described previously (Han et al., 2005).

A 378-bp fragment upstream of the *OsCycB1*;1 promoter was amplified by PCR from the rice genome and sequenced. The primer pair used was 5´AGCATTTCTGAGGAAGAAGT3´and 5´ATACAACTTTATTCTTCCCT3´. The PCR product of the 378-bp fragment was purified with use of the TIAN Quick Oligo Purification Kit (TIANGEN, China) and labeled with use of the Biotin 3´ End DNA Labeling Kit (PIERCE). Eight pairs of synthetic oligonucleotides containing optimal and mutant derivatives of the binding site for OsMYB3R-2 were labeled with use of the Biotin 3´ End DNA Labeling Kit (PIERCE). The 50-µl reaction mixture was mixed

gently with the following components: ultrapure water (25 µl), 5×TdT reaction buffer (10 µl), unlabeled oligo (1 µM, 5 µl), biotin-11-dUTP (5 µM, 5 µl), and diluted TdT (2 U/µl, 5 µl). Anneal oligos were mixed with equal amounts of labeled complementary oligos and denatured at 90□ for 1 min, then slowly cooled and incubated at melting temperature for 30 min, and stored at -20□. Frozen annealed oligos were thawed on ice for immediate use. The oligonucleotides were as follows: RT1WT (5´AATACCAGTCCCCAACGGCTAGTTTCAACC3´ and 5´GGTTGAAACTAGCCGT-TGGGGACTGGTATT3´); RT2WT (5´GCTAGGAGAGCTGGCAACCTCCGCAGCGG-G3´ and 5´CGATCCTCTCGACCGTTGGAGGCGTCGCCC3´); RT3 WT (5´CGC-CACCCTACCCAACGGCTCTATTCCCCT3´ and 5´AGGGGAATAGAGCCGTTGGG-TAGGGTGGCG3´); RT1WTmut1 (5´AATACCTTTCCCCAACGGCTAGTTTC AACC3´ and 5´GGTTGAAACTAGCCGTTGGGGAAAGGTATT3´); RT1WTmut2 (5´ AATACCAGATCCCAACGGCTAGTTTCAACC3´ and 5´GGTTGAAACTAGCCGTTG -GGATCTGGTATT3´); RT1WTmut3 (5´AATACCAGTCCTTAACGGCTAGTTTCAA -CC3´ and 5´GGTTGAAACTAGCCGTTAAGGACTGGTATT3´); RT1WTmut4 (5´AA-TACCAGTCCCCTTCGGCTAGTTTCAACC3´ and 5´GGTTGAAACTAGCCGAAGGG-GACTGGTATT3´); RT1WTmut5 (5´AATACCAGTCCCCAATTGCTAGTTTCAACC3´ and 5´GGTTGAAACTAGCAATTGGGGACTGGTATT3´).

Standard reaction mixtures (20 µl) for EMSA contained 2 µl purified proteins, 2 µl biotin-labeled annealed oligonucleotides, 2 µl 10×binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 1 µl 50% glycerol, 1 µl 1% NP-40, 1 µl 1 M KCl, 1 µl 100 mM $MgCl_2$, 1 µl 200 mM EDTA, 1 µl 1µg/µl poly (dI-dC) and 8 µl ultrapure water.

The reactions were incubated at room temperature (25□) for 20 min. The 10% native polyacrylamide gel was prepared and pre-run in 0.5×TBE buffer (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA, pH 8.3) for 30-60 min at 100 V before loading the samples, then the gel was run at room temperature in 0.5×TBE buffer at 100 V for 60 min until the bromophenol blue dye reached three-fourths of the gel. The gels were sandwiched and transferred to N^+ nylon membrane (Millipore) in 0.5×TBE buffer at 380 mA in a 4□ refrigerator for 60 min. When the transfer was complete, the membrane was placed with the bromophenol blue side up on a dry paper towel until the buffer on the membrane surface absorbed into the membrane and then was cross-linked for 10-15 min with the membrane face down on a transilluminator equipped with 312-nm bulbs. The detection of biotin-labeled DNA by chemiluminescence followed the manual of the LightShift® Chemiluminecent EMSA Kit (20148, PIERCE).

Flow cytometry of cell cycle progression

T_2-generation seeds of *OsMYB3R-2* transgenic rice were sterilized with 0.15% mercuric chloride and germinated on the filter with sterilized water at 28□ in the dark for 7 days. To obtain seedlings at the same stage as that of WT and *OsMYB3R-2* transgenic lines, the transgenic seeds with overexpressed *OsMYB3R-2* were germinated 16 h earlier than those of WT and antisense lines under the same conditions in petri dishes (diameter, 20 cm). All the GUS-positive seedlings of WT and *OsMYB3R-2* transgenic rice were assigned in equal quantity and subjected to 28□ or

4□ for 24 h, respectively. Samples of cell nuclei were prepared as described by Galbraith (Galbraith et al., 1983). Root apical tips (1 mm) were excised, immediately chilled on ice, and chopped with a single-edged razor blade in a glass petri dish (diameter, 5 cm). Chopping buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, and Triton X-100 [1 mg/ml], pH 7.0) was used to release the cells from the chopped tissues. The DNA content of individual transgenic cells was determined by flow cytometry. Cell nuclei were prepared for FACSAria™ by staining with 2 μg/ml DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride). Each sample was prepared three times and subjected to FACS Caliber cytometry three times (BD Corp). 10,000 nuclei were designed to be measured per analysis.

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Figure legends

Figure 1. Identification of *OsMYB3R-2* transgenic rice and its expression pattern

A, Northern blot assay of rice transgenic plants. Total RNA isolated from wild-type (WT) or transformed plants underwent hybridization with a α-^{32}P-dCTP-labeled probe of *OsMYB3R-2* cDNA as described in “Materials and methods”. B, Real-time RT-PCR of the expression of *OsMYB3R-2* in antisense lines. C and D, Southern blot assay of transformed rice plants. Genomic DNA isolated from wild-type (WT) or transformed plants were digested with *Eco*RI (E) or *Hin*dIII (H). The blot was hybridized with the open reading frame of the *GUS* gene labeled with α-^{32}P-dCTP. OL3, OL5, OL7, OL8 and AL1, AL2, AL4, AL5 representing overexpression (O) and antisense (A) lines of *OsMYB3R-2* transgenic rice, respectively. E, Expression pattern of *OsMYB3R-2 in vivo.* GUS staining to show expression pattern of *OsMYB3R-2 in vivo* in various tissues from the T_1 generation of *OsMYB3R-2* promoter::*GUS* transgenic rice. a: root, b: young internode, c: mature internode, d: node, e: mature leaf, f: lamina joint, g: leaf sheath, h: flower, i: immature seed.

Figure 2. Phenotype analysis of T_2 generation of *OsMYB3R-2* transgenic rice

A, The germination and growth of *OsMYB3R-2* transgenic rice at 30□. Shelled seeds of rice at 0 d; germinating seeds at 1, 2 and 3 d; young seedlings transferred to light (12-h light/12-h dark, 30□/26□) at 4 d and 6 d. B, Two-week-old seedlings (T_2

generation) of *OsMYB3R-2* transgenic rice. C, Transgenic overexpressing seedlings *OsMYB3R-2* at tillering stage for 35 days after germination. d: day; WT: wild type; OL3, OL5, OL7: *OsMYB3R-2*-overexpressing rice; AL1, AL2, AL4: *OsMYB3R-2*-antisense rice; Bars=10 cm.

Figure 3. Tolerance response of the *OsMYB3R-2* transgenic lines to cold stress

A, Wild-type and *OsMYB3R-2* transgenic 2-week-old rice seedlings at the same stage before the 2□ treatment. B, Seedlings were grown in the greenhouse for 2 weeks after 2□ treatment for 72 h. C, Survival rate of seedlings grown for 2 weeks in the greenhouse after 2□ treatment for 72 h. D, Time course for cold treatment in survival rate of seedlings grown for 2 weeks in the greenhouse. WT: wild type; OL3, OL5 and OL7: *OsMYB3R-2*-overexpressing lines; AL1, AL4 and AL5: *OsMYB3R-2*-antisense lines. The standard error bars are from three independent replications in the same experiment. The phenotype was confirmed by further experiments that were repeated more than 4 times.

Figure 4. Gene expression patterns and free proline level of transgenic rice plants in response to cold

A, Expression pattern of *OsCPT1* in wild-type and *OsMYB3R-2* transgenic rice at room temperature (25□) or low temperature (4□). *Tubulin* was used as an internal control. B, Cellular free proline level. Data represent means and SEs of experiments performed in triplicate. WT: wild type; RT: room temperature (25□); OL3, OL5 and

OL7: *OsMYB3R-2*-overexpressing lines; AL1, AL4 and AL5: *OsMYB3R-2*-antisense lines; FW: fresh weight of materials. Standard error bars are from three independent replications of the same experiment. The proline content determination was confirmed by experiments that were repeated twice. The expression pattern of *OsCPT1* to cold stress was confirmed with two independent experiments.

Figure 5. Transcription activation analysis of OsMYB3R-2 protein

A, Different pGBKT7-OsMYB3R-2 vector constructs. The truncated cDNA fragments of OsMYB3R-2 were sequenced and inserted into the *Nde*I-*Pst*I sites, with ATG added at the end of *Nde*I site in every forward primer. B, The corresponding positions of transformed yeast thalli daubed on the plates. C, **a** The transformed yeast thalli grew on the SD/-His/-Trp plates with solid SD medium. **b** X-Gal activation detection of transformed yeast thalli on the SD/-His/-Trp plates with solid SD medium shown in a. **c** The transformed yeast thalli grew on the SD/-Ade/-Trp plates with solid SD medium. **d** X-Gal activation detection of transformed yeast thalli on the SD/-Ade/-Trp plates with solid SD medium shown in c. **e** The transformed yeast thalli grew on the SD/-His/-Ade/-Trp plates with solid SD medium. **f** X-Gal activation detection of transformed yeast thalli on the SD/-His/-Ade/-Trp plates with solid SD medium shown in e. CK: pGBKT7 vector used as control, Numbers at the left of 5A: 1 represents full-length OsMYB3R-2 protein, 2 to 14 represent different truncated OsMYB3R-2 protein fragments. Numbers at the right of 5A represent the positions of different truncated OsMYB3R-2 protein fragments. Broken line represents the deleted 351-449

amino acid fragment of OsMYB3R-2. The transcription activation of OsMYB3R-2 was confirmed twice.

Figure 6. DNA binding affinity of OsMYB3R-2 protein

A, The alignment of MSA-like sequences shown in the promoters of type-B cyclin genes: rice *OsCycB1*;1 and *OsKNOLLE2*, *Arabidopsis cyc1bAt* (Day et al., 1996) and *cyc2aAt* (Ferreira et al., 1994), and tobacco *NtCYM* (Ito et al., 1997), *NACK1* and *NACK2* (Ito et al., 1998) encoding kinesin-like proteins. The boxed 11-bp sequences share high homology with each other. The nucleotide positions are numbered from the transcription start sites. The motifs of binding sites of c-Myb (Howe and Watson, 1991) and v-Myb (Grotewold et al., 1994) are also shown. B, Probes and competitors used in EMSA. BP: a 378-bp fragment of *OsCycB1*;1 promoter upstream of the transcription start site ATG; RT1: an MSA *cis*-acting element of BP; RT2: an MSA trans-acting element of BP; RT3: an MSA *cis*-acting element of *OsKNOLLE2* promoter upstream of the transcription start site ATG; RT1mut: mutant of RT1 motif; BPWT, RT1WT: competitors of biotin labeled probes of BP and RT1, respectively. C, D and E, EMSA assays of OsMYB3R-2 protein. Binding reaction mixtures were incubated with the probes and mock-translated product (Mock: probe + no protein) or in vitro-synthesized OsMYB3R-2, GST as control in the presence or absence of a 200-fold molar excess of unlabeled oligonucleotide competitors. DNA binding affinity of OsMYB3R-2 was confirmed by experimentally twice.

Figure 7. Expression patterns of cyclin genes in the *OsMYB3R-2* transgenic

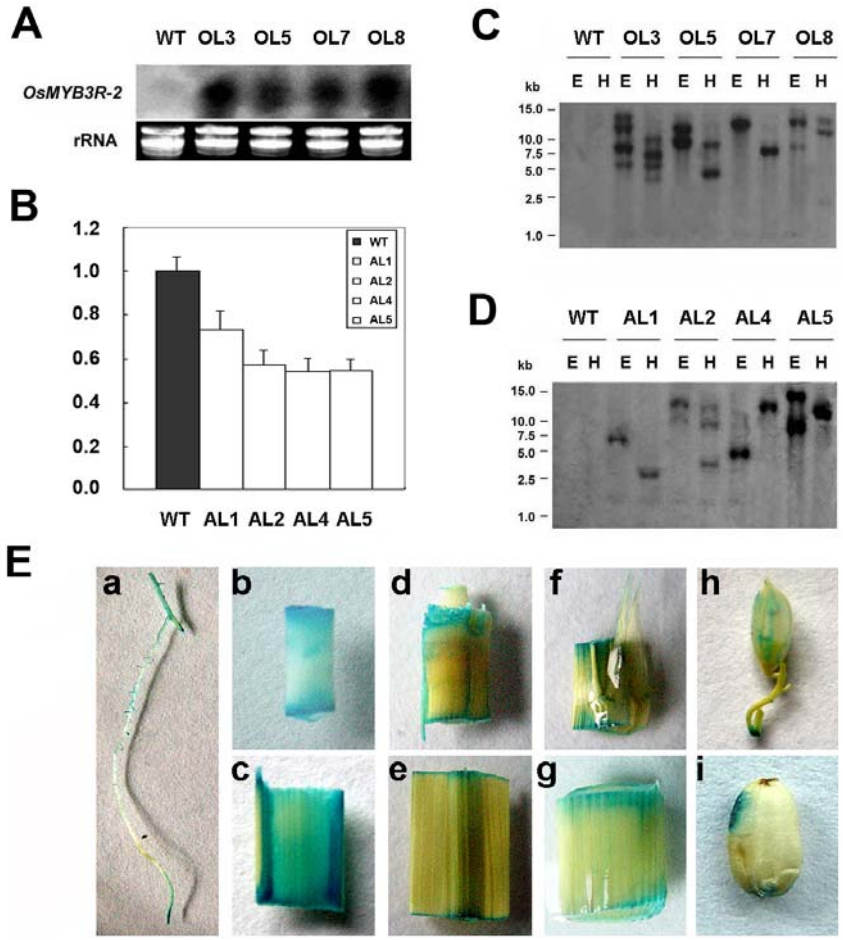
rice, to cold response and survival ratio and cellular free proline in the *OsCycB1*;1 transgenic rice lines.

A. Expression patterns of cyclin responsive genes in wild-type and *OsMYB3R-2* transgenic rice under room temperature (25□) or cold treatment (4□) for 24 h. The method of obtaining seedlings at the same stage is described in “Materials and methods”. *Tubulin* was used as an internal control. WT: wild type; OL5, OL7 and OL8: *OsMYB3R-2*-overexpressing lines; AL1, AL4: *OsMYB3R-2*-antisense lines. The experiments on the response of cyclin genes to cold were repeated twice at least. Data represent means and SEs of experiments performed in triplicate. B. The response of the *OsCycB1*;1 transgenic rice lines to cold stress. WT: wild type; OE: *OsCycB1*;1-overexpressing lines; RNAi: *OsCycB1*;1-RNAi lines; BT: before the cold treatment (3□) which is a control without cold treatment; AT: grown 1 week after the cold treatment (3□) for 72 h. C, The determination of cellular free proline in *OsCycB1*;1 transgenic rice lines. WT: wild type; OE: *OsCycB1*;1-overexpressing lines; RNAi: *OsCycB1*;1-RNAi lines; BT: before the cold treatment (2□); AT: after the cold treatment (2□) for 24 h. FW: fresh weight of materials. Standard error bars are from three independent replications.

Figure 8. Cell cycle progression response to cold in flow cytometry assay in the *OsMYB3R-2* transgenic lines

A, Wild type (WT) at 28℃. B, Wild type at 4℃. C, Overexpressing line 7 (OL7) of OsMYB3R-2 transgenic rice at 28℃. D, OL7 at 4℃; Seedlings 5 days after

germination were treated with low temperature (4℃) or room temperature (control, 28℃) for 24 h. Cell nuclei of 10,000 taken from the root apical meristem were stained with DAPI (1 μg/ml) and analyzed by flow cytometry. E, Cell mitotic index in root apical meristem in rice. Numbers on the top of the black histograms represent the percentage of decreased in the mitotic index at 4℃. Numbers 2C, 4C represent the DAPI signals that correspond to nuclei with different DNA contents. The standard error bars are from three independent replications at the same experiment. Flow cytometry determination was repeated twice.



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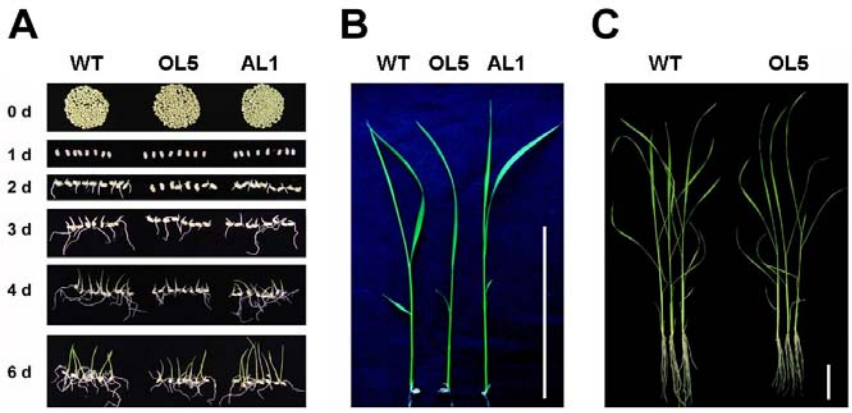

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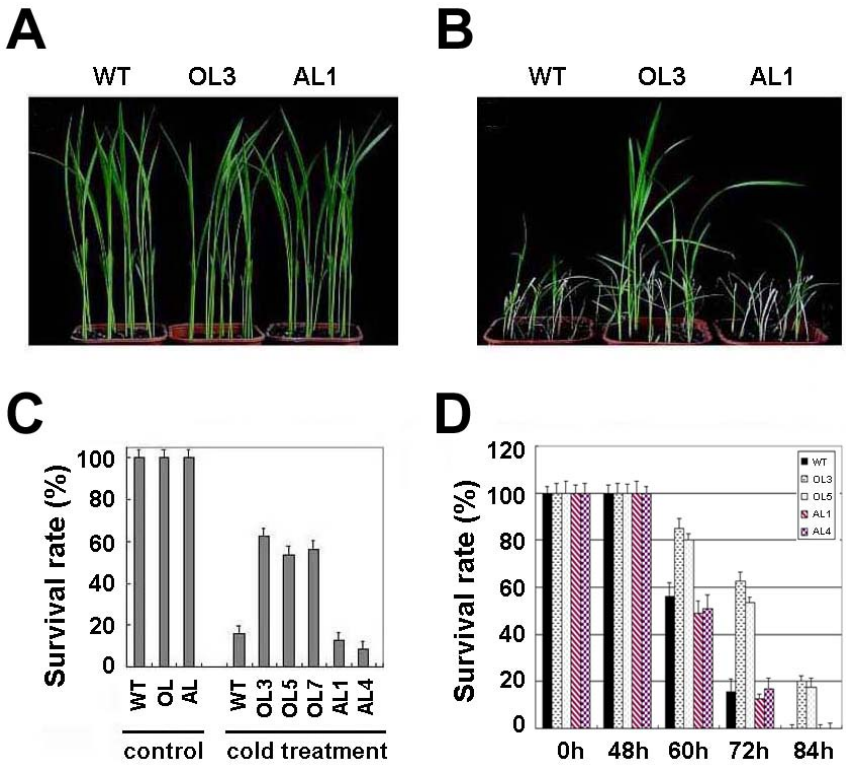

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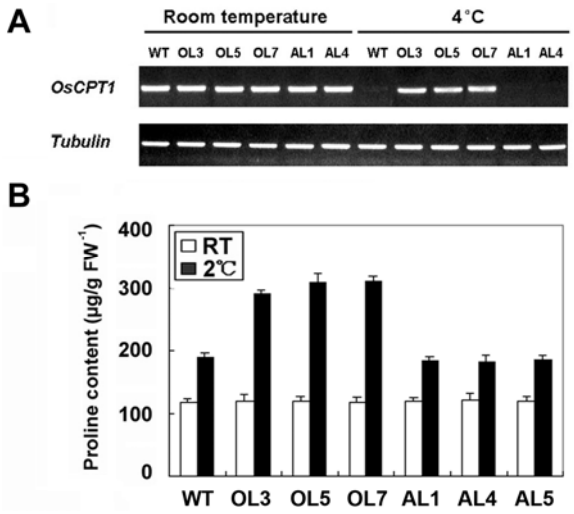

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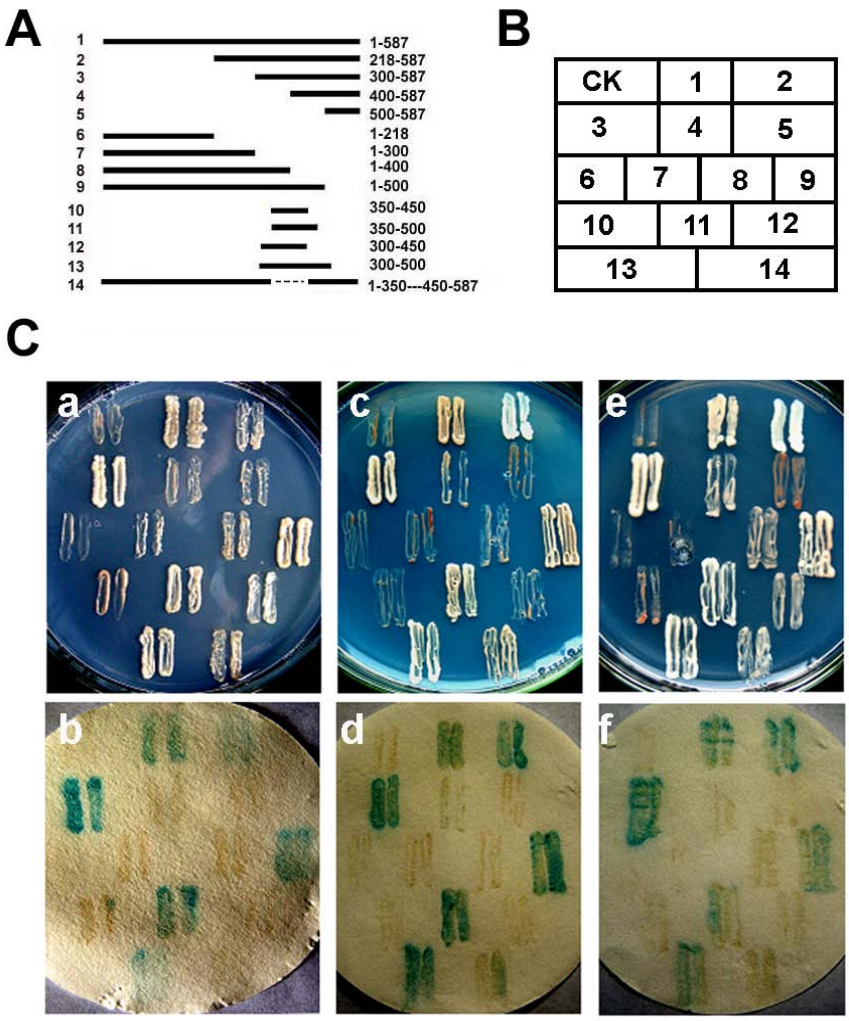

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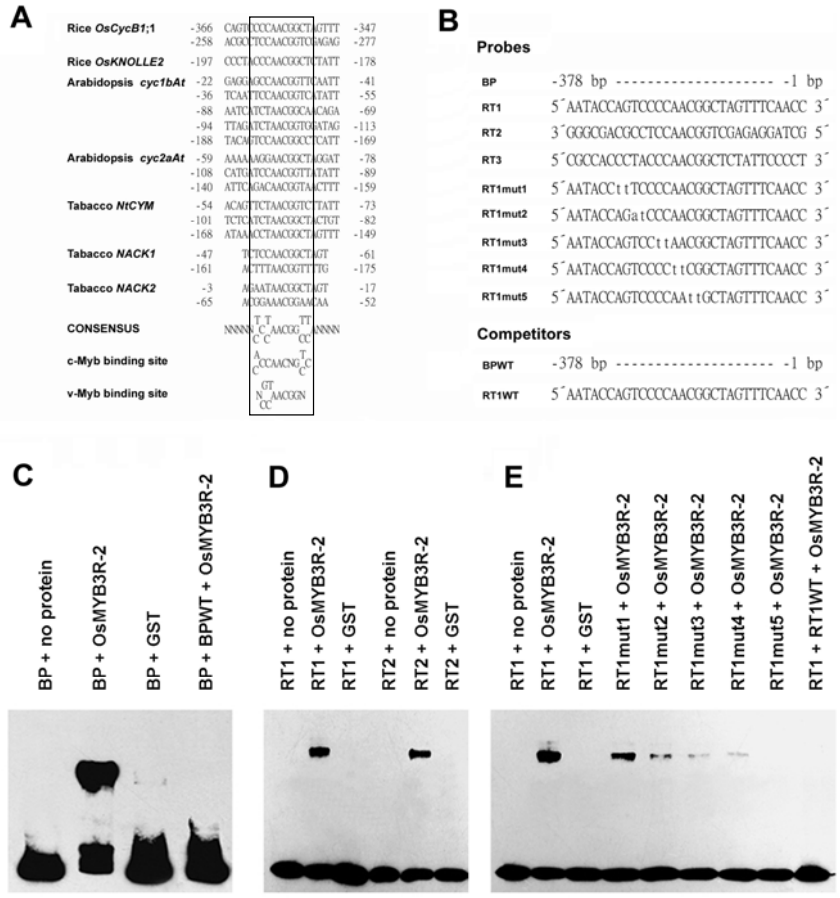

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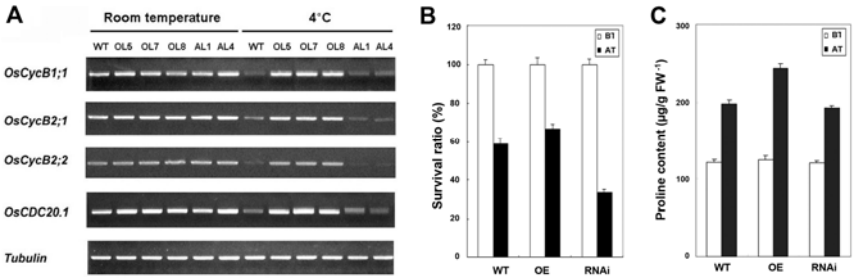

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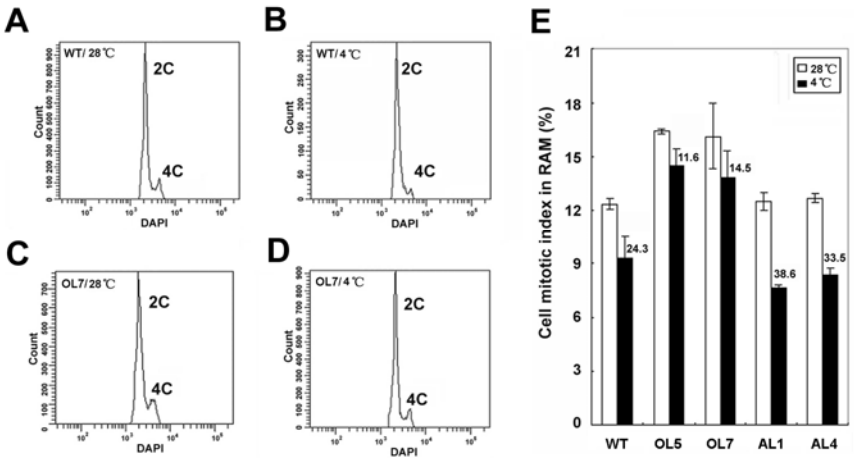

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