# The OsMYB30 Transcription Factor Suppresses Cold Tolerance by Interacting with a JAZ Protein and Suppressing β-Amylase Expression[1][OPEN]

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Cold stress is one of the major limiting factors for rice (*Oryza sativa*) productivity. Several MYB transcriptional factors have been reported as important regulators in the cold stress response, but the molecular mechanisms are largely unknown. In this study, we characterized a cold-responsive R2R3-type MYB gene, *OsMYB30*, for its regulatory function in cold tolerance in rice. Functional analysis revealed that overexpression of *OsMYB30* in rice resulted in increased cold sensitivity, while the *osmyb30* knockout mutant showed increased cold tolerance. Microarray and quantitative real-time polymerase chain reaction analyses revealed that a few β-amylase (*BMY*) genes were down-regulated by OsMYB30. The *BMY* activity and maltose content, which were decreased and increased in the *OsMYB30* overexpression and *osmyb30* knockout mutant, respectively, were correlated with the expression patterns of the *BMY* genes. OsMYB30 was shown to bind to the promoters of the *BMY* genes. These results suggested that OsMYB30 exhibited a regulatory effect on the breakdown of starch through the regulation of the *BMY* genes. In addition, application of maltose had a protective effect for cell membranes under cold stress conditions. Furthermore, we identified an OsMYB30-interacting protein, OsJAZ9, that had a significant effect in suppressing the transcriptional activation of OsMYB30 and in the repression of *BMY* genes mediated by OsMYB30. These results together suggested that OsMYB30 might be a novel regulator of cold tolerance through the negative regulation of the *BMY* genes by interacting with OsJAZ9 to fine-tune the starch breakdown and the content of maltose, which might contribute to the cold tolerance as a compatible solute.

As one of the major crops, rice (*Oryza sativa*) is widely grown in tropical, subtropical, and temperate regions. It also represents a model species for functional genomics studies of temperature stress tolerance in crops. For rice seedlings, low temperature in early spring is one of the major environmental factors limiting growth and agricultural production (Chinnusamy et al., 2007; Su et al., 2010; Cramer et al., 2011); thus, it is important to reveal the molecular mechanisms of the cold responses in rice for the genetic improvement of cold tolerance.

It has been known that various transcription factors were involved in the regulation of cold responses, including some members from the DREB and MYB families (Yamaguchi-Shinozaki and Shinozaki, 2006; Zhang et al., 2011). In Arabidopsis (*Arabidopsis thaliana*) and rice, the CBF/DREB1-dependent cold response pathway has been shown to play a predominant role in cold tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; Chinnusamy et al., 2007; Zhou et al., 2011). A previous transcriptome study in Arabidopsis indicated that 12% of the cold stress response genes were regulated by the CBF regulators and at least 28% of them were not regulated by the CBF regulators, suggesting that both the CBF/DREB-dependent and CBF/DREB-independent pathways play dominant roles in cold stress responses (Fowler and Thomashow, 2002).

MYB transcription factors constitute one of the largest gene families, with approximately 198 members in Arabidopsis and 183 members in rice (Yanhui et al., 2006). However, only a limited number of MYB genes have been identified for their functions in the regulation of stress response or tolerance. To cope with cold stress, rice has evolved various mechanisms, including the induction of key transcription factors such as CBF/DREBs, glutathione peroxidase, and glutathione *S*-transferase (GST), which act as the main reactive oxygen species scavengers, and the abscisic acid (ABA) signaling pathway (J. Zhao et al., 2015). Lines of evidence revealed that the function of the *MYB* genes in the cold stress response may depend on the CBF/DREB pathways. MYB15, which functioned as a negative regulator of CBF/DREB1, could be suppressed by the SUMO E3 ligase SIZ1 conferring cold tolerance (Miura

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et al., 2007). Another group also reported a pathway containing CBF and its upstream regulators ICE1 and ICE2, and their results showed that MYB96 regulated the HEPTAHELICAL PROTEIN, which activated the CBF pathway through interacting with ICE1 and ICE2 (Lee and Seo, 2015). MYBS3, which conferred cold tolerance, repressed the well-known DREB1/CBF pathway (Su et al., 2010). DREB1 responded quickly and transiently while MYBS3 responded slowly during cold stress treatment, suggesting that the MYBS3 and DREB1/CBF pathways may act sequentially and complementarily during the cold stress response (Su et al., 2010). Meanwhile, several studies showed that some MYBs regulate cold tolerance independently of the classical DREB/CBF-dependent cold response pathway. Ma et al. (2009) found that, besides the regulation of the DREB/CBF pathway, OsMYB3R-2 also could regulate the progress of the cell cycle during cold stress conditions through up-regulation of a mitosis-specific activator *OsCycB1;1*. Another example is *OsMYB4*, which was studied by different groups. Overexpression of *OsMYB4* could increase cold tolerance in Arabidopsis, apple (*Malus domestica*), and *Osteospermum ecklonis* (Vannini et al., 2004; Pasquali et al., 2008; Laura et al., 2010). In rice, a complex network of OsMYB4 was dissected based on commonalities between the global transcriptomes of *OsMYB4* overexpression plants before and after exposure to chilling stress conditions (Park et al., 2010). Various transcription factors, such as MYB, ERF, bZIP, NAC, ARF, and CCAAT-HAP, were included in the network. However, the OsMYB4 network was independent of the DREB/CBF pathway (Park et al., 2010). In addition, several members belonging to the MYB family, including OsMYB2 (Yang et al., 2012), OsMYB511 (Huang et al., 2015), CMYB1 (Duan et al., 2014), and OsMYB48-1 (Xiong et al., 2014), also were reported for their roles in cold stress regulation, but their molecular mechanisms have not been clearly revealed.

A previous study reported that starch content and soluble sugars in leaves were responsive to various environmental stresses, but the complex starch synthesis and degradation processes were not completely revealed (Lu et al., 2005; Lu and Sharkey, 2006), and only a few studies on starch degradation were reported in rice (Kitajima et al., 2009; Yun et al., 2011; Hakata et al., 2012; Hirose et al., 2013). With the discovery of the maltose transporter MEX1, researchers considered that maltose was one of the predominant forms that was exported to the cytosol for further metabolism (Niittylä et al., 2004; Lu et al., 2005; Lu and Sharkey, 2006), and the key role in starch digestion was attributed to $\beta$-amylase (BMY; Orzechowski, 2008). The first member isolated in Arabidopsis was named *BAM5* (also called *BMY1* or *RAM1*; Monroe and Preiss, 1990; Monroe et al., 1991). Nine genes encoding BMY-like proteins were investigated in Arabidopsis, and the results suggested that both BAM1 and BAM3 contributed to starch breakdown, and BAM4 facilitated starch breakdown independently of BAM1 and BAM3 (Fulton et al., 2008; Monroe et al., 2014). In rice, only two BMY isozymes have been identified in seeds (Mitsunaga et al., 2001), and one study analyzed the BMY family briefly (Koide et al., 2011). Several studies have shown that *BMY* genes were responsive to abiotic stresses in Arabidopsis. *BMY7* (*BAM1*) and *BMY8* (*BAM3*) were induced by heat and cold shock stress, and the induced expression was correlated with maltose accumulation (Kaplan and Guy, 2004, 2005). The same group demonstrated that, in vitro, maltose had a protective effect on the stability of proteins, membranes, and the photosynthetic electron transport chain during temperature shock. BMY8 also was reported by the same group for its role in photochemical efficiency with maltose accumulation under freezing stress conditions. In addition, BAM1 could trigger starch degradation in guard cells and mesophyll cells under osmotic stress conditions (Valerio et al., 2011), and a recent study confirmed BAM1's role in osmotic stress and found that BAM3 was involved in cold stress tolerance (Monroe et al., 2014). However, the regulation of the *BMY* genes remains largely unknown, especially in response to abiotic stresses.

JAZ proteins, a subgroup of the plant-specific TIFY family that contains a conserved amino acid pattern of TIF[F/Y]XG in their protein sequences (Vanholme et al., 2007; Ye et al., 2009; Bai et al., 2011), have been characterized as critical regulators of jasmonic acid (JA) signaling in Arabidopsis (Chini et al., 2007; Thines et al., 2007). JAZ proteins could repress the bHLH-MYB complex or MYB protein to suppress stamen development and seed production (Song et al., 2011; Qi et al., 2015). *OsJAZ9* (also named *OsTIFY11a*) could be induced by drought, salinity, cold, and JA treatment (Ye et al., 2009). The same group reported that overexpression of *OsJAZ9* resulted in increased salt tolerance and confirmed that OsJAZ9 acted as a repressor in the JA signaling pathway (Wu et al., 2015). However, the role of OsJAZ9 in cold stress regulation remains unknown.

In this work, we reported the identification of an R2R3-MYB gene, *OsMYB30*, which controls cold sensitivity in rice, and three *BMY* genes encoding BMY enzymes were confirmed as target genes of OsMYB30. OsMYB30 negatively regulated the *BMY* genes at the transcriptional level in response to cold stress, and the total BMY activity and maltose content also were decreased in the *OsMYB30* overexpression (*OsMYB30*-OE) plants. Additionally, maltose was shown to have a role in protecting cell membranes under cold stress conditions in rice. We further identified an OsMYB30-interacting protein, OsJAZ9, which was up-regulated by OsMYB30 at the transcriptional level and conferred the negative regulation dependent on OsMYB30. The major finding of this study is that OsMYB30 could negatively regulate cold tolerance by suppressing the *BMY* genes via interaction with OsJAZ9.

## RESULTS

### Identification and Expression Characterization of *OsMYB30*

In view of the potential roles of the R2R3 MYB genes in cold stress regulation, we chose one cold-responsive *MYB* gene (LOC_Os02g41510) from the rice microarray database (GSE71680) and named it *OsMYB30* according to GRASSIUS (http://grassius.org/grasstfdb.html; Yilmaz et al., 2009). We applied quantitative real-time PCR (qPCR) to inspect the expression profile of *OsMYB30* under multiple hormone treatments and abiotic stresses at the rice seedling stage. The results showed that *OsMYB30* was strongly up-regulated by cold stress conditions, suggesting that it may participate in the cold stress response in rice (Fig. 1A). It was also induced to some extent by JA and heat and submergence stresses. However, it was slightly repressed by exposure to ABA, salt, and drought stress treatment (Fig. 1A). We repeated this experiment with different levels of abiotic stresses and observed that, under the same type of abiotic stress or hormone treatment, the expression pattern of *OsMYB30* was similar (Supplemental Fig. S1A).

In order to investigate the tissue expression pattern of *OsMYB30*, the transcript level was checked in 17 organs and tissues of rice. We found that *OsMYB30* was expressed in most organs and tissues, but the expression levels were relatively lower at the four stages of panicle development and relatively higher in the lemma, palea, and secondary branches (Supplemental Fig. S1B). This expression profile was confirmed through a GUS staining test using $P_{OsMYB30}$:*GUS* transgenic plants (Supplemental Fig. S1C).

To determine the subcellular localization of OsMYB30, the full protein sequence of OsMYB30 was fused to the GFP reporter gene driven by the cauliflower mosaic virus (CaMV) 35S promoter. The GFP-OsMYB30 and cyan fluorescent protein (CFP)-GHD7 were cotransformed into rice protoplasts, since GHD7 has been confirmed

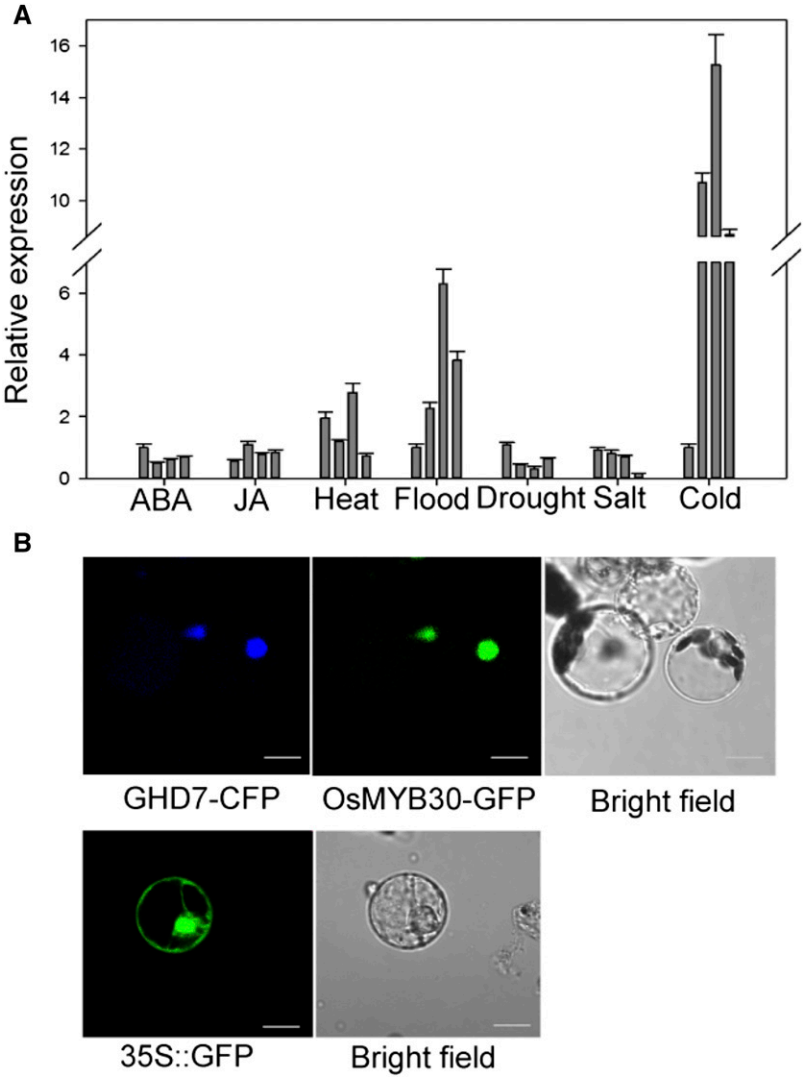


**Figure 1.** Expression profiles and subcellular localization of OsMYB30. A, Expression levels of *OsMYB30* under hormone and abiotic stress treatments, including ABA (time course, 0, 1, 3, and 12 h), JA (0, 1, 3, and 6 h), heat (0 and 10 min, 1 h, and 3 h), flooding (0, 6, 12, and 24 h), drought (0, 1, 2, and 3 d), salt (0, 2, 6, and 12 h), and cold (0, 6, 12, and 24 h). Seedlings at the four-leaf stage were treated with ABA (spraying 100 $\mu$M $L^{-1}$ ABA on the leaves), JA (spraying 100 $\mu$M $L^{-1}$ JA on the leaves), heat (42°C growth chamber), flooding (submerged with water), drought (stopping the water supply), salt (irrigation with 200 mM NaCl solution), or cold (4°C growth chamber). Error bars indicate the SE based on three biological replicates. B, OsMYB30 colocalized with the GHD7 transcription factor in the nucleus of rice protoplasts. 35S::GFP was transformed as a control. Bars = 10 $\mu$m.

to be a nuclear protein (Xue et al., 2008). The results showed that GFP fluorescence overlapped with CFP fluorescence and indicated that OsMYB30 was localized in the nucleus (Fig. 1B).

### Overexpression of *OsMYB30* Decreased Cold Tolerance While Disruption of *OsMYB30* Increased Cold Tolerance

To reveal the biological function of *OsMYB30*, we constructed an overexpression vector of this gene driven by LEAP (Supplemental Fig. S2A). LEAP is the promoter of the late embryogenesis-abundant protein gene *OsLEA3-1*, which showed constitutively high expression (Xiao et al., 2007). The *OsMYB30*-OE vector was transformed into Zhonghua 11 (ZH11) rice. Three independent transgenic plants (OE2, OE8, and OE28) showing overexpression of *OsMYB30* (Supplemental Fig. S2B) were selected for cold tolerance evaluation, since this gene was strongly induced by cold stress. The transgenic plants (ZH11 background) and wild-type ZH11 were grown on one-half-strength Murashige and Skoog (MS) medium and in soil, and the seedlings were then transferred to a 4°C chamber at the four-leaf stage. The *OsMYB30*-OE plants displayed more obvious leaf rolling than ZH11 (Fig. 2A; Supplemental Fig. S3A). After 7 d of exposure to cold stress conditions followed by recovery, about 5% to 18% of the *OsMYB30*-OE plants survived, while more than 60% of the ZH11 plants survived (Fig. 2B). It has been known that electrolyte leakage could reflect the damage of cell membranes, and the maintenance of cell membrane integrity and stability has been reported as a major contributing factor for cold tolerance (Whitlow et al., 1992; Morsy et al., 2005). Therefore, we examined the electrolyte leakage of the overexpression plants and ZH11 during cold stress conditions. The results showed that the electrolyte leakage increased more rapidly in the *OsMYB30*-OE plants than in the ZH11 plants during exposure to prolonged cold stress conditions (Fig. 2C). These results suggested that OsMYB30 may negatively regulate cold tolerance in rice.

To further validate the function of *OsMYB30*, a T-DNA insertion mutant, *osmyb30* (Huayang background), was obtained from the Rice T-DNA Insertion Sequence Database. The flanking sequence analysis showed that the T-DNA was inserted in the second exon, and the transcript level of *OsMYB30* was null in the mutant compared with the wild-type Huayang

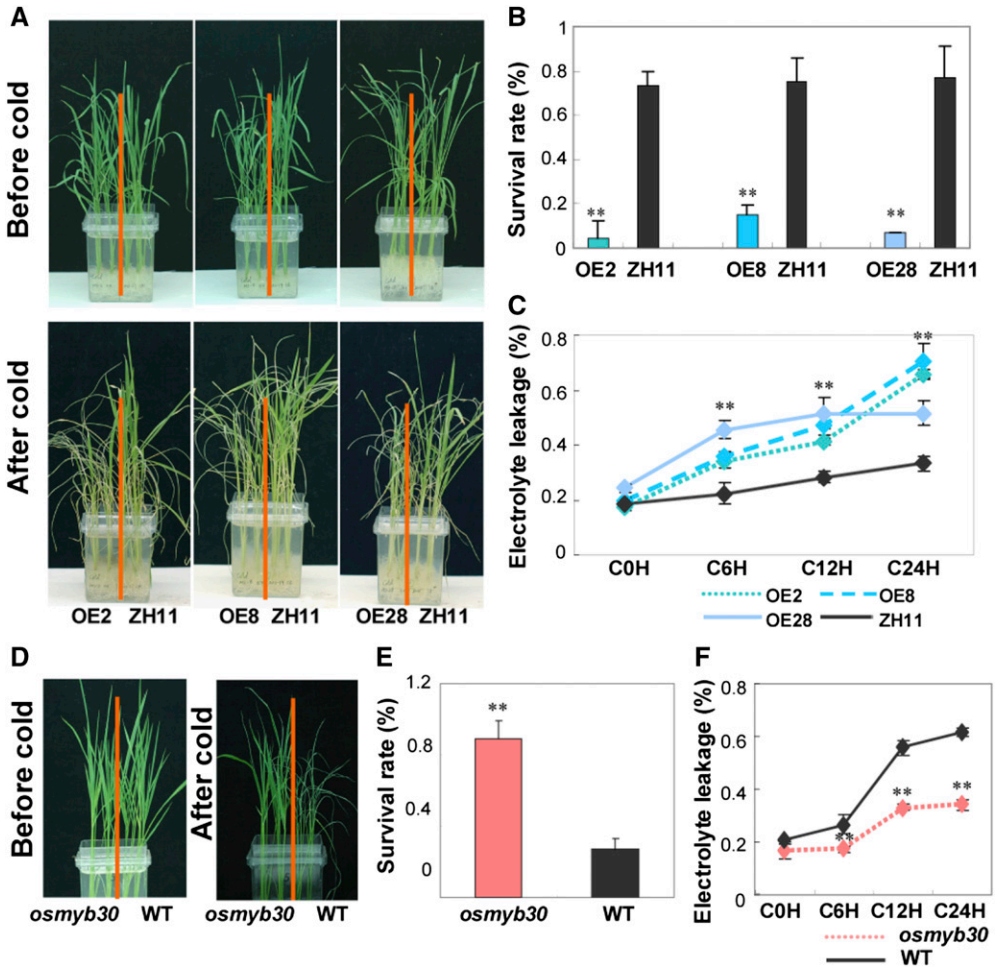


**Figure 2.** Phenotypes of *OsMYB30*-OE plants and the *osmyb30* mutant under cold stress conditions (4°C). A, Seedlings from three overexpression lines (OE2, OE8, and OE28) were grown on one-half-strength MS medium with ZH11 plants as a control. B, Survival rates of *OsMYB30*-OE plants and ZH11 after cold stress treatment. Error bars indicate the SE based on three replicates. C, Electrolyte leakage (percentage of total electrolyte leakage) of ZH11 and overexpression plants under cold stress treatment. Error bars indicate the SE of three replicates. **, $P < 0.01$ by Student's *t* test. D, Seedling phenotypes of the homozygous mutant line of *osmyb30* grown in MS medium with the wild-type Huayang (WT) as a control. E, Survival rates of the homozygous mutant plants and the wild type during cold stress treatment. Error bars indicate the SE of three replicates. **, $P < 0.01$ by Student's *t* test. F, Electrolyte leakage of homozygous mutant plants of *osmyb30* and the wild type under cold stress treatment. Error bars indicate the SE of three technical replicates. **, $P < 0.01$ by Student's *t* test.

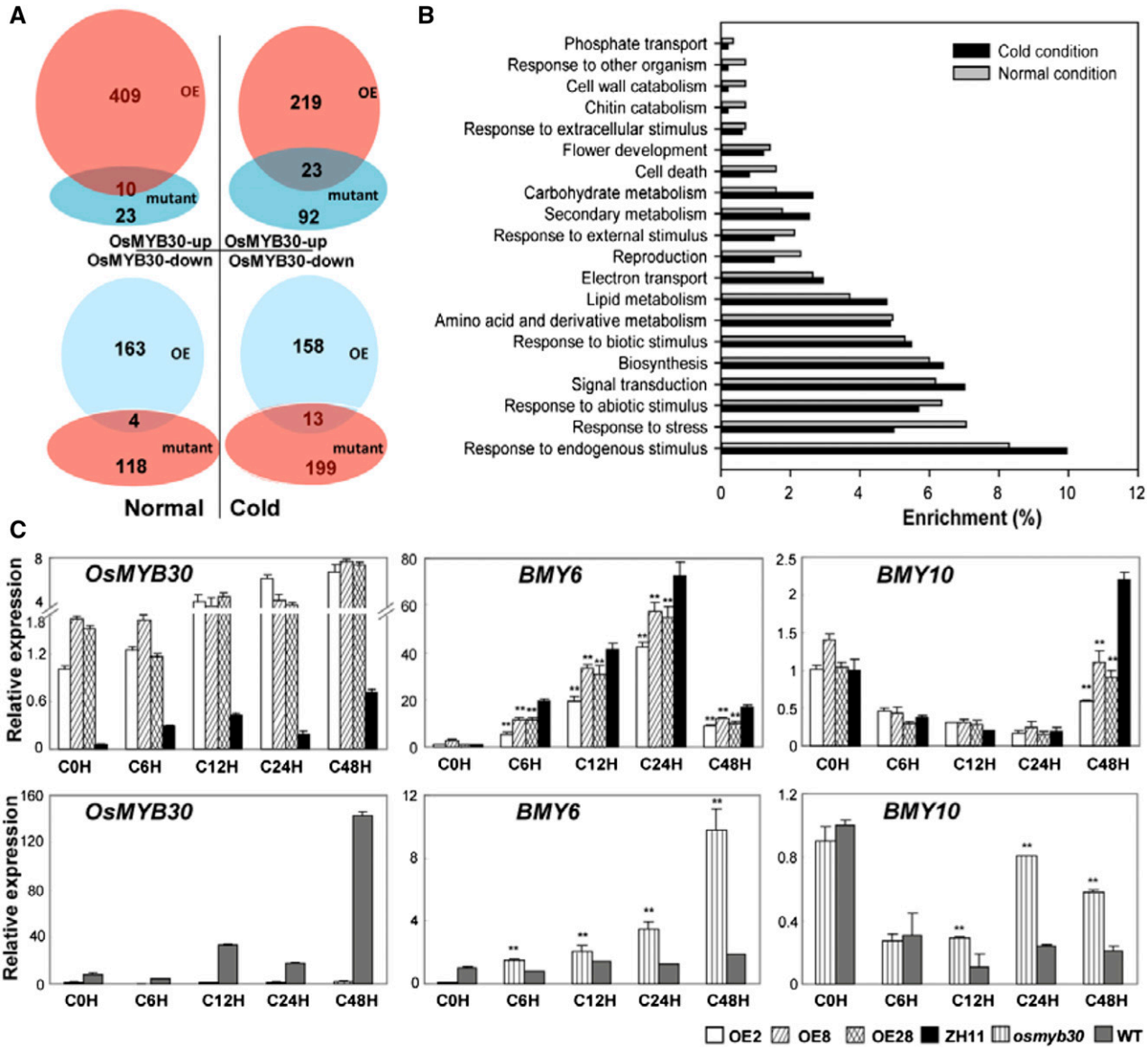


**Figure 3.** Microarray analysis of the *OsMYB30*-OE and mutant plants before and after cold stress treatment. A, Venn diagram for the number of genes with expression levels affected in the overexpression and mutant plants before and after cold stress treatment. OsMYB30-up indicates genes up-regulated in the overexpression plants or down-regulated in the mutant; OsMYB30-down indicates genes down-regulated in the overexpression plants or up-regulated in the mutant. B, Gene Ontology classification of genes up- or down-regulated by OsMYB30 with a threshold of expression change 1.8-fold or greater or 0.6-fold or less. C, Expression patterns of *OsMYB30* and the two *BMY* genes (*BMY6* and *BMY10*) in *OsMYB30*-OE and the *osmyb30* mutant under cold stress conditions, with ZH11 and Huayang as controls, respectively. Error bars indicate the SE of three replicates. **, $P < 0.01$ by Student's *t* test. WT, Wild type.

(Supplemental Fig. S2C). Under normal conditions, we did not observe any phenotypic differences between *osmyb30* and the wild type. Under the same cold stress treatment (exposure to 4°C for 7 d) as for the three independent *OsMYB30*-OE lines described above, the wilting phenotype of *osmyb30* appeared later than in the wild type when grown in both the medium and soil (Fig. 2D; Supplemental Fig. S3B), and the mutant had a significantly higher (80%) survival rate than the wild type (less than 20%; Fig. 2E). The electrolyte leakage, which was significantly lower in the mutant, was consistent with the phenotype (Fig. 2F). These results further demonstrated that OsMYB30 might have a negative role in cold stress tolerance in rice.

### Transcriptome Analysis of *OsMYB30*-OE and Mutant Plants

To reveal the regulatory mechanism of OsMYB30 in cold sensitivity, we first checked the expression levels

of cold-related genes from the classical cold response pathways, including *DREB/CBF* (Yamaguchi-Shinozaki and Shinozaki, 2006; Chinnusamy et al., 2007), *MYBS3* (Su et al., 2010), *MYB3R-2* (Ma et al., 2009), and *OsMYB4* (Park et al., 2010). To our surprise, none of these genes showed obvious differences in their expression levels between the overexpression (or mutant) plants and the wild type (Supplemental Fig. S4). These results suggested that the regulatory role of OsMYB30 in the cold stress response may not be related to these genes. Next, we employed a microarray to find differentially expressed genes (DEGs) in *OsMYB30*-OE (comparing two independent overexpression plants with ZH11) and the *osmyb30* mutant (comparing two independent homozygous mutant plants with Huayang) under normal conditions and cold stress treatment for 6 h (GSE83912). In order to validate the microarray data, we randomly selected 12 DEGs and checked their expression levels using qPCR, and the results matched well with the microarray data (Supplemental Fig. S5). With a threshold ratio of 1.8-fold and 0.6-fold change for up- and down-regulation, respectively, hundreds of genes were affected in *OsMYB30*-OE and the *osmyb30* mutant (Fig. 3A). In the *OsMYB30*-OE plants, 419 and 167 genes were up- and down-regulated, respectively, under normal conditions; 242 and 171 genes were up- and down-regulated, respectively, under cold stress conditions. In the *osmyb30* mutant, 122 and 33 genes were up- and down-regulated, respectively, under normal conditions; 212 and 115 genes were up- and down-regulated, respectively, under cold stress conditions. We selected genes changed in the *OsMYB30*-OE plants for Gene Ontology analysis (Fig. 3B). The enriched DEGs mainly belong to the following categories of biological processes: response to endogenous stimulus, response to stress, response to abiotic stress, and signal transduction. Noticeably, the number of DEGs belonging to carbohydrate metabolism and lipid metabolism also was enriched significantly after cold stress treatment.

Among all of the DEGs with the opposite tendency of expression change between *OsMYB30*-OE and the *osmyb30* mutant (Supplemental Files S1–S4), three *BMY* genes (LOC_Os07g35880, LOC_Os07g35940, and LOC_Os02g03690), which were down-regulated in *OsMYB30*-OE plants and slightly up-regulated in the *osmyb30* mutant, caught our attention because these types of genes have been reported to be involved in the cold response in Arabidopsis (Kaplan and Guy, 2004, 2005). In rice, 10 *BMY* genes have been predicted, and four of them were named *OsBamy1* to *OsBamy4* (Koide et al., 2011). The first *BMY* gene isolated in Arabidopsis was named *BAM5* (*BMY1* or *RAM1*; Monroe and Preiss, 1990; Monroe et al., 1991). For simplicity and clarification, we adopt *BMY* for the name of the *BMY* gene family in rice, and we list the correspondence between the *BMY* genes in this study and the published *OsBMY* genes in Supplemental File S5. We checked the expression of the 10 *BMY* genes in rice by qPCR and found that *BMY6* and *BMY10* (detected in the microarray; LOC_Os02g03690) were down-regulated in *OsMYB30*-OE plants but up-regulated in the *osmyb30* mutant after cold stress treatment (Fig. 3C). *BMY1* (detected in the microarray; LOC_Os07g35880) was down-regulated in the *OsMYB30*-OE plants but was not up-regulated in the *osmyb30* mutant after cold stress treatment, while the other *BMY* genes were not significantly different (Supplemental Fig. S6), except *BMY2* (detected in the microarray; LOC_Os07g35940) and *BMY8*, which were difficult to amplify in the qPCR using the ground-up tissue of seedlings. These results indicated that the expression of three *BMY* genes (*BMY2*, *BMY6*, and *BMY10*) may be negatively regulated by OsMYB30.

### OsMYB30 Regulates BMY Activity and Maltose Content

Since the expression profiles suggested that OsMYB30 may be involved in the regulation of the *BMY* genes during the cold stress response, we further measured BMY activity and maltose content in ground-up tissue to determine whether the physiological index is related to *BMY* gene expression. The results showed that the BMY activity in the *OsMYB30*-OE plants was decreased significantly compared with that in ZH11 after cold stress treatment for 6 and 12 h (Fig. 4A), whereas the *osmyb30* mutant exhibited significantly higher BMY activity than the wild type (Fig. 4B).

To further confirm whether the BMY activity is correlated with its catalytic product, soluble sugar and starch content were measured. The results revealed that, after a prolonged cold stress treatment, maltose showed a dramatic increase and then declined quickly in the wild type (ZH11 and Huayang; Fig. 4, C and D). However, we did not observe such a dramatic increase in the three overexpression plants, which suggested that starch degradation might be affected due to the repression of BMY activity. Although both the *osmyb30* mutant and the wild type displayed a dramatic increase, the maltose content in the *osmyb30* mutant was higher than that in the wild type, which may be the result of the higher BMY activity in the mutant (Fig. 4, C and D). Maltose has been suggested to be involved in the production of cytosolic Suc and Glc (Lu and Sharkey, 2004; Sharkey et al., 2004), so we further measured Suc, Glc, and Fru. The change tendencies of these sugars after cold treatment were very similar to that of maltose, except for Suc, which showed a slow increase. Nevertheless, no significant difference was observed for the content of the three sugars between the *OsMYB30*-OE plants and the control plants or between the mutant and the wild type (Supplemental Fig. S7).

A previous study confirmed the primary function of BMY in starch breakdown in plants (Kossmann and Lloyd, 2000), and *AtBMY8* RNA interference (RNAi) lines in Arabidopsis with decreased *AtBMY8* transcript level showed a starch-excess phenotype (Kaplan and Guy, 2005), so we further investigated the dynamic change of starch after cold stress treatment. The results revealed different change patterns of starch content in the two rice genotypes (ZH11 and Huayang), and we

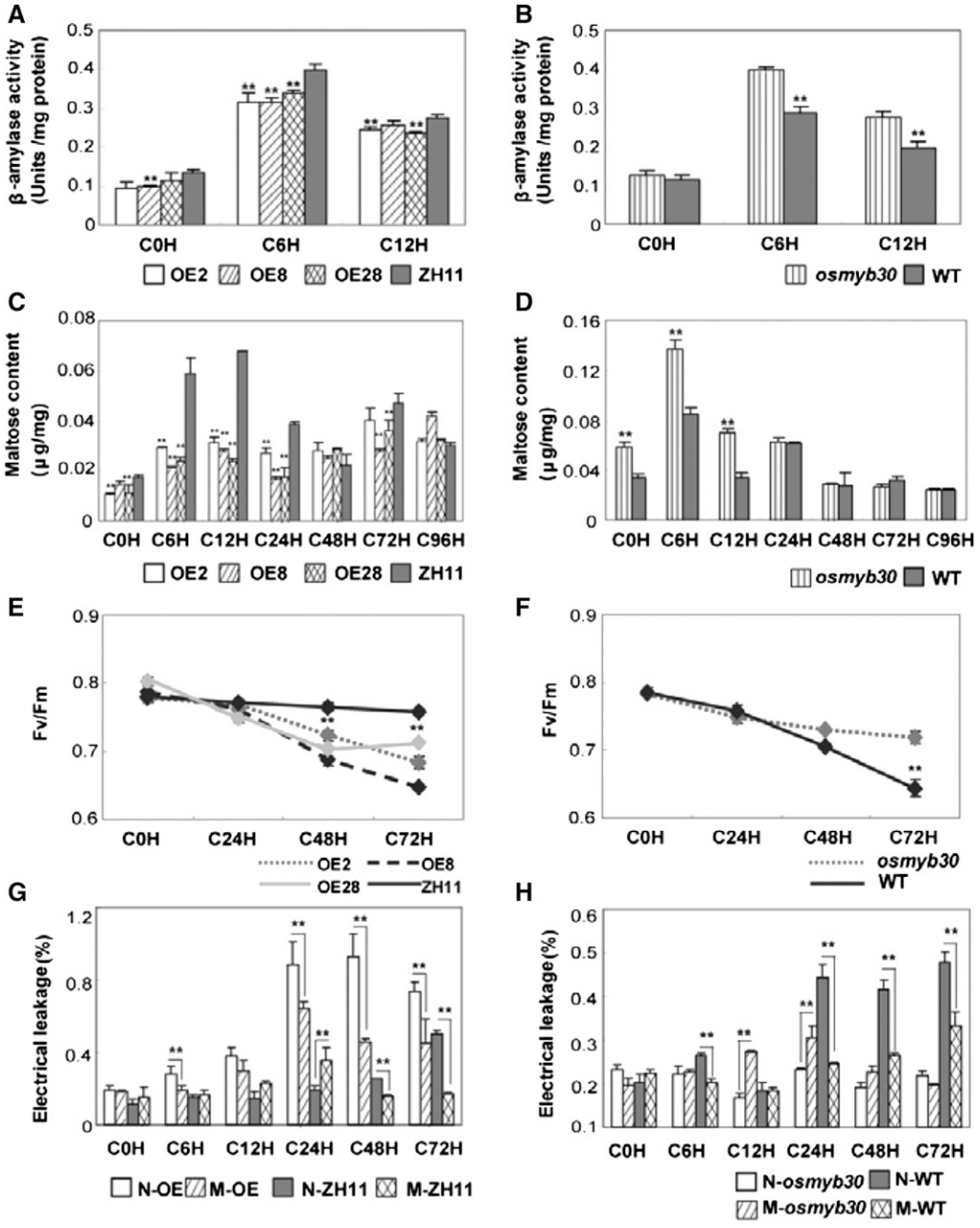


**Figure 4.** OsMYB30 decreases maltose content by repressing BMY activity. A and B, BMY activity in the *OsMYB30*-OE plants (A) and the *osmyb30* mutant (B) at the seedling stage under cold stress treatment. C and D, Maltose contents in *OsMYB30*-OE (C) and *osmyb30* mutant (D) seedlings using Suc as an internal reference. E and F, $F_v/F_m$ ratio in the *OsMYB30*-OE plants (E) and the *osmyb30* mutant (F) under cold stress conditions. G and H, Electrolyte leakage analyses in the maltose treatment. Electrolyte leakage was determined in the *OsMYB30*-OE plants (G) and the *osmyb30* mutant (F) under cold stress conditions (N) and cold stress plus 2.78 mM maltose treatment (M). Electrolyte leakage is presented as the ratio of R1 to R2, where R1 is the initial conductivity and R2 is the conductivity of the boiled leaf segments. Error bars indicate the SE of three biological replicates. **, $P < 0.01$ by Student's *t* test. WT, Wild type.

found significant starch accumulation in the *OsMYB30*-OE plants after 72 h of cold stress treatment (Supplemental Fig. S8). Since starch accumulation also may be related to its synthesis, we examined the expression levels of 10 starch synthesis-related genes, which are highly expressed in vegetative and source tissues (Fu and Xue, 2010), in the *OsMYB30*-OE and *osmyb30* mutant plants by qPCR. The results indicated that, after cold stress treatment, four genes (*OsAGPL3*, *OsSSIIIb*, *OsSSIIb*, and *OsSSIIc*) were slightly up-regulated in the *OsMYB30*-OE plants but were down-regulated in the *osmyb30* mutant plants (Supplemental Fig. S9),

suggesting that OsMYB30 also may be involved in the regulation of starch synthesis. These data suggested that OsMYB30 played a regulatory role in the metabolism of starch, at least including the regulation of the *BMY* genes, which resulted in significant changes of maltose content in the *OsMYB30*-OE and *osmyb30* mutant plants.

### Maltose May Contribute to Cell Membrane Protection during Cold Stress Treatment

According to previous studies, the increase in the maltose content could help in the protection of the photosynthetic electron transport chain (Kaplan and Guy, 2004, 2005), in which the chlorophyll fluorescence $F_v/F_m$ ratio has been used as an indicator to quantify cold tolerance in Arabidopsis, rice, and other plants (Baker and Rosenqvist, 2004; Ehlert and Hincha, 2008; Mishra et al., 2014; Thalhammer et al., 2014). We measured the $F_v/F_m$ ratio after cold stress treatment, and the results showed that the $F_v/F_m$ ratio was decreased significantly in the three *OsMYB30*-OE plants compared with ZH11 (Fig. 4E), and it was significantly higher in the *osmyb30* mutant than in the wild-type plants (Fig. 4F). This result was consistent with the difference in the maltose content mentioned above.

It has been reported that maltose and Glc are two major forms of sugars from starch degradation in Arabidopsis (Lu and Sharkey, 2006), and maltose provided much more protection than Glc as a chloroplast stromal compatible solute in vitro (Kaplan and Guy, 2004). Therefore, we wondered whether maltose would contribute to cold tolerance in rice. Two-week-old seedlings of *OsMYB30*-OE and the *osmyb30* mutant were transferred from normal growth conditions to a 4°C chamber with or without 2.78 mM maltose added in the one-half-strength MS medium. The seedlings treated with maltose for 3 d were sampled for measurements of electrolyte leakage and expression levels of *BMY* genes (Fig. 4, G and H; Supplemental Fig. S10). The data showed that, in the *OsMYB30*-OE plants treated with prolonged cold stress, electrolyte leakage was increased, but the increase was significantly weakened after cold stress treatment when maltose was added, although it was still higher than the electrolyte leakage in the ZH11 plants. Even in the wild-type ZH11, the electrolyte leakage was lower after cold stress treatment at day 3 when maltose was added (Fig. 4G). The effect of exogenous maltose application was not obvious for the *osmyb30* mutant, except for a significant difference at 12 h after cold stress treatment. However, electrolyte leakage in the wild-type Huayang, which was a relatively cold-sensitive genotype, was decreased significantly when maltose was applied (Fig. 4H), and the effect was more obvious than that in the wild-type ZH11, which was a relatively cold-tolerant genotype. In the cold treatment with the addition of maltose, the expression of *BMY6* and *BMY10* was up-regulated (Supplemental Fig. S10), suggesting that the maltose treatment may partially increase *BMY* gene expression during cold stress treatment. These results suggested that maltose may play a protective role for the cell membrane under cold stress conditions in rice.

We further selected seven rice accessions that showed significant differences in cold tolerance to examine the general protection effect of maltose in cold stress tolerance. In general, the protection effect, in terms of reducing electrolyte leakage, was more obvious in the cold-sensitive accessions such as Dongjing, Nipponbare, MH63, and Zhonghan5 than in the cold-tolerant accessions such as KY131 and Huhan3 (Supplemental Fig. S11). These results indicated that maltose may have a protection effect during cold stress treatment in rice, but the effect may be genotype dependent.

### OsMYB30 Binds to the Promoter of the *BMY* Genes

The above results demonstrated that OsMYB30 regulated maltose content through changing the expression levels of the *BMY* genes. We further examined whether OsMYB30 could directly regulate the *BMY* genes. Sequence analysis indicated that the MYB-specific cis-elements were enriched in the *BMY* gene promoters. We performed a yeast one-hybrid assay to examine whether OsMYB30 could bind to the promoter of the *BMY* genes. The transformants of pGBDT7-OsMYB30 along with pHIS2-P*BMY2*, pHIS2-P*BMY6*, and pHIS2-P*BMY10* were able to grow on the synthetic dextrose (SD)/-Trp/-Leu/-His medium with 50 mM 3-amino-1,2,4-triazole (3-AT), which was the same with the positive control (containing pGBDT7-53 and pHIS2-P53), whereas the negative control (containing pGBDT7-OsMYB30 and pHIS2-P53) could not grow on the medium with 3-AT (Fig. 5A). An electrophoretic mobility shift assay (EMSA) was performed to confirm whether the OsMYB30 protein binds directly to the promoters of the *BMY* genes. The probes from the *BMY2*, *BMY6*, and *BMY10* promoters were labeled with FAM and incubated with the OsMYB30 protein produced in *Escherichia coli*. The recombinant OsMYB30 bound to the promoters containing the MYB-specific cis-element (Fig. 5B).

Then, chromatin immunoprecipitation (ChIP)-qPCR was used to confirm whether OsMYB30 binds to the *BMY* promoters in vivo. The chromatin fractions from the *osmyb30* mutant and the wild type under normal or cold stress conditions were isolated and incubated with the anti-OsMYB30 antibody (immunoprecipitation samples) or without the antibody (input samples) for ChIP assay. The *osmyb30* mutant was used as a negative control. The precipitated products were analyzed by qPCR with seven primers covering different promoter regions of *BMY2*, *BMY6*, and *BMY10*. The levels of seven qPCR products in the wild-type plants before or after cold stress treatment were enriched to various degrees but were not enriched in the *osmyb30*

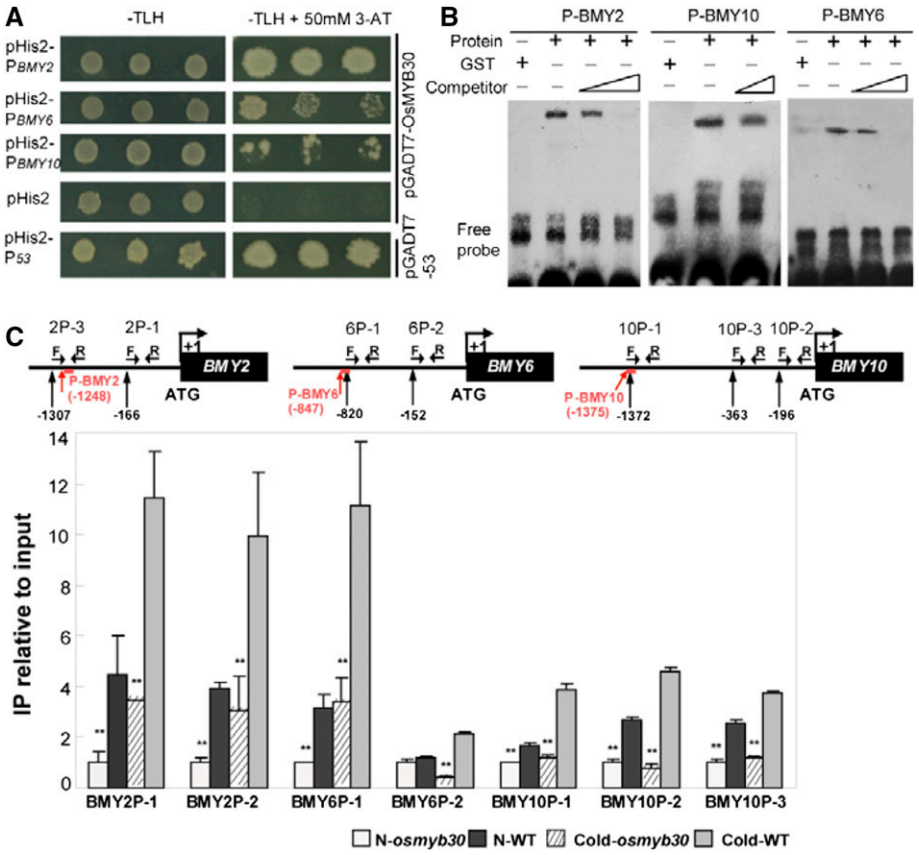


**Figure 5.** OsMYB30 interacts with BMY promoters in vitro and in vivo. A, OsMYB30 bound to the promoters of the *BMY* genes in yeast cells through a yeast one-hybrid assay by growing the plasmids (pHis2-$P_{BMY}$ plus pGADT7-OsMYB30) on selective medium (SD-Trp-Leu-His) containing 50 mM 3-AT along with the negative control (pHis2 plus pGADT7-OsMYB30) and the positive control (pHis2-P*53* plus pGADT7-53). B, EMSA was carried by using the OsMYB30::GST protein and the *BMY* promoters as probes labeled with 5′ FAM. Competition for the labeled sequences was tested by adding different concentrations of unlabeled probes. The positions of the probes are indicated with red lines in C. C, ChIP-qPCR to confirm OsMYB30 binding to the *BMY* gene promoter. The fragments for ChIP-qPCR are indicated by forward (F) and reverse (R) primers; vertical black and red arrows indicate the positions of forward primers and probes, respectively. N and Cold indicate normal growth and cold stress conditions, respectively. The results are represented as relative values of immunoprecipitation (IP) relative to input. The immunoprecipitation and input samples were the chromatin from the four samples (N-*osmyb30*, N-WT, Cold-*osmyb30*, and Cold-WT) precipitated with and without OsMYB30 antibody, respectively. The *osmyb30* mutant was used as a negative control. Error bars indicate the SE of three replicates. **, $P < 0.01$ by Student's *t* test.

mutant (Fig. 5C). These results indicated that OsMYB30 indeed regulated the *BMY* genes through direct binding to their promoters.

### OsJAZ9 Interacts with OsMYB30 and Confers Negative Regulation

It has been reported that JAZ proteins could repress the bHLH-MYB complex or MYB (Song et al., 2011; Qi et al., 2015), and *OsJAZ9* was identified as a repressor in jasmonate signaling and is induced by cold stress treatment in rice (Ye et al., 2009; Wu et al., 2015). Therefore, we examined whether OsJAZ9 could interact with MYB30. In the bimolecular fluorescence complementation (BiFC) assay (Fig. 6A), a yellow fluorescent protein (YFP) signal was observed in the nucleus when cYFP-OsMYB30/OsJAZ9-nYFP was transformed into rice protoplasts. A coimmunoprecipitation (co-IP) assay in vivo showed that the OsJAZ9-MYC could be detected by MYC antibody in the GFP-immunoprecipitated elution protein from the rice protoplast transformed with OsMYB30-GFP plus OsJAZ9-MYC (Fig. 6B). In addition, a pull-down assay in vitro (Fig. 6C) indicated that OsMYB30-GST but not GST could be pulled down by the beads containing OsJAZ9-His. These three different experiments confirmed the interaction between OsMYB30 and OsJAZ9. We observed that *OsJAZ9* showed higher expression levels in the three *OsMYB30*-OE plants under cold stress conditions (Fig. 7A) and lower expression levels in the *osmyb30* mutant (Supplemental Fig. S12), indicating that OsMYB30 may positively regulate the

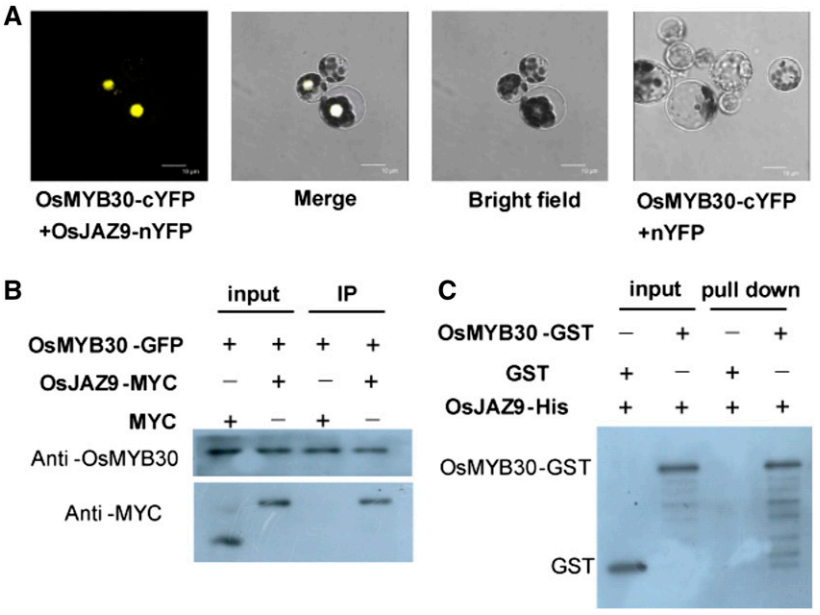


**Figure 6.** OsMYB30 interacts with OsJAZ9. A, BiFC assay indicates that OsMYB30 interacted with OsJAZ9 in rice. YFP fluorescence was detected in rice leaves with combinations of cYFP-OsMYB30 and OsJAZ9-nYFP. The combination of cYFP-OsMYB30 plus pVYNE was used as a negative control. Bars = 10 $\mu$m. B, Co-IP assay to verify the interaction of OsMYB30 and OsJAZ9 in vivo. OsMYB30-GFP was cotransformed with OsJAZ9-MYC (pVNYE) or the vector (pVNYE as a negative control) in rice protoplasts. Total protein extracts from the transient assay expressing OsMYB30-GFP plus OsJAZ9-MYC or OsMYB30-GFP plus the MYC-tag were immunoprecipitated (IP) using GFP beads and blotted with anti-OsMYB30 and anti-MYC antibodies. C, Pull-down assay to verify the interaction between OsMYB30 and OsJAZ9 in vitro. OsMYB30-GST fusion protein or GST alone was incubated with OsJAZ9-His in His beads. OsMYB30-GST but not GST was pulled down by the beads containing OsJAZ9-His. Western blot was used with anti-GST antibody.

expression level of *OsJAZ9*. We further validated the function of *OsJAZ9* in cold sensitivity. Two independent *OsJAZ9*-OE lines (*OsJAZ9*-OE13 and *OsJAZ9*-OE8) displayed more rapid leaf rolling than the wild type (ZH11). However, two independent *OsJAZ9* suppression transgenic lines (*OsJAZ9*-RNAi3 and *OsJAZ9*-RNAi5) showed no significant phenotypic difference compared with ZH11 (Supplemental Fig. S13A), which might be due to the inadequate suppression of this gene (Wu et al., 2015). The electrolyte leakage in the *OsJAZ9*-OE13 and *OsJAZ9*-OE8 plants increased more rapidly than in ZH11 during the prolonged cold stress treatment (Supplemental Fig. S13B). Interestingly, the expression levels of *BMY6* and *BMY10* also were repressed in the *OsJAZ9*-OE plants under cold stress conditions (Fig. 7B), but in the *OsJAZ9* suppression transgenic lines, only *BMY6* was significantly up-regulated (Supplemental Fig. S13C). These results suggested that OsJAZ9, as an interaction protein of OsMYB30, also might be involved in the regulation of cold sensitivity in rice, but the influence was limited.

We applied a dual-luciferase transient assay in rice protoplasts to examine whether the OsMYB30-OsJAZ9 interaction is involved in the OsMYB30-dependent transcriptional regulation. OsMYB30 and OsJAZ9 were fused with 35S-GAL4DB and 35S as effectors, and the promoters of *BMY* genes (*BMY2*, *BMY6*, and *BMY10*) were fused to the CaMV 35S promoter and firefly luciferase as reporters. The data showed that OsMYB30 alone had obvious activation activity (3-fold higher than the control vector) but no effect in suppressing the *BMY* promoters (Fig. 7C). However, when OsJAZ9 was cotransformed, we observed a significant suppression effect on the transcriptional activation activity (with a reduction of 50%) and a significant repression effect (with a reduction to 30%–50%) on the activation of the *BMY* promoters (Fig. 7C), suggesting that OsJAZ9 might facilitate the suppression of OsMYB30 transcriptional activation and, thus, confer the repression on the *BMY* promoters. These results together suggested that OsJAZ9 may function as a repressor to participate in the OsMYB30-mediated negative regulation on most of the *BMY* genes through interacting with OsMYB30.

## DISCUSSION

### OsMYB30 Negatively Regulates Cold Tolerance

According to previous studies, CBF/DREBs are important components in the cold stress response in which CBF1/DREB1B and CBF3/DREB1A are positive regulators, while CBF2/DREB1C is a negative regulator (Novillo et al., 2004). There have been quite a few studies on MYB transcription factors whose functions either depend on the CBF/DREB pathway, such as MYB15 (Agarwal et al., 2006; Miura et al., 2007), MYB96 (Lee and Seo, 2015), and MYBS3 (Su et al., 2010), or do not depend on the CBF/DREB pathway, such as OsMYB3R-2 (Ma et al., 2009) and OsMYB4 (Laura et al., 2010). Among them, MYB15 negatively regulated freezing tolerance through repression of the expression of the *CBF* genes (Agarwal et al., 2006), while MYB96 positively regulated freezing tolerance by CBF-COR activation (Lee and Seo, 2015). Besides the CBF/DREB-dependent mechanisms in cold tolerance, a unique regulation model of OsMYB3R-2, which acted as a positive regulator, has been verified. OsMYB3R-2 regulated the progress of the cell cycle during cold stress conditions via the up-regulated mitosis-specific activator *OsCycB1;1* (Ma et al., 2009). To date, no MYB has been reported as a negative regulator of cold tolerance in rice. In this study, we identified a novel R2R3-MYB gene, *OsMYB30*. OsMYB30 acted as a

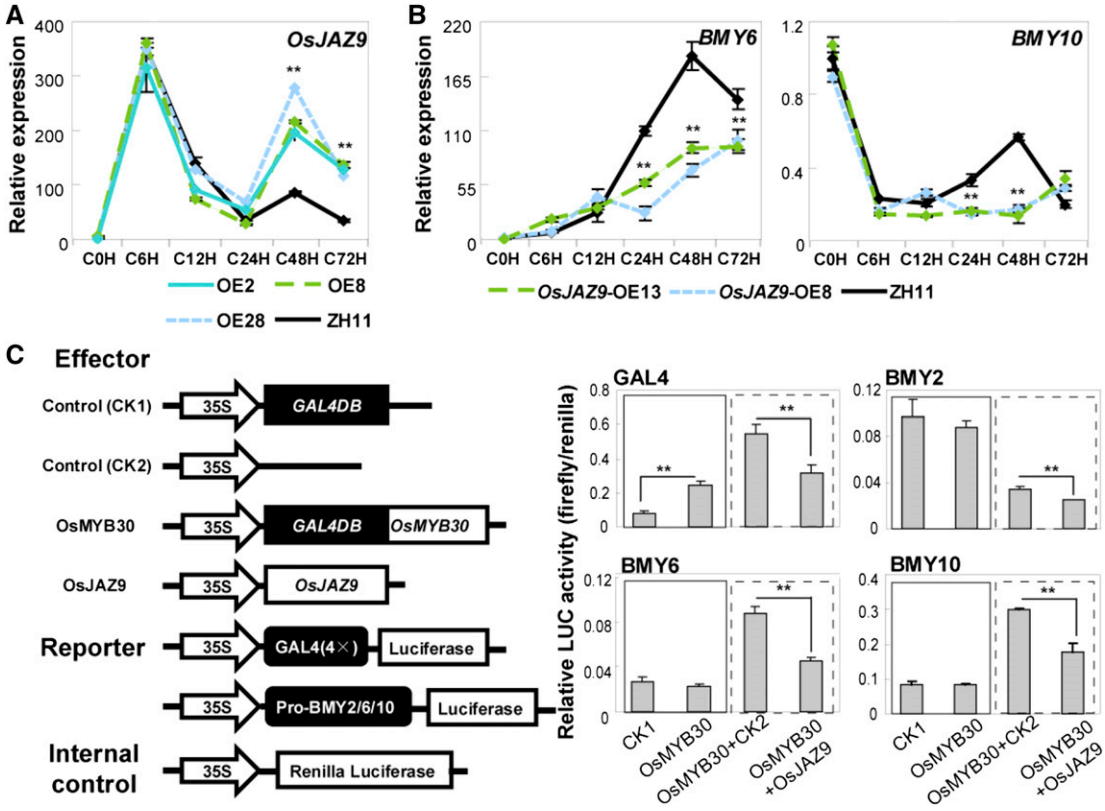


**Figure 7.** OsJAZ9 participates in the cold stress response and contributes to the negative regulation of OsMYB30. A, Expression levels of *OsJAZ9* in the *OsMYB30*-OE lines (OE2, OE8, and OE28) under cold stress conditions. ZH11 is the wild-type control of the overexpression lines. B, Expression levels of *BMY6* and *BMY10* in the *OsJAZ9*-OE (OE8 and OE13) plants under cold stress conditions. Error bars indicate the SE of three replicates. C, Effect of OsMYB30 on promoters of the *GAL4* and *BMY* genes using a dual-luciferase transient assay in rice protoplasts with or without OsJAZ9 cotransformed. The main components of the vectors are displayed on the left. On the right, the effect of OsMYB30 on the promoters is compared with the control CK1, which is indicated by the solid line box. The effect of OsJAZ9 on the promoters, given the presence of OsMYB30, was evaluated based on the comparison of OsMYB30 + OsJAZ9 and OsMYB30 + CK2, which is indicated by the dotted line box. GAL4, BMY2, BMY6, and BMY10 represent reporter constructs containing the corresponding promoters. Error bars indicate the SE of three replicates. **, $P < 0.01$ by Student's *t* test.

negative regulator of cold tolerance (Fig. 2) through repressing the expression of the *BMY* genes for control of the content of maltose, which had a protective role during cold stress treatment (Figs. 3 and 4). In addition, we found that the repressor protein OsJAZ9 interacted with OsMYB30 and participated in the OsMYB30-mediated repression of the *BMY* genes (Fig. 7). Thus, we proposed a model of cold tolerance regulation in rice mediated by the negative regulator OsMYB30 (Fig. 8) that represents an important development in deepening our understanding of the regulation mechanism of MYB proteins in cold stress tolerance.

Based on our results, the negative regulation of *BMY* genes may be one of the cold-responsive pathways mediated by OsMYB30. Since the expression level of *BMY* genes (Fig. 3C), the total amylase activity (Fig. 4, A and B), and the maltose content (Fig. 4, C and D) were correlated with the physiological index (membrane leakage and $F_v/F_m$) under cold stress conditions (Figs. 2 and 4, E and F). And these results could at least partially explain the phenotypic changes in cold sensitivity of the overexpression plants or cold tolerance in mutant plants. Nevertheless, some of the *BMY* genes were induced by the cold stress (Fig. 3C), which was unexpected given the suppression effect of OsMYB30 on these genes. The expression levels of these *BMY* genes may be regulated by other transcription factors in addition to OsMYB30.

### Complexity of OsMYB30 Regulation in Starch and Sugar Metabolism

Recent advances in understanding starch metabolism have led to a simplified model proposed for starch synthesis and degradation in Arabidopsis (Kötting et al., 2010). However, the regulation of starch metabolism remains poorly understood in cereal crops, including rice. From our results, the expression level of four members (*OsAGPL3*, *OsSSIIIb*, *OsSSIIb*, and *OsSSIIc*)

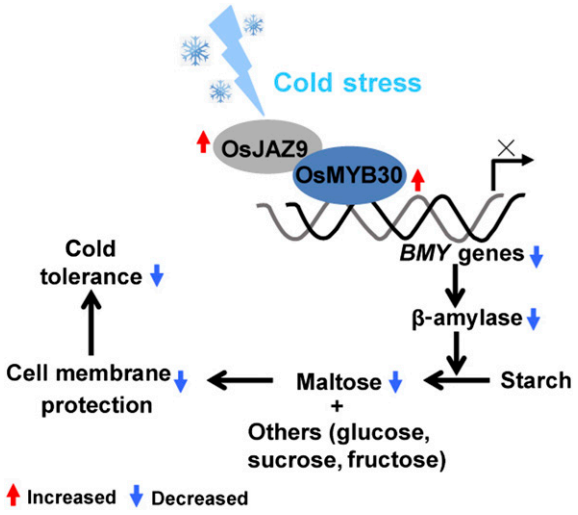


**Figure 8.** Schematic model of the OsMYB30 regulation mechanism in the cold stress response. Both *OsMYB30* and *OsJAZ9* were induced by cold stress. OsMYB30 directly bound to the promoter of the rice *BMY* genes. The interaction with OsJAZ9 may help OsMYB30 achieve the negative regulation on *BMY* genes. The *BMY* genes were repressed by OsMYB30-OsJAZ9 at the transcriptional level and BMY activity was decreased, then starch degradation and maltose accumulation were inhibited, resulting in the cell membrane protection being weakened, finally enhancing cold sensitivity.

of 10 starch synthesis enzymes (Fu and Xue, 2010) were up-regulated by OsMYB30 (Supplemental Fig. S9), and this may contribute to the starch accumulation observed after 72 h of cold stress treatment (Supplemental Fig. S8), while the repression of the *BMY* genes also may contribute to the starch accumulation, indicating that OsMYB30 may be involved in the starch regulation network. According to the microarray analysis, the number of genes involved in carbohydrate metabolism increased significantly after cold stress treatment (Fig. 3B). For example, four reported starch synthesis genes, *OsSSI*, *OsPHOL*, *OsDPE1*, and *OsDPE2*, were down-regulated by OsMYB30 during cold stress treatment (Ohdan et al., 2005), while two known genes related to starch degradation, *ADH1* and *RAMY3D*, were down-regulated and up-regulated, respectively, during cold stress treatment (Hwang et al., 1999; Takahashi et al., 2014). It remains to be determined whether OsMYB30 is involved in the regulation of these genes in starch or other carbohydrate metabolism pathways.

So far, our understanding of the physiological function of maltose in the stress response is limited. One study in Arabidopsis showed that maltose was a predominant form of soluble sugar exported from chloroplasts at night (Lu and Sharkey, 2006). Another study suggested that maltose could protect the photosynthetic electron transport chain during cold stress treatment (Kaplan and Guy, 2004, 2005). It has been generally accepted that the maintenance of cell membrane integrity and stability is critical for cold stress tolerance (Whitlow et al., 1992; Morsy et al., 2005), and conductivity can directly reflect damage to the cell membrane. Our results showed the relevance between maltose content and conductivity during cold stress treatment (Figs. 2, C and G, and 4, C and D). Furthermore, the exogenous application of maltose led to a reduced increase in conductivity in cold-sensitive plants such as *OsMYB30*-OE plants and some cold-sensitive rice accessions, suggesting that maltose could reduce the damage to the cell membrane. Besides this, the chlorophyll fluorescence $F_v/F_m$ ratio, which has been adopted as another physiological indicator reflecting the photosynthetic electron transport chain during cold stress treatment (Rosinski and Atchley, 1998; Baker and Rosenqvist, 2004; Ehlert and Hincha, 2008; Mishra et al., 2014; Thalhammer et al., 2014), was decreased in the rice plants with lower maltose accumulation (Fig. 4, E and F). This result was similar to previously reported results (Kaplan and Guy, 2004, 2005). Thus, we speculated that maltose may play an important role in maintaining cell membrane integrity and stability under the cold stress condition in rice.

It has been reported that maltose was involved in carbohydrate metabolism for the production of cytosolic Glc and Suc during starch degradation in Arabidopsis (Lu and Sharkey, 2004; Sharkey et al., 2004; Kaplan and Guy, 2005). But in this study, the *OsMYB30*-OE plants with decreased maltose content under cold stress treatment showed no obvious change in Suc, while Glc was increased slightly at 24 and 48 h after cold stress treatment. According to the proposed model of Arabidopsis starch degradation in chloroplasts (Kaplan and Guy, 2005; Kötting et al., 2010), we proposed that OsMYB30 may repress the pathway of starch degradation to produce maltose through the regulation of the *BMY* genes and slightly affect Glc production through unknown pathway(s), but for Suc, which was the final product of maltose and Glc in the cytosol, the synthesis of Suc may be regulated by different regulators, which await further investigations.

### OsJAZ9 May Facilitate the OsMYB30-Mediated Negative Regulation of Cold Stress Tolerance

A repression motif is sufficient for transcriptional repression. For example, a known conserved ERF motif was identified in ERF transcriptional repressors, and the MYB repressor UIF1 was found to contain this motif (Ohta et al., 2001; Moreau et al., 2016). In addition, a novel repression motif different from the ERF motif was identified in AtMYBL2, which acted as a negative regulator of anthocyanin biosynthesis in Arabidopsis (Matsui et al., 2008). However, no reported repression motif was found in OsMYB30; thus, we proposed that the OsMYB30-mediated repression of the *BMY* genes may require some other partner. Previous studies have revealed that JAZ proteins with transcriptional repression roles interacted with bHLH, MYB, and CBF/DREB1 to influence stamen development, seed production, and

abiotic tolerance (Song et al., 2011; Hu et al., 2013; Qi et al., 2015; Wu et al., 2015). Here, we observed that OsJAZ9, which functioned as a repressor (Ye et al., 2009; Wu et al., 2015), could interact with OsMYB30, which was verified by three different experimental techniques (Fig. 6). It should be noted that *OsJAZ9* was strongly induced by cold stress treatment (a few hundred-fold induction; Fig. 7A), and this induction also was detected in a chip data set from our group based on eight rice accessions with differences in cold tolerance (GSE71680). Additionally, leaves of *OsJAZ9*-OE plants (OE13 and OE8) rolled more quickly than leaves of ZH11 plants, although the survival rate of the seedlings showed no differences (Supplemental Fig. S13). We suspected that OsJAZ9 may negatively regulate the target genes of OsMYB30, including *BMY6* and *BMY10*, through interaction with OsMYB30. As expected, the expression levels of *BMY6* and *BMY10* were indeed repressed significantly in *OsJAZ9*-OE plants during cold stress conditions (Fig. 7B). However, the *OsJAZ9* suppression plants showed no phenotypic difference after cold stress, except for the up-regulation of *BMY6* (Supplemental Fig. S13C). As the previous study indicated, the *OsJAZ9* expression level was suppressed to only 40% of the level in the wild type (Wu et al., 2015). It is possible that such a moderate suppression was not enough to cause obvious phenotypic change but could affect the suppression of some target genes of OsMYB30. Moreover, the genetic effects of the overexpression of *OsJAZ9* alone may not be strong enough to cause serious phenotypic differences in terms of the survival rate after cold stress treatment.

More importantly, OsJAZ9 may be required for the transcriptional repression function of OsMYB30. When cotransformed with OsJAZ9, the activation of OsMYB30 on the *BMY2*, *BMY6*, and *BMY10* promoters was sharply reduced compared with OsMYB30 alone (Fig. 7C). On the other hand, OsMYB30 alone showed 3-fold activation, and this activation was weakened with a reduction of 50% when OsJAZ9 was cotransformed. These results supported that OsJAZ9 could help in enhancing the negative regulation and weaken the activation activity of OsMYB30. Taken together, OsJAZ9 may act as a repressor by targeting transcriptional factors like OsMYB30 and participate in the cold stress response through the regulation of downstream genes, including the *BMY* genes.

In conclusion, we have identified a negative regulation mechanism for cold tolerance in rice, which was mediated by OsMYB30 and its interaction protein OsJAZ9 through the regulation of the *BMY* genes. These findings also have expanded our understanding of the MYB family in cold stress regulation in plants.

## MATERIALS AND METHODS

### Plant Materials and Stress Treatments

The genomic sequence of *OsMYB30* was amplified from the *japonica* rice (*Oryza sativa*) variety IRAT109, digested by *Sal*I and *Kpn*I, and cloned into the pCAMBIA1301H vector driven by the *OsLEA3-1* promoter LEAP (Xiao et al., 2007). The *japonica* rice variety ZH11 was used as a transformation recipient.

To investigate the transcript levels of *OsMYB30* under hormone treatments, ZH11 seedlings were grown in a greenhouse (14-h-light/10-h-dark conditions) with temperatures of 28°C and 25°C for the light and dark conditions, respectively. Seedlings at the four-leaf stage were treated with ABA (spraying 100 $\mu$M $L^{-1}$ ABA on the leaves), JA (spraying 100 $\mu$M $L^{-1}$ JA on the leaves), heat (42°C growth chamber), flooding (submerging the seedlings completely with water), drought (stopping the water supply), salt (irrigation with 200 mM NaCl solution), or cold (4°C growth chamber). The experiment was repeated once with treatments of the same concentrations of ABA and JA added in the medium and different levels of abiotic stresses, including heat (40°C growth chamber), flooding (submerging two-leaf stage seedlings with water), drought (exposing the seedlings in the air without water supply), salt (irrigation with 150 mM NaCl), and cold (10°C growth chamber). The hormone- or stress-treated seedlings were sampled at the designated times.

To test the cold stress tolerance of *OsMYB30* and *OsJAZ9* transgenic or mutant plants at the seedling stage, the positive transgenic plants were selected by germinating on one-half-strength MS medium containing 25 mg $L^{-1}$ hygromycin. The ZH11 (wild-type), *osmyb30* mutant, and Huayang (wild-type) seeds were germinated on normal one-half-strength MS medium. After germination, the seedlings were transplanted to normal one-half-strength MS medium (12 seedlings each, three repeats) and in soil (20 seedlings each, three repeats) in a greenhouse with control plants in the same pot (half of each). The seedlings were transferred to a 4°C chamber at the four-leaf stage. After 3 to 7 d, depending on the performance of the seedlings, the plants were transferred back to normal conditions for recovery, and the survival rates were recorded.

For the maltose treatment under cold stress conditions, the growth conditions were essentially the same as mentioned above. Seedlings of *OsMYB30* plants were grown on one-half-strength MS medium with control plants in the same pot (12 seedlings each) with three repeats, and seedlings of seven accessions (24 seedlings each) also were grown on the one-half-strength MS medium. They were subjected to cold stress treatment (4°C chamber) at the four-leaf stage, and 40 mL of 2.78 mM $L^{-1}$ (10 mg $mL^{-1}$) maltose was added in the medium (cold plus maltose treatment); for the cold treatment only, distilled water was added instead of maltose. The experiment began with the addition of maltose and lasted for 3 d in a 4°C chamber. The seedlings (not including roots) were sampled for electrolyte leakage and gene expression analyses at the designated times.

### Quantification of Gene Expression

Total RNA was extracted using Trizol reagent (Invitrogen). The first-strand cDNA was reverse transcribed using SuperScript reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qPCR was conducted on the 7500 Real-Time PCR System (Applied Biosystems) using SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's instructions. The rice *ACTIN1* gene was used as the internal control. The relative expression level was determined as reported previously (Livak and Schmittgen, 2001). For the GUS assay of the $P_{OsMYB30}$::*GUS* transgenic plants, seven tissues or organs were collected for GUS staining as described previously (Ning et al., 2010).

### Subcellular Localization and Biochemical Assays in Yeast

To confirm the subcellular localization of OsMYB30, the full-length cDNA of *OsMYB30* was cloned into the pM999-33 vector driven by the CaMV 35S promoter. The fusion construct *35S*::*OsMYB30*::*GFP* was cotransformed with *35S*::*GHD7*::*CFP*, which was used as a nuclear marker, into rice protoplasts prepared from etiolated shoots by polyethylene glycol treatment. The construct *35S*::*GFP* was used as a control. After transformation for 12 to 16 h, the fluorescence signal was observed with a confocal microscope (Leica).

For the yeast one-hybrid assay, we used the Matchmaker one-hybrid system following the manual instruction (Clontech). The promoter fragments of the *BMY* genes containing the MYB-specific cis-element were amplified and fused to the *HIS3* mini promoter in the pHIS2 vector to generate the reporter constructs, designated as pHIS2-P*BMY2*, pHIS2-P*BMY6*, and pHIS2-P*BMY10*. The full-length *OsMYB30* was fused to the GAL4 activation domain in pGBDT7-Rec2, which was cotransformed with reporter constructs into the yeast strain Y187. pGADT7-53 and pHIS2-P*53* were cotransformed as positive controls, while pGBDT7-OsMYB30 and pHIS2-P*53* were used as negative controls. The following confirmation of the DNA-protein interactions was analyzed on SD/-Trp/-Leu/-His medium with 50 mM 3-AT.

### Microarray Analysis

Seedlings of two independent overexpression plants and the corresponding wild-type ZH11, and two independent homozygous *osmyb30* mutants and the

wild-type Huayang, were sampled (20 plants each) for the microarray experiment. The results were confirmed by qPCR for 12 randomly chosen genes (Supplemental Fig. S5). The microarray analysis was conducted according to the protocol from the Affymetrix GeneChip service (CapitalBio). The differentially expressed genes (with the change of relative expression levels 1.8-fold or greater or 0.6-fold or less) in the overexpression and mutant plants compared with the wild types were selected for MAS 3.0 molecule annotation analysis (http://bioinfo.capitalbio.com/mas3) with a significance threshold of $P < 0.01$.

## EMSA

To produce the OsMYB30 protein, the full-length cDNA was inserted into the pGEX-4T-1 expression vector (GE Healthcare) at the *Eco*RI and *Sal*I restriction sites and expressed in *Escherichia coli* (DE3 cells from GE Healthcare). The GST-tagged fusion protein was purified with a Glutathione HiCap Matrix (Qiagen; 139302891). Briefly, 5′ FAM oligonucleotides were synthesized by Shanghai Sangon (Supplemental File S6). The double-stranded oligonucleotides were generated by mixing with an equal amount of the complementary single-stranded oligonucleotides for 2 min at 95°C and cooled down to 25°C. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific), and purified GST-fused OsMYB30 was preincubated with the binding buffer [1 $\mu$g of poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, and 5 mM $MgCl_2$] at room temperature for 20 min. Then, nonlabeled probe was added into the mixture for the competition assay. After 20 min at room temperature, 1 $\mu$L of 5′ FAM-labeled probe (10 $\mu$M $L^{-1}$) was added and incubated for 20 min at room temperature. The samples were run on a 6% PAGE gel with 0.5× Tris-borate-EDTA buffer in the dark for 1 h at 4°C. The fluorescence signal was captured by FLA-5100 (Fuji).

## Physiological and Biochemical Measurements

The electrolyte leakage measurement was performed according to the previous study (Lv et al., 2016). Briefly, three fully expanded leaves at the four-leaf stage from three seedlings were cut into segments of the same size and immersed in 8 mL of double distilled water in a 10-mL tube for 24 h with shaking. The initial conductivity (R1) was measured with a model DDS-IIA device (Shanghai Leici Instrument). Then, the tube with leaf segments was boiled and the conductivity was determined (R2). The relative electrolyte leakage was calculated as the ratio of R1 to R2.

The chlorophyll measurements ($F_v/F_m$ ratio) were performed with a modulated chlorophyll fluorescence instrument (PAM-2500; Walz). Leaves of plants at the tillering stage, at which the leaves had enough width (about 1 cm) to measure, were sampled and immersed in 1% agarose for overnight dark treatment. Five leaf segments were measured for each line of *OsMYB30* transgenic plants to read the ratio.

The total activity of the BMY enzymes was determined according to a previous study (Kaplan and Guy, 2004) using the BMY Assay Kit (Megazyme) with little modification. Briefly, 0.2 g of leaf powder sample from ground-up tissues of four-leaf stage seedlings, frozen in liquid nitrogen, was prepared in 0.5 mL of $\beta$-buffer A after extraction. We used 0.1 mL of supernatant for the $\alpha$-amylase enzyme activity assay (no difference was observed; data not shown), and 0.1 mL of supernatant was added into 0.1 mL of reaction substrate for the BMY enzyme activity assay.

The determination of starch content was performed using the HCl hydrolysis-3,5-dinitrosalicylic acid method. The leaf tissues of seedlings were sampled at the four-leaf stage at the designated times under the cold stress condition (4°C chamber). Briefly, 20 mg of lyophilized fine powder of leaves from four-leaf stage seedlings was added into 3 mL of 2.5 mol $L^{-1}$ KOH (40°C), and the starch was dissolved totally with incubation at 40°C for 5 min. Then, the mixture was added in 1.5 mL of 0.4 mol $L^{-1}$ NaAc and a moderate volume of HCl to regulate the pH at 4.75. Finally, 60 $\mu$L of amyloglucosidase (Sigma-Aldrich) was added, the mixture was incubated at 60°C for 45 min (shaking every 5 min), and the supernatant was collected after centrifugation for the determination of Glc. A total of 50 $\mu$L of supernatant in a 10-fold dilution was mixed with 100 $\mu$L of 3,5-dinitrosalicylic acid solution. After incubating the reaction in a boiling water bath for 5 min, 350 $\mu$L of distilled water was added, and the absorbance was measured at 540 nm. The starch content in the samples was calculated through the standard curve of Glc.

The measurement of sugar content was performed as described previously with minor modifications (Tan et al., 2011). The soluble sugar in four-leaf stage seedlings was measured at the designated times under the cold stress condition (4°C chamber). Briefly, 10 mg of lyophilized fine powder from ground-up seedlings was incubated with 0.9 mL of chloroform at 37°C for 45 min, and after that, 0.9 mL of double distilled water was added and shaken at 37°C for 45 min. After centrifugation at 3,000*g* for 30 min at 4°C, 10 $\mu$L of the supernatant was added to 25 $\mu$g $mL^{-1}$ labeled Suc as an internal standard and dried in a lyophilizer. The dried sample was resuspended in 50 $\mu$L of methoxyamine hydrochloride for 1 h at 50°C with brief sonication. Then, 50 $\mu$L of BSTFA (including 1% TMCS; Sigma-Aldrich) was added with incubation for 1 h at 50°C. These products were then used for the subsequent gas chromatography-mass spectrometry analysis.

## ChIP Assays

We sampled the seedling leaf tissues from the *osmyb30* mutant and the wild type for chromatin extraction under normal and cold stress conditions, which were named N-*osmyb30*, N-WT, Cold-*osmyb30*, and Cold-WT, respectively. The rabbit polyclonal antibody by Yi Ji Sheng Wu (http://www.immunogen.com.cn/) was used for the immunoprecipitation. The OsMYB30 antibody was against the full-length protein (Supplemental Fig. S14). The ChIP assay was performed as described previously (Zong et al., 2013). Briefly, samples from four-leaf stage seedlings were fixed by 1% formaldehyde under vacuum for 30 min and frozen in liquid nitrogen; 3 g of powder for each sample was used for chromatin isolation. Isolated chromatin was sheared to approximately 100 to 500 bp by Diagenode Bioruptor. Then, the soluble chromatin was isolated and preabsorbed by DNA/protein A-agarose (Sigma-Aldrich). The sample immunoprecipitated with anti-OsMYB30 antibody (immunoprecipitation sample) and the sample subjected to the same operation without antibody (input sample) were used for quantification of the precipitated DNA using qPCR. The enriched levels of specific DNA in the immunoprecipitation samples were calculated relative to the levels in the corresponding input samples. The specific primers are listed in Supplemental File S6.

## Transient Expression Assays in Rice Protoplasts

The isolation and transformation of rice protoplasts were performed according to the previous polyethylene glycol-mediated method (Xie and Yang, 2013; Wu et al., 2015). Briefly, the protoplasts were isolated in digestion solution including 10 mM MES (pH 5.7), 0.6 M mannitol, 1 mM $CaCl_2$, 3 mM $\beta$-mercaptoethanol, 0.1% BSA, 0.75% Cellulase R10 (Yakult Pharmaceutical), and 0.75% Macerozyme R10 for 5 h under dark conditions. The following collection and incubation were performed in W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 5 mM KCl, and 125 mM $CaCl_2$) at room temperature, then the protoplasts were filtered through a sieve mesh and resuspended in 4 mM MES, 0.6 mannitol, and 15 mM $MgCl_2$ after centrifugation at 100*g* for 5 min. Each transformation contained 10 $\mu$L of different plasmid, 100 $\mu$L of protoplasts, and 110 $\mu$L of polyethylene glycol-$CaCl_2$ solution (0.6 M mannitol, 100 mM $CaCl_2$, and 40% polyethylene glycol 4000). After incubation at room temperature for 10 min, 440 $\mu$L of W5 solution was added to stop the process. Protoplasts were cultured in 800 $\mu$L of 4 mM MES, pH 5.7, 0.6 mannitol, and 4 mM KCl after collection at 100*g* for 5 min on 24-well culture plates. After 12 h, protoplasts were collected for the following dual-luciferase activity assay according to the manual instructions (Promega; Dual-Luciferase Reporter Assay System).

## Protein Interaction Analysis

For the interaction of OsMYB30 and OsJAZ9 in vivo, we performed a BiFC assay in the rice protoplast system with the pVYNE vector according to a previous study (Waadt et al., 2008; Tang et al., 2012). A co-IP assay was performed using a transient assay based on the rice protoplast system with the isolation and transformation procedures as described previously (Tang et al., 2016; Zong et al., 2016). The two transformations (OsMYB30-GFP plus OsJAZ9-MYC and OsMYB30-GFP plus MYC) were 10-fold amplified. After an incubation period in the dark for 16 h, the protoplasts were collected and resuspended in 600 $\mu$L of co-IP buffer (50 mM, pH 8, Tris-HCl, 150 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1× Protease Inhibitor Cocktail [Roche]). The protoplast proteins were collected from the supernatant after centrifugation at 12,000 rpm for 15 min, and 10% of the supernatant was used as the input sample. A total of 40 $\mu$L of GFP-Trp beads (Chromotek) and the supernatant from the two transformations were mixed and incubated with gentle rolling at 4°C for 4 h. Then, the beads were resuspended in 100 $\mu$L of 2× Laemmli buffer after washing with co-IP buffer five times. The immunocomplexes were analyzed by western blot using anti-OsMYB30 (Yi Ji Sheng Wu) and anti-MYC (ABclonal; AE038) antibodies.

For the interaction assay in vitro, a pull-down assay was performed as described previously (Y. Zhao et al., 2015) with little modification. Equal volumes of GST or OsMYB30-GST and OsJAZ9-His recombinant proteins were incubated for 6 h at 4°C in 500 $\mu$L of His-binding buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, and 20 mM imidazole), then 100 $\mu$L of Ni-NTA agarose (Qiagen; 133223438) was added into the mixture, and this was incubated for 3 h at 4°C. After extensive washing and boiling, the pull-down proteins were detected by an anti-GST antibody (Abcam; ab19256).

## Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *OsMYB30*, AK112056.1; *OsJAZ9*, AK070649.1; *BMY2*, AP014963.1; *BMY6*, AK287595.1; *BMY10*, AK120049.1; and *ACTIN1*, AK100267.1. The chip data have been deposited in the NCBI Gene Expression Omnibus with accession numbers GSE71680 and GSE83912.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Expression patterns of *OsMYB30* under abiotic stresses and in different tissues and organs.

**Supplemental Figure S2.** Expression levels of *OsMYB30* in the overexpression plants and the *osmyb30* mutant using qPCR.

**Supplemental Figure S3.** Phenotypes of the *OsMYB30*-OE plants and the *osmyb30* mutant under cold stress conditions with seedlings grown in soil.

**Supplemental Figure S4.** Expression patterns of cold tolerance-related genes under cold stress conditions.

**Supplemental Figure S5.** Validation of chip data results through qPCR for 12 randomly chosen genes.

**Supplemental Figure S6.** Expression patterns of seven other *BMY* genes under cold stress conditions.

**Supplemental Figure S7.** Suc, Glc, and Fru contents in *OsMYB30*-OE and *osmyb30* mutant plants.

**Supplemental Figure S8.** Starch content in *OsMYB30*-OE and the *osmyb30* mutant at the seedling stage during cold stress treatment.

**Supplemental Figure S9.** Expression levels of 10 starch synthesis genes before and after cold stress treatment in *OsMYB30*-OE and *osmyb30* mutant plants.

**Supplemental Figure S10.** Expression levels of *BMY6* and *BMY10* in *OsMYB30*-OE and ZH11 plants under cold stress treatment and cold stress plus 2.78 mM maltose treatment.

**Supplemental Figure S11.** Electrolyte leakage of seven accessions at the seedling stage under cold stress conditions and cold stress plus 2.78 mM maltose treatment.

**Supplemental Figure S12.** Expression levels of the *OsJAZ9* gene in *osmyb30* mutant plants before and after cold stress treatment.

**Supplemental Figure S13.** Phenotypes of the *OsJAZ9*-OE plants, *OsJAZ9* suppression plants, and ZH11 under cold stress conditions.

**Supplemental Figure S14.** Western blot of the antibody against OsMYB30.

**Supplemental File S1.** Genes up-regulated by OsMYB30 under normal conditions.

**Supplemental File S2.** Genes up-regulated by OsMYB30 under cold stress conditions.

**Supplemental File S3.** Genes down-regulated by OsMYB30 under normal conditions.

**Supplemental File S4.** Genes down-regulated by OsMYB30 under cold stress conditions.

**Supplemental File S5.** Accession numbers of the *BMY* genes in rice.

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

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