Journal of Experimental Botany, Vol. 63, No. 7, pp. 2541–2556, 2012
doi:10.1093/jxb/err431 Advance Access publication 2 February, 2012
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice

An Yang[1,2], Xiaoyan Dai[1] and Wen-Hao Zhang*[,1]

[1] State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, the Chinese Academy of Sciences, Beijing 100093, PR China

[2] Graduate University of the Chinese Academy of Sciences, Beijing 100049, PR China

* To whom correspondence should be addressed. E-mail: whzhang@ibcas.ac.cn

Received 1 August 2011; Revised 3 December 2011; Accepted 6 December 2011

Abstract

MYB-type transcription factors play a diverse role in plant development and response to abiotic stress. This study isolated a rice R2R3-type MYB gene, *OsMYB2*, and functionally characterized its role in tolerance to abiotic stress by generating transgenic rice plants with overexpressing and RNA interference *OsMYB2*. Expression of *OsMYB2* was up-regulated by salt, cold, and dehydration stress. OsMYB2 was localized in the nucleus with transactivation activity. No difference in growth and development between the *OsMYB2*-overexpressing and wild-type plants was observed under normal growth conditions, but the *OsMYB2*-overexpressing plants were more tolerant to salt, cold, and dehydration stresses and more sensitive to abscisic acid than wild-type plants. The *OsMYB2*-overexpressing plants accumulated greater amounts of soluble sugars and proline than wild-type plants under salt stress. Overexpression of *OsMYB2* enhanced up-regulation of genes encoding proline synthase and transporters. The *OsMYB2*-overexpressing plants accumulated less amounts of H_2O_2 and malondialdehyde. The enhanced activities of antioxidant enzymes, including peroxidase, superoxide dismutase, and catalase, may underlie the lower H_2O_2 contents in *OsMYB2*-overexpressing plants. There was greater up-regulation of stress-related genes, including *OsLEA3, OsRab16A*, and *OsDREB2A*, in the *OsMYB2*-overexpressing plants. Microarray analysis showed that expression of numerous genes involving diverse functions in stress response was altered in the *OsMYB2*-overexpressing plants. These findings suggest that *OsMYB2* encodes a stress-responsive MYB transcription factor that plays a regulatory role in tolerance of rice to salt, cold, and dehydration stress.

Key words: Abiotic stress, MYB, *OsMYB2*, oxidative stress, proline, rice, soluble sugars.

Introduction

Plants are sessile organisms and are frequently exposed to variable environmental stress that adversely affect plant growth and agricultural production. To cope with the stress, plants have evolved efficient mechanisms to sense and rapidly adapt to stressed conditions. As the phytohormone abscisic acid (ABA) plays a crucial role in the adaptive response of plants to abiotic stresses, the pathways leading to the adaptation to stress can be divided into two major categories: ABA-dependent and ABA-independent pathways (Xiang *et al.*, 2008). During the response and adaptation to abiotic stress, there are many changes in biochemical and physiological processes. These include accumulation of osmolytes and cryoprotectants such as sugar and proline (Xin and Browse, 1998) to facilitate osmo-regulation and prevent oxidative damage due to disruption of reactive oxygen species (ROS) homeostasis (Suzuki and Mittler, 2006). In addition, many genes are activated, leading to accumulation of numerous proteins involved in resistance to abiotic stress, such as late embryogenesis abundant (LEA; Ma *et al.*, 2010). Activation of the genes can protect plants from oxidative damage due to osmotic stress, ionic toxicity, and oxidative stress (Bartels, 2005). The expression of stress-induced genes is largely regulated by specific transcription factors (Hu *et al.*,

2008). Among these transcription factors, members of the APETELA2 (AP2), bZIP, NAC, and MYB families have been well characterized for their regulatory roles in the response of plants to abiotic stress (Hu *et al.*, 2008; Takasaki *et al.*, 2010). There have been a number of studies demonstrating that transgenic plants overexpressing genes encoding transcription factors can greatly enhance their tolerance to various abiotic stresses such as salinity, cold, and drought (Dubouzet *et al.*, 2003; Vannini *et al.*, 2004; Nakashima *et al.*, 2007; Xiang *et al.*, 2008; Song *et al.*, 2011).

MYB transcription factors occur widely in animals, plants, and fungi (Lippold *et al.*, 2009). They were first identified as oncogenes in animals, where their function is linked to control of the cell cycle (Ito *et al.*, 2001). MYB proteins contain one, two, or three imperfect repeats (51–53 amino acids) in their DNA-binding domain, and they are further classified into three subfamilies, type MYBR2R3, type MYBR1R2R3, and MYB-related, depending on the number of repeats in their MYB domains (Stracke *et al.*, 2001; Chen *et al.*, 2006). Among the MYB proteins in plants, the MYB family with the two-repeat (R2R3) is the most common one (Stracke *et al.*, 2001). There are 126 and 109 R2R3-type MYB proteins in *Arabidopsis* and rice, respectively (Chen *et al.*, 2006). The involvement of *Arabidopsis* MYB proteins in the regulation of secondary metabolism, control of cellular morphogenesis, and regulation of the meristem and the cell cycle has been demonstrated (Kranz *et al.*, 1998).

More recently, R2R3-type MYB proteins are reported to be involved in responses of plants to environmental stress. For instance, AtMYC2 and AtMYB2 proteins play important roles as transcription factors in ABA-dependent gene expression under drought and salt stress (Abe *et al.*, 2003). Denekamp and Smeekens (2003) reported that AtMYB102 is a key component to integrate signalling pathways in responses of *Arabidopsis* to wounding, osmotic stress, and ABA. Several studies also revealed that MYB proteins in *Arabidopsis* (AtMYB44, AtMYB60, and AtMYB61) are involved in regulation of stomatal aperture in response to drought stress (Cominelli *et al.*, 2005; Liang *et al.*, 2005; Jung *et al.*, 2008). In addition, AtMYB15 was found to negatively regulate the induction of cold-responsive genes in an ABA-independent way (Agarwal *et al.*, 2006). Lippold *et al.* (2009) reported that AtMYB41 controls the short-term transcriptional responses to osmotic stress.

Three MYB proteins have been reported to be involved in response of rice to abiotic stress. For instance, overexpression of *OsMYB4* significantly confers tolerance to chilling and freezing stress in transgenic *Arabidopsis* (Vannini *et al.*, 2004; Pasquali *et al.*, 2008). Ma *et al.* (2009) reported that *OsMYB3R-2* participates in the cold signalling pathway by targeting the cell cycle and a putative DREB/CBF. Moreover, a recent study revealed that *OsMYBS3* is essential for conferring tolerance of rice plants to cold stress (Su *et al.*, 2010). Among these characterized MYB proteins, OsMYB4 is the only R2R3-type protein, while OsMYB3R-2 and MYBS3 are R1R2R3-type and MYB-related proteins, respectively. Furthermore, studies on rice MYB proteins have mainly focused on their roles in response to cold stress, while little is known about the role of MYB proteins in response of rice to other abiotic stress, such as salt and dehydration stress.

This study isolated a R2R3-MYB transcription factor, designated *OsMYB2*, in rice. The role of *OsMYB2* in response of rice plants to salt, cold, and dehydration stress was characterized by generating transgenic plants with overexpressing and RNA interference (RNAi) *OsMYB2*.

Materials and methods

Plant material, growth conditions, and stress treatments

Seeds of rice cultivar Zhonghua 10 (*Oryza sativa* L.) were surface-sterilized by incubation for 3 min in 75% ethanol, followed by 10 min in 0.1% $HgCl_2$, and then washed thoroughly with sterile water. The sterilized seeds were germinated on half-strength Murashige and Skoog (1/2 MS) agar (0.6%, w/v, agar; pH 5.8) in darkness for 2 days. Thereafter the germinated seedlings were grown in a greenhouse at 28/25 °C (day/night) with a 14-h photoperiod. Two-week-old seedlings were treated with varying chemicals and abiotic stresses after the MS agar was washed off. Chemical treatments were conducted by exposing the seedlings to 1/2 MS medium containing 100 μM abscisic acid (ABA), 100 μM indoleacetic acid, 100 μM salicylic acid, or 10 μM brassinosteroids for 5 h and sampled for further analysis. For treatment with salt stress, 2-week-old seedlings were submerged into 1/2 MS medium containing 200 mM NaCl and sampled at varying periods after treatments. For cold and dehydration treatments, the 2-week-old seedlings were exposed to 2 °C and 20% PEG solution, respectively, and sampled at 0, 5, and 10 h after treatments.

Subcellular localization and transactivation assay

The whole coding sequence of *OsMYB2* was ligated with *Xba*I and *Kpn*I-digested pBI221 vector to generate pBI221-*OsMYB2-GFP* containing an *OsMYB2-GFP* fusion construct under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter. The construct was confirmed by sequencing and used for transient transformation of onion (*Allium cepa*) epidermis via a gene gun (Bio-Rad). After 24 h of incubation, GFP fluorescence in transformed onion cells was observed under a confocal microscope (Zeiss, Germany).

To determine the transactivation activity, the open reading frame of *OsMYB2* was generated by PCR amplification, cloned into *Kpn*I and *Xba*I sites, and fused in-frame to the GAL4 DNA binding domain in the vector pGBKT7 (Invitrogen). The fused construct of pGBKT7-*OsMYB2* was transformed into AH109 cells by the lithium acetate-mediated method. The transformed yeast strain was plated on SD/–Trp medium at 28 °C for 2 days. Yeast transformants from SD medium lacking Trp were then transferred and streaked onto solid SD agar lacking Trp/His/Ade (SD/–Trp/–His/–Ade) to score the growth response after 3 days. For the colony-lift filter assay (X-gal assay), the yeast was transferred to Whatman filter paper plus X-gal for transcription activation activity analysis within 8 h. Transcription factor *OsNAC5* was used as a positive control.

Vector construction and plant transformation

The full-length cDNA of *OsMYB2* were amplified from rice with the primers 5′-CGCGGATCCATGGACATGGCGCACGAGAG-3′ (*Bam*HI site underlined) and 5′-CGGGGTACCTCACACGGCGGCCTGGGTGG-3′ (*Kpn*I site underlined). The product was ligated into pGEM-T Easy vector (Promega) and sequenced. Then the *OsMYB2* fragment digested from pGEM-T

Easy-*OsMYB2* was cloned into the *Kpn*I-*Bam*HI sites of a pUN1301 vector to obtain the pUN1301-*OsMYB2* construct. *OsMYB2* was driven by an ubiquitin promoter in the construct and a GUS marker was carried in the vector pUN1301 as described previously (Ge *et al.*, 2004). The pUN1301-*OsMYB2* construct was electroporated into *Agrobacterium tumefaciens* EHA105 and then introduced into rice embryonic calli by *A. tumefaciens* EHA105-meditated methods (Xu *et al.*, 2005). *OsMYB2* transgenic rice plants were selected in 1/2 MS medium containing 75 mg l^{-1} hygromycin (Roche, Germany).

The RNAi plasmid was constructed as described by Wang *et al.* (2004). Briefly, a 314-bp fragment of *OsMYB2* was amplified using the primers 5′-GGGGTACCACTAGTGAGCTGTCGAGCAC-CACG-3′ (*Kpn*I and *Spe*I sites underlined) and 5′-CGGGATCC-GAGCTCTGGTCGTCCTCCATGCTC-3′ (*Bam*HI and *Sac*I sites underlined). Gene transformation protocols were used as described above.

Southern blot

Genomic DNA extracted from 14-d-old seedlings was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, and blotted onto a nylon membrane (Hybond–XL; Amersham Pharmacia Biotech) under alkaline conditions. α-^{32}P-dCTP-labeled *GUS* amplified from pUN1301 was used as a probe for hybridization. The membrane was exposed to X-ray film (Eastern Kodak) at –80 °C for 5 d.

Determination of tolerance to salt, cold, and dehydration stress

Two-week-old seedlings of both wild-type and *OsMYB2* transgenic rice were submerged into 1/2 MS medium supplemented with 200 mM NaCl for 2 days. Then the plants were transferred into the incubation solution without NaCl for an additional 4 days. For cold stress, 2-week-old seedlings of wild-type and *OsMYB2* transgenic rice were subjected to treatment at 2 °C for 3 days and then transferred into a greenhouse at a temperature of 28/25 °C (day/night) with a 14-h photoperiod for 1 week. For dehydration stress, wild-type and *OsMYB2* transgenic plants were exposed to 1/2 MS medium containing 20% PEG for 2 days and recovered in normal growth conditions for 1 week.

Wild-type and transgenic plants grown in soil were used to determine the stress tolerance. For treatment with salt stress, 40-d-old plants grown in pots were watered with 100 mM NaCl for 10 days and then recovered for another 8 days. For dehydration treatment, plants grown in pots for 60 days were subjected to drought stress for 7 days by withholding water and then were re-watered for 10 days. For cold stress, plants grown in pots under normal conditions for 30 days were exposed to 2 °C for 4 days and then recovered for another 6 days.

Determination of proline and soluble sugars

Proline contents in rice leaves were determined by the method described previously (Bates *et al.*, 1973; Song *et al.*, 2011). Total soluble sugar content was measured following the methods used previously (Baily, 1958; Song *et al.*, 2011).

Determination of H_2O_2 and malondialdehyde

Hydrogen peroxide was measured as described previously (Alexieva *et al.*, 2001) with some modifications. Briefly, 1 g leaf sample was ground with 0.1% trichloroacetic acid and centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was used to measure H_2O_2 spectrophotometrically. The reaction mixture consisted of 1 ml of the extracted supernatant, 1 ml of K_2PO_4 buffer, and 2 ml of 1 M KI. The reaction was developed for 1 h in darkness and the absorbance was measured at 390 nm.

Malondialdehyde (MDA) content in rice leaves was determined following the protocols described by Song *et al.* (2011). Briefly, rice leaves were homogenized in 5 ml of 10% trichloroacetic acid containing 0.25% thiobarbituric acid. The mixture was incubated in water at 95 °C for 30 min and the reaction was stopped in an ice bath. The mixture was centrifuged at 10,000 *g* for 20 min and the absorbance of the supernatant was measured at 450, 532, and 600 nm.

Determination of peroxidase, superoxide dismutase, and catalase activity

Two-week-old plants were exposed to 200 mM NaCl for 24 h. Rice leaves (approx. 0.50 g) were ground thoroughly with a cold mortar and pestle in 50 mM potassium phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15000 *g* for 20 min at 4 °C. The supernatant was crude enzyme extraction. The activities of peroxidase (POD; EC 1.11.1.7), superoxide dismutase (SOD; EC 1.15.1.1), and catalase (CAT; EC 1.11.1.6) were measured using the protocols described by Miao *et al.* (2010).

RNA isolation and real-time reverse-transcription PCR

Total RNA was isolated from leaves using TRIzol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). The total RNA was reverse-transcribed into first-strand cDNA with M-MLV reverse transcriptase (Promega). Real-time reverse-transcription (RT)-PCR was performed in an optical 96-well plate with a real-time Mx3000P PCR system. Each reaction contained 7.5 µl of 2×SYBR Green Master Mix reagent, 0.5 µl cDNA samples, and 0.6 µl of 10 µM gene-specific primers in a final volume of 15 µl. The thermal cycle used was 95 °C for 5 min and 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The primers were: for *OsMYB2*, 5′-GGGCTGAAACGCACAGGCAAGA-3′ and 5′-CTGCTTGGCGTGCTTCTGC-3′; for J033099M14, 5′-CTCAA ATCAAGGCGTCAACTAAGA-3′ and 5′-TTTGTCAATATATA CGTGGCATATACCA-3′; for J033031H21, 5′-CGCCCCTC CCCGTATCT-3′ and 5′-AGGAATGCGGCAACAAGTG-3′; for 03g44230, 5′-AGGGACGATGGAGTTCTAAAGCT-3′ and 5′-G GGATTCCAAAGGCAAAAAGA-3′; for 07g01090, 5′-GAG-GAGGCTACCTGACT GTCAAC-3′ and 5′-GCTCATGAAGT CGCCAAGGA-3′ (Xiang *et al.*, 2007); for *OsLEA3*, 5′-CGGCAG CGTCCTCCAAC-3′, 5′-CGGTCATCCCCAGCGTG-3′; for *OsDREB2A*, 5′-GCTGCACATCAGCACCTTCA-3′, 5′-TCCTGC ACCTCAGGGACTAC-3′; for *OsRab16A*, 5′-CACACCACAG-CAAGAGCTAAGTG-3′ and 5′-TGGTGCTCC ATCCTGCT-TAAG-3′; and for *actin*, 5′-ACCACAGGTATTGTGTTGGACT C-3′ and 5′-AGAGCATATCCTTCATAGATGGG-3′. The relative quantification (Delta-Delta CT) was used to evaluate quantitative variation between the replicates. The amplification of *actin* (accession no. AB047313) was used as an internal control to normalize data.

Microarray analysis

Total RNA was isolated from wild-type and *OsMYB2*-overexpressing plants using TRIzol reagent. All processes for labelling, hybridization, and washing were performed through Affymetrix custom service (Capitalbio) by the protocols given at http://www.affymetrix.com/support/technical/mannual/exprssion-mannual.affx. Normalization was performed according to the standard Affymetrix protocols to allow the comparison of the samples. Expression of genes with changes of more than 3-fold was taken as significantly different. Two biological replicates were used in the present study.

Statistics

All data were analysed by analysis of variance using SAS statistics program. Statistical differences are referred to as significant when $P < 0.05$.

Results

Isolation and characterization of OsMYB2

OsMYB2 (accession no. AK120551) cDNA was isolated from a salt-stressed rice microarray. The gene comprises 1645 nucleotides with a 990-bp open reading frame. It encodes a putative protein of 329 amino acids with a calculated molecular mass of 36.0 kD and a pI of 4.59. A Blastp search indicated that it is an R2R3-Myb protein with two imperfect repeat sequences in its MYB domain (Supplementary Fig. S1, available at *JXB* online).

A phylogenetic tree based on the full-length amino acid sequences of rice MYB proteins was constructed (Supplementary Fig. S1). The resulting trees contained six clusters, which were named as C6, C12, C17, C20, C25, and Cr28 according to the classification by Zhang *et al.* (2011). OsMYB4 (LOC_Os04g43680) clustered with LOC_Os02g41510, LOC_Os10g33810 in C25. OsMYB3R-2 (LOC_Os01g62410) clustered in C6, which was composed of four R1R2R3-MYB proteins. OsMYBS3 (AAN63154) is a MYB-related protein and clustered in Cr28. OsMYB2 (LOC_Os3g20090) clustered with another five members in C12, representing the functional cluster of the regulation of stamen development. According to the phylogenetic tree, OsMYB2 protein had the highest similarity with LOC_Os07g48870, a R2R3-MYB protein with unknown function.

OsMYB2 is located in the nucleus and has transactivation activity in yeast

To examine the subcellular localization of *OsMYB2*, the open reading frame of *OsMYB2* was fused to the 5′-terminus of the *GFP* reporter gene under the control of the CaMV 35S promoter. The recombinant constructs of the *OsMYB2-GFP* fusion gene and *GFP* alone were introduced into onion (*A. cepa*) epidermal cells by particle bombardment. The results showed that the *OsMYB2-GFP* fusion protein was specifically localized in the nucleus, whereas *GFP* alone showed ubiquitous distribution in the whole cell (Fig. 1A).

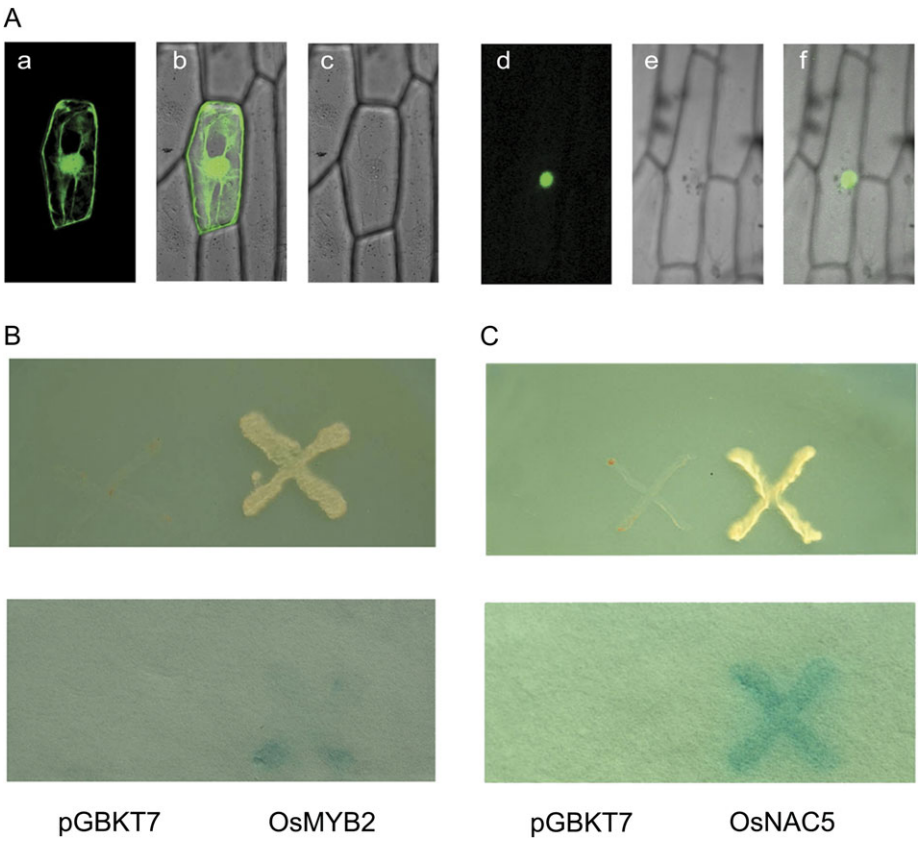

Fig. 1. Subcellular localization and transactivation analysis of *OsMYB2*. (A) Nuclear localization of *OsMYB2*. Confocal images of onion epidermis cells under the GFP channel showing the constitutive localization of GFP (a) and nuclear localization of *OsMYB2*-GFP (d). The confocal images (b and e) are of the same cells in (a) and (d) with transmitted light. The merged images (c and f) are of (a) and (b) and (d) and (e), respectively. GFP or *OsMYB2*-GFP fusion was driven by the control of the cauliflower mosaic virus 35S promoter. Onion epidermal peels were bombarded with DNA-coated gold particles and GFP expression was visualized 24 h later. (B) Transactivation assay of *OsMYB2* in the yeast strain AH109. Fusion protein of the GAL4 DNA-binding domain and *OsMYB2* were expressed in yeast strain AH109. The vector pGBKT7 was expressed in yeast as a control. The culture solution of the transformed yeast was dropped onto SD plates without tryptophan, histidine, or adenine. The plates were incubated for 3 days (upper) and then subjected to β-galactosidase assay (lower). (C) Transactivation assay of *OsNAC5* in the yeast strain AH109.

To test whether *OsMYB2* has transcription activity, *OsMYB2* was fused in-frame to GAL4 DNA-binding domain in the pGBKT7 vector and the fusion constructs pBD-*OsMYB2* were transformed into the yeast strain AH109. The transcription factor *OsNAC5*, which has been shown to have transactivation activity and localize in nucleus, was used as a positive control during this assay (Takasaki *et al.*, 2010; Song *et al.*, 2011). As shown in Fig. 1B and C, only the transformants containing pBD-*OsMYB2* and pBD-*OsNAC5* grew normally on SD/–Trp/–His/–Ade medium exclusively and exhibited the activity of β-galactosidase reporter gene upon addition of X-gal on Whatman filter paper. Thus these results confirm that *OsMYB2* is a transcription activator.

Expression profile of OsMYB2

The response of *OsMYB2* expression to salt, cold, and dehydration stress was monitored by real-time RT-PCR. An increase in the *OsMYB2* transcript was observed after 30 min of exposure to salt stress. The salt stress-induced increase in the *OsMYB2* transcript peaked after 5 h of salt stress, and thereafter the transcript declined gradually under salt stress (Fig. 2A). A similar increase in the *OsMYB2* transcript was also observed when rice seedlings were exposed to low temperature (2 °C) or osmotic stress (20% PEG) (Fig. 2B). In addition, treatment of rice seedlings with ABA also led to an increase in expression of *OsMYB2* (Fig. 2C). In contrast, exogenous application of salicylic acid reduced the expression of OsMYB2, while no effect of indoleacetic acid and brassinosteroids on the OsMYB2 transcript was observed. *OsMYB2* was detected in roots, shoots, leaves, and flowers under non-stressed conditions, with the expression being greatest in leaves, followed by roots and shoots (Fig. 2D). The strong induction of this gene by abiotic stress prompted this study to check its promoter sequence (1500 bp upstream from the transcription start site) by searching the promoter sequence against the PLACE database (http://www.dna.affrc.go.jp/PLACE/). The promoter of *OsMYB2* contains stress-responsive related *cis*-elements, such as ABRE and MYB and MYC recognition sites (Supplementary Fig. S2).

Overexpression of OsMYB2 *enhanced tolerance to salt, cold, and dehydration stress*

To study the function of *OsMYB2*, an overexpressing construct and an RNAi construct, under the control of an ubiquitin promoter and CaMV 35S respectively, were transformed into rice 'Zhonghua 10' and several transgenic lines were obtained. The transgenic rice lines were confirmed by hygromycin selection, GUS staining, and real-time RT-PCR. Compared with the untransformed wild-type rice, the abundance of the *OsMYB2* transcript was higher in the *OsMYB2*-overexpressing lines (OE2, OE3, OE5, OE8, and

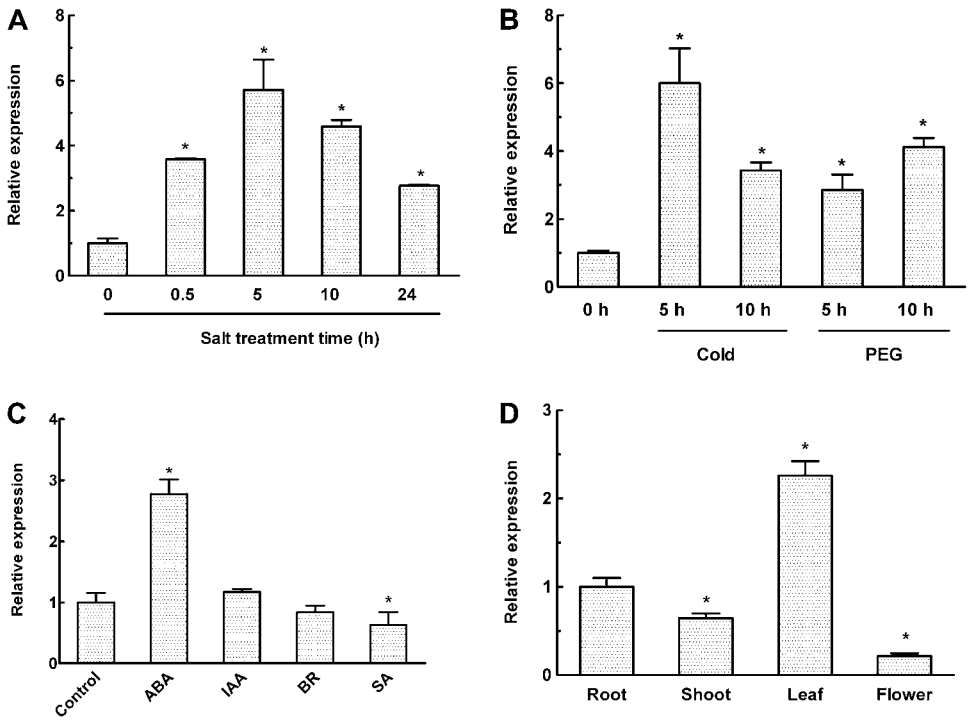

Fig. 2. Real-time reverse-transcription (RT) PCR analysis for the expression of *OsMYB2* in rice. (A and B) Time course of *OsMYB2* expression during salt (A) and cold and PEG (B) treatments. (C) Expression of *OsMYB2* under various hormone treatments. (D) Expression of *OsMYB2* in different tissues. Total RNAs were prepared from 14-d-old seedlings of wild-type rice after the above treatments and then reverse-transcribed. The resultant cDNAs were used as templates for real-time RT-PCR and *actin* was used as an internal control. Data are mean±SE of three biological replicates. Asterisks indicate statistically significant differences ($P < 0.05$) from controls (0 h in A and B, root in D).

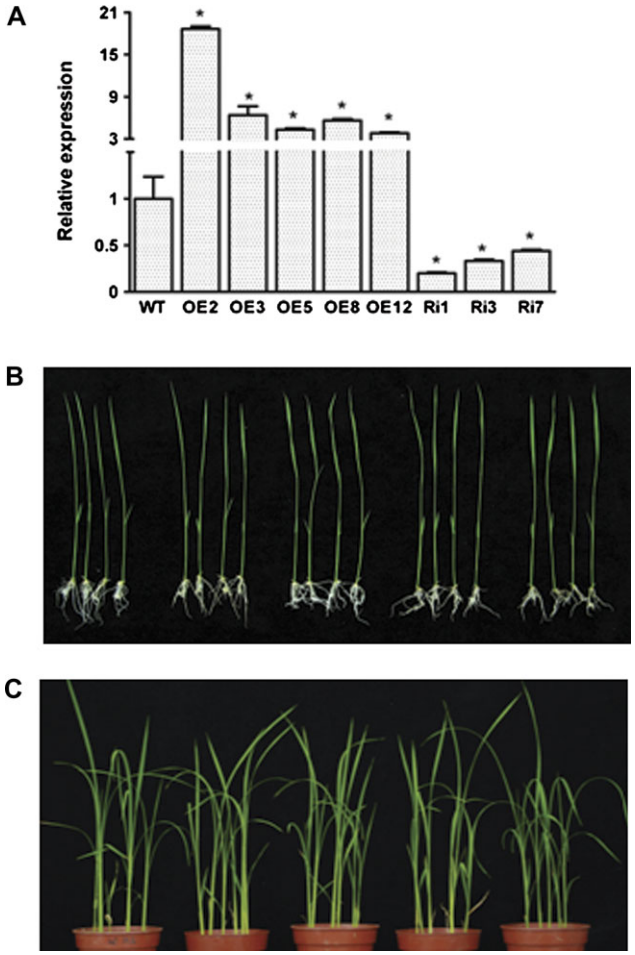

Fig. 3. Molecular characterization and phenotypes of *OsMYB2* transgenic rice. (A) *OsMYB2* expression in wild-type and transgenic rice. Total RNAs from 14-d-old wild-type and transgenic rice plants were isolated, reverse-transcribed, and analysed by real-time reverse-transcription PCR. *Actin* was used as an internal control. Error bars are based on three replicates. (B) The phenotypes of the T3 generation of wild-type and transgenic plants after growing on 1/2 MS medium for 14 days. (C) The phenotypes of the T3 generation of wild-type and transgenic plants after growing in soil for 30 days. Data are mean±SE of three biological replicates. Asterisks indicate statistically significant differences ($P < 0.05$) between wild-type (WT) and transgenic lines (OE and Ri).

OE12) and lower in the *OsMYB2*-underexpressing lines (Ri1, Ri3, and Ri7) than that of wild-type plants (Fig. 3A). To examine whether the phenotypes of transgenic lines differ from their wild-type, homozygous T3 progeny of the transgenic lines and the wild type were grown on 1/2 MS medium and in soil in the greenhouse. No differences in phenotypes among wild-type, overexpressing, and RNAi plants were observed when grown under normal, non-stressed conditions (Fig. 3B, C). T3 progeny of the transgenic lines (OE2, OE3, Ri1, and Ri3) were chosen based on their expression levels of *OsMYB2* and seed availability to further study the physiological function of *OsMYB2*. Southern blotting with a *GUS* gene probe was performed to confirm integration of exogenous *OsMYB2* into the rice genome, using DNA digested with *Eco*RI. Different hybridized patterns to the *GUS* probe were observed, indicating that *OsMYB2* was integrated into the rice genome and that the four transgenic lines were independent (Supplementary Fig. S3).

The involvement of *OsMYB2* in salt, cold, and osmotic stress was investigated by exposing wild-type and the transgenic plants grown in hydroponic solution with NaCl, low temperature, and PEG. There was no difference between transgenic and wild-type plants when grown under normal, non-stressed conditions in hydroponic solution (Fig. 4A). Phenotypically, most *OsMYB2*-overexpressing seedlings remained green and showed continuous growth, whereas both wild-type and RNAi seedlings showed severe leaf rolling and wilting after exposure to the salt, cold, and osmotic stress (Fig. 4B-D). In addition, the two transgenic rice lines overexpressing *OsMYB2* grown in soil also exhibited greater tolerance to NaCl, cold, and drought stress than wild-type and RNAi plants (Fig. 4E-J). This study also determined the survival rate for wild-type and transgenic plants grown in both hydroponic solution and soil challenged with salt, cold, and osmotic stress. As shown in Fig. 5A-C, the survival rate of overexpressing lines was significantly higher than that of wild-type and RNAi seedlings when exposed to salt stress (200 mM NaCl for 2 d), cold stress (5 °C for 3 d), and osmotic stress (20% PEG6000 for 2 d). The survival rates of the two over-expressing lines higher than those of wild-type and RNAi plants were also observed when rice seedlings grown in soil were challenged by salt, cold, and drought stress (Fig. 5D-F).

Overexpression of OsMYB2 *altered sensitivity of seed germination and growth to salt stress and ABA*

Given that expression of *OsMYB2* was sensitive to ABA (Fig. 2C), this study further investigated the effects of salt stress and ABA on seed germination of wild-type, *OsMYB2*-overexpressing, and RNAi seeds. Exposure of both wild-type and transgenic seeds to NaCl reduced their germination rate (Fig. 6A). However, germination of *OsMYB2*-overexpressing seeds was less inhibited by NaCl than that of wild-type and RNAi seeds. For example, seed germination rate of the two *OsMYB2*-overexpressing lines (OE2, OE3) was 81% and 86% when incubated in the presence of 100 mM NaCl, while germination rate for wild-type and RNAi (Ri1, Ri3) seeds was found to be 51%, 49%, and 53% under the identical conditions, respectively. The effect of NaCl on seedling growth was also examined. In the saline medium containing 150 mM NaCl, the *OsMYB2*-overexpressing plants exhibited faster growth and their shoots were significantly longer than wild-type plants (Fig. 6B, C).

In contrast to salt stress, seed germination of *OsMYB2*-overexpressing lines was more sensitive to ABA than that of wild-type and RNAi lines, such that wild-type and RNAi lines had higher seed germination rate than

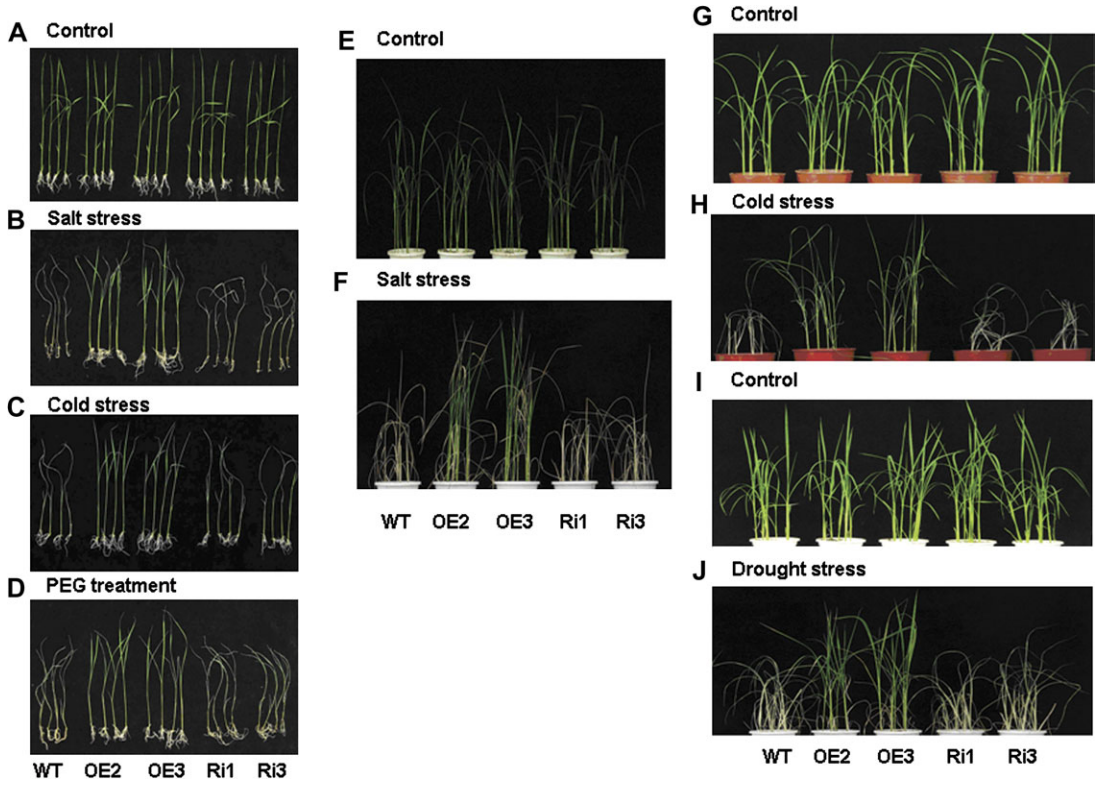

Fig. 4. Effect of salt, cold, and dehydration stress on wild-type (WT) and transgenic (OE and Ri) rice plants. (A–D) Phenotypes of wild-type and transgenic rice plants grown on 1/2 MS medium for 14 days and then under normal conditions (A), salt stress (200 mM NaCl for 2 days and normal conditions for 4 days) (B), cold stress (2 °C for 3 days and normal conditions for 7 days) (C), and treatment with 20% PEG for 2 days and recovery for 1 week (D). (E–J) Phenotypes of wild-type and transgenic rice plants grown in soil under normal conditions (E, G, I), subjected to 100 mM NaCl for 10 days and then recovered for another 8 days (F), exposed to 2 °C for 4 days and then recovered for another 6 days (H), and exposed to drought stress for 7 days and then re-watered for 10 days (J). Age of seedlings: 40 d ((E and F), 30 d (G and H) and 60 d (I and J).

OsMYB2-overexpressing lines when ABA was present in the incubation medium (Fig. 6D). Like seed germination, growth of *OsMYB2*-overexpressing seedlings was more inhibited by ABA than that of wild-type and RNAi seedlings, as shown by a shorter length of *OsMYB2*-overexpressing seedlings than wild-type and RNAi seedlings when grown in the presence of ABA (Fig. 6E, F). No difference in shoot length of wild-type and the transgenic plants grown in control medium was found.

OsMYB2-*overexpressing plants accumulated greater amounts of proline and soluble sugars*

Accumulation of proline and soluble sugars to facilitate osmoregulation is a common adaptive mechanism for tolerance of plants to abiotic stress. To test whether the enhanced tolerance of *OsMYB2*-overexpressing plants to salt stress is related to the capacity to accumulate proline and soluble sugars, the effect of salt stress on contents of proline and soluble sugars in wild-type and transgenic plants was investigated. There was no significant difference in proline contents between wild-type and transgenic plants under non-stressed, control conditions (Fig. 7A). An increase in proline content was observed upon exposure to salt stress in both wild-type and transgenic plants. However, the increase in proline content in the *OsMYB2*-overexpressing plants was significantly higher than in wild-type plants. Similar to proline, no significant difference in content of soluble sugars between wild-type and transgenic plants was observed under control conditions (Fig. 7B). However, the two *OsMYB2*-overexpressing lines accumulated greater amounts of soluble sugars than wild-type plants when these plants were exposed to 200 mM NaCl for 2 d.

To further elucidate the mechanism by which *OsMYB2*-overexpressing plants accumulate greater amounts of proline than wild-type plants under salt stress, the effects of salt stress on expression of genes responsible for proline biosynthesis and proline transport were investigated. As shown in Fig. 7C and D, treatment with salt stress led to a greater increase in transcripts of proline biosynthesis genes (Δ-1-pyrroline-5-carboxylate synthase genes, J033099M14 and J033031H21) in the *OsMYB2*-overexpressing lines than in the wild-type and RNAi lines. A similar greater up-regulation of the two genes encoding putative proline transport (03g44230 and 07g01090) in the two *OsMYB2*-overexpressing lines than in the wild-type and RNAi lines was also observed in response to salt stress (Fig. 7E, F).

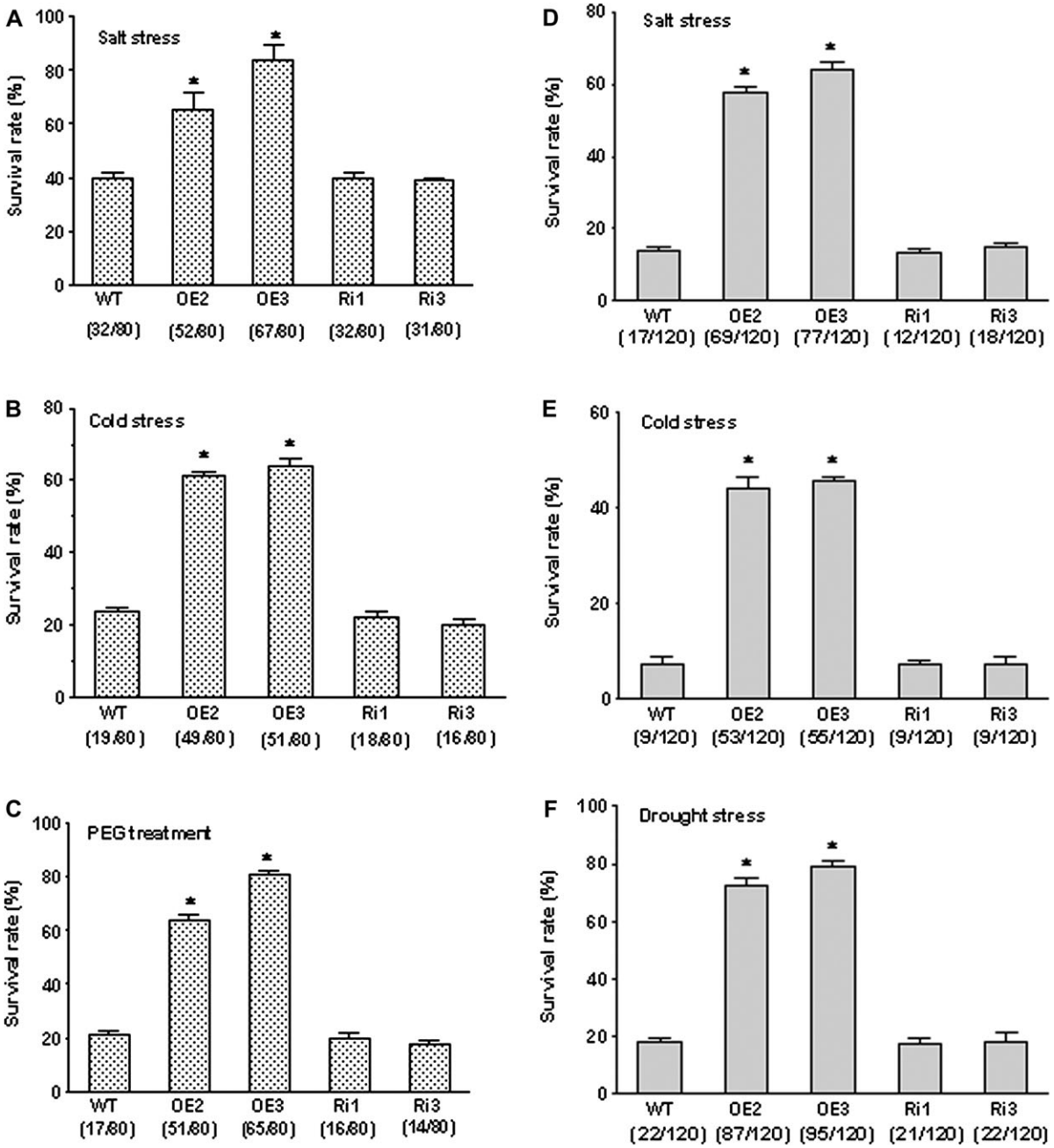

Fig. 5. Effect of salt, cold, and dehydration stress on survival rates of wild-type and transgenic rice plants, corresponding to the plants and treatments as shown in Fig. 4. (A–C) Plants grown on 1/2 medium. (D–F) Plants grown in soil. Data are mean±SE of three replicates with total seedling number of 80 for all stress treatments. Values in parentheses are the numbers of survived seedlings/total seedlings used to calculate the survival rate. Asterisks indicate statistically significant differences ($P < 0.05$) between wild-type (WT) and transgenic lines (OE and Ri).

OsMYB2-*overexpressing plants accumulated less* H_2O_2 *and MDA under salt stress*

The effects of NaCl on H_2O_2 and MDA contents in wild-type and transgenic rice were investigated and no significant differences in H_2O_2 and MDA contents were found in the absence of NaCl in the incubation medium (Fig.8A, B). There were marked increases in H_2O_2 and MDA contents in both wild-type and transgenic plants upon exposure to 200 mM NaCl. However, the salt stress-induced increases in H_2O_2 and MDA contents were less in the *OsMYB2*-overexpressing plants than those in the wild-type and RNAi plants. These results indicate that overexpression of *OsMYB2* confers greater tolerance of the oxidative stress associated with salt stress.

The lower content of H_2O_2 in the *OsMYB2*-overexpressing plants under salt stress prompted this study to test whether the difference in H_2O_2 accumulation between wild-type and the *OsMYB2*-overexpressing lines resulted from differences in the activities of the major antioxidant enzymes. Under normal conditions, activities of POD, SOD, and CAT were

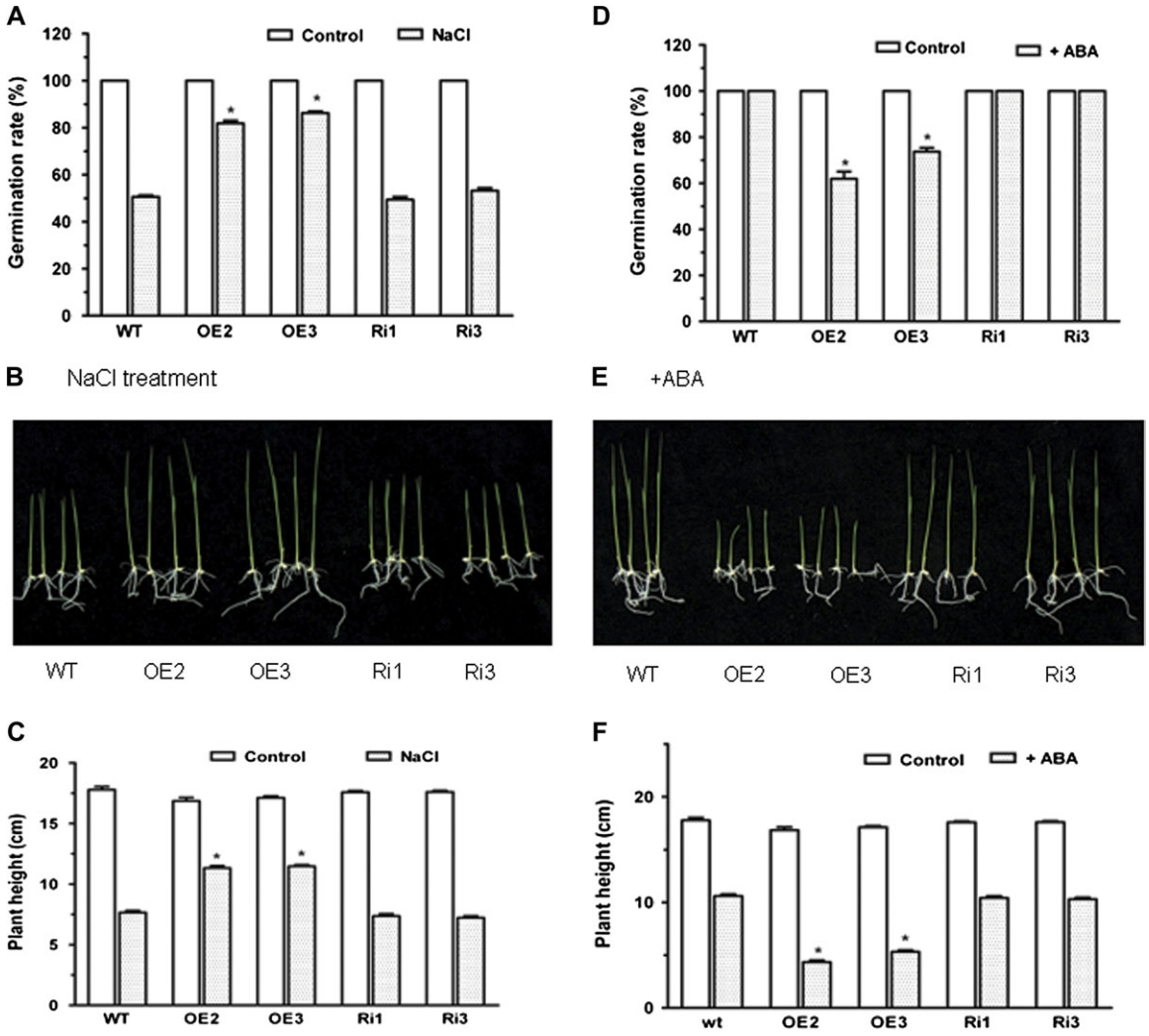

Fig. 6. Responses of seed germination and seedling growth to treatment with NaCl and abscisic acid (ABA). (A–C) Responses to NaCl treatment: (A) germination rates: T3 generation seeds were soaked in distilled water for 1 day and then allowed to germinate on sterile-water-saturated filter paper with 100 mM NaCl for 5 days; (B) phenotypes: seeds were allowed to germinate in darkness for 2 days, and then transferred to 1/2 MS medium containing 150 mM NaCl under 28/25 °C (day/night) with a 14-h photoperiod for 12 days; (C) shoot heights of seedlings under normal and 150 mM NaCl conditions. (D–F) Responses to ABA treatment: (D) germination rates: T3 generation seeds were soaked in distilled water for 1 day and then allowed to germinate on sterile-water-saturated filter paper with 4 μM ABA for 5 days; (E) phenotypes: seeds were allowed to germinate in darkness for 2 days, and then transferred to 1/2 MS medium containing 4 μM ABA under 28/25 °C (day/night) with a 14-h photoperiod for 12 days; (F) shoot heights of seedlings under normal and 4 μM ABA conditions. Data are mean±SE of three replicates. Asterisks indicate statistically significant differences ($P < 0.05$) between wild-type (WT) and transgenic lines (OE and Ri).

comparable among wild-type, *OsMYB2*-overexpressing, and RNAi plants (Fig.8C-E). There were marked increases in activities of these enzymes for the rice seedlings upon exposure to salt stress. However, the salt stress-induced increases in activities of POD, SOD, and CAT were higher in the *OsMYB2*-overexpressing plants than the wild-type and RNAi plants (Fig. 8C-E). In contrast to *OsMYB2*-overexpressing plants, activities of these enzymes in the *OsMYB2*-RNAi lines did not differ from the wild-type plants under salt-stressed conditions (Fig.8C-E). These results imply that overexpression of *OsMYB2* confers a more efficient antioxidant system to counteract oxidative stress under saline conditions.

Comparison of expression profiles between wild-type and OsMYB2*-overexpressing rice plants*

Up-regulation of several genes such as *OsLEA3*, *OsRab16A*, and *OsDREB2A* can contribute to enhanced tolerance of plants to salt stress (Zhang *et al.*, 2009). To test whether these genes are also involved in the enhanced tolerance of *OsMYB2*-overexpressing plants to salt stress, real-time RT-PCR was used to study the effect of salt stress on the expression levels of these genes in wild-type and transgenic plants. When treated with 200 mM NaCl, a significant up-regulation of the salt-responsive genes was observed in both wild-type and transgenic plants, with higher expression levels in the

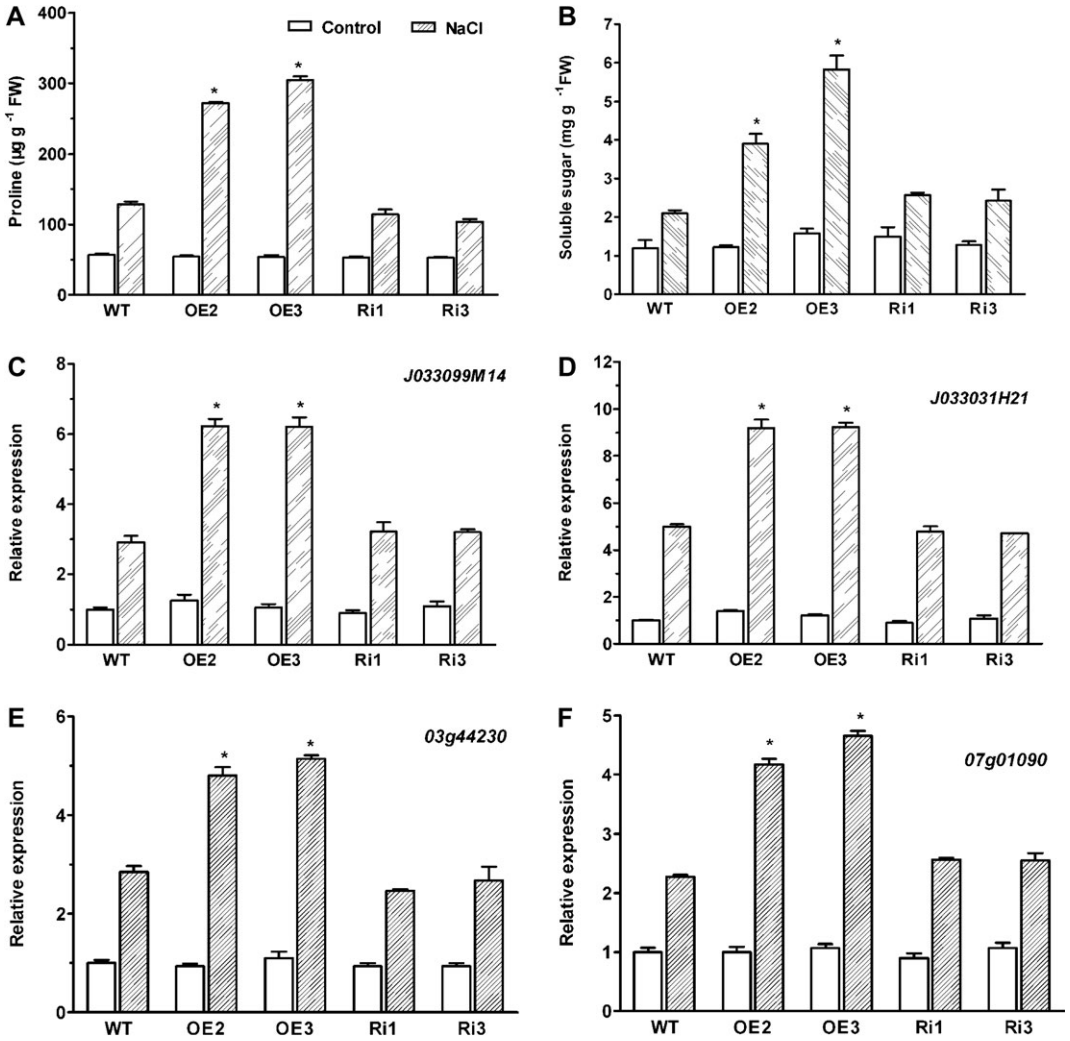

Fig. 7. Effect of salt stress on contents of proline and soluble sugars and gene expression in wild-type and transgenic rice plants. (A and B) Wild-type and transgenic rice seedlings of 14-d-old were exposed to 200 mM NaCl for 2 days and then collected for determination of proline (A) and soluble sugars (B) contents. (C–F) Expression levels of putative proline synthase genes (*J033099M14* and *J033031H21*; C and D) and transporter genes (*03g44230* and *07g01090*; E–F) in transgenic and wild-type plants. Total RNA was extracted from the 14-d-old rice seedlings grown under control and salt stress (200 mM NaCl) conditions for 24 hours. The transcript levels were measured by real-time reverse-transcription PCR. *Actin* was used as an internal control. Data are mean±SE of three replicates. Asterisks indicate statistically significant differences ($P < 0.05$) between wild-type (WT) and transgenic lines (OE and Ri). The accession numbers of the sequences of *J033099M14*, *J033031H21*, *03g44230*, and *07g01090* are AK102633, AK101230, AK067118, and AK0666298.

OsMYB2-overexpressing plants than in the wild-type and RNAi plants (Fig. 9). No difference in expression of these genes between wild-type and RNAi plants was found in response to salt stress (Fig. 9)

To identify the downstream genes targeted by *OsMYB2*, whole-genome expression profiling was performed using the Affymetrix Rice GeneChip array, which contains 55,515 probe sets. Relative change was determined by normalizing the data for the transgenic line (OE3) relative to wild-type plants. Total RNA was isolated from 14-d-old seedlings of the transgenic line and the wild type grown under normal conditions. A total of 519 genes was up-regulated (greater than 3-fold changes) and 423 genes were down-regulated (greater than 3-fold changes) in the overexpression line compared with wild-type plants (Supplementary Tables S2 and S3). The detected genes were mainly involved in stress response (Fig. 10A). For example, genes encoding stress-related functional proteins, such as dehydrin proteins, LEA proteins, and stress-related regulatory factors such as transcription factor, protein kinase, and phosphatase, were greatly affected by overexpression of *OsMYB2*. The microarray data was further validated by real-time RT-PCR. Twelve genes representing different expression profiles were analysed, of which all exhibited expression patterns comparable to that obtained from the microarray data (Fig. 10B). A high degree of concordance was observed between the results generated by the two methods (Fig. 10C).

Discussion

In the rice genome, there are 183 MYB-encoding genes with diverse roles in developmental processes and defence

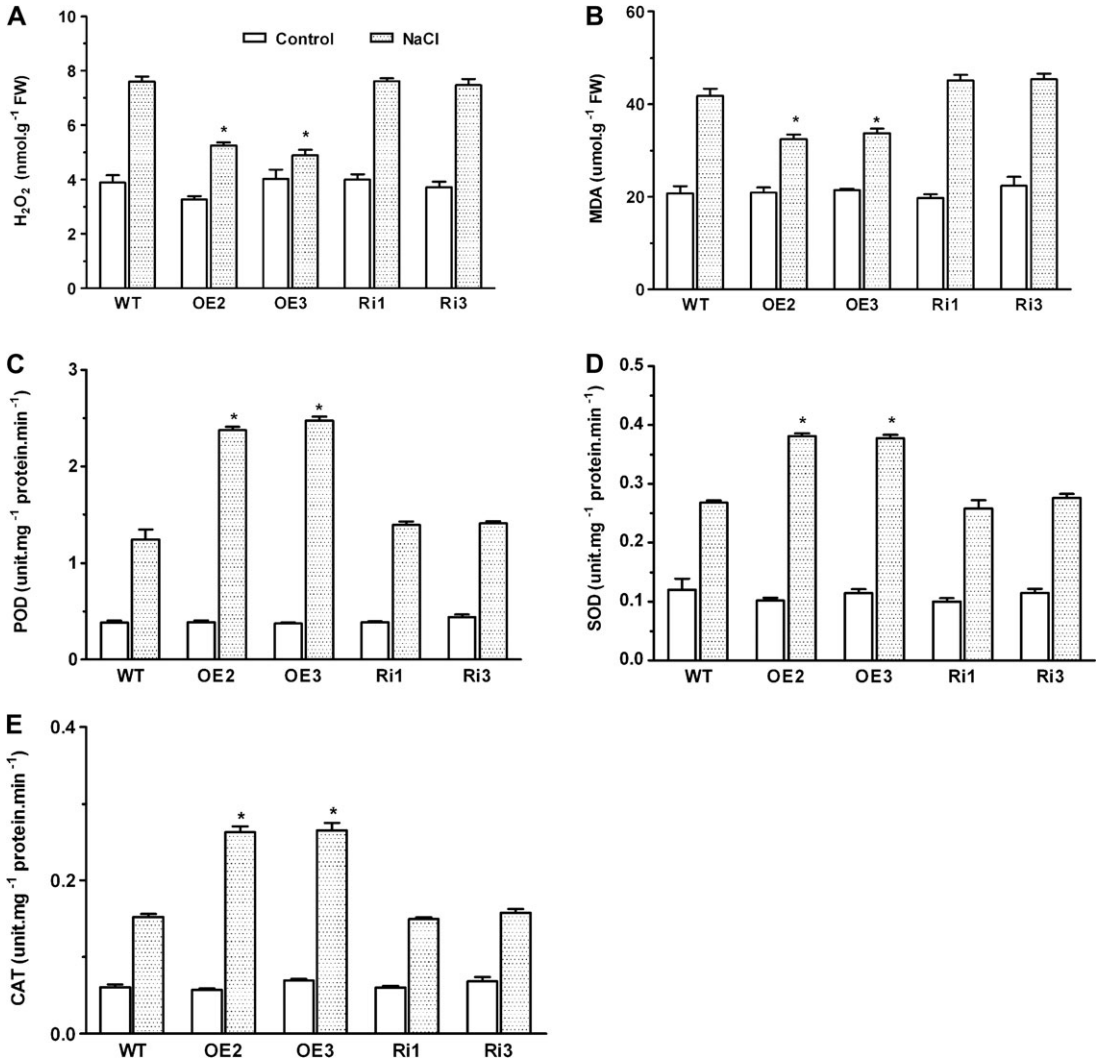

Fig. 8. Effect of salt stress on contents of oxidants (A and B) and antioxidant enzymes (C–D) in wild-type and transgenic rice plants. (A and B) Plants were exposed to 200 mM NaCl for 2 days before determination of H_2O_2 (A) and malondialdehyde (MDA) (B). (C–E) were exposed to 200 mM NaCl for 24 hours and before determination of peroxidase (POD; C), superoxide dismutase (SOD; D), and catalase (CAT; E). Data are mean±SE of three replicates. Asterisks indicate statistically significant differences ($P < 0.05$) between wild-type (WT) and transgenic lines (OE and Ri).

responses (Chen *et al.*, 2006). There is emerging evidence to support that many MYB proteins are involved in response and an adaptation to abiotic stress. For instance, the involvement of MYB proteins in tolerance of rice to cold stress has been demonstrated by overexpression of *OsMYB4* (Vannini *et al.*, 2004), *OsMYB3R-2* (Dai *et al.*, 2007; Ma *et al.*, 2009), and *OsMYBS3* (Su *et al.*, 2010) in *Arabidopsis* and rice, which conferred tolerance to cold stress at the seedling stage. The present study isolated a novel R2R3-type MYB gene, *OsMYB2*, and demonstrated that overexpression of *OsMYB2* greatly conferred tolerance of rice to salt, cold, and dehydration stress. This study also found that overexpression of *OsMYB2* led to greater accumulation of soluble sugars and proline due to up-regulation of genes responsible for proline synthesis and transport, and less accumulation of H_2O_2 and MDA under salt stress. These metabolic changes due to overexpression of *OsMYB2* would allow plants for effective osmo-regulation and less oxidative damage under salt stress, thus conferring tolerance to salt stress. In addition to these metabolic changes, overexpression of *OsMYB2* also led to changes in expression of numerous genes involved in stress response as revealed by the microarray data (Fig. 10). These results also showed that the transcripts of salt-responsive genes such as *OsLEA3*, *OsRAB16A*, and *OsDREB2A* were higher in the *OsMYB2*-overexpressing plants than in wild-type plants under salt stress (Fig. 9), suggesting that the improved tolerance of transgenic plants overexpressing *OsMYB2* may result from direct regulation of these stress-responsive genes by *OsMYB2*. Identification of targets of *OsMYB2* and unravelling their signalling network may shed some light on the molecular mechanism underlying the OsMYB2-dependent tolerance to salt stress.

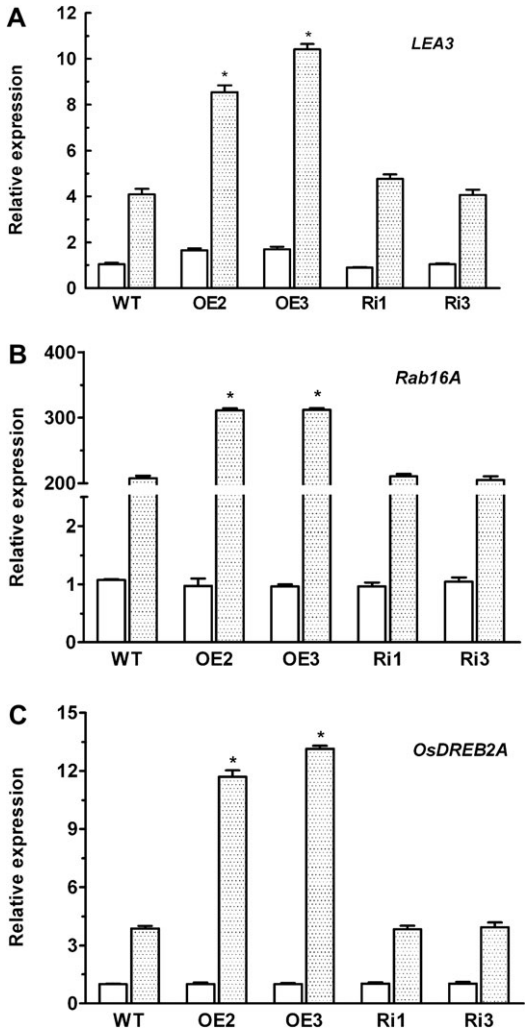

Fig. 9. Expression levels of some salt-responsive genes in wild-type and transgenic plants. (A) *OsLEA3*; (B) *OsRab16A*; (C) *OsDREB2A*. Total RNA was extracted from the 14-d-old rice seedlings grown under control (open columns) and salt stress (200 mM NaCl, filled columns) conditions for 24 hours. The transcript levels were measured by real-time reverse-transcription PCR. *Actin* was used as an internal control. Data are mean±SE of three replicates. Asterisks indicate statistically significant differences ($P < 0.05$) between wild-type (WT) and transgenic lines (OE and Ri).

This study constructed a phylogenetic tree with a total of 31 MYB proteins and clustered them into six groups following the protocols used by Zhang *et al.* (2011). It has been suggested that the clusters C12, C20, and C25 are involved in stress response (Zhang *et al.*, 2011). Cluster C6 was composed of four members with three MYB repeats, such as OsMYB3R-2. Four MYB-related proteins were grouped in Cr28. OsMYB2, together with AtMYB2, was clustered in C12, which has been implicated in stamen development (Zhang *et al.*, 2011). *AtMYB2*, which encodes a R2R3-type MYB transcription factor, has been shown to regulate salt- and dehydration-responsive genes (Urao *et al.*, 1993; Abe *et al.*, 2003). Yoo *et al.* (2005) demonstrated that a specific calmodulin isoform mediates salt-induced Ca^{2+} signalling through activation of *AtMYB2*, conferring tolerance of *Arabidopsis* to salt stress. These results suggest that the cluster C12 may also participate in the abiotic stress signalling in addition to stamen development.

The expression of *OsMYB2* was induced rapidly by cold such that its expression peaked after exposure to cold for 5 h and the expression declined thereafter (Fig. 2B). In contrast, cold-induced up-regulation of *OsMYB3R-2* is sustained up to 72 h of exposure to cold (Dai *et al.*, 2007). In addition, the present study found that the expression of *OsMYB2* was also up-regulated by salt and dehydration stress (Fig. 2A, B). These findings are in contrast to *OsMYB4* and *OsMYBS3*, which are induced by cold stress exclusively (Vannini *et al.*, 2004; Su *et al.*, 2010). Therefore, OsMYB2 is a novel R2R3-type MYB transcription factor in terms of its activation by abiotic stresses compared with the identified MYB proteins in rice.

To evaluate the role of *OsMYB2* in tolerance to abiotic stress, this study generated transgenic plants with over-expression and underexpression of *OsMYB2* by *Agrobacterium*-mediated transformation (Fig. 3). One important finding is that *OsMYB2*-overexpressing plants exhibited a higher survival rate than wild-type or RNAi plants grown in both hydroponic solution and soil when exposed to salt, cold, and dehydration stress (Figs. 4, 5). As far as is known, this is the first report demonstrating that a rice MYB protein confers tolerance to multiple abiotic stresses.

To elucidate the mechanisms responsible for the enhanced tolerance of *OsMYB2*-overexpressing plants to salt stress, several experiments were conducted to monitor changes in physiological processes associated with plant tolerance to salt stress. Accumulation of compatible solutes such as soluble sugars (Garg *et al.*, 2002; Gupta and Kaur, 2005) and free proline (Liu and Zhu, 1997; Armengaud *et al.*, 2004) is a common phenomenon in response to abiotic stress. The accumulated soluble sugars and free proline act as osmolytes to facilitate osmo-regulation, thus protecting plants from dehydration resulting from salt stress by reducing water potential of plant cells. In addition, proline can also function as a molecular chaperone to stabilize the structure of proteins as well as play a role in regulation of the antioxidant system (Hare *et al.*, 1999; Szekely *et al.*, 2008). These compatible solutes have been reported to play a predominant role in transgenic plants with enhanced tolerant to abiotic stress (Xiang *et al.*, 2007; Xu *et al.*, 2008). This study found greater accumulation of soluble sugars and free proline in the *OsMYB2*-overexpressing plants, which may partially account for the higher tolerance of *OsMYB2*-overexpressing plants to salt stress (Fig. 7B). In this context, similar increases in soluble sugar and proline levels have been demonstrated in transgenic plants with overexpressing *OsMYB4* and *OsMYB3R-2* under conditions of cold stress (Vannini *et al.*, 2004; Pasquali *et al.*, 2008; Ma *et al.*, 2009).

Overexpression of *OsMYB4* confers tolerance to chilling and freezing by up-regulating the cold-regulated (COR) gene and increasing the proline level in *Arabidopsis* (Vannini *et al.*, 2004) and enhances tolerance to cold and drought by accumulating compatible solutes in transgenic apple (*Malus*

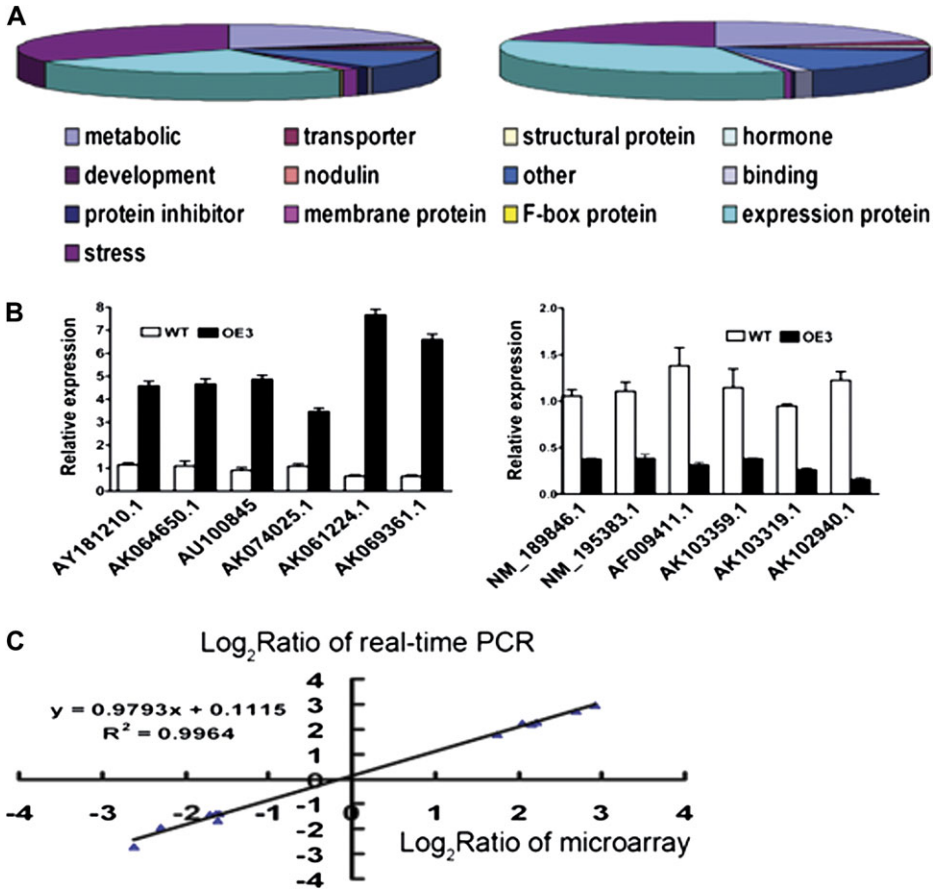

Fig. 10. Global analysis of gene expression in *OsMYB2*-overexpressing rice (transgenic line OE3). (A) Predicted functions of the proteins encoded by up-regulated genes (left) and down-regulated genes (right). (B) Relative expression in the wild type and the transgenic line of six up-regulated genes (left) and six down-regulated genes (right) selected from the microarray data and confirmed by real-time reverse-transcription (RT) PCR. (C) Correlation between data obtained from microarray and RT-PCR data. Data are mean±SE of three replicates.

pumila) (Pasquali *et al.*, 2008). A recent study also demonstrated that overexpression of *OsMYB3R-2* in rice leads to an increase in proline content and enhances the tolerance to cold (Ma *et al.*, 2009). Thus, one important mechanism underlying the enhanced tolerance of over-expression of rice *MYB* genes may be accounted for by the enhanced accumulation of compatible solutes.

To further elucidate the mechanism underlying the greater accumulation of proline in the *OsMYB2*-over-expressing plants under salt stress, this study examined the changes in proline synthase and proline transporter genes in *OsMYB2*-overexpressing, RNAi, and wild-type plants in response to salt stress at the transcriptional level. Up-regulation of proline synthase and proline transporter genes has been reported in *OsCIPK03*- and *OsCIPK12*-overexpressing plants, leading to greater amounts of proline in these transgenic rice than in the wild type under conditions of cold and dehydration stress (Xiang *et al.*, 2007). The present results demonstrate that expression of proline synthase and proline transporter genes was higher in *OsMYB2*-overexpressing plants than in wild-type plants under salt stress, suggesting that the higher proline contents in *OsMYB2*-overexpressing plants are likely to result from the greater up-regulation of these genes under salt stress (Fig. 7C-F). However, no significant difference in soluble sugars and free proline contents was found between the *OsMYB2*-overexpressing and wild-type plants under normal growth conditions, despite the fact that the constitutive promoter was used to drive the gene. This pattern is similar to that of other genes involved in abiotic stresses, such as *OsZFP252*, *OsCIPK03*, and *OsCIPK12* (Xiang *et al.*, 2007; Xu *et al.*, 2008). One possible explanation is that other stress-responsive regulators are required to activate *OsMYB2*-dependent, stress-responsive genes under stressed conditions.

In addition to the genes encoding proline synthase and transporters, this study also found that overexpression of *OsMYB2* led to higher expression of stress-related genes such as *OsLEA3*, *OsRab16A*, and *OsDREB2A* (Fig. 9). Both *OsLEA3* and *OsRab16A* belong to the late embryogenesis abundant genes, which have been implicated in response to many abiotic stress (RoyChoudhury *et al.*, 2007; Xiao *et al.*, 2007; Xiang *et al.*, 2008). *OsRab16A* encodes a group-2 late embryogenesis abundant protein and is significantly up-regulated by salt stress and ABA (Mundy and Chua, 1988) and transgenic tobacco plants overexpressing *OsRab16A* confer enhanced tolerance to salt stress (RoyChoudhury *et al.*, 2007). *OsLEA3* belongs to

group 3 of the *LEA* gene family and is induced by dehydration, salt stress, and ABA (Xiao *et al.*, 2007); for instance, under field conditions, overexpression of *OsLEA3* in rice plants can significantly enhance the dehydration tolerance (Xiao *et al.*, 2007). *OsDREB2A* is a stress-responsive, DRE/CRT gene that is involved in dehydration and salt stress (Dubouzet *et al.*, 2003) and constitutive expression of *Arabidopsis DREB2A* and maize *ZmDREB2A* confers enhanced tolerance of transgenic *Arabidopsis* plants to drought stress (Sakuma *et al.*, 2006; Qin *et al.*, 2007). The present observations that overexpression of *OsMYB2* led to a greater up-regulation of *OsLEA3*, *OsRab16A*, and *OsDREB2A* and enhanced tolerance to abiotic stress suggest that *OsMYB2* may regulate the expression of *LEA* genes through the *OsDREB2A*-dependent signalling pathway. Moreover, this study also found by analysing the microarray data that overexpression of *OsMYB2* altered the expression levels of a large number of genes and that many of the up- or down-regulated genes in the *OsMYB2*-overexpressing plants are predicted to be involved in stress tolerance. These results indicate that *OsMYB2* play an important role in stress tolerance in rice by regulating a number of downstream genes that are closely associated with plant tolerance to abiotic stress.

The elevated concentrations of ROS can damage cellular structures and macromolecules, leading to cell death (Mittler, 2002). Malondialdehyde is widely recognized as a marker for lipid peroxidation (RoyChoudhury *et al.*, 2007). The present study found that the contents of H_2O_2 and MDA in *OsMYB2*-overexpressing plants were markedly lower than in the wild-type and *OsMYB2*-RNAi plants under conditions of salt stress (Fig. 8A, B). The less accumulation of H_2O_2 under salt stress may result from an enhanced capacity for scavenging ROS, and the finding that POD, SOD, and CAT activities were higher in the *OsMYB2*-overexpressing plants than in the wild-type and *OsMYB2*-RNAi plants (Fig. 8, C-E) is in line with this proposition. The greater tolerance of *OsMYB2*-overexpressing plants to salt stress found in this study may be accounted for, at least in part, by mitigating oxidative damage due to suppression of ROS production.

Expression of *OsMYB2* was up-regulated by exogenous ABA, and transgenic plants overexpressing *OsMYB2* were more sensitive to exogenous ABA than the wild type in terms of seed germination and seedling growth (Fig. 6). Those findings are in contrast to other reported rice MYB genes in the literature. For example, expression of *OsMYB4* is insensitive to ABA (Vannini *et al.*, 2004). Overexpression of *OsMYB3R-2* in *Arabidopsis* renders it less sensitive to ABA (Dai *et al.*, 2007). Expression of *OsMYBS3* in leaves is suppressed by exogenous application of ABA, while its expression in roots is not responsive to ABA (Su *et al.*, 2010). The mechanism by which ABA regulates *OsMYB2* in response of rice plants to salt stress warrants further investigation. These results suggest that MYB proteins play diverse roles in the ABA-dependent signalling transduction pathways.

Another interesting observation in this study is that the wild-type and RNAi plants showed no differences in terms of their response to the abiotic stress examined and metabolic changes induced by salt stress. This result may be because some other MYB proteins complement the function of OsMYB2. A similar observation has been reported in *OsZFP252*-knockdown lines (Xu *et al.*, 2008). For example, overexpression of *OsZFP252* in rice enhances accumulation of free proline and soluble sugars and improves the expression of stress-responsive genes, thus conferring *OsZFP252*-overexpressing rice with more tolerance to salt and drought stress than *OsZFP252*-antisense and wild-type rice (Xu *et al.*, 2008). However, no difference in tolerance to salt and drought stress was observed between *OsZFP252*-knockdown lines and wild-type plants. Alternatively, it is possibile that the residual levels of *OsMYB2* in the underexpressing RNAi plants may be sufficient for *OsMYB2* functioning.

In summary, this study identified a transcription factor *OsMYB2* that functions as positive regulator to mediate tolerance of rice seedlings to salt, cold, and dehydration stress. Overexpression of *OsMYB2* led to greater accumulation of compatible osmolytes, such as soluble sugars, free proline, and LEA proteins in rice, and suppressed the accumulation of MDA and H_2O_2 under conditions of salt stress. The up-regulation of *OsMYB2* may allow rice plants to effectively osmo-regulate their water potential by accumulating compatible solutes and minimize oxidative damage to plants under abiotic stress. More importantly, overexpression of *OsMYB2* in rice seedlings did not affect their phenotypes under control conditions. Therefore, OsMYB2 provides a promising tool for improving the tolerance of rice to abiotic stress in general and to salt stress in particular.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Structure of the OsMYB2 protein and a phylogenetic tree of MYB proteins.

Supplementary Fig. S2. Distribution of stress-related *cis*-elements in the *OsMYB2* promoter region.

Supplementary Fig. S3. Southern blot analysis of independent transgenic rice lines.

Supplementary Table S1. Primers used in real-time RT-PCR to verify the expression pattern of differentially expressed genes from the microarray experiment.

Supplementary Table S2. Genes up-regulated by overexpression of *OsMYB2*.

Supplementary Table S3. Genes down-regulated by overexpression of *OsMYB2*.

Acknowledgements

This work was supported by the National Science Foundation of China (30788003 and 30870188) and the Chinese Academy of Sciences (KSCX1-YW-03).

References

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2003. *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB)

function as transcriptional activators in abscisic acid signaling. *The Plant Cell* **15,** 63–78.

Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK. 2006. A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *Journal of Biological Chemistry* **281,** 37636–37645.

Alexieva V, Sergiev I, Mapelli S, Karanov E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell and Environment* **24,** 1337–1344.

Armengaud P, Thiery L, Buhot N, Grenier-De March G, Savoure A. 2004. Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. *Physiologia Plantarum* **120,** 442–450.

Bailey RW. 1958. Reaction of pentoses with anthrone. *Biochemical Journal* **68,** 669–672.

Bartels DSR. 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Science* **24,** 23–58.

Bates LS, Waldren RP, Teare ID. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil* **39,** 205–207.

Chen Y, Yang X, He K, *et al*. 2006. The MYB transcription factor superfamily of *Arabidopsis*: expression analysis and phylogenetic comparison with the rice MYB family. *Plant Molecular Biology* **60,** 107–124.

Cominelli E, Galbiati M, Vavasseur A, Conti L, Sala T, Vuylsteke M, Leonhardt N, Dellaporta SL, Tonelli C. 2005. A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. *Current Biology* **15,** 1196–1200.

Dai X, Xu Y, Ma Q, Xu W, Wang T, Xue Y, Chong K. 2007. Overexpression of an R1R2R3 MYB gene, *OsMYB3R*-2, increases tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis*. *Plant Physiology* **143,** 1739–1751.

Denekamp M, Smeekens SC. 2003. Integration of wounding and osmotic stress signals determines the expression of the *AtMYB102* transcription factor gene. *Plant Physiology* **132,** 1415–1423.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2003. *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *The Plant Journal* **33,** 751–763.

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ. 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proceedings of National Academy of Sciences USA* **99,** 15898–15903.

Ge L, Chen H, Jiang JF, Zhao Y, Xu ML, Xu YY, Tan KH, Xu ZH, Chong K. 2004. Overexpression of *OsRAA1* causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower, and root development and root response to gravity. *Plant Physiology* **135,** 1502–1513.

Gupta AK, Kaur N. 2005. Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Bioscience* **30,** 761–776.

Hare PD, Cress WA, van Staden J. 1999. Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. *Journal of Experimental Botany* **50,** 413–434.

Hu H, You J, Fang Y, Zhu X, Qi Z, Xiong L. 2008. Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice. *Plant Molecular Biology* **67,** 169–181.

Ito M, Araki S, Matsunaga S, Itoh T, Nishihama R, Machida Y, Doonan JH, Watanabe A. 2001. G2/M-phase-specific transcription during the plant cell cycle is mediated by c-Myb-like transcription factors. *The Plant Cell* **13,** 1891–1905.

Jung C, Seo JS, Han SW, Koo YJ, Kim CH, Song SI, Nahm BH, Choi YD, Cheong JJ. 2008. Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*. *Plant Physiology* **146,** 623–635.

Kranz HD, Denekamp M, Greco R, *et al*. 1998. Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *The Plant Journal* **16,** 263–276.

Liang YK, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM. 2005. AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. *Current Biology* **15,** 1201–1206.

Lippold F, Sanchez DH, Musialak M, Schlereth A, Scheible WR, Hincha DK, Udvardi MK. 2009. AtMyb41 regulates transcriptional and metabolic responses to osmotic stress in *Arabidopsis*. *Plant Physiology* **149,** 1761–1772.

Liu J, Zhu JK. 1997. Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of *Arabidopsis*. *Plant Physiology* **114,** 591–596.

Ma HS, Liang D, Shuai P, Xia XL, Yin WL. 2010. The salt- and drought-inducible poplar GRAS protein SCL7 confers salt and drought tolerance in *Arabidopsis thaliana*. *Journal of Experimental Botany* **61,** 4011–4019.

Ma Q, Dai X, Xu Y, Guo J, Liu Y, Chen N, Xiao J, Zhang D, Xu Z, Zhang X, Chong K. 2009. Enhanced tolerance to chilling stress in *OsMYB3R-2* transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. *Plant Physiology* **150,** 244–256.

Miao BH, Han XG, Zhang WH. 2010. The ameliorative effect of silicon on soybean seedlings grown in potassium-deficient medium. *Annals of Botany* **105,** 967–973.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Sciences* 7: 405–410.

Mundy J, Chua NH. 1988. Abscisic acid and water-stress induce the expression of a novel rice gene. *The EMBO Journal* **7,** 2279–2286.

Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K. 2007. Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. *The Plant Journal* **51,** 617–630.

Pasquali G, Biricolti S, Locatelli F, Baldoni E, Mattana M. 2008. *Osmyb4* expression improves adaptive responses to drought and cold stress in transgenic apples. *Plant Cell Reports* **27,** 1677–1686.

Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K. 2007. Regulation

and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. *The Plant Journal* **50,** 54–69.

RoyChoudhury A, Roy C, Sengupta DN. 2007. Transgenic tobacco plants overexpressing the heterologous *lea* gene *Rab16A* from rice during high salt and water deficit display enhanced tolerance to salinity stress. *Plant Cell Reports* **26,** 1839–1859.

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2006. Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *The Plant Cell* **18,** 1292–1309.

Song SY, Chen Y, Chen J, Dai XY, Zhang WH. 2011. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. *Planta* **234,** 331–345.

Stracke R, Werber M, Weisshaar B. 2001. The *R2R3-MYB* gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4,** 447–456.

Su CF, Wang YC, Hsieh TH, Lu CA, Tseng TH, Yu SM. 2010. A novel MYBS3-dependent pathway confers cold tolerance in rice. *Plant Physiology* **153,** 145–158.

Szekely G, Abraham E, Cseplo A, *et al*. 2008. Duplicated *P5CS* genes of *Arabidopsis* play distinct roles in stress regulation and developmental control of proline biosynthesis. *The Plant Journal* **53,** 11–28.

Suzuki N, Mittler R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum* **126,** 45–51.

Takasaki H, Maruyama K, Kidokoro S, Ito Y, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K, Nakashima K. 2010. The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. *Molecular and Genetic Genomics* **284,** 173–183.

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K. 1993. An *Arabidopsis myb* homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *The Plant Cell* **15,** 1529–1539.

Vannini C, Locatelli F, Bracale M, Magnani E, Marsoni M, Osnato M, Mattana M, Baldoni E, Coraggio I. 2004. Overexpression of the rice *Osmyb4* gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. *The Plant Journal* **37,** 115–127.

Wang Z, Xu Y, Jiang R, Xu Z, Chong K. 2004. A practical vector for efficient knockdown of gene expression in rice (*Oryza sativa L.*). *Plant Molecular Biology Reports* **22,** 409–417.

Xiang Y, Huang Y, Xiong L. 2007. Characterization of stress-responsive *CIPK* genes in rice for stress tolerance improvement. *Plant Physiology* **144,** 1416–1428.

Xiang Y, Tang N, Du H, Ye HY, Xiong LZ. 2008. Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. *Plant Physiology* **148,** 1938–1952.

Xiao B, Huang Y, Tang N, Xiong L. 2007. Over-expression of a *LEA* gene in rice improves drought resistance under the field conditions. *Theorectical and Applied Genetics* **115,** 35–46.

Xin Z, Browse J. 1998. Eskimo1 mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proceedings for National Academy of Sciences USA* **95,** 7799–7804.

Xu DQ, Huang J, Guo SQ, Yang X, Bao YM, Tang HJ, Zhang HS. 2008. Overexpression of a TFIIIA-type zinc finger protein gene *ZFP252* enhances drought and salt tolerance in rice (*Oryza sativa* L.). *FEBS Letters* **582,** 1037–1043.

Xu ML, Jiang JF, Ge L, Xu YY, Chen H, Zhao Y, Bi YR, Wen JQ, Chong K. 2005. *FPF1* transgene leads to altered flowering time and root development in rice. *Plant Cell Reports* **24,** 79–85.

Yoo JH, Park CY, Kim JC, *et al*. 2005. Direct interaction of a divergent CaM isoform and the transcription factor, MYB2, enhances salt tolerance in *Arabidopsis*. *Journal of Biological Chemistry* **280,** 3697–3706.

Zhang L, Tian LH, Zhao JF, Song Y, Zhang CJ, Guo Y. 2009. Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. *Plant Physiology* **149,** 916–928.

Zhang L, Zhao G, Jia J, Liu X, Kong X. 2011. Molecular characterization of 60 isolated wheat MYB genes and analysis of their expression during abiotic stress. *Journal of Experimental Botany* doi:10.1093/jxb/err264.

Zhao MG, Tian QY, Zhang WH. 2007. Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. *Plant Physiology* **144,** 206–217.