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RESEARCH PAPER

A wheat R2R3-MYB gene, *TaMYB30-B*, improves drought stress tolerance in transgenic *Arabidopsis*

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Abstract

The MYB-type proteins are involved in various processes of plant growth, development, and stress response. In a previous work, a polyethylene glycol (PEG) stress-induced gene, *TaMYB30*, which encodes a R2R3-type MYB protein was identified in wheat. In this study, the isolation and functional characterization of the *TaMYB30* gene are reported. Three homologous sequences of *TaMYB30* were isolated from hexaploid wheat and designated as *TaMYB30-A*, *TaMYB30-B*, and *TaMYB30-D* genes based on the localizations of these three sequences to chromosomes 2A, 2B, and 2D, respectively. The expression levels of these three genes were similar under PEG stress conditions, and *TaMYB30-B* was selected for further analysis. The TaMYB30-B protein was localized to the nucleus where it activated transcription. The detailed characterization of *Arabidopsis* transgenic plants that overexpress the *TaMYB30-B* gene revealed that the TaMYB30-B protein can improve drought stress tolerance during the germination and the seedling stages. It was also found that overexpression of *TaMYB30-B* resulted in altered expression levels of some drought stress-responsive genes and changes in several physiological indices, which allow plants to overcome adverse conditions. These results indicate that the TaMYB30-B protein plays important roles in plant stress tolerance, and modification of its expression may improve drought stress tolerance in crop plants.

Key words: Drought stress, MYB, overexpression, transcription factor, transgenic, wheat.

Introduction

Drought is an abiotic stress that adversely affects plant growth and productivity. To prevent drought stress in wheat, a large amount of water is needed for the irrigation of farmland, which exacerbates the shortages of fresh water resources. Therefore, the identification and characterization of key genes that mediate plant responses to drought stress provide a powerful method to engineer or select for crop plants with enhanced tolerance to drought stress (Kasuga *et al*., 1999; Valliyodan and Nguyen, 2006). Regulatory proteins act as early responders to environmental signals and trigger global changes in stress-related genes; the functions of regulatory proteins can also be gradually amplified through signal transduction cascades. Therefore, regulatory proteins have become an important topic in the study of plant responses to abiotic stresses (Seki *et al*., 2002; Kreps *et al*., 2002; Ahuja *et al*., 2010; Hirayama and Shinozaki, 2010). Transcription factors (TFs) are important regulatory proteins that control the expression of target genes by specifically binding to the *cis*-acting elements within the regulatory regions. Based on differences in the DNA-binding domains, the TFs can be classified into different families. Reports have indicated that numerous TF families, such as MYB, DREB, NAC, and WRKY, are directly or indirectly involved in the regulation of plant response to drought stress (Singh *et al*., 2002; Huang *et al*., 2010). In plants, the MYB TFs comprise one of the largest gene families.

The MYB proteins are characterized by a MYB DNA-binding domain in their N-terminus that is composed of one or more imperfect tandem repeat(s) (Pabo and Sauer, 1992; Riechmann *et al*., 2000). According to the number and position

of the MYB repeats, plant MYB proteins are classified into four major groups: (i) the 4R-MYB group, which has four adjacent MYB repeats; (ii) the 3R-MYB (R1R2R3-MYB) group, with three repeats; (iii) the R2R3-MYB group, with two adjacent repeats; and (iv) the MYB-related proteins, which always contain one repeat or two separated repeats (Rosinski and Atchley, 1998; Jin and Martin, 1999; Dubos *et al*., 2010). In plants, the MYB TFs are integrally involved in various developmental processes, such as the control of primary and secondary metabolism, the regulation of cell development and the cell cycle, the transduction of hormone signals, and responses to biotic and abiotic stresses (Stracke *et al*., 2001; Du *et al*., 2009; Dubos *et al*., 2010; Feller *et al*., 2011).

Many plant MYB genes that are involved in plant responses to diverse abiotic stresses have been identified and functionally characterized. The AtMYB2 protein was shown to act as an activator to regulate the expression of abscisic acid (ABA)-inducible genes under drought stress conditions in *Arabidopsis* (Urao *et al*., 1996; Abe *et al*., 2003). The overexpression of *AtMYB15* results in enhanced drought tolerance and sensitivity to ABA (Ding *et al*., 2009). The AtMYB44/AtMYBR1 protein regulates ABA-mediated stomatal closure under abiotic stresses (Jung *et al*., 2008), and the AtMYB60 protein controls stomatal closure and root growth in response to drought stress (Oh *et al*., 2011). The AtMYB62 protein is involved in the response to phosphate starvation (Devaiah *et al*., 2009), and the AtMYB96 protein mediates ABA signalling in response to abiotic stresses (Seo *et al*., 2009, 2011; Seo and Park, 2011). In rice, the OsMYB2 protein is involved in salt, cold, and dehydration tolerance (Yang *et al*., 2012). The overexpression of *OsMYB4* improves the cold and freezing tolerances of transgenic plants (Vannini *et al*., 2004; Pasquali *et al*., 2008; Park *et al*., 2010; Soltesz *et al*., 2012). The *OsMYB3R-2* gene enhances stress tolerance in rice and *Arabidopsis* (Dai *et al*., 2007; Ma *et al*., 2009). In wheat, *TaMYBsdu1* is up-regulated during salt and drought stresses and differentially regulated between salt-tolerant and salt-sensitive genotypes (Rahaie *et al*., 2010). The *TaMYB1* gene encodes a R2R3-MYB protein that is involved in responses to abiotic and ABA stresses (Lee *et al*., 2007). Another R2R3-MYB protein, TaMYB2A, improves tolerance to multiple abiotic stresses in transgenic *Arabidopsis* plants (Mao *et al*., 2011). Overexpression of *TaPIMP1* confers enhanced resistance to biotic and abiotic stresses in transgenic tobacco plants (Liu *et al*., 2011), while overexpression of *TaMYB33* increases salt and drought tolerance in *Arabidopsis* plants (Qin *et al*., 2012), and the ectopic expression of *TaMYB73* improves the tolerance of transgenic *Arabidopsis* plants to salinity stress (He *et al*., 2012).

In previous works, a large-scale sequencing project of TF genes was conducted in wheat because it is an important global crop, and the stress-responsive MYB genes in this plant were screened. A polyethylene glycol (PEG) stress-induced gene was identified and named *TaMYB30* (Zhang *et al*., 2012). In this study, the isolation and functional characterization of the *TaMYB30* gene are reported. Detailed characterization of the *TaMYB30-B* gene, which is a member of the *TaMYB30* homologues, revealed the potential roles of *TaMYB30* in tolerance to drought stress.

Materials and methods

Plant materials

Wild and cultivated wheat lines of different ploidy levels were used to amplify the genomic and cDNA sequences of *TaMYB30*. The *Triticum urartu* accession UR206 (original code no. 1010015) was kindly provided by Mr Reader from the John Innes Centre, Norwich, UK. The *Aegilops tauschii* accession Y2282 (original code AL8/78) was generously provided by Dr Mingcheng Luo, UC Davis. The *Aegilops speltoides* accession Y2006 and Chinese Spring (CS) were from the authors' laboratory.

The CS nulli-tetrasomic (NT) lines were used to determine the chromosomal locations of each gene. *Arabidopsis* (*Arabidopsis thaliana*) Columbia-0 was used for transgenics of *TaMYB30*.

PEG stress treatment of wheat

Wheat cv. Hanxuan 10 (drought resistance) was used for the PEG treatment. Ten-day-old seedlings grown in Hoagland's liquid culture at 22 °C under a 16 h light/8 h dark photoperiod were transferred to new Hoagland's liquid culture that contained 16.1% PEG (–0.5 MPa). The root samples were harvested at 0, 1, 3, 6, 12, 24, 48, and 72 h after the transfer.

Cloning and sequence analysis of TaMYB30 *gene members*

The following TaMYB30FL primers were designed according to the cDNA sequences of *TaMYB30* (JF951913) to cover the open reading frame (ORF) and were used to amplify the genomic and cDNA sequences in UR206 (AA genome), Y2006 (SS genome), Y2282 (DD genome), and CS (ABD genome): MYB30FL-F, 5'-CGCCCAAACTCCAAAC-3' and MYB30FL-R, 5'-GCTTGGAGCTGGATACG-3'. The PCR products were cloned into pEASY-T1 vectors (TransGen) and sequenced with an ABI 3730XL 96-capillary DNA analyzer (Lifetech). In order to clone all the homologous sequences of *TaMYB30*, 96 clones in the recombinant plasmid for hexaploid wheat and 24 clones of diploid ancestors used for sequencing were randomly picked.

The amino acid sequences of the TaMYB30 homologous proteins were downloaded from the GenBank website. Sequence similarity analysis was performed by the MegAlign program in DNAStar software. The complete amino acid sequences of MYB proteins were used to construct phylogenetic trees. Sequence alignment was performed by ClustalW using BioEdit software and adjusted manually. The Neighbor–Joining tree was constructed using the MEGA5.1 program (Tamura *et al*., 2011), and the internal branch support was estimated with 1000 bootstrap replicates. Sequence logos for the R2 and R3 MYB repeats were obtained by submitting the multiple alignment sequences of the TaMYB30 members and their homologues to the website http://weblogo.berkeley.edu/logo.cgi (Crooks *et al*., 2004).

Promoter isolation and cis*-acting regulatory element analysis*

To isolate the promoter of the *TaMYB30* genes, the genomic DNA sequence of *TaMYB30* was used in a BLAST search of the scaffolds of the *A. tauschii* (DD, D genome donor species of common wheat) draft sequence (unpublished data). Based on the sequences that were identified from the scaffold, gene-specific primers were designed to cover the 1500 bp of sequence upstream of the start codon; these primers were used to isolate the promoter sequences from the hexaploid wheat CS. The *cis*-acting regulatory elements were predicted using tools in the PlantCARE database (Lescot *et al*., 2002).

Chromosomal locations of the three TaMYB30 *members*

Based on the nucleotide sequence polymorphisms of the *TaMYB30* genes, gene-specific primers were designed as follows: TaMYB30-A-F, 5'-TGTACCTCCCTCTTCCGACG-3'andTaMYB30-A-R,5'-TCCTCT TCACGAGACCACCC-3'; TaMYB30-B-F, 5'-GGGCTAAGGATGAT GAACTATTG-3' and TaMYB30-B-R, 5'-GGGTTGCCGAGAATCCT

G-3; and TaMYB30-D-F, 5'- AGAAACACCAGCATCCA-3' and TaMYB30-D-R, 5'-CTATCTTGGGCAACCTC-3'. These primers were used to distinguish the homologous *TaMYB30* genes from different genomes. Genomic DNA samples that were prepared from the CS NT lines served as templates for the PCR amplifications. The PCR parameters were as follows: 95 °C for 5 min; 32 cycles of 95 °C for 30 s, different denaturing temperatures (60 °C for the TaMYB30-A and TaMYB30-B primers and 56 °C for the TaMYB30-D primers) for 30 s, and 72 °C for 30 s; and a final step at 72 °C for 5 min. The amplified PCR products were separated by electrophoresis on a 2% agarose gel.

Subcellular location of theTaMYB30-B–green fluorescent protein (GFP) fusion protein

The full-length coding sequence of *TaMYB30-B* without the stop codon was amplified from plasmid cDNA by PCR using gene-specific primers that contained *Sal*I and *Bam*HI restriction sites; the sequences of these primers were as follows: 5'-GTCGACATGGCGACCGGCCCCG-3 and 5'-GGATCCAAGGCTATTAAGAAGAGCCCTTT-3, where the *Sal*I and *Bam*HI sites are underlined, respectively. For the expression of the 35S::TaMYB30-B–GFP fusion protein, the PCR product was cloned adjacent to the *Cauliflower mosaic virus* (CaMV) 35S promoter in the pJIT163-GFP vector. The construct was confirmed by sequencing and transformed into onion epidermal cells via a Gene Gun. The transformed onion cells were observed under a confocal microscope (Nikon).

Transactivation assay in yeast cells

The yeast strain AH109 *Saccharomyces cerevisiae*, containing the *His3*, *Ade2* reporter genes with GAL4-binding elements in the promoter, and the vector pDEST32 (ProQuest™ Two-Hybrid System with Gateway® Technology, Lifetech, Cat. 10835), containing the GAL4 DNA-binding domain (BD) and *Leu2* reporter gene, were used for testing the transactivation of *TaMYB30*. The sequences of different lengths of *TaMYB30-B* were cloned into the pDEST32 vector by using the Gateway system. The constructs of *pDEST32* that fused different sequences of *TaMYB30-B*, the negative control *pDEST32* vector alone, and the positive control *pGAL4* were transformed into AH109, according to the protocol of the manufacturer, in Leu– medium. After ~2 d, the positive transformants verified by PCR were plated on Leu– and Leu–His–Ade– medium, respectively. The transcriptional activation activities were evaluated according to their growth status.

Generation of the TaMYB30-B *transgenic* Arabidopsis *plants*

The cDNA sequence containing the full-length coding region of *TaMYB30-B* was amplified from plasmid cDNA by PCR using the following gene-specific primers containing attB sites(underlined): 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGCGACCGGCCCCGATGGCGACCGGC CCCGAT-3 and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCAAAGGCTATTAAGAAGAGCCC-3. The PCR product was cloned into the Gateway plant expression vector pLEELA containing the CaMV 35S promoter. The construct was then sequenced and transferred into *Agrobacterium* GV3101::Pmp90RK. The *Arabidopsis* plants were transformed by the floral dip method (Clough and Bent, 1998).

Germination assays

For the germination assay, 100–150 seeds from each of the transgenic and wild-type (WT) plants were surface-sterilized in 10% (v/v) NaClO containing 0.2% (v/v) Triton X-100 for 15 min, followed by washing five times with sterilized distilled water. The surface-sterilized seeds were sown on Murashige and Skoog (MS) medium plates with various concentrations of mannitol (0–500 mM). The percentage of germinated seeds was calculated based on the number of seedlings that reached the cotyledon stage at 2 weeks (Saleki *et al*., 1993).

Drought stress treatments of transgenic Arabidopsis

The surface-sterilized seeds were sown on MS medium plates with 3% (w/v) sucrose and 0.8% agar (w/v). Plates were sealed with parafilm and placed in the dark at 4 °C for 2 d before being transferred to a growth chamber under a long-day photoperiod (16 h light/8 h dark) at 22 °C.

Ten-day-old seedlings were planted in a 4 cm deep rectangular plate filled with a 1:1 mixture of vermiculite and humus, and well watered. The seedlings were not watered on the following days until they were rewatered when they were fully affected by drought stress. The phenotypes before and after treatment were surveyed and photographed. The drought stress treatment experiment was performed in triplicate.

Determination of the water loss rate

For the determination of water loss, the leaves of 3-week-old transgenic and WT seedlings were detached and weighed immediately. The samples were incubated at 23 °C with 60% relative humidity and weighed at the designated times. The water loss rate was calculated based on the initial fresh weight of the samples. Ten plants of each transgenic and WT line were used in this assay, and each measurement was repeated three times.

Measurements of the proline content, malondialdehyde (MDA) content, soluble sugar content, and relative electrolyte leakage

Seven-day-old seedlings were grown on MS plates, transferred onto new media plates containing plain MS or MS supplemented with 300 mM mannitol, and grown for 1 week. The seedlings were harvested and measured. All the measurements were repeated three times, and the Student's *t*-test was used for statistical analysis.

For measurements of the proline content, ~0.5 g of seedlings was homogenized in 10 ml of 3% sulphosalicylic acid and centrifuged. The resulting 2 ml extract was incubated with 2 ml of ninhydrin reagent, which contained 2.5% (w/v) of ninhydrin, 60% (v/v) of glacial acetic acid, and 40% of 6 M phosphoric acid, and 2 ml of glacial acetic acid, at 100 °C for 40 min. The reaction was terminated in an ice bath, and 5 ml of toluene was added; the samples were then vortexed. The reaction mixture was incubated at 23 °C for 24 h and the proline content was calculated as described previously (Bates *et al*., 1973).

For measurements of the MDA content, ~0.5 g of seedlings was homogenized in 2 ml of a chilled reagent, which was composed of 0.25% (w/v) TBA (thiobarbituric acid) in 10% (w/v) TCA (trichloracetic acid), and then centrifuged at 10 000 rpm for 20 min. The supernatant was heated at 95 °C for 30 min, quickly cooled on ice, and then centrifuged at 10 000 rpm for 20 min. The absorbances at 532 nm (A_{532}), 600 nm (A_{600}), and 450 nm (A_{450}) were measured using a spectrophotometer. The MDA content was calculated with the equation: C (MDA content)=6.45 (A_{532}–A_{600})–0.56×A_{450} (Hodges *et al*., 1999; Cui and Wang, 2006).

To determine the soluble sugar content, seedlings were collected and immediately dehydrated in a refrigerated vacuum evaporator under 8.1 kPa of air pressure at –60 °C for 24 h. Each sample was then incubated on an 80 °C heat source until a constant dry weight was achieved. Each resulting sample contained 0.1 g of dry material and was boiled in 4 ml of double-distilled water for 4 min. The extracted filtrates were transferred to volumetric flasks, and double-distilled water was added to a final volume of 5 ml. The total soluble sugars were measured as fructose equivalents using the anthrone colorimetric assay (Yemm and Willis, 1954) at 620 nm with a spectrophotometer (Yemm and Willis, 1954; Zhang *et al*., 2010).

Using a conductivity detector, the electrolyte leakage was evaluated by measuring the relative conductivity of the solution that contained the samples. The seedlings were vacuum-infiltrated in deionized water for 20 min. After 2 h, the conductivities (C1) of the solutions were determined. The seedlings were boiled for 15 min in deionized water and cooled to room temperature. The conductivities (C2) of the solutions were then determined. The values of the C1 to C2 (C1/C2) ratios were calculated and used to evaluate the relative electrolyte leakage (Cao *et al*., 2007).

Quantitative real-time PCR

Total RNA was extracted from *Arabidopsis* or wheat seedlings using the TRIZOL reagent and treated with DNase I. For each sample, 10 μg of total RNA was used to synthesize first-strand cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen). For quantitative real-time PCR experiments, each reaction contained 10 μl of 2× SYBR® Premix Ex Taq™ (TaKaRa), 3 μl of 2.0 μM gene-specific primers, 0.4 μl of 50× ROX References Dye, and 2.0 μl of cDNA in a final volume of 20 μl. The PCR parameters were the following: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 31 s; and a dissociation step at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The *Arabidopsis* actin (NM_179953) gene was used as a reference gene. The reactions were performed using the ABI Prism 7300 real-time PCR system (Lifetech) and repeated three times. A quantitative analysis was performed using the $2^{-\Delta\Delta CT}$ method. The primers used for quantitative real-time PCR are listed in Supplementary Table S1 available at *JXB* online.

Results

Molecular features and structures of the TaMYB30 *genes*

To investigate further the roles of the *TaMYB30* genes in the regulation of drought stress tolerance, the genomic and cDNA sequences of the *TaMYB30* homologues were isolated from the available diploid wheat genomes A (UR206), S (Y2006), and D (Y2282), and the hexaploid wheat ABD genomes (CS). A single sequence was isolated from each of the diploid wheat genomes and designated as the *TruMYB30* gene from UR206, the *AesMYB30* gene from Y2006, and the *AetMYB30* gene from Y2282. From the hexaploid wheat plants, three highly homologous sequences were identified in CS. By comparing these sequences with the diploid *TaMYB30* gene sequences, the three hexaploid sequences were assigned to the A, B, and D genomes and named the *TaMYB30-A*, *TaMYB30-B*, and *TaMYB30-D* genes, respectively. The three hexaploid genes share 98.4–99% identity at the cDNA level from the start codon to the termination codon and 97.3–98.5% identity at the amino acid level.

The structure of the *TaMYB30* gene was assessed by aligning the genomic sequences with the cDNA sequences of *TaMYB30*. Twelve introns, with a length ranging from 78 bp to 891 bp, were identified in the *TaMYB30* genomic sequences (Fig. 1). Based on comparisons between the three genomes, the sequence lengths of some introns varied significantly between different genomes (Supplementary Table S2 at *JXB* online).

The 1500 bp sequence upstream of the start codon of *TaMYB30* was defined as the promoter region and used to analyse the *cis*-acting regulatory elements in this study. Drought-responsive *cis*-elements (DRE/C-repeat), A-boxes, G-boxes, and other functional motifs were identified (Supplementary Table S3 at *JXB* online).

Homologous sequences of the TaMYB30 protein in other species

Previous work identified one homologous TaMYB30 sequence in rice and two homologous TaMYB30 sequences in *Arabidopsis*. In this study, the presence of TaMYB30 homologues was searched for in other species, including barley, *Brachypodium*, sorghum, grapes, and poplar. Several homologous R2R3-MYB proteins with unknown function were revealed. Phylogenetic analysis of the protein sequences of TaMYB30 and the homologues produced a phylogenetic tree that contained two clades; these clades branched distinctly for the dicot and monocot plant sequences (Fig. 2A). The barley protein BAJ92519.1 shared the closest relationship with the TaMYB30 proteins, and the sequence identities between these proteins ranged from 88.8% to 89.5%. The other proteins shared different levels of sequence identity with the TaMYB30 proteins and ranged from 22.9% to 57.8% (Supplementary Table S4 at *JXB* online). The R2R3-MYB proteins exhibited significant sequence conservation within the MYB domain regions. To gain insight into the nature of the R2R3-MYB domains of TaMYB30 proteins and their homologues, sequence logos were produced to examine the level of conservation in the R2 and R3 repeats of the R2R3-MYB proteins at each residue position. The results revealed that 31 (59.6%) and 38 (74.5%) of the conserved amino acids were identical in the R2 and R3 MYB repeat regions, respectively, among all the MYB proteins; the residues located in other positions displayed varying levels of conservation (Fig. 2B). The MYB repeat always contains three regularly spaced tryptophan residues, which participate in the interaction between the MYB protein and specific DNA sequences (Ogata and Nishimura, 1995). A previous study indicated that the third tryptophan residue of the TaMYB30 protein in the R3 repeat was replaced by the amino acid phenylalanine (Zhang *et al*., 2012). In this study, it was found that the third tryptophan residue in the R3 repeat of all the TaMYB30 members and their homologues was replaced by the same amino acid, phenylalanine.

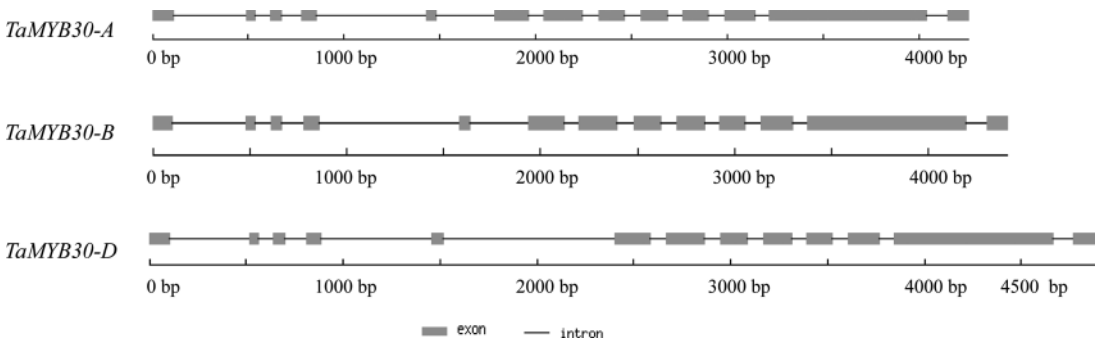

Fig. 1. The gene structure of the *TaMYB30* genes. The position and length of the exons and introns of the *TaMYB30* genes are displayed schematically. The rectangles indicate the exons, and the black lines indicate the introns. (This figure is available in colour at *JXB* online.)

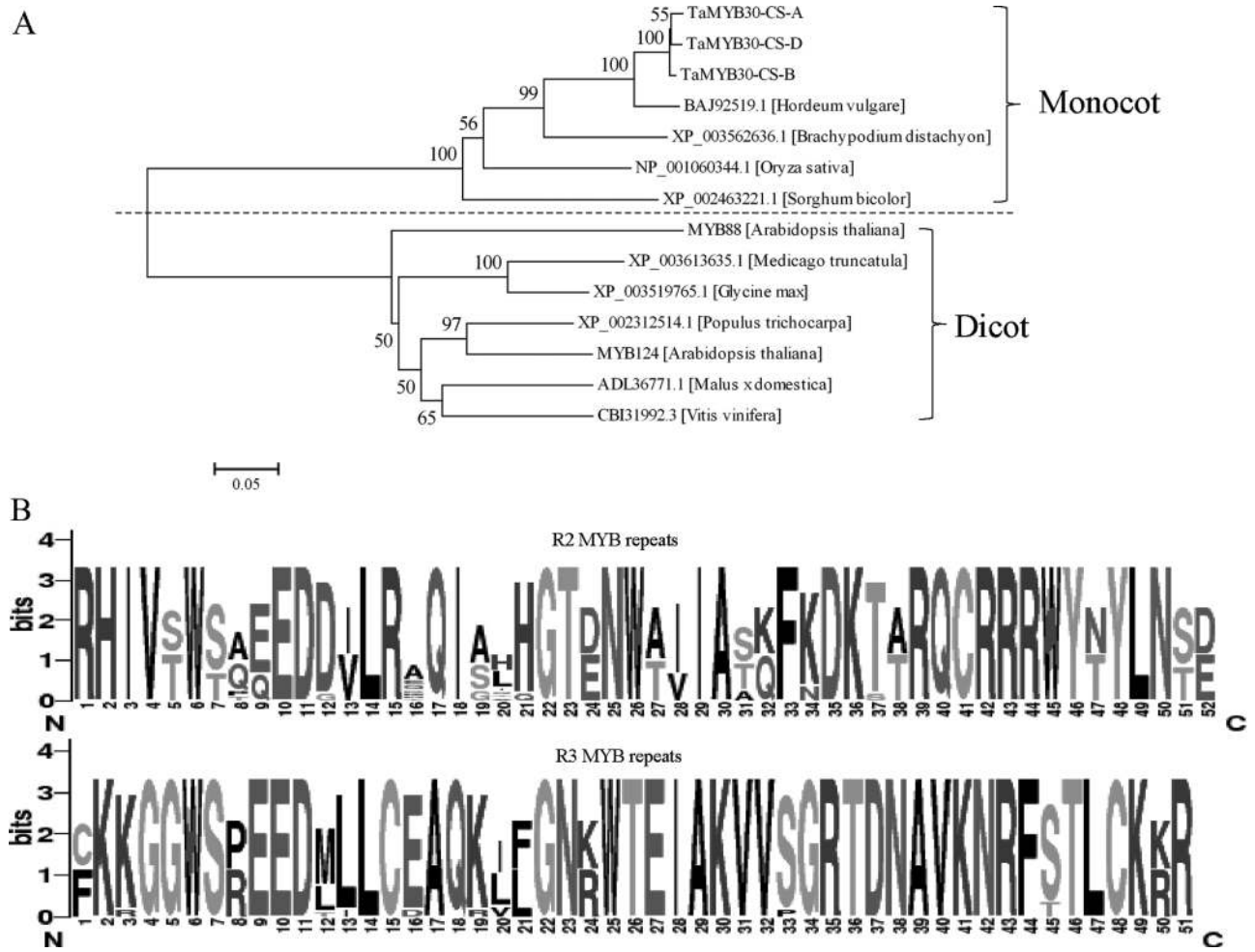

Fig. 2. Phylogenetic relationships and sequences logos for the MYB domains of TaMYB30 proteins and their homologues. (A) Phylogenetic relationships of TaMYB30 proteins and their homologues. The divergence of the clades between the monocots and dicots is labelled by the dotted lines. (B) The sequence logos for the R2 and R3 repeats of TaMYB30 and their homologues. The overall height of each stack indicates the conservation of the protein sequence at that amino acid position, whereas the height of letters within each stack represents the relative frequency of the corresponding amino acid. (This figure is available in colour at *JXB* online.)

Chromosomal locations of the TaMYB30 *genes*

The chromosome assignments of the *TaMYB30-A*, *TaMYB30-B,* and *TaMYB30-D* genes were determined using the CS NT lines and gene-specific primers to amplify each *TaMYB30* gene. The amplification results showed that the primers specific to the *TaMYB30-A* gene did not result in PCR products for NT2A2B and NT2A2D. The primers specific to the *TaMYB30-B* gene did not produce products for NT2B2A and NT2B2D, and the primers specific to the *TaMYB30-D* gene did not show products for NT2D2A and NT2D2B. Therefore, the *TaMYB30-A*, *TaMYB30-B*, and *TaMYB30-D* genes were mapped to chromosomes 2A, 2B, and 2D, respectively (Fig. 3).

The three TaMYB30 *genes have similar expression levels under osmotic stress conditions*

Previous research revealed that the expression of *TaMYB30* was strongly induced by osmotic stress. However, the expression patterns of the three individual genes were unknown. Thus, gene-specific primers were used in quantitative real-time PCR experiments to detect the relative expression levels of the *TaMYB30-A*, *TaMYB30-B*, and *TaMYB30-D* genes in the root

Fig. 3. Chromosomal locations of the *TaMYB30* genes. For the *TaMYB30-A* gene, no PCR-amplified product was generated from the templates lacking chromosome 2A (NT2A2B and NT2A2D). For *TaMYB30-B*, no PCR-amplified product was generated from the templates lacking chromosome 2B (NT2B2A and NT2B2D). For *TaMYB30-D*, no PCR-amplified product was generated from the templates lacking chromosome 2 (NT2D2A and NT2D2B).

tissues of wheat plants exposed to PEG-induced osmotic stress. The quantitative real-time PCR results showed that the expression levels of these three genes were similar (Supplementary Fig. S1 at *JXB* online), which indicated that the *TaMYB30-A*, *TaMYB30-B*, and *TaMYB30-D* genes might play similar roles in drought stress tolerance. In this study, the *TaMYB30-B* gene was selected for further functional analyses.

The TaMYB30-B protein localizes to the nucleus and acts as a transcriptional activator

To provide further evidence for the potential role of TaMYB30 in transcriptional regulation, the subcellular localization of TaMYB30-B was determined. The full-length sequence of *TaMYB30-B* was fused to the *GFP* sequence in the pJIT163-GFP vector. The recombinant constructs of the *TaMYB30-B–GFP* fusion gene or the *GFP* gene alone were transformed into onion epidermal cells via particle bombardment. When the transformed cells were observed by confocal microscopy, the GFP fluorescence of the TaMYB30-B–GFP fusion protein was found to accumulate exclusively in the nucleus, whereas the GFP fluorescence of the GFP alone was distributed throughout the whole cell. These data indicate that the TaMYB30-B protein is a nuclear-localized protein (Fig. 4).

As a TF, TaMYB30-B protein may function as a transcriptional activator. To verify this hypothesis, the sequences that code the full-length protein, the N-terminus of 433 and 333 amino acids, the C-terminus of 333 and 233 amino acids, and the middle 33 amino acids of TaMYB30 were fused to the GAL4 DNA-binding domain (pBD) in the pDEST32 vector, respectively, resulting in the constructs *pDEST32-1–733*, *pDEST32-1–433*, *pDEST32-1–333*, *pDEST32-400–733*, *pDEST32-501–733*, and *pDEST32-401–433*. The constructs were transferred into the yeast strain AH109 and each of the resulting yeast transformants was tested for the ability to activate transcription of the reporter gene and to promote yeast growth on the selection medium lacking leucine, histidine, and adenine. The yeast transformants containing the *GAL4* gene and the *pBD* vector alone were used as the positive and negative controls, respectively. As shown in Fig. 5, all transformants grew well on medium lacking leucine. On the selection medium, the yeast transformants harbouring the *GAL4* gene, *pDEST32-1–733*, *pDEST32-1–433*, *pDEST32-401–733*, and *pDEST32-401–433* constructs grew well, whereas the yeast transformants containing the *pBD* alone, *pDEST32-1–333*, and *pDEST32-501–733* could not grow on this medium. These results indicated that the TaMYB30-B protein contains a transcriptional activation domain in amino acids 401–433.

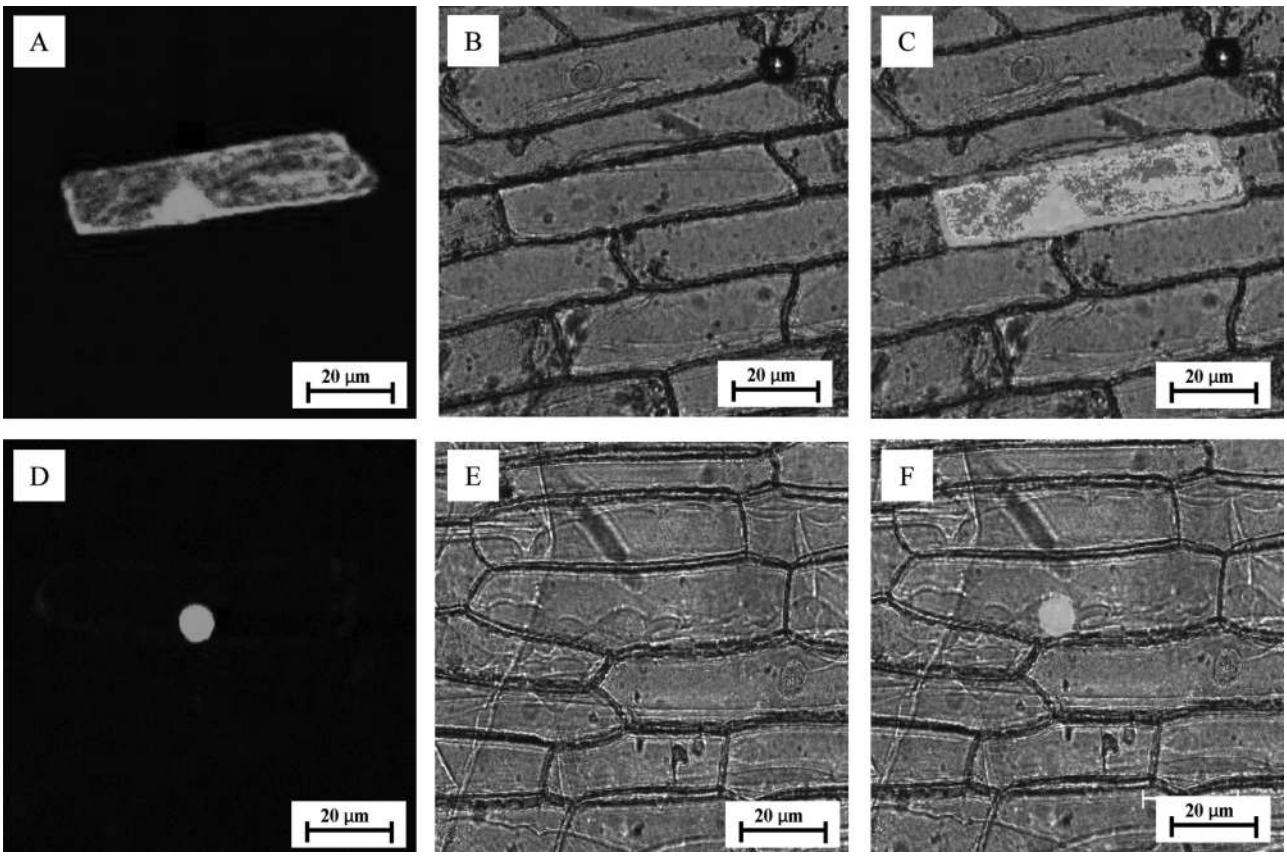

Fig. 4. Subcellular localization of the TaMYB30-B protein in onion epidermal cells. GFP alone or the TaMYB30-B–GFP fusion proteins driven by the CaMV 35S promoter were transiently expressed in onion epidermal cells and observed under a laser scanning confocal microscope. The dark fluorescence (A and D), the cell outline (B and E), and the combination photographed in bright field (C and F) are shown. (This figure is available in colour at *JXB* online.)

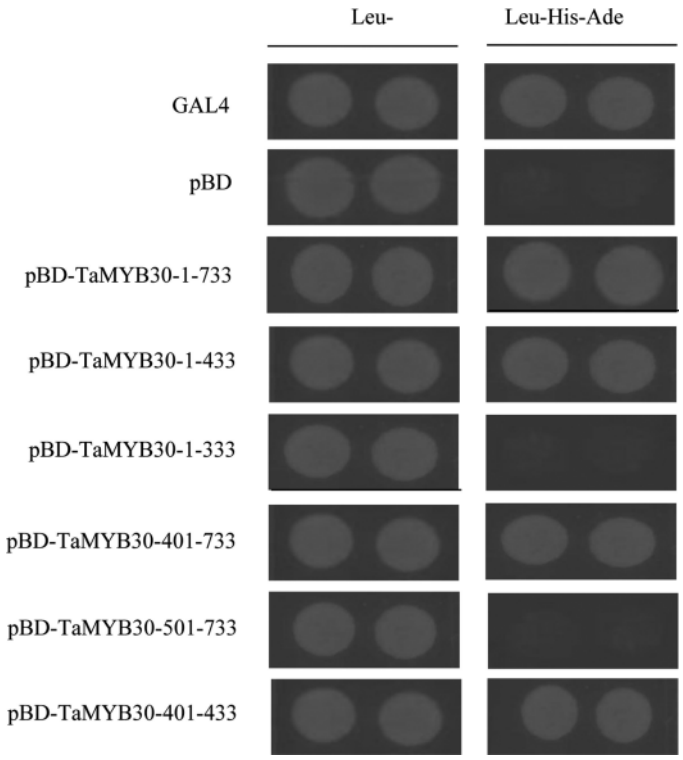

Fig. 5. Transcriptional activity of the TaMYB30-B protein in yeast. Test of transcriptional activity of the full-length and truncated fragments of TaMYB30-B. The *pDEST30* vector alone and *GAL4* were used as negative and positive controls. The numbers indicate the position and length of truncated fragments of TaMYB30-B. (This figure is available in colour at *JXB* online.)

Generation of transgenic Arabidopsis *lines overexpressing the* TaMYB30-B *gene*

The role of the *TaMYB30-B* gene in plant stress tolerance was investigated in *Arabidopsis* plants transformed with a construct containing the *TaMYB30-B* coding sequence under the control of the CaMV 35S promoter. Ten T_3 homozygous transgenic lines were obtained, and the expression level of *TaMYB30-B* was tested in each of these transgenic lines using semi-quantitative PCR and quantitative real-time PCR. Three transgenic lines, L2, L3, and L9, showed high expression levels of *TaMYB30-B* and were selected for further analysis (Supplementary Fig. S2 at *JXB* online). Constitutive overexpression of stress-up-regulated genes could lead to a slower growth rate or alteration of the flowering date. Therefore, transgenic plants were phenotyped from germination to maturity. There were no evident morphological differences between the transgenic and WT lines in terms of their growth rate, flowering times, seedling sizes, primary root lengths, and lateral root numbers (data not shown).

Examination of the seed germination of these lines on media containing various concentrations of mannitol

The germination of seeds from transgenic *Arabidopsis* lines was tested using treatments with different concentrations of mannitol. Under normal conditions, nearly 100% of the seeds from the *Arabidopsis TaMYB30-B* transgenic and WT lines germinated.

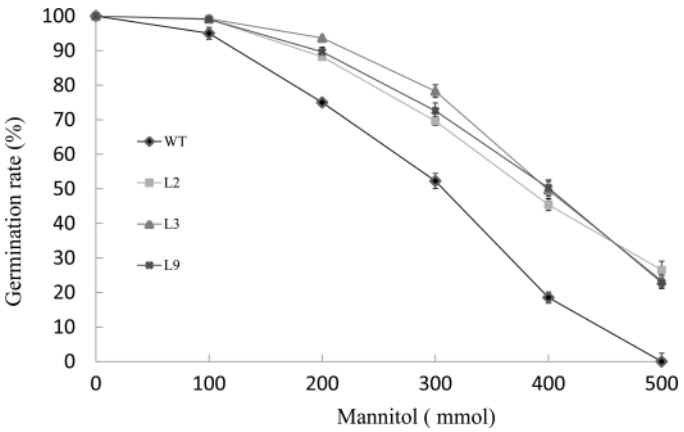

Fig. 6. Germination of *TaMYB30-B* transgenic and WT seeds using various mannitol concentrations. The percentage of germinated seeds was calculated based on the number of seedlings that reached the cotyledon stage at 2 weeks. The data shown are the means of three replicates of 100–150 seeds. The error bars indicate the SD.

However, when the *TaMYB30-B* transgenic lines were sown on MS medium containing different mannitol concentrations, these lines displayed 4–31.8% higher levels of seed germination than the WT lines. For example, in the 100 mM mannitol treatment, 95% of seeds from the WT line germinated, whereas an average of 99% of the seeds from the three transgenic lines germinated. When the mannitol concentration was increased to 500 mM in the medium, most seeds of the WT line could not germinate, whereas an average of 24.27% of the seeds from the *TaMYB30-B* transgenic lines germinated (Fig. 6). Taken together, these results indicated that the *TaMYB30-B* transgenic *Arabidopsis* lines were tolerant to mannitol-induced stress during seed germination.

The performance of TaMYB30-B *transgenic* Arabidopsis *plants under drought stress*

To validate further that the *TaMYB30-B* gene functions in plant tolerance to drought stress, the performance of *TaMYB30-B* transgenic *Arabidopsis* plants was investigated in soil by depriving the *TaMYB30-B* transgenic *Arabidopsis* seedlings of water. No evident morphological differences were observed between the *TaMYB30-B* transgenic lines and the WT lines in the first few days of water deprivation. However, by the 30th day of water deprivation, all WT plants exhibited severe symptoms of water loss and significant wilting; only a little green colour was visible in some leaves. In contrast, most of the *TaMYB30-B* transgenic lines were green at this time point, and only slight wilting was observed in some of the *TaMYB30-B* transgenic leaves. After 30 d of water deprivation, all the plants were re-watered. All the *TaMYB30-B* transgenic plants exhibited normal growth after 3 d of watering, but the WT plants died (Fig. 7).

Changes in physiological traits under stress conditions

The water loss of plants was assessed by measuring the fresh weights of detached leaves from the three *TaMYB30* transgenic

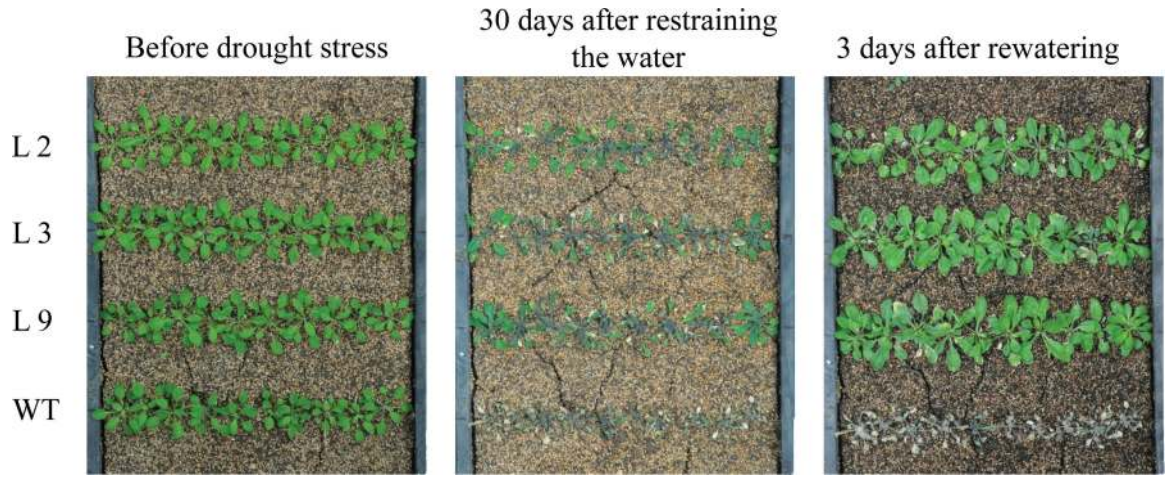

Fig. 7. Effect of *TaMYB30-B* overexpression on drought tolerance in transgenic *Arabidopsis*. Ten-day-old seedlings grown on MS medium plates were planted in a 4 cm deep rectangular plate filled with a 1:1 mixture of vermiculite and humus, and well watered. The seedlings were not watered on the following days; the seedlings that were fully affected by drought stress were re-watered. The phenotypes before and after treatment were surveyed and photographed. The drought stress experiment was performed in triplicate. (This figure is available in colour at *JXB* online.)

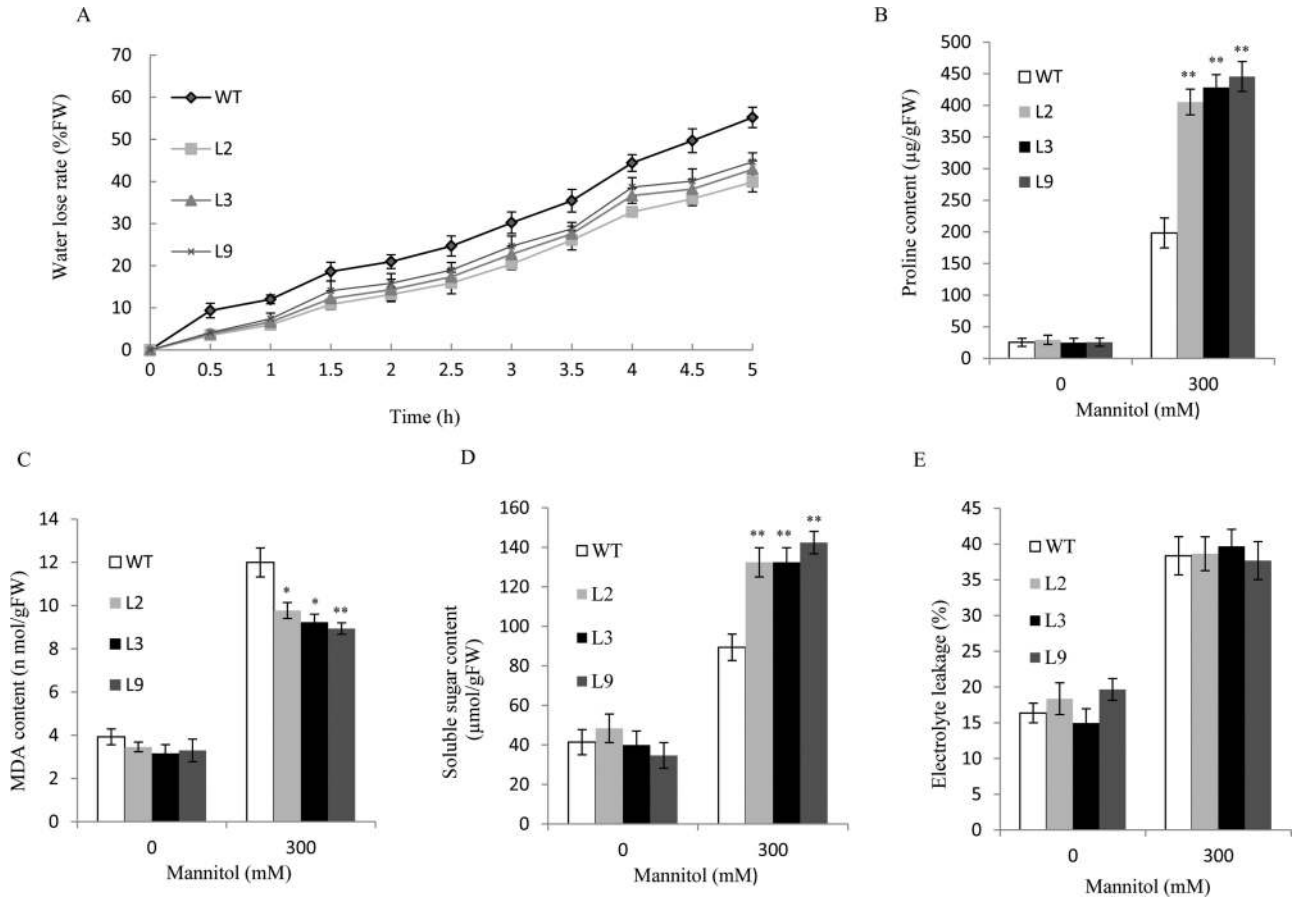

Fig. 8. Physiological changes associated with the abiotic stress response in *TaMYB30-B*-transgenic *Arabidopsis*. (A) Water loss rate of detached leaves. (B) Proline content in seedlings. (C) MDA content in seedlings. (D) Soluble sugar content in seedlings. (E) Relative electrolyte leakage of seedlings. The data represent the means of three replicates. The error bars indicate the SD; * and ** indicate significant differences in comparison with the WT lines at the $0.01 < P < 0.05$ and $P < 0.01$ levels, respectively, using the Student's *t*-test.

plants and the WT plants at the designated times. Compared with the WT plants, the three *TaMYB30* transgenic plants displayed lower rates of water loss at each time point after leaf detachment (Fig. 8A). Furthermore, the improved drought tolerance of the *TaMYB30-B* transgenic plants was correlated with changes in the proline, MDA, and soluble sugar contents of these plants. As shown in Fig. 8B, under normal growth conditions, the proline contents found in the *TaMYB30-B* transgenic plants and the WT plants were similar. When the plants were exposed to mannitol stress, the proline content was significantly higher in the *TaMYB30-B* transgenic plants than in the WT plants. The MDA content is also an important parameter related to plant responses to abiotic stress (Chen and Murata, 2002). The results indicated that the MDA content in the *TaMYB30-B* transgenic plants and the WT plants that were grown under normal conditions was similar. However, when the plants were grown under mannitol-induced stress conditions, the MDA content was lower in the *TaMYB30-B* transgenic plants than in the WT plants (Fig. 8C). Alterations in soluble sugar content are also reported to be important for the plant response to stress conditions. When grown under normal conditions, the *TaMYB30-B* transgenic plants and the WT plants contained nearly equivalent amounts of soluble sugar. However, when grown under stress conditions, the soluble sugar content in *TaMYB30-B* transgenic plants was significantly higher than for WT plants (Fig. 8D). We also measured electrolyte leakage levels of the *TaMYB30-B* transgenic and WT *Arabidopsis* plants, and no differences were observed between these plants under normal or stress conditions for this parameter (Fig. 8E).

Altered expression of drought stress-responsive genes in transgenic TaMYB30 *plants*

The molecular mechanism underlying the response of *TaMYB30-B* transgenic plants to drought stress was investigated through an examination of the expression level of seven known drought stress-responsive genes in *TaMYB30-B* transgenic plants grown under normal conditions by quantitative real-time PCR. Compared with the WT plants, two genes, *RD29A* and *ERD1*, exhibited a significantly higher expression level in the *TaMYB30-B* transgenic plants (Fig. 9). However, the expression of five other genes, *DREB2A*, *RD26*, *RD29B*, *MYB2*, and *RD20A*, was unchanged in the transgenic plants (Fig. 9). It was also found that the expression levels of the two up-regulated genes, *RD29A* and *ERD1*, were correlated with the expression levels of the *TaMYB30-B* gene in each transgenic line (Fig. 9; Supplementary Fig. S2 at *JXB* online).

Discussion

In plants, the transcripts of regulatory proteins that include various families of TFs, such as the AP2/EREBP and NAC families, and several classes of the zinc finger protein family are induced by abiotic stress. These TFs might function to regulate signal transduction pathways in response to drought, cold, or high salinity stress, and also confer stress tolerance by the regulation of stress-responsive gene expression. The MYB gene family is a large family encoding regulatory proteins with multiple functions (Dubos *et al*., 2010). Recently, the functions of the MYB gene family have been widely studied in different species, especially in the model plants *Arabidopsis* and rice (Dubos *et al*., 2010). Wheat is the most widely grown crop in the world. Although several shotgun sequencing approaches have been performed on the large and complex wheat genomes, its large size and hexaploid nature will make it harder and slower to link the sequence to a map and carry out the annotation. Therefore, relatively limited wheat MYB genes have been discovered. In previous work, 60 *MYB* genes were isolated from wheat and the *TaMYB30* gene induced by PEG-induced osmotic stress was identified (Zhang *et al*., 2012). In this study, the three gene sequences of *TaMYB30* in bread wheat were isolated and a series of experiments were conducted to explore the function of this gene in drought stress tolerance. Phylogenetic analysis of the TaMYB30 sequences and those of its homologues revealed a phylogenetic tree that branched into two distinct clades representing the dicots and monocots, which suggests that the sequence of TaMYB30 was

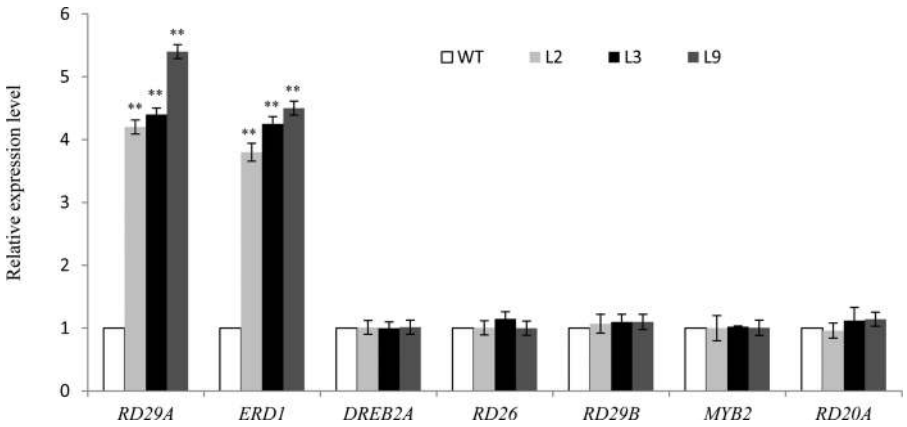

Fig. 9. Expression levels of drought stress response genes. Gene-specific primers were used for the detection of the relative transcript levels of the drought stress-responsive genes. The data represent the means of three replicates. The error bars indicate the SD; * and ** indicate significant differences in comparison with the WT lines at the $0.01 < P < 0.05$ and $P < 0.01$ levels, respectively, using the Student's *t*-test.

drastically changed after the divergence of dicots and monocots from their last common ancestor.

Using microarray and other experimental methods, a group of stress-inducible genes, which were potentially important for cold, salt, and drought tolerance, have been identified; most of these genes encode several families of TFs, such as the DREB/CBF, ERF, MYB, AREB/ABF, NAC, and HDZip TF families (Chen *et al*., 2002; Kreps *et al*., 2002; Singh *et al*., 2002; Huang *et al*., 2010; Golldack *et al*., 2011). Studies have demonstrated that overexpressing the stress-inducible TF genes in transgenic plants is an effective strategy for improving the abiotic stress tolerance of crops (Flowers, 2004; Valliyodan and Nguyen, 2006; Bhatnagar-Mathur *et al*., 2008; Cattivelli *et al*., 2008; Hussain *et al*., 2011*a*; Morran *et al*., 2011). Transgenic plants were produced that overexpressed the *TaMYB30-B* gene in *Arabidopsis*. One significant finding is that the *TaMYB30-B* transgenic plants exhibited greater levels of seed germination under osmotic stress, and the seedlings exhibited enhanced tolerance to drought stress. This finding suggested that the the *TaMYB30* gene is involved in regulating the plant response to drought stress, which indicates that this gene has prospects in plant improvement.

Drought stress could induce various physiological responses in plants (Seki *et al*., 2007). To explore the possible mechanisms of TaMYB30-B that are responsible for improving drought tolerance, several experiments were conducted to monitor the changes in physiological processes associated with plant response to stress. In this work, it is reported that the water loss rate was lower in detached leaves from the *TaMYB30-B* transgenic *Arabidopsis* seedlings than the water loss rate found in control leaves, which is consistent with the results reported recently for the wheat WRKY gene family (Niu *et al*., 2012). Proline is one of the most common osmolytes in plants, and proline accumulation is associated with plant responses to stress conditions (Bais and Ravishankar, 2002; Urano *et al*., 2009; Gill and Tuteja, 2010; Hussain *et al*., 2011*b*). Soluble sugars have also been implicated in drought stress tolerance in plants. Studies have correlated the presence of particular soluble sugars with the acquisition of stress tolerance (Kerepesi and Galiba, 2000; Farrant, 2010; Pinheiro and Chaves, 2011). A greater accumulation of free proline and soluble sugars was found in the *TaMYB30-B* transgenic plants than in the WT plants grown under stress conditions, which suggests that proline and soluble sugars are factors that contribute to the tolerance of *TaMYB3-B* transgenic plants to drought stress. To survive in poor environmental conditions, plants have developed flexible mechanisms to alter their physiological status in response to various abiotic stresses. Although these changes are not the only way that plants cope with desiccation, these physiological changes are considered important factors in plant tolerance to stress. Taken together, the changes in the physiological parameters that were detected in the *TaMYB30-B* transgenic plants were beneficial for plant responses to adverse conditions.

Although the transfer of a single stress-inducible gene has been demonstrated to be a simple and practical method of enhancing stress tolerance in plants, it is clear that the transferred gene should function in the signal transduction pathways by regulating the expression of downstream target genes. In this study, the expression of *RD29A* and *ERD1* was significantly induced in the transgenic plants overexpressing *TaMYB30-B*. *RD29A* and *ERD1* are two stress-inducible genes that function in the ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki, 1993*a*, *b*; Simpson *et al*., 2003). A previous study indicated that the expression of *TaMYB30* was strongly induced by PEG stress but was not affected by exogenous ABA treatment (Zhang *et al*., 2012). These results suggested that TaMYB30-B functions in an ABA-independent pathway and that the enhanced drought stress tolerance of the *TaMYB30-B* transgenic *Arabidopsis* plants is at least partially due to increases in the expression of *RD29A* and *ERD1*.

Discovering stress-responsive genes is significant for breeding stress-tolerant wheat through transgenic approaches. In this work, a PEG stress-induced gene was identified and its functions in drought stress in *Arabidopsis* were detected. Compared with wheat, *Arabidopsis* needs a shorter growth period and is it easy to transfer genes, which make it a simple and rapid system to verify the function of possible tolerance genes that are screened from wheat. This case study can serve as an example for identification of further stress-responsive genes in wheat.

In conclusion, a wheat *MYB* gene, *TaMYB30-B*, which encodes a nuclear-localized protein and acts as a transcriptional activator, was characterized. Based on the performance of *TaMYB30-B* transgenic plants, it is proposed that overexpression of *TaMYB30-B* leads to improved drought tolerance through an integrated effect of the regulation of stress-responsive genes and changes in some physiological traits which were triggered. These results enhance our understanding of the roles of wheat MYB TFs in plant responses to abiotic stresses and provide a candidate gene for wheat improvement.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Expression patterns of the TaMYB30 genes. Gene-specific primers were used for the examination of the individual expression levels of the three TaMYB30 genes.

Figure S2. Expression of TaMYB30-B in different transgenic Arabidopsis lines. The TaMYB30-B specific primers were used for the expression analysis of TaMYB30-B.

Table S1. Gene-specific primers used for quantitative real-time PCR.

Table S2. Diversity of sequence composition and length in three genomic sequences of each intron.

Table S3. Cis-acting regulatory elements predicted in the promoter of TaMYB30.

Table S4. Sequence identities of the TaMYB30 proteins and their homologues.

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