

A R2R3-MYB transcription factor gene in common wheat (namely *TaMYBsm1*) involved in enhancement of drought tolerance in transgenic *Arabidopsis*

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Abstract We isolated the *TaMYBsm1* genes, encoding R2R3-type MYB proteins in common wheat, aimed to uncover the possible molecular mechanisms related to drought response. The *TaMYBsm1* genes, *TaMYBsm1-A*, *TaMYBsm1-B* and *TaMYBsm1-D*, were isolated and analyzed from the common wheat cultivar Shimai 15. Their expression patterns under PEG 6000 and mannitol were monitored by semi-quantitative RT-PCR and β -glucuronidase (Gus) assay. The function of *TaMYBsm1-D* under drought stress in transgenic *Arabidopsis* plants was investigated, and the germination rate, water loss rate, as well as the proline and malondialdehyde (MDA) content were compared with that in wild type (WT) plants. The expression of three downstream genes (DREB2A, P5CS1 and RD29A) in *TaMYBsm1-D* transgenic plants was analyzed. The R2R3-MYB domains of the MYBsm1 proteins were highly conserved in plants. In addition, the *TaMYBsm1* proteins were targeted to the nucleus and contained transcriptional activation domains (TADs). Gus assay and semi-quantitative RT-PCR analysis demonstrated that the *TaMYBsm1* genes were up-regulated when the

wheat was treated by PEG and mannitol. Compared with WT plants, the germination rates were much higher, but the water loss rates were much lower in *TaMYBsm1-D* over-expression plants. *TaMYBsm1-D* transgenic plants showed distinct higher proline contents but a lower MDA content than the WT plants. The three downstream genes were highly expressed in *TaMYBsm1-D* transgenic plants. We concluded from these results that *TaMYBsm1* genes play an important role in plant drought stress tolerance through up-regulation of DREB2A, P5CS1 and RD29A. The increase of proline content and decrease of MDA content may also be involved in the drought response.

Keywords Drought stress · *MYBsm1* · Transcription factor · Common wheat · *Arabidopsis* plants · Meng-jun Li

Introduction

The MYB proteins constitute a large family of transcription factors (TFs), which are functionally diverse and present in all eukaryotes (Dubos et al. 2010). The MYB proteins are characterized by a highly conserved MYB domain generally with 1–4 imperfect amino acid sequence repeats. According to the number of adjacent repeats of the DNA-binding domains SWI3, ADA2, N-CoR, and TFI-IIB (SANT), MYB proteins are divided into four groups, R2R3-MYB, 3R-MYB, 4R-MYB and 1R-MYB (Dubos et al. 2010).

The R2R3-MYB proteins are the largest group of MYB TFs in plants (Feller et al. 2011). The large family of plant-specific R2R3-MYB has been proved to associate with evolution of plant resistance to environmental stress (Dubos et al. 2010). Many R2R3-MYB proteins are involved in drought responses in plants. Several

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Table 1 Specific primers used in the study of the *TaMYBsm1* genes

| Primer name | Upstream primer (5'–3') | Downstream primer (5'–3') |
|--------------|--|---|
| NP | 5'CTGCCAATCTCCAGTACGTG3' 5'TCACTGCTTCACTCTGCAGC3' | 5'TCATGCAAACCTCGCAGAGC3' 5'GACTACTGACGCCGTACAG3' |
| TaMYBsm1FL | 5'CCGCCATTGACCTATCCT3' | 5'CAAGCTAGGGAATTCAGGTTA/GC3' |
| DW1 | 5'CATTATACGCCACTACGCCAG3' | 5'CCATTTGGGCAATCACTATGAC3' |
| DW2 | 5'GGCAGGATACAGGATTGGAC3' | 5'CAAGGGACACCAGAAAACGAC3' |
| TRA1 | 5'CGGAATTCATGGGGAGGCCACCTTCT3' | 5'CGGGATCCTGCAAACCTCGCTCAGCAG3' |
| TRA2 | 5'CGGAATTCATGGGGAGGCCACCTTCT3' | 5'CGGGATCCTGCAAACCTCGCAGAGCAG3' |
| WT1 | 5'AGAACACTGTTGTAAGGCTCAAC3' | 5'GAGCTTTACTGCCTCGAACATGG3' |
| SQA | 5'GTGTACTTGAGGGTAATGGTGG3' | 5'CTTGCTGTTATTCTCTTCTCAC3' |
| SQB | 5'CGGGCACGTGTCTCAGAGC3' | 5'CCCTCATCGCAGTGTGTTCT3' |
| SQD | 5'GTGTACTTGAGGGTATTGATGG3' | 5'GAAACGACCTTTTCTGGCTAC3' |
| TaMYBsm1pro1 | 5'CGGAATTCTGACTGGGACAGTGGGATTC3' | 5'CCAAGCTTGGATGGGGGAGACGAGAC3' |
| TaMYBsm1pro2 | 5'CGGAATTCTGACTGGGACAGTGGGATTC3' | 5'TGCACTGCAGCCTTACACCTTAGCGTACC3' |
| TaMYBsm1TR | 5'GGGGTACCCCGCCATTGACCTATCCT3' | 5'CGGGATCCCAAGCTAGGGAATTCAGGTTA/GC3' |
| β-Actin | 5'TCGCTGACCGTATGAGCAAAG3' | 5'TGTGAACGATTCTCGGACCTG3' |
| DREB2A | 5'CTGGAGAATGGTGCAGGAAGA3' | 5'CAGATAGCGAATCCTGCTGTTGT3' |
| P5CS1 | 5'GCGCATAGTTTCTGATGCAA3' | 5'TGCAACTTCGTGATCCTCTG3' |
| RD29A | 5'ATCACTTGGTCCACTGTTGTTCT3' | 5'ACAAAACACACATAAACATCCAAAGT3' |

R2R3-MYB TFs in response to drought stress have been isolated and studied. For example, AtMYB15 functions as a TF involved in improvement of drought tolerance in *Arabidopsis* (Manu et al. 2006). AtMYB41 is reported to negative regulate of transcriptional response to osmotic stress (Lippold et al. 2009), *OsMYB2* encodes stress-responsive MYB TFs to regulate dehydration response (Yang et al. 2012). The expression of TaMYB1 in roots is strongly related to the wheat plant responses to oxygen concentration stresses (Lee et al. 2007). TaMYBsd1 is markedly up-regulated in the leaf and root of wheat under long-term drought stress (Rahaie et al. 2010). TaMYB33 enhances salt and drought tolerance, partly through reconstruction of osmotic balance and detoxification of reactive oxygen species (Qin et al. 2012; Zhang et al. 2012b). TaMYB30-B and TaMYB19-B can improve drought stress tolerance of the *Arabidopsis* transgenic plants (Zhang et al. 2012b, 2014).

Wheat is one of the most important crops worldwide. Its production is severely affected by drought stress. A crop simulation model showed that the wheat production has reduced by 4.6 % during 1961 and 1980 in China (Song and Dong 2006), and the loss presented an upward tendency from 1962 to 2010 (Cao et al. 2014). Therefore, identification of drought response genes will help to elucidate the molecular mechanisms of plant drought response and tolerance. In this study, we firstly reported the isolation and characterization of three homeologous copies of the *TaMYBsm1* genes, including *TaMYBsm1-A*, *TaMYBsm1-B* and *TaMYBsm1-D*. Correlation between the expression of

the genes encoding wheat TaMYBsm1 and drought was evaluated,

Materials and methods

Plant material and wheat BAC library

T. aestivum cv. Shimai 15 was used to clone the genomic DNA and cDNA sequences of *TaMYBsm1*. The nullisomic/tetrasomic lines of the Chinese Spring (CS NT) lines were used to determine the chromosomal location of *TaMYBsm1*. *Arabidopsis thaliana* Columbia-0 was used to generate *TaMYBsm1*-promoter (*TaMYBsm1-A*, *TaMYBsm1-B* and *TaMYBsm1-D*) transgenic lines.

Isolation of full-length cDNAs and genomic DNAs of the *TaMYBsm1* genes

Based on expressed sequence tag (EST, GenBank: CA653725) sequence, nested PCR primers (NP, Table 1) were designed to screen wheat cv. Shimai 15 BAC library. BAC pool plasmids were used as template and amplified for 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s.

Sequencing primers were designed based on known sequences of the *TaMYBsm1* genes. BAC plasmids were used as template for sequencing on ABI 3730XL DNA Analyzer to acquire 5' and 3' ends of *TaMYBsm1*. These steps were repeated until the genomic DNA sequences of

the *TaMYBsm1* genes were obtained, including their 5' flanking sequences.

The primers (*TaMYBsm1FL*, Table 1) used to isolate full-length cDNAs and genomic DNAs of the *TaMYBsm1* genes were designed based on the predicted *TaMYBsm1* genomic DNA sequences. The Premix PrimerSTAR HS (TaKaRa, Dalian, China) was used in the PCR amplification. The PCR program included 98 °C for 30 s, followed by 30 cycles of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and then a final extension step at 72 °C for 7 min.

To obtain full length cDNA sequences of the *TaMYBsm1* genes, total RNA was extracted from two-week-old wheat seedlings using RNAiso reagent (TaKaRa, Dalian, China). mRNA was isolated using polyAtractmRNA isolation system III (Promega). One microgram of mRNA was reverse transcribed by using PrimerScript™ 1st Strand cDNA Synthesis Kit. The PCR products were cloned into the pMD18-T plasmid (TaKaRa, Dalian, China). The positive clones were picked randomly and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) on ABI 3730XL DNA Analyzer. The 5' UTR and 3' UTR of the *TaMYBsm1* genes were identified using the 5'/3' RACE Kit (Roche) with oligo (dT) as primer.

Chromosomal location of the *TaMYBsm1* genes

The primers were used to amplify *TaMYBsm1* genes in the 42 CS NT lines in order to determine chromosomal location of *TaMYBsm1*. The primers, DW1 (Table 1) for *TaMYBsm1-A* (512 bp) and *TaMYBsm1-D* (717 bp) and DW2 (Table 1) for *TaMYBsm1-B* (573 bp), were designed based on the promoter sequences of *TaMYBsm1* by using Primer premier 6.0 (Premier, Canada) software. The DNA of CS NT lines was used as template for 30 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s.

Sequence analysis

Sequence assembly and coding region prediction were performed using Lasergene SeqMan II Module (DNASTar; <http://www.DNAStar.com>). Multiple sequence alignments were analyzed by ClustalW 1.83 software (<http://www.ch.embnet.org/software/ClustalW.html>). Cluster analysis was conducted by MEGA4.1. A bootstrap analysis was carried out and the robustness of each cluster was verified in 1,000 replications. Sequences were shaded using the Box-Shade program (http://www.ch.embnet.org/software/BOX_form.html).

Transactivation assay of the *TaMYBsm1* genes

The transactivation assay was performed according to literature previously described. The *TaMYBsm1-A*,

TaMYBsm1-B, and *MYBsm1-D* constructs were produced via PCR reaction using full length *TaMYBsm1* cDNA sequences. The primers, TRA1 for *TaMYBsm1-A* and *TaMYBsm1-B* and TRA2 for *TaMYBsm1-D* are listed in Table 1. The PCR products were cloned in pMD18-T plasmid (TaKaRa, Dalian, China) and were digested with EcoRI and BamHI. The digested constructs were cloned with the same restrict enzyme site of the pGBKT7 vector (Clontech). The constructs were transformed to yeast strain Y190 (MATa, HIS3, lacZ, rp1, leu2, cyhr2) and then the transformant was selected from a selection medium (SD/-Trp) including 25 mM 3-amino-1, 2, aminotriazole (3-AT). The selected transformant was cultured in the selection medium (SD/-Trp-His) including 25 mM 3-AT for 1 day, and a filter-lift assay was performed for blue color development.

Subcellular localization assay

The full-length cDNA sequences of the *TaMYBsm1* genes were amplified by PCR using the primers TRA1 and TRA2 (Table 1), and were cloned in-frame into the binary vector pEGAD between the EcoRI and BamHI sites. The positive transformants were identified by enzyme cutting and sequencing. The vectors were introduced into *Nicotiana benthamiana* leaves through *Agrobacterium tumefaciens* mediated (strain EHA101) transformation. The epidermal cell layers of tobacco leaves expressing the green fluorescent protein (GFP)-*TaMYBsm1* fusions were assayed for fluorescence using a confocal microscope (Leica SP8).

Semi-quantitative reverse transcription-PCR

The resulting cDNA was used as template for 30 cycles of 20 s at 95 °C, 35 s at 62 °C. Ten microliters of the PCR product was electrophoresed and visualized by ethidium bromide staining. Wheat tubulin was used as a loading control. The primers, WT1 for wheat tubulin, SQA for *TaMYBsm1-A*, SQB for *TaMYBsm1-B* and SQD for *TaMYBsm1-D*, are listed in Table 1.

Construction of *TaMYBsm1pro*: Gus and Gus assay

The promoter sequences of the *TaMYBsm1* genes were amplified using BAC clone plasmids as templates. The primers, *TaMYBsm1pro1* for amplification of the promoter regions of *TaMYBsm1-A*, *TaMYBsm1-D* and *TaMYBsm1pro2* for amplification of the promoter region of *TaMYBsm1-B*, are listed in Table 1. PCR products were firstly cloned in pMD18-T plasmids (TaKaRa, Dalian, China) confirmed by sequencing, then the positive transformants were digested with BamHI and Hind III and cloned into pCAMBIA1381Z to generate *TaMYBsm1pro*:Gus.

The vectors were transformed into *Arabidopsis* mediated by *A. tumefaciens* strain GV3101 by the floral dipping method (Clough and Bent 1998). Then the expression patterns of *TaMYBsm1* genes under 16 % PEG 6000 and 400 mM dehydrant mannitol treatments were monitored by semi-quantitative RT-PCR and Gus staining. Briefly, the 12-day seedlings tested were infiltrated in Gus staining solutions and incubated at 37 °C for 4–8 h, the stained seedlings were then cleared in 70 % ethanol and photographed.

Construction of CaMV35S:*TaMYBsm1-D* vector and plant transformation

The open reading frame (ORF) of the *TaMYBsm1-D* was amplified using primers TaMYBsm1TR (Table 1). The amplified products were digested by KpnI and BamHI and then cloned into the vector pCAMBIA2300, in which *TaMYBsm1-D* was driven by the CaMV35S promoter; the resultant vector was named as CaMV35 s:*TaMYBsm1-D*. Then the vector was transformed into *Arabidopsis* mediated by *A. tumefaciens* strain GV3101 using the floral dipping method (Clough and Bent 1998). Three homozygous transgenic *Arabidopsis* lines were confirmed by PCR and RT-PCR, and were selected for further study.

Drought tolerance analysis of the *TaMYBsm1-D* transgenic *Arabidopsis* plants

For drought stress assay, the CaMV35 s:*TaMYBsm1-D* transgenic *Arabidopsis* plants together with the wild type (WT) control were grown under normal conditions for 4 weeks and stopped watering for another 2 weeks. To observe the adaptability of *TaMYBsm1-D* transgenic plants against to drought, and to detect if the response is reversible, we re-watered the plants for 2 days for observation. All the tests were photographed to record their phenotypic changes.

Physiological characteristics of the *TaMYBsm1-D* transgenic *Arabidopsis* plants

Arabidopsis seeds of the transgenic *Arabidopsis* plants and the WT plants used in the germination assays were planted in MS medium containing 15 % PEG 6000. Seeds with radicle tip fully expanding the seed coat were regarded as germination. The percentage of germinated seeds was scored as the germination rate.

The transgenic *Arabidopsis* plants and the WT seedlings grown for 4 weeks under normal conditions were used for water-loss assay. Their aerial parts were collected to record their fresh weight at room temperature (40 % relative humidity), afterwards, they were weighed per hour until no obvious differences were detected between two adjacent time points. To record their final dry weight,

they were dried in an oven at 85 °C for 24 h. The water-loss rate was calculated on the basis of the initial weight of the plants. Sixteen plants in each line were pooled for measurement.

The transgenic *Arabidopsis* plants and the WT seedlings were cultured for 4 weeks at normal conditions, then they were drought-stressed for 2 weeks, their rosette leaves were collected for free proline and malondialdehyde (MDA) measurement. The proline content (mg/g) was quantified by the ninhydrin acid reagent method using proline as the standard (Bates et al. 1973). MDA content was determined as described previously (Draper and Hadley 1990). For statistical analysis, thirty seedlings were measured for individual WT and transgenic *Arabidopsis* lines, and Student's *t* test was performed.

Real-time quantitative PCR assay of the drought-stress-related genes in the *TaMYBsm1-D* transgenic *Arabidopsis* plants

Two-week-old seedlings of CaMV35 s:*TaMYBsm1-D* transgenic *Arabidopsis* plants together with the WT control were treated with 16 % PEG 6000 for 24 h. Three downstream drought-stress-related genes (DREB2A, P5CS1 and RD29A) were selected for real-time quantitative PCR assay. The primers used to amplify these genes in real-time quantitative PCR analysis are listed in Table 1. The *Arabidopsis* β -actin gene was amplified as internal control. The cDNA product was used as template for 40 cycles of 30 s at 95 °C, 5 s at 95 °C, 34 s at 60 °C. The quantitative analysis was performed using the $2^{-\Delta\Delta Ct}$ method.

Results

Cloning of the *TaMYBsm1* genes

The dehydration-induced EST-CA653725, annotated as *TaMYBsm1*, was identified in wheat expression profile. To further determine the function of the *TaMYBsm1* genes in the regulation of drought tolerance in common wheat, we screened the hexaploid wheat genomic DNA library. Eight genomic BAC clones were isolated and three highly homologous genomic sequences (*TaMYBsm1-A*, *TaMYBsm1-B* and *TaMYBsm1-D*) were identified, then their corresponding DNA and cDNA sequences of the *TaMYBsm1* genes were confirmed by PCR amplification.

The *TaMYBsm1* genes were located on wheat chromosomes 7A, 7B and 7D, respectively (Fig. 1). The three promoter sequences of each of the *TaMYBsm1* genes were highly identical to sequences on chromosomes 7A, 7B and 7D, respectively. All these results indicated that *TaMYBsm1* was located on the short arm of chromosome 7.

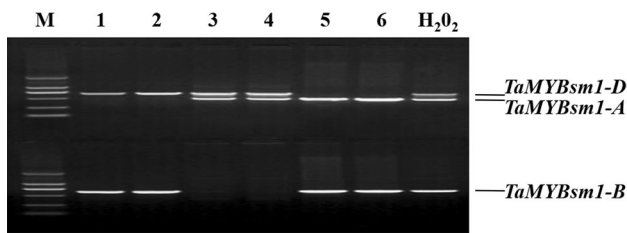


Fig. 1 Chromosomal location of the *TaMYBsm1* genes. *M* marker (D2000), *1* nulli-tetrasomic stocks (NT) of Chinese Spring 7A7B, *2* NT7A7D, *3* NT7B7A, *4* NT7B7D, *5* NT7D7A, *6* NT7D7B, *CS* Chinese Spring

All the *TaMYBsm1* genes had ORFs of 975 bp, each of which encoded an R2R3-MYB of 324 amino acids. The three genes share 95.0–96.1 % identity at the nucleotide level and 93.2–94.8 % identity at the amino acid level. The *TaMYBsm1* genes had identical structure, containing two introns and three exons.

Cluster analysis of plant MYBsm1 proteins

Using *TaMYBsm1* as the query sequences, sixteen *TaMYBsm1* homologs were identified. Cluster tree showed that plant MYBsm1 proteins were divided into two divergent branches, corresponding to monocot and dicot plant sequences (Fig. S1a). The *AeMYBsm1* of *Aegilops tauschii* is identical to *TaMYBsm1-D*. Only 2 of 324 amino acids in the *TuMYBsm1* of *Triticum urartu* were different from *TaMYBsm1-A*. The results were in consistent with chromosomal locations of the *TaMYBsm1* genes.

Multiple alignment revealed that 35 (76 %) and 32 (73 %) of amino acids were identical in the R2 and R3 MYB repeat regions, respectively, among all the plant MYBsm1 proteins (Fig. S1b). These results indicating that the R2R3-MYB domains were highly conserved in plant MYBsm1 proteins.

Autoactivation in the heterologous yeast system

The transcriptional activations of the *TaMYBsm1* proteins were investigated via yeast-based transactivation assay. The full length of the *TaMYBsm1* genes were fused to the GAL4 DNA-binding domain in the pGBKT7 plasmids. When the constructed plasmids were induced in yeast Y190 strain containing *GAL1* promoter and *HIS3* and *lacZ* reporter genes, β -galactosidase activity was observed in yeast cells harboring *TaMYBsm1-A*, *TaMYBsm1-B*, *TaMYBsm1-D*, and *TaNAC6*, but not in yeast cells containing pGBKT7 plasmids (Fig. 2). The results indicated that each of the *TaMYBsm1* proteins may contain a transcriptional activation domain.

Subcellular localization of the TaMYBsm1 proteins

To investigate the subcellular location of the *TaMYBsm1* proteins, we made three constructs harboring a GFP-*TaMYBsm1* fusion protein under the control of the constitutive CaMV35S promoter, respectively. The generated constructs were introduced into *N. benthamiana* leaf epidermal cells via *A. tumefaciens*-mediated infiltration (Sparkes et al. 2006). The fluorescent signals from the constructs of GFP:*TaMYBsm1-A*, GFP:*TaMYBsm1-B* and GFP:*TaMYBsm1-D* were all observed in the nucleus of *N. benthamiana* mesophyll cells, indicating that all the *TaMYBsm1* genes have localized in the nucleus (Fig. 3).

Expression analysis of the TaMYBsm1 genes in common wheat

The expression patterns of the *TaMYBsm1* genes under 16 % PEG 6000 were monitored by semi-quantitative RT-PCR (Fig. 4a–c). The results showed that the *TaMYBsm1* genes were induced by 16 % PEG 6000 treatment in the root and leaf of wheat. *TaMYBsm1* transcripts accumulated

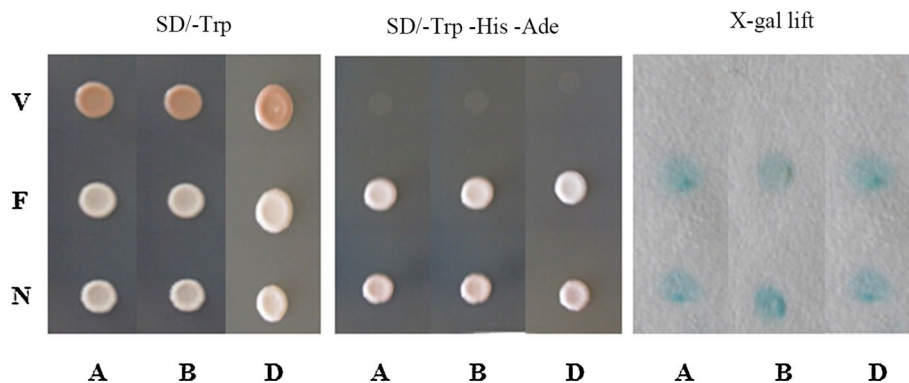
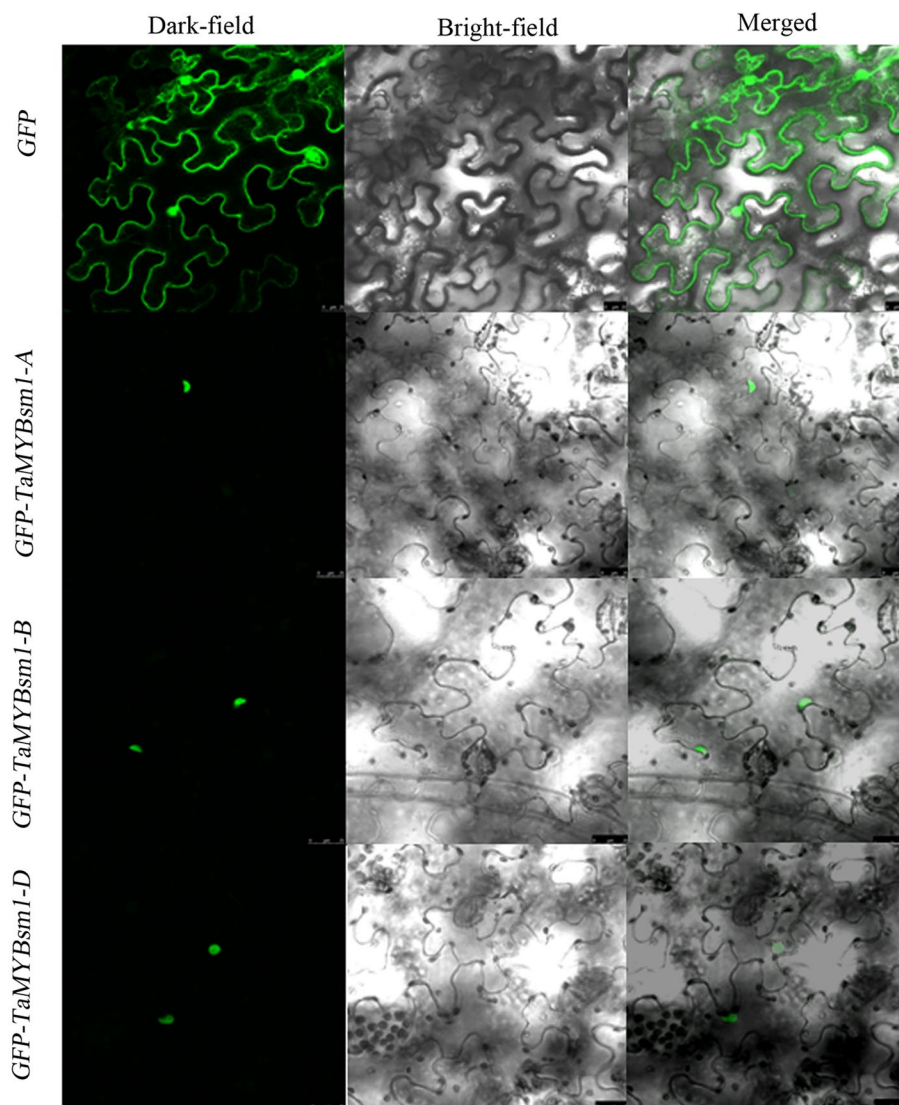


Fig. 2 Transactivation assay of *TaMYBsm1* proteins in yeast cells. The transformed yeast cells were grown on selective medium SD/-Trp (left) and SD/-Trp-His-Ade (middle). An X-gal lift assay was carried out using SD/-Trp-His plate (right). *V* yeast transformed with the

pGBKT7 vector, *F* yeast transformed with the *TaMYBsm1* genes, *N* yeast transformed with *T. aestivum NAC6* (*TaNAC6*), *A* *TaMYBsm1-A*, *B* *TaMYBsm1-B*, *D* *TaMYBsm1-D*

Fig. 3 Subcellular localization of the TaMYBsm1 proteins. *Left* images, *dark-field* microscopy; *Middle* images, *bright-field* microscopy; *Right* images, the combination of the *left* and *middle* images



at the late stage of PEG treatment in root (Fig. 4a) and at the early stage in leaf (Fig. 4b). The *TaMYBsm1* genes were mainly expressed in roots, leaves and stamens of wheat under normal conditions. The expression of The *TaMYBsm1* genes in roots is stronger than those in other organs (Fig. 4c), indicating that the *TaMYBsm1* genes mainly functioned in wheat roots.

Using PCR amplification, the 1,709–2,144 bp sequences upstream of the start code of the *TaMYBsm1* genes were amplified. Many motifs related to dehydration stress were predicted on PLACE (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>). The promoter region of *TaMYBsm1-B* is distinct from that of *TaMYBsm1-A* and *TaMYBsm1-D*. Compared to *TaMYBsm1-A*, two dehydration stress related motifs were deleted at the same position on the promoter region of *TaMYBsm1-D* (Fig. 5). Gus assay was performed in transgenic *Arabidopsis* plants expressing Gus gene under the control of the *TaMYBsm1* promoters

(*TaMYBsm1-A* promoter:Gus, *TaMYBsm1-B* promoter:Gus and *TaMYBsm1-D* promoter:Gus) to examine the expression of the *TaMYBsm1* genes under 16 % PEG 6000 and 400 mM mannitol treatment (Fig. 4d). Results showed that Gus proteins accumulated when the *Arabidopsis* plants expressing three *TaMYBsm1* promoter:Gus were treated by PEG and mannitol.

Overexpression of *TaMYBsm1-D* improves drought tolerance of transgenic *Arabidopsis* plants

The function of *TaMYBsm1-D* in drought stress tolerance was investigated in *Arabidopsis* plants. Three transgenic lines were selected for further study. All the transgenic plants together with the WT control grown for 3 weeks under normal conditions, all the plants grown well and no obvious phenotypic changes were observed.

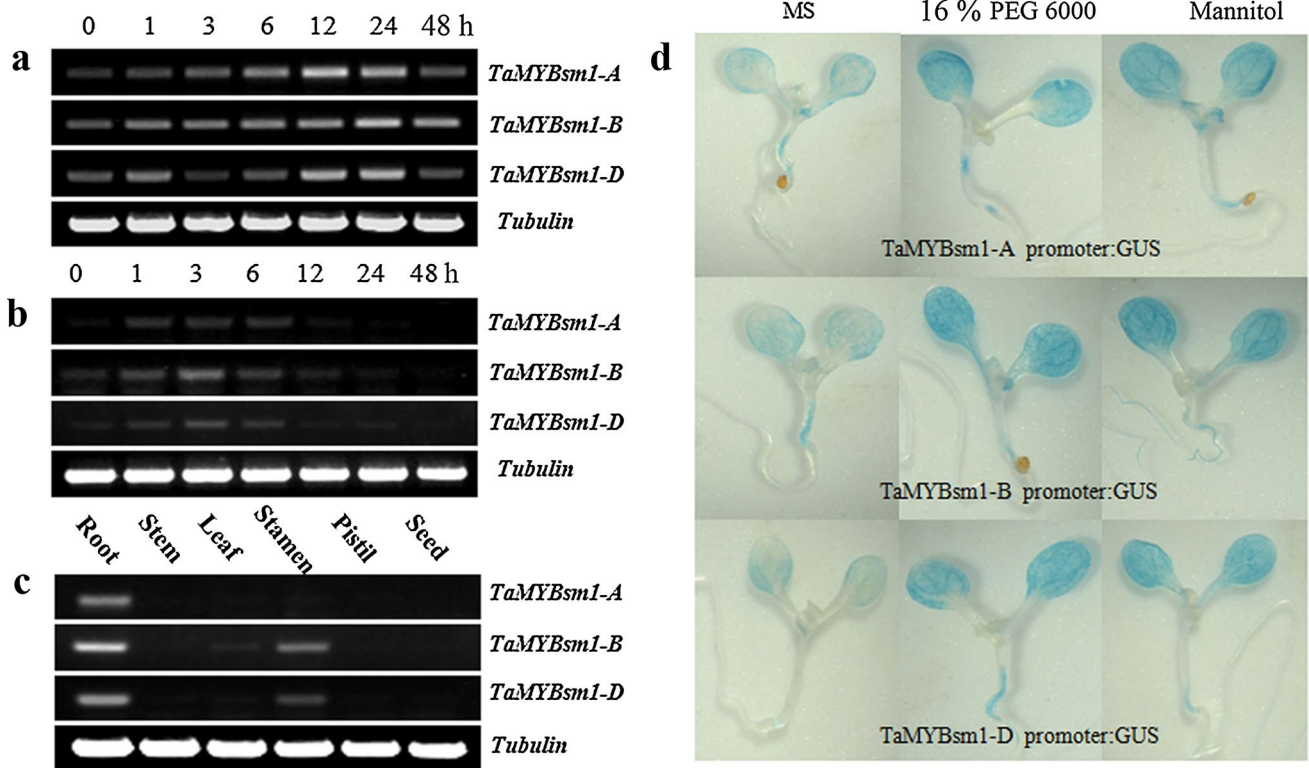


Fig. 4 Expression analyses of the *TaMYBsm1* genes. **a** wheat root, **b** wheat leaf, **c** different organs of wheat, **d** Gus staining of *TaMYBsm1*pro:Gus *Arabidopsis* plants. The seedlings were treated by 16 % PEG 6000 and 400 mM mannitol for 12 h before Gus staining

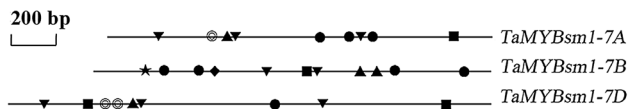


Fig. 5 Cis-acting regulatory elements of promoter regions of the *TaMYB39* genes. Filled triangle ABRELATERD1, concentric circle ACGTATERD1, inverted filled triangle MYB1AT, filled diamond MYB2AT, filled circle MYBCORE, filled star MYCATERD1, filled square MYCATRD2

The drought tolerance of *TaMYBsm1-D* transgenic *Arabidopsis* plants was investigated in soil under water deprivation conditions during the seedling stage. After stopping watering for 2 weeks, all WT plants (n = 16, 100 %) exhibited severe symptoms of water loss and wilting. On the contrary, the wilting symptoms in the *TaMYBsm1* overexpression *Arabidopsis* transgenic lines were reduced and the majority of leaves were green at this time point. After re-watering, most of the WT plants died (n = 12.75 %), while a number (n = 4.92 %) of *TaMYBsm1-D* transgenic plants exhibited normal growth after 2 days of re-watering (Fig. 6a).

When growing in MS medium containing 16 % PEG 6000, the germination rates of *TaMYBsm1-D* overexpression plants were much higher than those of WT plants

(Fig. 6b). Compared with the WT plants, the *TaMYBsm1-D* transgenic plants displayed lower rates of water loss at all the time points (1, 2, 3, 5, 7 h) after 4 weeks under normal conditions (Fig. 6c). When exposed to mannitol stress, the *TaMYBsm1-D* transgenic plants under dehydration showed distinct higher proline contents but lower MDA content than the WT plants (Fig. 6d, e). Taken together, these results suggested that there might be an association between the overexpression of *TaMYBsm1-D* and drought tolerance in transgenic *Arabidopsis* plants.

Expression of downstream genes in *TaMYBsm1-D* transgenic plants

In order to investigate whether the expression levels of drought stress-related genes were altered or not in *TaMYBsm1-D* transgenic plants, three drought stress-related genes (DREB2A, P5CS1 and RD29A) were chosen for analysis by real-time quantitative PCR. As a result, these genes exhibited a significantly higher expression level in the *TaMYBsm1-D* transgenic plants than WT (Fig. 7). These changes suggested that *TaMYBsm1-D* functioned in drought response through up-regulating the downstream drought stress-related genes.

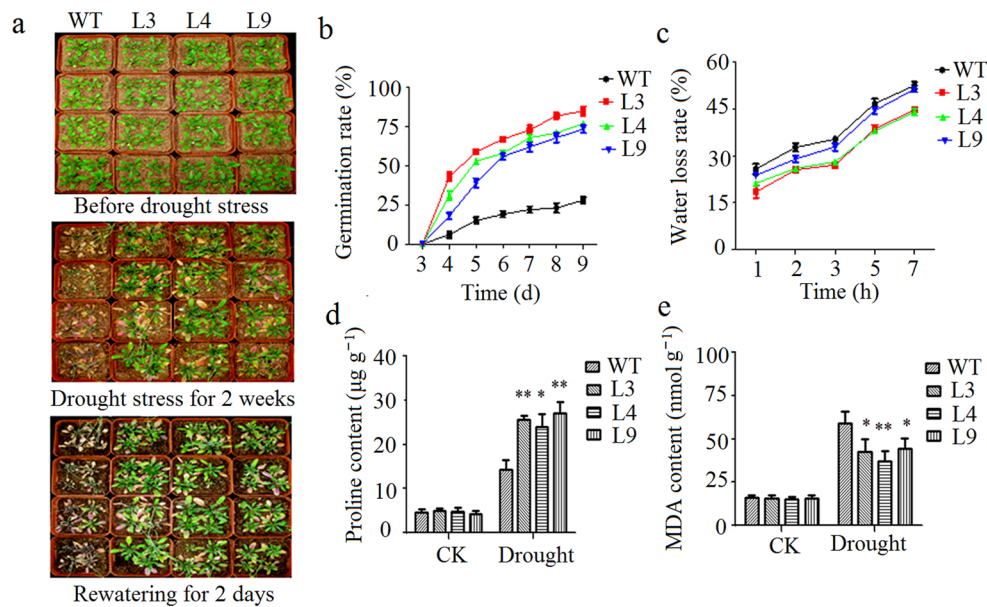


Fig. 6 Transgene identification and drought stress response analysis in WT and transgenic plants. **a** Phenotypic changes of transgenic *Arabidopsis* plants before/after drought stress. **b** Effect of drought stress on the germination of seeds. **c** water-loss assay of all the transgenic *Arabidopsis* plants and WT control. **d** proline content. **e** MDA contents were measured before/after drought stress. WT

wild type Columbia-0; L3, L4, and L9, *TaMYBsm1-D* overexpression transgenic lines. CK normal condition. The asterisks indicate the statistically significant differences between the WT control and the transgenic *Arabidopsis* lines determined through student's *t*-tests (* $P < 0.05$, ** $P < 0.001$)

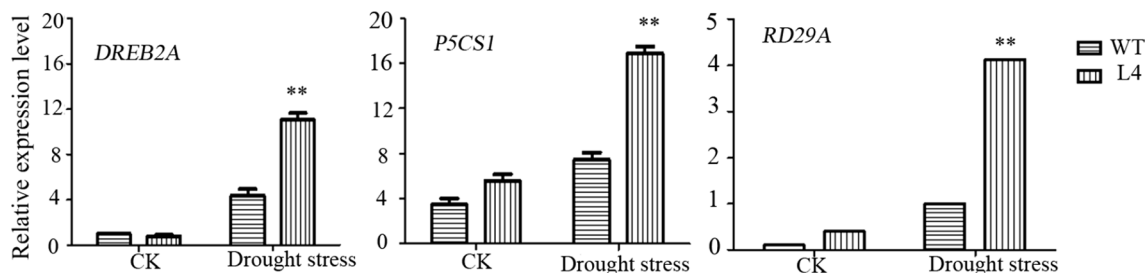


Fig. 7 Expression levels of three genes related to drought stress in transgenic *TaMYBsm1-D* *Arabidopsis* plants. Gene-specific primers were used for the detection of the relative transcript levels of the drought stress-responsive genes. The data represent the means of three replicates. WT Columbia-0, L4 *TaMYBsm1-D* overexpression

transgenic line. CK normal condition. The asterisks indicate the statistically significant differences in the comparisons between the WT control and the transgenic *Arabidopsis* lines which were determined through student's *t*-tests (* $P < 0.05$, ** $P < 0.001$)

Discussion

Drought stress is a major limited factor that influences the growth and development of plants. Therefore, isolating the genes involved in drought stress tolerance and cultivating plant varieties with enhanced drought stress tolerance are important for plant breeding. MYB gene family is one of the largest families encoding regulatory proteins with multiple functions (Dubos et al. 2010). In recent years, some R2R3-MYB genes have been described to be associated with drought responses and tolerance in some species,

especially in rice and *Arabidopsis*, but only a few works have been reported in common wheat (Lee et al. 2007; Liu et al. 2011; Mao et al. 2011; Rahaie et al. 2010; Zhang et al. 2012a, b, 2014).

In this study, the function of the *TaMYBsm1* genes was analyzed by screening the wheat genomic DNA library in transgenic *Arabidopsis*. The *TaMYBsm1* genes (*TaMYBsm1-A*, *TaMYBsm1-B* and *TaMYBsm1-D*) were successfully isolated, and the potential function of these genes is closely related to plant drought tolerance. Based on variation of the *TaMYBsm1* promoter sequences, the

TaMYBsm1 genes were mapped on wheat chromosomes 7A, 7B, and 7D, named as *TaMYBsm1-A*, *TaMYBsm1-B* and *TaMYBsm1-D*, respectively. Gus staining indicated that the expressions of the *TaMYBsm1* genes were up-regulated by dehydration stress in transgenic *Arabidopsis*. Semi-quantitative RT-PCR experiments showed that the *TaMYBsm1* genes were induced by 16 % PEG 6000, but showed different expression profiles in roots and leaves of common wheat. The results indicated the *TaMYBsm1* genes were the drought stress response genes, and differently expressed in different plant organs.

TaMYBsm1 N-terminal regions contained R2 and R3 domains with the conserved amino acid motifs R2 [-W-(X19)-W-(X19)-W-] and R3 [-F-(X18)-W-(X18)-W-] that are known to be essential for DNA binding (Dubos et al. 2010). In this study, the R2R3-MYB domains of plant MYBsm1 homologs exhibited significant sequence conservation and showed no difference between monocot and dicot plants. These results indicated that plant R2R3-MYB domains of the MYBsm1 proteins played important roles in their functions. Many R2R3-MYB proteins have transcriptional activation domains (TADs), that has been firstly identified in ZmC1 (Goff et al. 1991). Transcriptional activity was identified in all TaMYBsm1 proteins by yeast-based transactivation assay in our study. The promoter region of *TaMYBsm1-B* is distinct from that of *TaMYBsm1-A* and *TaMYBsm1-D*. Compared to *TaMYBsm1-7A*, two dehydration stress related motifs (MYBCORE) were deleted at the same position on the promoter region of *TaMYBsm1-D*. Under the dehydration stress, the TFs activate RNA polymerase II through targeting on the cis-element, induce expression of target genes to resist against the stress. However, this regulation depends on an interaction of a series of IFs. For example, MYB IFs were reported to interact with basic/helix-loop-helix (bHLH) for environmental stress response (Singh 1999). Though the promoter elements of the three *TaMYBsm1* genes were different as reported in our study, these elements may play a key role in co-regulation of drought stress. The plant TaMYBsm1 proteins branched into two distinct groups on the cluster tree corresponding to monocot and dicot plants, because of the difference of the C-terminal regions which drastically changed after the divergence of dicots and monocots from their common ancestor. Therefore, the MYBsm1 proteins may exhibit different transcriptional activation in monocot and dicot plants.

Arabidopsis seedlings of overexpressing *TaMYBsm1-D* exhibited enhanced tolerance to drought stress. Under drought stress, the transgenic plants had higher seed germination rate and lower water loss rate than the non-transgenic plants. In order to uncover the related molecular factors involved in the drought tolerance of *TaMYBsm1-D* in transgenic *Arabidopsis*, we detected physiological indicators of

stress tolerance. Physiological indicators, such as MDA and proline, could be used as a reference for evaluation of plant stress tolerance. It has been reported that rapid damage, such as drought stress will damage the membrane of plant cells (Liu et al. 2016; Wang et al. 2016). The leakage of membrane is caused by the enhancement of free radical, which will result in lipid peroxidation (Mirzaee et al. 2013). MDA would produce during this process. Therefore, MDA is widely recognized as a marker for lipid peroxidation (RoyChoudhury et al. 2007). At the early period of drought, MDA substantially produced for response until the plant could not undergo biosynthesis. Then the content of MDA is decreased. Proline is compatible solutes that accumulate in plants in response to abiotic stress (Armen-gaud et al. 2004; Chyzykova and Palladina 2005; Zhang et al. 2016). Under drought stress, transgenic *Arabidopsis* plants accumulated more proline but less MDA than that in the WT plants. It may be that proline is a factor that contributes to drought tolerance capabilities of the *TaMYBsm1-D* transgenic plants. Low MDA content provides the transgenic plants with more growth advantages.

Stress-responsive genes play important roles in regulating the defense response pathways. Some major stress-responsive genes in transcriptional regulatory networks in response to abiotic stresses have been identified in *Arabidopsis*. The expression profiles of these genes could provide important information for uncovering the mechanisms of *TaMYBsm1-D* in drought stress response and tolerance. In this study, the three downstream genes *DREB2A*, *P5CS1* and *RD29A* were significantly up-regulated in transgenic *Arabidopsis* plants under drought stress. In *Arabidopsis*, *DREB2A* was induced by dehydration and high-salt stress (Liu et al. 1998; Nakashima et al. 2000; Sakuma et al. 2002). Overexpression of *DREB2A* activated the expression of many stress-inducible genes and resulted in significant drought stress tolerance (Sakuma et al. 2006). Under drought stress, *TaMYBsm1* overexpression increased *DREB2A* expression level and the capabilities of transgenic *Arabidopsis* drought stress tolerance were improved by the activation of many stress-inducible genes. P5CS catalyzes the rate-limiting step of proline biosynthesis. P5CS1 is necessary for proline accumulation under osmotic stress (Székely et al. 2008). We also found the proline was significantly increased in transgenic plant in our study. Therefore, overexpression of P5CS improved proline level and increased tolerance to osmotic stress (Kishor et al. 1995). Due to up-regulation of *P5CS1*, a large amount of proline accumulated in *TaMYBsm1* overexpression *Arabidopsis* plants which enhanced the drought stress tolerance of the transgenic plants. *RD29A* is induced by desiccation and has at least two cis-acting elements, one involved in the ABA-associated response to desiccation and the other induced by changes in osmotic potential (Shinozaki and

Yamaguchi-Shinozaki 2007). The results above suggested that the enhanced drought tolerance of the *TaMYBsm1-D* transgenic *Arabidopsis* plants is at least partially due to up-regulating expression of downstream genes related to drought stress including *DREB2A*, *P5CS1* and *RD29A*. We thought that the drought stress stimulates overexpression of *DREB2A*, which significantly activated the expression of many stress-inducible genes, such as *P5CS1* and *RD29A*. However, this study limited to analyze the transcriptional regulation of these genes to uncover the stress response at the protein level. We will perform a further study to explain the regulation mechanism in translational level.

In conclusion, overexpression of *TaMYBsm1* enhances the drought tolerance of transgenic plants. Up-regulation of *DREB2A*, *P5CS1* and *RD29A* genes play a key role in drought tolerance.

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