

Identification of *MYB* transcription factor genes and their expression during abiotic stresses in maize

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Abstract

The *MYB* transcription factor superfamily is a large gene family that plays central roles in developmental processes and defence responses in plants. Unlike in *Arabidopsis*, only few members of the *R2R3-MYB* gene family have been functionally well characterized in maize, especially in abiotic stress-response pathways. Subgroup-specific conserved motifs outside the *MYB* domain may reflect functional conservation. A comparative genomics study using *Arabidopsis* abiotic stress-responsive *MYB* protein sequences identified 46 *ZmMYB* genes that may be involved in abiotic stress responses of *Zea mays*. An expression pattern analysis of the 46 *ZmMYB* genes under abiotic stress treatments was used to identify 22 *MYB* genes that were induced by one or more of the stress treatments. *ZmMYB30* was highly up-regulated under the four stress treatments. The ectopic expression of *ZmMYB30* in transgenic *Arabidopsis* plants promoted salt-stress tolerance and also increased the expression of a number of abiotic stress-related genes, allowing the plants to overcome adverse conditions.

Additional key words: abscisic acid, low temperature, osmotic stress, salinity, *Zea mays*.

Introduction

Abiotic stresses, such as drought, salinity, and cold, negatively affect vegetative and reproductive growth and the productivity of plants, including maize (Habben *et al.* 2014, Harrison *et al.* 2014, Lobell *et al.* 2014). Therefore, the identification and characterization of key genes that mediate maize responses to abiotic stresses, as well as the study of the molecular mechanisms involved, will provide important information necessary to engineer or select maize having an enhanced tolerance to abiotic stresses (Hoang *et al.* 2014).

In recent years, several proteins that play roles in maize abiotic stress resistance, especially in drought resistance, have been identified. These proteins have been classified into two major categories, transcription factors, such as ZmbZIP72, ZmWRKY33, and ZmSNAC1, and protein kinases, such as ZmMPK17, ZmCPK4, and ZmMKK1 (Lu *et al.* 2012, Pan *et al.* 2012, Ying *et al.* 2012, Jiang *et al.* 2013, Li *et al.* 2013, Cai *et al.* 2014,

Zhang *et al.* 2014, Zhou *et al.* 2016).

Transcription factors (TFs) are major players in regulating plant responses against adverse abiotic factors (Hoang *et al.* 2014). Among them, the large *MYB* family participates in plant responses to various stressors as was verified in model plant species, such as *Arabidopsis thaliana* and rice (Li *et al.* 2015). In addition to the *MYB* proteins in model species, several other *MYB* proteins are involved in drought responses in crops. In wheat, *TaPIM1* is up-regulated during drought stress, and transgenic tobacco expressing this gene displays enhanced tolerance (Zhang *et al.* 2012b). The expression of *TaMYB30* and its homologs is induced in response to polyethylene glycol (PEG) stress, and the over-expression of *TaMYB30-B*, one of three homologs, improves the drought tolerance of transgenic *Arabidopsis* (Zhang *et al.* 2012a). In cotton, *GbMYB5* is positively involved in plant adaptation to drought stress (Chen *et al.* 2015).

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Abbreviations: ABA - abscisic acid; CaMV - cauliflower mosaic virus; DBD - DNA binding domain; ML - maximum likelihood; MS - Murashige and Skoog; NJ - neighbor-joining; ORFs - open reading frames; PEG - polyethylene glycol; qPCR - quantitative PCR; TFs - transcription factors; WT - wild type.

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However, less is known about the function of the MYB family in maize. Using a genomic comparative analysis, 157 *R2R3-MYB* genes were identified in maize (Du *et al.* 2012), but the stress-response-related *MYB* genes have not been determined until now.

The aim of this study was to identify the stress-response-related *MYB* genes and we used the following three approaches: 1) comparative analysis to reveal

potential stress response related *MYB* genes; 2) expression pattern analysis on these potential genes under stress treatment to find candidate genes; and 3) functional analysis on the candidate gene(s). This first comprehensive study of the involvement of the *MYB* gene family in maize stress responses could provide valuable data for the further exploration of the functions of this significant gene family in this plant.

Materials and methods

Plants, growth conditions, and stress treatments: The seeds of maize (*Zea mays* L. cv. B73) were germinated on moistened gauze under dark at 28 °C. The seedlings were then transferred to Hoagland's liquid medium to grow at a temperature of 28 °C, an air humidity of 70 %, a 16-h photoperiod, and an irradiance of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Seedlings at the three-leaf stage were used in subsequent experiments. To follow effects of high salinity, drought, and ABA, the seedlings were treated with 200 mM NaCl, 20 % (m/v) polyethylene glycol (PEG6000), and 100 μM ABA, respectively. The third-leaf tissues were collected at 0, 3, 6, 12, 24, and 48 h. For the cold treatment, three-leaf stage seedlings were transferred from 28 to 6 °C, and the leaf samples were harvested at 0, 3, 6, 12, 24, and 48 h. The stress-treatment experiments were repeated three times.

Identification of putative abiotic stress related *MYB* genes in maize: There are many abiotic stress related *MYB* genes in other species, such as *Arabidopsis*, rice, and wheat. Using the amino acid sequences of these *MYBs* as query sequences (a total of 35 proteins; see Table 1 Suppl.); we performed a search with the *BLASTP* algorithm on the *NCBI* website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). Maize (taxid: 4577) was selected in the organism column. The query results that were greater than 57 % in query coverage column were selected for further analyses. All of the *BLASTP* results were screened using the following procedure: firstly, the redundant *MYB* proteins and proteins with incomplete open reading frame were discarded; and secondly, all of the proteins were subjected to multiple alignment analyses by *ClustalW* (www.ebi.ac.uk/clustalw/) and a phylogenetic tree analysis to exclude the *MYB* proteins that did not fit the tree. To verify the reliability of our results, the functional and structural domains of these candidate maize *MYB* proteins were predicted using *PROSITE* profiling (<http://www.expasy.org/tools/scanprosite/>) and *SMART* analysis (<http://smart.embl-heidelberg.de/>).

Phylogenetic analysis: The amino acid sequences of *Arabidopsis* (21 *R2R3-MYB* members), maize (46 *R2R3-MYB* members), and 14 other *MYB* proteins from other species, including rice and wheat, were

downloaded from *TAIR* and *NCBI* databases. The names and amino acid sequences of the proteins are listed in Table 1 Suppl. The complete amino acid sequences of the *MYB* proteins were used to construct phylogenetic trees. Sequence alignments were performed with *ClustalW* using the web platform (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). A neighbor-joining (NJ) tree and maximum likelihood (ML) tree were constructed individually using the *MEGA6* program (Tamura *et al.* 2013), and internal branch support was estimated with 1 000 bootstrap replicates.

Conservative analysis of potential protein motifs within and outside the maize *R2R3-MYB* DNA-binding domains (DBDs): To analyze the features of the *MYB* domain of the stress-responsive *R2R3-MYB* proteins, the sequences of the R2 and R3 *MYB* repeats in the 57 *R2R3-MYB* proteins (22 from maize, 21 from *Arabidopsis*, and 14 proteins from other species) were aligned with the *ClustalW* method using *BiOEdit* software and adjusted manually. The sequence logos for the R2 and R3 *MYB* repeats were obtained by submitting the multiple alignment sequences to the website <http://weblogo.berkeley.edu/logo.cgi>. To identify potential protein motifs outside the maize *MYB* domain, the full amino acid sequences of 57 proteins were analyzed using the motif discovery software *MEME Suite 4.11.1* (<http://meme-suite.org/tools/meme>), with the following parameter settings: the distribution of motifs, 0 or 1 per sequence; maximum number of motifs to find, 20; minimum width of motif, 6; maximum width of motif, 50; and the motif must be present in all members within the same subgroup. In addition, only motifs with e-value #1e-10 were kept for further analyses. Subsequently, the *MAST* program was used to search for detected motifs in protein databases.

Real-time quantitative PCR (qPCR) analysis: Total RNA was extracted using *TRIzol* reagent from leaves of maize and *Arabidopsis thaliana* L. (cv. Columbia, 30-d-old) and treated with DNase I. For each sample, 3 μg of total RNA was used to synthesize first-strand cDNA with *SuperScript*^{II} reverse transcriptase (*Invitrogen*, Waltham, USA). For real-time qPCR, each reaction contained

10 mm³ of 2× SYBR Green PCR Master Mix (Roche, Basel, Switzerland), 2.0 mm³ of cDNA (10-fold dilution), and 1 mm³ of gene-specific primers (2.0 μ M) in a final volume of 20 mm³. The thermal cycling condition were 95 °C for 2 min; followed by 40 cycles at 95 °C for 20 s, at 57 °C for 30 s, and at 72 °C for 31 s. The maize and *Arabidopsis Actin* genes were used as references, and the reactions were performed using the *ABI Prism 7000* real-time PCR system (Applied Biosystems, Waltham, USA). The reactions were repeated three times, with three biological replicates each. The quantitative analysis used the $2^{-\Delta\Delta Ct}$ method. The gene-specific primers for the 46 maize *MYB* genes and *Arabidopsis* stress-responsive genes can be found in Table 4 Suppl.

Transformation of *ZmMYB30* in *Arabidopsis*: The full-length coding sequence (ATG to TAA) of *ZmMYB30* was amplified from maize leaf cDNA by PCR using primers 5'-GAAGGTACCATGGCGTCGTCTCCGTCTG-3' and 5'-AGGTCTAGACTACTCGATCTTGGCCATACCCATC-3', which contain *KpnI* and *XbaI* enzyme digestion sites. The PCR product was inserted in the pCAMBIA1300 vector with a 35S cassette (35S enhancer + 35S promoter + multiple cloning site + terminator) by *KpnI* and *XbaI* enzyme digestion. The construct was confirmed by sequencing and then transformed into

Agrobacterium strain GV3101. The *Arabidopsis* *ZmMYB30* ectopic expression transgenic lines (OE) were generated using an *Agrobacterium*-mediated transformation protocol and screened using 20 mg dm⁻³ hygromycin (Roche). The empty vector was also transformed into *Arabidopsis* (Columbia ecotype) to produce control transgenic (CK) plants.

Salt treatment of transgenic *Arabidopsis* plants: The surface-sterilized seeds from three OE lines (OE-1, OE-2, OE-3), CK, and wild-type plants were sown on Murashige and Skoog (MS) medium-containing plates with 1 % (m/v) sucrose and 0.8 % (m/v) agar, and MS plates independently supplemented with 50, 100, and 150 mM NaCl. The plates were placed at 4 °C for 2 d and then moved to a vertical position under a 16-h photoperiod, irradiance of 120 μ mol m⁻² s⁻¹, and a temperature of 22 °C. Three days after growth in the control and salt-treated media, the percentages of radicle emergence were carefully counted to describe the seed germination rates. The root lengths of the seedlings were measured in the germinated lines after being grown vertically on normal media for 7 d and then transferred to the salt media. The root lengths were recorded 3 d after being transferred. The salt-treatment experiment was repeated three times.

Results

In general, orthologous proteins from different species share similar structures and functions. To uncover the putative abiotic stress-responsive MYB TF(s) in maize, we used full length sequences of well-known abiotic stress-response-related MYB proteins from *Arabidopsis*, wheat, and rice as queries (Table 1 Suppl.), and a *BLASTP* algorithm-based search was performed to reveal the maize MYB proteins having high similarities. After discarding the redundant protein and the incomplete open reading frame-containing proteins, 46 maize R2R3-MYBs were identified. Using the neighbor-joining method in *MEGA6* and the ML method in *PhyML*, we performed a phylogenetic reconstruction containing the *Arabidopsis* defence-related R2R3-MYBs (21 members), 14 well-characterized R2R3-MYBs from other plant species and their 46 orthologs in maize. The topology of phylogenetic trees was illustrated in Fig. 1 Suppl.

The phylogenetic comparative analysis of these abiotic stress-related R2R3-MYBs (putative in maize) in different plant species revealed considerable diversification and conservation. In the phylogenetic tree, there are nine clades and most of the clades contained members of orthologous genes belonging to maize, *Arabidopsis*, and/or other plant species, such as Clades 6, 7, and 9. However, Clades 3 and 8 are maize specific, having only maize genes.

The expression profiles of the 46 maize *MYB* genes under different abiotic stresses (200 mM NaCl, 20 % PEG6000, 6 °C, and 100 μ M ABA) were screened to find stress-induced genes using the real-time PCR. As a whole, 22 genes were detected that responded to at least one treatment, including 14 genes that responded to exogenous ABA (Fig. 1), 6 genes that responded to low temperature (Fig. 2), 14 genes that responded to NaCl (Fig. 3), and 13 genes that responded to PEG (Fig. 4). Among these genes, 16 responded to more than one treatment (Table 3 Suppl.). For example, *ZmMYB30* was induced by four kinds of treatments, while *ZmMYB53* was only induced by cold stress. In the ABA-, PEG- and salt-stress treatments, the expressions of most genes peaked 12 or 24 h after treatment and then decreased; while in the cold treatment, the expression patterns of *ZmMYB30*, *ZmMYB53*, *ZmMYB95* and *ZmMYB139* were different, with the peak values being delayed until 48 h.

After the expression pattern analysis, the amino acid sequences of 22 stress-induced *ZmMYB* proteins and 35 MYB proteins involved in stress response from other species were entered in the query box to determine any conserved motifs using *MEME*. In the DBD, similar to counter-parts in other plant species, the basic regions of these 22 maize R2R3-MYB domains contained on average 108 basic residues with a rare frequency of

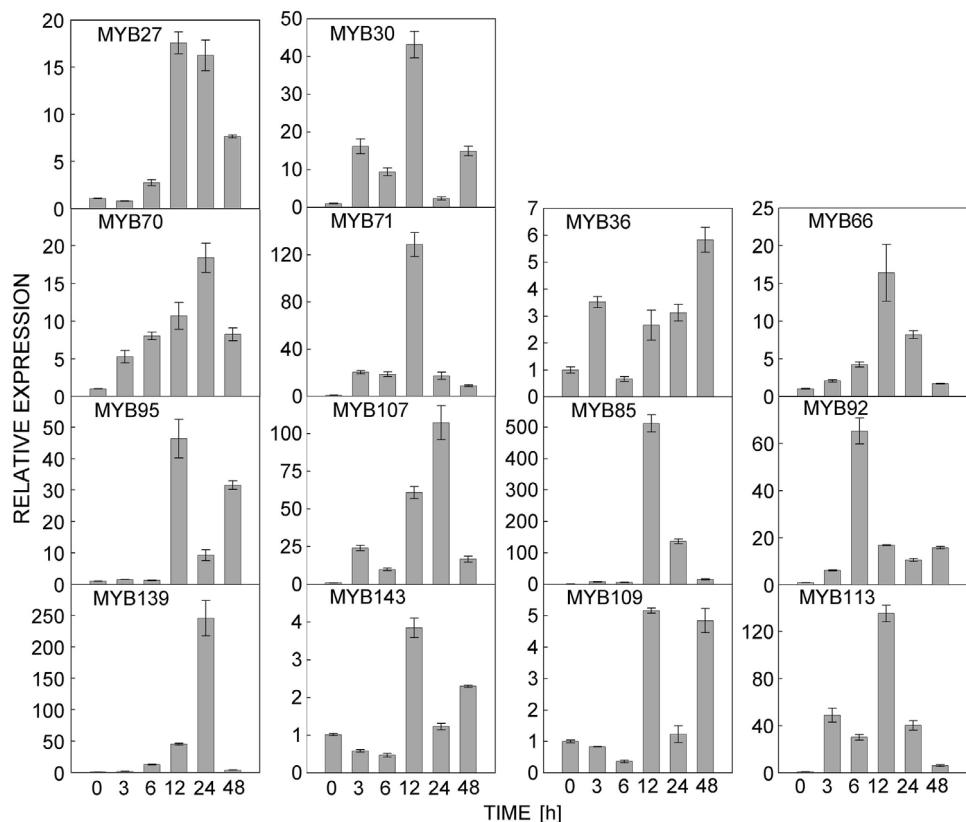


Fig. 1. Expression patterns of 14 ABA-responsive *ZmMYB* genes under 100 μ M ABA treatment. The ABA-responsive *ZmMYB* genes were identified by real-time qPCR and *Actin* gene was used as reference. Maize seedlings without treatment was referred as control (0 h) and set to 1. Means \pm SD, $n = 3$.

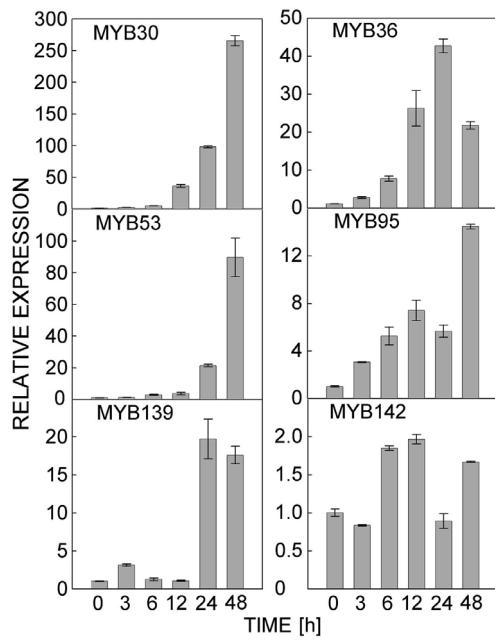


Fig. 2. Expression patterns of 6 cold-responsive *ZmMYB* genes under low temperature (6 °C) treatment. For other detail see Fig. 1.

deletion or insertion ($> 2\%$). On the contrary, the region outside the DBD was the most divergent, both in protein length and amino acid composition. The R2 and R3 MYB repeats had similar structures (Fig. 6), including a series of evenly distributed and highly conserved tryptophan (Trp/W) residues (Fig. 2 Suppl.). The five Trp residues, including three in the R2 repeat and two in the R3 repeat at particular positions, are highly conserved in plant MYB DBDs. In addition to the conserved Trp sites, many other residues were also highly conserved.

In the C-termini of these MYB proteins, other conserved motifs with variable lengths (6 - 50 amino acids) were detected. Seven motifs were identified, and three motifs had more than six MYB protein members (Fig. 2 Suppl.). Motifs 2 and 4 had 10 members, and motif 6 had 6 members. The proteins sharing a motif are always classified into the same subgroup. For instance, 10 MYB proteins (AtMYB70, AtMYB44, ZmMYB085, TaMYB1, ZmMYB142, AtMYB77, AtMYB73, ZmMYB095, ZmMYB030, and ZmMYB109) shared the C-terminal Motif 2 (Fig. 2 Suppl.) from the same clade 2 of the phylogenetic tree (Fig. 1 Suppl.). These subgroup-specific motifs may provide additional functional

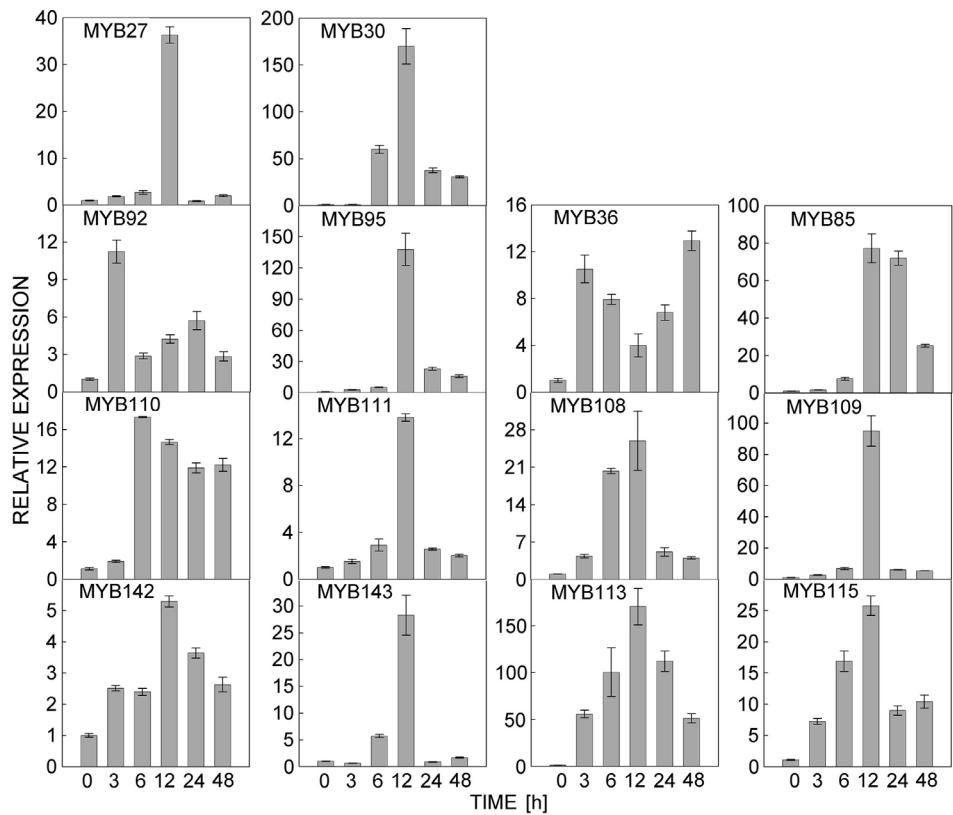


Fig. 3. Expression patterns of 14 salt-responsive *ZmMYB* genes under salt treatment (200 mM NaCl). For other detail see Fig. 1.

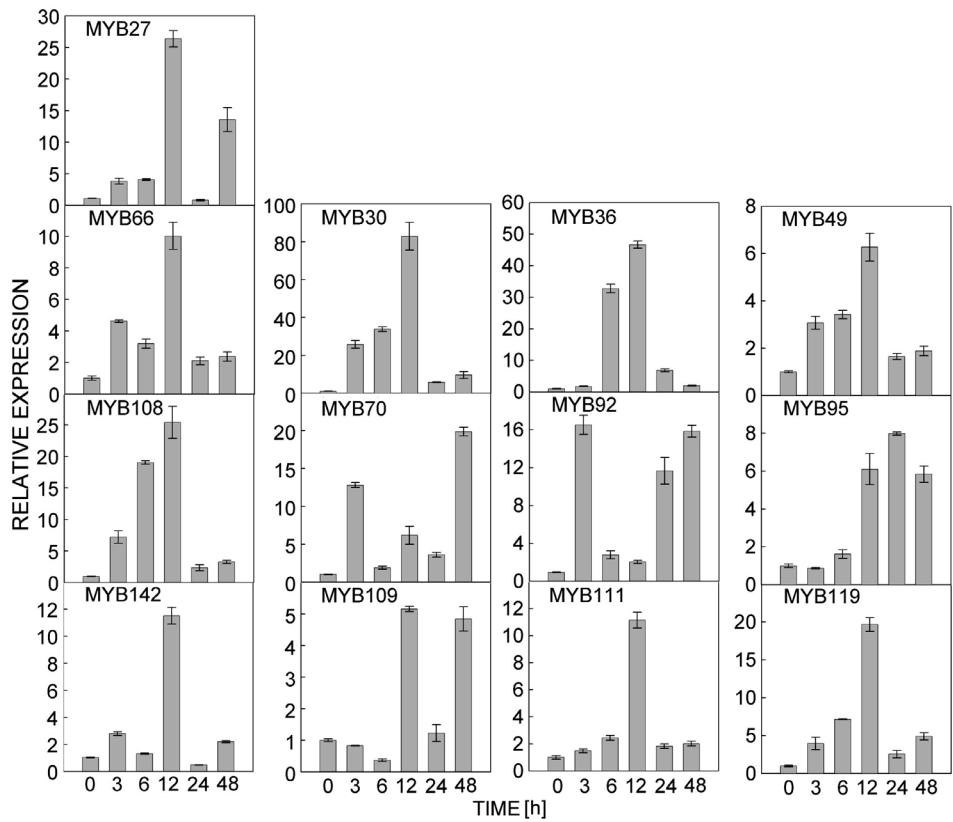


Fig. 4. Expression patterns of 13 drought-responsive *ZmMYB* genes under osmotic stress (20 % PEG). For other detail see Fig. 1.

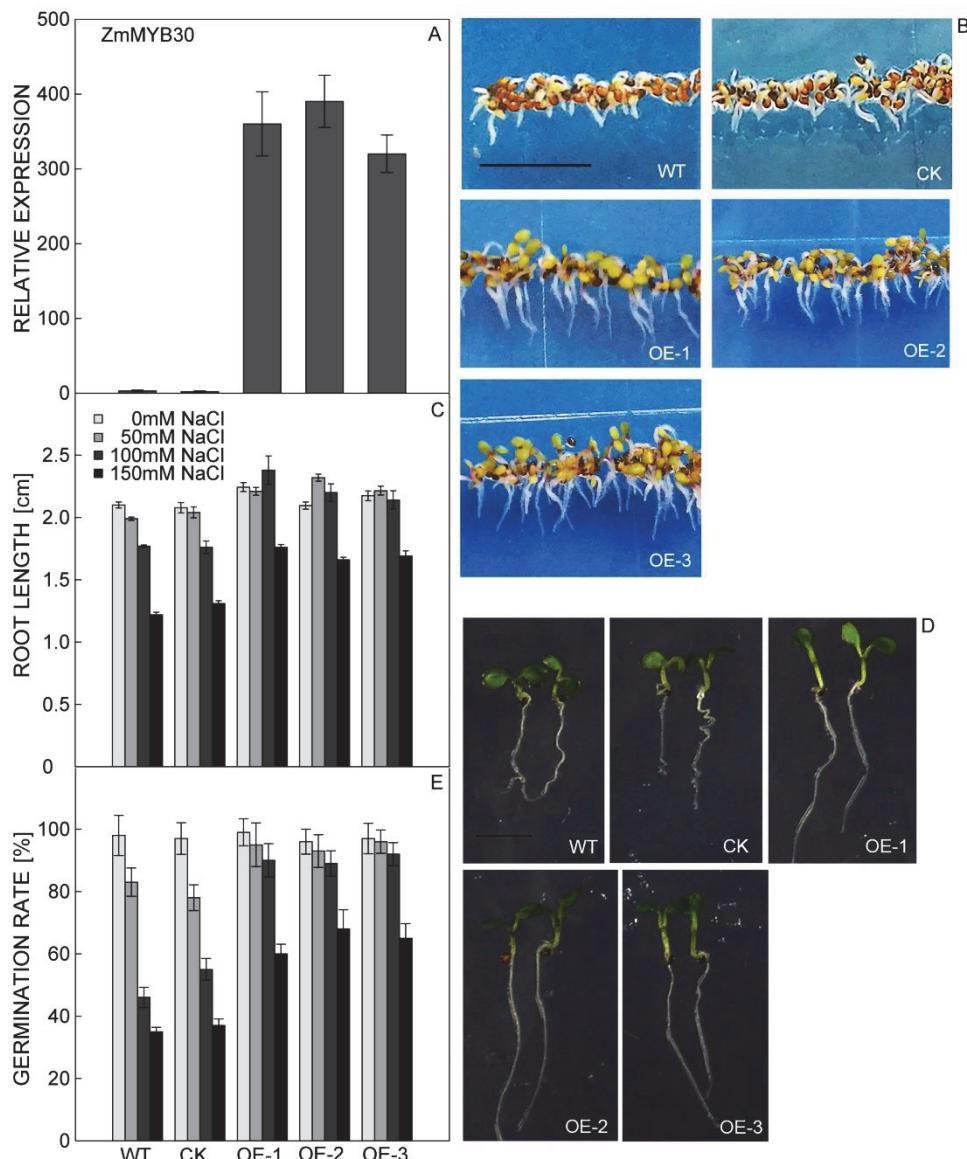


Fig. 5. *A* - The expression of *ZmMYB30* in three ectopic transgenic *Arabidopsis* lines (OE1, OE2, and OE3), control (CK), and wild type (WT) plants determined by real-time qPCR. *B* to *E* - Germination and growth of WT, CK, OE1, OE2, and OE3 on MS media with NaCl (*B* - after growth on 100 mM NaCl for 4 d, bar = 1 cm; *C*,*E* - root length and germination rate on media with different NaCl concentrations; *D* - seedlings after growth on MS medium for 7 d and then transferred to MS medium containing 100 mM NaCl for another 3 d, bar = 0.5 cm). Means \pm SDs, $n = 3$.

properties and enabled us to determine the regulatory specificity of the proteins.

To determine the functions of maize *MYB* genes in plant abiotic stress responses, we selected the *ZmMYB30* gene for further experimental analysis because it was induced under all four treatments and its expression was higher than that of other genes. *ZmMYB30* was ectopically expressed in *Arabidopsis* under the control of the cauliflower mosaic virus 35S promoter. Using a real-time PCR analysis, we found that the expression of *ZmMYB30* in the three OE lines was much higher than in the control plant (Fig. 5A). For the salt-tolerance analysis of OE, three transgenic lines of T2 progeny (OE-1, OE-2, and

OE-3), CK, and wild type (WT) plants were grown under normal conditions and high salinity (50, 100, and 150 mM NaCl). Under normal conditions, no obvious differences in the phenotypes among WT, CK, and OE seedlings were observed. All of the plants germinated simultaneously and there were no differences among the root lengths. When plants were grown on MS plates containing 50, 100 and 150 mM NaCl, the three transgenic OE seeds germinated earlier than both WT and CK seeds (Fig. 5B,C). The root lengths of all three OE transgenic seedlings were longer than those of the CK and WT plants (Fig. 5D,E). These results indicate that the ectopic expression of *ZmMYB30* enhanced the salt

tolerance in *Arabidopsis*. To investigate the molecular mechanism underlying the responses of OE plants to salt stress, the expression of 14 known stress-responsive genes in OE plants grown under normal conditions were examined by real-time qPCR. Compared with the CK plants, eight genes, *DREB2A*, *RD20*, *ABI5*, *RD29A*, *ATGolS2*, *ABF3*, *MYB2*, and *RD29B*, exhibited significantly higher expression in the three OE plants (Fig. 6). However, the expression of six other genes, *RbohD*, *RD22*, *RAB18*, *P5CS1*, *LEA14*, and *RbohF*, were

unchanged or slightly increased in the transgenic plants (Fig. 6A). Numerous genes, such as *AtNXH1*, *AtNXH2*, *AtHTK1*, *AtCHX16*, *AtMPK4*, *AtMPK6*, *AtSOS1*, *AtSOS2*, and *AtSOS3* play roles in coping with and surviving under salt-stress (Park *et al.* 2016). We also detected the expression of these salinity responsive genes in OE plants by real-time qPCR and all nine genes were upregulated, especially *AtSOS1* and *AtSOS3* (the expression of them was more than 2 fold higher than in control plants; Fig. 6B).

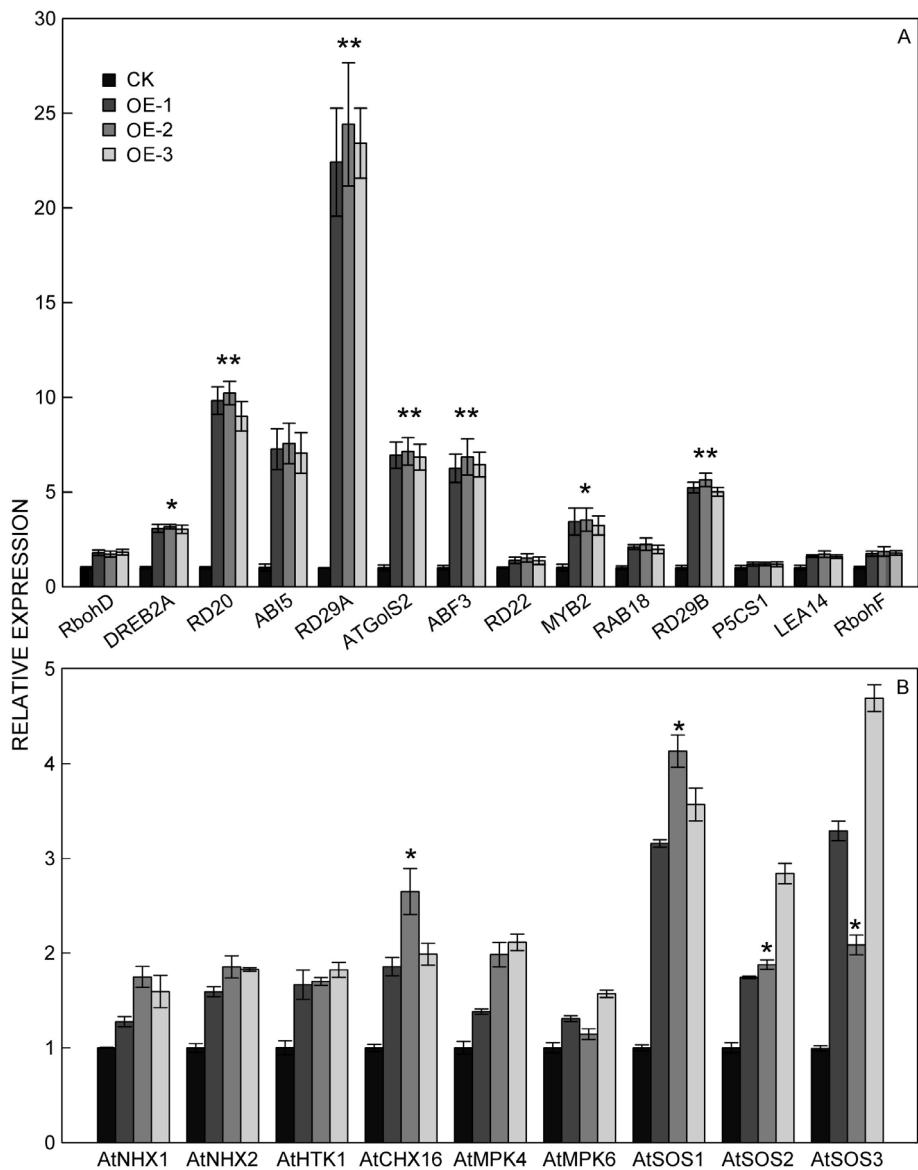


Fig. 6. Expression of stress-responsive genes in control (CK) and ectopic transgenic lines (OE1, OE2, and OE3) of *Arabidopsis*. RNA was extracted from leaf samples after 30-d growth on 100 mM NaCl and reverse transcribed to synthesize cDNA. The *Actin* gene was used as an internal control. A - Expression pattern of fourteen genes induced by both NaCl and PEG. B - Expression pattern of nine known salt stress responsive genes. Means \pm SDs, $n = 3$, * and ** - significant differences between CK and transgenic lines $0.01 < P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test).

Discussion

The important roles of MYB TFs in stress tolerance have been demonstrated in model plants and many crops. In maize, previous studies identified 157 *R2R3-MYB* TFs. A comprehensive computational analysis was performed to yield an overview of the *R2R3-MYB* gene family in maize, including the phylogeny, expression patterns, and structural and functional characteristics (Du *et al.* 2012), but the expression patterns under different stress treatments were not explored. Here, we focused on the *MYB* genes having putative abiotic stress-responsive functions. Using a comparative analysis, we identified 46 *MYB* genes that are orthologs of stress-resistance *MYB* genes from other species, including *Arabidopsis*, rice, and wheat. In the phylogenetic tree, the *ZmMYB* genes that share the same clade may have similar functions to the homologous proteins of other species.

The expression patterns of all 46 maize *MYB* genes under different abiotic stress conditions were studied. In total, 22 genes that responded to different stress treatments were discovered, of which 16 responded to at least two treatments, indicating that they were major factors involved in the cross-talk among different signal transduction pathways in responses to abiotic stresses. Different stress treatments stimulated the expression of the same genes. Four treatments shared three genes, *ZmMYB30*, *ZmMYB36*, and *ZmMYB95*. The PEG treatment, which mimics drought stress conditions, shared nine responsive genes with the NaCl treatment, and eight genes with the ABA treatment. The NaCl treatment shared nine responsive genes with the ABA treatment. Of the six genes responding to cold, only *ZmMYB53* was unique. The other five genes also responded to other treatments (Table 3 Suppl.). The ABA is an important factor that participates in regulatory networks involved in abiotic stress responses. The signal-transduction pathways are divided into ABA dependent and ABA independent (Shinozaki and Yamaguchi-Shinozaki, 2007). The 14 ABA responsive genes, except *ZmMYB71*, responded to other stress treatments. Thus, we could preliminary classify the genes that responded to drought, salt, and cold stresses into two types, playing roles in the ABA-dependent or ABA-independent signal-transduction pathways. For example, in the drought

treatment, *ZmMYB49*, *ZmMYB111*, *ZmMYB119*, and *ZmMYB142* may participate in the ABA-independent signalling pathway, while the other nine genes may participate in the ABA-dependent signalling pathway. However, these predictions need further experimental verification.

In this study, 22 maize MYB proteins were demonstrated to be stress-induced proteins. However, the functions of almost all of these proteins remain unknown. *ZmMYB30*, whose expression was strongly induced by all treatments, was selected for a further functional analysis through an ectopic expression in *Arabidopsis thaliana*. Because the expression *ZmMYB30* could be highly induced by salt treatment, we determined the salt tolerance potential of OE lines. Really, the OE seedlings exhibited an enhanced salt tolerance during germination. This finding suggested that the *ZmMYB30* gene is involved in responses to salinity stress, which is in agreement with its expression pattern.

Some parts of the transcriptional regulatory networks in abiotic stress responses have been identified in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 2007). The investigation of the expression profiles of major stress-responsive genes and salt-responsive genes in these networks could provide important information for uncovering the mechanisms of *ZmMYB30* in salt-stress response and tolerance (Park *et al.* 2016). Our results showed that the expression of eight stress-responsive genes and nine salt-responsive genes were higher in the OE plants. The enhanced salt-stress tolerance of the OE plants is at least partially due to the increased expression of these genes.

In the phylogenetic tree, the *ZmMYB30* gene was clustered in the same clade with *Arabidopsis* genes *AtMYB44* and *AtMYB77*. It was reported that these two genes have multiple roles in stress response through ABA signalling pathway (Jaradat *et al.* 2013, Zhao *et al.* 2014). According to the expression pattern of *ZmMYB30* under stress and preliminary results of functional analysis, *ZmMYB30* may be a key player in maize stress response. The potential roles of *ZmMYB30* will be further investigated in our ongoing study.

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