PPB

Research article

Expression of the maize MYB transcription factor ZmMYB3R enhances drought and salt stress tolerance in transgenic plants

Jiandong Wu[1], Yingli Jiang[1], Yani Liang, Long Chen, Weijun Chen, Beijiu Cheng[*]

National Engineering Laboratory of Crop Stress Resistance, College of Life Science, Anhui Agricultural University, Hefei, Anhui, 230036, China

ABSTRACT

MYB proteins are major transcription factors that play significant roles in plant defenses against various stresses. However, available information regarding stress-related *MYB* genes in maize is minimal. Herein, a maize *MYB* gene, *ZmMYB3R*, was cloned and functionally characterized. Subcellular localisation analysis showed that ZmMYB3R is localised to the nucleus. Yeast one-hybrid results revealed that ZmMYB3R has trans-activation activity, and a minimal activation domain at the C-terminus spanning residues 217–563. Gene expression analysis suggested that *ZmMYB3R* was induced by drought, salt and abscisic acid (ABA). Transgenic *Arabidopsis* plants overexpressing *ZmMYB3R* displayed enhanced growth performance and higher survival rates, elevated catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) enzyme activities, increased sensitivity to ABA, and regulation of the stomatal aperture, suggesting that *ZmMYB3R* enhances tolerance to drought and salt stress. qRT-PCR assays revealed elevated expression levels of stress/ABA genes in transgenic plants following stress treatments. Moreover, transgenic plants accumulated higher ABA content than wild-type plants under drought and salt stress conditions. Collectively, these results indicate that ZmMYB3R is a positive transcription factor that enhances tolerance to drought and salt stress via an ABA-dependent pathway. The findings may prove useful for engineering economically important crops.

1. Introduction

Plants live in constantly changing environments that often impose constrains on growth and development. In particular, drought and high salinity are serious environmental stresses that can cause stomatal closure, generate reactive oxygen species (ROS), and decrease photosynthetic activity, subsequently disturbing plant growth and development, and even resulting in the death of crops (Zhu, 2002, 2016).

Plants have evolved various pathways and regulatory mechanisms to adapt to the negative impacts of abiotic stress. Both abscisic acid (ABA)-dependent and ABA-independent pathways are involved in the perception and signaling of drought and salt stress (Yoshida et al., 2014). For example, *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) serves as an important ABA synthesis regulatory protein that can be induced by drought and salt treatment in *Arabidopsis* (Xiong and Zhu, 2003). Among these regulatory mechanisms, regulatory proteins play an important role in enhancing abiotic stress tolerance of plants by activating expression of defense-associated genes (Shinozaki et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). Typical examples of such regulatory proteins are transcription factors (TFs) that control the

expression of target genes by binding their promoters.

With the advancement of transgenic methods and genome sequencing, a growing body of research has demonstrated that manipulating TFs such as bZIP, WRKY, MYB, HSF, and NAC family members can enhance abiotic stress tolerance by activating stress response signal transduction pathways in transgenic plants (Chen et al., 2015; Wu et al., 2016; Cai et al., 2015; Cao et al., 2017; Jiang et al., 2018). The MYB transcription factor family is widely distributed in both monocotyledons and dicotyledons. Members of this family are characterized by the highly conserved MYB domain of 52 amino acid residues at the N-terminus (Du et al., 2009). Based on the number of adjacent repeats in their MYB domains, MYB TFs can be classified into three main subgroups; R1R2R3-MYB, R2R3-MYB, and R1-MYB. Recently, a few MYB4R subfamily members, which contain four repeats, have also been identified in plant, but their exact functions remain obscure (Dubos et al., 2010).

MYB TFs were first identified in *Zea mays*, and many have since been discovered in various plant species (Dubos et al., 2010). To date, 198, 155, 244 and 200 MYB members have been identified in *Arabidopsis*, rice, soybean and maize, respectively (Du et al., 2012a, 2012b;
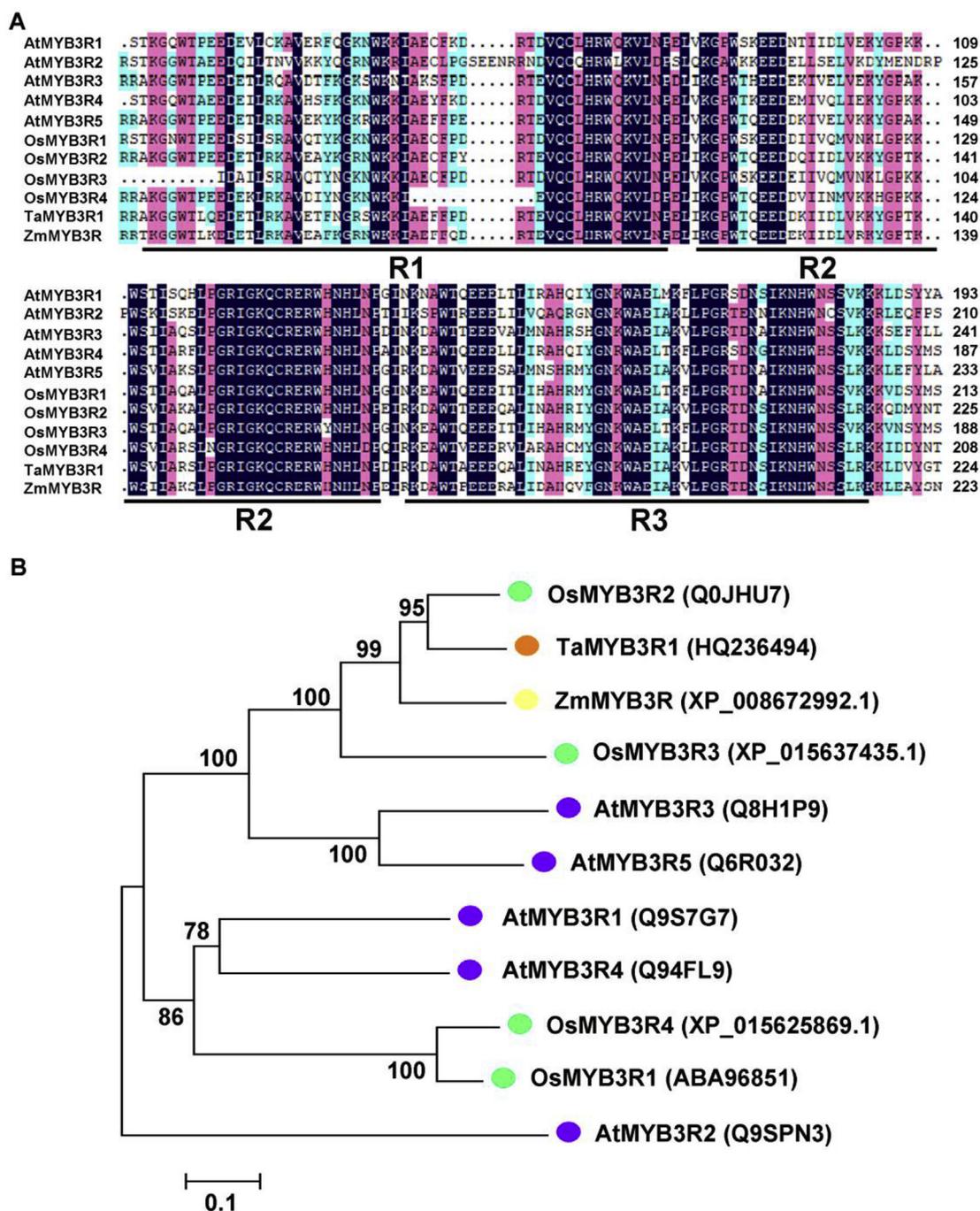
Fig. 1. Sequence analysis and phylogenetic relationships of 3R-MYB genes. A. Sequence alignment of conserved motifs of 3R-MYB genes. The conserved repeat residues (R1, R2 and R3) are indicated by straight lines. **B.** Phylogenetic tree constructed using MEGA4.0 by the N-J method. Bootstrap values (above 50%) from 1,000 replicates are indicated at each node.

Katiyar et al., 2012; He et al., 2016). Among these, R2R3-MYB constitute the largest subgroup, and diverse critical functions of R2R3-MYB genes have been reported, mostly related to regulating plant-specific biological processes including determination of cell shape, responses to biotic stresses, regulation of primary and secondary metabolism, and developmental processes (Salomoni et al., 1997; Meissner et al., 1999; Dubos et al., 2010). Additionally, R2R3-MYB genes are also involved in increasing tolerance to abiotic stress. For example, overexpression of *Triticum aestivum MYB33* (*TaMYB33*) increases salt and drought tolerance (Qin et al., 2012), while cotton MYB transcription factor (*GbMYB5*) is positively involved in plant adaptive response to drought stress (Chen et al., 2015). By contrast, the 3R-MYB group constitutes a

smaller subfamily in plants, and to date, only five, four, and one 3R-MYB members has been reported in *Arabidopsis*, rice and wheat, respectively (Ma et al., 2009; Cai et al., 2015). These proteins share conserved functions with animal MYB proteins, which play a key role in controlling cell cycle processes (Ito et al., 2001). Interestingly, 3R-MYB members are also reported to play an important role in enhancing tolerance to multiple abiotic stress in wheat and rice. Overexpression of *OsMYB3R-2* enhances tolerance to numerous abiotic stresses in *Arabidopsis* (Dai et al., 2007; Ma et al., 2009), and heterologous expression of *TaMYB3R1* increases drought and salt tolerance in transgenic *Arabidopsis* plants (Cai et al., 2015).

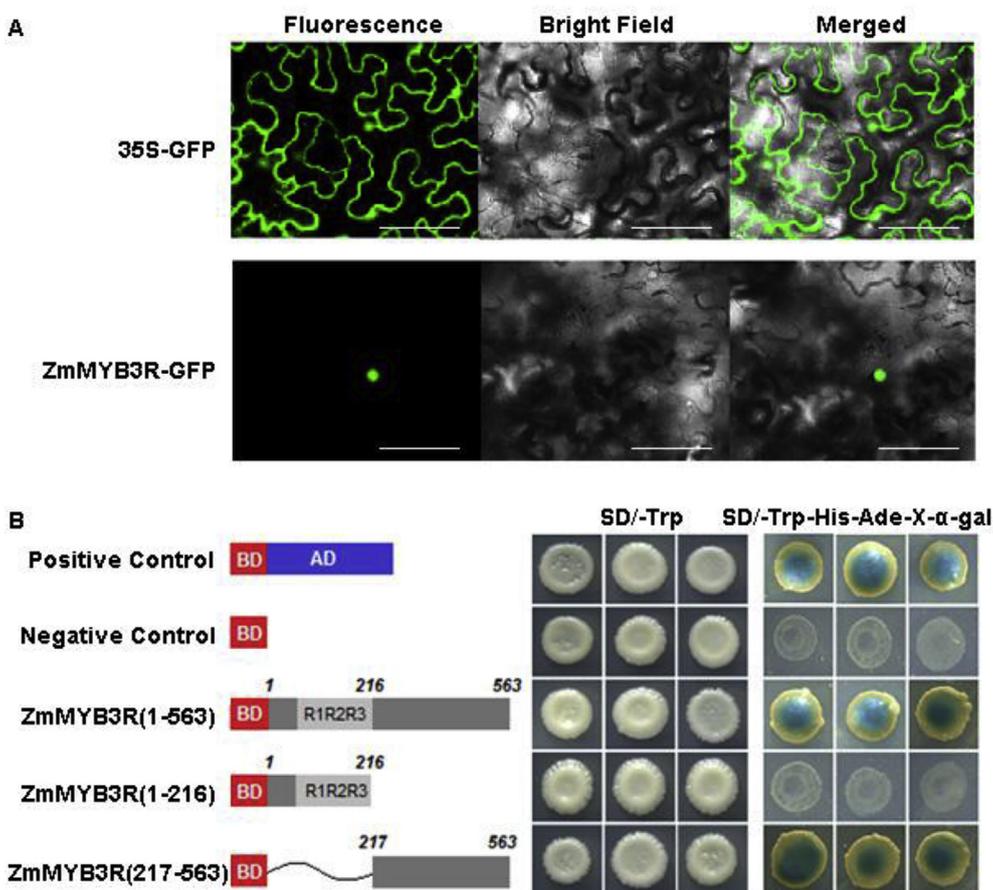Maize (*Zea mays* L.) is a major global food crop, but maize

Fig. 2. Subcellular localisation and transactivation analysis of ZmMYB3R. A. Subcellular localisation of the ZmMYB3R-GFP fusion protein in *Nicotiana benthamiana* leaf epidermal cells. Scale bar = 50 μm. **B.** Transactivation activity analysis of full-length ZmMYB3R and different fragments in yeast. Negative control = pGBKT7; positive control = pGBKT7-53 + pGADT7-T. Scale bar = 1 cm).
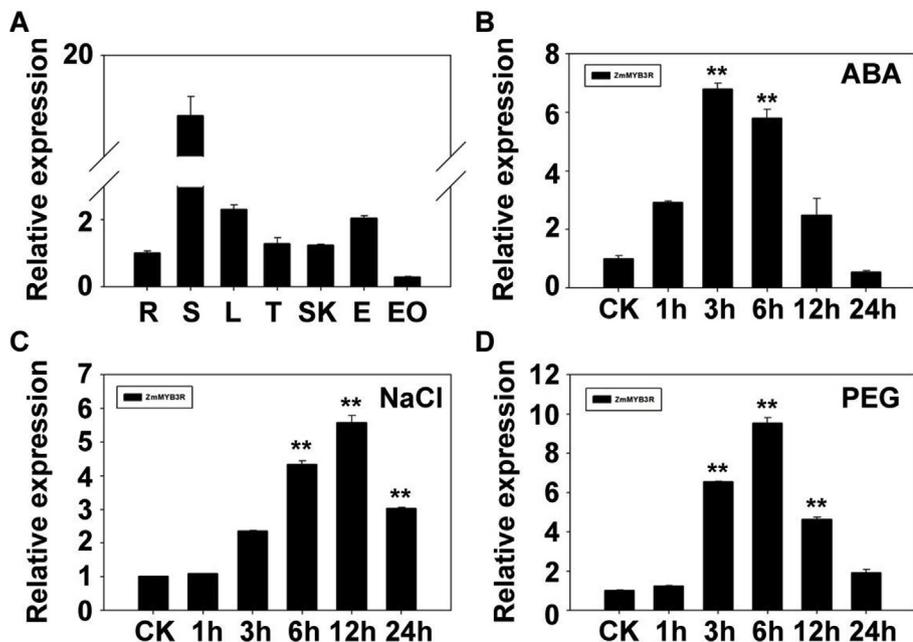

Fig. 3. Expression profiles of *ZmMYB3R* genes. A. Tissue-specific expression patterns of the *ZmMYB3R* gene. R, roots; S, stems; L, leaves; T, tassels; SK, sick; E, ears; EO, embryos. **B – D.** Expression analysis of *ZmMYB3R* in response to different stress treatments (B, 100 μM ABA; C, 250 mM NaCl; D, 10% PEG6000). **Indicates statistical significance compared to the control group ($p < 0.01$).

production is seriously constrained by climatic conditions, especially drought stress. The significant contributions of MYB TFs in enhancing tolerance to abiotic stress has inspired the cloning of MYB genes from maize for potential applications in molecular breeding, and 200 MYB genes have been identified in the maize genome (Du et al., 2012a). However, little is known about the role of maize MYB genes in the responses to abiotic stress, especially 3R-MYB genes. Therefore, it is

necessary to determine their functions for both academic and applied research aimed at enhancing abiotic stress tolerance.

In the present study, the gene encoding the novel 3R-MYB TF *ZmMYB3R* was cloned from the *Zea mays* inbred line B73. Expression pattern analysis showed that *ZmMYB3R* can be strongly induced by drought, salt stress, and ABA treatment, implying that *ZmMY3R* may have an important role in plant stress responses. To illuminate the
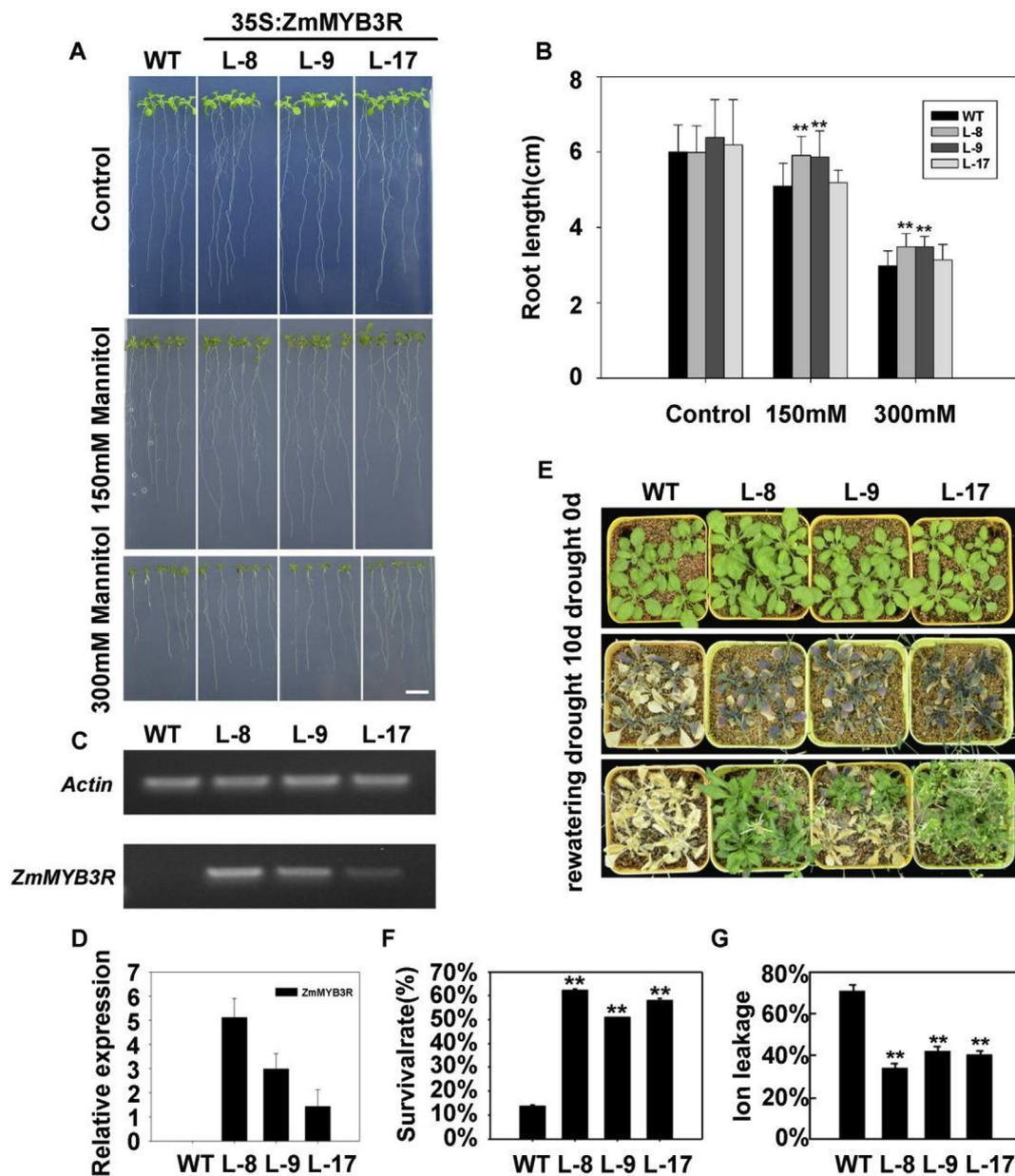
Fig. 4. *ZmMYB3R* **transgenic lines display enhanced drought stress tolerance**. **A.** Seeds of wild-type (WT) and transgenic lines were germinated and transferred to new MS agar plates supplemented with different concentrations of mannitol. **B.** Root elongation in WT and *ZmMYB3R* transgenic lines exposed to 0, 150 mM and 300 mM mannitol treatment. **C and D.** Expression pattern of *ZmMYB3R* in transgenic *Arabidopsis* seedlings assessed by RT-PCR and qRT-PCR. **E.** Phenotype of WT and transgenic plants before and after drought treatment for 10 days followed by 5 days of recovery (rewatering). **F.** Survival rate of WT and *ZmMYB3R* transgenic lines after rewatering. **G.** Ion leakage in WT and ZmMYB3R transgenic lines after drought stress treatment. Results are presented as mean values of three replicates ± SD (n > 50 each). Scale bar = 1 cm **Indicates statistical significance compared to the control group ($p < 0.01$).

biological functions, we investigated ectopic expression of *ZmMYB3R* in transgenic plants and characterized its role in mediating abiotic stress tolerance. Ectopic expression of *ZmMYB3R* in transgenic plants significantly enhanced tolerance to both drought and salt stresses. Therefore, our results suggest that *ZmMYB3R* plays a positive role in plant stress responses, and serves as a candidate gene for the improvement of crops against drought and salt tolerance in future breeding programs.

2. Materials and methods

2.1. Plant materials and stress treatments

Growth conditions for maize, *Nicotiana benthamiana* and *Arabidopsis*

thaliana were as described previously (Wu et al., 2016; Jiang et al., 2018). In brief, maize (inbred line B73) seedlings were grown in the greenhouse (14 h light/10 h dark, temperature = 30 °C) for tissue collection and stress treatments. For analysis of gene expression, roots, stems, leaves, tassels, sick, ears, and embryos from a single maize growth cycle were collected for RNA extraction. For salt stress treatment, seedlings were treated with 250 mM NaCl solution. For PEG stress treatment, seedlings were treated with 10% polyethylene glycol (PEG) 6000 solution. ABA was sprayed at a concentration of 100 μM for ABA treatments.

Wild-type (WT) *N. benthamiana*, *A. thaliana* (ecotype Columbia, Col-0) and transgenic seedlings were grown in a growth chamber under a 12 h light/12 h dark photoperiod with a light intensity of 150 μmol s^{-1} m^{-2} (PAR) at plant height, a relative humidity of 35% by
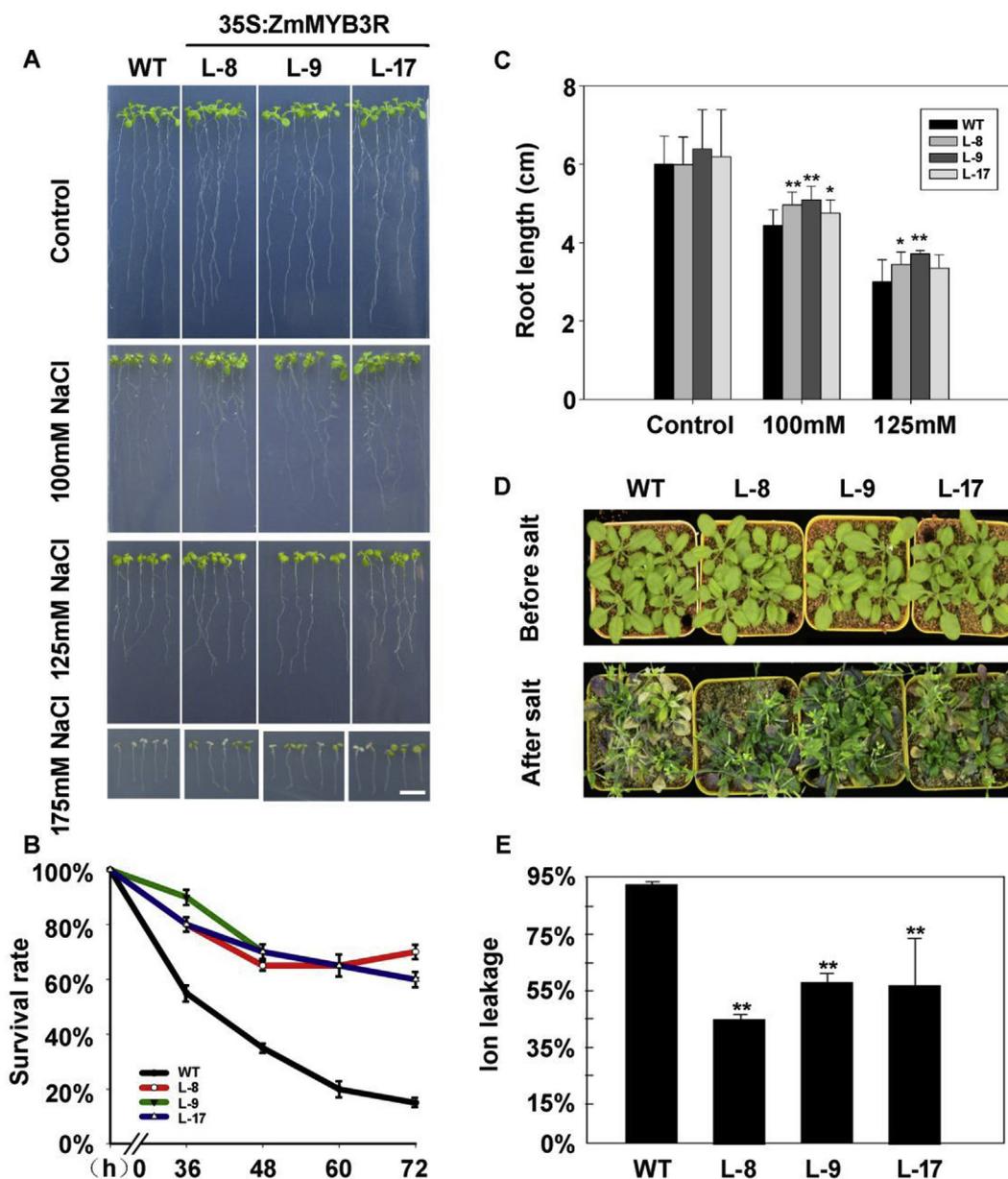
Fig. 5. *ZmMYB3R* **transgenic lines exhibit increased tolerance to salt stress. A.** Seeds of WT and transgenic lines were germinated and transferred to new MS agar plates supplemented with different concentrations of NaCl. **B.** Survival rates of WT and transgenic plants under 175 mM NaCl treatment. **C.** Root elongation in WT and ZmMYB3R transgenic lines exposed to 0, 100 mM or 125 mM NaCl treatment. **D.** Phenotype of WT and transgenic plants following salt stress. **E.** Ion leakage in WT and *ZmMYB3R* transgenic lines after salt stress. Results are presented as mean values of three replicates $\pm$ SD (n > 50 each). Scale bar = 1 cm **Indicates statistical significance compared to the control group ($p < 0.01$).

day and 60% by night, and a temperature of 20–23 °C.

2.2. Multiple sequence alignment and phylogenetic analysis

Amino acid sequences of different 3R-MYB proteins were analyzed by ClustalX software (Thompson et al., 1997), and conserved motifs of 3R-MYB proteins were defined by Pfam (PF00642; http://pfam.sanger.ac.uk/) and SMART (Sm00356; http://smart.embl-heidelberg.de/) (Peng et al., 2012).

The neighbor joining (NJ) method was applied to construct phylogenetic trees in MEGA (version 4.0) (Tamura et al., 2007). Bootstrap analysis was performed with 1000 replicates, and all other parameters were default values.

2.3. Subcellular localisation and transcriptional activation activity analysis of ZmMYB3R

The coding region of ZmMY3R was amplified using PCR primers listed in Table S1. Plasmids pCAMBIA1305-GFP (negative control) and pCAMBIA1305-ZmMYB3R-GFP for green fluorescent protein (GFP) expression were constructed and separately introduced into *Agrobacterium tumefaciens* strain EHA105. Leaves of 30-day-old WT tobacco (*N. benthamiana*) plants were injected with *A. tumefaciens* cells harboring either pCAMBIA1305 or pCAMBIA1305-ZmMYB3R-GFP. Leaves were then harvested and observed under a Zeiss Microsystems LSM 710 laser scanning confocal microscope at 48–72 h after injection/treatment.

The MATCHMAKER GAL4 yeast one-hybrid system was employed according to the manufacturer's instructors (Clontech, Dalian, China). The full-length open reading frame (ORF) of ZmMYB3R and two truncated fragments were separately cloned into the pGBKT7 vector. Empty
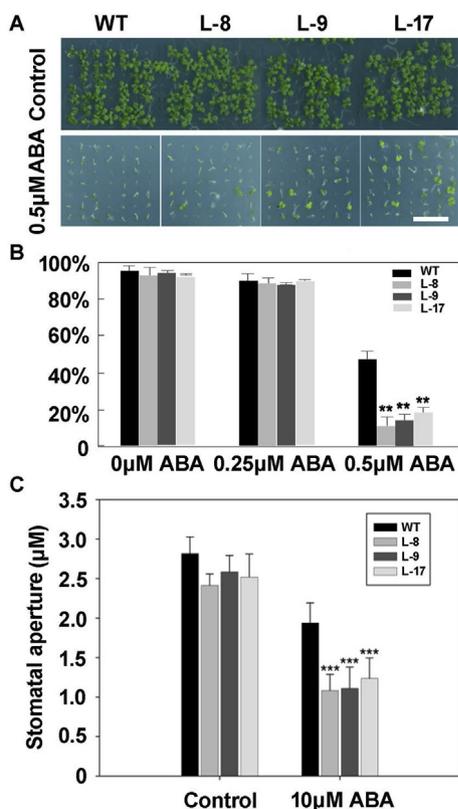
Fig. 6. *ZmMYB3R* **enhances stomatal closure and increases ABA sensitivity. A.** Germination phenotype of WT and *ZmMYB3R* transgenic *Arabidopsis* seeds on MS medium containing 0.5 μM ABA (n = 50). Scale bar = 1 cm. **B.** Germination rate of WT and *ZmMYB3R* transgenic *Arabidopsis* seeds (n = 50). **C.** Comparison of stomatal aperture in WT and transgenic plants with or without application of 10 μM ABA. Results are presented as mean values of three replicates ± SD (n > 50 each). ***Indicates statistical significance compared to the control group (p < 0.001).

pGBKT7 vector was used as a negative control, and the interaction between pGBKT7-53 and pGADT7-T served as a positive control. These constructs were transformed into the yeast strain AH109 and subjected to selection on SD/Trp$^-$/His$^-$/Ade$^-$/X-α-gal medium plates at 30 °C for 3–5 days.

2.4. Transformation of ZmMYB3R into Arabidopsis

The full-length coding sequence of *ZmMYB3R* was amplified by PCR and inserted into the pCAMBIA1301 vector. The resulting p1301a-ZmMYB3R construct was introduced into *Agrobacterium* strain GV3101 for *Arabidopsis* transformation using the floral dip method (Clough, 2005). Seeds were collected and sown on Murashige and Skoog (MS) agar medium containing 25 μg mL^{-1} hygromycin for selection. Twenty transgenic plants overexpressing *ZmMYB3R* were produced and further confirmed by β-glucuronidase (GUS) staining of seedlings and RT-PCR analysis. Three T2 generation homozygous lines (Lines 8, 9 and 17) were used for further functional analysis.

2.5. RNA isolation and quantitative real-time PCR

Total RNA samples were extracted from *Arabidopsis* and different tissues of maize using RNAiso plus (TaKaRa, Dalian, China). Quantitative RT-PCR (qRT-PCR) was conducted using an ABI PRISM 7500 sequence detection system with a preincubation at 95 °C for 5 min, followed by 41 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 60 s, and extension at 60 °C for 10 min. Relative differences in expression were calculated by the 2$^{-\Delta\Delta Ct}$ method as described

previously (Jiang et al., 2018). PCR primer sequences are listed in Table S1. All experiments were conducted with two biological and three technical replicates.

2.6. Phenotypic analysis of transgenic Arabidopsis plants

Seeds of homozygous WT and transgenic *Arabidopsis* lines were placed in MS solid medium at 4 °C for 3 days, then transferred to a growth chamber as described above. For mannitol and NaCl stress experiments, 3-day-old *Arabidopsis* seedlings were transferred to MS agar plates supplemented with different concentrations of mannitol or NaCl, respectively, cultured vertically for 10 days, and the root length was measured and photographed. For drought treatments, 14-day-old strong, healthy *Arabidopsis* seedlings grown in soil were not watered for 10 days and, then rewatered for 5 days. For salt treatments, the 14-day-old strong, healthy *Arabidopsis* seedlings grown in soil were watered with 250 mM NaCl solution for two weeks. After stress treatment, relative electrolyte leakage values were measured to estimate relative electrolyte leakage according to the method described by Wu et al. (2016).

2.7. Measurement of catalase (CAT), peroxidase POD) and superoxide dismutase (SOD) activities and ABA content

WT and transgenic plants were cultured in the chamber for 20 days, and watering of seedlings was stopped or replaced with watering using 250 mM NaCl for 10 days for drought or salt stress treatments, respectively. The activities of CAT, POD and SOD were spectrophotometrically measured using 0.5 g samples in 5 mL extraction buffer containing 0.05 M phosphate buffer. CAT and SOD activities were analyzed according to the instructions (Maehly and Chance, 1954; Beauchamp and Fridovich, 1971). POD activity was measured as the absorbance at 470 nm (Polle and Otter, 1994). For ABA content determination, 1 g samples were collected from WT and three transgenic lines, extracted with 10 ml isopropyl alcohol-hydrochloric acid buffer solution and 20 ml dichloromethane, and the ABA content was measured using a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method as described previously (Liu et al., 2014).

2.8. ABA sensitivity analysis and stomatal aperture assay

Seeds of WT and homozygous transgenic *Arabidopsis* lines were cultivated in the growth chamber on MS medium supplemented with 0, 0.25 μM or 0.5 μM ABA as described above, and the germination rate was scored after 7 days. The stomatal pore size was measured as described previously (Lim and Lee, 2016). Leaves of WT and transgenic *Arabidopsis* seedlings were floated in solution buffer containing 0.2 mM CaCl$_2$, 10 mM KCl and 10 mM MESKOH (pH 6.15) under light conditions for 2 h, then transferred to the solution buffer supplemented with 10 μM ABA for 2 h. Stomatal aperture was recorded and photographed using a Nikon Eclipse 80i microscope (Japan).

3. Results

3.1. Isolation and sequence analysis of ZmMYB3R

In recent years, 3R-MYB proteins in wheat and rice have been reported to play important roles in the responses to multiple abiotic stresses (Ma et al., 2009; Cai et al., 2015). However, the exact functions of maize 3R-MYB members remain obscure. Amino acid sequences of the conserved 3R domain (220 aa) of *Arabidopsis*, wheat and rice 3R-MYB proteins were used as queries in BLASTp searches of homologous sequences in the maize genome, and one 3R-MYB member was identified and designated *ZmMYB3R* (GRMZM2G081919_T02). *ZmMYB3R* consists of a complete ORF of 1692 bp encoding a protein of 563 amino
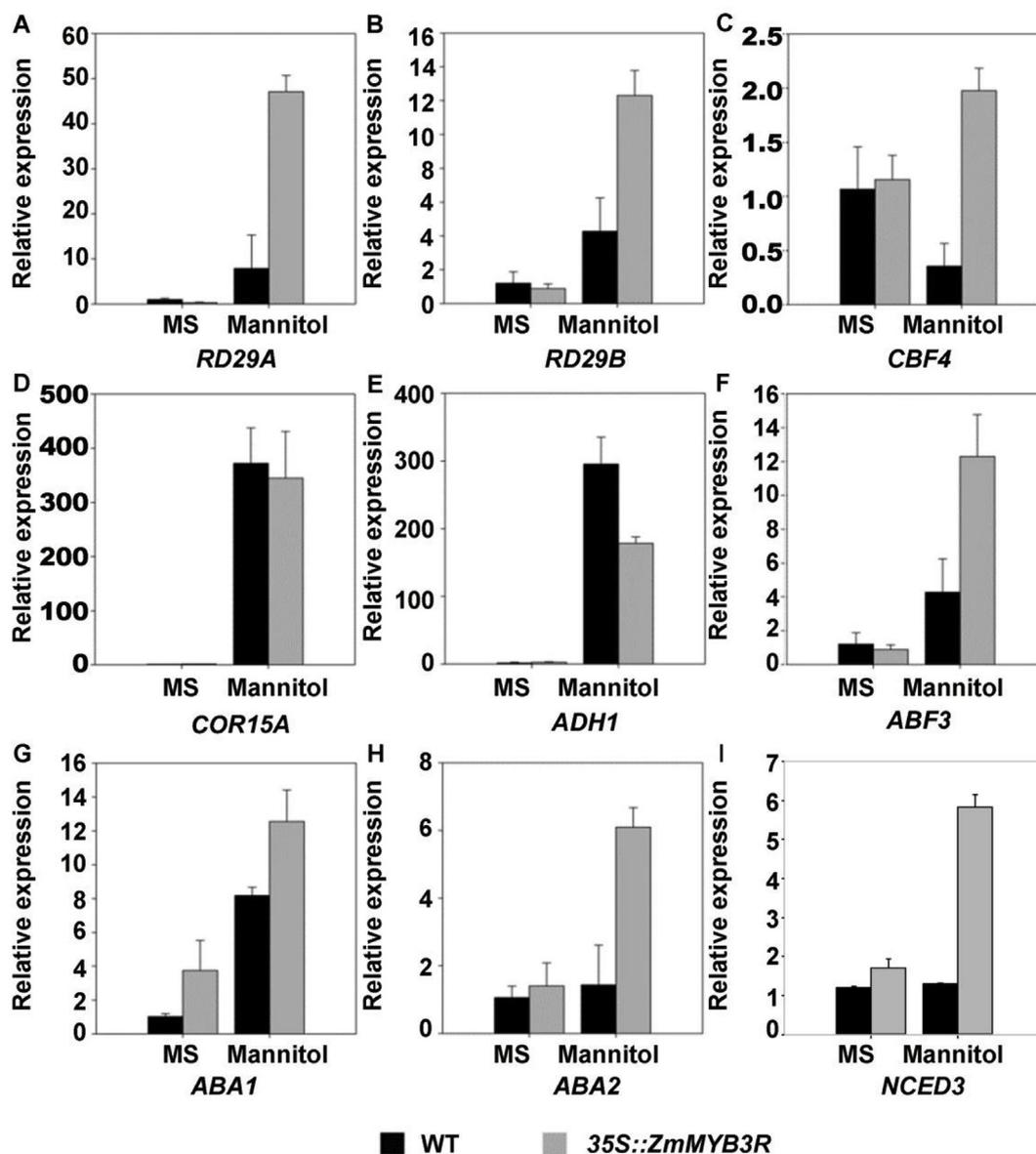
Fig. 7. Expression levels of stress-responsive genes in WT and ZmMYB3R-expressing transgenic *Arabidopsis* plants. qPCR analysis of marker genes in WT and transgenic plants under drought conditions (300 mM mannitol treatment for 7 days) vs. controls. Each column shows the mean of three individual seedlings, and bars represent the standard error of the mean.

acids with a predicted molecular weight of 61.66 kDa and a calculated isoelectric point of 8.33. Sequence alignment revealed that *ZmMYB3R* belongs to the 3R-MYB transcription factor subgroup, and includes three typical conserved adjacent repeats in the MYB domain, similar to other known 3R-MYB protein family members (Fig. 1A). A phylogenetic tree based on protein sequences was constructed to explore the relationships between ZmMYB3R and other plant MYB3R members. The results showed that ZmMYB3R clustered with TaMYB3R1 and Os-MYB3R2, which are known to be involved in abiotic stress responses (Fig. 1B), indicating that ZmMYB3R may play an important role in responses to abiotic stress.

3.2. ZmMYB3R is localised in the nucleus and possesses transcriptional activation activity

In order to investigate the subcellular localisation of the ZmMYB3R protein, recombinant plasmid p1305-35S-ZmMYB3R-GFP was generated and transfected into tobacco (*N. benthamiana*) leaf epidermal cells. The fluorescence signal from the ZmMYB3R-GFP fusion protein was mainly detected in the nucleus under confocal laser scanning microscopy, whereas the GFP control was detected in both the nucleus and cytoplasm (Fig. 2A). These results demonstrated that ZmMYB3R is a nuclear protein.

To analyze the transcriptional activity of ZmMYB3R, yeast one-hybrid assays were performed. Full-length ZmMYB3R, and N− and C-terminal domains, were separately inserted into the pGBKT7 vector, and the resulting constructs were transformed into yeast strain AH109. The results showed that all transformed yeast strains grew well on SD/-Trp medium. Yeast strains carrying the positive control vector (pGBKT7-53 + pGADT7-T), the full-length ZmMYB3R (ZmMYB3R residues 1–563) or the C-terminus of ZmMYB3R (ZmMYB3R residues 217–563) grew well and appeared blue on the SD/Trp⁻/His⁻/Ade⁻/X-α-gal selection medium, whereas cells containing the negative control vector (pGBKT7) or the N-terminus of ZmMYB3R (ZmMYB3R residues 1–216) did not grow (Fig. 2B). These results indicate that ZmMYB3R is a transcriptional activator, and the active site is mainly localised in the C-terminus of ZmMYB3R.
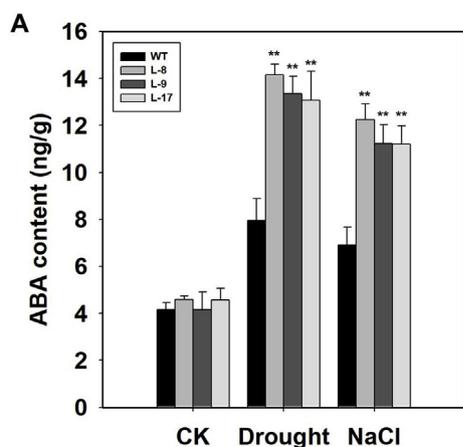
Fig. 8. Measurements of ABA content in plants after drought and NaCl treatment. ABA content was measured in WT and transgenic lines after drought treatment for 10 days or 2 weeks with 250 mM NaCl, respectively. Each column represents an average of three replicates, and bars indicate standard deviation (SD). ** and * indicate significant differences in comparison with controls at p < 0.01 and p < 0.05, respectively.

3.3. Expression analysis of ZmMYB3R

Next, qRT-PCR was performed to analyze the expression pattern of *ZmMYB3R* in different maize tissues, and following various stress treatments. Tissue expression analysis results showed that *ZmMYB3R* was ubiquitously expressed in all sampled tissues, with highest expression in leaves and stems (Fig. 3A). Induced expression pattern assays indicated that *ZmMYB3R* was upregulated by PEG6000, NaCl, and ABA treatments (Fig. 3B, C, D). These results suggest that ZmMYB3R may be involved in plant stress responses, similar to other known 3R-MYB proteins such as TaMYB3R1 and OsMYB3R2.

3.4. Expression of ZmMYB3R enhances tolerance to drought and salt stress in Arabidopsis

The expression pattern results suggest that ZmMYB3R may play a key role in drought stress. To investigate the functions of ZmMYB3R in plants, we constructed the pCAMBIA1301a-ZmMYB3R vector driven by the CaMV 35S promoter. Twenty homozygous transgenic *Arabidopsis* lines were obtained by the floral dip method. Three independent lines (L8, L9 and L17) exhibiting different expression levels by RT-PCR and qRT-PCR were chosen for all subsequent experiments (Fig. 4C and D).

To investigate the functions of ZmMYB3R, WT and three *ZmMYB3R* transgenic lines were placed on MS agar plates supplemented with 150 mM or 300 mM mannitol. Under normal conditions, there were no differences in plant morphology between WT and *ZmMYB3R* transgenic *Arabidopsis* (Fig. 4A). However, *ZmMYB3R* transgenic *Arabidopsis* displayed longer roots than WT plants following mannitol treatment (Fig. 4A and B). Moreover, drought treatment and rewatering experiments revealed that only a few leaves in *ZmMYB3R* transgenic plants were rolled and wilted, whereas the majority of leaves in WT plants were rolled and wilted, even after rewatering (Fig. 4E). In addition, survival rates in transgenic lines were significantly higher than that of the WT line after rewatering (Fig. 4F). Similarly, the phenotype was also analyzed in L8, L9 and L17 transgenic plants under high salinity conditions. WT plants and the three ZmMYB3R transgenic lines were placed on MS agar plates supplemented with 100 mM, 125 mM or 175 mM NaCl. The results showed that transgenic plants displayed longer roots following treatment with 100 mM or 125 mM NaCl, compared to WT plants (Fig. 5A, C). Under 175 mM NaCl, the survival rate of transgenic plants was 64% (survival rate averaged from three different lines), compared to only 15% for WT plants after NaCl treatment

for 72 h (Fig. 5B). Moreover, when WT and transgenic plants were subjected to 250 mM NaCl solution for 2 weeks, the leaves of control lines were wilted, yellowed, and senescent, whereas most leaves of the transgenic tobacco lines remained green (Fig. 5D).

In addition, some important physiological parameters, including relative electrolyte leakage and the activities of CAT, POD and SOD enzymes, were measured before and after drought and salt stress treatments, respectively. Relative electrolyte leakage in ZmMYB3R transgenic plants was lower than in WT plants (Figs. 4G and 5E). Additionally, the activities of all three antioxidant enzymes were elevated in transgenic lines compared to WT plants after drought and salt treatments (Fig. S1). Taken together, these results suggest that ZmMYB3R improved tolerance to drought and salt in transgenic *Arabidopsis*.

3.5. Arabidopsis lines ectopically expressing ZmMYB3R are hypersensitive to ABA and display increased stomatal closure

Application of exogenous ABA could up-regulate the expression of *ZmMYB3R* (Fig. 3B), indicating that *ZmMYB3R* may be involved in the ABA pathway. To verify this hypothesis, we measured the seed germination rates of WT and transgenic plants. Under normal conditions, there were no significant differences in seed germination rates. However, 47% of WT seeds germinated, but only 11% of *ZmMYB3R* transgenic *Arabidopsis* seeds germinated in the presence of 0.5 μM ABA (Fig. 6A and B). These results suggest that transgenic *Arabidopsis* seeds were hypersensitive to ABA.

Under drought stress, ABA promotes stomatal closure (Desikan et al., 2004; Gao et al., 2018). Herein, *ZmMYB3R* transgenic plants displayed ABA hypersensitivity and enhanced drought tolerance compared to WT plants. We therefore investigated whether the transgenic plants enhanced drought tolerance by regulating stomatal closure. We analyzed stomatal movement in response to ABA in WT and transgenic plants. The stomatal aperture of leaves in transgenic plants was much smaller than in WT plants under 10 μM ABA treatment, however, there were no obvious differences in stomatal aperture between WT and transgenic plants under normal conditions (Fig. 6C). These results suggest that ectopic expression of ZmMYB3R enhances drought tolerance by controlling stomatal closure.

3.6. Altered expression of stress/ABA-responsive genes in ZmMYB3R transgenic plants

To further explore the molecular mechanisms of ZmMYB3R in stress responses, the expression levels of stress/ABA-responsive genes involved in responses to abiotic stress were assessed in transgenic *Arabidopsis* plants. Under normal conditions, similar expression levels were observed for WT and transgenic lines for most of the analyzed genes. However, expression levels of stress/ABA-responsive genes RD29A, RD29B, ABF3, ABA1 and NCED3 (Ergen et al., 2009) in transgenic plants were higher than in WT plants after drought and salt treatments (Fig. 7, Fig. S2). These results indicate that ZmMYB3R enhances drought and salt tolerance via an ABA-dependent pathway. To verify this possibility, we measured the ABA content of WT and transgenic line under different conditions. There were no significant differences in ABA content between WT and transgenic line under normal conditions. However, after drought or salt treatments, the ABA content in transgenic lines was higher than in WT plants (Fig. 8). Taken together, our results suggest that ZmMYB3R is an important TF that enhances tolerance to drought and salt stress via an ABA-dependent pathway.

4. Discussion

Drought and high salinity are major abiotic stresses that inhibit plant growth and affect crop production. Thus, in recent years, a

growing body of studies have focused on explaining the functional mechanisms underpinning the responses to drought and salt stress in plants. Manipulating TFs by genetic engineering has great potential since these often regulate the expression of marker genes related to abiotic stress defense responses (Nakashima et al., 2014). MYB proteins are an important family of TFs involved in regulating the expression of abiotic stress-responsive genes (Dubos et al., 2010). In the present study, we isolated and characterized a novel 3R-MYB gene, ZmMYB3R, from maize. In accordance with the putative role of 3R-MYB proteins as TFs, ZmMYB3R includes three adjacent repeats in the MYB domain at the N-terminus. In addition, most of TFs are localised in nucleus and possess transactivation activity. Our results showed that ZmMYB3R is indeed localised in the nucleus, and ZmMYB3R displayed transcriptional activator activity and has a transactivation domain located at its C-terminus, consistent with a previous study (Cai et al., 2011) and the classical features of MYB TFs. These results demonstrate that ZmMYB3R is a novel 3R-MYB TF in maize.

3R-MYB members constitute a small gene family in plants compared to the larger R2R3-MYB subgroup; there are five, four, three and one 3R-MYB TFs in the *Arabidopsis*, rice, tobacco and wheat genome, respectively (Dai et al., 2007; Ma et al., 2009; Cai et al., 2015). A number of studies showed that R2R3-MYB TFs are involved in abiotic stress responses (Munns and Tester, 2008; Ding et al., 2009; Chen et al., 2015). However, except for OsMYB3R-2 and TaMYB3R1, the functions of most of 3R-MYB proteins remain obscure. OsMYB3R-2 plays an important role in enhancing tolerance to multiple abiotic stresses in *Arabidopsis* (Dai et al., 2007; Ma et al., 2009). Overexpression of Ta-MYB3R1 has been shown to increase drought and salt tolerance in *Arabidopsis* (Cai et al., 2015). Interestingly, phylogenetic tree analysis showed that ZmMYB3R is closely related to OsMYB3R-2 and Ta-MYB3R1, suggesting ZmMYB3R may play a conserved role during stress tolerance in plants.

ZmMYB3R significantly improved tolerance to salt and drought stress in transgenic *Arabidopsis*, as evidenced by better growth performance and a higher survival rate under stress conditions (Fig. 4 − 6), indicating that ZmMYB3R also functions as a positive stress-responsive TF in salt and drought stress tolerance in maize. Interestingly, line L-17 did not display enhanced resistance to mannitol, but it did exhibit increased resistance to drought. One possible explanation is that mannitol treatment cannot fully replicate an actual drought environment. In addition, various physiological traits in transgenic *Arabidopsis* appear to reflect the mechanisms underpinning abiotic stress tolerance. Firstly, CAT, POD and SOD can quickly accumulate under drought and salt conditions to minimize oxidative damage (Farooq et al., 2012; Diaz-Vivancos et al., 2013). Herein, ZmMYB3R transgenic lines exhibited high CAT, POD and SOD enzyme activities following stress treatments, which could help to maintain low intracellular ROS levels. Second, drought and salt stress can both promote ABA synthesis in plants (Ingram and Bartels, 1996). ABA is an important phytohormone that regulates the stomatal aperture, plant growth and development, and stress tolerance (Yoshida et al., 2014). Stomatal pores are involved in drought tolerance by controlling the utilization of water (Murata et al., 2015). Our observations showed that ectopic expression of ZmMYB3R in *Arabidopsis* led to higher ABA content and ABA hypersensitivity, which could increase the closure of stomata to minimize water loss and thereby enhance stress tolerance. Therefore, increased ABA content and sensitivity in transgenic lines may explain the enhanced stress tolerance. Third, both ABA-dependent and ABA-independent pathways are involved in drought and salt stress in *Arabidopsis* (Tran et al., 2007). In ABA-dependent pathways, MYB family TFs are major regulatory factors (Shinozaki and Yamaguchi-Shinozaki, 1997). For example, TaMYB3R1 regulates the expression of stress-responsive genes via an ABA-dependent pathway in *Arabidopsis* (Cai et al., 2015). Similarly, in the present work, the ABA content and expression levels of stress/ABA genes *RD29A*, *RD29B*, *ABF3*, *ABA1* and *NCED3* were higher in ZmMYB3R transgenic lines than in WT plants after drought and salt treatments,

suggesting that ZmMYB3R may function as a positive regulator of abiotic stress tolerance via an ABA-dependent pathway. Interestingly, expression levels of ABA-independent pathway genes *COR15* and *ADH1* were also elevated in transgenic lines following stress treatment compared with normal conditions. These results suggest that *ZmMYB3R* is a positive TF for stress responses that functions via an ABA-dependent pathway. In addition, constitutive overexpression of different stress-related TFs often results in the upregulation of downstream stress-responsive genes under both control and stress conditions. Interestingly, the 35S:ZmMYB3R lines did not show any significant effect on the expression of the selected target genes under control conditions. Consistently, over-accumulation of ABA in transgenic lines only occurred under mannitol or salt stress conditions. A possible reason is that *ZmMYB3R* has a positive role in enhancing abiotic stress only under stress conditions. Transgenic maize lines would be needed to clarify this hypothesis in the future.

In conclusion, we cloned and characterized *ZmMYB3R* from maize. Ectopic expression of *ZmMYB3R* resulted in increased ABA content and decreased ABA sensitivity by regulating the expression of various ABA/ stress-responsive genes, suggesting that ZmMYB3R mediates abiotic stress tolerance via an ABA-dependent signaling pathway.

Contributions

BC and JW conceived this project. JW and YJ designed experiments. YJ, YL, LC and WC performed experiments. JW and YJ analyzed the data and wrote the manuscript. All authors discussed the contents of the manuscript and approved the submission.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Abbreviations

ABA	abscisic acid
ROS	reactive oxygen species
TFs	transcription factor
PEG6000	polyethylene glycol 6000
WT	wild-type
Col-0	ecotype Columbia
NJ	neighbor joining
GFP	green fluorescent protein
ORF	open reading frame
qRT-PCR	quantitative real-time PCR
CAT	catalase
POD	peroxidase
SOD	superoxide dismutase

Appendix A. Supplementary data

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.plaphy.2019.02.010.

References

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–286.

Cai, H., Tian, S., Dong, H., Guo, C., 2015. Pleiotropic effects of TaMYB3R1 on plant development and response to osmotic stress in transgenic *Arabidopsis*. Gene 558, 227–234.

Cai, H., Tian, S., Liu, C., Dong, H., 2011. Identification of a MYB3R gene involved in drought, salt and cold stress in wheat (Triticum aestivum L.). Gene 485, 146–152.

Cao, H., Wang, L., Nawaz, M.A., Niu, M., Sun, J., Xie, J., Kong, Q., Huang, Y., Cheng, F., Bie, Z., 2017. Ectopic expression of pumpkin NAC transcription factor CmNAC1 improves multiple abiotic stress tolerance in arabidopsis. Front. Plant Sci. 8, 2052–2065.

Chen, T., Li, W., Hu, X., Guo, J., Liu, A., Zhang, B., 2015. A cotton MYB transcription factor, GbMYB5, is positively involved in plant adaptive response to drought stress. Plant Cell Physiol. 56, 917–929.

Clough, S.J., 2005. Floral dip: agrobacterium-mediated germ line transformation. Methods Mol. Biol. 286, 91–102.

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

Desikan, R., Cheung, M.K., Bright, J., Henson, D., Hancock, J.T., Neill, S.J., 2004. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J. Exp. Bot. 55, 205–212.

Diaz-Vivancos, P., Faize, M., Barba-Espin, G., Faize, L., Petri, C., Herna´ndez, J., 2013. Ectopic expression of cytosolic superoxide dismutase and ascorbate peroxidase leads to salt stress tolerance in transgenic plums. Plant Biotechnol. J 11, 976–985.

Ding, Z., Li, S., An, X., Liu, X., Qin, H., Wang, D., 2009. Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. J. Genet. Genom. 36, 17–29.

Du, H., Feng, B.R., Yang, S.S., Huang, Y.B., Tang, Y.X., 2012a. The R2R3-MYB transcription factor gene family in maize. PLoS One 7 (6), e37463.

Du, H., Yang, S.S., Liang, Z., Feng, B.R., Liu, L., Huang, Y.B., 2012b. Genome-wide analysis of the MYB transcription factor superfamily in soybean. BMC Plant Biol. 12, 106–125.

Du, H., Zhang, L., Liu, L., Tang, X.F., Yang, W.J., Wu, Y.M., Huang, Y.B., Tang, Y.X., 2009. Biochemical and molecular characterization of plant MYB transcription factor family. Biochemistry 74, 1–11.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., Lepiniec, L., 2010. MYB transcription factors in *Arabidopsis*. Trends Plant Sci. 15, 573–581.

Ergen, N.Z., Thimmapuram, J., Bohnert, H.J., Budak, H., 2009. Transcriptome pathways unique to dehydration tolerant relatives of modern wheat. Funct. Integr. Genom. 9, 377–396.

Farooq, M., Hussain, M., Wahid, A., Siddique, K., 2012. Drought stress in plants: an overview. Plant Responses to Drought Stress 1–33.

Gao, Y., Wu, M., Zhang, M., Jiang, W., Ren, X., Liang, E., Zhang, D., Zhang, C., Xiao, N., Li, Y., Dai, Y., Chen, J., 2018. A maize phytochrome-interacting factors protein ZmPIF1 enhances drought tolerance by inducing stomatal closure and improves grain yield in Oryza sativa. Plant Biotechnol. J. 16, 1375–1387.

He, Q., Jones, D.C., Wei, L., Xie, F., Ma, J., Sun, R., 2016. Genome-Wide identification of R2R3-MYB genes and expression analyses during abiotic stress in Gossypium raimondii. Sci. Rep. 6, 22980.

Ingram, J., Bartels, D., 1996. The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 377–403.

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

Jiang, Y., Zheng, Q., Chen, L., Liang, Y., Wu, J., 2018. Ectopic overexpression of maize heat shock transcription factor gene ZmHsf04 confers increased thermo and salt-stress tolerance in transgenic *Arabidopsis*. Acta Physiol. Plant. 40, 9–22.

Katiyar, A., Smita, S., Lenka, S.K., Rajwanshi, R., Chinnusamy, V., Bansal, K.C., 2012. Genome-wide classification and expression analysis of myb transcription factor families in rice and arabidopsis. BMC Genomics 13, 544–562.

Lim, C.W., Lee, S.C., 2016. Pepper protein phosphatase type 2C, CaADIP1 and its interacting partner CaRLP1 antagonistically regulate ABA signalling and drought response. Plant Cell Environ. 39, 1559–1575.

Liu, J., Ding, J., Yuan, B., 2014. Magnetic solid phase extraction coupled with in situ derivatization for the highly sensitive determination of acidic phytohormones in rice leaves by UPLC-MS/MS. Anaiyst 139, 5605–5613.

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

Maehly, A.C., Chance, B., 1954. The assay of catalases and peroxidases. Methods Biochem. Anal. 1, 357–425.

Meissner, R.C., Jin, H., Cominelli, E., Denekamp, M., Fuertes, A., Greco, R., 1999. Function search in a large transcription factor gene family in *Arabidopsis*: assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. Plant Cell 11, 1827–1840.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681.

Murata, Y., Mori, I.C., Munemasa, S., 2015. Diverse stomatal signaling and the signal integration mechanism. Annu. Rev. Plant Biol. 66, 369–392.

Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 2014. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. Front. Plant Sci. 5 (170), 170.

Peng, X., Zhao, Y., Cao, J., Zhang, W., Jiang, H., Li, X., Ma, Q., Zhu, S., Cheng, B., 2012. CCCH-type zinc finger family in maize: genome-wide identification, classification and expression profiling under abscisic acid and drought treatments. PLoS One 7 (7), e40120.

Polle, A., Otter, T., 1994. Apoplastic peroxidases and lignification in needles of Norway spruce (picea abies L.). Plant Physiol. 106, 53–60.

Qin, Y., Wang, M., Tian, Y., He, W., Han, L., Xia, G., 2012. Over-expression of TaMYB33 encoding a novel wheat MYB transcription factor increases salt and drought tolerance in *Arabidopsis*. Mol. Biol. Rep. 39, 7183–7192.

Salomoni, P., Perrotti, D., Martinez, R., Franceschi, C., Calabretta, B., 1997. Resistance to apoptosis in CTLL-2 cells constitutively expressing c-Myb is associated with induction of BCL-2 expression and Myb-dependent regulation of bcl-2 promoter activity. Proc. Natl. Acad. Sci. U.S.A. 94, 3296–3301.

Shinozaki, K., Yamaguchi-Shinozaki, K., 1997. Gene expression and signal transduction in water-stress response. Plant Physiol. 115, 327–334.

Shinozaki, K., Yamaguchi-Shinozaki, K., Seki, M., 2003. Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. 6, 410–417.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Tran, L.S., Nakashima, K., Shinozaki, K., Yamaguchi-Shinozaki, K., 2007. Plant gene networks in osmotic stress response: from genes to regulatory networks. Methods Enzymol. 428, 109–128.

Wu, J., Zhou, W., Gong, X., Cheng, B., 2016. Expression of ZmHDZ4, a maize homeodomain-leucine zipper I gene, confers tolerance to drought stress in transgenic rice. Plant Mol. Biol. Rep. 34, 845–853.

Xiong, L., Zhu, J., 2003. Regulation of abscisic acid biosynthesis. Plant Physiol. 133, 29–36.

Yamaguchi-Shinozaki, K., Shinozaki, K., 2005. Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci. 10, 88–94.

Yoshida, T., Mogami, J., Yamaguchi-Shinozaki, K., 2014. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr. Opin. Plant Biol. 21, 133–139.

Zhu, J., 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53, 247–273.

Zhu, J., 2016. Abiotic stress signaling and responses in plants. Cell 167, 313–324.