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Research article

Function analysis of *ZmNAC33*, a positive regulator in drought stress response in *Arabidopsis*

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ABSTRACT

Drought significantly affects plant growth and has devastating effects on crop production, NAC transcription factors respond to abiotic stresses by activating gene expression. In this study, a maize NAC transcription factor, *ZmNAC33*, was cloned and characterized its function in *Arabidopsis*. Transient transformation in *Arabidopsis* leaves mesophyll protoplasts and trans-activation assays in yeast showed that *ZmNAC33* was localized in the nucleus and had transactivation activity. qRT-PCR analysis showed that *ZmNAC33* in maize was induced by drought, high salinity and abscisic acid (ABA) stress. Promoter analysis identified multiple stress-related cis-acting elements in the promoter region of *ZmNAC33*. In *ZmNAC33* transgenic *Arabidopsis*, germination rates were higher than in wild type plants under ABA and osmotic stress at the germination stage, and overexpression lines exhibited higher survival rates and higher antioxidant enzyme activities compared with wild type under drought stress. These results indicate that *ZmNAC33* actes as a positive regulator in drought tolerance in plants.

1. Introduction

Drought and salinity are major forms of stress that affect plant growth and can have devastating effects on crop production. Plants have evolved a series of mechanisms to cope with such environmental adversities (Lu et al., 2012; Sakuraba et al., 2015; Wang et al., 2015). In response to drought stress, plants activate a number of defence mechanisms that function to increase tolerance to water deficit (Le et al., 2011). The response to abiotic stresses requires the production of important functional proteins, such as those involved in the synthesis of osmoprotectants, and regulatory proteins operating in signal transduction pathways, such as kinases and transcription factors (TFs) (de Oliveira et al., 2011). TFs are key proteins that regulate gene expression at the transcriptional level by interacting with promoter elements of genes, resulting in increased expression of many functional genes (Shiriga et al., 2014). Several TF families have been found to play important roles in plant stress tolerance, such as the DREB (dehydration responsive element binding protein), MYB (v-myb avian myeloblastosis viral oncogene homolog), and NAC (NAM, ATAF1/2, CUC2) families (Tran et al., 2004; Hao et al., 2010; Jeong et al., 2010). These TFs separately or cooperatively affect the expression of various downstream genes, and are components of gene networks for stress adaptation (Wang et al., 2015). Recent work also revealed that some NAC TFs function in abiotic stress-responsive signaling, and the molecular mechanisms of these TFs have been extensively studied in maize (Sakuraba et al., 2015).

NAC genes encode plant-specific transcriptional regulators that constitute a large transcription factor family in plants; for example, maize has 124 NAC members (Fan et al., 2014). NAC family proteins have a highly conserved N-terminal NAC binding domain and a highly variable C-terminal domain, which generally operates as a transcriptional activator or repressor (Aida et al., 1997; Ernst et al., 2004; Nuruzzaman et al., 2010; Hussain et al., 2017). These proteins act by binding to cis-acting promoter elements, thereby providing the trigger for the initiation of target gene' transcription (Nakashima et al., 2009; Wang et al., 2016).

NAC genes have been implicated in a wide range of plant developmental processes, including shoot apical meristem development (Olsen et al., 2005), reactive oxygen species (ROS) production and cell death (Yan et al., 2018), and secondary cell wall biosynthesis (Zhang et al., 2018a,b). Moreover, a number of studies implicated NAC genes in various stress and defense responses (Wei et al., 2016). Drought-

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inducible NAC genes were initially studied in *Arabidopsis* and were found to improve stress tolerance when overexpressed (Fujita et al., 2004). For example, *ANAC019*, *ANAC055* and *ANAC072* were first discovered from a dataset of multiple stress-responsive genes in *Arabidopsis*, and overexpression of these genes led to enhanced drought tolerance (Tran et al., 2004; Bu et al., 2008). *Arabidopsis NAC016* was found to be involved in drought stress (Sakuraba et al., 2015), and *ANAC017*, *ANAC082* and *ANAC090* were found to impact salicylic acid and ROS production (Kim et al., 2018). Rice and maize NAC genes have also been shown to confer tolerance to abiotic stress. For example, transgenic *Arabidopsis* plants overexpressing *OsNAC063* showed enhanced tolerance to high-salinity and osmotic stress and *OsNAC5* transgenic plants had improved tolerance to high salinity (Yokotani et al., 2009; Takasaki et al., 2010). Overexpression of *ZmNAC84* in tobacco improved drought tolerance (Zhu et al., 2016), and *ZmSNAC1* can enhance tolerance to dehydration in transgenic *Arabidopsis* (Lu et al., 2012). *ZmNAC111* is associated with maize seedling drought tolerance (Mao et al., 2015), *ZmNAC55* can enhance tolerance to drought and lead to hypersensitivity to ABA at the germination stage in transgenic *Arabidopsis* (Mao et al., 2016), and *ZmNAC88* is important for ABA-induced antioxidant defense against drought stress (Zhu et al., 2016). Therefore, NAC genes represent one of the key regulatory gene families involved in plant development and abiotic stress responses (Wei et al., 2016). These studies support the view that NAC type TFs might be part of the general framework of drought and salt adaptation by connecting or regulating subsets of linear adaptive pathways.

In this study, a NAC transcription factor gene *ZmNAC33* was cloned from maize inbred line Ji853. Gene expression pattern analysis demonstrated that *ZmNAC33* was up-regulated by dehydration, ABA, and salt treatments. We show that overexpression of *ZmNAC33*, a positive effector of stress response, enhances abiotic stress tolerance in *Arabidopsis*.

2. Materials and methods

2.1. Cloning and bioinformatics analysis of ZmNAC33

To clone novel *NAC* genes in maize, the *ATAF1* protein sequence was used as a query in a Blastp search against the Maize Genetics and Genomics Database (MaizeGDB; http://www.maizegdb.org/). The gene *ZmNAC33* (accession No. GRMZM2G014653) from MaizeGDB was identified. To obtain the complete coding sequence (CDS) of *ZmNAC33*, the DNA sequence of *ZmNAC33* was used as the query in a Blast search against the maize EST library (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Highly homologous EST sequences were identified and assembled using DNA Star software to obtain a longer sequence. A full-length cDNA sequence containing the open reading frame (ORF), 5′- and 3′-untranslated regions (UTRs) was obtained. Based on this result, primers were designed to amplify the putative sequence from cDNA synthesized from RNA extracted from the leaves of inbred line Ji853. The PCR conditions for amplifying *ZmNAC33* were as follows: 2 min at 94 °C; 35 cycles of 10 s at 98 °C, 30 s at 60 °C, and 30 s at 68 °C; and then 5 min at 68 °C. The primers are listed in Supplemental Table 1. The PCR product was cloned into a pEASY Blunt Simple vector (Transgen, China) and transfected into *Escherichia coli* DH5α competent cells. Finally, the target sequence in positive strains was sequenced. The cDNA and genomic DNA of sequences of *ZmNAC33* were aligned to identify the exons and introns in the genomic sequence. The ORF of the *ZmNAC33* gene was analyzed using online software (https://www.ncbi.nlm.nih.gov/orffinder/), and domains were identified using the Pfam (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) tools to confirm that *ZmNAC33* is a NAC gene. The theoretical molecular weight and isoelectric point were calculated using website ExPASy (https://web.expasy.org/protparam/).

2.2. Bioinformatic analysis of the ZmNAC33 promoter

The promoter sequence of *ZmNAC33* was amplified from the genomic DNA of inbred line Ji853. The PLACE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify cis-acting elements in the 2,000 bp of sequence upstream from the translation initiation codon (ATG) of *ZmNAC33* (Lescot et al., 2002).

2.3. Plant materials and stress treatments

Seeds of the maize inbred line Ji853 were germinated in vermiculite and cultivated under a 16 h light/8 h dark cycle at 28 °C. Seedlings at the three-leaf stage were subjected to four stress treatments: osmotic stress (PEG), high salinity (NaCl), high alkalinity (Na_2CO_3) and ABA. For PEG and ABA treatments, the roots of seedlings were immersed in solutions containing 20% (w/v) PEG6000 and 100 μM ABA, respectively. For high salinity and high alkalinity treatments, the seedlings were irrigated with 200 mM NaCl solution and 100 mM Na_2CO_3 solution, respectively. Samples were collected from five seedlings after 0, 6, 12 and 24 h of treatment with PEG, Na_2CO_3, NaCl or ABA. All collected samples were immediately frozen in liquid nitrogen and stored at −80 °C for future analysis.

2.4. RNA extraction and gene expression analysis

Total RNA was extracted from various stress-treated materials (see above) using the HiPure Plant RNA Mini kit (Magen, China) according to manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Invitrogen). Quantitative real time-PCR (qRT-PCR) was performed using a LightCycler 480 (Roche). Reactions were carried out in a 10 μl volume containing 2 μl diluted cDNA, 200 nM each primer, and 5 μl SYBR Premix EX Taq II (TaKaRa) with the following conditions: 10 s at 95 °C, 30 s at 60 °C, followed by 40 cycles. The specificity of each primer pair was verified by melting curve analysis. The expression of Actin (Genbank: GRMZM2G014653) was used as an internal control. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used for quantification and the variation in expression was estimated from three biological replicates. The primer pairs used for qRT-PCR analysis are listed in Supplemental Table 1.

2.5. Subcellular localization of ZmNAC33

To investigate the subcellular localization of *ZmNAC33*, the coding region of *ZmNAC33* was amplified using a pair of primers (Supplemental Table 1). The PCR products were digested with *Hind* III and *Sma* I and inserted into the super pCAMBIA 1300 vector to generate the ZmNAC33-GFP fusion protein. The marker gene, *AtFIB1* (AT5G52470), was as a positive control. After sequencing confirmation, mesophyll protoplasts were prepared from *Arabidopsis* leaves, the recombinant plasmid was introduced into the protoplasts using the DNA-PEG-calcium transfection method (Yoo et al., 2007). The pCAMBIA 1300 vector was used as a negative control. Finally, transformed mesophyll protoplasts were examined by fluorescence microscopy (Leica TCS SP5) after incubation at 21 °C overnight in the light.

2.6. Transactivation activity analysis of ZmNAC33 in yeast

ZmNAC33 was examined for the presence of an activation domain using a yeast assay system. The full-length CDS of *ZmNAC33* was amplified with the primers listed in Supplemental Table 1. The PCR products were digested with *EcoR*I and *BamH*I and were then cloned into vector pGBKT7 according to the manufacturer's protocol. The yeast strain Y2H Gold (Clontech) was transformed with the vector, and then plated on selective synthetic dropout (SD) medium (SD/-Trp). The

empty pGBKT7 vector was used as a negative control, plates were incubated at 30 °C for 3 d. For transactivation activity analysis, the tansformants were selected to spot separately on SD medium (SD/-Trp) with X-α Gal, plates were incubated at 30 °C for 3 d before photographing.

2.7. Transformation of Arabidopsis

To obtain transgenic *Arabidopsis* plants, the ZmNAC33-GFP plasmid (see above) was transfected into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis* plants were then transformed with this strain using floral dipping method (Clough and Bent, 1998). For selection of transformants, T_1 seeds were plated on 1/2 Murashige and Skoog (MS) medium containing 1% sucrose and 50 mg/mL hygromycin B. Homozygous T_3 plants were used for further analysis.

2.8. Stress tolerance of seed germination

Fifty seeds of the wild type (WT) and *ZmNAC33*-overexpression plants were sown on 1/2 MS medium supplemented with or without 0.5 μM ABA or 350 mM mannitol. Plates were chilled at 4 °C for 2 days to synchronize germination and moved to 21 °C with a 16 h light/8 h dark cycle. The seeds were regarded as germinated when the cotyledons obviously expanded and turned green. Germination was scored on day 5 after germination, with three replicated assays. The Student's t-test function in SPSS 20.0 was used to assess the difference between WT and overexpression plants.

2.9. Stress tolerance assays of plants overexpressing ZmNAC33

The tolerance of WT and *ZmNAC33*-overexpression plants to various stresses was evaluated. Seeds were sowned on 1/2 MS medium (1% sucrose) and stratified at 4 °C for 2 d. After stratification, seeds were germinated in a growth chamber under a 16 h light/8 h dark cycle at 21 °C for an additional 5 d. Five-day-old seedlings were transferred to 1/2 MS medium supplemented with 400 mM mannitol to impose osmotic stress and were vertically grown for 7 d. Root length was scored after 7 d of growth on vertical plates. For the drought tolerance assays, 7-day-old plants germinated on 1/2 MS medium were transferred into pots containing a soil mixture (vermiculite: humus = 1:2). Water was withheld from 21-day-old plants to impose drought stress. After withholding water for 21 days, watering was resumed to allow the plants to recover. Five days later, the number of surviving plants was recorded. At least 72 plants for each line were compared with WT plants in each test, and statistical analysis was based on data obtained from three independent experiments. The Student's t-test function in SPSS20.0 was used to assess the difference between WT and overexpression plants.

2.10. Measurement of leaf water loss

For the water loss assay, rosette leaves were detached from 3-week-old seedlings and placed on paper at 21 °C (humidity 45–55%) to impose water deficiency. Meanwhile changes in rosette leaf shape were observed during this treatment. The weight of rosette leaves was measured at 0.5 h intervals. Water loss rates were measured using 10 plants each of WT and 35S-*ZmNAC33* overexpression plants with three replicated assays.

2.11. Expression analyses of relevant marker genes and measurement of drought responsive physiological indices

To detect the expression of relevant marker genes in WT and *ZmNAC33*-overexpression plants under drought stress, the leaves of seedlings were detached after being subjected to drought stress for 10 d. Total RNA was extracted from leaves using the HiPure Plant RNA Mini kit (Magen, China), and qRT-PCR analysis was performed to examine the expressions of the marker genes *DREB2A* (AT5G05410), *AIB1* (AT2G46510), *SAG13* (AT2G29350), and *COR47* (AT1G20440); Actin was used as the internal control gene. Three independent biological experiments were performed. Primer pairs used are listed in Supplemental Table 1. To detect changes in physiological indices under drought stress, leaves were collected from plants before and 10 d after stress treatment; the enzyme activities of MDA, SOD and PRO were measured. For extraction of MDA, SOD and PRO, were sampled with the corresponding detection kit (MDA-2-Y, SOD-2-Y, PRO-2-Y, Suzhou Comin, China).

3. Results

3.1. Sequence characterization of ZmNAC33

NAC TFs contribute to abiotic stress tolerance, and NAC members from *Arabidopsis* have been shown to enhance plant drought or salt tolerance. For example, *ATAF1* remarkably enhances plant tolerance to drought in *Arabidopsis*, and transgenic rice plants overexpressing *ATAF1* have significantly improved salt tolerance and insensitivity to ABA (Wu et al., 2009; Liu et al., 2016). To obtain a novel stress-related *NAC* gene in maize, a Blastp search against MaizeGDB was performed using the ATAF1 protein sequence as a query, four putative NAC genes were found, a sequence alignment showed that *ATAF1* shared 68%, 64%, 60% and 55% amino acid identity with *ZmNAC33*, *ZmNAC20* (GRMZM2G180328), *ZmNAC49* (GRMZM2G347043) and *ZmNAC4* (GRMZM2G079632), respectively. A putative candidate NAC gene, *ZmNAC33*, with high homology to NAC family members of rice and *Arabidopsis* was found; *ZmNAC33* shares 68% identity with *ATAF1* and shares the highest percent identity (81%) with *OsNAC2* and *OsNAC6* in rice. Analysis of the putative *ZmNAC33* protein sequence using the SMART and Pfam tools revealed that it contains a typical NAC domain in the N-terminal region. These findings indicate that *ZmNAC33* is a putative stress-responsive NAC transcription factor. So, to clone *ZmNAC33*, the full-length cDNA was amplified from the maize inbred line Ji853 by PCR. Sequence analysis revealed that the ORF of *ZmNAC33* is 882 bp, and encodes a protein of 293 amino acid residues with a predicted molecular mass of 32.4 kD and pI value of 8.46. Analysis of the *ZmNAC33* promoter sequence (~2000bp upstream from transcription start site) using the Plant CARE database revealed, as expected, that five putative plant stress response-related cis-acting elements were present in the promoter; such as ABRE (ABA-responsive element), DRE (Dehydration-responsive element), LTRE (low temperature responsive element), MYB (MYB recognition site) and MYC (MYC recognition site) (Fig. 1A). This result suggests that *ZmNAC33* may play a notable role in the response to drought stress in maize.

3.2. Response of ZmNAC33 to abiotic stresses

We assayed the expression of *ZmNAC33* under various abiotic stresses in maize seedlings. As expected, *ZmNAC33* was highly expressed in roots under salt stress (200 mM NaCl), 100 μM ABA, high alkalinity (Na_2CO_3) and osmotic stress (PEG). The expression levels of *ZmNAC33* peaked at 12 h for PEG, 24 h for NaCl, and 24 h for ABA (Fig. 2). *ZmNAC33* was significantly up-regulated by PEG and ABA; the expression level of *ZmNAC33* increased by more than 18 and 16 fold at in roots after 12 h of PEG and ABA treatment, respectively. *ZmNAC33* was slowly induced under salt stress and a small increase in expression was observed after 6 h, after which expression continuously increased. *ZmNAC33* was slowly induced under alkaline stress, the expression levels of *ZmNAC33* peaked in roots at 12 h, then expression decreased at 24 h *ZmNAC33* expression rapidly increased under osmotic stress, with the highest expression observed at 12 h after treatment, then decreased gradually. *ZmNAC33* transcript rapidly accumulated in response to ABA, with high levels of expression observed at 6 h after ABA treatment. Then expression decreased at 12 h, but increased at 24 h. The

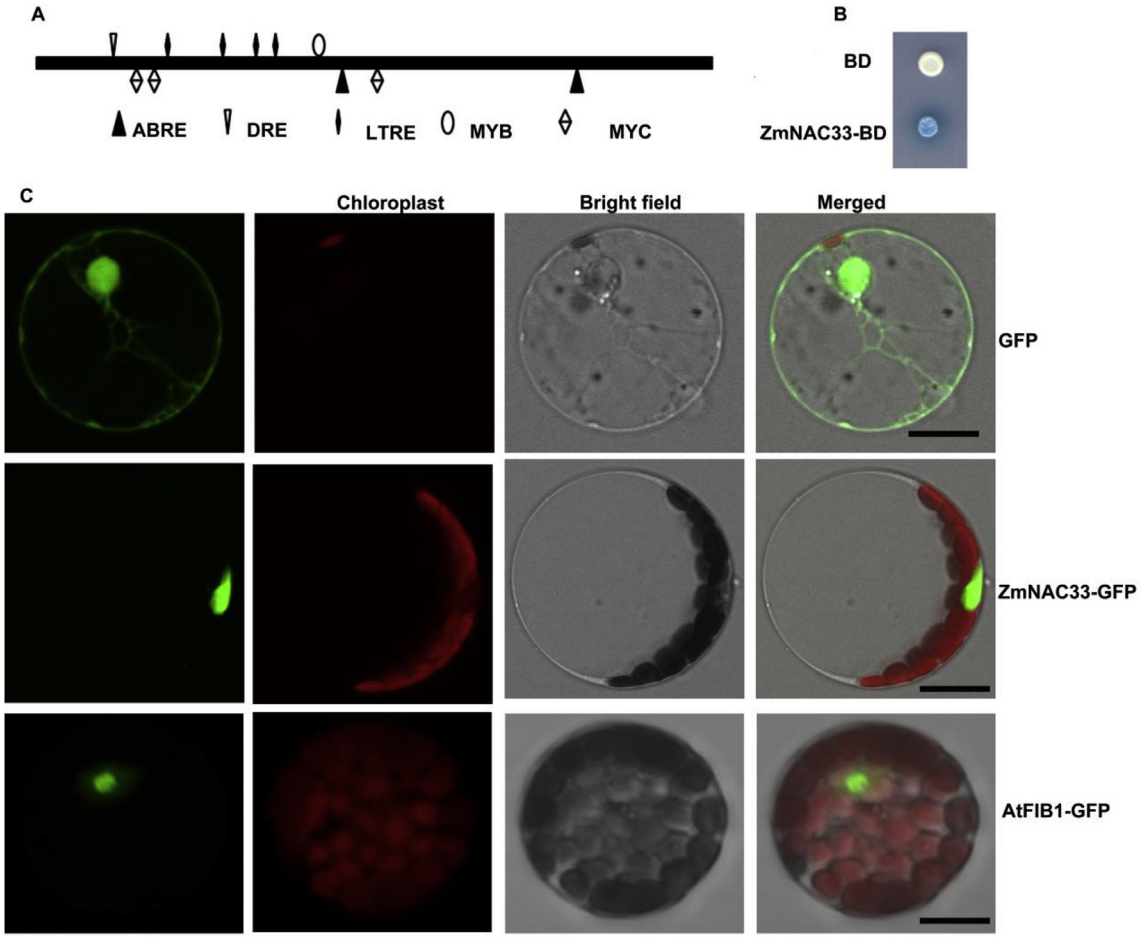

Fig. 1. Analysis of the subcellular localization, transactivation activity and promoter of *ZmNAC33*. A, Distribution of several stress-related cis-elements in the promoter region (2 kb upstream of the translational start site) of *ZmNAC33* of inbred line Ji853. ABRE, ABA-responsive element; DRE, Dehydration-responsive element; LTRE, low-temperature responsive element; MYB, MYB recognition site; MYC, MYC recognition site. B, Transactivation activity assay of *ZmNAC33* in yeast strain Y2H Gold. Yeast cultures transformed the pGBKT7 plasmid or plasmid ZmNAC33, were diluted and inoculated on SD medium (SD/-Trp) with X-α Gal. C, Subcellular localization of ZmNAC33 in *Arabidopsis* protoplast cells. The middle panels show the localization of ZmNAC33-GFP in *Arabidopsis* protoplast cells in a transient assay, the bottom panels show the localization AtFIB1-GFP (marker gene) as a positive control, while the upper panels show the localization of GFP as a control. Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

change in *ZmNAC33* expression levels in leaves was small under osmotic stress, salt stress, ABA and alkaline stress. Taken together, these results showd that *ZmNAC33* is induced by salt, ABA, alkaline and drought stress and was a positive regulation under abiotic stresses.

3.3. Characteristics of localization and transactivation activity of *ZmNAC33*

To investigate whether ZmNAC33 has transactivation activity, the *ZmNAC33* CDS was fused to the GAL4 DNA-binding domain in the pGBKT7 vector. Yeast strain Gold was transformed with this vector and then dropped onto SD-Trp/X-α Gal selection media. All transformants grew well on the selection medium, confirming that *ZmNAC33* had transactivation activity (Fig. 1B). To determine the subcellular localization of *ZmNAC33*, the full-length CDS of *ZmNAC33* was fused with GFP and placed under the control of the cauliflower mosaic virus 35S promoter, and the resulting vector, ZmNAC33-GFP, was transformed into *Arabidopsis* protoplasts, the same vector containing only GFP was used as a control. Confocal imaging showed that the control GFP protein was located in the cytoplasm and nucleus, whereas ZmNAC33-GFP was observed only in the nucleus (Fig. 1C), positive control AtFIB1 showed nucleus localization, demonstrating that ZmNAC33 localized in the cell nucleus and possibly functions as a TF.

3.4. Germination sensitivity of seeds from *ZmNAC33* overexpression plants to ABA and osmotic stress

To investigate the germination sensitivity of seeds from *ZmNAC33* overexpression plants under osmotic (induced by mannitol) and ABA stress, three independent overexpression lines (OE1, OE3, OE13) were chosen for further analysis, the seeds of WT and *ZmNAC33* overexpression plants were sown on medium containing ABA or mannitol. There was no difference between the seed germination rates of WT and overexpression plants under normal conditions (Fig. 3A). In the presence of 350 mM mannitol, percent germination ranged from 41% to 57% for the overexpression lines, while the percent germination for WT was 29% (Fig. 3B). Under ABA treatment, the percent germination ranged from 30 to 40% for overexpression lines, compared with 14.7% for the WT. The germination of seeds from both WT and the *ZmNAC33* overexpression plants was inhibited, but the inhibition of WT seed germination was much more severe than that of *ZmNAC33* overexpression plant seed germination. Thus, *ZmNAC33* overexpression plants are resistant to ABA and osmotic stress during the germination stage.

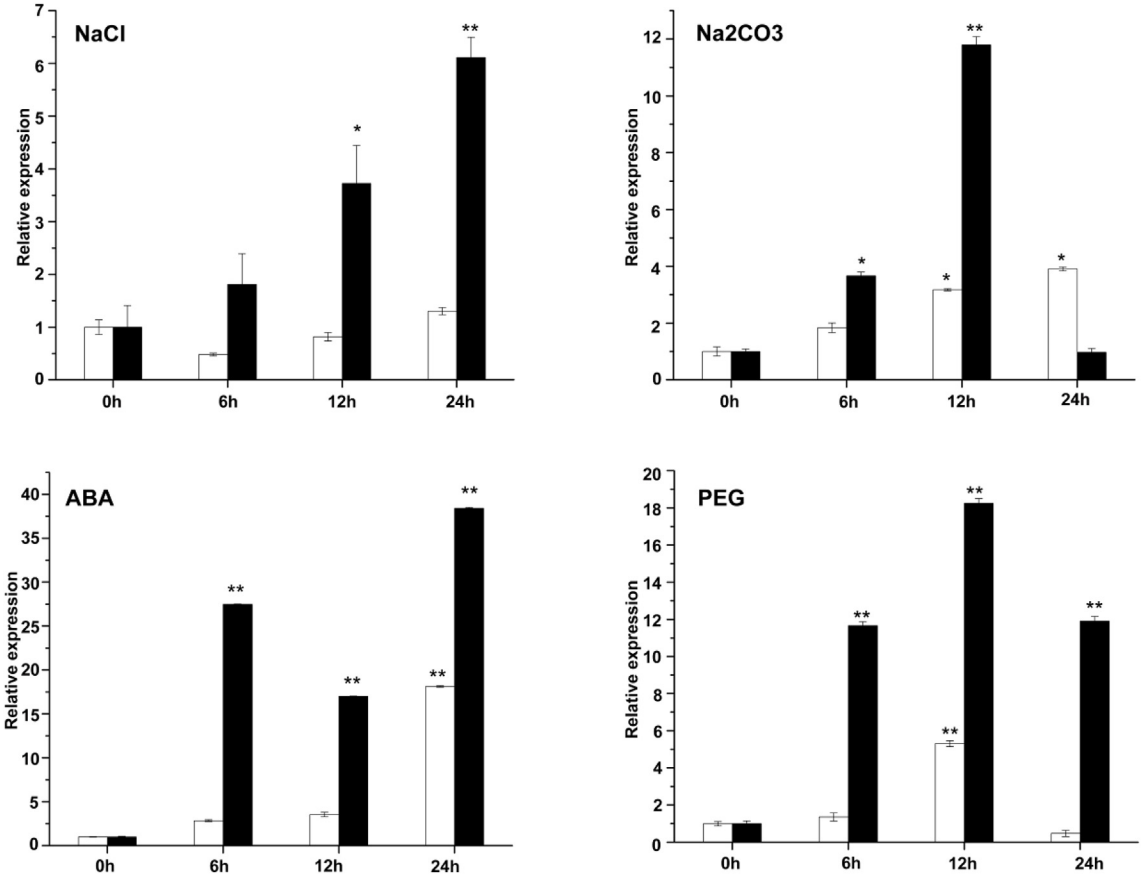

Fig. 2. Expression patterns of *ZmNAC33* under abiotic stress in maize. Expression of *ZmNAC33* in leaves (white bars) and roots (black bars) after treatment with NaCl, ABA, PEG and Na_2CO_3. *, **: means differ from the control value at, respectively, $P < 0.05$ and $P < 0.01$.

3.5. *ZmNAC33* enhanced the tolerance under drought stress in transgenic *Arabidopsis*

ZmNAC33 overexpression plants exhibited no differences in growth from WT under normal conditions (Fig. 4A). To analyze the effect of *ZmNAC33* overexpression under drought stress, WT and overexpression plants were grown for 3 weeks in soil, then water was withheld for 21 d. After withholding water for 10 d, phenotypic differences between the WT and overexpression plants were observed. Leaf shrinking was substantially delayed in the overexpression plants compared with WT. After the plants were subjected to drought stress for 14 d, most WT plants were visibly wilted, whereas the leaves of overexpression plants remained green, leaf rolling and shrinking were observed in a small number of plants. WT and most overexpression plants wilted by 21d; however, most of the overexpression plants recovered after rewatering (Fig. 4A). Overexpression plants showed stronger growth recovery. Further analyses showed that the survival rate of the overexpression lines (62.2–81% recovered after rewatering) was significantly higher than that of WT plants (Fig. 4B). These results show that overexpression of *ZmNAC33* could significantly enhance drought tolerance.

To further assess the sensitivity of *ZmNAC33* overexpression plants to osmotic stress during the post-germination stage, root elongation inhibition was analyzed under osmotic stress. Overexpression plant seedlings (5-day-old) were transferred to 1/2 MS medium with or without 400 mM mannitol. After 7 d, the growth of WT under mannitol-induced osmotic stress was more severely inhibited than that of overexpression plants (Fig. 5A). The roots of overexpression plants were longer than those of WT under mannitol stress (Fig. 5B), but overexpression plants exhibited no significantly differences in root length from WT under osmotic stress ($P < 0.05$). Taken together, the results indicate that overexpression plants promotered resistance to drought stress in *Arabidopsis*. Our results suggest that *ZmNAC33* acts as a positive regulator of drought stress and may contribute to plant tolerance under drought stress.

3.6. Overexpression of *ZmNAC33* improves the drought tolerance of transgenic *Arabidopsis* plants by increasing the activities of antioxidant enzymes

Drought stress can cause the accumulation of harmful ROS that could lead to damage of plant cells, DNA and protein. Antioxidant enzymes such as MDA, PRO and SOD are involved in the ROS scavenging system, which protects plants from oxidative damage (Wang et al., 2018; Yang et al., 2018). We analyzed the activities of antioxidant enzymes in WT and overexpression plants under drought stress conditions, and found that the levels of PRO enzyme activity in the overexpression plants were remarkably higher than those of WT (Fig. 6). The SOD activities in the three overexpression lines were significantly higher than those in WT after 10 d of drought stress. In addition, the MDA activities in overexpression lines were higher than those in WT plants. These results suggest that overexpression of *ZmNAC33* improves drought tolerance by increasing the activities of antioxidant enzyme.

3.7. Water loss assay

To further verify drought resistance of overexpression plants, which were associated with transpiration, the phenotypes of detached leaves were examined after air-drying them in a 21 °C environment. The fresh weights of leaves of WT and overexpression plants were measured after a half hour at room temperature. After 5 or 6 h, the leaves of WT had severely curved, whereas the leaves of overexpression plants displayed a slightly curled phenotype. After 1 h, the leaf weight of OE1 and OE3

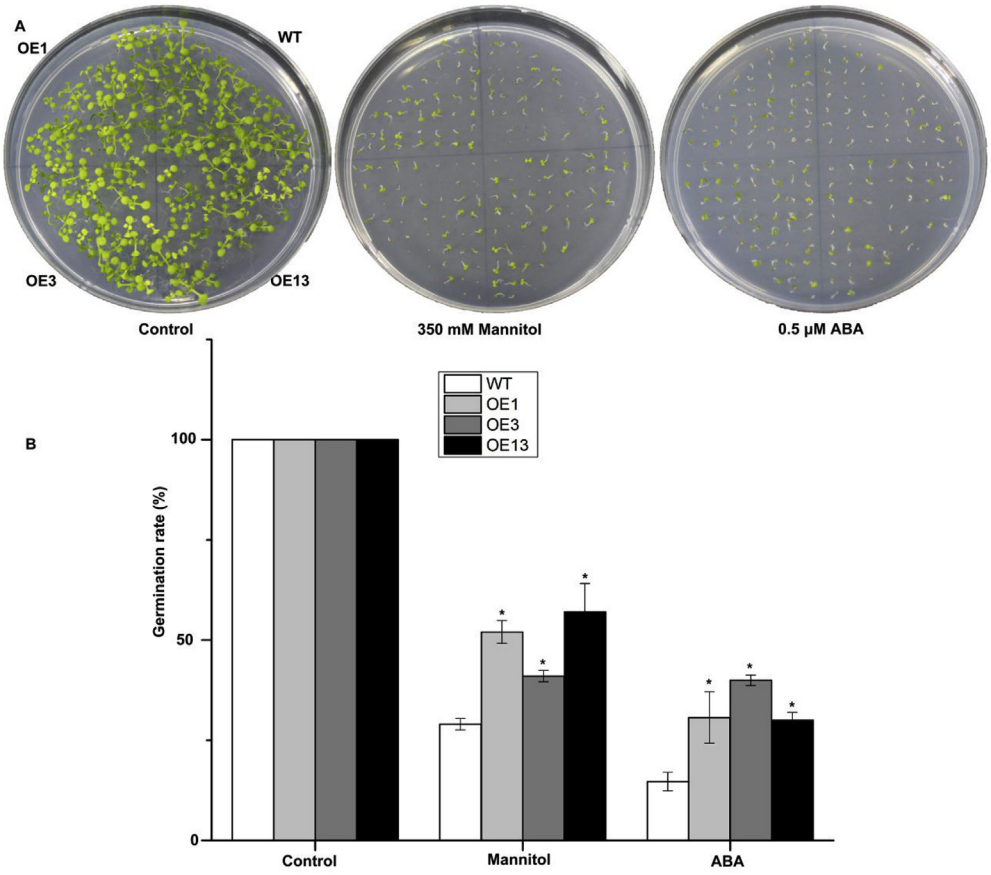

Fig. 3. Analysis of the seed germination rates of WT and *ZmNAC33* overexpression lines. A, The WT and *ZmNAC33* overexpression line seeds were grown on 1/2 MS media and containing 0.5 μM ABA or 350 mM mannitol. B, Seed germination rates were recorded before photographing. * significantly different at $P < 0.05$ (Student's t-test).

plants decreased at the slowest rate compared with WT. Overall, water loss rate assays revealed that overexpression plants had a lower rate of water loss at each time point (Fig. 7), thus rendering *ZmNAC33* overexpression plants more tolerant to drought stress.

3.8. *ZmNAC33* regulates the expression of stress-responsive genes under drought stress

Drought assays indicated that *ZmNAC33* overexpression plants had enhanced tolerance to drought. To evaluate differences in the expression levels of four stress-responsive genes of in *ZmNAC33* overexpression plants, qRT-PCR experiments were conducted. Four ABA signal transduction pathway-related genes, *AIB1*, *DREB2A*, *SAG13* and *COR47* were selected for further this analysis. The qRT-PCR results showed that the transcript levels of these four genes were significantly higher in both stressed and non-stressed transgenic plants than in WT. The relative expression levels of *ABI5* and *DREB2A* drastically increased; expression levels increased by 143.17-fold and 65.87-fold in OE3 line, respectively (Fig. 8). The expression levels of *ABI1* were slightly increased compared with those in WT. The relative expression levels of *SAG13* lightly decreased. These results show that exogenously expressed *ZmNAC33* regulates the expression of different endogenous *Arabidopsis* genes involved in abiotic stress tolerance and suggest that *ZmNAC33* enhances drought tolerance in *Arabidopsis* by regulating the expression of ABA-related and dehydration-responsive genes.

4. Discussion

Many genes that are related to drought stress have been identified in maize and *Arabidopsis*, the proteins encoded by these stress-induced genes protect cells not only through the production of important metabolic proteins (functional proteins) but also by regulating signal transduction genes (regulatory proteins) during the stress response (Takasaki et al., 2010). TFs operate as master switches of transcription, and so represent a critical component of gene regulation in response to abiotic stresses including salt, drought and osmotic. NAC family genes in particular are thought to play an important role in stress responses (Podzimska-Sroka, O'Shea et al., 2015). In this study, we characterized a stress-responsive gene, *ZmNAC33,* which encodes a nuclear-localized NAC-type DNA-binding protein and determined its subcellular localization. NAC domain of other NAC TFs has been shown to determine nuclear localization and interaction with other proteins (Sakuraba et al., 2015). NAC TFs have been implicated in interactions with other proteins, studies of ANAC suggested that NAC interactions may also regulate pathways controlled by the plant stress hormone ABA (Ernst et al., 2004). *ANAC016* binds strongly to the *AREB1* promoter and downregulates its expression under drought stress (Sakuraba et al., 2015). Because *ZmNAC33* localized in the cell nucleus, it may interact with other proteins. *ZmNAC33* may regulate drought stress-responsive pathways through similar mechanisms.

Sequence analysis showed *ZmNAC33* shares high identity with the reported stress-responsive NAC *ATAF1*, *OsNAC2* and *OsNAC6* (Mao et al., 2014; Chen et al., 2015). In a previous study of the HKI577 (drought tolerant) and HKIPC3-3 (drought susceptible) maize inbred lines subjected to dehydration stress, *ZmNAC33* was found to be up-regulated in the tolerant line and down-regulated in the susceptible line (Shiriga et al., 2014). Here, we found that *ZmNAC33* was strongly induced by PEG, ABA and NaCl stress compared with other NAC genes. *ZmNAC20* expression was increased in roots after 3 h under PEG treatment, then peaked at 12 h with a 6.3-fold higher expression than in the untreated sample, and declined thereafter. *ZmNAC20* increased and accumulated to the peak at 6 h with a 6.2-fold in roots under ABA treatment, and declined thereafter. *ZmNAC20* increased and accumulated to the peak at 12 h with a 1.6-fold in roots under NaCl treatment.

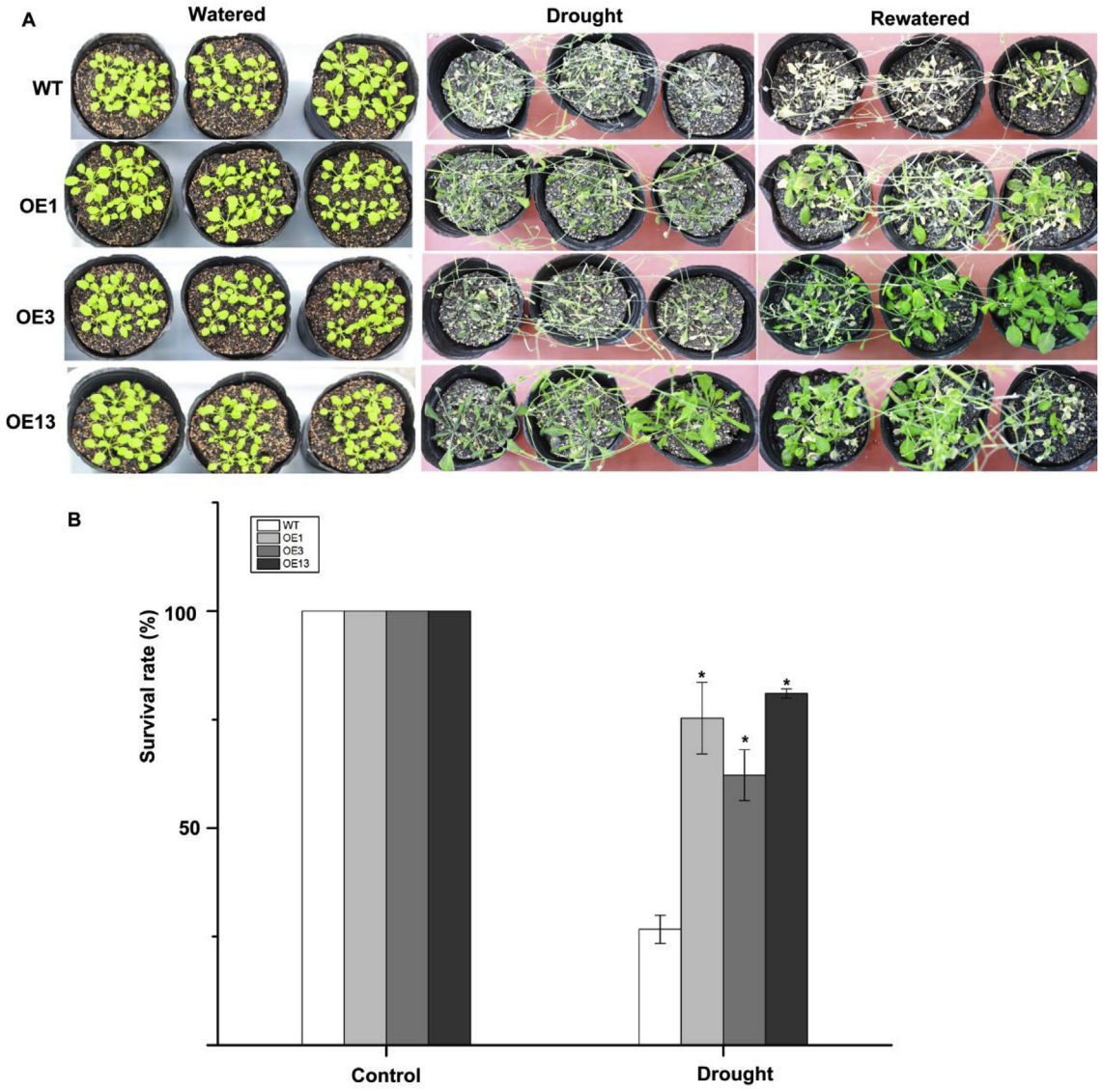

Fig. 4. Transgenic *Arabidopsis* plants overexpressing *ZmNAC33* have enhanced tolerance to drought. A, Phenotypes of *ZmNAC33* overexpression lines and WT were photographed after water dehydration for 21 d followed by rewatering for 5 d. B, Survival rates of WT and transgenic lines under drought stress. * significantly different at $P < 0.05$ (Student's t-test).

ZmNAC49 expression was increased to the highest level (4.4-fold) in roots after 12 h under PEG treatment, followed by a decrease. For ABA treatment, *ZmNAC49* expression was peaked at 24 h with 9.3-fold, and declined thereafter. *ZmNAC20* increased and accumulated to the peak at 12 h with a 1.5-fold in roots under NaCl treatment. *ZmNAC4* expression was increased to the highest level (2.1-fold) in roots after 6 h under PEG treatment, followed by a decrease. For ABA treatment, *ZmNAC4* expression was peaked at 6 h with 6.2-fold, and declined thereafter. *ZmNAC20* increased and accumulated to the peak at 24 h with a 2.8-fold in roots under NaCl treatment. *ZmNAC33* expression were significantly up-regulated under PEG, ABA and NaCl treatments compared with three NAC genes (data not shown). These results suggest that *ZmNAC33* responds to many stresses and may function in multiple stress processes, which is similar to the functions reported for other NAC genes.

A major *cis*-acting element in the promoter regions of ABA-responsive gene, the ABRE was found to be important for the expression of genes in response to ABA (Ying et al., 2012). Consistent with the induction of *ZmNAC33* by multiple stresses, the *ZmNAC33* promoter was found to contain several types of stress-responsive elements, such as the ABRE, DRE, and MYB and MYC TF recognition sites. The seed germination rates and root lengths of *Arabidopsis* plants overexpressing *ZmNAC33* were higher than those of WT under mannitol or ABA treatment. Reducing water loss from leaves is crucial for plant tolerance to drought stress, and leaves of plants overexpressing *ZmNAC33* had a lower water loss rate than those WT. WT and *ZmNAC33*-overexpression plants under drought stress, the leaves of seedlings were detached after being subjected to drought stress for 10 d, *ATAF1* expression was conducted by qRT-PCR. *ATAF1* expression was 3.6-fold higher expression than in the untreated sample in WT under drought stress, the expression levels of *ATAF1*increased from 2.3 to 3-fold in *ZmNAC33*-overexpression plants under drought stress. Just as *Arabidopsis ATAF1* study, overexpression *ATAF1* plants in *Arabidopsis* were survival ratio 94% and wild type plants were survival ratio 32% under drought stress (data not shown). Coincidentally, *ATAF1* from *Arabidopsis* exert similar functional effects, as they enhanced plant tolerance to drought (Wu et al., 2009). Overexpression *ZmNAC33* in *Arabidopsis* may functioned overexpression *ATAF1* plants. These results consistent with the drought tolerance phenotype of the overexpression lines.

The exposure of plants to various adverse environments, results in

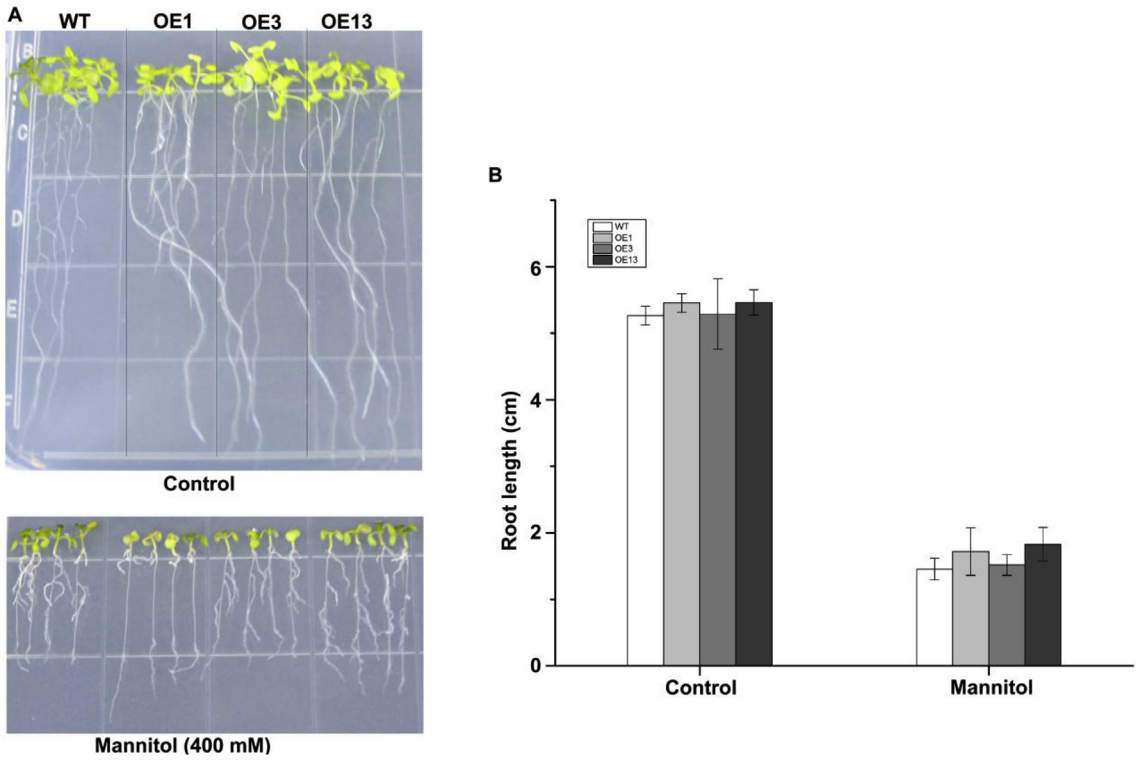

Fig. 5. Sensitivity of *ZmNAC33* overexpression plants to mannitol. A, Effects of mannitol on growth. B, Phenotypic comparison of root lengths in the presence and absence of mannitol, overexpression plants exhibited no significantly differences in root length from WT under osmotic stress ($P < 0.05$).

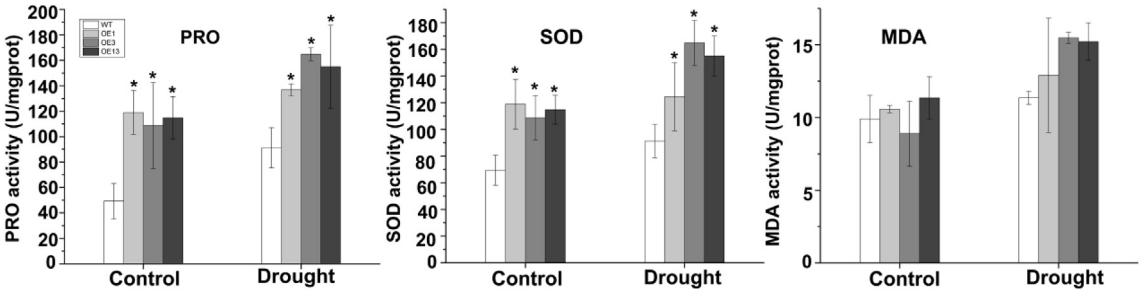

Fig. 6. Activities of antioxidant enzymes in WT and *ZmNAC33* overexpression plants. The MDA, PRO and SOD activities of WT and three overexpression lines were measured at 10 d after growth under drought stress or normal conditions. *: means differ from the control value ($P < 0.05$).

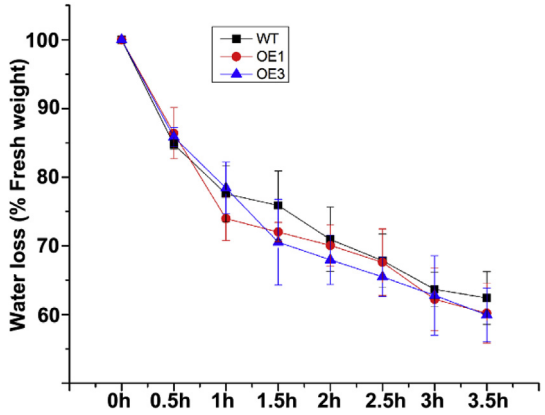

Fig. 7. Leaf water loss assay of WT and *ZmNAC33* overexpression plants. Leaf weight was measured at the indicated time point (n = 20), curves were drawn based on the data from three replicated experiments.

the accumulation of ROS; generation of oxidative stress; and damage to protein synthesis and stability, cellular macromolecules and membrane lipids in plant cells (Wang et al., 2017). A number of studies have previously reported the involvement of NAC TF family members in ROS and salicylic acid responses (Kim et al., 2018). The antioxidant enzymes, SOD, MDA and PRO, are known to play important roles in ROS scavenging, which influences the cellular ROS levels, such as PRO, SOD and MDA, form a complex antioxidative defense system that is vital for plant survival under abiotic stress conditions (Mittler, 2002). Thus, antioxidant enzymes levels have been used to estimate ROS-caused damage (Apse and Blumwald, 2002). We found that the antioxidant enzyme activities were higher in the *ZmNAC33* overexpression plants than in WT under drought stress, which suggests that *ZmNAC33* enhances the tolerance of plants to drought stress by increasing antioxidant enzyme activities and decreasing ROS levels.

Overexpression of *NAC* genes in *Arabidopsis* can enhance drought resistance. Consistent with this, different endogenous genes associated with drought stress response were found to be upregulated in transgenic *Arabidopsis* plants overexpressing *ZmNAC33*, and these plants showed more tolerance to drought stress and higher survival rates compared with the WT (Saad et al., 2013). In overexpression plants the ABA-

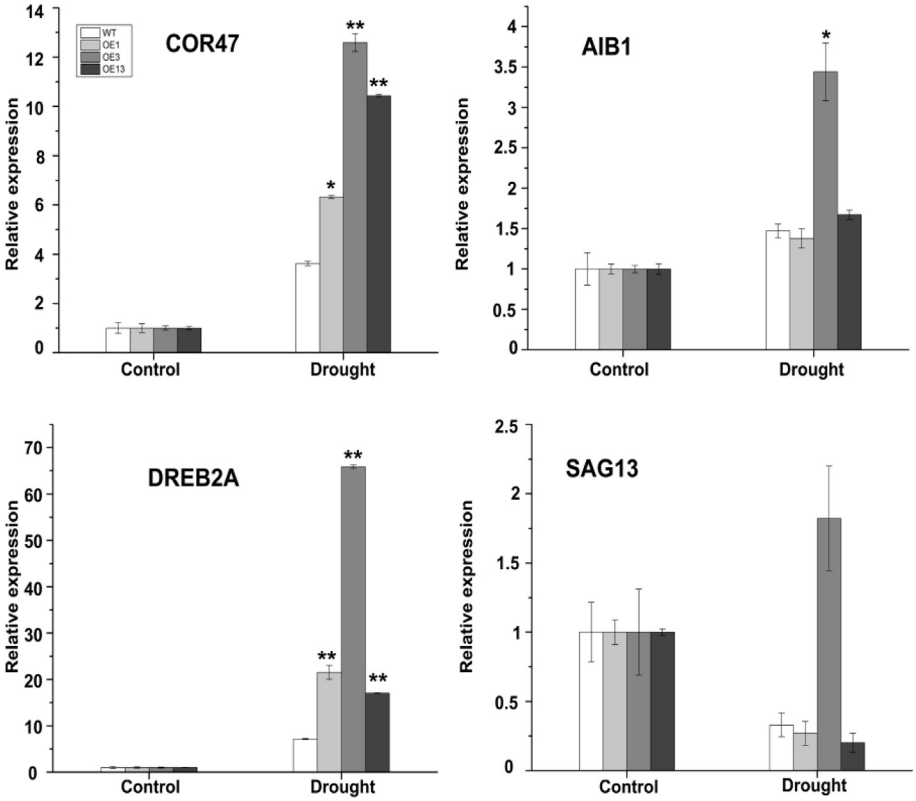

Fig. 8. Comparison of the relative transcript levels of *AIB1*, *COR47*, *SAG13* and *RD29B* in WT and *ZmNAC33* overexpression plants after 10 d of growth under control or drought stress conditions. *,**: means differ from the control value at, respectively, $P < 0.05$ and $P < 0.01$.

responsive marker genes *ABI1* and *COR47*, *DREB2A* were up-regulated compared with WT under drought stress. These genes have been reported to be involved in stress resistance. Thus, up-regulation of stress-related genes may explain the increased drought tolerance of *Arabidopsis* plants overexpressing *ZmNAC33*.

In conclusion, we elucidated the functions of *ZmNAC33* in drought stress responses. *Arabidopsis* plants overexpressing *ZmNAC33* had improved osmotic and ABA stress responses during seed germination and enhanced drought stress tolerance. Crop breeding to develop varieties tolerant to drought has emerged as one of the most urgent aims for modern agriculture. Plants have evolved several adaptations to survive drought. For example, drought stress triggers the accumulation of the ABA, and metabolites and amino acids (proline and glutamine) (Sakuraba et al., 2015). Our study of *ZmNAC33* clearly points to the great potential of exploiting the larger variability in the NAC family for targeted breeding efforts in crop plants (Podzimska-Sroka, O'Shea et al., 2015).

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Author contribution

Wenping Liu performed the experiments, analyzed the data and wrote the manuscript., Qian Zhang helped with RT-PCR analysis and data interpretation. Biligen-Gaowa Zhao and Chunxiao Zhang were involved in plant growth and drought treatment experiments. Qing Chao conducted the molecular experiments. Shufang Li and Fengxue Jin analyzed the data. Baichen Wang provided all financial support, and Baichen Wang, Xiaohui Li and Deguang Yang were involved in developing research ideas, experimental design and writing the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.038.

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