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Research article

ZmNAC55, a maize stress-responsive NAC transcription factor, confers drought resistance in transgenic *Arabidopsis*

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ABSTRACT

Abiotic stress has been shown to significantly limit the growth and productivity of crops. NAC transcription factors play essential roles in response to various abiotic stresses. However, only little information regarding stress-related *NAC* genes is available in maize. Here, we cloned a maize NAC transcription factor ZmNAC55 and identified its function in drought stress. Transient expression and transactivation assay demonstrated that ZmNAC55 was localized in the nucleus and had transactivation activity. Expression analysis of *ZmNAC55* in maize showed that this gene was induced by drought, high salinity and cold stresses and by abscisic acid (ABA). Promoter analysis demonstrated that multiple stress-related *cis*-acting elements exist in promoter region of *ZmNAC55*. Overexpression of *ZmNAC55* in *Arabidopsis* led to hypersensitivity to ABA at the germination stage, but enhanced drought resistence compared to wild-type seedlings. Transcriptome analysis identified a number of differentially expressed genes between *35S::ZmNAC55* transgenic and wild-type plants, and many of which are involved in stress response, including twelve qRT–PCR confirmed well-known drought-responsive genes. These results highlight the important role of ZmNAC55 in positive regulates of drought resistence, and may have potential applications in transgenic breeding of drought-tolerant crops.

1. Introduction

As sessile organisms, plants often encounter the adverse environment, such as drought, high salinity and extreme temperature, that frequently constrain their growth and development (Valliyodan and Nguyen, 2006). In order to combat and survive in these environmental adversities, plants have evolved a series mechanisms that involved in changes at whole-plant, tissue, cellular, physiological, and molecular levels (Shinozaki et al., 2003). The molecular mechanisms underlying plant adaptation to environmental stresses have been researched intensively, and transcriptional regulation of gene expression play an important role in this process. Transcription factors (TFs) and their corresponding *cis*-regulatory sequences act as molecular switches for gene expression, regulating their temporal and spatial expression (Badis et al., 2009). In plants, numerous transcription factors have been identified to involved in the regulation of plant stress responses, such as DREB, CBF, bZIP, zinc-finger, MYB, and NAC transcription factors (Shinozaki et al., 2003; Fujita et al., 2004).

NAC (NAM, ATAF1/2, CUC2) transcription factors family is a plant-specific transcription factor superfamily and is present in a broad diversity of plants (Olsen et al., 2005; Puranik et al., 2012). Consistent with their TF functions, members of the NAC family commonly harbor a highly conserved DNA-binding domain at their N termini, which comprise approximately 150 amino acid residues that are divided into five subdomains (A–E) (Olsen et al., 2005). However, the C-terminal regions of NAC family members are highly varied, suggesting that the C-terminal regions may have a role in determining specificity of binding to their respective target genes (Olsen et al., 2005). NAC TFs are associated with diverse biological processes, including cell division (Kim et al., 2006), shoot apical meristem development (Nikovics et al., 2006), secondary wall development (Mitsuda et al., 2005), lateral root development (Xie et al., 2000), leaf senescence (Guo and Gan, 2006; Kim et al.,

Abbreviations: TF, transcription factor; NAC, NAM, ATAF1/2 and CUC2; ABA, abscisic acid; ORF, open reading frame; GFP, green fluorescent protein; CaMV, cauliflower mosaic virus; qRT-PCR, quantitative reverse transcriptase PCR; WT, wild-type; ABRE, ABA-responsive element; DRE, dehydration-responsive element; CRT, C-repeat element; LTRE, low temperature responsive element; MYBRS, MYB recognition site; MYCRS, MYC recognition site.

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2009; Kim et al., 2013), and response to many biotic and abiotic stresses (Tran et al., 2004; Puranik et al., 2012; Nakashima et al., 2012).

Recently, considerable attention has been focused on the role of NAC TFs in the drought stress response (Lee et al., 2012; Nakashima et al., 2012; Puranik et al., 2012; Xu et al., 2013; Sakuraba et al., 2015). Some members have been identified and studied in model plants such as *Arabidopsis*, and crops such as soybean, rice, wheat and maize, and found to play important roles in drought stress signaling pathways. Three *Arabidopsis* NAC proteins, ANAC019, ANAC055 and ANAC072 were identified as significantly improving drought resistance in transgenic plants (Tran et al., 2004). In rice, when the stress-responsive *NAC* gene *SNAC1* was introduced into rice, the transgenic plants displayed significantly enhanced tolerances to multiple abiotic stresses (Hu et al., 2006). Several ABA-dependent NAC TFs in rice, including OsNAC5, OsNAC6 and OsNAC10, enhance drought tolerance in transgenic plants by regulating distinct target genes successively (Puranik et al., 2012; Nakashima et al., 2012). Recently, three novel NAC members from wheat, TaNAC2, TaNAC67, and TaNAC29 were characterized for their enhanced multi-abiotic stress tolerance in *Arabidopsis* (Mao et al., 2012, 2014; Huang et al., 2015). For soybean, the drought-inducible gene *GmNAC2* functions as a negative regulator in abiotic stress, whereas *GmNAC20* overexpression in plants induces enhanced salt and freezing tolerance (Hao et al., 2011; Jin et al., 2012). A maize NAC gene, *ZmSNAC1*, is strongly induced by drought, cold, high salinity, and abscisic acid (ABA) treatments. Overexpression of *ZmSNAC1* in *Arabidopsis* induces enhanced drought tolerance (Lu et al., 2012). Recently, *ZmNAC111* has been identified as important in maize drought tolerance; increasing *ZmNAC111* expression in transgenic maize improves water use efficiency and upregulation of drought-responsive genes under water stress (Mao et al., 2015). These studies indicate that NAC TFs have important roles in response to drought stress and that their overexpression can improve the drought tolerance of transgenic plants.

Although maize is one of the most planted crops world-wide, its productivity is frequently hampered by water scarcity. Thus, increasing drought tolerance is a priority target in maize breeding programs. In this study, a novel NAC transcription factor gene *ZmNAC55* was cloned from maize. Gene expression analysis demonstrated that *ZmNAC55* was upregulated by drought, high salinity and cold stresses and by ABA. Furthermore, ZmNAC55 is localized in the nucleus and functions as a transcriptional activator. *ZmNAC55*-overexpressing transgenic *Arabidopsis* showed enhanced sensitivity to ABA in the germination stage, and exhibited enhanced drought resistence through regulating many stress-responsive genes. Our results suggest that *ZmNAC55* play an important role in drought stress response and is expected to be used in genetically modified crops.

2. Materials and methods

2.1. Plant materials and stress treatments

Seeds of the maize inbred line B73 (*Zea mays* L. cv B73, from Northwest A & F University, China) were surface-sterilized in 1‰ (v/v) Topsin-M (Rotam Crop Sciences Ltd.) for 10 min. Then they were washed in deionized water and germinated on wet filter paper at 28 °C for 3 days. The germinated seeds were placed in a nutrient solution (0.75 mM K_2SO_4, 0.1 mM KCl, 0.25 mM KH_2PO_4, 0.65 mM $MgSO_4$, 0.1 mM EDTA-Fe, 2.0 mM $Ca(NO_3)_2$, 1.0 mM $MnSO_4$, 1.0 mM $ZnSO_4$, 0.1 mM $CuSO_4$, 0.005 mM $(NH_4)_6Mo_7O_{24}$) for hydroponic cultivation with a 16-h light/8-h dark cycle at 28 °C. Four kinds of treatments: drought, high salinity, cold and abscisic acid (ABA) were separately applied to three-leaf stage B73 seedlings. The high salinity and ABA treatments were conducted by culturing the seedlings in 200 mmol/L of NaCl and 100 μmol/L of ABA culture solutions, respectively. For drought treatment, the seedlings were placed on a clean bench and subjected to dehydration (28 °C, relative humidity of 40–60%). For cold treatment, seedlings were transferred to a growth chamber at a temperature of 4 °C. Leaves from a minimum of three seedlings were collected after 0, 5, 10 and 24 h for drought, high salinity and cold treatments; 0, 2, 5 and 10 h for ABA treatment. Field grown maize plants (*Zea mays* L. cv B73, from Northwest A & F University, China) were used for measuring organ-specific expression patterns of *ZmNAC55*. Young root, stem, and leaf (at the three-leaf stage), mature root, stem, and leaf (at anthesis stage), tassel, anther and ear were collected. All collected samples were immediately frozen into liquid nitrogen and stored at −80 °C for RNA extraction.

2.2. RNA extraction and quantitative real-time PCR

Total RNA was isolated using TRIZOL reagent (Biotopped, China) according to the manufacturer's instructions. RNA was treated with RNase-free DNase I (TaKaRa, China) to remove any genomic DNA contamination. First-strand cDNA was synthesized from 1 μg of total RNA using Recombinant M-MLV reverse transcriptase (Promega, USA). Quantitative real time-PCR (qRT-PCR) was performed in optical 48-well plates using an ABI7300 Thermo-cycler (Applied Biosystems, USA). Reactions were carried out in 10 μl volume, containing 1 μl diluted cDNA, 200 nM gene-specific primers, and 5 μl SYBR Premix Ex Taq II (TaKaRa) with the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. The specificity of the amplicon for each primer pair was verified by melting curve analysis. The expression of *ZmUbi-2* (UniProtKB/TrEMBL; ACC:Q42415) was used as an internal control. The $2^{-\Delta\Delta Ct}$ quantification method (Livak and Schmittgen, 2001) was used, with the variation in expression being estimated from three biological replicates. The primer pairs used for qRT-PCR analysis are listed in Supplemental Table 1.

2.3. ZmNAC55 isolation and sequence analysis

The gene sequence of *ZmNAC55* was downloaded from the 5b.60 version of the maize genome sequence database (http://www.maizegdb.org/). To obtain the full-length open reading frame (ORF) by the reverse transcription polymerase chain reaction (RT-PCR) approach, two primers were used (sense: 5′-ATGGGTCCTGAATCAGCT-3′ and antisense: 5′-TCAGAAGGGGCCCAACCCC-3′). The PCR conditions for amplifying *ZmNAC55* were as follows: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1.5 min at 72 °C; and then 10 min at 72 °C. The PCR products were purified and cloned into the pBluescript SK (pSK, Clontech) cloning vector for sequencing.

Multiple alignment of amino acid sequences was constructed using ClustalW (Chenna et al., 2003), using the Gonnet protein weight matrix, with multiple alignment gap opening/extension penalties of 10/0.5 and pairwise gap opening/extension penalties of 10/0.1. Phylogenetic tree was constructed with MEGA 5.0 software (Tamura et al., 2011) using the neighbor-joining (NJ) method. Bootstrapping was carried out on 1000 replicates with the pairwise deletion option.

2.4. In silico promoter sequence analysis

To identify putative *cis*-acting regulatory elements in the promoter region of *ZmNAC55*, the 1500 bp upstream of the coding region of *ZmNAC55* was selected as promoter sequence, and downloaded from the 5b.60 version of the maize genome sequence

database (http://www.maizegdb.org/). This sequence was used to search the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

2.5. Subcellular localization of ZmNAC55

The coding region of *ZmNAC55* without a terminator codon (TGA) was amplified using a pair of primers containing *Xba* I or *BamH* I sites (sense: 5′-TTTCTAGAATGGGTCCTGAATCAGCT-3′ and antisense: 5′-TTCCTAGGGAA GGGGCCCAACCCC-3′). The PCR products were digested with *Xba* I and *BamH* I and inserted into the *Xba* I and *BamH* I sites of the modified PBI121 (Clontech, USA) vector to generate ZmNAC55::GFP fusion protein. After sequencing confirmation, the recombinant plasmid and GFP alone were introduced into the *Agrobacterium tumefaciens* strain GV3101. These plasmids were then introduced into the onion (*Allium cepa*) epidermal cells by *Agrobacterium*-mediated method. After 24 h incubation on the MS medium in darkness at 25 °C, GFP fluorescence in transformed onion cells were observed using a confocal microscope (Olympus, FluoViewTM FV300, Japan).

2.6. Transactivation assay

For transactivation analysis of ZmNAC55 in yeast cell, the yeast strain AH109 (Clontech) was transformed with the appropriate bait vectors. The full-length coding sequences of *SNAC1*, *ZmSNAC1* and *ZmNAC55* were amplified using three pairs of primers with *Eco*RI- and *BamH* I sites. The primers are listed in Supplemental Table 1. The PCR products were digested with *Eco*Rland *BamH* I and were then cloned into the GAL4 binding domain vector pGBKT7 according to the manufacture's protocol (Clontech). Empty pGBKT7 vector was used as a negative control. The cell concentration of yeast transformants was adjusted to an OD_{600} of 0.1, and then plated on various selective plates, SD/-T, SD/-T-H, SD/-T-H-A, to compare their survival. Plates were incubated at 28 °C for 3 days before photographing.

2.7. Drought tolerance assay of transgenic Arabidopsis plants

The coding region of *ZmNAC55* was amplified from maize inbred line B73 by PCR with the following primers: 5′-TTTGCGGCCGCATGGGTCCTGAATCAGCT-3′ and 5′-TTGAGCTCTCA-GAAGGGGCCCAACCCC-3′. The PCR products were digested with *Not* I and *Xho* I and inserted into the pGreen vector (Qin et al., 2008) under the *CaMV 35S* promoter. The constructed plasmid was transformed into the GV3101 *Agrobacterium tumefaciens* strain containing the pSoup helper plasmid. *Arabidopsis thaliana* ecotype *Col-0* was transformed by *Agrobacterium*-mediated transformation. For selection of transformants, T_1 seeds were plated on MS medium containing 2% sucrose and 50 mg/mL kanamycin. Homozygous T_3 plants were used for phenotypic analysis. For the drought tolerance assays, seven-day-old plants germinated on MS medium were transferred into pots containing 150 gg 2:1 mixture of Jiffy mix and vermiculite. Thirty two-day-old plants growing under favorable water conditions were exposed to drought stress. Water was withheld from the plants for 14 days. Watering was then resumed to allow the plants to recover. Six days later, the number of surviving plants was recorded. At least 64 plants of each line were compared with wild-type (WT) plants in each test, and statistical data were based on data obtained from three independent experiments. The student's *t*-test function in MS Excel was used to assess the difference between wild-type and transgenic plants.

2.8. Stomatal aperture analysis

Stomatal apertures were measured as described previously (Pei et al., 1997). Leaves of similar size and age were sampled from wild-type and *35S::ZmNAC55* transgenic plants that had been subjected to drought for 10 d as described above. Rosette leaves were floated in solutions containing 30 mM KCl, 10 mM Mes-Tris, pH 6.15, and exposed to light for 3 h. A light microscope (Olympus ix71, Tokyo, Japan) was used to examine the stomata on epidermal strips obtained from rosette leaves. The width and length of stomatal pores, as determined by the software Image J (http://rsbweb.nih.gov/ij), were used to calculate stomatal apertures (ratio of width to length). The student's *t*-test function in MS Excel was used to assess the difference between wild-type and transgenic plants.

2.9. Germination assay

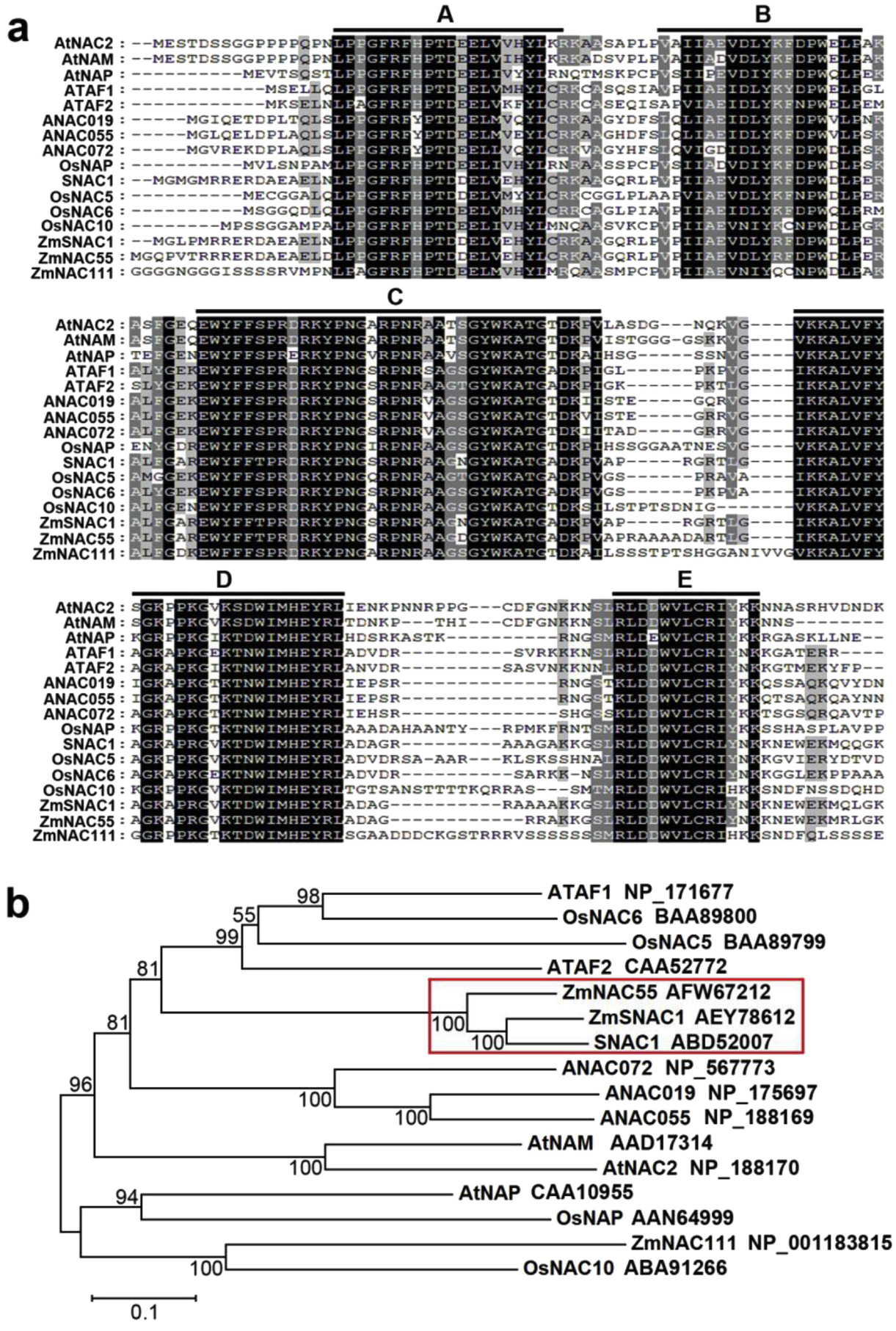
The wild-type and *35S:ZmNAC55* transgenic plants were grown in parallel and harvested. Seeds obtained from these plants were planted on 1/2 × MS plates containing 1% sucrose and were supplemented with or without different concentrations of ABA. Plates were chilled at 4 °C in the dark for 3 days for stratification and moved to 22 °C with a 16-h-light/8-h-dark cycle. The seeds were regarded as germinated when the radicles protrude from the seed coat. Germination (emergence of radicals) was scored on the 3rd day after germination, with three replicated assays. The student's *t*-test function in MS Excel was used to assess the difference between wild-type and transgenic plants.

2.10. Water loss measurement

Water loss rates were measured using 8 plants each of wild-type and *35S::ZmNAC55* transgenic plants. Three-week-old soil-grown plants were detached from roots and weighed immediately (fresh weight, FW), the plants were then left on the laboratory bench (humidity, 45–50%, 20–22 °C) and weighed at designated time intervals (desiccated weights). The proportions of fresh weight loss were calculated relative to the initial weights. Plants were finally oven-dried for 24 h at 80 °C to a constant dry weight (DW). Water loss was represented as the percentage of initial fresh weight at each time point. Three replicates were performed for each line. The student's *t*-test function in MS Excel was used to assess the difference between wild-type and transgenic plants.

2.11. RNA-seq analysis of transgenic Arabidopsis plants

For *Arabidopsis* RNA-seq analysis, pooled tissues from ten three-week-old *Arabidopsis* seedlings were collected from transgenic and wild-type plants under normal condition, to conduct the RNA-seq analysis. Total RNA was isolated using TRIZOL reagent (Biotopped, China) and RNA integrity was evaluated using a Bioanalyzer 2100 (Agilent). The 100-bp paired-end Illumina sequencing was conducted at Berry Genomics (Beijing, China). An average of 3 gigabases of raw data were generated for each sample. Differential gene expression was determined using Strand NGS 2.0 software. Enrichment analysis of gene ontology of biological pathways (GOBPs) was performed using the DAVID software program (Huang et al., 2009) to compute *P*-values that indicate the significance of each GOBP being represented by the genes. GOBPs with $P < 0.01$ were identified as enriched processes. Heat map was generated using Treeview software (http://rana.lbl.gov/EisenSoftware.htm). The hierarchical clustering was created using the default complete linkage method included in the Cluster software package (Eisen et al., 1998). qRT-PCR of selected genes that were determined to be critical to drought tolerance was performed to verify the RNA-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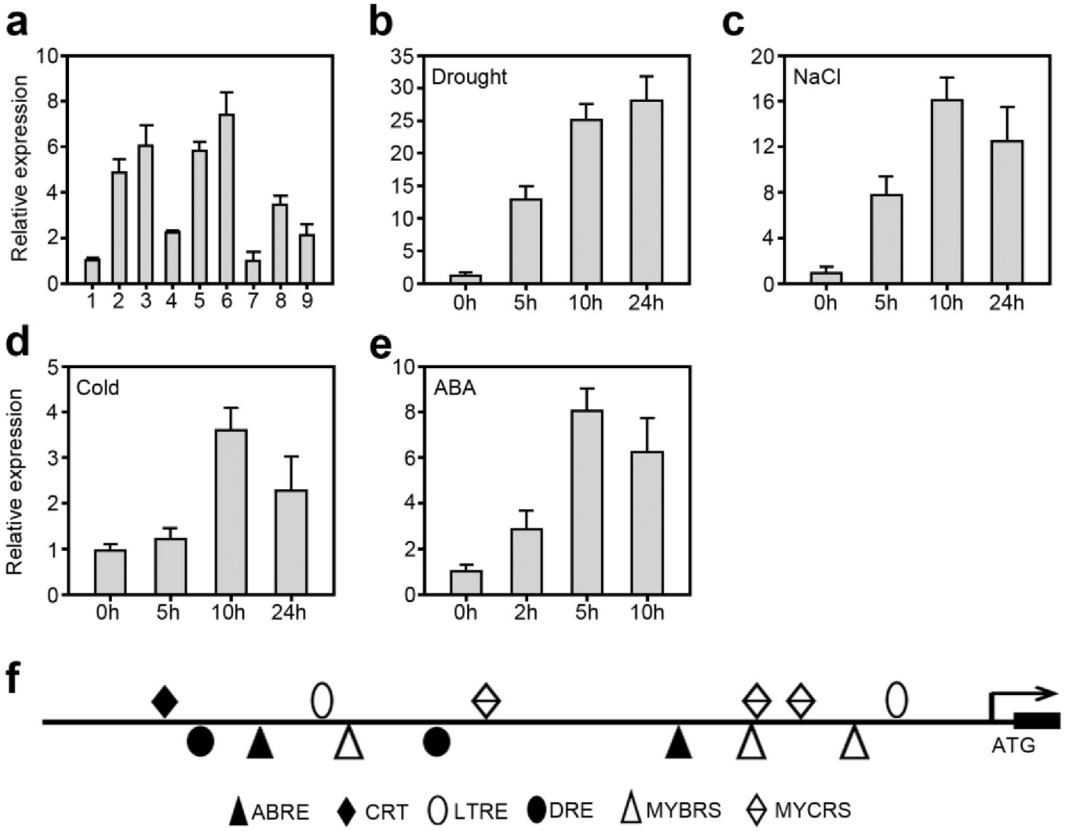

Fig. 2. The expression patterns of *ZmNAC55* in maize. (**a**) The expression profiles of *ZmNAC55* in different tissues under normal conditions. 1–3: root, stem and leaf at the seedling stage, respectively; 4–9: root, stem, leaf, tassel, anther and ear at the anthesis stage, respectively. (**b**–**e**) The expression pattern of *ZmNAC55* under drought, high salinity, cold and ABA treatments. The error bars indicate standard deviations derived from three independent biological experiments. (**f**) Distribution of several stress-related *cis*-elements in the promoter region (~1.5 kb) of *ZmNAC55*. ABRE, ABA-responsive element; DRE, Dehydration-responsive element; CRT, C-repeat element; MYBRS, MYB recognition site; MYCRS, MYC recognition site.

seq data. The primers are listed in Supplemental Table 1.

3. Results

3.1. ZmNAC55 encodes a plant-specific NAC transcription factor

In this study, we cloned a *NAC* gene from maize. This gene has been designated as *ZmNAC55* (GenBank ID: AFW67212) in previous study according it's chromosomal localization (Voitsik et al., 2013). The full length open reading frame (ORF) of *ZmNAC55* is 1311 bp, and encodes a polypeptide of 436 amino acid residues with a predicted molecular mass of 48.02 kD and pI value of 8.23. Sequence alignment revealed that ZmNAC55 contains a typical NAC structure with a conserved NAC domain including five subdomains (A, B, C, D, E), consistent with NAC conserved domain characteristics (Fig. 1a). Phylogenetic analysis revealed that ZmNAC55 belongs to a subgroup that also contains several stress-responsive NAC proteins, such as SNAC1, OsNAC6 and ZmSNAC1 (Lu et al., 2012; Nakashima et al., 2012) (Fig. 1b). ZmNAC55 showed high sequence similarity to members of the SNAC1 clade. According to NCBI blastp results, the amino acid sequence of ZmNAC55 was similar to that of ZmSNAC1 with an identity of 79% and to that of SNAC1 with an identity of 76%. All of these findings implied that ZmNAC55 is a new member of plant-specific NAC transcription factor family.

3.2. ZmNAC55 expression is induced by abiotic stresses

Temporal and spatial expression analysis revealed that *ZmNAC55* had relatively higher expression levels in the leaf and stem, with the highest expression level occurring in the leaf. However, *ZmNAC55* expressed at very low levels in the root, silk, tassel, and ear (Fig. 2a). To investigate the response of *ZmNAC55* to abiotic stresses, we performed quantitative real-time PCR (qRT-PCR) using RNA isolated from drought-, NaCl-, cold- and ABA-treated maize plants. The results showed that the expression of *ZmNAC55* was induced significantly by drought, high salinity (200 mM NaCl) and ABA (100 μM), but was induced weakly by cold (4 °C) (Fig. 2b–e). *ZmNAC55* was upregulated throughout the testing period by drought, high salinity, cold and ABA, while it was observed to plateau at the ten hour under high salinity, cold and ABA treatments, with the corresponding maxima being 16.28-, 3.77-, and 8.11-fold greater than the control (0 h), respectively (Fig. 2c–e).

Fig. 1. NAC domain sequence alignment and phylogeny of ZmNAC55 with orthologs. (**a**) NAC domain sequence alignment of ZmNAC55 and NAC members from other plant species. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. The locations of the five highly conserved amino acid motifs (A–E) are indicated by black lines. (**b**) Phylogenetic relationship between ZmNAC55 and typical stress-responsive NAC proteins. The multiple sequence alignment was performed using ClustalW, and the phylogenetic tree was constructed by MEGA5.0 using the neighbor-joining method. The numbers at each node indicate the percentage of bootstrap values from 1000 replicates. The scale bar indicates the relative amount of change along branches. The accession numbers are shown to the right of protein names.

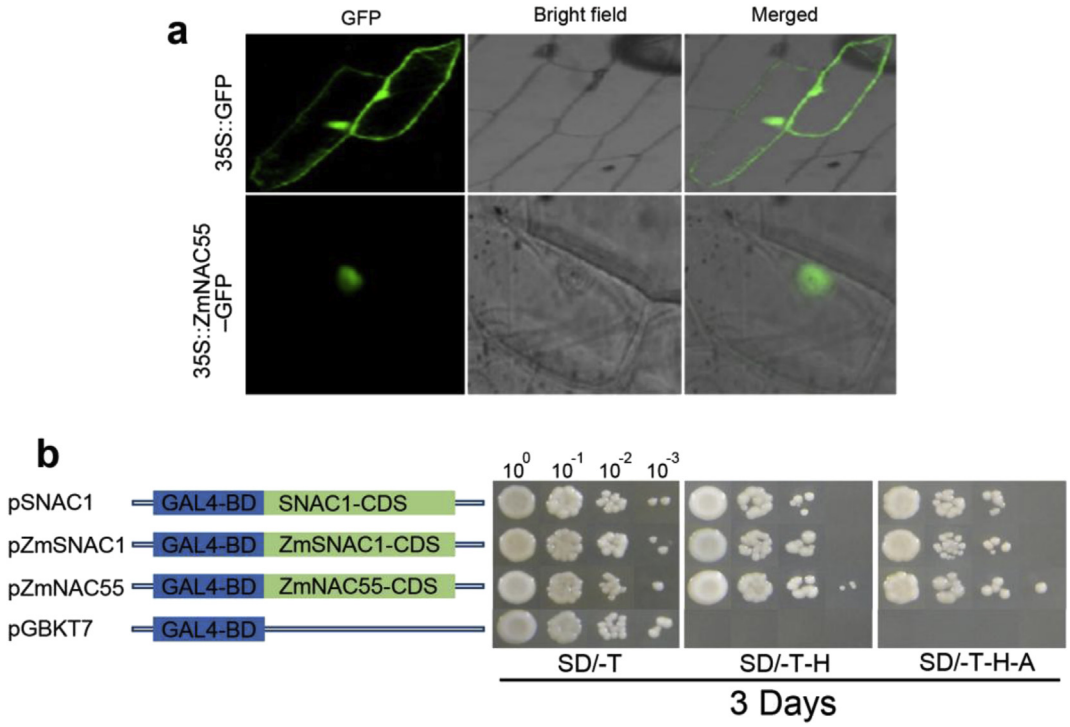

Fig. 3. Subcellular localization and transactivation activity assay of ZmNAC55. (**a**) Subcellular localization of *ZmNAC55*. The bottom panels show the localization of GFP-ZmNAC55 in onion cells in a transient assay, while upper panels show the localization of GFP as a control. (**b**) Transactivation activity assay of ZmNAC55 in yeast strain AH109. Cultures of the AH109 yeast, transformed with a plasmid containing *SNAC1*, *ZmSNAC1* and *ZmNAC55* genes, were diluted and inoculated on to a synthetic dropout (SD) media without tryptophan (SD/-T), without tryptophan and histidine (SD/-T-H), or without tryptophan, histidine, and adenine (SD/-T-H-A). Photos were taken 3 days after inoculation for the plates.

The increased expression levels of *ZmNAC55* observed under abiotic stresses led us to exam its promoter region (~1500 bp upstream from transcription start site). As expected, a number of stress response-related *cis*-acting elements were present in the *ZmNAC55* promoter, such as ABRE (ABA-responsive element), DRE (Dehydration-responsive element), CRT (C-repeat element), LTRE (low temperature responsive element), MYBRS (MYB recognition site) and MYCRS (MYC recognition site) (Yamaguchi-Shinozaki and Shinozaki, 2005) (Fig. 2f). This analysis, combined with stress-induced expression profiles of *ZmNAC55*, strongly suggests that *ZmNAC55* has broad functions in abiotic stress response and may play a prominent role in maize's response to drought stress in particular.

3.3. *ZmNAC55 is localized in the nucleus and exhibits transactivation activity*

Transcription factors are typically localized in cell nuclei where they perform DNA binding and transcriptional activation roles. To determine the subcellular localization of ZmNAC55, we transformed ZmNAC55-GFP construct under the control of the cauliflower mosaic virus (CaMV) 35S promoter into onion epidermis cells using *Agrobacterium*-mediated method. A strong green fluorescence signal of transformed cells carrying ZmNAC55-GFP was observed in the nuclei, while the control GFP protein was located in the cytoplasm and nuclei (Fig. 3a), demonstrating the nucleus localization of ZmNAC55.

In order to investigate whether this deduced protein had transactivation activity, the full-length CDS of ZmNAC55 and its two homologous fragments (SNAC1 and ZmSNAC1) were fused to the GAL4 DNA-binding domain (DBD) in the pGBKT7 vector. These constructs were transformed into yeast strain AH109, serially diluted, and then dropped on -Trp, -Trp/-His, and -Trp/-His/-Ade SD selection media. All transformants grew well on the selection medium, confirmed that the full-length ZmNAC55 had transactivation activity (Fig. 3b).

3.4. *Overexpression of ZmNAC55 enhances drought resistance in transgenic Arabidopsis*

The prominent expression of *ZmNAC55* under drought stress (Fig. 2b) prompted us to analyze its potential role in drought resistance. Three independent transgenic lines (*OE1*, *OE2* and *OE3*) were chosen for further analysis based on their expression levels (Fig. 4b). Wild-type (WT) and *35S::ZmNAC55* transgenic plants were grown for 3 wk in soils before water was withheld for 14 d. Most WT plants wilted, whereas the *35S::ZmNAC55* transgenic plants remained turgid and their leaves remained green after rewatering (Fig. 4a). When data were analyzed from three different experiments, 75–81% of the *35S::ZmNAC55* transgenic plants recovered from the stress after rewatering, which was significantly higher than for WT plants (Fig. 4c). Next, we measured the stomatal apertures of leaves from WT and *35S::ZmNAC55* plants grown in soil. Under normal conditions, the stomatal aperture index of WT plants was 0.43, and those of *OE1*, *OE2* and *OE3* plants were 0.44, 0.41 and 0.41, respectively (Fig. 4e). After 10 d of drought stress, the stomatal aperture indices of *OE1*, *OE2* and *OE3* plants decreased to 0.25, 0.18 and 0.16 (Fig. 4e), which was significant smaller than that of the WT. Consistent with these results, detached leaves of *35S::ZmNAC55* transgenic plants lost water much more slowly than those of WT plants after dehydration (Fig. 4d), suggesting that WT may have depleted the soil water more rapidly than *35S::ZmNAC55* transgenic plants, and thus wilted more quickly. Taken together, these data clearly demonstrate that overexpression of *ZmNAC55*

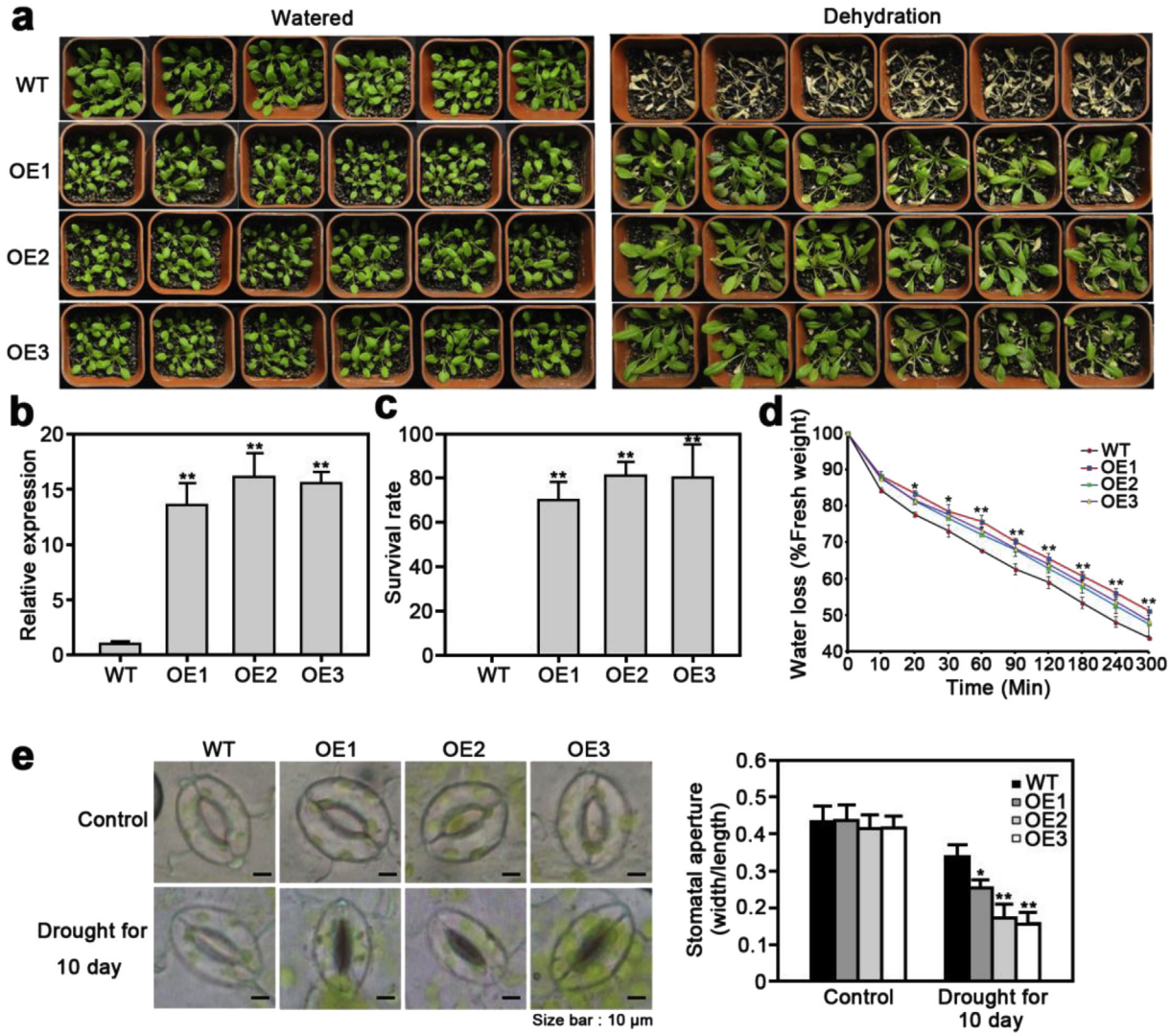

Fig. 4. Phenotype of the *35S:ZmNAC55* transgenic *Arabidopsis*. (**a**) Drought tolerance of transgenic *Arabidopsis* plants overexpressing *ZmNAC55*. Photographs were taken before and after the drought treatment followed by a six-day period of re-watering. Wild-type (WT) and *OE1*, *OE2* and *OE3* transgenic plants are shown. (**b**) qRT-PCR analysis of *ZmNAC55* transcript levels in the three independent lines. (**c**) Statistical analysis of survival rates after the drought-stress treatment. The average survival rates and standard errors were calculated based on data obtained from three independent experiments. Significant differences were determined by a *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$. (**d**) Water loss from detached rosettes of WT and *35S::ZmNAC55* transgenic plants. Water loss was expressed as the percentage of initial fresh weight. Values are means from 8 plants for each of three independent experiments. Significant differences were determined by a *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$. (**e**) Stomatal closure of WT and *35S::ZmNAC55* transgenic plants. Values are mean ratios of width to length. Error bars represent standard errors of three independent experiments ($n = 45$). Bars, 10 μm.

can confer drought resistance on transgenic *Arabidopsis*.

3.5. Overexpression of ZmNAC55 increases plant sensitivity to ABA during germination

To examine changes in the response of *35S::ZmNAC55* transgenic plants to ABA, we investigated the ABA sensitivity of the transgenic plants. WT and *35S::ZmNAC55* transgenic plants were germinated on MS medium supplemented with 0 μM, 0.5 μM and 1 μM ABA, respectively, and the germination rate was compared in the following 7 days. It is observed that *35S::ZmNAC55* transgenic plants exhibited lower germination rate than WT when exposed to ABA conditions (Fig. 5a,b). Consistently, the subsequent greening rate of *35S::ZmNAC55* transgenic plants seedlings was also lower than WT when exposed to ABA conditions (Fig. 5a,c). The results suggest that, in comparison to WT plants, overexpression of *ZmNAC55* makes transgenic *Arabidopsis* hypersensitive to ABA.

3.6. Global expression changes in ZmNAC55 overexpression plants

RNA sequencing was performed in order to further elucidate the mechanisms of improved drought resistance mediated by the overexpression of *ZmNAC55*. We compared the transcriptome of the *35S::ZmNAC55* transgenic and WT plants under normal condition. A total of 1026 and 763 genes were found to be upregulated and downregulated by twofold in the transgenics compared with WT, respectively (Fig. 6a,b; Supplemental Table 2). Biological pathways responsive to water deprivation, abscisic acid, hormone and abiotic stimuli were greatly enriched among these identified upregulated genes, whereas those responsive to hydrogen peroxide, oxidative stress and secondary metabolic process were especially enriched among the downregulated genes (Fig. 6c). We hypothesized that these transcriptomic changes may contribute to the early reduction in water loss, quick stomatal closure of transgenic *Arabidopsis* plants under drought stress.

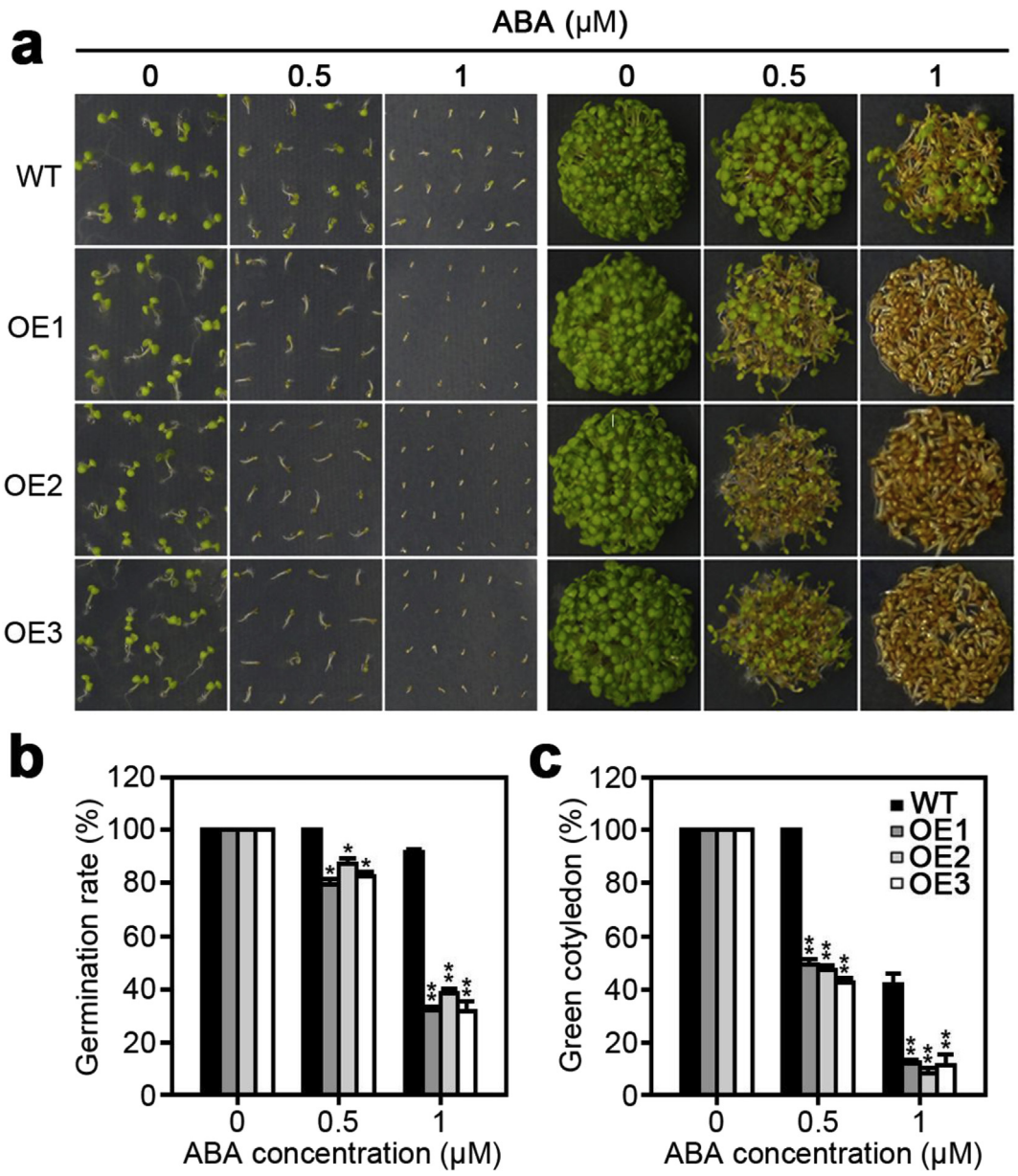

Fig. 5. ABA sensitivity of *35S:ZmNAC55* transgenic lines at the germination stage. (**a**) Growth of WT and *35S::ZmNAC55* seedlings on Murashige and Skoog (MS) medium containing 0.5 μM and 1 μM ABA. Germination was scored by the appearance of radicals and plant images were obtained 7 d after placing seeds on the MS plates. (**b**–**c**) Statistical analysis of germination rates (**b**) and seedling greening rates (**c**) based on data obtained from three independent experiments using 100 seeds in each experiment. Significant differences were determined by a *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

There were 63 genes related to water deprivation and ABA response were significantly upregulated (FC > 2; $P < 0.001$) in *35S::ZmNAC55* transgenic plants, compared with the WT. Most of these genes contain copies of NAC recognition core sequence (CACG) (Tran et al., 2004; Hu et al., 2006) in their promoters, suggesting that they might be direct target genes of ZmNAC111 (Table 1). We selected twelve well-known drought-responsive genes, such as *DREB2A* (Sakuma et al., 2006), *RD29A* (Nakashima et al., 2006), *RD29B* (Nakashima et al., 2006), *LEA14* (Jia et al., 2014), *RD26* (Fujita et al., 2004), *RD17* (Nylander et al., 2001), *PP2CA* (Yoshida et al., 2006), *ZAT10* (Mittler et al., 2006), *RAB18* (Nylander et al., 2001), *ANAC019* (Tran et al., 2004), *NCED3* (Iuchi et al., 2001), and *RD20* (Aubert et al., 2010), that were upregulated in transgenic plants to evaluate their expression in transgenic and WT plants grown under normal and drought conditions by qRT–PCR analysis (Fig. 7). The results were consistent with the microarray results, indicated that the transcription of these genes may be positively regulated by ZmNAC55. Taken together, these data strongly suggest that the expression of many stress-responsive genes was increased to enhance the drought resistance of the transgenic plants.

4. Discussion

4.1. ZmNAC55 is a stress-responsive NAC transcription factor

NAC TFs constitute one of the largest families of transcription factors in plants, whose members participate in many regulatory and developmental processes. They have been well investigated in *Arabidopsis* and rice (Puranik et al., 2012; Nakashima et al., 2012). In

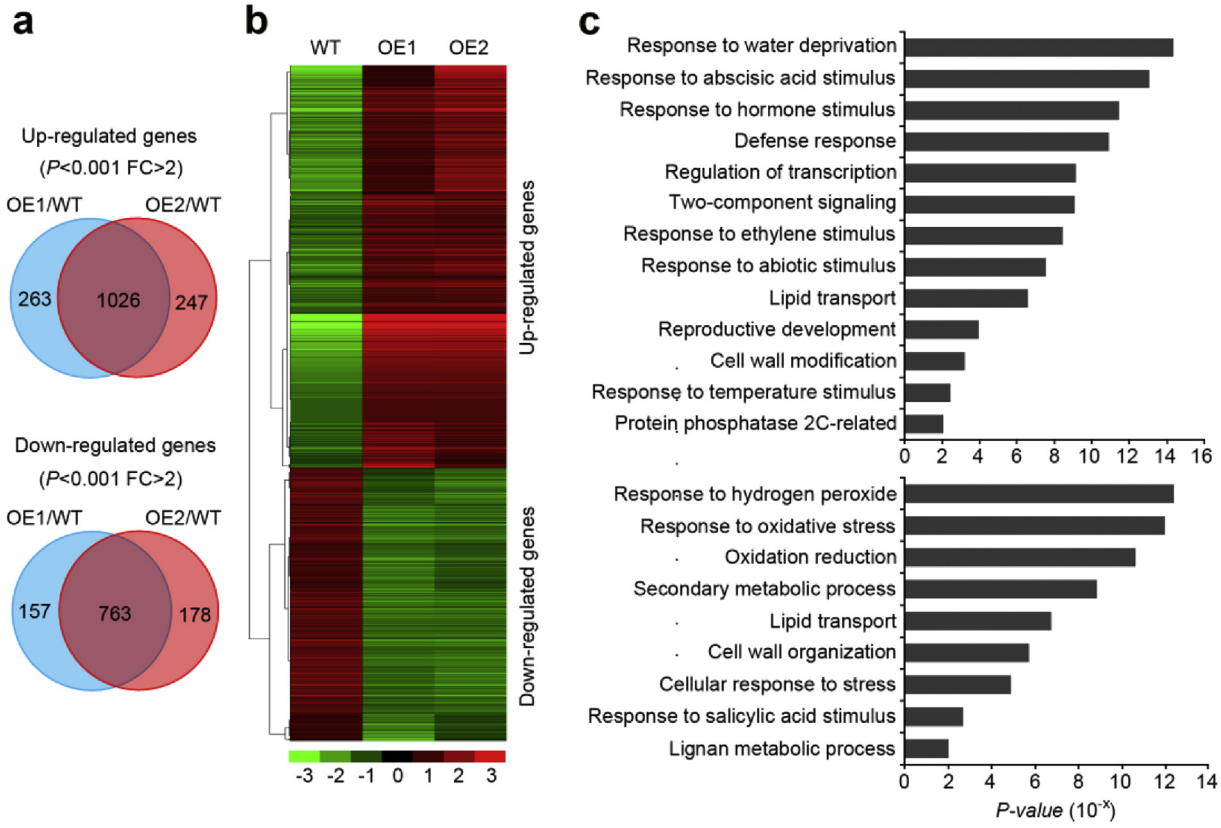

Fig. 6. Transcriptomic analysis of the *35S:ZmNAC55* transgenic *Arabidopsis* under normal condition. (**a**) Venn diagrams of up or downregulated genes in *OE1* and *OE2* plants relative to WT plants using a significant cutoff of $P < 0.001$, and a fold change (FC) > 2. (**b**) Hierarchical clustering of differentially expressed genes in the transgenic lines relative to WT plants. The data was obtained from the $\log_2$ value of the normalized level of gene expression. The indicated scale is the $\log_2$ value of the normalized level of gene expression. (**c**) Gene ontology of biological pathways enriched in the transgenic lines based on the up or downregulated genes.

maize, more than 116 predicted *NAC* genes have been identified recently (Voitsik et al., 2013). The typical NAC proteins share a well-conserved N-terminal NAC domain (~150 amino acids) and a diversified C-terminal transcription regulatory (TR) region (Olsen et al., 2005). In the present study, sequence analysis showed that ZmNAC55 contains a typical NAC conserved domain located in the N-terminal region, which can be divided into five subdomains (A–E, Fig. 1a). Subcellular localization analysis revealed that ZmNAC55 was localized in the nucleus (Fig. 3a) and transactivation assay demonstrated that ZmNAC55 functions as a transcriptional activator (Fig. 3b). These results are consistent with the previous research that the other *NAC* genes function as transcriptional activators (Hu et al., 2006; Lu et al., 2012). Moreover, ZmNAC55 has a significant sequence similarity with ZmSNAC1 (~79% sequence identity), SNAC1 (~76% sequence identity), and ATAF1 (~69% sequence identity), which makes them fall into the same subgroup in phylogenetic analysis (Fig. 1b). In this subgroup, *OsNAC5*, *OsNAC6*, *OsNAC10*, *SNAC1* and *ZmSNAC1* are strongly induced by abiotic stresses. Overexpression of these stress-responsive genes could significantly enhance stress tolerance (Hu et al., 2006; Lu et al., 2012; Nakashima et al., 2012).

For another, expression analysis showed that the *ZmNAC55* gene was induced by drought, high salinity, cold and exogenous ABA treatments (Fig. 2b–e). After sequence analysis, several kinds of stress-responsive elements were found in the promoter of *ZmNAC55* (Fig. 2f). ABRE, DRE, MYBRS and MYCRS can be recognized by the presence of AREB, DREB, MYB and MYC transcription factors, respectively (Yamaguchi-Shinozaki and Shinozaki, 2005). These transcription factors, in turn, have been shown to be involved in both ABA signaling and a variety of stress responses. The enrichment of these stress-responsive elements in the *ZmNAC55* promoter sequence may thus explain why *ZmNAC55* expression was responsive to stress. Taken together, these analysis combined with our experimental results, significantly indicating that ZmNAC55 is a stress-responsive NAC transcription factor, and might play an important role in response to various environmental stresses, especially in response to drought stress.

4.2. *ZmNAC55 functions in ABA signaling and confers the drought resistence of transgenic plants*

The prominent expression pattern under drought stress imply that *ZmNAC55* gene may play important role in the regulation of plant response to drought stress. To further identify the function of *ZmNAC55* in response to drought stress, we overexpressed *ZmNAC55* in transgenic *Arabidopsis*. The transgenic plants showed significantly improved drought resistance compared with WT plants (Fig. 4). Analysis of the transpiration rate of detached leaves indicated that the rate of water loss of the transgenic plants was lower than that of WT plants (Fig. 4d). Consistently, the stomatal close faster in transgenic plants than that of WT plants under drought conditions (Fig. 4e). These data provided evidence that ZmNAC55 can enhance transgenic plants drought resistence.

The plant response to abiotic stresses involves both ABA-dependent and ABA-independent signaling pathways (Agarwal and Jha, 2010). Our data demonstrated that overexpression of *ZmNAC55* made transgenic plants more sensitive to exogenous ABA than WT plants (Fig. 5). This increased sensitivity to ABA might be a

Table 1
The list of upregulated genes in *35S:ZmNAC55* transgenic *Arabidopsis* (FC > 2.0, $P < 0.001$) that involved in water deprivation and ABA stimulus response. The sequence, CACG, was used as the core NAC-recognition sequence (NACRS). The number of CACG motifs in the 1000-bp-promoter region of these genes is shown.

Gene ID	Gene symbol	FC	Description	CACG	Response
AT4G25490	CBF1	794.20	dehydration-responsive element-binding protein 1B	2	Drought, ABA
AT4G28520	CRU3	222.48	cruciferin 3	4	ABA
AT5G59310	LTP4	142.55	non-specific lipid-transfer protein 4	2	Drought, ABA
AT4G25480	DREB1A	101.60	dehydration-responsive element-binding protein 1A	2	Drought, ABA
AT5G51990	CBF4	72.29	dehydration-responsive element-binding protein 1D	2	ABA
AT4G21440	MYB102	65.32	MYB-like 102	3	ABA
AT3G14440	NCED3	36.39	9-cis-epoxycarotenoid dioxygenase NCED3	2	Drought, ABA
AT2G21490	LEA	35.64	dehydrin LEA	1	Drought
AT2G47770	TSPO	29.55	tryptophan-rich sensory protein-like protein	1	Drought, ABA
AT1G15360	SHN1	28.51	ethylene-responsive transcription factor WIN1	1	Drought
AT5G44120	CRA1	20.26	12S seed storage protein CRU4	3	ABA
AT1G27730	ZAT10	14.27	zinc finger protein STZ/ZAT10	2	Drought, ABA
AT5G45340	CYP707A3	13.75	abscisic acid 8'-hydroxylase 3	3	Drought, ABA
AT1G48130	PER1	12.44	1-Cys peroxiredoxin PER1	4	Drought
AT2G40170	GEA6	12.22	Em-like protein GEA6	7	Drought, ABA
AT5G52300	RD29B	11.98	desiccation-responsive protein 29B	6	Drought, ABA
AT5G52300	LTI65	11.63	CAP160 protein	3	Drought, ABA
AT3G51810	EM1	10.37	Em-like protein GEA1	3	ABA
AT1G03880	CRU2	10.18	cruciferin 2	2	ABA
AT5G62470	MYB96	8.33	myb domain protein 96	3	Drought, ABA
AT4G05100	MYB74	7.39	myb domain protein 74	3	ABA
AT1G56600	GolS2	7.30	galactinol synthase 2	5	Drought, ABA
AT1G32640	MYC2	6.85	transcription factor MYC2	4	Drought, ABA
AT2G38470	WRKY33	6.84	putative WRKY transcription factor 33	2	Drought
AT1G52890	ANAC019	6.54	NAC domain-containing protein 19	8	Drought, ABA
AT5G64750	ABR1	6.49	ethylene-responsive transcription factor ABR1	4	Drought, ABA
AT5G05410	DREB2A	6.07	dehydration-responsive element-binding protein 2A	6	Drought
AT1G05510	OBAP1A	5.70	uncharacterized protein	4	ABA
AT3G15210	ERF4	5.65	ethylene-responsive transcription factor 4	2	ABA
AT5G37770	TCH2	5.64	calcium-binding protein CML24	1	ABA
AT3G11020	DREB2B	5.29	dehydration-responsive element-binding protein 2B	2	Drought
AT2G41010	CAMBP25	5.22	calmodulin binding protein 25	2	Drought
AT2G17840	ERD7	5.17	senescence/dehydration related protein	3	Drought
AT1G20450	ERD10	4.97	dehydrin ERD10	4	Drought, ABA
AT3G11410	PP2CA	4.96	protein phosphatase 2C 37	4	Drought, ABA
AT2G33380	RD20	4.80	caleosin-related protein	7	Drought, ABA
AT5G59220	HAI1	4.45	protein phosphatase	5	Drought, ABA
AT5G52310	RD29A	4.25	desiccation-responsive protein 29A	4	Drought, ABA
AT3G57530	CPK32	4.11	calcium-dependent protein kinase 32	4	ABA
AT2G36270	ABI5	4.08	protein abscisic acid-insensitive 5	8	Drought, ABA
AT5G01540	LECRKA4.1	3.97	Lectin-domain containing receptor kinase A4.1	1	ABA
AT2G46510	AIB	3.87	transcription factor ABA-INDUCIBLE bHLH-TYPE	4	ABA
AT3G19580	ZF2	3.75	zinc-finger protein 2	3	Drought, ABA
AT5G59320	LTP3	3.72	non-specific lipid-transfer protein 3	2	Drought, ABA
AT2G35930	PUB23	3.70	E3 ubiquitin-protein ligase PUB23	2	Drought
AT1G29395	COR413IM1	3.58	cold regulated 314 inner membrane 1	4	Drought, ABA
AT1G47510	5PTASE11	3.12	Type I inositol-1,4,5-trisphosphate 5-phosphatase 11	2	ABA
AT3G47600	MYB94	3.05	myb domain protein 94	2	ABA
AT4G27410	RD26	3.04	NAC domain-containing protein 72	5	Drought, ABA
AT3G61890	HB-12	3.02	homeobox-leucine zipper protein ATHB-12	3	Drought, ABA
AT1G01470	LEA14	2.96	putative desiccation-related protein LEA14	8	Drought
AT3G14050	RSH2	2.82	RelA-SpoT like protein RSH2	2	ABA
AT3G23920	BAM1	2.81	beta-amylase 1	4	Drought
AT5G66400	RAB18	2.56	dehydrin Rab18	7	Drought, ABA
AT4G17615	CBL1	2.46	calcineurin B-like protein 1	4	Drought, ABA
AT3G28910	MYB30	2.43	myb proto-oncogene protein	2	ABA
AT3G52450	PUB22	2.41	E3 ubiquitin-protein ligase PUB22	2	Drought
AT4G34390	XLG2	2.32	extra-large GTP-binding protein 2	7	ABA
AT1G18570	MYB51	2.31	myb domain protein 51	1	ABA
AT3G45640	MPK3	2.12	extracellular signal-regulated kinase 1/2	2	Drought, ABA
AT2G46270	GBF3	2.09	G-box binding factor 3	4	ABA
AT1G20440	RD17	2.06	dehydrin COR47	2	Drought, ABA
AT5G25370	PLDALPHA3	2.02	phospholipase D zeta	1	Drought, ABA

result of lower transpiration rates and faster stomatal closure in transgenic plants, leading to lower water loss and enhanced drought resistence. In addition, the expression levels of several ABA signal pathway genes, such as *RD17* (Nylander et al., 2001), *PP2CA* (Yoshida et al., 2006), *RAB18* (Nylander et al., 2001), *NCED3* (Iuchi et al., 2001), were shown to be much higher in transgenic plants than in WT plants (Fig. 7 and Table 1). These results, combined with the presence of ABRE element in *ZmNAC55* promoter region (Fig. 2f) and the observed induction of *ZmNAC55* expression following ABA treatment (Fig. 2e), led us to hypothesize that ZmNAC55 may be involved in ABA-dependent signaling pathway in response to abiotic stresses.

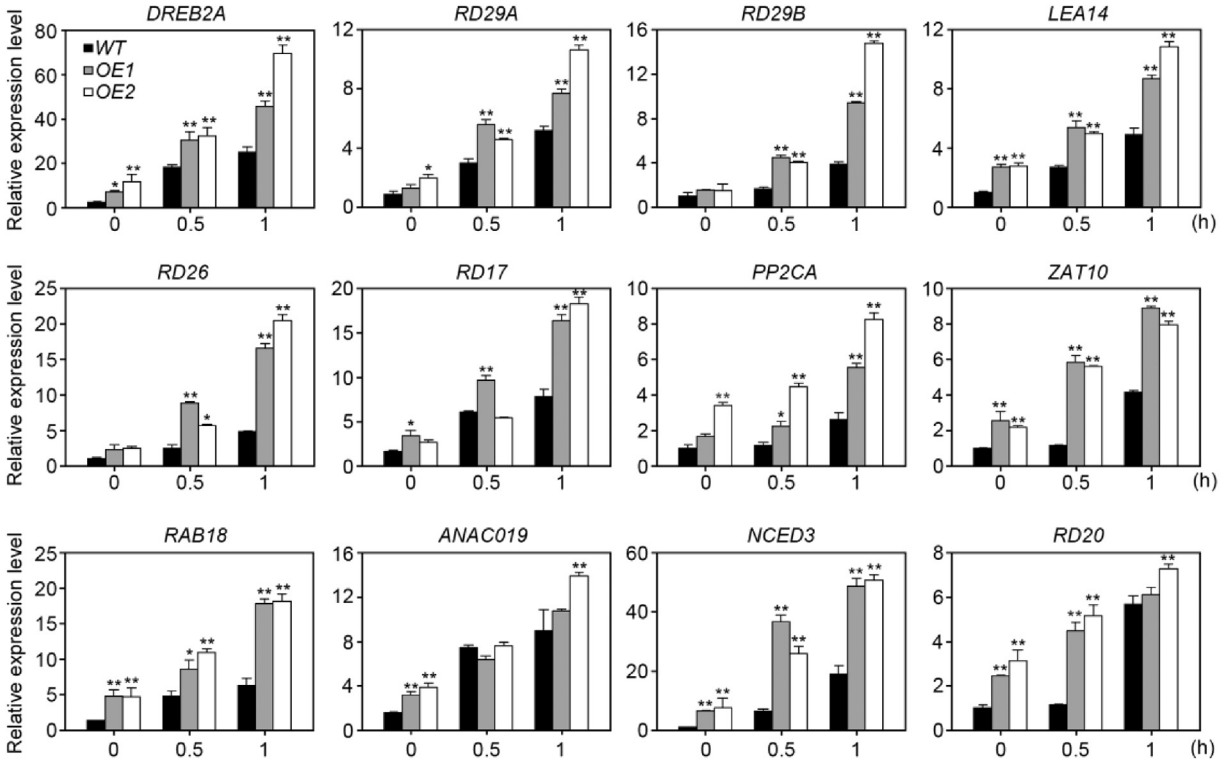

Fig. 7. qRT–PCR verification of increased expression of genes involved in plant drought response in the transgenics under normal and drought conditions. The error bars indicate standard deviations derived from three independent biological experiments. Significant differences were determined by a *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

4.3. *ZmNAC55 regulates stress-responsive gene expression*

As a transcription factor, ZmNAC55 may regulate the transcription of downstream genes in response to drought stress. Our RNA sequencing data demonstrated that overexpression of *ZmNAC55* can significantly change the expression levels of numerous genes (Fig. 6a,b). Among them, 1026 genes were significantly upregulated (FC > 2; $P < 0.001$) in transgenic plants and these genes were enriched in a number of stress-related biological processes, including water deprivation and ABA response (Fig. 6c). We selected some well-known drought- or ABA-responsive genes (*DREB2A*, *RD29A*, *RD29B*, *LEA14*, *RD26*, *RD17*, *PP2CA*, *ZAT10*, *RAB18*, *ABI5*, *NCED3* and *RD20*) that showed changes in transcript levels in the transcriptome analysis and confirmed their transcript levels by qRT–PCR (Fig. 7). The results confirmed that all of these genes were upregulated in the transgenic plants.

NAC TFs mainly recognize the consensus *cis*-acting element CACG, or NAC-recognition sequences (NACRSs) (Tran et al., 2004; Hu et al., 2006). As might be expected, 63 upregulated genes related to water deprivation and ABA response contain copies of NAC recognition core sequence (CACG) in their promoters (Table 1), suggesting that they might be direct target genes of ZmNAC55. About one third of these upregulated genes encoded transcription factors. For example, it was reported that *DREB2A* is induced by drought, and overexpressing *DREB2A* resulted in significant drought tolerance in transgenic *Arabidopsis* plants (Sakuma et al., 2006). Overexpressing *NAC019*, *RD26* and *ATHB12* also showed increased tolerance to drought stress (Tran et al., 2004; Fujita et al., 2004; Ré et al., 2014). It is also noticed that some ABA-responsive genes were upregulated in transgenic plants, such as *ZAT10*, *RD20*, *RAB18* and *COR47*, overexpression of these genes can significantly increased drought tolerance of transgenic plants (Nylander et al., 2001; Mittler et al., 2006; Aubert et al., 2010). Members of the PP2C family have been proposed to mediate drought tolerance (Singh et al., 2010). In this study, one PP2C member, PP2CA was upregulated in transgenic plants. 9-*cis*-epoxycarotenoid dioxygenase (NCED) is thought to be a key enzyme in ABA biosynthesis. Here we demonstrate that the expression of an *NCED* gene of *Arabidopsis*, *AtNCED3*, was upregulated in transgenic plants. *Arabidopsis* plants overexpressing *AtNCED3* showed a reduction in transpiration rate from leaves and an improvement in drought tolerance (Iuchi et al., 2001). Taken together, *ZmNAC55* triggered the constitutive expression of many stress-related genes. These genes could be considered as direct or indirect downstream genes of *ZmNAC55* and further works still need to uncover the *ZmNAC55* related signal transduction during abiotic stress.

In conclusion, our results showed that ZmNAC55 is a stress-responsive NAC transcription factor in maize. Overexpression of *ZmNAC55* in *Arabidopsis* resulted in a lower rate of water loss and significantly increased drought resistance via an ABA-dependent pathway. This study suggest that *ZmNAC55* functions as a positive regulator of drought stress and may have great promise for the genetic improvement of stress tolerance in crops.

Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

H.D.M. and L.J.Y. conceived and designed the experiments. H.D.M., L.J.Y. and Z.J.L. performed the experiments. H.D.M., L.J.Y., Z.J.L., R.H. and H.L. analyzed the data. H.D.M. and L.J.Y. wrote the paper. All authors read and approved the final manuscript and have no conflicts of interest with regard to this research or its funding.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.04.018.

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