Plant Growth Regul (2017) 83:301–312
DOI 10.1007/s10725-017-0295-y

ORIGINAL PAPER

Genome-wide identification, classification and expression analysis of NAC family of genes in sorghum [*Sorghum bicolor* (L.) Moench]

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Received: 16 January 2017 / Accepted: 20 June 2017 / Published online: 3 July 2017

Abstract NAC transcription factors are involved in many biological processes via regulation of downstream target gene expression and play essential roles in regulation of plant growth and improving plant tolerance to abiotic stress. NAC transcription factors have been studied in various species, but little information is available regarding these factors in sorghum. Genome-wide investigation of potentially abiotic stress related sorghum NAC-type genes was performed. A total of 145 non-redundant *NAC* genes (*SbNAC1* –*SbNAC145*) were identified in the sorghum genome. These genes were distributed unevenly across the 10 chromosomes, and were divided into 16 groups based on sequence similarity. Gene structure analysis indicated that most *SbNAC* genes contained three exons and two introns, and had ten putative conserved motifs. Phylogenetic analysis indicated that the *SbNAC* genes with similar motif distributions were clustered into the same branch. Seven *SbNAC* genes, which were grouped into the stress-related subgroup, were isolated and have been confirmed to have transcriptional activity in yeast. *SbNAC* genes showed differential expression patterns over time in response to dehydration, salinity, cold, and phytohormone abscisic acid stress treatments, thus suggesting essential roles in plant responses to abiotic stress. In the germination stage, *SbNAC56* overexpression transgenic lines exhibited significantly enhanced hypersensitivities to ABA, NaCl and D-Mannitol. This may infer that *SbNAC56* may play essential roles in plants response to abiotic stresses in ABA dependent signaling pathway. Here, we present a comprehensive overview of the *SbNAC* genes and provide a foundation for future functional research regarding their biological roles in sorghum stress tolerance, even in the regulation of plant growth.

Keywords NAC · Transcription factor · Sorghum · Abiotic stress

Abbreviations

NAC	*N*AM, *A*TAF and *C*UC transcription factor
TF	Transcription factor
TM	Transmembrane
qRT-PCR	Quantitative real-time polymerase chain reaction
SD	Synthetic dropout
Chr	Chromosomes

Introduction

In the natural environment, plants are constantly exposed to a variety of abiotic and biotic stresses, such as salinity, drought, cold, wounding, and pathogen infection, which restrict plant growth and development (Ji et al. 2014). To cope with these adverse conditions, plants have evolved multiple adaptive strategies at the physiological, biochemical, and molecular levels (Broun 2004). Accumulating evidence indicates that numerous transcription factors (TFs),

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Electronic supplementary material The online version of this article (doi:10.1007/s10725-017-0295-y) contains supplementary material, which is available to authorized users.

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such as NAC (*N*AM, *A*TAF1/2 and *C*UC2), MYB, AP2/EREB, and bZIP, play essential roles in stress response via binding to the *cis*-acting elements in the promoter regions of target genes (Guo et al. 2015).

NAC proteins comprise one of the largest families of plant-specific TFs and have been considered the key regulators in plant responses to abiotic and biotic stresses (Zhao et al. 2016). NAC proteins share a common highly conserved DNA-binding domain in the N-terminal region, which consists of five subdomains (A–E). In contrast, a quite divergent sequence in the C-terminus was considered to be the transactivation domain (Ooka et al. 2003).

Increased evidence indicated NAC TFs play essential roles in plant growth and development. Moreover, functional studies of NAC proteins have demonstrated that they are involved in responses to drought, salinity, and cold stresses. For example, *ANAC019, ANAC055*, and *ANAC072* expression in *Arabidopsis* was induced by salinity, drought, and the phytohormone abscisic acid (ABA), respectively. Overexpression of these three *NAC* genes markedly enhanced the drought stress tolerance in transgenic *Arabidopsis* (Tran et al. 2004). Furthermore, *ANAC096* has been reported to be involved in the drought stress response through binding to ABA-responsive elements in the promoter regions of target genes (Xu et al. 2013). In wheat, the expression of *TaNAC67* was induced by ABA, as well as by salt and drought stresses, which suggested that it may play important roles in the abiotic stress response in the ABA signaling pathway (Mao et al. 2014). Overexpression of wheat *TaNAC2, TaNAC2a*, and *TaNAC69* significantly improved abiotic stress tolerance in transgenic plants (Mao et al. 2012; Xue et al. 2006, 2011). Under conditions of extreme temperature, ABA, gibberellic acid, and polyethylene glycol (PEG) stress treatments, 6 of 55 selected *NAC* genes in Chinese cabbage were significantly induced (Liu et al. 2014). *Solyc07g063410, Solyc04g009440, Solyc01g094490*, and *Solyc10g047060* in tomato were significantly upregulated by low temperature, PEG, and high salinity treatments (Su et al. 2015). In addition, several NAC members have been identified in chickpea. Overexpression of *CarNAC3, CarNAC6* and *CarNAC4* in chickpea significantly enhanced salt and drought tolerance in transgenic plants (Movahedi et al. 2015; Yu et al. 2016). Drought, methyl jasmonic acid (MeJA), and salicylic acid (SA) stress treatments increased the transcription levels of 13 *Miscanthus NAC* genes. *MlNAC5* and *MlNAC9* conferred enhanced drought stress tolerance and ABA hypersensitivity in transgenic *Arabidopsis* (Yang et al. 2015; Zhao et al. 2016). Recently, a stress-responsive *NAC* gene, *ZmNAC55*, was identified in maize and reportedly functions in drought stress tolerance (Mao et al. 2016). Salinity, cold, wounding, MeJA, and ABA induced the expression of *PpNAC2* and *PpNAC3*, which clustered with *Arabidopsis ATAF1* and *ATAF2*, respectively. A miniature inverted repeat transposable element (MITE) insertion in the *ZmNAC111* promoter negatively regulated drought stress tolerance in maize (Pascual et al. 2015; Mao et al. 2015).

Sorghum originated in northern Africa, now it is a tropical crop with a good ecological plasticity, being grown worldwide in the semiarid areas of Africa, India, Southeastern Asia and Australia (Endre et al. 2017; Kebede et al. 2001; Oprea et al. 2015). Its tolerance to drought and extreme temperatures make it is a typical drought-resistant model crop, which is important for production and research. Sorghum expresses a series of stress-related genes under various environmental stresses. To date, little information regarding *NAC* genes in sorghum has been reported, although NAC TFs are involved in regulating both abiotic and biotic stress responses, which pose challenges to the characterization of NAC family proteins in sorghum. In the present study, 145 SbNAC members were identified in sorghum and classified according to their amino acid sequence identity. Seven of them were isolated from sorghum XGL-1. Phylogenetic analysis, gene structure, chromosomal locations, transactivation activity, prokaryotic expression, expression profiles, and seeds germination were examined. This study will provide a basis for future clarification of their functions in sorghum stress responses.

Materials and methods

Plant material and growth conditions

The inbred sorghum line XGL-1 was provided by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. Compared to other tested sorghum genotypes, sorghum XGL-1 was considered as an outstanding drought stress resistant genotype according to our prior trial information in Xinjiang province. XGL-1 provided a relative ideal genetic background to clarify the molecular mechanisms of drought stress resistance.

Seeds were rinsed several times. Plants were grown at 28 °C with 16 h of light, and 22 °C with 8 h of darkness in a greenhouse.

Identification and bioinformatics analysis

Two approaches were used to identify the all putative NAC proteins in sorghum. First, the amino acid sequences of SbNAC proteins were downloaded from the Plant Transcription Factor Database version 4 (PlantTFDB; http://planttfdb.cbi.pku.edu.cn/). Then, "NAC" was used as a query to search against the Joint Genome Institute (JGI) annotation database (https://phytozome.jgi.doe.gov/).

Multiple alignments were performed with Clustal X (ver. 1.83) (Thompson et al. 1997). A phylogenetic tree was constructed by the neighbor-joining method with 1000 replicates on each node using MEGA 4.0 (Saitou 1987; Tamura et al. 2007). The structures of *SbNAC* genes were analyzed by Gene Structure Display Server 2.0 (http://gsds1.cbi.pku.edu.cn/). MEME online software (version 4.11; http://meme-suite.org/) was used to analyze the amino acid motifs that are represented by colored boxes.

Theoretical molecular weight (Mw) was calculated with online software (http://web.expasy.org/compute_pi/). TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the transmembrane (TM) domains. Chromosome location images were generated by MapChart software (version 2.2). Potential *SbNAC* gene pairs were identified with multiple sequence alignment.

Abiotic stress treatments

Seeds of the inbred sorghum line XGL-1 were grown in pots filled with vermiculite. For cold stress treatment, sorghum seedlings at the 3-leaf stage were transferred to a low-temperature (4 °C) growth chamber. For salt, drought, and ABA stress treatments, sorghum seedlings were transferred to Hoagland solution supplemented with 200 mM NaCl, 20% PEG, and 100 mM ABA, respectively. Shoots were separately harvested at 0, 1, 3, 6, and 24 h. Plant samples were frozen in liquid nitrogen and stored at −80 °C until analysis.

RNA extraction and quantitative real-time PCR analyses

Total RNA was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The first-strand cDNA was synthesized using a Thermo Scientific RevertAid™ First Strand cDNA Synthesis Kit (#K1622; Waltham, MA). qRT-PCR was performed using SYBR® Ex Taq™ (Takara, Dalian, China) and carried out with a LightCycler® 96 SW 1.1 PCR Detection System (Roche Diagnostics, Penzberg, Germany). The *18SrRNA* gene was used as an internal control. The relative expression level for each gene was calculated with the $2^{-\Delta\Delta CT}$ method. These experiments were repeated with at least three biological replicates.

Transactivation activity analysis

Transactivation activity analysis was performed in the yeast strain *AH109* (Clontech, Palo Alto, CA). The full-length cDNAs were amplified by PCR with forward and reverse primers containing *Eco*RI and *Bam*HI sites, respectively, and inserted into the corresponding sites of the pGBKT7 vector. Subsequently, the pGBKT7 vector (control) and pGBKT7—SbNAC6/SbNAC26/SbNAC46/SbNAC56 were separately transformed into yeast strain *AH109*. The transformants were spotted on synthetic dropout (SD) medium SD/-Trp, SD/-Trp/-His, SD/-Trp/-His/10 mM (20 mM/30 mM) 3-AT and SD/-Trp/-His/-Ade for 3 days at 30 °C, and β-galactosidase (X-Gal) activity was measured as described by Jin (Jin et al. 1997).

Prokaryotic expression of SbNACs

To investigate the functions of the *SbNAC* genes, the full-length cDNAs of *SbNAC6, SbNAC26*, and *SbNAC56* were inserted into the pET40b (+) vector to construct fusion vectors. The empty pET40b (+) vector was used as a negative control. The constructs were transformed into Rosetta (DE3) cells. The fusion proteins could be induced by 0.5 mmol/L IPTG for 8 h at 23 °C. The proteins were identified by SDS-PAGE as described by Li et al. (2012).

Arabidopsis transformation and seeds germination assay

To analyze its biological functions, *SbSNAC56-pCambia3301* recombinant vector was transformed into *Arabidopsis* Col-0 wild-type plants via Agrobacterium tumefaciens strain GV3101 and T3 homozygous were obtained. The WT and *SbSNAC56* overexpression transgenic lines were screened on MS medium plates that containing NaCl (150, 175 Mm), D-Mannitol (350, 400 mM), phytohormone ABA (1.5, 2.0 μM). The germination ratio of seeds was calculated. All experiments were repeated at least three times, and 45–50 seeds were used in each comparison.

Results

Identification of *NAC* genes in sorghum

The *SbNAC* members in sorghum genome DNA were identified in the PlantTFDB version 4. Initially, 180 putative *NAC* transcription factor genes were obtained. Then, 46 redundant SbNAC proteins were determined by multiple sequence alignment. Furthermore, "NAC" was used as a key word to search for NAC domain-containing proteins in the U.S. Department of Energy (DOE) JGI database, and 53 putative NAC transcription factor genes were obtained. Finally, 145 non-redundant SbNAC proteins, including seven membrane-bound proteins, were identified and used for further study (Fig. S1).

Specific primers were designed to amplify the full-length cDNAs of seven stress-related *SbNAC* genes: *SbNAC6, SbNAC17, SbNAC26, SbNAC46, SbNAC56,*

SbNAC58, and *SbNAC73* (GenBank accession numbers: KY348374, KY348378, KY348375, KY348376, KY348377, KY348379, and KY348380, respectively), which ranged in size from 873 to 1209 bp and encoded peptides of 290 to 402 amino acids (Table S1).

Phylogenetic, structural, and motif analyses of *SbNACs*

To clarify the phylogenetic relationships among the *SbNAC* genes, an unrooted phylogenetic tree was constructed. *SbNAC* members were clustered together and showed similar motif distributions. Most conserved motifs were found within the N-terminal region of the NAC domain, which suggested that these motifs may be essential for the biological function of NAC proteins. In addition, some well-known stress-responsive *NAC* genes were analyzed in the phylogenetic tree. Based on the results, sorghum *NAC* genes could be divided into 16 subgroups. According to the phylogenetic relationship, *SbNAC6, SbNAC26*, and *SbNAC56* belonged to the OsNAC3 subfamily, *SbNAC58* belonged to the ATAF subfamily, and *SbNAC46, SbNAC17*, and *SbNAC73* were clustered into the NAP subfamily (Fig. 1). And *SbNAC6* was defined as the paralogous gene of *SbSNAC1* in sorghum (the sequence similarity is 99.38%). Previous studies have confirmed that OsNAC3, ATAF, and NAP are a stress-related subgroup of the NAC superfamily.

Subsequently, we performed gene structure analysis to support the phylogeny reconstruction (Fig. 2a, b). Consistent with the results of phylogenetic analysis, *SbANAC*

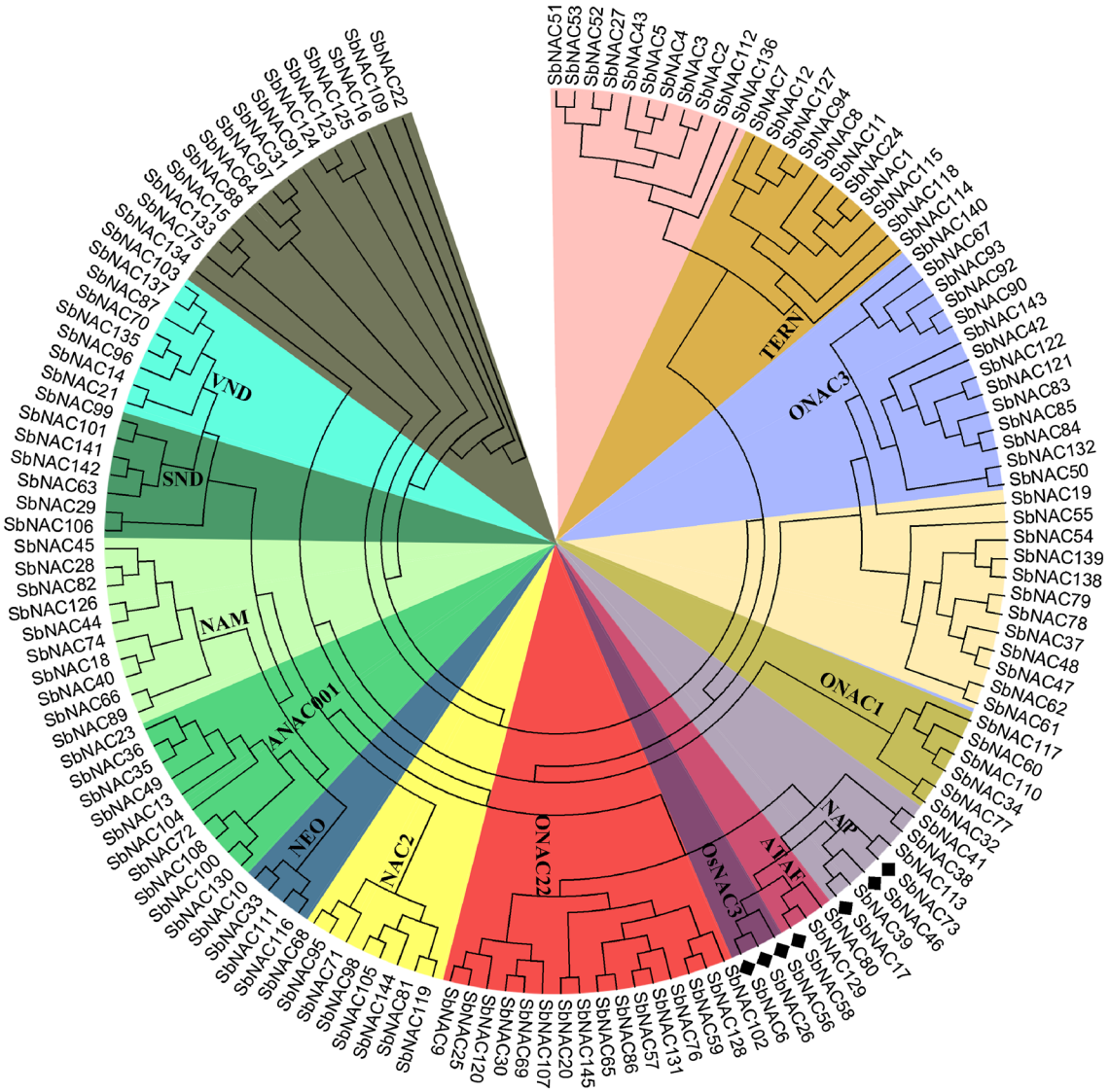

Fig. 1 Evolutionary analysis of SbNAC genes and some reported NAC proteins. 16 subfamilies were shown in *different colors*. (Color figure online)

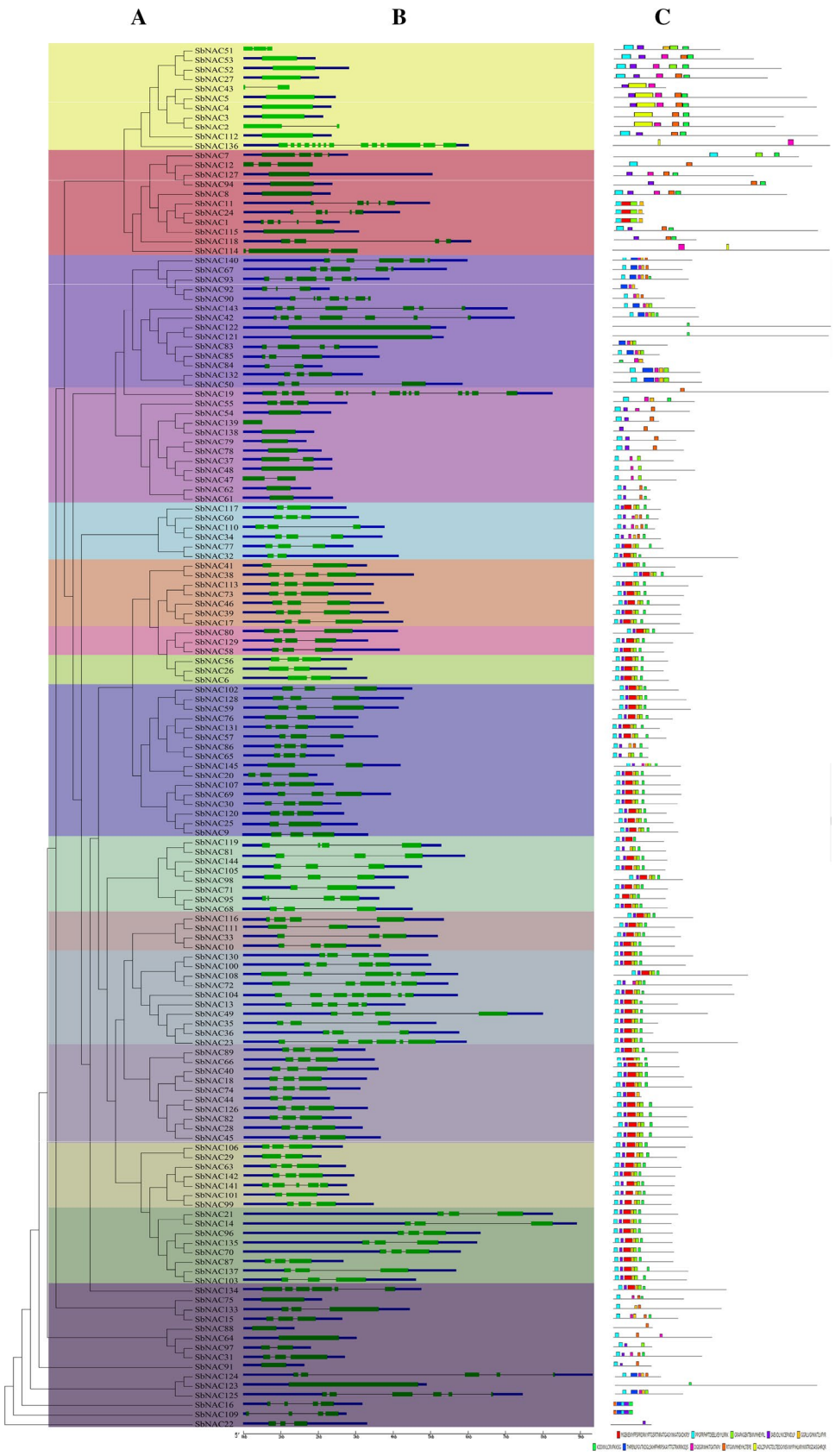

Fig. 2 Phylogenetic relationship, gene structure analysis, and motif distributions of *SbNAC* genes. **a** A phylogenetic tree was constructed by the neighbor-joining method with 1000 replicates on each node. **b** Exons and introns are indicated by *green rectangles and gray lines*, respectively. **c** Amino acid motifs in the SbNAC proteins (1–10) are represented by *colored boxes*. The *black lines* indicate relative protein lengths. (Color figure online)

genes that clustered in the same subgroup showed similar gene structures, especially with regard to the numbers of exons and introns.

To further determine the diversification of the NAC family in sorghum, 10 distinct conserved motifs were identified in sorghum NAC proteins (Fig. 2c). The NAC members with a close phylogenetic relationship shared common motif compositions. This gene structural similarity inferred similar biological functions in NAC proteins.

Chromosomal location and gene duplication of *SbNACs*

A total of 145 *SbNAC* genes were mapped on sorghum chromosomes (Chr) and showed an uneven distribution. Each of the SbNACs' name was given according to its physical position from the top to the bottom on the sorghum chromosomes 1 to 10. Chr1 and Chr3 contained the largest number of *SbNAC* genes (24 and 22 genes, respectively), while Chr8 contained the least (9 genes). Chr4 and Chr5 contained 10 *SbNAC* genes each (Fig. 3). As shown in Fig. 3, 19 (13%) paralogs have been identified in the sorghum *NAC* gene family, indicating the evolutionary relationship of these *NAC* members. Gene duplication was confirmed to play a major role in the expansion of gene families.

SbNACs exhibit transactivation activity

NAC-type proteins are characterized by a conserved N-terminal DNA-binding domain and transcriptional activation or repression region in the C-terminal (Hao et al. 2011; Ooka et al. 2003). Multiple sequence alignment revealed that the putative transcriptional activation region located in the C-terminus of these seven SbNAC proteins, which belong to OsNAC3, ATAF and NAP subfamily, respectively. To determine the SbNAC proteins transactivation activity, four NAC genes were selected randomly and the recombinant plasmids pGBKT7–SbNAC6/SbNAC26/SbNAC46/SbNAC56 were transformed into yeast strain *AH109*. Different transformants expressing fusion proteins in the yeast strain grew well on SD/-Trp, SD/-Trp/-His, SD/-Trp/-His/10 mM (20/30 mM) 3-AT, and SD/-Trp/-His/-Ade medium. The four NAC proteins showed transactivation activity. In contrast, transformants with the empty pGBKT7 vector (negative control) failed to grow. The results of yeast-based reporter and X-gal activity assays confirmed that *SbNAC6, SbNAC26, SbNAC46*, and *SbNAC56* had transcriptional activation activity in yeast (Fig. 4a).

The β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. Compared with the negative control (pGBKT7), four SbNAC proteins showed enhanced β-galactopyranoside activity in yeast Y187, among which, SbNAC56 showed the highest activity (Fig. 4b).

Expression of prokaryotic recombinant plasmid

Prokaryotic expression analyses indicated that the fusion proteins expressing pET40b-SbNAC6/SbNAC26/SbNAC56 were induced by IPTG treatment. SDS-PAGE analysis indicated that the Mw of these three SbNAC proteins were approximately 35.09, 31.90, and 34.76 kDa, respectively.

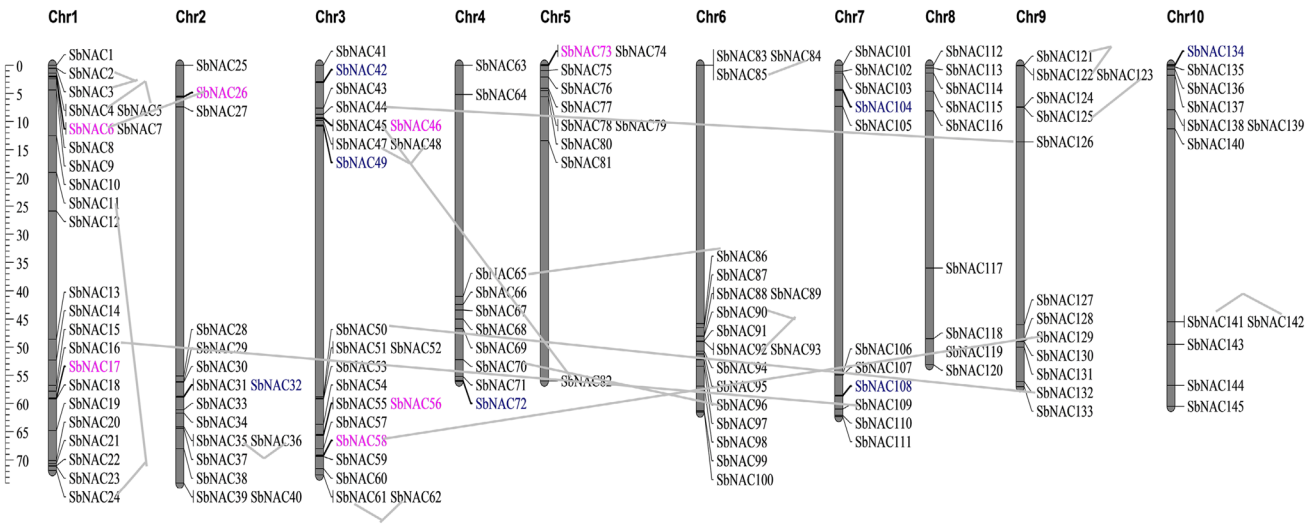

Fig. 3 Chromosomal location and gene duplicates of sorghum *NAC* genes. Seven stress-related and seven membrane-associated *SbNACs* are indicated in *pink* and *purple*, respectively. (Color figure online)

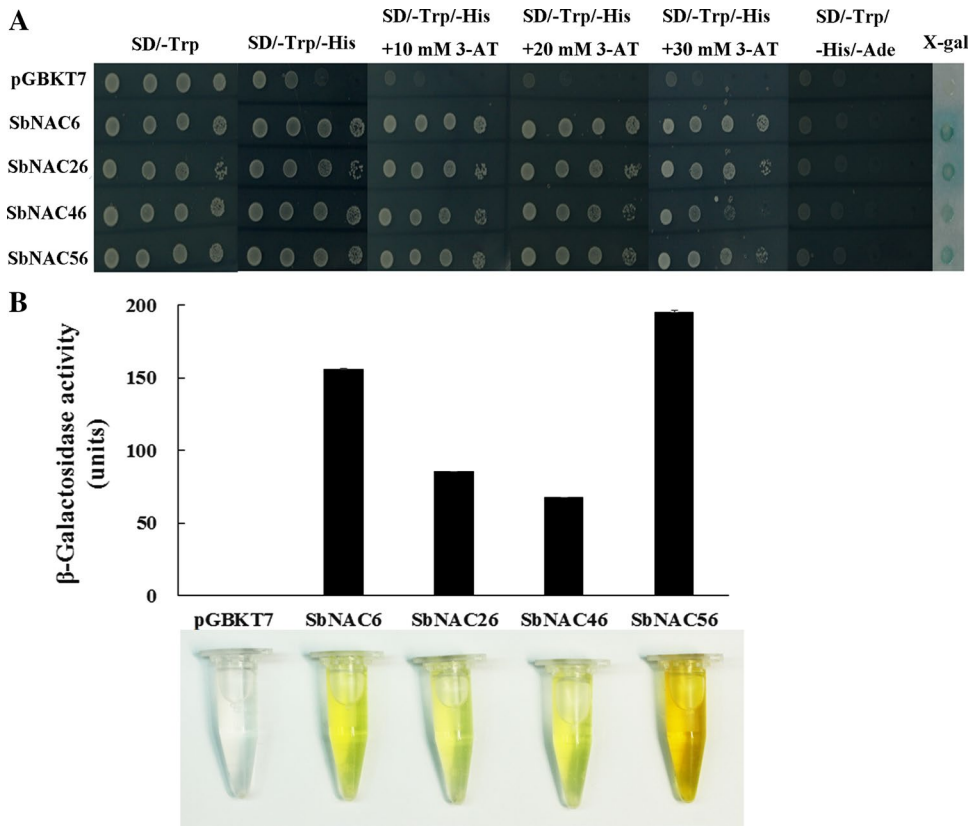

Fig. 4 Transactivation activity analysis of selected *SbNAC* genes in yeast. Transformants were streaked on SD/-Trp, SD/-Trp/-His, SD/-Trp/-His+10 mM (20/30 mM 3-AT), and SD/-Trp/-His/-Ade for examination of growth. **a** β-galactosidase activity assay was performed using X-α-Gal staining. **b** The β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. The *error bars* indicate standard deviation ($n=3$)

Expression patterns of *SbNAC* genes under different stress treatments

To explore the sorghum *NAC* gene functions in abiotic stress responses, the expression patterns of seven selected *SbNAC* genes, that localized in stress-related subgroups OsNAC3, ATAF and NAP, were analyzed. *SbNAC* genes were expressed at relatively higher levels under ABA and cold stress treatments (Fig. 5c, d).

As shown in Fig. 5a, the seven *SbNAC* genes were not significantly induced by control treatment (Hoagland solution). *SbNAC17, SbNAC46, SbNAC56, SbNAC58*, and *SbNAC73* were strongly induced by both PEG and ABA treatments. The transcript level peaked at 6 h, and was then downregulated at 24 h (Fig. 5b, c). Under conditions of cold stress, the expression levels of these selected SbNACs were commonly upregulated, among which, *SbNAC17* and *SbNAC73* were induced with relative higher expression levels (Fig. 5d). *SbNAC17, SbNAC46*, and *SbNAC73* exhibited a sustained dehydration stress response, and reached the highest level at 24 h (Fig. 5e). The transcripts of SbNACs were all increased in response to salt stress and reached the highest level at 3 h, then decreased at 6 h; among them, *SbNAC6, SbNAC26*, and *SbNAC58* showed the highest transcript levels (Fig. 5f).

SbNAC56 transgenic plants were hypersensitive to ABA and osmotic stresses

Abscisic acid (ABA) is synthesized under drought stress conditions (Shinozaki et al. 2003) and exogenous application of ABA could induce a number of dehydration responsive genes (Zhu 2002). The expression of *SbNAC56* was strongly induced by ABA, PEG and NaCl treatments (Fig. 5c). This may infer that *SbNAC56* may play essential roles in plants response to osmotic stresses in ABA dependent signaling pathway. To confirm this hypothesis, *35S::SbNAC56* transgenic plants were generated. Under 1.5 and 2.0 μM ABA stress treatment, the germination rates of the WT and *35S::SbNAC56* transgenic lines were 54 and 4%, 48 and 4%, respectively. Whereas no significant differences were detected on ABA-free medium. Similar differences could also be observed under NaCl (150, 175 mM), D-Mannitol (350, 400 mM) treatments(Fig. 7). The significantly increased hypersensitivity to ABA and osmotic stresses indicated that *SbNAC56* may work in ABA dependent signaling pathways in response to osmotic stresses.

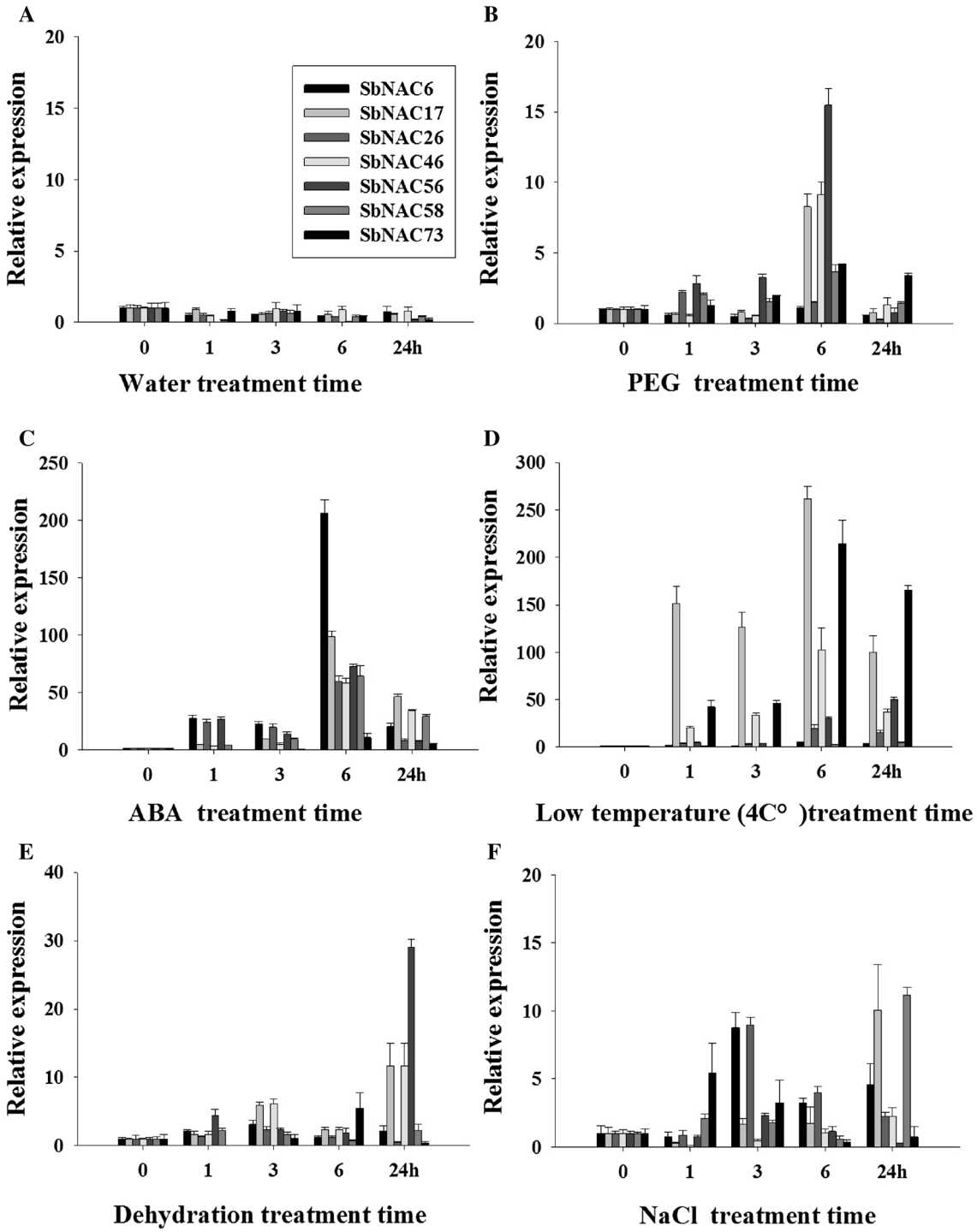

Fig. 5 qRT-PCR analysis of seven selected *SbNAC* genes. Relative expression profiles of seven selected *SbNAC* genes under (**a**) control, (**b**) PEG, (**c**) ABA, (**d**) cold, (**e**) dehydration, and (**f**) NaCl stress treatments. Values are means ± SD of at least three independent experiments

Discussion

To date, numerous NAC proteins have been identified in multiple species, including 104 in tomato (Xie et al. 2002), 204 in Chinese cabbage (Liu et al. 2014), 79 in grape (Wang et al. 2013), 163 in poplar (Hu et al. 2010), 157 in maize, 151 in rice, and 117 in *Arabidopsis* (Nuruzzaman et al. 2010). These proteins were shown to play essential roles in diverse aspects of plant growth and development, including modulation of leaf senescence (Guo and

Gan 2006), embryogenesis (Souer et al. 1996), lateral root development (He et al. 2005), and secondary wall synthesis (Mitsuda et al. 2005),JUB1 represses *GA3ox1* and *DWARF4 (DWF4)*, two key members involved in gibberellin (GA) and brassinosteroid (BR) biosynthesis, and regulated plant growth (Shahnejat-Bushehri et al. 2016, 2017). In Arabidopsis, NST2 together with SND1 and NST1 regulate the deposition of secondary walls in fibers of stems (Rui and Zheng. 2015). MiRNA164-directed cleavage of Arabidopsis *NAC1* affects lateral root development. Meanwhile, overexpression of maize *ZmNAC1*, a homologous gene of Arabidopsis *NAC1*, positively regulated lateral root development in Arabidopsis (Guo et al. 2005; Jing et al. 2012).

In addition, NAC proteins involved in plant defense responses to abiotic stresses. In this paper, a total of 145 *SbNAC* genes were identified through genome-wide analysis. Of these, 120 SbNAC genes had at least 1 intron, with the majority containing 2 introns (Fig. 2b). Putative conserved motifs were predicted, and 10 motifs were identified to explore the structural characteristics of the SbNACs. The conserved motifs were located in the N-terminal region of NAC proteins. Based on the results of phylogenetic analysis of SbNACs and some typical stress-related NAC TFs, seven predicted *NAC* genes were grouped into OsNAC3, NAP, and ATAF subfamilies (Fig. 1), which are reportedly involved in abiotic stress responses. In general, the NAC proteins with close phylogenetic relationships showed similar motif compositions. This will contribute to studies of the biological functions of NAC proteins (Fig. 2a, c).

Chromosomal location analysis indicated that *SbNAC* members were distributed on all 10 sorghum chromosomes. Some *NAC* genes often occurred in complexes. For example, *SbNAC* genes were clustered at the ends of the chromosome arms, including Chr2, Chr4, Chr5, and Chr6 (Fig. 3). Gene duplication may occur after the divergence of different species, and 19 NAC gene duplications were detected in sorghum. In this study, *SbNAC6* and *SbNAC26* were connected by a dashed line, as well as *SbNAC58* and *SbNAC129*. These duplicated genes showed significantly increased transcript levels under multiple abiotic stresses. Based on syntenic analysis, these *NAC* paralogs were retained and fixed with similar biological functions.

β-Galactosidase activity was examined to explore the transcriptional activation activity of sorghum *NAC* gene products. It has been reported that wheat *TaNAC4*, a homolog of *SbNAC56*, functions as a transcriptional activator. Significantly increased transcriptional activity of *SbNAC56* was particularly noticeable (Fig. 4a, b). While, *SbNAC6* performed increased β-galactosidase activity, suggesting the enhanced transcriptional activation (Fig. 4b). For its homolog gene in sorghum, *SbSNAC1* was confirmed to have relative higher β-galactosidase activity in the C terminus than in the other deletion regions. This indicated that *SbNAC6* functions as a transcriptional activator. To some extent, these stress-responsive *SbNACs* may have disparate regulatory mechanisms.

Prokaryotic expression analyses were presented to illustrate the Mw of these three SbNAC proteins. As shown in Fig. 6, the theoretical Mw values were consistent with the results of the SDS-PAGE analyses. For example, the theoretical Mw of pET40b-SbNAC6 was 35.42 kDa, while SDS-PAGE indicated 35.09 kDa.

The phytohormone ABA is synthesized under many conditions of environmental stress, including drought, cold, and high soil salinity (Wang et al. 2015). In this study, combined with phylogenetic analysis, we identified seven abiotic stress-responsive *NAC* genes in sorghum, among which *SbNAC17, SbNAC46*, and *SbNAC73* were significantly upregulated by various abiotic stresses and ABA treatment (Fig. 5). In comparison with the control treatment, the

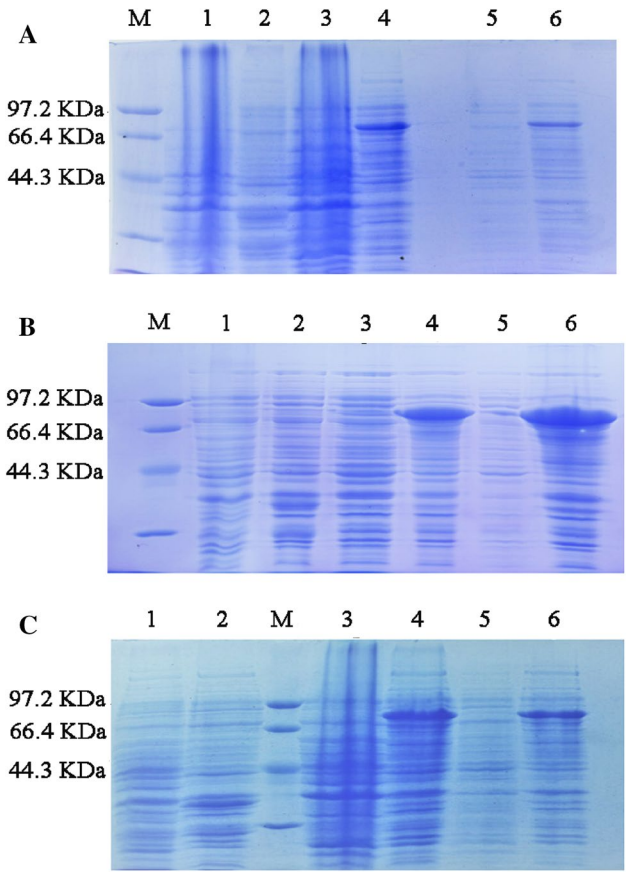

Fig. 6 The prokaryotic expression of SbNAC6, SbNAC26 and SbNAC56. **a** pET40b-SbNAC6, **b** pET40b-SbNAC26, **c** pET40b-SbNAC56. *M* markers; *lane 1* pET-40b vector was non-induced; *lane 2* pET-40b induced by IPTG for 8 h; *lane 3* SbNACs non-induced; *lane 4* SbNACs induced for 8 h at 23 °C; *lane 5* SbNACs supernatant after ultrasonication induced for 8 h at 23 °C; *lane 6* SbNACs inclusion body induced for 8 h at 23 °C

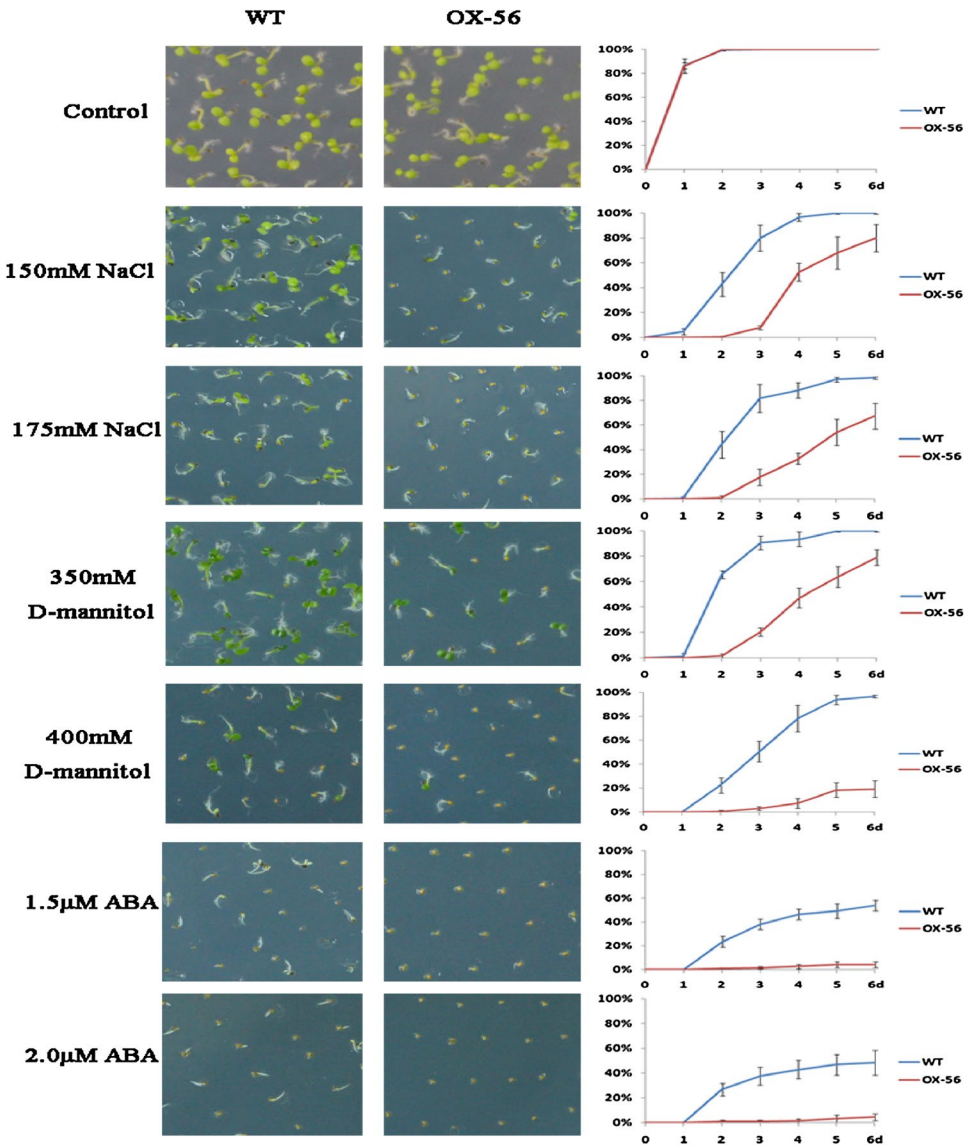

Fig. 7 Performance of *SbNAC56* overexpression lines germinated under ABA and osmotic treatments stress. Seeds were germinated on MS medium containing NaCl (150, 175 Mm), D-Mannitol (350, 400 mM) and ABA (1.5, 2.0 μM) for six days. The germination rates were calculated after planting on the plates. Data represent means ± SD of three replicates

expression levels of these seven *SbNAC* genes were commonly upregulated by at least 10-fold under conditions of ABA treatment (Fig. 5c). This suggested that these NAC members may play important roles in regulation of abiotic stress responses in the ABA signaling pathway. Under conditions of PEG, cold and salt stress treatments, the expression patterns of the selected SbNACs were indicative of the divergence in regulatory mechanisms (Fig. 5b, d–f). *SbNAC6*, a homolog of *ZmSNAC1* in maize and *SbSNAC1* in sorghum, both of which performed similar expression patterns (Lu et al. 2012, 2013), was also induced by salinity, dehydration, cold and ABA. Overexpression of *ZmSNAC1* and *SbSNAC1* enhanced the dehydration and drought stress tolerance in transgenic Arabidopsis. Previous studies inferred that SbNAC6 is likely to play a potential role in drought stress tolerance. Similarly, the transcript level of *SbNAC73*, an ortholog of wheat *TaNAC69-1*, was significantly increased by drought and salt treatments (Fig. 1) (Baloglu et al. 2012). The divergent expression patterns suggested that *SbNACs* may have various roles in regulation of abiotic stress signaling pathways, and may therefore be excellent candidates for improving abiotic stress resistance.

In this study, we detected the sensitivity to ABA at seed germination stage. Compared to the WT plants, the germination of transgenic plants was significantly inhibited by ABA, NaCl and D-Mannitol. These finding suggest that *SbNAC56* may function as a transcriptional activator in ABA-dependent signaling pathway in plant growth, development and response to osmotic stresses.

Overall, in order to mine the drought responsive NAC genes in sorghum and exam the potentially biological functions, we presented detailed descriptions of the sorghum NAC genes. Based on the results of phylogenetic analysis,

the 145 SbNAC TFs, along with some well-known stress-responsive NAC TFs in model plants, wheat, and soybean, were classified into 16 groups. Comprehensive analysis of the 145 SbNACs was presented and seven stress related candidates were isolated. Of these, four selected NAC proteins were confirmed to be transcriptional activators. Furthermore, the expression profiles of these seven SbNACs strongly implied diversity in their expression patterns under conditions of abiotic stress in the ABA-dependent pathway. The molecular mechanisms of sorghum NAC genes in abiotic stresses response will be examined in further studies. This work revealed the genomic landscape and expression patterns of seven sorghum NAC genes associated stress response. It can also provide the theoretical basis and candidate gene resources for crops genetic improvement, which will lay the foundation for further function analyses and breeding new varieties of crops.

Acknowledgements The work was supported by National Natural Science Foundation of China (Grant No. 31501314), DaBeiNong young teachers' Scientific Research Fund Project (Grant No. 1014115004/025), State Key laboratory of Crop Biology foundation (Grant No. 2015KF08) and the Scientific Research Project of Beijing Educational Committee (Grant No. KM201510020003).

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