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Genome-Wide Identification of NAC Transcription Factor Family and Functional Analysis of the Abiotic Stress-Responsive Genes in *Medicago sativa* L.

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Abstract
Plant-specific NAC (NAM, ATAF, and CUC2) transcription factors (TFs) are a large gene family in plants that have been shown to play important roles in regulating many developmental processes and abiotic stress resistance. Here, a total of 113 *Medicago sativa* NAC (MsNAC) TFs, known as MsNAC001 to MsNAC113, were identified and divided into 15 distinct subgroups. A comprehensive bioinformatics analysis of the MsNAC TFs is presented, including phylogenetic relationships, membrane-bound, and conserved motifs. Fourteen *MsNAC* genes grouped into stress-related subgroup were isolated, and the *cis*-elements and potentially biological functions of these genes were further investigated. Coupled with expression analysis and qRT-PCR testing, differential expression profiles over time in response to drought and salinity treatments were observed. In transgenic yeast, overexpression of *MsNAC001* and *MsNAC058* increased tolerance to salt (both) and drought (*MsNAC058*) stresses. Our findings will provide a starting point for the functional investigation and application of this gene family for crop improvement, especially in legume species.

Keywords *Medicago sativa* · NAC transcription factor · Phylogenetic relationships · Membrane-bound · Abiotic stress · Transgenic yeast

Introduction

Agricultural yields are affected by the environment in which the crop is exposed during development. Stress events pose a serious challenge for agricultural production around the world, causing annual losses estimated at billions of dollars (Mittler and Blumwald 2010). Stress adaptation is a complex event, as plants may be affected by stresses that occur at different stages of growth concurrently (Wang et al. 2003). To cope with stresses by surviving and completing their life cycles, plants have evolved complex adaptation and genetic mechanisms that regulate gene expression by tight transcriptional control and accurate signaling.

Transcription factors (TFs) are proteins that regulate various plant processes through binding specific to *cis*-regulatory sequences in the promoters of target genes (Puranik et al. 2013). The NAC (NAM, ATAF, and CUC) protein family is one of the largest groups of plant-specific TFs. The NAC TFs were originally identified from consensus sequences from petunia NAM, *Arabidopsis thaliana* ATAF1 and CUC2 (Nakashima et al. 2012). Typically, NAC TFs contain a highly conserved N-terminal DNA-binding domain, and research in *Arabidopsis* has indicated that there are at least five distinct types of DNA-binding domain for NAC TFs (Hisako Ooka et al. 2003). In addition to DNA binding, the unstable C-terminal transcriptional regulation region of NAC TFs can activate or repress transcription

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of multiple target genes (He et al. 2016; Le et al. 2011; Yamaguchi-Shinozaki amd Shinozaki 2005). In the crystal structure, the NAC domain of *Arabidopsis ANAC019* (Ernst et al. 2004) and rice *SNAC1* (Chen et al. 2011) revealed the existence of a new TF fold comprising a twisted anti-parallel β-sheet enclosed by a few helical elements. Some NAC proteins, which comprise a α-helical transmembrane (TM) motif, and are responsible for the anchoring to the plasma membrane, are classified as membrane associated and are referred to as NTL (NAC with Transmembrane Motif 1-like) TFs (Kim et al. 2010; Seo et al. 2008). Studies of NTL genes in *Arabidopsis*, rice, soybean, and maize indicated that most of the putative *NTL* genes are stress-responsive (Karanja et al. 2017; Kim et al. 2007, 2010; Li et al. 2016; Wang et al. 2016). Thus, identification and functional characterization of novel *NAC* genes will be helpful for understanding the potential stress response mechanisms in plants.

Increasing lines of evidence suggest that *NAC* genes play important roles in various plant physiological and developmental stages, including regulation of shoot apical meristem formation and vascular cell differentiation (Grant et al. 2010; Takada et al. 2001); adjacent embryonic, vegetative, and floral organs formation and separation (Mallory et al. 2004); grain protein, and iron and zinc content improvement (Velu et al. 2014; Zhu et al. 2019); leaf senescence (Ma et al. 2018); lateral root development (Chen et al. 2018; Guo et al. 2005); secondary wall formation (Yoshida et al. 2013; Zhang et al. 2018); hormone signaling (Mao et al. 2017; Ren et al. 2018); and biotic and abiotic stress responses regulations (Nakashima et al. 2007; Shao et al. 2015; Song et al. 2011). Recently, many *NAC* genes have been identified to participate in plant responses to drought and salinity stresses. Overexpression of *Arabidopsis ANAC019*, *ANAC055*, or *ANAC072* genes in transgenic plants resulted in the improvement of drought tolerance and modulated the expression of drought, high salinity, and ABA (abscisic acid) stress-inducible genes (Tran et al. 2004; Xu et al. 2013). The *Arabidopsis ATAF1* overexpression lines showed enhanced plant drought tolerance, and hypersensitive to high salinity, oxidative stress, ABA, and necrotrophic-pathogen infection (Wu et al. 2009). In transgenic rice, the *ONAC022* gene was observed to increase drought and salinity tolerance through modulating an ABA-mediated pathway (Hong et al. 2016). *SNAC1*-overexpressing cotton plants significantly increased drought and salt tolerance, transgenic cottons showed more vigorous growth, especially in terms of root development (Liu et al. 2014). *NAC6/SNAC2* and *OsNAC10* are drought-tolerance genes that belong to the ATAF subfamily, and overexpression of these genes enhanced drought and salt tolerance, and the rice grain yield under field drought conditions were improved in the *OsNAC10* transgenic rice (Hisako Ooka et al. 2003; Hu et al. 2006; Jeong et al. 2010). In wheat, overexpression of *TaNAC47* in *Arabidopsis* can enhance the drought, freezing, and salt resistance by activating the expression of downstream genes (Zhang et al. 2016). Considering their significance in various plant physiological and developmental stages, genome-wide analysis was performed to identify NAC TFs in many plants such as *Arabidopsis* (117 NAC genes) (Nuruzzaman et al. 2010), *Oryza sativa* (151) (Nuruzzaman et al. 2010), *Medicago truncatula* (97) (Ling et al. 2017), *Solanum tuberosum* L. (110) (Singh et al. 2013), *Glycine max* (152) (Le et al. 2011), *Nicotiana tabacum* (152) (Rushton et al. 2008), *Brachypodium distachyon* (101) (You et al. 2015), *Manihot esculenta Crantz* (96) (Hu et al. 2015), *Panicum virgatum* (251) (Yan et al. 2017), and so on.

Legumes (Fabaceae) as the second most important family of plant crops, generating approximately one-third of the world's primary crop yield (Benedito et al. 2008). Alfalfa (*Medicago sativa*), as one of the most-grown perennial forage legume worldwide, has high yield, nitrogen fixation capacity, and choice nutritional profiles (Min et al. 2017, 2019; Zhang et al. 2017). Recently, The Cultivated Alfalfa at the Diploid Level (CADL) Genome Blast Sever (https://www.alfalfatoolbox.org/abt_doblast/Home.gy?filterword=DOBLAST&function=function0) has been made available to the public, which provides an excellent opportunity for genome-wide analysis. To date, no overall analysis of the *NAC* gene family in alfalfa has been reported, except by Yong Xin Wang, who found a drought stress response gene named novel *Medicago sativa* NAC (Wang 2013). Given the significance of NAC TFs in the regulation of plant development, growth, and adaption to the abiotic stress, a genome-wide systematic analysis of alfalfa NAC family was performed. Finally, 113 *MsNAC* genes were identified and their phylogeny, membrane-bound structures, conserved motifs, and expression profiles were comprehensively studied. The expression profiles of *MsNAC* genes under drought and salt treatments were examined by real-time reverse transcription PCR (qRT-PCR). Furthermore, heterologous expression experiments in yeast cells indicated that *MsNAC001* and *MsNAC058* are key candidate genes for improving abiotic stress tolerance. The detailed results presented here would provide the insights for the further functional investigation and application of this gene family for crop improvement, especially in legume species.

Materials and Methods

Plant Growth, Treatments, and Tissues Collection

Alfalfa seeds (cultivar Zhongmu No. 1) were scarified, sterilized, and grown under greenhouse conditions (27/23 °C 14-h light/10-h dark, and 60%; relative humidity). Uniform seedlings were selected and hydroponically grown in an

aerated nutrient solution as described by Liu et al. (2017). For expression profiling of *MsNAC* genes under dehydration and salinity stresses, seedlings were transferred and grown in 20% PEG 6000 (dehydration) and 180 mM NaCl (salinity) for abiotic stress. Samples were collected at 0-h, 4-h, 8-h, and 24-h intervals. To reduce the circadian rhythm effects, samples from the control, drought, and salt conditions were grown in parallel and harvested at the same time after 24 h. Three independent roots were collected for each of the above treatments and were immediately frozen in liquid nitrogen and stored at -80 °C until used.

Database Mining and Identification of NAC Protein Family in Alfalfa

Searching for *NAC* genes in the alfalfa genome, 517 protein sequences encoding NAC TFs from *Arabidopsis, Oryza*, *M. truncatula*, and *Glycine max* were retrieved from Phytozome v12 (Goodstein et al. 2011). Then, these sequences were used to identify homologous peptides from alfalfa by using a Basic Local Alignment Search Tool algorithms (BLASTP) at CADL genome blast server. With the help of the PFAM databases (El-Gebali et al. 2018), all the potential MsNAC proteins identified from Hidden Markov Model (HMM) profile were searched, only if they contained the NAM domain (PF02365). Then the redundant sequences were removed using the decrease redundancy tool (web.expasy.org/decrease_redundancy).

Phylogenetic Tree Construction and Conserved Motifs Identification

To investigate the evolutionary relationships among alfalfa and *Arabidopsis*, multiple sequence alignment was performed using CluatalW2 program with default parameters. The software MEGA7 was employed to conduct the phylogenetic analysis by the NJ (neighbor-joining) method with 1000 bootstrap replicates (Tamura et al. 2011). The conserved motifs in full length NAC proteins were identified using Multiple Expectation Maximization for Motif Elicitation (MEME) program version 5.0.1, with the maximum number of motifs as 12 (Timothy et al. 2009). Moreover, the secondary structure of MsNAC domain was predicted by Promals3D web program (Lipman et al. 1989).

In Silico Sequence Analysis

The theoretical isoelectric point (pI) and molecular weight (Mw) of the MsNAC proteins were predicted using the ProtParam tool (http://web.expasy.org/protparam/). The number of transmembrane helices (TMs) was predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). *Arabidopsis* orthologs for alfalfa NAC proteins were identified using a BLASTP search against *Arabidopsis* proteins TAIR10 release (http://www.arabidopsis.org). The functional interacting networks of predicated stress response NAC proteins were integrated in STRING software with the confidence limits set at 0.400 (Franceschini et al. 2012).

Promoter *cis*-Element Analysis

The 1.5 kb sequence upstream from the translation start site of the predicated 14 predicated stress-related *MsNAC* genes were obtained from the CADL Genome Blast Sever. The PlantCARE online database was used to analyze the putative stress or hormone-responsive *cis*-acting regulatory elements (Lescot et al. 2002).

Expressed Sequence Tag Retrieval and Function Prediction of 14 Stress-Related *MsNAC* Genes

Expressed sequence tags (ESTs) corresponding to the *NAC* genes in alfalfa were isolated from the Alfalfa Gene Index and Expression Atlas Database database (http://plantgrn.noble.org/AGED/) using the BLAST program and the nucleotide sequences of MsNAC as queries. The expression data of different tissues were collected from two alfalfa phenotypes (*Medicago sativa* ssp. *Sativa* and *Medicago sativa* ssp. *falcata*). The MeV v4.9 software (http://www.mybiosoftware.com/) was used to normalize expression data and generate heatmap. Furthermore, the biological function of each putative MsNAC was accomplished via four public databases.

RNA Isolation and qRT-PCR Analysis

Total RNA was isolated using Sangon UNIQ-10 column Trizol total RNA extraction kit (TaKaRa, Dalian, China). RNA concentration test, DNase treatment, and cDNA synthesis were conducted according to previously described (Zhang et al. 2018). Fourteen gene-specific primer pairs were designed using the Primer3 software for qRT-PCR analysis (Table S1) (Rozen and Skaletsky 2000), and their specificity was confirmed by blasting the alfalfa CADL genome blast server. qRT-PCR reactions and data analysis were performed according to previous studies (Liu et al. 2017; Zhang et al. 2018). As an internal standard, the *Medicago actin* (AA660796) gene was selected to calculate the relative fold differences based on the comparative *Ct* method.

Expression Vector Construction and Stress Tolerance Tests of the Transgenic Yeast

Alfalfa (cultivar Zhongmu No. 1) cDNA was prepared using the above-mentioned method. The complete coding sequences of *MsNAC001* and *MsNAC058* were amplified

with specific primers (MsNAC001-F: CCGGAATTC ATGCAAGGAGCATTAGAATT (EcoR I), MsNAC001-R:CGGGATCCTTACTTGGGCAGGTACATAA (BamH I)) and (MsNAC058-F: CCGGAATTCATGCAAGGTGAA TTAGAATT (EcoR I), MsNAC058-CGGGATCCTCAAAA TGGCTTTTGTGGGT (BamH I)), respectively. The coding sequences of *MsNAC001* and *MsNAC058* were cloned into yeast expression pYES2 vector (Invitrogen, Carlsbad, USA). Subsequently, the pYES2-MsNAC001, pYES2-MsNAC058, and pYES2 only (vector control) plasmids were introduced into yeast strain INVSc1 (Invitrogen, USA) using a lithium acetate procedure according to the pYES2 vector kit instructions. The transformants were then selected on SC medium devoid of uracil at 30 °C. For the drought and salt tolerance assay was performed according to previously described methods (Li et al. 2014).

Results and Discussion

Identification of the *NAC* Gene Family and Phylogenetic Relationship Analysis in Alfalfa

To gain insight into the accurate size of the *MsNAC* TFs in alfalfa, the alfalfa genome database was screened by HMM profile and BLAST searches using *Arabidopsis,* rice, *M. truncatula*, and soybean NAC sequences as queries. Finally, a total of 113 NAC proteins were identified and all of them were confirmed to contain the NAC domain or NAM domain. In prior study, an in silico analysis was performed using RNA-seq data to reveal and systematize alfalfa TFs. Finally, 67 NAC TFs were identified (Postnikova et al. 2014). The number of NACs identified in alfalfa was smaller than those in other plants, such as rice (151, genome size ~372 Mb), *Populus trichocarpa* (163, ~443 Mb), soybean (152, ~978 Mb), and *Arabidopsis* (117, ~135 Mb), but bigger than *Vitis vinifera* (74, 487 Mb) and *M. truncatula* (97, 360 Mb). The alfalfa genome size (800–900 Mb) (Hong-Kyu et al. 2004) is approximately two points two times, one point six times, and nearly to that of the rice, *Vitis vinifera* and soybean genome, respectively, suggesting that there is no direct linkage between the number of *NAC* genes and the genome size in plants. Due to improper annotation, the existing identifies for *MsNAC* genes were highly disordered. Thus, a uniform nomenclature has been assigned to these 113 MsNAC proteins according to their numeric sorting as follows: MsNAC001-MsNAC113. Among the 113 MsNAC TFs, the relative molecular weight ranged from 14.97 kDa (MsNAC048) to 95.578 kDa (MsNAC022), and the pIs varied from 4.13 (MsNAC002) to 9.77 (MsNAC080) with 71 members showing $pI < 7$ and the remains with $pI > 7$ (Table S2), indicating that these different NAC proteins may work under different conditions. The amino acids length ranged from 130 (MsNAC048) to 848 (MsNAC022) with an average of 353.3 aa. MsNAC048 is the smallest protein, with a NAM domain that appears to be truncated and lacks subdomains E at the N-terminal end (Fig. S1). To study the evolutionary relationships between MsNAC proteins and *Arabidopsis*, an unrooted NJ phylogenetic tree was created (Fig. 1), and the MsNACs were classified into A and B two major groups. These two groups were further divided into 10 and 5 subgroups, respectively. Accordingly, to Ooka et al., *Arabidopsis* NACs were classified into two large groups, the MsNAC members of groups A and B showed high homology with the *Arabidopsis* NACs (Ooka et al. 2003).

Correlation Between Conserved Motif Analysis and Functional Predication

Previous studies have shown that the NAC proteins are characterized with a conserved DNA-binding region at the N-terminal, which is divided into five subdomains (A–E), and highly diversified C-terminal region that contains a transcriptional regulatory domain (Ooka et al. 2003). A multiple sequence alignment of 113 MsNAC proteins and three representative *Arabidopsis* NAC proteins (ANAC019, ANAC055, and ANAC072) was performed, and the results revealed that most MsNACs have the complete NAC domains (Fig. S1). However, some MsNACs such as MsNAC002, 048, 063, 086, and 107 lack one or more conserved subdomains; such NAC proteins may be characterized as NAC-like proteins according to the description of these proteins in rice, potato, and *B. distachyon* (Nuruzzaman et al. 2010; Singh et al. 2013; You et al. 2015).

Previously, there were 6, 10, and 12 subgroups for soybean, poplar, and potato, respectively (Hu et al. 2010; Le et al. 2011; Singh et al. 2013). In this study, 12 conserved motifs were predicted to examine the diversity of *MsNAC* genes (Fig. S2), the observations in our study indicate that NAC proteins in alfalfa pose a relatively high diversity (Fig. S3). In general, MsNAC proteins with similar motif composition tended to cluster together. All MsNACs contain at least one of the six main motifs (motif 2, 4, 1, 5, 3, and 7) that represent the subdomains A, B, C, D, and E, respectively. We further predicted the secondary structure of conserved motifs corresponding to five subdomains covering the entire NAC domain. All of them were found at the conserved N-terminal, but none in the diversified C-terminal ends, indicating that these motifs may be important for the function of NAC proteins, which is also found for NACs in potato (Ooka et al. 2003). Among the MsNACs in all groups, compared with subdomains B, C, and E, subdomains A and D were more tightly conserved, proving that the structure of A and D might have the most conserved and indispensable functions for MsNACs. It is worth noting that all MsNACs in group I shared motif

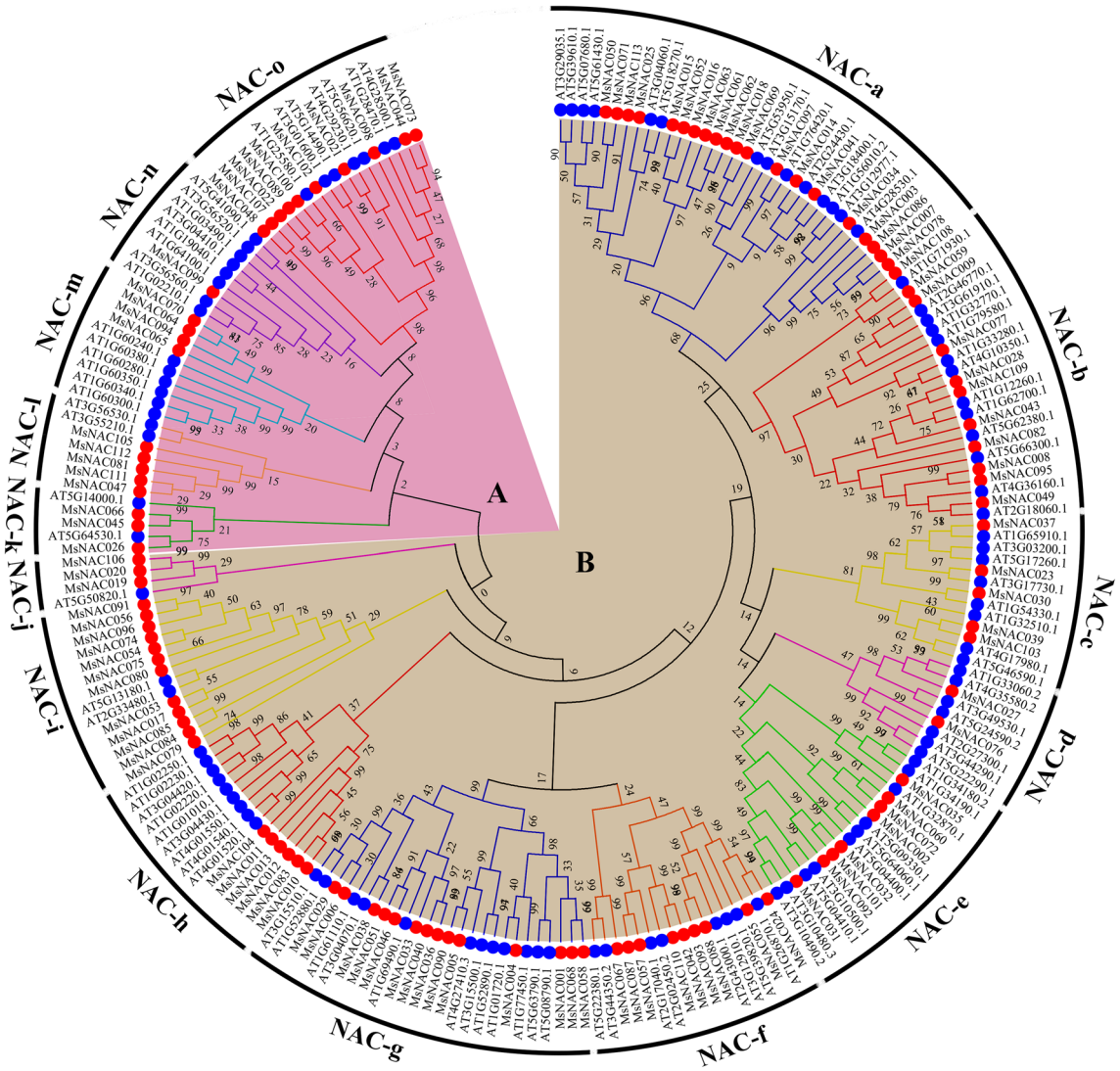

Fig. 1 Phylogenetic tree of NAC proteins from alfalfa and *Arabidopsis*. The phylogenetic tree was constructed using MEGA7 by the neighbor-joining method with 1000 bootstrap replicates. The tree was divided into two (A and B) subfamilies comprising 15 smaller subgroups. Members of alfalfa and *Arabidopsis* are denoted by circles with red and blue, respectively (Color figure online)

10, and were homologous to ANAC073, ANAC075, and ANAC088 in *Arabidopsis*, which are involved in secondary cell wall biosynthesis and DNA damage checkpoint, indicating that the motif 10 could be an important region in defining the function of these NACs in the process of cell wall biogenesis and DNA damage response. Recently, study has shown that the NAC domain monomer was characterized with a twisted anti-parallel β-sheet that flanked with an N-terminal α-helix on one side and a short helix on the other side (Olsen et al. 2005). In this study, a β-sheet in subdomains A and B was also found to be packed against a α-helix (Fig. S4). Moreover, two α-helices and six β-sheets were predicted, which is also similar to previous reports (Ernst et al. 2004; Singh et al. 2013).

Membrane-Bound MsNAC Subfamily

It has been established that NAC membrane-bound TFs (MTFs) have been implicated in plant response to abiotic stresses (Kim et al. 2010; Lee et al. 2012; Seo et al. 2008). The dormant form of membrane-associated NAC TFs is activated by degrading their cytoplasmic anchors and then enters the nucleus, where it regulates the expression of target genes (Kim et al. 2010). In this study, we identified 7 (6.2%) MsNAC proteins containing α-helical TMs by TMHMM server (Table 1). In soybean, 9 and 2 GmNACs have been predicted to contain single and two TMs, respectively (Le et al. 2011). On the other hand, only one TM was identified in *Arabidopsis* and rice NAC MTFs (Kim et al. 2007, 2010; Singh et al. 2013). In this study, 5 of the 7 identified MsNAC

Table 1 Predicted membrane-bound MsNAC proteins, their protein length, number of predicted TMHs, position of NAM domain, and transmembrane locates

Gene name	Seq. ID	Protein length (aa)	Number of predicted TMHs	NAM domain sequence	Transmembrane sequences
MSNAC028	MSAD_022233.t1	638	1	26–152	610–632
MSNAC029	MSAD_023548.t1	417	1	77–205	27–49
MSNAC034	MSAD_027023.t1	613	1	9–136	588–610
MSNAC037	MSAD_027503.t1	571	1	20–146	540–562
MSNAC039	MSAD_030599.t1	673	2	7–133	578–597, 651–670
MsNAC080	MSAD_099416.t1	523	2	19–144	348–370, 438–457
MsNAC100	MSAD_242157.t1	577	1	6–133	552–574

MTFs were found to contain a single TM, and the remaining two (MsNAC039 and MsNAC080) contained two TMs. Like *Arabidopsis*, rice, soybean, and other model plants NAC MTFs, all the identified MsNAC MTFs in this study were located at the C-terminal, except MsNAC036, which contains a TM at its N-terminal (Fig. S5).

Furthermore, a phylogenetic tree of the NAC MTFs from alfalfa (7), *Arabidopsis* (18), rice (5), *M. truncatula* (8), soybean (11), and maize (7) was constructed to illustrate the evolutionary relationship between heterologous NTLs. Six clades were recognized, and of these, three (clades III and VI) were specific to the dicotyledonous species (Fig. 2), the inference was that the NTLs within a clade probably diversified and expanded after the monocot–eudicot split. The most (13) NTLs were clustered together in Clades V, followed by 11, 9, 9, 8, and 6 in Clade I, II, IV, III, and VI, respectively. A MEME-based analysis showed that MsNTLs clustered within one clade shared a similar kind of motif content, indicating functional similarities among members of the same subgroup (Fig. 2). Notably, all NAC MTFs in clades I and II shared the specific motif 6, in clades III, IV, V, and VI shared the specific motif 7, and it would be interesting to further verify their functions using experimental approaches. Furthermore, a multiple sequence alignment verified that all NAC MTFs encoded the highly conserved NAC domain at their N terminus, in which five subdomains (A to E) were identified (Fig. S6). In *Arabidopsis*, the function of at least four NTLs (NTL4, NTL6, NTL8, NTL9, and NTL13/NTM2) has been proven to link external signals, such as osmotic, salt, and cold stresses (Le et al. 2011; Liang et al. 2015). In maize, NAC MTFs displayed a variety of both conserved and distinct functions in response to abiotic stress (Wang et al. 2016). Other studies also showed that NAC MTFs are activated by membrane-associated proteases in the endoplasmic reticulum by posttranslational modifications when plants suffer environmental stresses (Bhattacharjee et al. 2017; Li et al. 2016). Thus, it is feasible that the MsNAC MTFs may play a significant role in nuclear localization and downstream stress-responsive gene expression that may serve as an adaptive strategy for legume plants to survive under adverse environmental cues.

Characterization of Abiotic Stress Response Related MsNAC Genes

Increasing evidence has suggested that phylogenetic analysis can provide clues into the functional prediction, which could be subsequently prioritized for further planta functional studies (Le et al. 2011; Van Ha et al. 2014; You et al. 2015). Previous studies have reported that several *NAC* genes are well described in terms of their important roles in responses to abiotic stress (Nuruzzaman et al. 2013; Pascual et al. 2015; Shao et al. 2015). To further predict and distinguish the function of *MsNAC* genes, an NJ tree was constructed using 147 NAC TFs, including the 34 well investigated plant NAC proteins at the molecular level (response to single dehydration and salinity, or multiple-stresses) and 113 MsNAC proteins (Fig. S7, Table S3). As shown in Fig. S7, 22 (75.86%) of the 29 dehydration-related NAC genes and 20 (76.92%) of the 26 salinity-related NAC genes were clustered into one group with 14 *MsNAC* genes, indicating these MsNAC proteins might be involved in the alfalfa stress response. One of our main interests for performing phylogenetic analysis of *MsNAC* genes was to predicate their potential roles in response to dehydration and salinity in alfalfa that could be subsequently select candidate *MsNAC* genes that may respond to diverse environmental stress.

To further investigate our selected stress-related MsNAC proteins, four public databases were used to predict MsNAC protein annotations with an e-value cut-off of 1e-10. The annotation results showed that these MsNAC proteins are possibly involved in hyperosmotic salinity, water deprivation, plant hormone signaling, and growth regulation (Table S4). Furthermore, the STRING softer was used to determine the functional and physical relationships of 14 MsNAC proteins predicted to be stress related through an *Arabidopsis* association model (Fig. 3). Of these 14 MsNAC proteins, the homologous gene matches the highest bit score by default, which identified 8 (ATAF1,

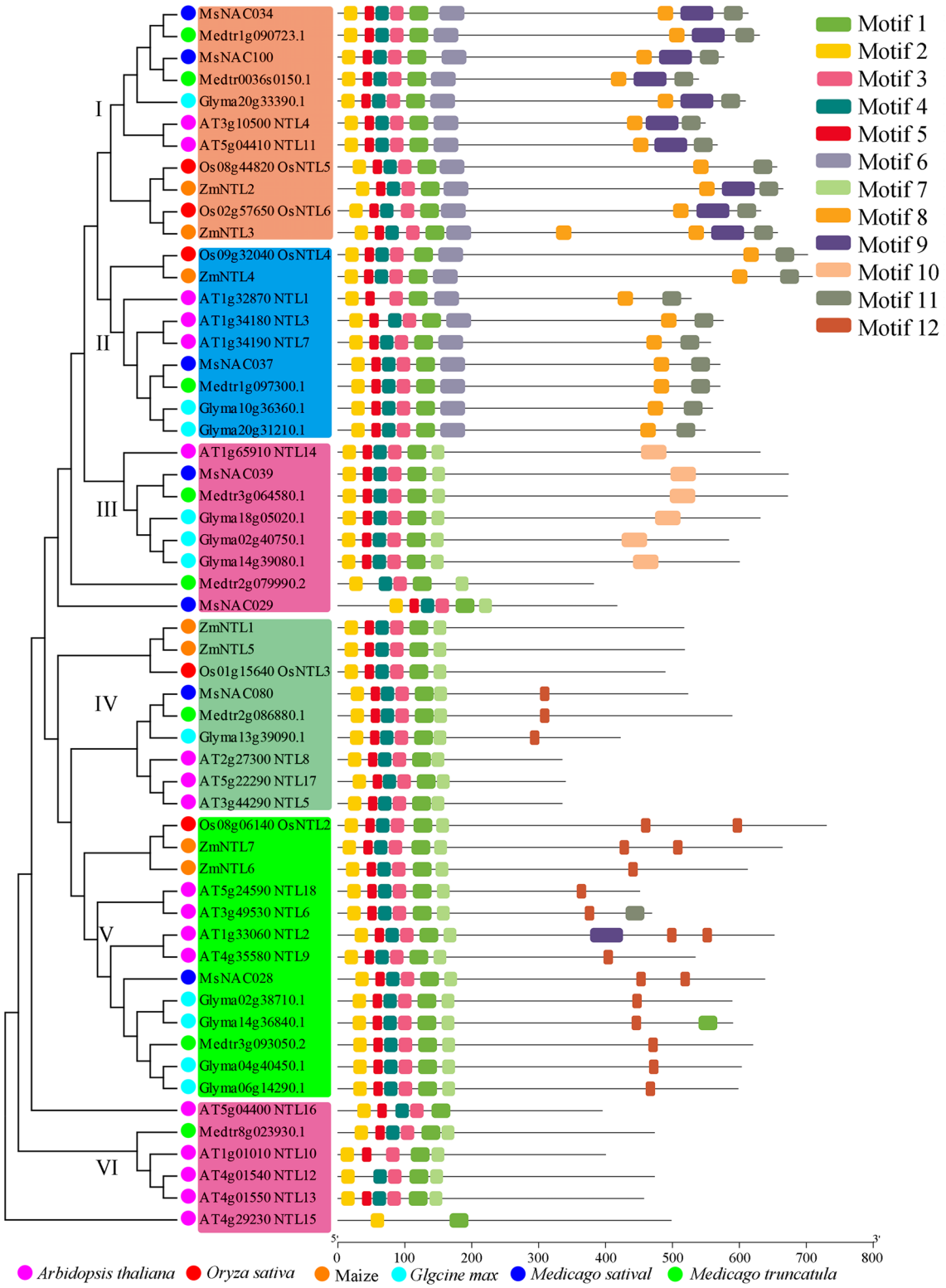

Fig. 2 Phylogenetic relationships of membrane-bound NAC proteins from alfalfa with proteins from *Arabidopsis*, *M. truncatula*, rice, soybean, and maize. The phylogenetic tree was constructed using MEGA7 by the neighbor-joining method with 1000 bootstrap replicates. The tree was divided into six phylogenetic subgroups according to the kinds of motif

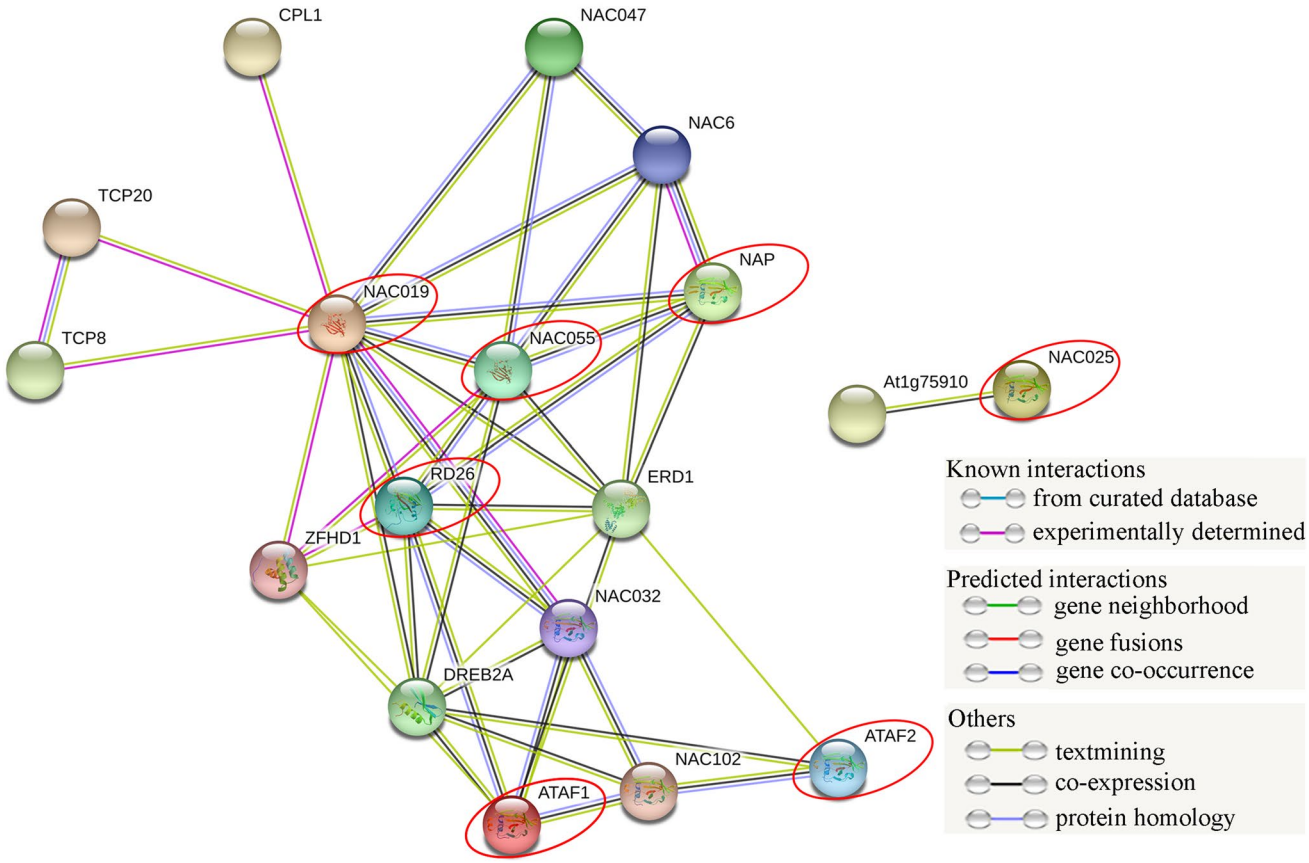

Fig. 3 Protein interaction network for 14 stress-related MsNAC proteins based on these orthologs in *Arabidopsis*. Red lines indicate proteins that are predicted to interact with more than four other NAC proteins. The highly matched seven ortholog *NAC* genes are showed in red ellipse (Color figure online)

ATAF2, RD26, NAC019, NAC025, NAC027, NAC055, and NAP) high confidence interactive proteins involved in the NAC family networks in *Arabidopsis*. A previous study found that overexpression of ATAF1 in *Arabidopsis* increased plant sensitivity to ABA, salt, and oxidative stresses, and remarkably enhanced plant drought tolerance (Liu et al. 2016; Wu et al. 2009). A stress-responsive NAC (SNAC) subfamily comprises seven genes as following: *ANAC019*, *ANAC055*, *RD26*, *ATAF1*, ATAF2, *ANAC102*, and *ANAC032*, and it has been suggested that SNAC-A subfamily genes regulate the expression of abiotic stress-responsive genes. For example, ANAC019, ANAC055, and RD26 were induced by drought, high salinity, ABA, and MeJA, plants individually overexpressing these genes showed a significant increase in salt or drought tolerance (Nakashima et al. 2012; Takasaki et al. 2015). The functional annotations of MsNAC proteins were obtained based on other proteins with known biological functions, which provides a reference for predicting the potential regulatory roles of MsNAC proteins in alfalfa.

Characterization of Putative *cis*-Regulatory Elements in the Stress-Related *MsNAC* Genes

The *cis*-acting regulatory elements are specific motifs existing in the promoter regions of genes functioning as binding sites, which play important roles in response to stresses through regulate gene transcription in plant (Nakashima et al. 2014). In addition, phytohormones, such as ABA, salicylic acid (SA), ethylene (ET), and jasmonic acid (JA), are also essential for plant adaptation abiotic stresses (Santner and Estelle 2009; Wu et al. 2017). The −1500 bp upstream promoter regions of *MsNAC* genes were scanned in the PlantCARE database, fifteen *cis*-acting elements were used in this study, including six stress-responsive (MBS, HSE, ARE, LTR, TC-rich repeats, and Box-W1) and nine hormone-responsive (TCA-element, ABRE, GARE-motif, CGTCA-motif, P-box, ERE, TATC-box, TGA-element, and TGACG-motif) *cis*-acting elements (Table S5). The analysis indicates that all these genes promoter regions contain at least two kinds of environmental stress signal responsiveness and three kinds of phytohormone-related *cis*-elements.

The number of *cis*-elements in the promoter regions of the *MsNAC* genes is variable. For example, *MsNAC036* and *MsNAC040* promoter have 25 *cis*-elements, whereas *MsNAC046* only contains ten *cis*-elements (Table S6).

Expression Analysis of Abiotic Stress-Related *MsNAC* Genes in Various Tissues

Tissue-specific expression patterns are useful data as they can determine whether a gene plays a role in defining the precise nature of individual tissue. Several *NAC* genes have been demonstrated to play essential roles in regulating plant tissues development at different growth stages (Kusano et al. 2005; Mitsuda et al. 2007; Yang et al. 2011). To investigate the overlapping and tissue-specific expression profiles, the expression patterns of fourteen stress-related *MsNAC* genes in six tissues were analyzed using the public alfalfa gene expression atlas (Fig. 4). The heatmap showed that the expression patterns of two alfalfa varieties have a highly similarity, indicating these may possess the same transcript abundance between two alfalfas. Clustering analysis of the expression data indicates that *MsNACs* possess highly distinct transcript abundance in different tissues. Of these 14 *MsNAC* genes, 5, 9, 10, 6, 4, and 6 *MsNACs* had high expression levels (value > 2.25) in leaf, flower, ES (elongating stem internodes), PES (post-elongation stem internodes), root, and nodule, respectively, in *Medicago sativa* ssp. *Sativa*. *MsNAC004, MsNAC058,* and *MsNAC068* genes showed an overall coverage among the six tissues, suggesting that these genes might play a role in plant growth and development. Furthermore, some *MsNAC* genes showed tissue-specific expression patterns, as *MsNAC033* and *MsNAC058* in leaf; *MsNAC005*, *MsNAC006,* and *MsNAC038* in the flower (Fig. 4), suggesting they might play key roles in specific tissue development or function (Liu et al. 2014). This phenomenon was also observed for the *NAC* genes in other plants, such as *Arabidopsis*, rice, chickpea, and soybean (Fang et al. 2008; Le et al. 2011; Van Ha et al. 2014). Moreover, several studies have indicated that overexpression of tissue-specifically expressed *NAC* genes can promote the development of particular tissue, such as *NAC1* promotes lateral root development in *Arabidopsis* (Xie et al. 2000). *SND1 and VND7* are two *Arabidopsis* NAC domain TFs that are master regulators of secondary wall biosynthesis in fibers and vessels, respectively (Zhong et al. 2010). Together, the tissue expression pattern of *MsNAC* genes identified in our study would provide useful information for further investigation of alfalfa development.

Validation of *MsNAC* Expression Profiles Under Abiotic Stress

Medicago truncatula as model plant has been selected to study functional genomics of legumes. Because of the sequence conservation between *M. truncatula* and alfalfa are relatively high, we downloaded the microarray data of homologous genes in *M. truncatula*, and evaluated their expression patterns under drought and salt treatments in root tissue (Fig. 5). Overall, expression of most *MsNAC* genes was significantly up-regulated under both treatments, and under different salt density (180 mmol and 200 mmol) treatments *MsNAC* genes have similar expression patterns. For example, the *M. truncatula* homologous genes *MsNAC004*, *-005*, *-006*, *-046,* and *-090* were up-regulated significantly

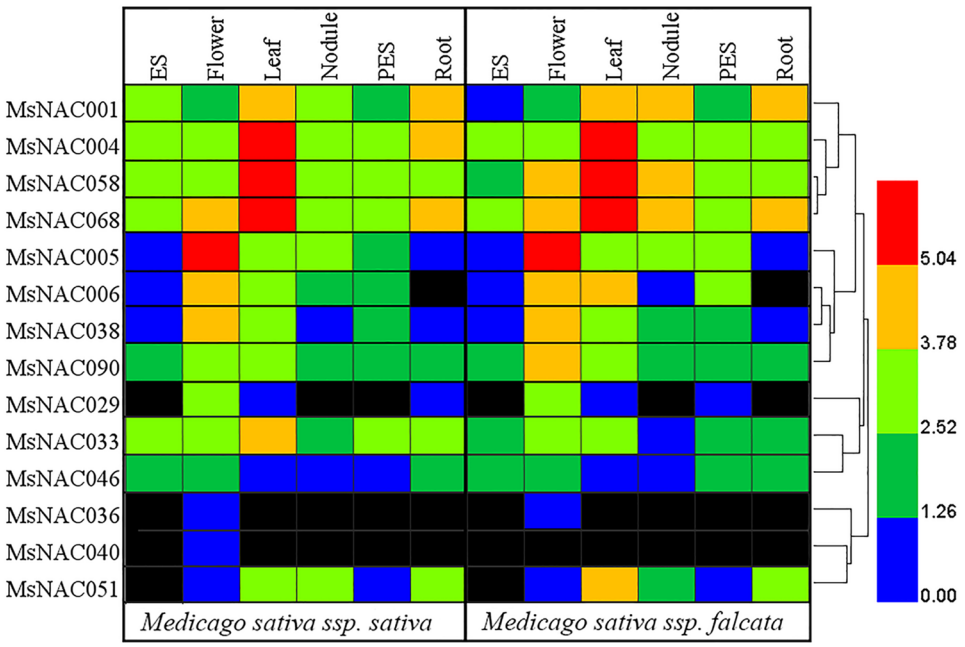

Fig. 4 Heatmap representation and hierarchical clustering of the *MsNAC* genes in various alfalfa tissues. The transcript data of six tissues were used to construct the expression patterns of *MsNAC* genes. The bar at the right of the heat map represents the relative expression values

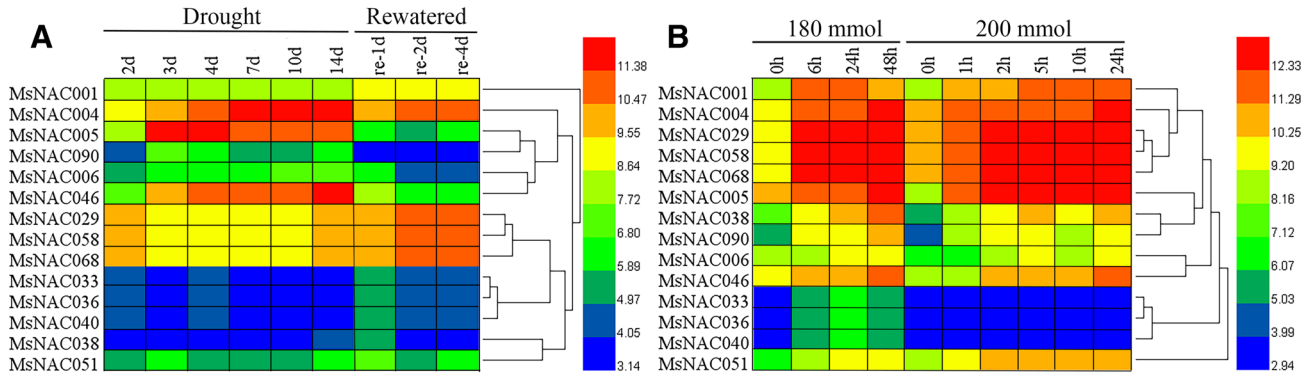

Fig. 5 Heatmap representation and hierarchical clustering of the *MsNAC* homologous genes in *M. truncatula* during **a** drought and **b** salinity stress treatments. The microarray data were reanalyzed and the relative expression values were $\log_2$ transformed. The bar at the right of the heat map represents the relative expression values

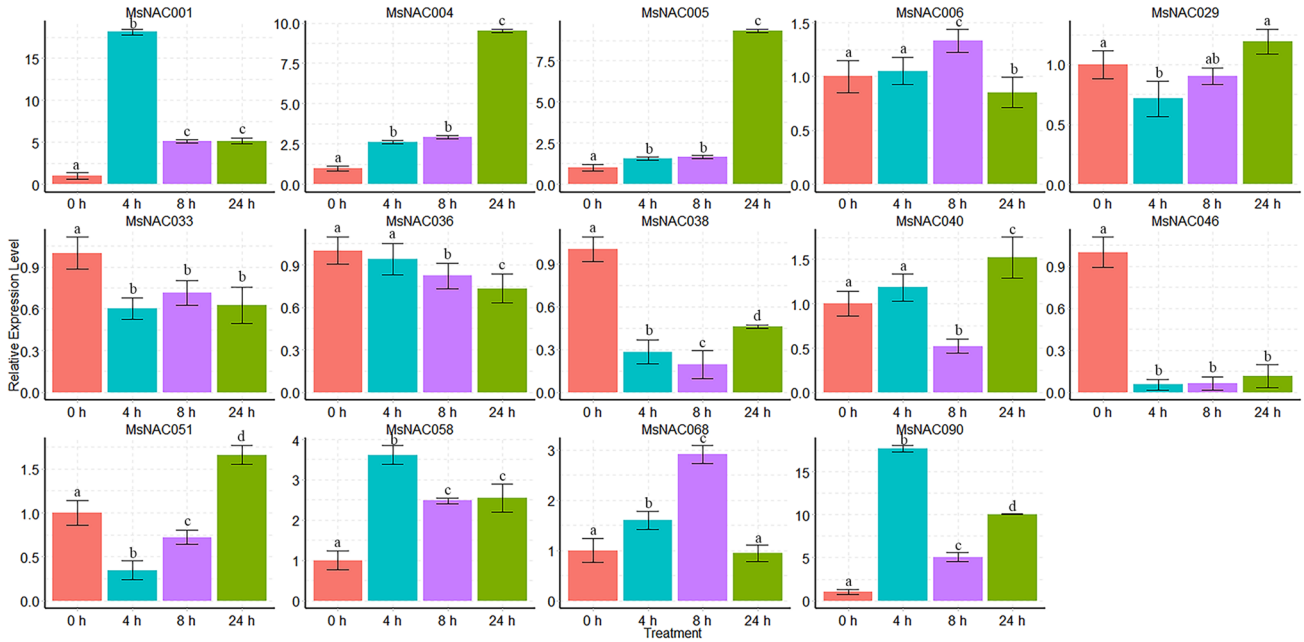

Fig. 6 The relative expression ratios of fourteen representative *MsNAC* genes under drought conditions (under drought treatments for 0, 4, 8, and 24 h) have been calculated with respect to reference (*Actin* gene). Different letters indicate significant differences between different treatment time ($P < 0.05$). The name of the gene is written on the top of each bar diagram (error bars indicate the standard deviation from three replicates)

under drought and salt conditions. Some *MsNAC* genes may utilize opposing regulatory mechanisms under abiotic stresses, such as *MsNAC029*, *-058,* and *-068* that were down-regulated under drought conditions, whereas these genes were up-regulated significantly under salt stress.

The expression patterns of candidate stress-response *MsNAC*s were further measured by qRT-PCR under drought and salt treatments (Figs. 6 and 7). Among the fourteen abiotic stress response related *MsNAC* genes, some showed similarly expression patterns between drought and salt treatments, such as *MsNAC001*, *-004*, *-005*, *-058*, *-068*, and *-090*, were continuously up-regulated under both of two stresses. Wang et al. (2013) cloned a new NAC TF from alfalfa, named *Medicago sativaNAC*, which could be induced by high salinity, drought and ABA, and transgenic *Arabidopsis* possessed a better drought tolerance than the wild-type, which shared a highly similarity with *MsNAC046* (Wang 2013). While *MsNAC046* was significantly down-regulated at all treated time points under drought and salt stresses. These differences might be due to differences of

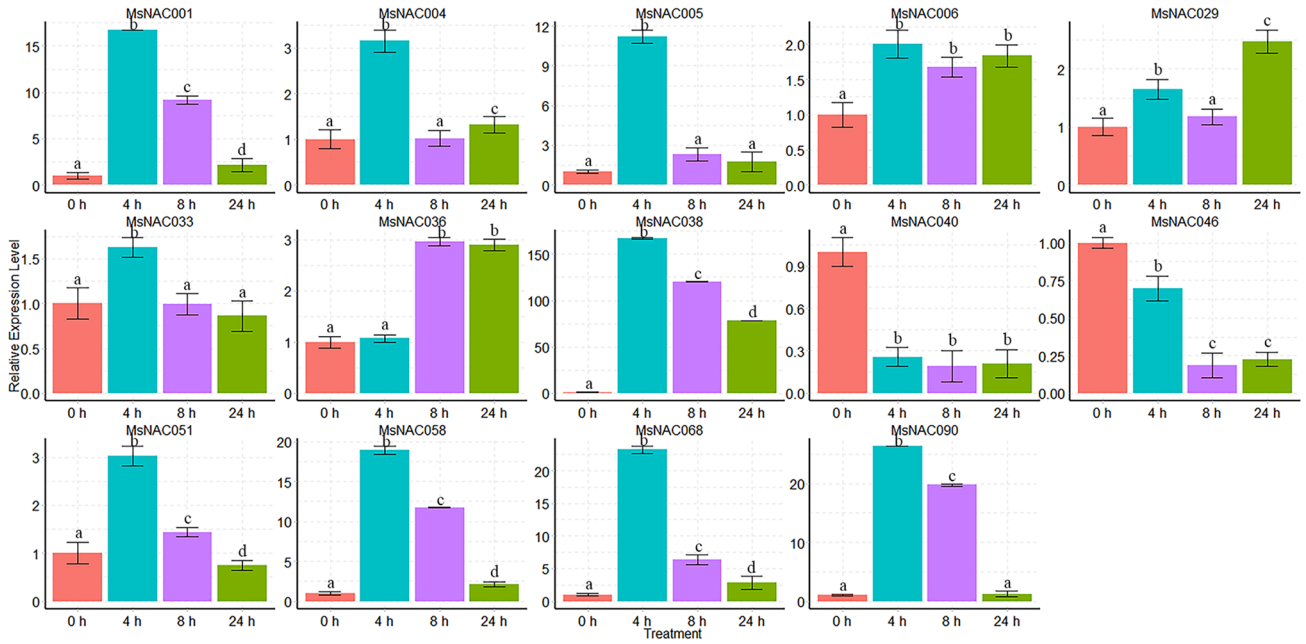

Fig. 7 The relative expression ratios of representative *MsNAC* genes under salinity conditions (under salinity treatments for 0, 4, 8, and 24 h) have been calculated with reference (*Actin* gene). Different capital letters indicate significant differences between different treatment time ($P < 0.05$). The name of the gene is written on the top of each bar diagram (error bars indicate standard deviation)

testing sample (*Medicago sativaNAC* gene was validated using leaf). The *MsNAC029*, *-033*, *-036,* and *-038* possessed opposite expression profiles in salt conditions compared to drought, suggesting that these genes may have different response mechanisms between these two stresses. Previous study performed a functional analysis of *ONAC095* in drought and cold stress, and the result showed that it is a positive regulator of cold response but a negative regulator of drought response in rice (Huang et al. 2016). Expression of *MsNAC051* under salt stress, and *MsNAC006* under drought increased initially, whereas *MsNAC040* and *-051* were firstly down-regulated and then up-regulated under drought conditions (Figs. 6 and 7). These results also suggested that some genes in the NAC family may utilize opposing regulatory mechanisms in response to abiotic stresses.

Transgenic Yeast to Analysis Drought and Salt Stresses

To investigate the possible role of *MsNAC* genes in salt and drought stresses, we heterologously overexpressed two selected proteins (MsNAC001 and MsNAC058) in yeast strain INVSc1 using the pYES2 vector. We examined the effects of MsNAC001 and MsNAC058 on the survival of yeast cells exposed to 5 M NaCl and 30% PEG. As shown in Fig. 8, there is no difference in survival rates between the MsNACs transgenic and the control yeast under

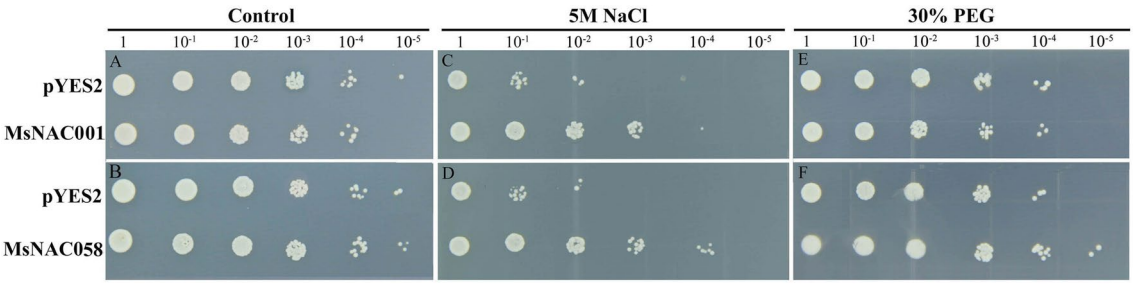

Fig. 8 Stress tolerance test of *MsNAC001* and *MsNAC058* genes in yeast cells. The transformed yeast INVSc1 harboring MsNAC001, MsNAC058, and empty pYES2 were diluted into different ratio and grown on SC-Ura selective medium. **a**, Bb Non-stress, **c**, **d** 5 M NaCl for 36 h, **e**, **f** 30% PEG6000 for 36 h, respectively

non-stress conditions. After cultured in 5 M NaCl for 36 h, MsNAC001-transformed line survived well, but the control line was intensely inhibited, while the yeast cells viability was reduced in MsNAC001-transformed cells grown on 30% PEG for 36 h compared with control. The result agrees with the expression patterns of homologous gene in *M. truncatula*, which utilize opposing regulatory under both stresses (Fig. 5). Therefore, MsNAC001 may decrease drought resistance in yeast cells, and indicated that MsNAC001 proteins conferred salt tolerance to yeast cells, but not tolerant to drought tolerance. MsNAC058 transformed survived better than the control under salt and drought stresses, especially under salt stress, which shared 83% identity with ATAF1. Previous study has showed that the overexpression of ATAF1 in *Arabidopsis* increased plant sensitivity salt, and exhibited significantly improved salt tolerance in transgenic rice (Liu et al. 2016).

Conclusion

Overall, 113 putative *MsNAC* TFs were identified in alfalfa. The phylogenetic relationships, conserved motifs, and membrane-bound of *MsNAC* genes were evaluated. To mine the abiotic responsive *NAC* genes in alfalfa, a phylogenetic analysis was performed along with 34 well-investigated stress-responsive NAC TFs. A comprehensive analysis of the 113 MsNAC was presented and 14 abiotic stress response-related candidates were isolated. The functional annotation, regulatory network, and expression profiles of these 14 *MsNACs* strongly implied diversification and important roles under conditions of abiotic stress. Furthermore, overexpression of MsNAC001 and MsNAC058 in yeast cells increased tolerance to salt (both) and drought (MsNAC058) stresses. Considering the limited functional understanding of *MsNAC* in alfalfa, our findings will provide theoretical basis and candidate gene resources for subsequent studies of gene cloning and functional characterization of *MsNAC* members in alfalfa.

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Author Contributions WL and XM conceived and designed research. XM, ZZ, and XW performed the experiments. XM, XJ, and WL analyzed the data. XM, WL, NB, and YW wrote the manuscript. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflicts of interest The authors declare that there are no conflicts of interest.

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