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RESEARCH ARTICLE

Genome-wide analysis and expression patterns of the NAC transcription factor family in *Medicago truncatula*

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Abstract NAC transcription factor (TF) family proteins are expressed in various developmental stages and following various stresses. NAC TFs are involved in mediating various physiological functions of plants and participate in various signaling pathways under biotic or abiotic stress. The present study provided a comprehensive functional analysis of members of the MtNAC TF family. Via screening of *Medicago truncatula* genome information, we identified 97 MtNAC TFs in *M. truncatula* and compared the phylogenetic analysis of 14 conserved groups with their Arabidopsis and rice counterparts. The NAC TFs were categorized into 14 groups based on their conserved motifs and gene structure. The predicted *M. truncatula* NAC genes were distributed among eight chromosomes, and in addition, we found that these genes showed mass gene duplication. Through expression profiling of RNA-seq data analysis, we determined that NAC family members were expressed significantly under different abiotic stresses. This indicates that the NAC TF shows different functions in *M. truncatula*. Together, this genome-wide analysis of the NAC gene family in *M. truncatula*, could be applied to improving stress tolerance in plants.

Keywords NAC transcription factors · Phylogenetic analysis · Expression profile · *Medicago truncatula*

Introduction

Alfalfa is an important perennial forage legume species. It is a high—yielding perennial grass species that has high nutritional value and nitrogen fixation capacity. It is an important germplasm resource in the world. They make a contribution to modern society end economy construction (Samac et al. 2006; Sanderson et al. 2004; Yang et al. 2011).

The NAC domain is a highly conserved amino acid motif in one of the largest groups of plant-specific transcription factors (TFs). No apical meristem (NAM) was the first characterized NAC protein found in petunias, and Arabidopsis transcription activation factor (ATAF) and cup-shaped cotyledon (CUC) were found in Arabidopsis (Tran et al. 2004; Zhong et al. 2006). Several NAC proteins have been identified in other plant species, including rice, wheat, and soybean (Hussey et al. 2015).

The NAC TF family members have a highly conserved NAC domain about 150 amino acids, which include N-terminal ends containing five subdomains (A–E) and highly variable domain at C-terminal ends (Ernst et al. 2004). At their C-terminal end, the basic region also contains α-helical transmembrane motifs (TMs) (Puranik et al. 2012). The special structure is related to specific biotic functions, and the NAC TFs are involved in biotic and abiotic stress processes (Mao et al. 2012). These genes influence plant growth, enhance the absorption of mineral elements, and improve crop nutrition and quality. The NAC TFs are involved in mediating a variety of physiological activities in plants, such as auxin conduction and

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cell apoptosis (Yoshii et al. 2010). When plants are affected by biotic or abiotic stress, NAC TFs participate in various signaling pathways to cope with these adverse conditions (Pinheiro et al. 2009).

Earlier studies have shown that NAC TFs play an important regulatory role in plants subjected to abiotic stress including salinity, drought, cold, or abscisic acid (ABA) (Olsen et al. 2005). For example, over accumulation of ANAC (019, 055, 072) in Arabidopsis lead to enhanced stress tolerance in transgenic plants (Bu et al. 2008). The gene ANAC062 is involved in the cold stress signal regulation (Yang et al. 2014). OsNAC5 is a senescence-associated ABA-dependent NAC TF. OsNAC045 and OsNAC063 can enhance drought and salt tolerance in rice (Xu et al. 2015). The expression level of OsNAC19 is increased after *Magnaporthe grisea* infection to regulate the defense responses in rice (Nuruzzaman et al. 2015). NAC proteins are involved in responses to viral infections during plant vegetative development. In soybean, GmNAC11 and GmNAC20 are involved in responses to low temperature, and overexpression of these two genes enhanced low temperature tolerance (Hao et al. 2011).

GmNAC1-6 are differentially expressed during seed development and other physiological processes in *Medicago sativa*. A novel *M. sativa* NAC transcription factor has been characterized during drought stress. In *M. truncatula*, the MtNAC969 is involved in root system architecture by several pathways.

With the rapid development of high-throughput sequencing technologies, several NAC members have been studied in model plants such as *A. thaliana* (117), *Glycine max* (152), *O. sativa* (151), and *Vitis vinifera* (74) (Le et al. 2011; Nuruzzaman et al. 2010; Ooka et al. 2003; Wang et al. 2013). The theoretical basis for the present study was provided by the completed *M. truncatula* genome sequence and previous studies on NAC TFs.

NAC proteins are found in most plant species, but their research is poorly understood in *M. truncatula*. *M. truncatula* is an excellent model organism for leguminous plants due to small genome and high genetic transformation efficiency (Bell et al. 2001). Legumes are the second most important family of crop plants after Poaceae and significantly contribute to agricultural production (Graham and Vance 2003). For efficient agricultural production, it is necessary to enhance crop resistance to abiotic stress and therefore the study of NAC TF is prerequisite for improving stress tolerance in plants (Chen et al. 2015).

In this study, we used bioinformatic approach to analyse the NAC family in *M. truncatula*. We have performed comprehensive study of gene structures, motif composition, chromosomal locations, gene duplication, sequence homologies, and expression patterns during different stresses. The transcriptome information was used to characterize the functions of these TFs during abiotic stress. The results of the present study will be helpful for future investigations to enhance the stress tolerance of plants.

Materials and methods

Identification of NAC gene information

The Hidden Markov Model (HMM) profiles of the NAM domain PF02365 were downloaded from the Pfam database (Punta et al. 2011). HMM searched NAM (PF02365) domains from the *M. truncatula* protein database with values (*e*-value) cut-off at 1.0 (Johnson et al. 2010). The integrity of the NAM domain was determined using the online program SMART (http://smart.embl-heidelberg.de/) with an e-value < 0.1 (Letunic et al. 2012). In addition, the three fields (length, molecular weight, and isoelectric point) of each NAC protein were predicted by the online ExPasy program (http://www.expasy.org/tools/) (Rueda et al. 2015).

Phylogenetic analysis and motif prediction

To investigate the phylogenetic relationship of the NAC gene families in Arabidopsis, rice, and *M. truncatula*, NAC protein sequences were downloaded from phytozomes (http://www.phytozome.org) (Goodstein et al. 2012). NAC TFs were aligned using the BioEdit program. A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA6 program (Tamura et al. 2011). Bootstrapping was performed with 1000 replications. The online MEME analysis used to identify the unknown conserved motifs (http://meme.ebi.edu.au/meme/intro.html) using the following parameters: site distribution: zero or one occurrence (of a contributing motif site) per sequence, maximum number of motifs: 25, and optimum motif width ≥ 6 and ≤ 200 (Bailey et al. 2015). Detailed information of *M. truncatula* NAC proteins can be found in Table A1.

Gene structure and chromosomal localization

The whole-genomic sequence of *M. truncatula* and the summary of gene localization information were downloaded from the phytozome database (http://phytozome.jgi.doe.gov/medicago.php). The genomic sequence for each NAC gene was extracted from the whole-genomic sequence according to gene localization information using a programmed Perl script. A gene structure display server program (http://gsds.cbi.pku.edu.cn/index.php) was used to

display the *M. truncatula* NAC gene structures (Guo et al. 2007). Duplications between the NAC genes were identified and complemented using the PGDD database (http://chibba.agtec.uga.edu/duplication/), and were identified as tandem duplications (TD). Ideograms were created using Circos (Krzywinski et al. 2009).

Transcriptome analysis of the NAC gene in different tissues and under five abiotic stress

M. truncatula transcriptome data in different tissues during development were downloaded from the NCBISRA database (http://www.ncbi.nlm.nih.gov, Accession numbers SRX099057–SRX099062). The transcriptome data were derived from six tissues, including roots, nodules, blades, buds, seedpods, and flowers. *M. truncatula* transcriptome data under different abiotic stresses were downloaded from the NCBISRA database (http://www.ncbi.nlm.nih.gov, Accession numbers SRX1056987–92) (Li et al. 2009). The transcriptome data were derived under six stress factors, including cold, freezing, drought, salt, and high levels of ABA. We performed RNA-seq to detect the expression levels of NAC TF genes under different stresses, including cold, freezing, drought, salt and ABA. Clean reads from six samples were mapped to the *M. truncatula* genome sequencing using Samtool (Li et al. 2009). Tophat and Cufflinks were used to analyze RPKM (Trapnell et al. 2012). The RPKM values for NAC genes were utilized for generating the heatmap and k-means clustering using R (software) (Gentleman et al. 2004).

Plant material and treatments

M. truncatula (Jemalong) A17 was used in this study. Seeds were planted in a soil and sand mixture (3:1), germinated, and irrigated with half-strength Hoagland solution once every 2 d. The seedlings were grown in the following environmental conditions: temperature of 18 °C (night) and 24 °C (day), and relative humidity of 60–80%. The seedlings that germinated after 8 weeks were subjected to the following environmental conditions: temperatures of 4 (cold) or −8 °C (freezing), treated with 300 mM mannitol (drought) or 200 mM NaCl solution (salt), and the seedling leaves were sprayed with 100 μM ABA solution (ABA). Control and treated seedlings were harvested 3 h after treatment. All samples were frozen in liquid nitrogen and stored at −80 °C until use.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

The transcriptome sequencing analysis was validated and quantified by qRT-PCR. Primers were designed according to NAC cds with Primer Express 3.0 software (Untergasser et al. 2012), the primer pairs are listed in Table A1. Total RNA were extracted with an RNA prep pure Plant Kit (Tiangen, Beijing, China), and cDNA was synthesized from total RNA using a Rever Tra Ace (Toyobo, Shanghai, China), and qRT-PCR was performed in an ABI 7300 Real-time Detection System (Applied Biosystems, Foster City, CA, USA). The thermal profile for SYBR green RT-PCR consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, and a final dissociation at 95 °C for 15 s, followed by one cycle at 60 °C for 20 s and one cycle at 95 °C for 15 s. To confirm that a single PCR product was amplified and detected, a dissociation curve analysis of amplification products was performed at the end of each PCR reaction. After amplification, data were analyzed with ABI 7300 SDS software (Applied Biosystems, USA). The comparative CT method (2-△△Ct method) was used to analyze the expression level of different genes. All of the samples were tested in triplicate, and the experiments were performed on three biological replicates.

Results

Genome-wide identification of NAC family genes in *M. truncatula*

Searching for NAC genes in the *M. truncatula* genome, all proteins of the *M. truncatula* genome from phytozomes were annotated (Town, 2006). Finally, 97 non-redundant and complete NAC-domain-containing protein sequences were selected for further analysis—the amino acid sequence length was between 54 and 672 (average length 341.3)—and named from MtNAC1 to MtNAC97 based on the coordinate order on *M. truncatula* chromosomes information, including protein properties in Table 1 (Committee, 1999). To further understand NAC TF functions, these target genes were downloaded from database of *M. truncatula*.

Phylogenetic analysis and identification of additional motifs

To understand the evolutionary history among MtNAC genes, we constructed a phylogenetic tree for *M. truncatula*, *A. thaliana* (dicot), and *O. sativa* (monocot) (Zhu et al. 2012). From the results, NAC proteins can be divided into 14 subfamilies, characterized by highly conserved motifs with the exception of the NAC domain motif A. The motif pattern was clustered in the same way as the subgroup pattern, and therefore the results demonstrated our phylogenetic clustering results were accurate. The MtNAC

Table 1 List of all MtNAC genes information identified in the *Medicago truncatula* genome

Gene name	Gene ID	Chromosome location	Length (aa)	Family group	PI	Molecular weight (kDa)
MtNAC1	Medtr0036s0150	scaffold0036:60708-55826	539	XIII	4.51	60,016.90
MtNAC2	Medtr2157s0010	scaffold2157:1006-209	266	VII	9.01	30,458.60
MtNAC3	Medtr1g008740	chr1:1057858-1061279	362	I	9	40,725.70
MtNAC4	Medtr1g045470	chr1:17058321-17056452	325	XI	5.91	37,435.10
MtNAC5	Medtr1g053575	chr1:22598992-22597045	323	XI	4.74	37,448.70
MtNAC6	Medtr1g069805	chr1:30480393-30484636	275	I	5.88	31,799.80
MtNAC7	Medtr1g087190	chr1:39063064-39062900	54	XIII	6.01	6,453.50
MtNAC8	Medtr1g090720	chr1:40728357-40734069	482	VI	6.42	53,997.70
MtNAC9	Medtr1g090723	chr1:40735770-40738910	630	XIII	4.42	70,012.50
MtNAC10	Medtr1g093670	chr1:42006623-42004062	345	X	5.1	39,407.20
MtNAC11	Medtr1g093680	chr1:42018089-42012426	481	X	4.81	54,345.30
MtNAC12	Medtr1g096430	chr1:43438375-43439601	339	II	6.53	39,409.80
MtNAC13	Medtr1g097300	chr1:43891021-43886555	571	XIII	4.58	64,727.80
MtNAC14	Medtr2g014680	chr2:4232801-4236401	308	X	6.76	34,805.90
MtNAC15	Medtr2g062730	chr2:26494431-26498275	344	II	6.85	39,737.40
MtNAC16	Medtr2g064090	chr2:27141403-27145406	346	III	5.13	39,985.10
MtNAC17	Medtr2g064470	chr2:29175407-29181087	306	I	8.07	34,667.20
MtNAC18	Medtr2g068880	chr2:28613602-28616289	312	XII	6.56	35,958.10
MtNAC19	Medtr2g068920	chr2:28628371-28630420	291	XII	6	33,721.50
MtNAC20	Medtr2g078700	chr2:32897238-32899105	381	I	8.17	42,221.90
MtNAC21	Medtr2g079990	chr2:33727407-33729223	354	IX	7.05	40,053.90
MtNAC22	Medtr2g080010	chr2:33761666-33764021	354	VII	8.85	39,359.90
MtNAC23	Medtr2g086690	chr2:36452124-36453846	249	XIII	6.61	28,467.60
MtNAC24	Medtr2g086880	chr2:36530061-36538039	589	III	8.82	66,653.20
MtNAC25	Medtr2g090735	chr2:38885938-38887846	285	X	8.72	32,439.60
MtNAC26	Medtr2g093810	chr2:40013370-40012105	323	VII	8.79	37,217.80
MtNAC27	Medtr3g064580	chr3:29101701-29105835	672	III	5.74	76,914.70
MtNAC28	Medtr3g070030	chr3:31349143-31346724	352	I	6.68	40,119.70
MtNAC29	Medtr3g070040	chr3:31359786-31357396	335	I	5.73	37,705.10
MtNAC30	Medtr3g088110	chr3:39954022-39955471	288	VIII	7.65	33,062.30
MtNAC31	Medtr3g093040	chr3:42540067-42535957	312	VI	5.86	35,072.70
MtNAC32	Medtr3g093050	chr3:42546323-42542510	292	VIII	5.94	33,796
MtNAC33	Medtr3g096140	chr3:43934316-43939062	336	XIII	4.8	37,484.70
MtNAC34	Medtr3g096400	chr3:44063892-44061980	208	IX	4.3	23,721.30
MtNAC35	Medtr3g096920	chr3:44370629-44372461	292	VIII	5.94	33,796
MtNAC36	Medtr3g098810	chr3:45276406-45275099	241	VI	8.84	27,405.90
MtNAC37	Medtr3g109340	chr3:50581341-50579226	358	I	8.14	40,490.20
MtNAC38	Medtr3g116070	chr3:54270247-54268085	353	I	6.64	40,385.60
MtNAC39	Medtr3g435150	chr3:11464191-11461627	303	I	9.22	34,581.40
MtNAC40	Medtr4g035590	chr4:12274680-12278280	354	II	6.9	41,163.80
MtNAC41	Medtr4g036030	chr4:12849080-12853325	346	II	5.41	40,428.90
MtNAC42	Medtr4g052620	chr4:19088028-19085634	261	XIII	5.69	29,816
MtNAC43	Medtr4g075980	chr4:29048799-29051425	299	XII	7.05	34,465.30
MtNAC44	Medtr4g078875	chr4:30504183-30506651	284	X	9.02	31,985.90
MtNAC45	Medtr4g081870	chr4:31873646-31871462	271	VII	6.51	31,265.30
MtNAC46	Medtr4g088245	chr4:34833163-34829374	318	X	5.66	35,933.20
MtNAC47	Medtr4g089135	chr4:35772138-35774193	344	VII	7.23	38,660.70
MtNAC48	Medtr4g094302	chr4:37671153-37672443	191	IX	4.82	22,243.60

Table 1 continued

Gene name	Gene ID	Chromosome location	Length (aa)	Family group	PI	Molecular weight (kDa)
MtNAC49	Medtr4g098630	chr4:40652597-40651083	318	III	5.52	36,214.40
MtNAC50	Medtr4g101680	chr4:42049132-42045198	364	II	6.25	42,406.90
MtNAC51	Medtr4g108760	chr4:45058271-45060202	359	I	8.56	40,556.60
MtNAC52	Medtr4g134460	chr4:56311697-56314294	434	XII	6.15	48,628
MtNAC53	Medtr5g012080	chr5:3572221-3576785	349	II	6.27	40,487
MtNAC54	Medtr5g014300	chr5:4775644-4779821	335	III	4.98	37,963.20
MtNAC55	Medtr5g021710	chr5:8449579-8452636	352	II	5.12	40565.4
MtNAC56	Medtr5g040420	chr5:17777239-17779842	391	XII	6.27	44699.2
MtNAC57	Medtr5g041940	chr5:18429509-18427695	260	VII	6.91	30,003.7
MtNAC58	Medtr5g053430	chr5:22012794-22017536	442	X	4.84	49,671
MtNAC59	Medtr5g069030	chr5:29216428-29220286	630	VI	4.82	70,313.2
MtNAC60	Medtr5g076850	chr5:32791723-32786597	644	III	5.37	74,164.4
MtNAC61	Medtr5g090970	chr5:39632529-39636258	311	XII	8.51	36,029.5
MtNAC62	Medtr6g011860	chr6:3555895-3560091	391	XII	6.94	44,591.2
MtNAC63	Medtr6g012670	chr6:3892753-3894308	321	I	5.01	37,140.5
MtNAC64	Medtr6g032770	chr6:10318538-10315903	379	I	5.35	43,087
MtNAC65	Medtr6g084430	chr6:31607391-31604165	325	I	7.18	36,958.40
MtNAC66	Medtr6g477900	chr6:28664418-28659313	239	IV	4.9	27,812.20
MtNAC67	Medtr7g005280	chr7:47539-49564	256	VI	9.15	27,812.20
MtNAC68	Medtr7g011120	chr7:2918496-2920585	310	I	5.84	35,229.10
MtNAC69	Medtr7g011130	chr7:2923772-2925970	352	I	5.66	40,054.40
MtNAC70	Medtr7g033320	chr7:11902390-11906760	501	X	4.84	56,139.70
MtNAC71	Medtr7g070140	chr7:25851047-25851665	137	VI	8.52	15,558.40
MtNAC72	Medtr7g070150	chr7:25852151-25851784	92	VI	10.07	10,692.10
MtNAC73	Medtr7g083330	chr7:32051182-32049757	194	V	9.12	22,900.10
MtNAC74	Medtr7g083360	chr7:32063997-32063118	155	V	9.14	18,494
MtNAC75	Medtr7g083370	chr7:32071800-32070846	143	V	6.08	17,385.60
MtNAC76	Medtr7g085220	chr7:32967009-32968969	340	VII	8.87	38,464.50
MtNAC77	Medtr7g085260	chr7:32990543-32992707	383	I	6.52	43,927.10
MtNAC78	Medtr7g097090	chr7:39019290-39014420	291	I	5.43	33,546.60
MtNAC79	Medtr7g100990	chr7:40734227-40732629	328	VII	7.69	37,535.30
MtNAC80	Medtr7g105170	chr7:42639031-42640463	257	XIII	5.75	29,660.10
MtNAC81	Medtr7g116460	chr7:48060498-48059630	206	XI	8.97	23,610.70
MtNAC82	Medtr8g023840	chr8:8690689-8692417	400	IV	5.47	45,950.20
MtNAC83	Medtr8g023860	chr8:8704958-8707169	419	IV	5.28	47,510
MtNAC84	Medtr8g023880	chr8:8712893-8717845	269	IV	5.56	30,475.30
MtNAC85	Medtr8g023900	chr8:8722035-8725267	470	IV	5.65	55,139.10
MtNAC86	Medtr8g023930	chr8:8738431-8747309	473	IV	6.74	55,551.40
MtNAC87	Medtr8g024480	chr8:9002671-9004590	434	II	6.06	48,952.90
MtNAC88	Medtr8g059170	chr8:20701024-20703544	329	IX	5.93	36,960.50
MtNAC89	Medtr8g063550	chr8:26582418-26578891	444	XIII	4.31	48,649.20
MtNAC90	Medtr8g069160	chr8:28951511-28948614	259	X	6.32	29,439.30
MtNAC91	Medtr8g076110	chr8:32226602-32223015	311	II	6.39	36,440.20
MtNAC92	Medtr8g093580	chr8:39133468-39136666	489	XIII	4.16	53,787.50
MtNAC93	Medtr8g093790	chr8:39242842-39241213	185	IX	4.52	21,408.70
MtNAC94	Medtr8g094580	chr8:39492258-39494179	285	VIII	6.08	32,727
MtNAC95	Medtr8g099750	chr8:40364682-40363158	227	VI	8.32	25,747.80
MtNAC96	Medtr8g102240	chr8:43013735-43018918	485	X	6.62	55,002.80

Table 1 continued

Gene name	Gene ID	Chromosome location	Length (aa)	Family group	PI	Molecular weight (kDa)
MtNAC97	Medtr8g467490	chr8:24258825-24255680	395	XII	8.43	44,571.40

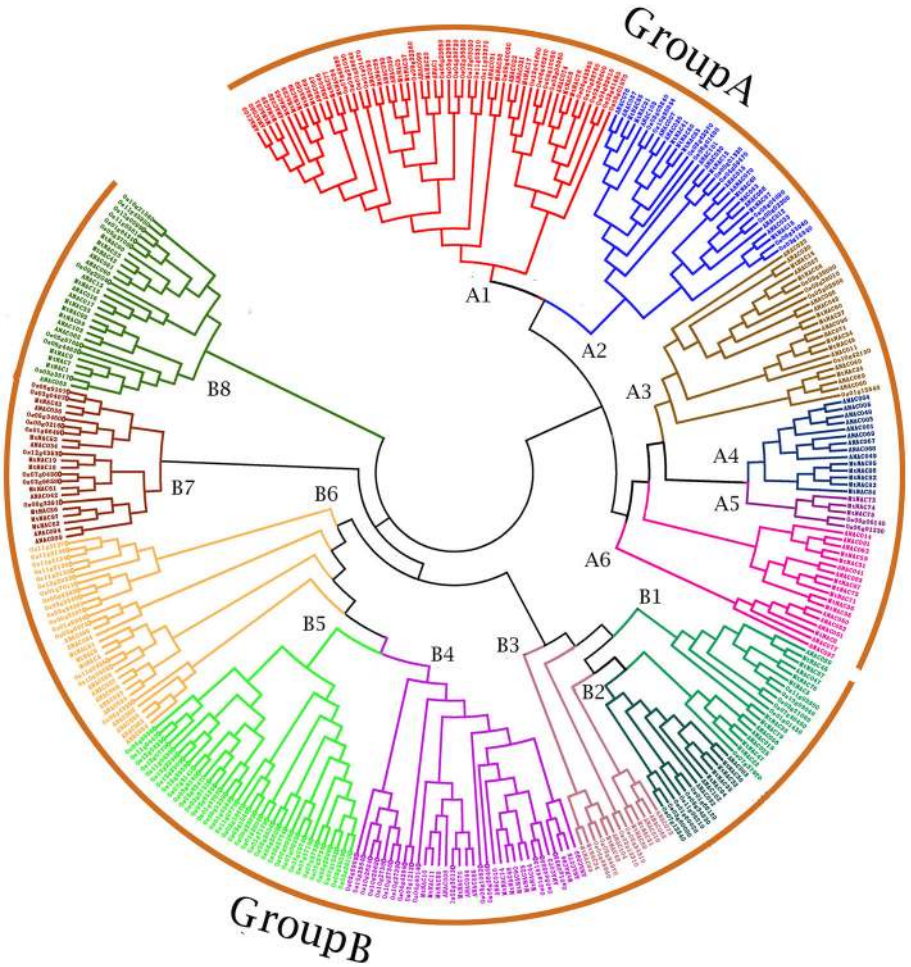


Fig. 1 Phylogenetic tree analysis of the NAC transcription factor amily in *Medicago truncatula*, *Arabidopsis thaliana* (dicot) and *Oryza sativa*. The phylogenetic tree was constructed using MEGA 6.0 by the neighbor-joining method. The Bootstrap value was 1000 replicates. The three plant-specific clusters were designated as *A* (*A1–A6*), *B* (*B1–B8*) and indicated in a specific *color* (color figure online)

proteins were divided into o two groups (A and B). The tree was divided into six groups in Group A (A1–6). Group B possessed eight phylogenetic subgroups (B1–8) (Fig. 1). Most of the subgroups had highly conserved motifs excluding the NAC domain motifs, such as Group (I–VI) belonging to (A1–6) Group (VII–XIII) belonging to (B1–8, except B5). The motifs in the NAC family proteins in *M. truncatula* were investigated using MEME software, revealing 25 conserved motifs (motifs 1–25), (Fig. 2). Thus, the results show that gene sequences belong to 14 groups.

In general, the clusters of NAC proteins had similar motif compositions and most of the conserved motifs were found in N-terminal. Motifs 1–20 were conserved in the NAC protein family (Fig. S1). Most groups possessed an N-terminal NAC domain that included Motif1-5 (Hu et al. 2010). Most of these proteins had a special motif in the C-terminal; Group I (28, 29, 51, 63, 68, and l69) possessed an NAC domain Motif 8, Group II possessed an NAC domain Motif 14, and Group VIII possessed a composite motif (19.20) in the C-terminal. Detailed information is listed in Table S2.

Gene structure, gene chromosomal location, and gene duplication events of MtNAC

We found the same groups members had the same or similar gene structures (Fig. 3). Groups I and II had two introns and the same CDS distribution in genes, and the last coding region was longer than the others. Most members

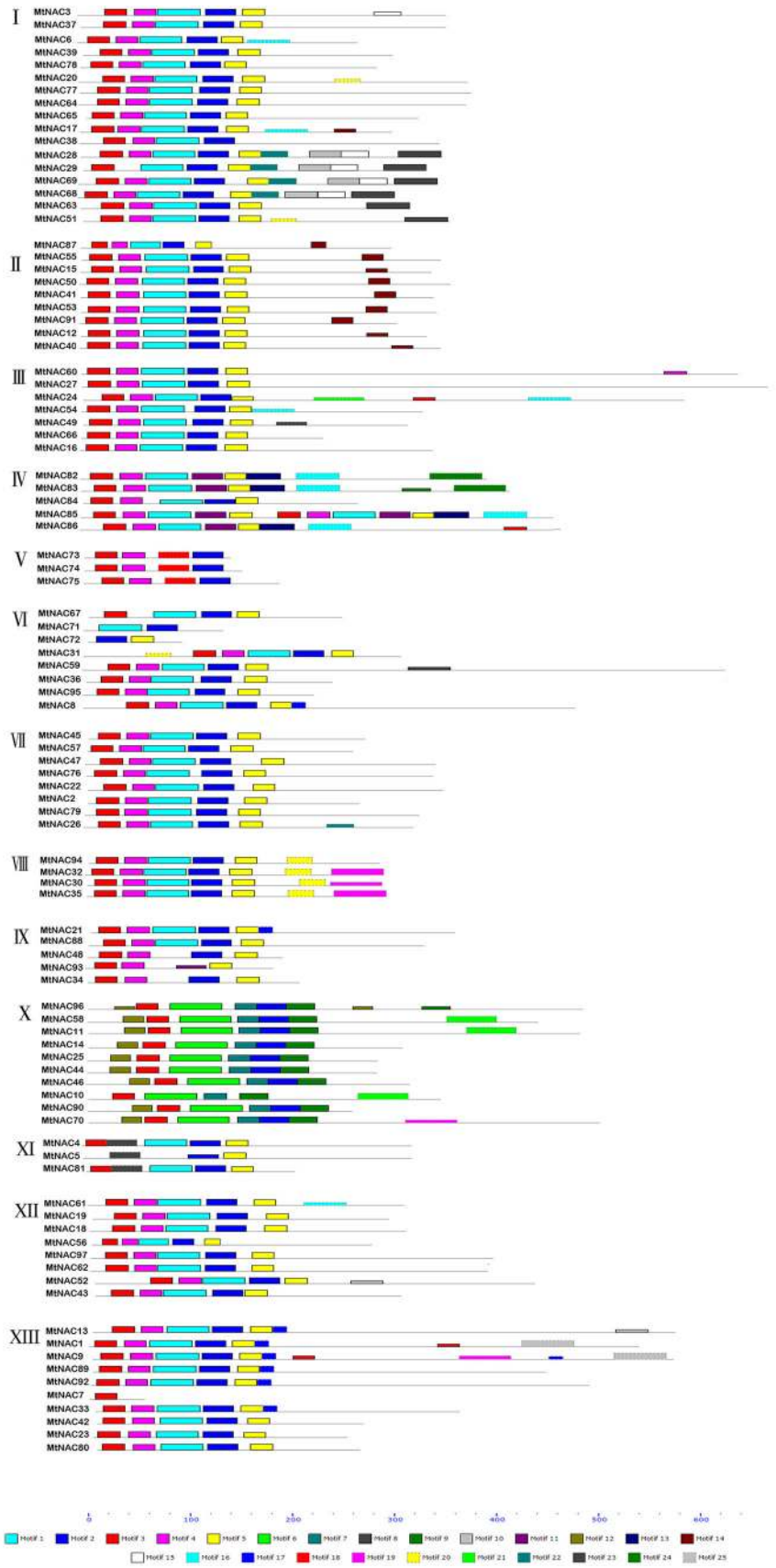
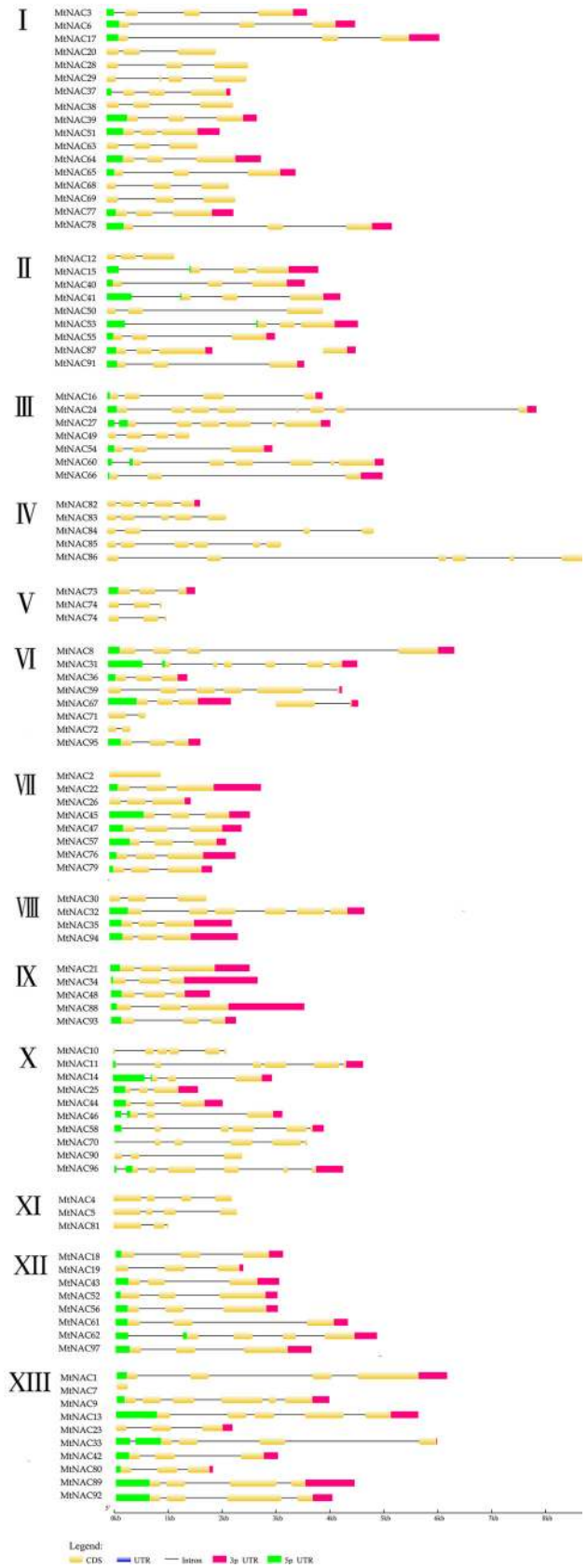


Fig. 2 Distribution of conserved motifs within MtNAC transcription factor family in *Medicago truncatula*. Summary for the distribution of conserved motifs identified from 93 MtNAC proteins by each group given separately. Each motif is represented by a number in *colored box* (color figure online)



◀**Fig. 3** Exon-intron structure analyses of MtNAC genes were performed by using the online tool GSDS. Lengths of exons and introns of each MtNAC gene were exhibited proportionally. Exon/intron organization of 97 MtNAC genes was depicted for each group. The exons and introns are represented by *box* and *lines*, respectively

had three introns and up/downstream in Group III, except MtNAC7. Groups IV and XI had large quantities of introns, Groups V and VI also showed obvious genetic structure characteristics with longer intron and shorter exon positions. Group VIII had numerous members, most members having three exon positions besides every area being longer. The other groups had shorter gene lengths and all groups had similar gene structures.

A total of 97MtNACs were located on eight chromosomes of *M. truncatula*, with 63 pairs of genes in tandem duplication. Different color links were used to distinguish gene indices of similarity. A total of 95 MtNAC genes could be located in eight chromosomes (1–8), and MtNAC1 and 2 could not be conclusively mapped on any chromosome. There were only 5 NAC genes in MtChr6, whereas others possessed at least 10 MtNAC genes. Multi-member groups were widely distributed among chromosomes, for example Group I was distributed among 6 chromosomes, excluding MtChr8 and MtChr5. Most chromosomes had more than six different groups. The result showed gene clusters and hot regions is produced by tandem duplications in MtNAC, for instance the MtNAC73–77 and MtNAC82–87 clusters on Chr1. Segmental duplication produced homologous NAC genes, which expanded the numbers of MtNAC genes in genome. For example, MtNAC6 and MtNAC78 from Group I were distributed on different chromosomes (MtNAC6, MtNAC78, MtNAC56 and 57), which were segmental duplication in *M. truncatula* (Fig. 4).

Expression profiles of MtNAC genes among different tissues

The heatmap showed that 40 NAC were expressed in all six tissues: Mt (35, 94, 17, 77, 89, 32, 13, 30, 45) were highly expressed in the root; Mt (34,14,47) were specifically expressed in buds; Mt (43, 87, 22, 59, 25, 49, 93, 60, 9, 8, 55, 1, 70) were highly expressed in seedpods; Mt (12, 11, 48, 91, 21, 76) were highly expressed in seedpods and flowers, and Mt (33, 58, 24, 92, 67, 44, 96, 73, 31) were highly expressed in roots and seedpods (Fig. 5).

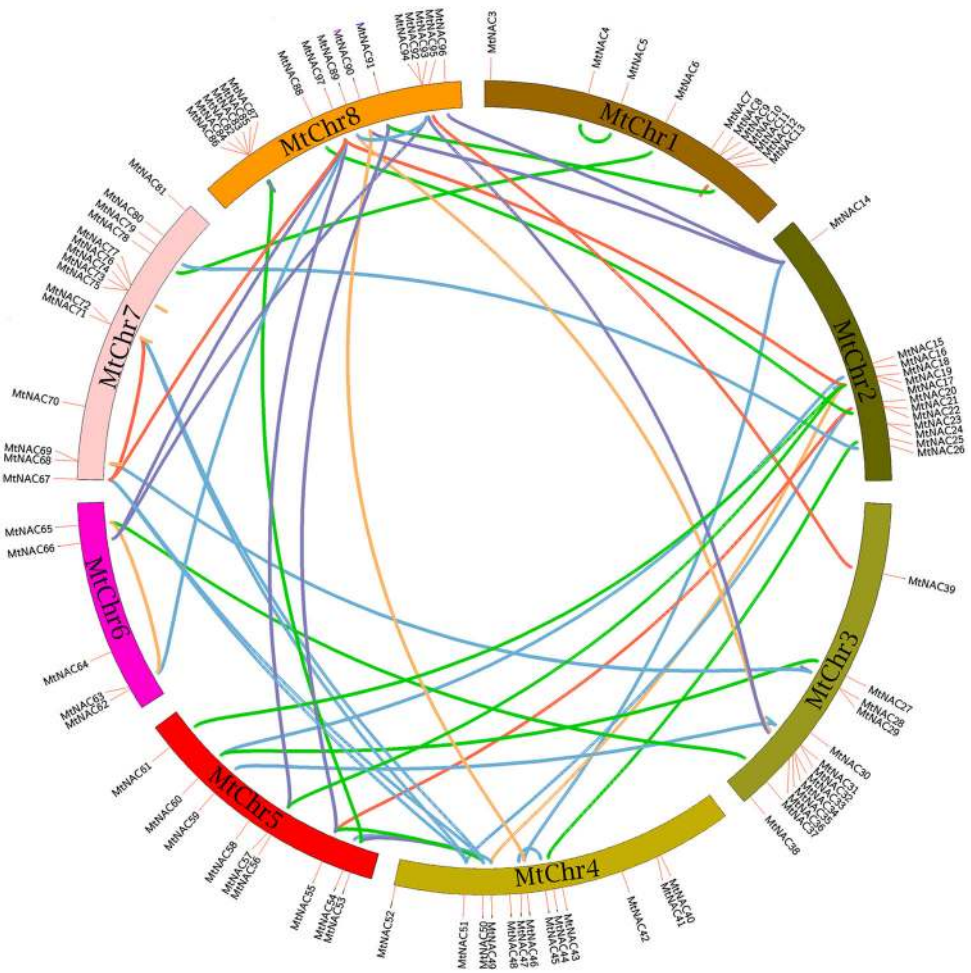


Fig. 4 Duplicated genes between different chromosomes or loci were linked with colored lines in the diagrams using Circos as described previously. Genes were identified using the BLASTP using parameters; e-value $\leq$ 1e $-$ 10 and minimum percent identity = 70%

Expression responses of MtNAC genes among abiotic stress

We used RNA-seq to analyse the expression of MtNAC genes under different stresses, such as cold-stress, freezing-stress, drought-stress, salt-stress and ABA-stress. We attempted to evaluate the 44 genes detected in at least one library (Fig. 6). Most genes were exclusively induced and partial genes were exclusively repressed. There were 17 genes up regulated under all five stresses (Fig. S2). Only MtNAC1 was downregulated under all five stresses. During cold stress, 6 genes showed no obvious change and 5 genes were repressed, whereas 33 genes were exclusively induced. In the freezing stress treatments, 8 genes were repressed and 36 were induced. In the drought conditions, 6 genes showed no obvious change, 5 genes were repressed, and 33 genes were exclusively induced. MtNAC (17, 21, 30, 32, 35, 67, and 94) were highly expressed and showed the greatest variation in most abiotic stresses. MtNAC (24, 58, 92, 8, 33, 25, 22, 87, 96, 77, 51, and 80) were specifically expressed in response to freezing and salt stresses. MtNAC (97, 16, 38, 39, and 82) showed very low expression in various stress conditions. MtNAC (13, 43, 9, 59, 88, 1, 89, 57, 45, and 76) were not expressed in response to abiotic stresses.

qRT-PCR of MtNAC genes in abiotic stress

To verify the authenticity of transcriptome data, we selected 12 genes to detect their expression profiles under 6 stress conditions using qTR-PCR (Fig. 7). The results of qRT-PCR were consistent with the transcriptome data. The results showed that MtNAC33 and 48 shared the same expression pattern under five different stress conditions. In addition, the expression of the two genes increased significantly in salt and drought conditions. MtNAC50 showed high expression levels only in cold- and salt-stress conditions. MtNAC57 and 73 were upregulated under all stresses, except freezing. MtNAC88, MtNAC92, and MtNAC94 expression only increased in cold stress. MtNAC95 expression increased in salt-, drought- and ABA-stress conditions. These results corroborate the findings of transcriptome data.

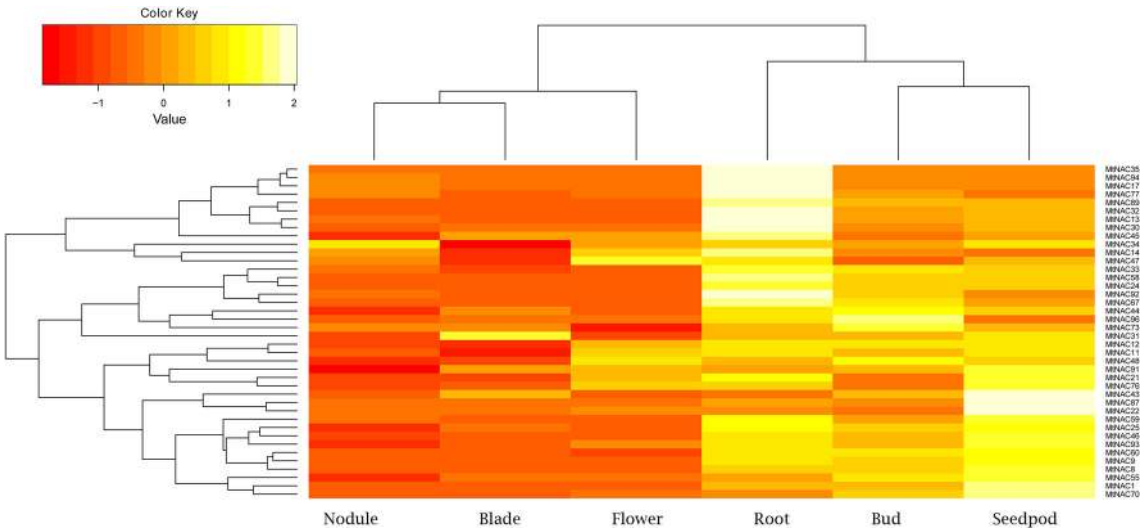


Fig. 5 Number of differentially expressed MtNAC genes involved in tissue development. Clustering of legume MtNAC genes according to their expression profiles in tissues including roots, nodules, blades, buds, seedpods and flowers

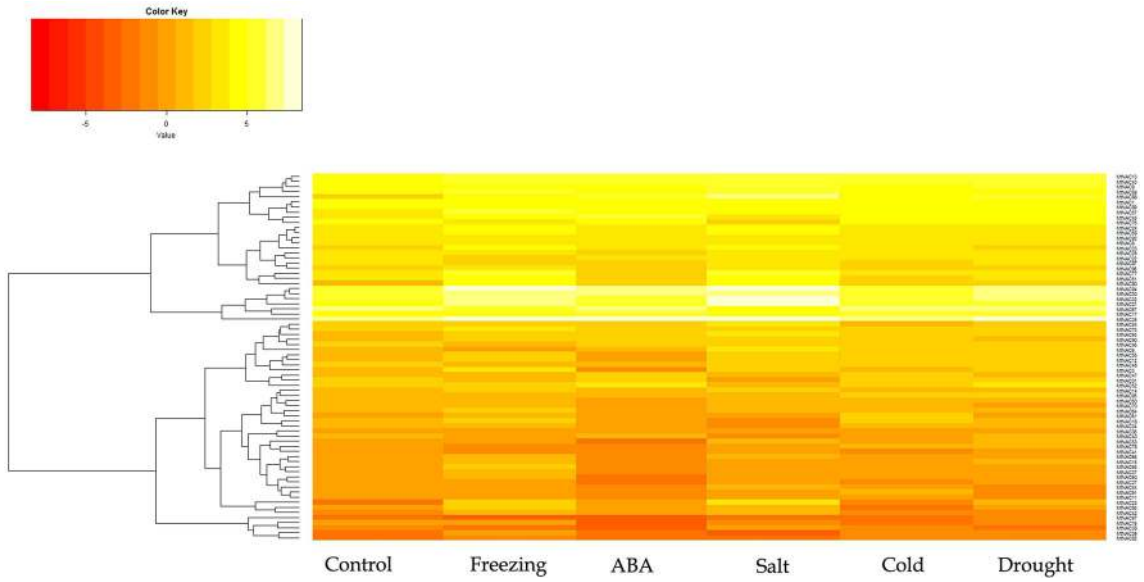


Fig. 6 Differential expression analysis of MtNAC genes involved in the response to abiotic stress. Heatmap of MtNAC genes expressed among five stresses. The relative expression values were log2 transformed using the R soft

Discussion

We performed a comprehensive in silico study and characterized 97 MtNAC genes in the Medicago genome (Table 1). The NAC TF gene family were surveyed in Arabidopsis (117), rice (151), soybean (152), grape (*Vitis vinifera*) (163), and tobacco (152) (Le et al. 2011; Nuruzzaman et al. 2010; Ooka et al. 2003; Wang et al. 2013).

The phylogenetic analysis classified the MtNACs into 13 groups with their AtNAC and OsNAC (Fig. 1). These members were widely distributed in different groups, but only B5 contained OsNACs (Cenci et al. 2014). Soybean showed similar results, which confirmed that differences appeared between monocots and dicots in the course of their evolutionary history (Le et al. 2011).

All NAC TFs have important conserved motifs for their function. We identified 25 motifs in MtNAC, motifs 1–5 are related to the NAC domain, and motifs (3, 4, 1, 2, 5) represent the A–E subdomain (De Zélicourt et al. 2012). Subdomain A is component of functional dimmer, subdomains C and D can bind to DNA terminal, and the divergent subdomains B and E play an important role in gene function. Subdomain E is NAC DB domain that has five

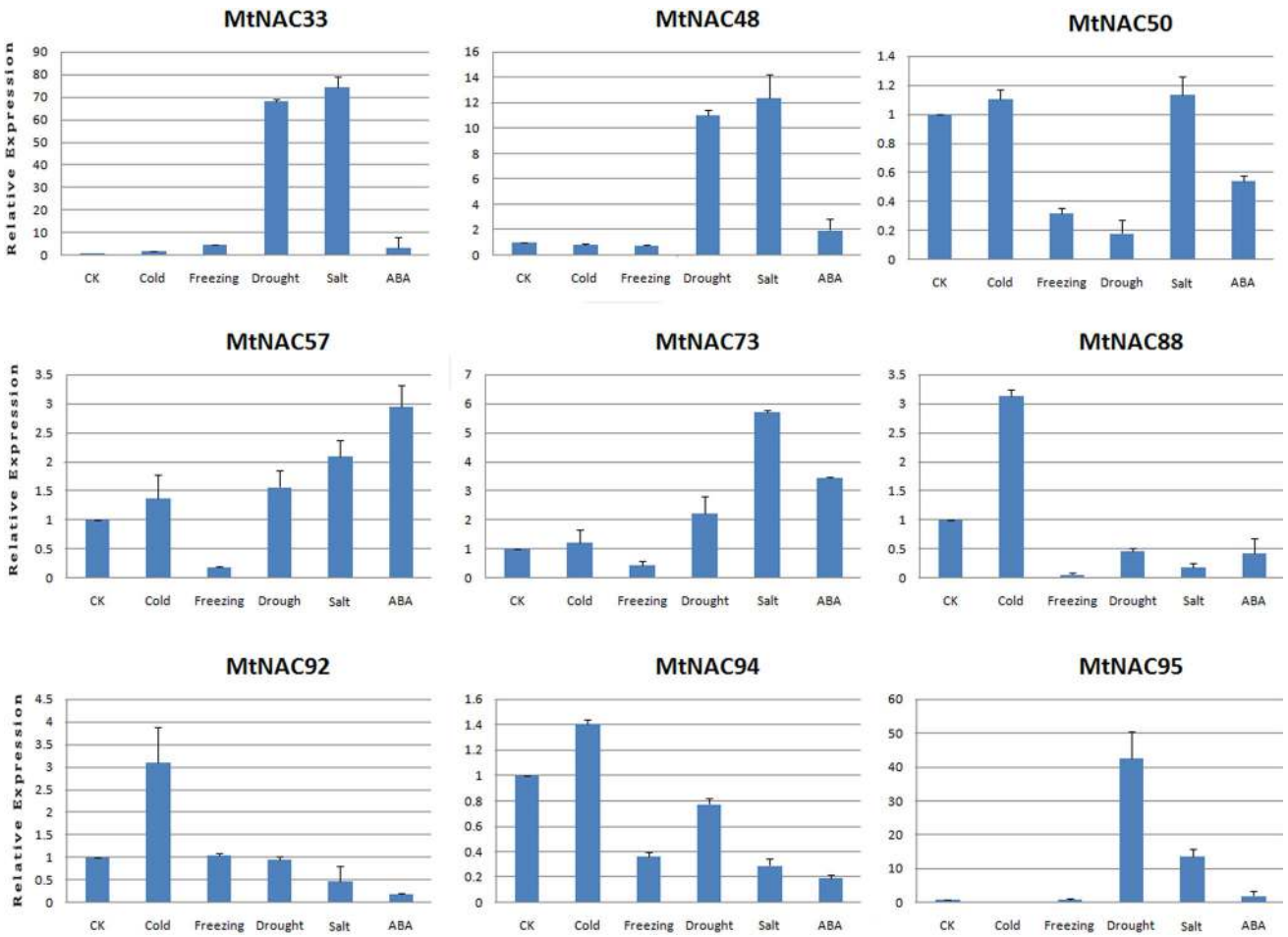


Fig. 7 qRT-PCR analysis reveals NAC genes under five stress conditions: cold, freezing, salt, drought, and ABA treatments compared to the controls. Stress treatments and time course are described in ''Materials and methods''

motifs. Interestingly, the function of NAC TFs can regulate downstream gene expression level through the complex interaction between the DB domain, NARD, and the activation domain. Additionally, plant physiology could also mediate transcriptional regulation by the NAC protein domain (Hao et al. 2010). NAC gene family promoter regions (NACBS) showed positive response to stress (JENSEN et al. 1997). The TR domain represents transcription activation repression and protein binding in the C-terminal. A single or few motifs constitute TR domains such as motifs (8, 14, 19, and 20).

The plants gene duplication phenomenon plays an important role in dealing with environmental change. However information about functionality of duplicated genes is still limited (Bowers et al. 2003).

This phenomenon increase the diversity of the gene family through changing gene expression (Friedman and Hughes 2003). The differential expresion pattern of genes may result in functional diversity (Soskine and Tawfik 2010).

However, the high number of TCP genes in *M. truncatula* was possibly caused by gene duplication. In the paralogous pairs, most TCPs shared conserved exons/introns and organization such as MtNAC4 and 5, and most paralogous pairs of MtTCPs exhibited conserved motif composition such as MtNAC14 and MtNAC46, with several motifs disappearing in some MtTCP members, such as motif 16 for MtTCP6-78. Several motifs were observed, such as motifs 9 and 12 for MtTCP14-96. These specific motifs may contribute to which paralogous member obtains a new function after gene duplication, and duplicated genes are changed by a series of synonymous or nonsynonymous mutations during evolution.

NAC family genes regulate different tissue development such as shoot, meristem and organ, therefore they have different expression patterns in plant (Kim et al. 2007). CUC1, CUC2, NAM, NACL, AT5G07680, and AT5G61430 regulate plant and meristem development through miR164 (Kim et al. 2009). NST1 and NST3 play

an important role in woody secondary walls regeneration. Most types of leaf vein are regulated by the VND7 in roots and shoots (Mitsuda et al. 2007). Mt (35, 94, 17, 77, 89, 32, 13, 30, and 45) were only highly expressed in root tissue (Fig. 5). MtNAC47 was only expressed in nodule tissue, and its homologous gene, RhNAC100 participates in flower petals cell expansion through ethylene regulatory pathway (Mitsuda et al. 2007). The closest homolog of MtNAC969 in Arabidopsis is AtNAP/ANAC029 (De Zélicourt et al. 2012). This gene takes part in floral and stamen formation through APETALA3 PISTILLATA regulating. This phenomenon demonstrate that NAC family genes may have significant role in root and flower formation in *M. truncatula.*

This study shows that NAC TFs are involved in various environmental stresses. The transcript of MtNAC30 had higher expressions in drought than normal condition and the homolog of the ANAC002 was previously upregulated during drought in Arabidopsis (Balazadeh et al. 2010). In our study MtNAC35 was upregulated during ABA treatment. The homolog of the MtNAC35 291 OsNAC19 has a role in ABA and ethylene (ETH) induction (Lin et al. 2007). Based on recent studies and abiotic stress expression data from genes, we found that MtNAC genes take part in diverse signaling pathways and stress responses. The SNAC1 transgenic lines possess higher drought and salt tolerance than wild plants in dry fields, and the expression of several MtNAC genes improved by drought and salt stress, such as MtNAC35 (You et al. 2014).

Homologous gene of MtNAC83, SsNAC23 was strongly induced at 4 °C, indicating a positive response to cold stress (Nogueira et al. 2005). MtNAC83 was also upregulated under cold (4 °C) conditions. The SiNAC improve the plant resistance by three different pathway, such as ABA-independent, JA and SA (Puranik et al. 2011). Several NAC genes help the plant to adapt to large environmental changes through promoting plant growth. Their versatility ensures the longevity and multiplication of plants (Puranik et al. 2012). Furthermore, our results suggest that Group VIII were more sensitive to abiotic stress, whereas Group VIII were active in tissue development (Figs. 5, 6). The results of our studies verify those of previous results. We used genome information and transcriptome data along with qRT-PCR validation to determine the function of a many NAC genes in *M. truncatula*. Understanding the gene function will help to provide useful information for genetic model systems. The information about NAC genes will be helpful for improving plant resistance and crop yield in the future. Furthermore, it can help us to understand the NAC transcription factors regulatory mode during adverse stress condition.

Conclusions

In summary, 97 putative NAC transcription factors were identified from the *M. truncatula* genome sequence, one of the most important model organisms for leguminous plants. We investigated the structure, phylogeny, and gene duplication of the conserved motifs and gene organization. Furthermore, the differential expression profile of MtNAC genes suggest their responsiveness to different stress. The present study shows that NAC TF family has responsiveness to abiotic stress (cold, freezing, drought, salt, and ABA) in *M. truncatula*.

The study provides advantageous information for understanding the molecular basis of the NAC TF family in *M. truncatula*, and the results can be used to engineer the plants with enhanced stress resistance. Further study of the NAC genes' function will be helpful for transgenic applications.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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