Physiol Mol Biol Plants (April 2020) 26(4):705–717
https://doi.org/10.1007/s12298-020-00770-w

RESEARCH ARTICLE

Genome-wide analysis of NAC transcription factor family in maize under drought stress and rewatering

Guorui Wang[1] · Zhen Yuan[1] · Pengyu Zhang[1] · Zhixue Liu[1] · Tongchao Wang[1] · Li Wei[1]

Received: 15 August 2019 / Revised: 26 December 2019 / Accepted: 22 January 2020 / Published online: 27 February 2020

Abstract The plant-specific NAC transcription factor (TFs) plays crucial role in plant growth as well as in stress resistance. In the present study, 87 *Zea mays* NAC TFs were obtained from the transcriptome analysis using drought-resistant maize inbred line Y882 as experimental material under PEG stress and rewatering treatment. Comprehensive analyses were conducted including genes structure, chromosomal localization, phylogenetic tree and motif prediction, *cis*-elements and expression patterns. The results showed that the 87 *ZmNAC* genes distributed on 10 chromosomes and were categorized into 15 groups based on their conserved gene structure and motifs. Phylogenetic tree analysis was also constructed referencing to the counterparts of *Arabidopsis* and rice, and the stress-related *cis*-elements in the promoter region were also analyzed. 87 *ZmNAC* genes exhibited different expression levels at 3 treatment points, indicating different response to drought stress. This genome-wide analysis of 87 *ZmNAC* genes will provide basis for further gene function detection.

Keywords *Zea mays* · NAC transcription factors · Drought · Expression pattern

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12298-020-00770-w) contains supplementary material, which is available to authorized users.

✉ Li Wei
weili-wtc@126.com

[1] Agricultural College of Henan Agricultural University, Zhengzhou 450046, Henan, China

Introduction

Maize is one of the most important food crop in China with strong adaptability, high economic value and widely utilization. In China, especially in the north and north–west region, the water resources are very limited and drought stresses with different degrees occur every year. Under drought stress, plants undergo various biochemical changes in gene or protein levels to adapt against adverse environmental conditions (Fang et al. 2015). If water stress does not exceed a certain threshold, then plants can actively produce a compensation mechanism to cope with water shortage (Shan 2003). Studies show that the recovery ability of plants after rewatering and drought resistance is more important than drought tolerance under drought stress (Kamoshita et al. 2004). The response of maize plants to rewatering after drought stress is the rapid growth (Bu et al. 2009), and many genes are involved in the process.

In regulating processes of plants, the expression of stress-related genes are largely governed by specific transcription factors. Research findings show that numerous transcription factors (TFs) play an essential roles in improving plant tolerance to abiotic stress (Puranik et al. 2012; Wang and Dane 2013). NAC (NAM, ATAF1,2 and CUC2) transcription factors belong to the plant-specific transcription factor family and functional studies demonstrate that NAC TFs involve in responses to drought, salinity, and cold stresses (Borrill et al. 2017; Kadier et al. 2017; Kou et al. 2014; Saidi et al. 2017; Sun et al. 2018). In *Arabidopsis*, *ATAF1* gene was induced by drought stress and ABA treatment. Overexpression of *ATAF1* in transgenic *Arabidopsis* plants resulted in enhanced drought tolerance (Wu et al. 2009). Overexpression of *TaNAC69* in transgenic wheat improved dehydration tolerance and enhanced the expression levels of genes up-regulated under

stress (Xue et al. 2011). Another wheat NAC transcription factor *TaNAC29* was involved in response to drought, salt and ABA treatments (Xu et al. 2015). Overexpression of *SNAC3* in rice showed an enhanced plant tolerance to high temperature and drought, whereas suppression of *SNAC3* by RNAi exhibited increased sensitivity to these stresses (Fang et al. 2015).

NAC transcription factors have been well studied in various species such as *Arabidopsis* (Capella et al. 2014), rice (Nuruzzaman et al. 2010), soybean (Dung Tien et al. 2011) and wheat (Borrill et al. 2017), but only a few NAC members have been analyzed in maize. *ZmSNAC1* and *ZmNAC55* are strongly induced by drought, cold, and ABA treatments, overexpression of *ZmSNAC1* and *ZmNAC55* in *Arabidopsis* induce enhanced drought tolerance, respectively (Lu et al. 2012; Mao et al. 2016). Based on the maize genome sequences from relative database, 148 and 128 maize NAC members were identified, respectively (Ge et al. 2015; Peng et al. 2015), Shiriga identified 152 NAC TFs in maize and selected 11 NAC genes for expressing analysis between a drought-tolerant genotype and a susceptible genotype during drought stress (Shiriga et al. 2014).

In the present study, we used 20% PEG6000 to simulate drought stress and carried out a transcriptome analysis using a drought-resistant maize inbreed line Y882 under PEG stress and rewatering treatment. The genes structure, chromosomal localization, gene ontology, phylogenetic tree and motif prediction, *cis*-elements and expression patterns were investigated. This study provides the basis for further function detection of NAC genes.

Materials and methods

Plant growth and stress treatments

Maize inbred line Y882 was used in this study. The initial experiment indicated that Y882 was a drought-resistant line (data unpublished). Seeds were surface sterilized and germinated in an incubator for 24 h at 28 °C. Seedlings were grown in a greenhouse with 14 h/10 h light/dark photoperiod, 60% relative humidity, and light intensity of 120 μmol m^{-2} s^{-1}. Seedlings were grown in half-strength modified Hoagland's nutrient solution (pH 5.8), which was refreshed every 3 days. Seedlings at the 3-fully expanded leaf stage were transferred to nutrient solution containing 20% polyethylene glycol PEG-6000 for stress treatment. Leaves were harvested at 60 and 96 h of the drought treatment and rewatered at 3 d (denoted as T60, T96 and TR3d, respectively). Control seedlings were grown under the same conditions. Three plants from three different containers of each treatment were used as biological replicates. All samples were immediately frozen in liquid nitrogen and stored at −80 °C.

RNA extraction, cDNA library construction and transcriptome sequencing

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The concentration and quality of the RNA were verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The construction of cDNA library and transcriptome sequencing was performed by Genedenovo Technology Company in Guangzhou, China. Equal amounts of total RNA extracted from the three replicate plants at each treatment point to construct the cDNA library using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® by protocols. Double-stranded cDNAs were synthesized using the reverse-transcriptase and random hexamer primers. The cDNA fragments were purified using QIA quick PCR kit and washed with EB buffer. Following the reparation of poly (A) addition and ligated to sequencing adapters, the fragments with the expected sizes were purified by agarose gel electrophoresis and enriched by PCR to construct the cDNA library. The cDNA library was sequenced on the Illumina sequencing platform (Illumina HiSeq™2500) using the paired-end technology.

Reads obtained from the sequencing machines were filtered and mapped to ribosome RNA database using short reads alignment tool Bowtie2 (Langmead and Salzberg 2012). The tools used for read alignment and expression quantification were TopHat2 (Kim et al. 2013) and Cufflinks (Trapnell et al. 2012), respectively. The gene expression level was normalized by using fragments per kilobase of transcript per million mapped reads (FPKM).

Identification of *ZmNAC* genes and differential gene expression (DEG) analysis

ZmNAC genes were identified from the transcriptome data and the whole-genomic sequence and gene localization information were downloaded from the Ensembl Plants database (http://plants.ensembl.org/index.html). The NAC domains were screened using Plant Transcription Factor Database v5.0 (http://planttfdb.cbi.pku.edu.cn/download.php). The chromosome location of NAC transcription factors were analyzed using MG2C software (http://mg2c.iask.in/mg2c_v2.0/?tdsourcetag=s_pcqq_aiomsg). The level of NAC genes expression was normalized by calculating the Fragments Per Kilobase of transcript per Million mapped reads (FPKM). The differentially expressed genes (DEGs) were identified with |log2 (fold change)| ≥ 1 and

FDR value < 0.05. Gene Ontology (Go) analysis was also conducted for gene functional classification.

Phylogenetic analysis and motif prediction

The phylogenetic tree was constructed in MEGA 6.0 using the neighbor-joining method (Bootstrap method: 1000), according to the classification method of NAC TFs in *Arabidopsis* and rice (Xu et al. 2015). The protein sequences of *ZmNAC* genes were downloaded from the Ensembl Plants database (http://plants.ensembl.org/index.html). The online MEME software was used to analyze the conserved motifs (http://meme-suite.org/) according to the default program, the maximum number of motif was 20.

cis-Elements analysis in the promoter regions of *ZmNAC* genes

Upstream regions (− 2000 bp) of *ZmNAC* genes were selected for *cis*-elements analysis using Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Five *cis*-elements were selected in this study including ABRE, MBS, TC-rich, CGTCA and DRE.

qRT-PCR analysis

qRT-PCR was performed on CFX96 real-time PCR (Bio-Rad). The specific primers were designed according to the gene sequence and listed in Table S1. Action *18S* (GenBank accession number: *AF168884.1*) was used as an internal control. Three technical replicates were analyzed for each gene. The relative expression levels of the candidate genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Identification of NAC family members in maize under PEG stress and rewatering

We screened the drought-rewatering transcriptome data and totally obtained 147 *ZmNAC* genes; then we eliminated 40 NAC genes from the total 147 *ZmNAC* genes, because the FPKM value of the 40 *ZmNAC* genes was zero, respectively, indicating that they were not responsive to drought stress and rewatering at any of three treatment point, and finally a total of 87 *ZmNAC* genes were identified. Comparing with the Plant Transcription Factor Database (V5.0), the coverage was 66%. The relevant biological information parameters such as protein sequences, Molecular weight, PI value, the conserved domain of 87 *ZmNAC* genes were downloaded from the Ensembl Plants and Plant TFDB v5.0 website and listed in Table 1. The proteins encoded by 87 *ZmNAC* genes ranged from 171 to 1400 amino acid (aa) residues in length, with the average 395 aa, the molecular weight (MW) varied from 19.9012 to 155.9226 kDa, the Isoelectric point (PI) ranged from 4.18 to 12.01.

The Gene Ontology (GO) analysis was conducted to predict the functions of proteins encoded by *ZmNAC* genes. The gene products were grouped into molecular function, biological process and cell component categories (Fig. 1). Comparing with the molecular function and cell component category, most of the 87 *ZmNAC* genes were involved in the regulation of biological process, such as the regulation of gene expression (GO:0010468), biosynthetic process (GO 0009058) and regulation of metabolic process (GO: 0050789).

Chromosomal locations and phylogenetic analysis

87 *ZmNAC* genes were distributed unevenly on the ten chromosomes of maize. There were eleven *ZmNAC* genes mapped on Chromosome 2 and 4, respectively, which was the largest number, followed by ten genes on chromosome 3, nine genes on chromosome 6 and 1, and eight genes on chromosome 7, 8, and 9, respectively; seven genes on chromosome 5, only six genes on chromosome 10. These genes were present in different regions of the chromosomes, most of them were located on both telomeric ends (Fig. 2). Most of the genes on chromosome 2 were positioned on the short arm, while *ZmNAC* genes on chromosome 3 and 10 were located on the long arms.

Using MEGA6.0 and the NJ method (Bootstrap = 1000), a phylogenetic tree was constructed, some of NAC genes selected from *Arabidopsis* and rice as reference. 87 NAC proteins were divided into 12 sub-groups, except *ZmNAC63*, *ZmNAC79*, *ZmNAC99*, *ZmNAC134* and *ZmNAC94*, such as NAM, OSNAC7, TIP, ONAC022, ATAF, SEN5, ONAC003, NAC1, ANAC011, NAC2, ANAC104 and AtNAC3 (Fig. 3), the sequence alignment of each sub-group was listed in Figure S1. Each group had different members, the biggest sub-group was ONAC022, including 16 *ZmNAC* genes, followed by ATAF, NAC1 and ONAC003. ANAC011 sub-family contained five members, they were *ZmNAC57*, *ZmNAC78*, *ZmNAC18, ZmNAC28* and *ZmNAC43*; the sub-group NAC2 had *ZmNAC112*, *ZmNAC38*, *ZmNAC120* and *ZmNAC26* four members. *ZmNAC63* and *ZmNAC79* was divided into one group, but we could not find the homologous corresponded to *Arabidopsis* and rice, the same with *ZmNAC99*, *ZmNAC134* and *ZmNAC94*.

Table 1 List of *ZmNAC* genes information identified in transcriptome analysis under PEG stress and rewatering

Gene name	Gene ID	Chromosome location	Length (aa)	Molecular weight (kDa)	PI	Introns
ZmNAC44	GRMZM2G011598	Chr 1: 54.263.185–54.265.683	373	40.5886	8.6697	2
ZmNAC43	GRMZM2G082709	Chr 1:100.385.980–100.387.952	343	37.3713	5.6565	3
ZmNAC50	GRMZM2G475014	Chr 1:180.369.693–180.371.167	371	43.1199	8.6473	2
ZmNAC48	GRMZM2G054252	Chr 1:197.545.631–197.549.052	231	24.7123	11.1472	2
ZmNAC7	GRMZM2G163251	Chr 1:288.351.666–288.353.564	368	40.7219	7.8768	2
ZmNAC49	GRMZM2G347043	Chr 1:297.601.946–297.603.764	312	34.8413	6.7249	1
ZmNAC78	GRMZM2G406204	Chr 1:4.341.041–4.351.530	664	73.3813	5.7927	5
ZmNAC11	GRMZM2G031001	Chr 1:54.034.211–54.036.320	433	47.6004	6.1945	2
ZmNAC53	GRMZM2G059428	Chr 1:7.490.367–7.492.305	325	35.6411	7.2937	2
ZmNAC126	GRMZM2G018436	Chr 2:154.629.678–154.631.157	322	35.3645	7.0337	2
ZmNAC5	GRMZM2G162739	Chr 2:163.612.209–163.613.834	303	33.8931	5.9449	1
ZmNAC24	GRMZM2G008374	Chr 2:196.582.504–196.584.317	388	42.4861	9.6988	2
ZmNAC35	GRMZM2G179049	Chr 2:198.198.555–198.200.085	285	30.5106	10.4877	2
ZmNAC103	AC212859.3_FG008	Chr 2:27.119.340–27.120.568	328	36.5449	6.4937	2
ZmNAC120	GRMZM2G176677	Chr 2:27.817.387–27.825.275	408	44.7543	5.0525	7
ZmNAC36	GRMZM2G081930	Chr 2:30.534.378–30.537.200	297	32.5494	5.8566	2
ZmNAC111	GRMZM2G450445	Chr 2:39.094.599–39.100.476	438	48.1764	6.0695	4
ZmNAC32	GRMZM2G009892	Chr 2:43.705.185–43.706.945	365	39.0406	7.362	2
ZmNAC76	GRMZM2G316840	Chr 2:50.875.247–50.876.743	211	22.8341	4.748	2
ZmNAC22	GRMZM2G156977	Chr 2:9.589.202–9.591.738	317	34.5429	7.635	2
ZmNAC108	GRMZM2G114850	Chr 3:122.367.956–122.372.020	338	36.1373	9.0111	2
ZmNAC90	AC203535.4_FG002	Chr 3:159.141.395–159.142.793	266	28.3918	7.9515	2
ZmNAC95	GRMZM5G813651	Chr 3:172.642.954–172.645.804	447	47.2793	9.1307	2
ZmNAC109	GRMZM2G014653	Chr 3:173.458.717–173.460.818	295	32.5442	8.4322	2
ZmNAC93	GRMZM5G832473	Chr 3:179.023.907–179.025.903	292	31.2086	4.6344	2
ZmNAC70	GRMZM2G312201	Chr 3:189.129.421–189.137.112	1400	155.9226	7.3037	6
ZmNAC94	GRMZM2G122615	Chr 3:212.680.911–212.681.900	329	36.0838	5.9373	0
ZmNAC82	GRMZM2G058518	Chr 3:213.845.082–213.849.487	323	35.3575	9.0907	2
ZmNAC66	GRMZM2G064541	Chr 3:37.808.012–37.817.360	517	57.5021	5.5862	3
ZmNAC16	GRMZM2G166721	Chr 3:6.101.834–6.112.905	436	48.5854	7.9203	4
ZmNAC40	GRMZM5G898290	Chr 4:128.778.456–128.780.327	348	38.0191	7.1616	2
ZmNAC101	GRMZM2G104078	Chr 4:133.589.483–133.593.214	399	44.9497	5.9164	3
ZmNAC17	GRMZM2G062009	Chr 4:145.569.635–145.572.065	294	31.8253	6.6	2
ZmNAC26	GRMZM2G113950	Chr 4:173.236.782–173.241.210	657	71.9765	4.3839	4
ZmNAC51	GRMZM2G140901	Chr 4:175.257.867–175.260.077	298	32.5553	6.6954	1
ZmNAC41	GRMZM2G439903	Chr 4:193.274.586–193.275.656	309	33.8418	6.8422	0
ZmNAC125	GRMZM2G123667	Chr 4:208153304–208156017	359	38.8424	5.1217	2
ZmNAC77	AC196475.3_FG005	Chr 4:33.810.533–33.814.584	661	73.7044	4.5225	6
ZmNAC115	GRMZM2G069047	Chr 4:39.731.374–39.733.815	379	41.2863	8.4057	2
ZmNAC105	GRMZM2G123246	Chr 4:40.262.262–40.265.308	368	40.6109	9.6501	2
ZmNAC75	GRMZM2G100583	Chr 4:48.975.342–48.977.969	261	27.7676	12.0128	2
ZmNAC133	GRMZM2G094067	Chr 5:177.041.057–177.042.174	225	24.4760	6.5758	2
ZmNAC13	GRMZM2G038073	Chr 5:184.855.226–184.858.638	399	44.9364	4.9022	3
ZmNAC60	GRMZM2G336533	Chr 5:2.977.575–2.979.101	436	48.0243	8.299	1
ZmNAC59	GRMZM2G100593	Chr 5:212.630.308–212.636.028	386	43.4753	9.061	6
ZmNAC104	GRMZM5G857701	Chr 5:220.828.387–220.830.479	296	32.4974	7.1764	1
ZmNAC113	GRMZM2G063522	Chr 5:44.701.128–44.705.362	305	34.1288	8.1747	2
ZmNAC74	GRMZM2G112548	Chr 5:5.453.473–5.454.938	336	37.2851	8.4435	2

Table 1 continued

Gene name	Gene ID	Chromosome location	Length (aa)	Molecular weight (kDa)	PI	Introns
ZmNAC21	GRMZM2G091490	Chr 6:68.998.881–69.001.722	367	39.4101	7.0947	2
ZmNAC8	GRMZM2G078954	Chr 6:120.615.193–120.635.200	672	73.5481	9.1013	11
ZmNAC3	GRMZM2G147867	Chr 6:151.568.742–151.571.658	420	45.1531	8.4449	2
ZmNAC20	GRMZM2G180328	Chr 6:151.942.943–151.944.950	339	35.8055	9.3501	2
ZmNAC112	GRMZM2G456568	Chr 6:151.995.956–152.000.206	452	50.4803	4.1803	3
ZmNAC31	GRMZM2G465835	Chr 6:153.483.310–153.484.444	272	30.2296	7.1793	2
ZmNAC54	GRMZM2G030325	Chr 6:3.928.244–3.931.456	380	41.8371	9.7379	2
ZmNAC123	GRMZM2G092465	Chr 6:4.344.874–4.346.980	418	43.9535	6.9341	2
ZmNAC42	GRMZM2G074358	Chr 6:85.809.798–85.811.340	326	36.5904	6.7984	1
ZmNAC79	GRMZM2G004531	Chr 7:138.223.556–138.229.618	714	78.2989	7.0494	2
ZmNAC56	GRMZM2G386163	Chr 7:138.832.493–138.835.958	882	98.2060	5.9215	2
ZmNAC63	GRMZM2G054277	Chr 7:142.080.504–142.083.844	202	22.8372	11.7003	1
ZmNAC18	GRMZM5G885329	Chr 7:151.788.597–151.792.933	171	19.9012	9.1496	2
ZmNAC122	GRMZM2G430849	Chr 7:177.373.961–177.376.027	395	42.0798	7.3831	1
ZmNAC2	GRMZM2G181605	Chr 7:179.273.873–179.275.691	318	35.3439	7.3601	2
ZmNAC4	GRMZM2G079632	Chr 7:21.927.733–21.929.407	300	33.6288	5.1564	1
ZmNAC73	GRMZM2G479980	Chr 7:4.825.498–4.826.987	358	39.9655	6.1938	1
ZmNAC38	GRMZM2G104400	Chr 8:104.776.612–104.781.353	445	49.5132	4.3657	3
ZmNAC88	GRMZM2G134687	Chr 8:105.572.654–105.574.982	425	45.2939	6.8603	3
ZmNAC134	GRMZM2G163843	Chr 8:155.137.898–155.138.968	356	38.2061	5.6107	0
ZmNAC9	GRMZM2G134073	Chr 8:165.320.638–165.322.096	259	27.2156	8.4232	2
ZmNAC23	GRMZM2G068973	Chr 8:176.104.082–176.105.842	308	34.5120	8.9735	2
ZmNAC47	GRMZM2G112681	Chr 8:21.509.525–21.515.943	494	54.9776	7.4882	4
ZmNAC97	GRMZM2G167492	Chr 8:4.801.455–4.804.054	518	57.3319	5.6171	4
ZmNAC118	GRMZM2G109627	Chr 8:7.349.376–7.352.005	398	42.4814	6.6486	2
ZmNAC81	GRMZM2G159500	Chr 9:109.567.045–109.568.953	348	38.4314	5.9744	2
ZmNAC45	GRMZM2G126936	Chr 9:148.682.940–148.684.179	281	30.5788	10.5925	1
ZmNAC99	GRMZM2G027309	Chr 9:149.085.887–149.093.085	413	46.7591	5.2229	12
ZmNAC39	GRMZM2G126817	Chr 9:154.824.112–154.825.475	317	34.8534	6.6821	2
ZmNAC57	GRMZM2G174070	Chr 9:157.106.289–157.122.699	687	76.1387	6.2411	5
ZmNAC86	GRMZM2G171395	Chr 9:23.151.861–23.156.737	435	47.0604	6.7506	4
ZmNAC46	GRMZM2G440219	Chr 9:28.126.449–28.127.152	349	40.2159	6.9691	1
ZmNAC117	GRMZM2G163914	Chr 9:28.909.570–28.912.421	612	66.8057	5.8317	5
ZmNAC15	GRMZM2G111770	Chr 10:126.186.003–126.189.175	438	47.6603	5.6337	3
ZmNAC65	GRMZM2G043813	Chr 10:135.223.915–135.225.952	293	32.3954	5.7558	1
ZmNAC67	GRMZM2G083347	Chr 10:14.594.105–14.596.141	259	27.8656	10.6607	1
ZmNAC25	GRMZM2G127379	Chr 10:2.262.454–2.264.529	475	51.2345	6.2523	2
ZmNAC110	GRMZM2G167018	Chr 10:60.226.179–60.228.837	282	31.1724	7.5318	2
ZmNAC61	GRMZM2G003715	Chr 10:77.529.347–77.533.684	664	73.6056	4.5672	6

Gene structure and motifs identification of *ZmNAC* genes

The number of introns and exons of 87 *ZmNAC* genes revealed significant diversity (Fig. 4). The same sub-group members had the same or similar gene structures. The members in NAC1 group had three exons; most of members had more than 4 exons in ANAC011 and ONAC003 sub-group. Most of NAC genes had 5′ or 3′ UTR region, except *ZmNAC79*, *ZmNAC103*, *ZmNAC46*, *ZmNAC41*, *ZmNAC31*, *ZmNAC134*, *ZmNAC94*, *ZmNAC8* and *ZmNAC56*. *ZmNAC41*, *ZmNAC134* and *ZmNAC 94* had no introns, accounting for 3.44%, 14 *ZmNAC* genes only had one introns, accounting for 16.10%, 45 *ZmNAC* genes had two introns, accounting for 51.72%, 7 *ZmNAC* genes

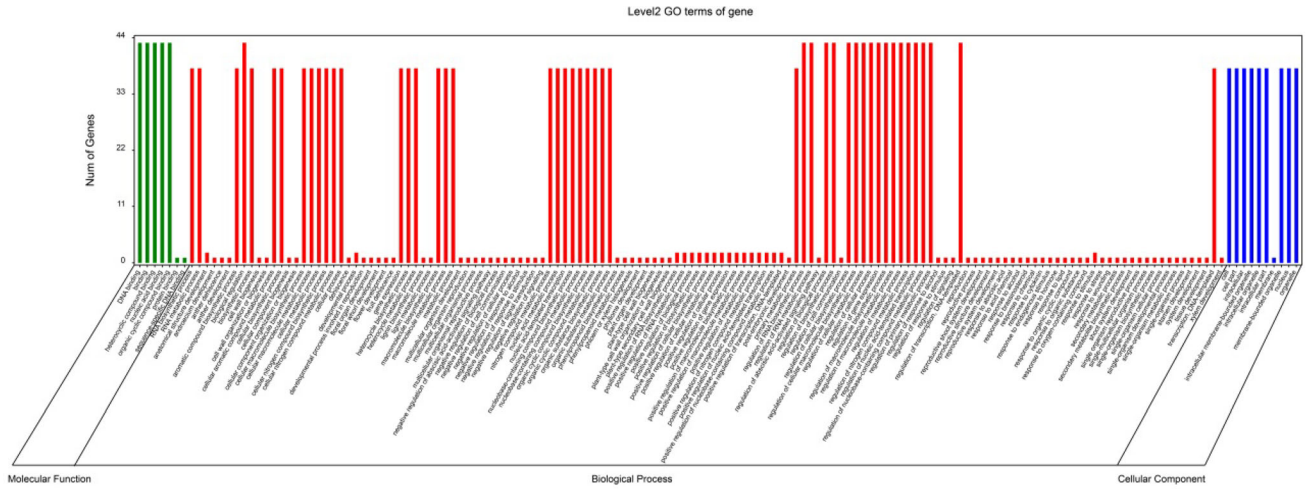

Fig. 1 Gene ontology (GO) analysis of *ZmNAC* genes. The green, red, and blue columns represent the molecular function, biological process and cellular component, respectively (colour figure online)

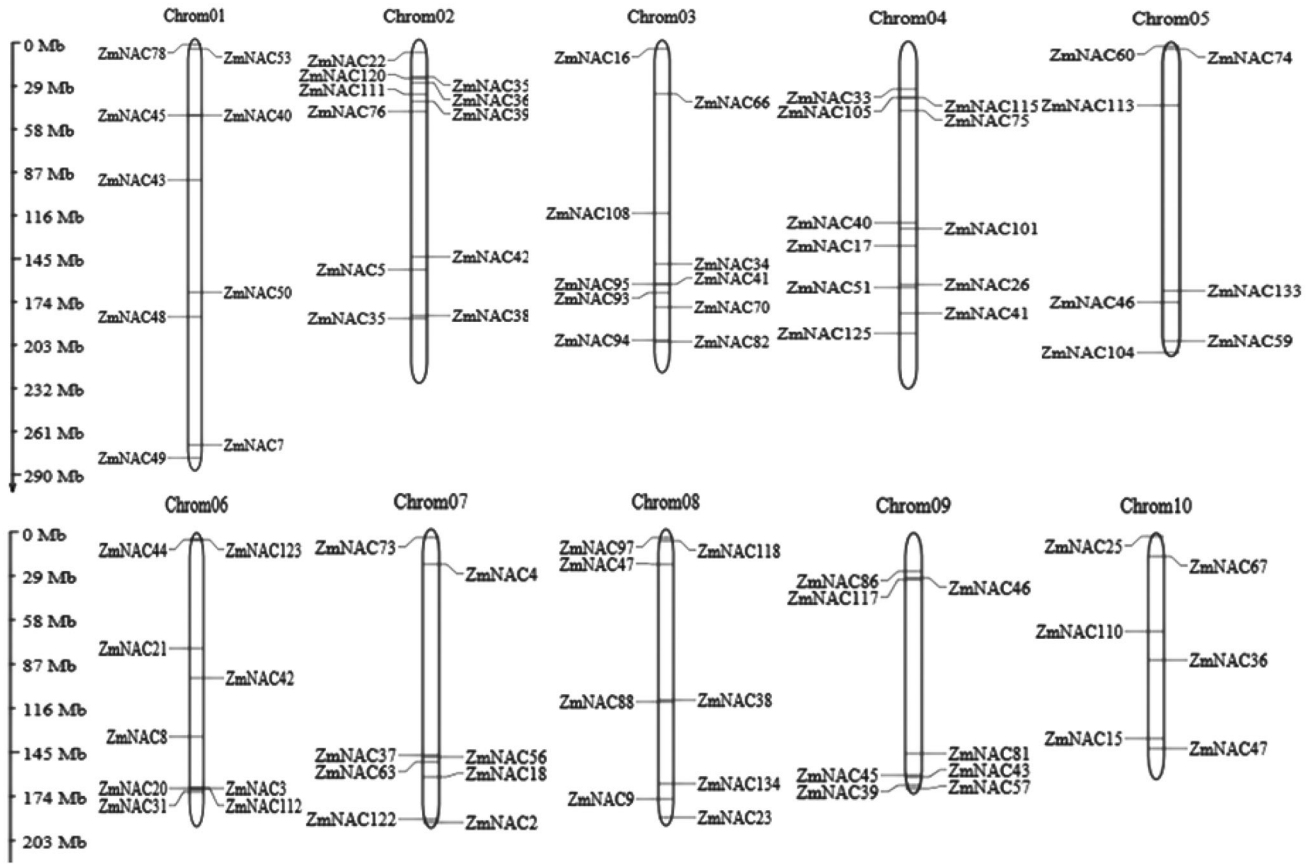

Fig. 2 Distributions of 87 *ZmNAC* genes on the 10 chromosomes of maize

contained three introns, and 16 *ZmNAC* genes contained 4-12 introns.

To further identify the diversity of 87 *ZmNAC* genes, putative motifs were predicted using MEME software (http://meme-suite.org/tools/meme). There was no motifs predicted in *ZmNAC79* and *ZmNAC63*, and the motifs of 85 *ZmNAC* genes were exhibited in Fig. 5. We could find that the motif pattern was clustered in the same way as the sub-families pattern, and the same clusters had the similar motifs compositions, indicating that the phylogenetic analysis results were accurate. Motif 1-6, and 11 were the common element in most of sub-families, except *ZmNAC99*

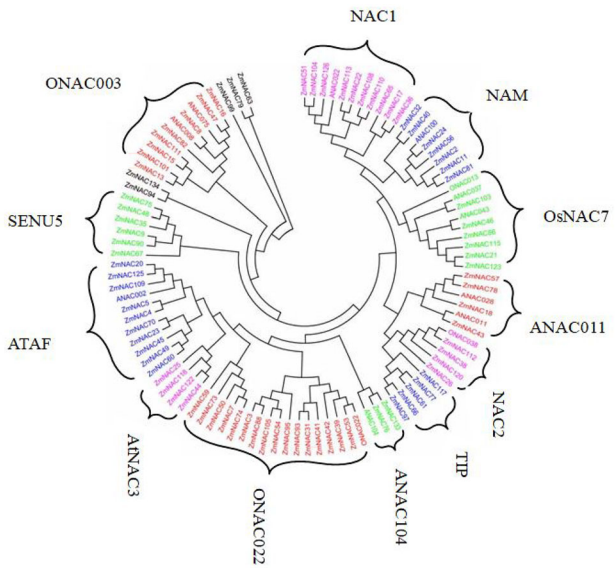

Fig. 3 Evolutionary relationship of 87 *ZmNAC* genes and some homologous proteins in *Arabidopsis thaliana* and rice

and ONAC003 sub-group. Motif 17 only appeared in *ZmNAC57*, *ZmNAC112*, *ZmNAC38* and *ZmNAC3*; Motif 20 appeared in *ZmNAC117*, *ZmNAC77* and *ZmNAC61*; Motif 16 appeared in *ZmNAC60*, *ZmNAC49*, *ZmNAC45* and *ZmNAC125*. Motif 7, 8, 9, 10, 12, 16 and 18 were appeared in ONAC003 sub-group members, and *ZmNAC99* was specific and only had six motifs.

Stress-related *Cis*-elements analysis in promoters of *ZmNAC* genes

In order to better understand the potential regulatory mechanisms of *ZmNAC* genes under PEG treatment and rewatering, we selected five *cis*-element ABRE, MBS, TC-rich, CGTCA, DRE and scanned the promoter regions (− 2000 bp upstream of the translation start site) of 87 *ZmNAC* genes, finally 39 *ZmNAC* genes were listed and the result was shown in Fig. 6. ABRE is the element with the most frequency and was found in 37 selected promoter regions among 39 *ZmNAC* genes; sixteen ABRE elements were found in *ZmNAC49*, eight ABRE were found in *ZmNAC43* and *ZmNAC54*, respectively. TC-rich *cis*- acting element was involved in defense and stress responsiveness, and was found in 14 selected *ZmNAC* genes, three were found in *ZmNAC78*. MBS was MYB binding site involved in drought-induced ability, and was found in 21 selected *ZmNAC* genes, five were found in *ZmNAC16* and *ZmNAC112*, respectively. The DRE element was specific induced under drought and osmotic stress and only was found in *ZmNAC77*. The CGTCA element was found in 34 selected gene promoter regions such as *ZmNAC42*, *ZmNAC17* and *ZmNAC21*.

Digital expression patterns of *ZmNAC* genes under PEG stress and rewatering

The expression patterns of 87 *ZmNAC* genes were different at 60 h, 96 h and 3 d treatment point (Fig. 7). At 60 h treatment point, the number of differentially expressed genes (DEGs) was 18, 12 genes were up-regulated and 6 genes were down-regulated; at 96 h point, the number of DEGs was 11, 5 genes were up-regulated and 6 genes were down-regulated; there were 3 up-regulated genes and 28 down-regulated genes at 3 d point. We also found that the number of up-regulated genes was decreasing, and the number of down-regulated genes was increasing, especially at 3 d point, indicating that the gene expression were more stimulated by rewatering after drought stress. There were 4, 5 and 6 same differentially expressed genes between CK60-vs-T60, CK96-vs-T96 and CK3d-vs-T3d, respectively, and the expression level reaching the significant level at the three treatment point were *ZmNAC36* and *ZmNAC93*.

Heatmap of 87 *ZmNAC* genes was also conducted according to the transcriptome result (Fig. 8). Under PEG treatment and rewatering, the expression patterns of 87 *ZmNAC* genes exhibited different trend. The expression of some of genes were up-regulated under drought stress, down-regulated after rewatering, such as *ZmNAC40*, *ZmNAC51* and *ZmNAC20*; some were down-regulated expression under drought stress while up-regulated expression after rewatering, such as *ZmNAC45, ZmNAC95* and *ZmNAC103*; the expression of some genes exhibited random pattern. Under drought stress, plants accordingly make much effort to adapt at molecular, cellular, physiological and metabolic levels in order to survive or avoid adverse effects, and many genes were involved in the process. The expression of drought-resistant genes would be up-regulated under drought stress, while their transcript level would be reduced when the plants were rewatered and returned to normal growth state, and vice versa. Our research goal is to find highly expressed under PEG treatment and down-expressed after rewatering, so *ZmNAC40*, *ZmNAC51*, *ZmNAC36*, *ZmNAC104*, *ZmNAC82*, *ZmNAC20, ZmNAC49* were selected as candidate genes.

Expression profiles of six *ZmNAC* genes under PEG stress and rewatering by qRT-PCR analysis

To further validate the accuracy of transcriptome result and explore the expression patterns of *ZmNAC* genes, six randomly selected *ZmNAC* genes were investigated under drought stress and rewatering using qRT-PCR analysis. As shown in Fig. 9, six *ZmNAC* genes were expressed at different times during PEG stress and rewatering. The expression of *ZmNAC118*, *ZmNAC122*, *ZmNAC49*, *ZmNAC25*, *ZmNAC20* and *ZmNAC74* was up-regulated at

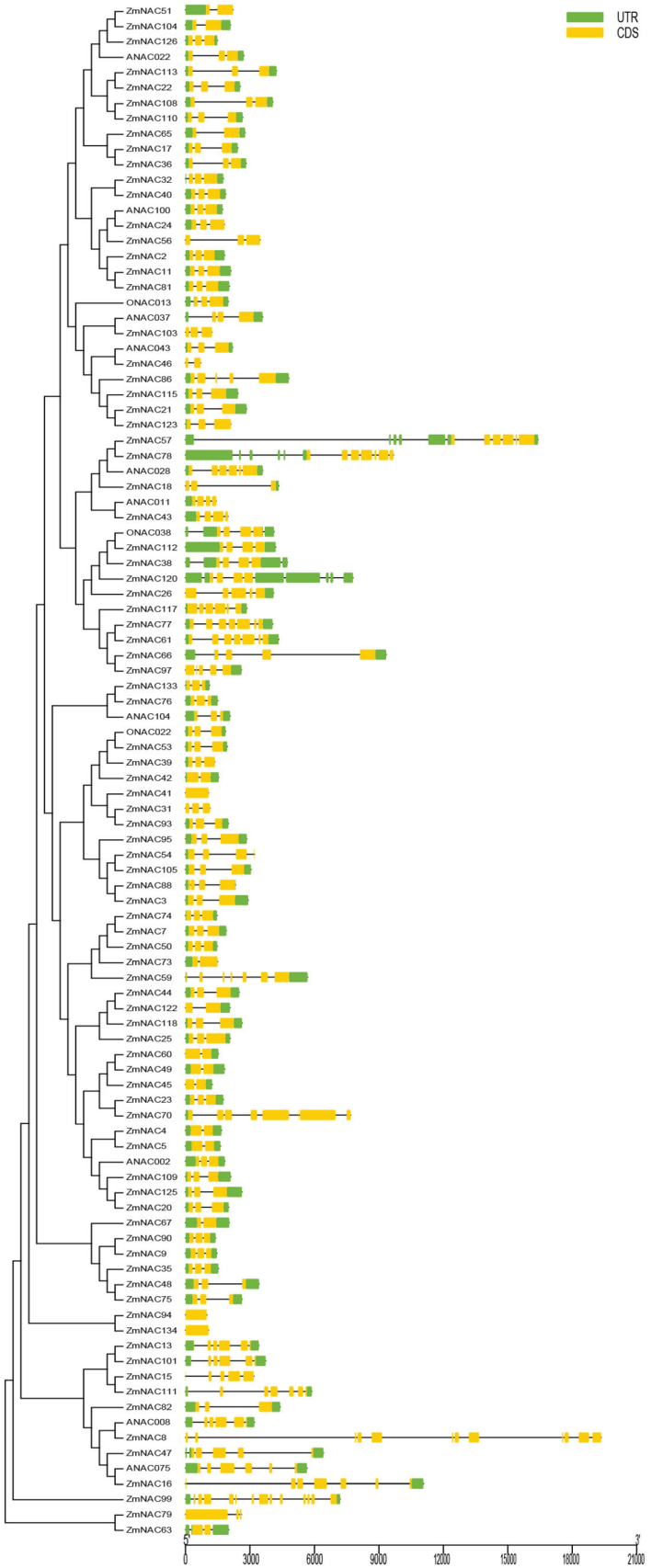

Fig. 4 Exon-intron structure analyses of 87 *ZmNAC* genes (colour figure online)

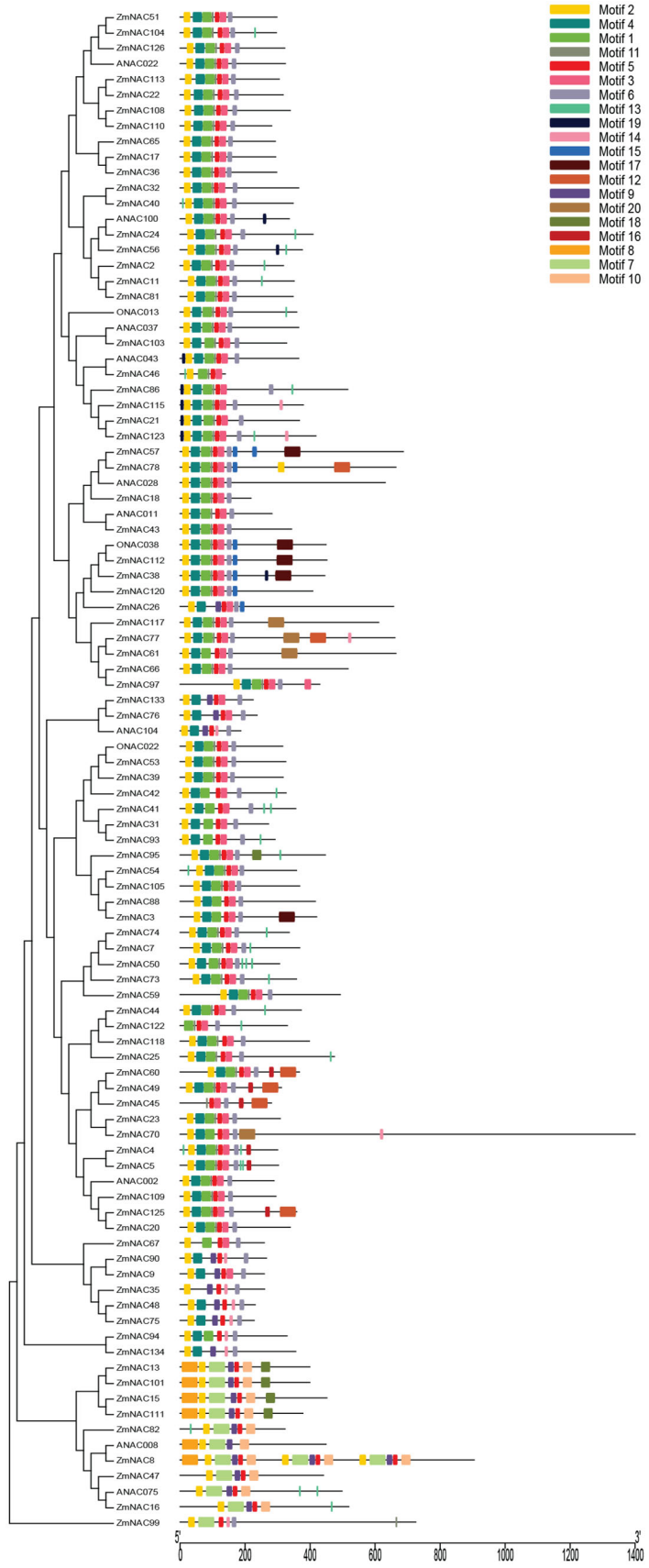

Fig. 5 Motif distributions of 87 *ZmNAC* genes (colour figure online)

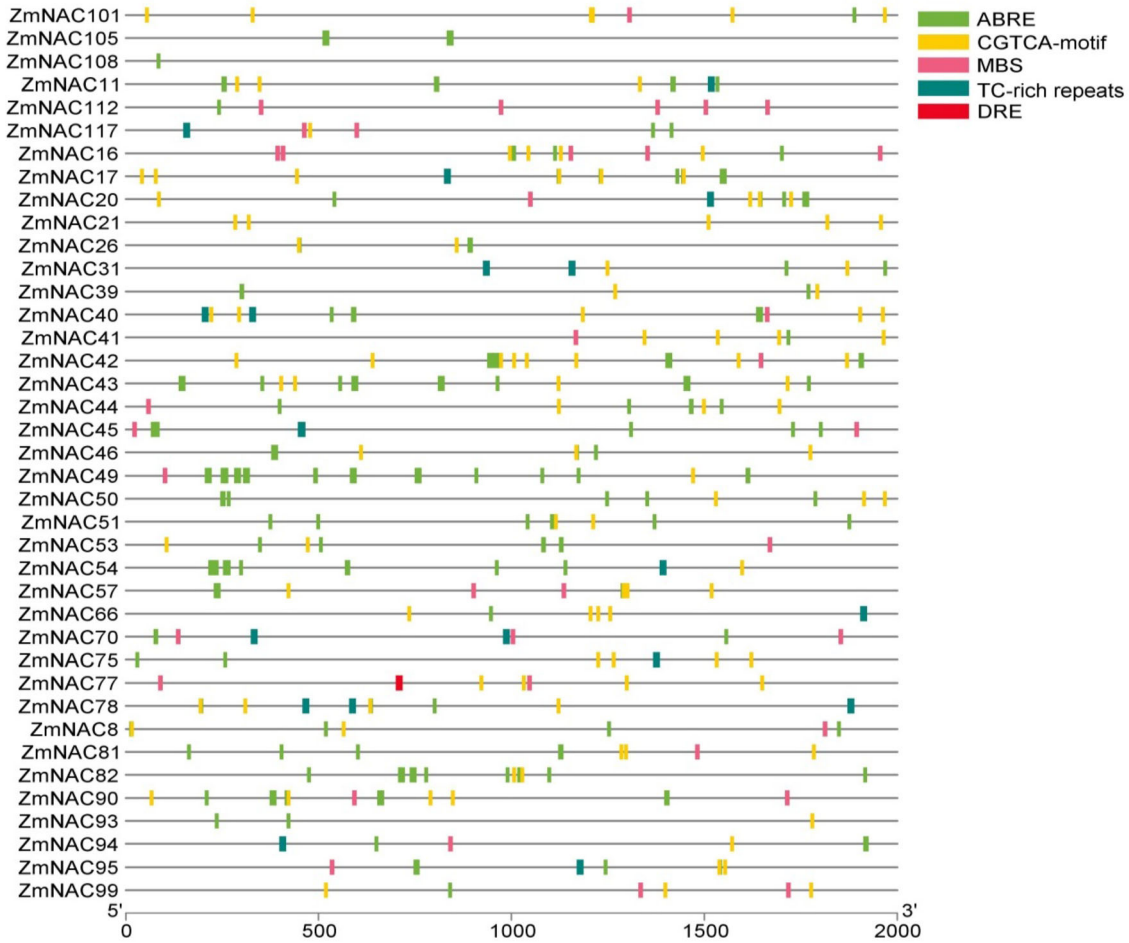

Fig. 6 *cis*-elements predication in the promoter regions of *ZmNAC* genes (colour figure online)

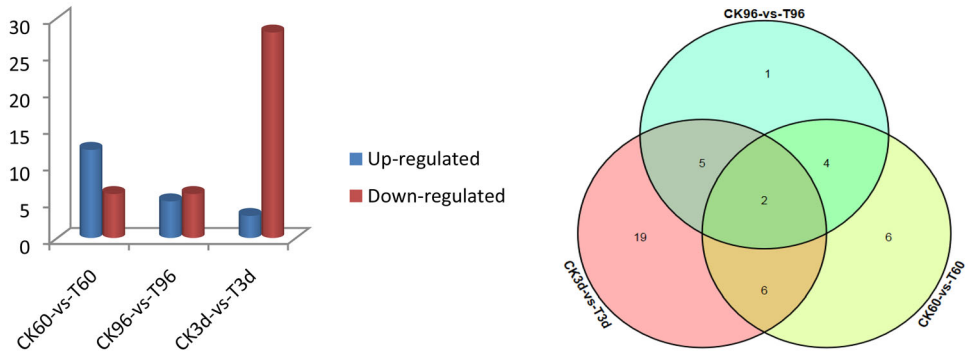

Fig. 7 Statistical analyses of differentially expressed *ZmNAC* genes under PEG treatment and rewatering

60 h and 96 h under PEG stress, then the transcript level was sharply reduced after rewatering at 3 d, which was consistent with the transcriptome result.

Discussion

Drought, like other environment stresses, has adverse effect on maize yield. As water resources becoming more limiting, discovering drought-resistant genes and cultivating drought-resistant lines become more urgent (Bruce et al. 2002). NAC is one of the largest plant- specific transcription factor families and play an essential role in plants' responses to abiotic stress (Nakashima et al. 2012; Shang et al. 2016; Yang et al. 2015). Genome-wide analysis of NAC family genes have been identified in *Arabidopsis*, rice, soybean, *Medicago truncatula* and white pear (Capella et al. 2014; Dung Tien et al. 2011; Gong et al. 2019; So and Lee 2019). Peng (Peng et al. 2015) and Ge

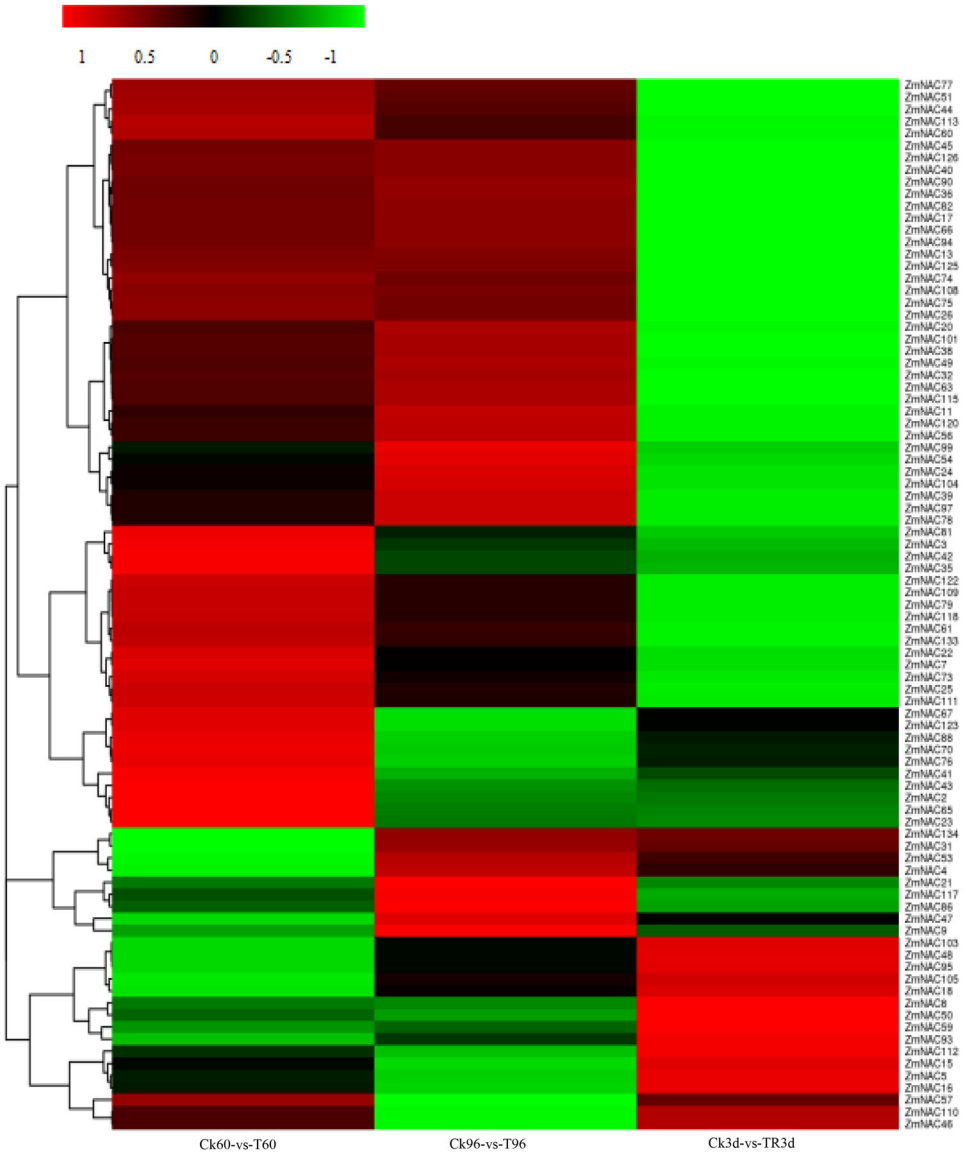

Fig. 8 Expression patterns of 87 *ZmNAC* genes under PEG stress and rewatering (colour figure online)

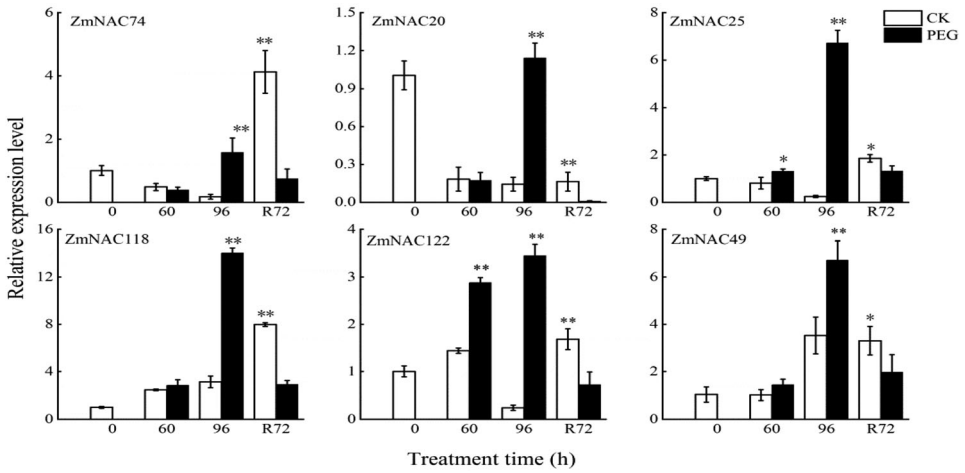

Fig. 9 Expression patterns of *ZmNAC* genes in response to drought treatment. Different lowercase letters indicate significant differences at $p < 0.05$ (Duncan's test)

(Ge et al. 2015) performed the genome-wide analysis of NAC gene family in maize, respectively, while their studies were based on the bioinformatics analysis. In this study, we focused on exploring *ZmNAC* genes with drought-resistance and conducted a high-throughput transcriptome, and obtained 87 *ZmNAC* genes that were responsive to drought stress and rewatering. GO analysis showed that 87 *ZmNAC* genes were mainly involved the regulation of biological process, such as the regulation of biological process, biosynthetic process and gene expression. The 87 *ZmNAC* genes were unevenly distributed on 10 maize chromosomes. It had been reported that the N-terminus of NAC proteins was a highly homologous region containing the DNA-binding NAC domain, which was approximately 150 amino acids in length and contained five conserved regions (A to E). Phylogenetic analysis showed that 87 *ZmNAC* genes were classified 12 sub-groups, with the exception of *ZmNAC63*, *ZmNAC79*, *ZmNAC99*, *ZmNAC134* and *ZmNAC94*. Ten *ZmNAC* genes belonged to ATAF subfamily and each sub-group had the homologous with *Arabidopsis* or rice, illustrating not only the accuracy of the phylogenetic tree, but also indicating that genes in the same sub-group have similar functions. The analysis of gene structure was also conducted, and the arrangement and number of introns and exons shed light on the evolution and origin of a given gene (Schwartz et al. 2009). Previous study showed that the expression of *ATAF1* was obviously induced by drought, high-salinity and abscisic acid (ABA) and the overexpression of *ATAF1* in *Arabidopsis* increased plant sensitivity to drought, ABA and salt (Liu et al. 2016; Wu et al. 2009); the overexpression of *ONAC003* in rice resulted in enhanced tolerance to high temperature, drought (Fang et al. 2015), which indicated that genes in the ATAF1 and ONAC003 subfamily might be involved in abiotic stress responses.

Cis-element analysis is an effective method to study potential transcriptional regulation of genes (Tran et al. 2004). Among them, ARBE was involved in the abscisic acid (ABA) and drought responsiveness, DRE especially indicated the drought and osmotic stress induction in maize, and MBS was MYB binding site involved in drought-induce ability. According to our result, DRE *cis*-element only appeared in *ZmNAC77*, 21 candidated genes contained MBS *cis*-element and 37 candidate genes contained ARBE *cis*-element. *ZmNAC40*, *ZmNAC95*, and *ZmNAC20* contained 4 kinds of *cis*-element, respectively. Through *cis*-element analysis, we concluded that *ZmNAC* genes were likely related to drought stress.

Under drought stress and rewatering, different genes exhibited different expression patterns. Under drought stress, the expressions of some genes were up-regulated and then decreased after rewatering, such as *ZmNAC49*, *ZmNAC94*, and *ZmNAC20*; some genes gave the opposite trend, the gene expression was down-regulated under drought stress, and then up-regulated after rehydration, such as *ZmNAC8*, *ZmNAC59*, and *ZmNAC50*. But the expressions of some genes does not follow a regular pattern, which is inconsistent with our research purpose. From the result, we also found that number of DEGs at 3 d was higher than that at 60 h and 96 h, and the number of up-regulated genes was less than that of down-regulated genes. Under drought stress, there were complicated reactions occurred in plants to adapt to the stress, and the expression of drought-resistant genes were motivated during drought stress. When plants were rewatered after drought stress, greater changes took placed reflecting in growth restored, and much genes were involved in the changes. To further explore the expression of *ZmNAC* genes, the expression analysis of six *ZmNAC* genes was performed using qRT-PCR, respectively, and the result revealed that under drought stress, *ZmNAC118*, *ZmNAC25*, *ZmNAC49*, *ZmNAC74*, *ZmNAC122* and *ZmNAC20* were up-regulated during PEG treatment, and the transcript levels were dropped sharply after rewatering at 3 d, further revealing the accuracy of the transcriptome result and *cis*-element analysis. This study provides solid basis for further genes function identification and resistant molecular breeding.

Acknowledgements The manuscript "Genome-Wide Analysis of NAC Transcription Factor Family in Maize under Drought Stress and Rewatering" was supported by the National Natural Science Foundation of China (No. 31471452), and the National Key Research and Development Program of China (No. 2017YFD0301106).

References

Borrill P, Harrington SA, Uauy C (2017) Genome-wide sequence and expression analysis of the NAC transcription factor family in polyploid wheat. G3 Genes Genomes Genet 7:3019–3029. https://doi.org/10.1534/g3.117.043679

Bruce WB, Edmeades GO, Barker TC (2002) Molecular and physiological approaches to maize improvement for drought tolerance. J Exp Bot 53:13–25. https://doi.org/10.1093/jexbot/53.366.13

Bu LD, Zhang RH, Han MM, Xue JQ, Chang Y (2009) The physiological mechanism of compensation effect in maize leaf by rewatering after draught stress. Acta Agric Boreal Occident Sin 18:88–92. https://doi.org/10.3969/j.issn.1004-1389.2009.02.020

Capella M, Re DA, Arce AL, Chan RL (2014) Plant homeodomain-leucine zipper I transcription factors exhibit different functional AHA motifs that selectively interact with TBP or/and TFIIB. Plant Cell Rep 33:955–967. https://doi.org/10.1007/s00299-014-1576-9

Dung Tien L, Nishiyama R, Watanabe Y, Mochida K, Yamaguchi-Shinozaki K, Shinozaki K, Lam-Son Phan T (2011) Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress. DNA Res 18:263–276. https://doi.org/10.1093/dnares/dsr015

Fang Y, Liao K, Du H, Xu Y, Song H, Li X, Xiong L (2015) A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. J Exp Bot 66:6803–6817. https://doi.org/10.1093/jxb/erv386

Ge SS, Tang GY, Bi YP, Liu ZJ (2015) Genome-wide identification and analysis of NAC gene family in maize. Shandong Agric Sci 47:1–6. https://doi.org/10.14083/j.issn.1001-4942.2015.02.001

Gong X, Zhao L, Song X, Lin Z, Gu B, Yan J, Zhang S, Tao S, Huang X (2019) Genome-wide analyses and expression patterns under abiotic stress of NAC transcription factors in white pear (*Pyrus bretschneideri*). BMC Plant Biol. https://doi.org/10.1186/s12870-019-1760-8

Kadier Y, Zu Y-y, Dai Q-m, Song G, Lin S-w, Sun Q-p, Pan J-b, Lu M (2017) Genome-wide identification, classification and expression analysis of NAC family of genes in sorghum *Sorghum bicolor* (L.) Moench. Plant Growth Regul 83:301–312. https://doi.org/10.1007/s10725-017-0295-y

Kamoshita A, Rodriguez R, Yamauchi A, Wade LJ (2004) Genotypic variation in response of rainfed lowland rice to prolonged drought and rewatering. Plant Prod Sci 7:406–420. https://doi.org/10.1626/pps.7.406

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. https://doi.org/10.1186/gb-2013-14-4-r36

Kou X, Wang S, Wu M, Guo R, Xue Z, Meng N, Tao X, Chen M, Zhang Y (2014) Molecular characterization and expression analysis of NAC family transcription factors in tomato. Plant Mol Biol Rep 32:501–516. https://doi.org/10.1007/s11105-013-0655-3

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. https://doi.org/10.1038/nmeth.1923

Liu Y, Sun J, Wu Y (2016) Arabidopsis ATAF1 enhances the tolerance to salt stress and ABA in transgenic rice. J Plant Res 129:955–962. https://doi.org/10.1007/s10265-016-0833-0

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods (San Diego, Calif) 25:402–408. https://doi.org/10.1006/meth.2001.1262

Lu M, Ying S, Zhang D-F, Shi Y-S, Song Y-C, Wang T-Y, Li Y (2012) A maize stress-responsive NAC transcription factor, ZmSNAC1, confers enhanced tolerance to dehydration in transgenic Arabidopsis. Plant Cell Rep 31:1701–1711. https://doi.org/10.1007/s00299-012-1284-2

Mao H, Yu L, Han R, Li Z, Liu H (2016) ZmNAC55, a maize stress-responsive NAC transcription factor, confers drought resistance in transgenic Arabidopsis. Plant Physiol Biochem 105:55–66. https://doi.org/10.1016/j.plaphy.2016.04.018

Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) NAC transcription factors in plant abiotic stress responses. Biochim Biophys Acta Gene Regul Mech 1819:97–103. https://doi.org/10.1016/j.bbagrm.2011.10.005

Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, Kikuchi S (2010) Genome-wide analysis of NAC transcription factor family in rice. Gene 465:30–44. https://doi.org/10.1016/j.gene.2010.06.008

Peng X, Zhao Y, Li X, Wu M, Chai W, Sheng L, Wang Y, Dong Q, Jiang H, Cheng B (2015) Genomewide identification, classification and analysis of NAC type gene family in maize. J Genet 94:377–390. https://doi.org/10.1007/s12041-015-0526-9

Puranik S, Sahu PP, Srivastava PS, Prasad M (2012) NAC proteins: regulation and role in stress tolerance. Trends Plant Sci 17:369–381. https://doi.org/10.1016/j.tplants.2012.02.004

Saidi MN, Mergby D, Brini F (2017) Identification and expression analysis of the NAC transcription factor family in durum wheat (*Triticum turgidum* L. ssp. durum). Plant Physiol Biochem 112:117–128. https://doi.org/10.1016/j.plaphy.2016.12.028

Schwartz S, Meshorer E, Ast G (2009) Chromatin organization marks exon-intron structure. Nat Struct Mol Biol 16:990–995. https://doi.org/10.1038/nsmb.1659

Shan L (2003) Issues of science and technology on water saving agricultural development in China. Agric Res Arid Areas. https://doi.org/10.3321/j.issn:1000-7601.2003.01.001

Shang H, Wang Z, Zou C, Zhang Z, Li W, Li J, Shi Y, Gong W, Chen T, Liu A, Gong J, Ge Q, Yuan Y (2016) Comprehensive analysis of NAC transcription factors in diploid *Gossypium*: sequence conservation and expression analysis uncover their roles during fiber development. Sci China Life Sci 59:142–153. https://doi.org/10.1007/s11427-016-5001-1

Shiriga K, Sharma R, Kumar K, Yadav SK, Hossain F, Thirunavukkarasu N (2014) Genome-wide identification and expression pattern of drought-responsive members of the NAC family in maize. Meta Gene 2:407–417. https://doi.org/10.1016/j.mgene.2014.05.001

So H-A, Lee J-H (2019) NAC transcription factors from soybean (*Glycine max* L.) differentially regulated by abiotic stress. J Plant Biol 62:147–160. https://doi.org/10.1007/s12374-018-0285-2

Sun H, Hu M, Li J, Chen L, Li M, Zhang S, Zhang X, Yang X (2018) Comprehensive analysis of NAC transcription factors uncovers their roles during fiber development and stress response in cotton. BMC Plant Biol. https://doi.org/10.1186/s12870-018-1367-5

Tran LSP, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16:2481–2498. https://doi.org/10.1105/tpc.104.022699

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7:562–578. https://doi.org/10.1038/nprot.2012.016

Wang Z, Dane F (2013) NAC (NAM/ATAF/CUC) transcription factors in different stresses and their signaling pathway. Acta Physiol Plant 35:1397–1408. https://doi.org/10.1007/s11738-012-1195-4

Wu Y, Deng Z, Lai J, Zhang Y, Yang C, Yin B, Zhao Q, Zhang L, Li Y, Yang C, Xie Q (2009) Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. Cell Res 19:1279–1290. https://doi.org/10.1038/cr.2009.108

Xu Z, Gongbuzhaxi Wang C, Xue F, Zhang H, Ji W (2015) Wheat NAC transcription factor TaNAC29 is involved in response to salt stress. Plant Physiol Biochem 96:356–363. https://doi.org/10.1016/j.plaphy.2015.08.013

Xue G-P, Way HM, Richardson T, Drenth J, Joyce PA, McIntyre CL (2011) Overexpression of TaNAC69 leads to enhanced transcript levels of stress up-regulated genes and dehydration tolerance in bread wheat. Mol Plant 4:697–712. https://doi.org/10.1093/mp/ssr013

Yang X, Wang X, Ji L, Yi Z, Fu C, Ran J, Hu R, Zhou G (2015) Overexpression of a *Miscanthus lutarioriparius* NAC gene MlNAC5 confers enhanced drought and cold tolerance in Arabidopsis. Plant Cell Rep 34:943–958. https://doi.org/10.1007/s00299-015-1756-2