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Identification of 7 stress-related NAC transcription factor members in maize (*Zea mays* L.) and characterization of the expression pattern of these genes

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ABSTRACT

NAC proteins are plant-specific transcription factors that play essential roles in plant development and various abiotic stress responses. A comprehensive analysis of maize NAC genes was performed in this study. A total of 157 non-redundant maize NAC genes including seven membrane-bound members were identified and found to be unevenly distributed on 10 maize chromosomes. Motif composition analysis indicated that the maize NAC proteins share three relatively conserved motifs in the NAC domain within the N-terminal region. Phylogenetic analysis of 157 maize NAC proteins accompanied by 117 NAC proteins from *Arabidopsis* and 151 from rice were presented. The NAC proteins evaluated were divided into two large groups including 18 subgroups. Gene duplication analysis indicated that gene loss occurred during maize evolution. Seven NAC members that belong to the same clade of maize NAC domain genes were isolated, and overlapping expression patterns were observed under various abiotic stresses, including low temperature, high salinity and dehydration, and phytohormone abscisic acid treatments. This suggested that NAC members function as stress-responsive transcription factors in ABA-dependent signaling pathways. Relatively higher expression levels of these selected maize NAC genes were detected in roots. The stress responsive NAC genes may have applications in molecular breeding to improve crop stress tolerance.

1. Introduction

Plant growth and agricultural productivity are constantly threatened by various environmental factors such as drought, high salinity, and extreme temperatures. Plants have evolved effective mechanisms to adapt to stress at molecular and physiological levels, thus enabling them to survive under negative stress conditions. Transcriptional regulation is an important mechanism underlying gene expression [1]. Regulatory proteins such as transcription factors activate or repress stress-related genes by interacting with relevant *cis*-elements present in gene promoters and play essential roles in stress responses.

Recently, plant-specific NAC, NAM, ATAF1/2, and CUC2 (NAC) transcription factors have received extensive attention for their important roles in stress responses [2]. Compared with the conserved NAC domain in the N-terminal region, the variable C-terminus functions as a transcriptional activator or repressor [3]. It has been reported that the N- and C- termini play essential roles in protein interactions [5,6].

To date, a variety of NAC members have been isolated from wheat, soybean, grapevines, *Solanum lycopersicum*, and Chinese cabbage [4,7]. NAC transcription factors regulate a series of biological processes and have numerous advantages in both biotic and abiotic stress signaling pathways [8]. The well-known rice NAC transcription factor SNAC1 confers enhanced drought and salt stress tolerance via transcriptional regulation of downstream NAC target genes, but shows no negative effect on plant growth or grain yield [9,10]. A number of ABA-dependent NAC transcription factors in rice, including *OsNAC5*, *ONAC052*, and *ONAC10*, enhance drought

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tolerance in transgenic plants by regulating distinct target genes successively [11–13]. Recently, two novel NAC members from wheat, designated as *TaNAC2* and *TaNAC67*, were characterized for their enhanced multi-abiotic stress tolerance in *Arabidopsis* [14,15]. Drought-inducible genes including *GmNAC2*, *GmNAC11*, and *GmNAC20* were identified in soybean. Functional analysis revealed that GmNAC2 functions as a negative regulator in abiotic stress, whereas *GmNAC20* overexpression in plants induced enhanced salt and freezing tolerance [16,17]. In addition, certain membrane-anchored NAC transcription factors such as NTL4 and NTL6 are still considered important in the plant response to abiotic stresses.

Previous studies have characterized NAC genes. In this study, by searching databases, 157 non-redundant NAC members from maize were identified. These NAC members showed a nearly equal division among 10 maize chromosomes, and seven of them were considered membrane-bound proteins due to the presence of a predicted trans-membrane (TM) motif. Due to their high expression level, seven selected NAC genes from these 157 NAC candidates may be used for evaluation of various abiotic stresses and hormone treatments, although they may have different roles in the plant response to abiotic stress based on their tissue-specific expression patterns at different developmental stages. The results of this study can be used for further functional characterization of the candidate stress responsive NAC genes in maize.

2. Materials and methods

2.1. Chromosomal location, phylogenetic and motif analyses of non-redundant NAC family genes in maize

To explore the NAC genes in maize, we search the online Plant Transcription Factor Database (TFDB) (http://planttfdb.cbi.pku.edu.cn); 191 maize NAC predicted protein sequences were extracted. Protein sequence alignment was performed to remove redundancies using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) database. The scores suggest that greater than 97% were considered non-redundant NAC sequences. The specific positions of each putative ZmNAC gene in the maize genome were retrieved from the B73 maize sequencing database (http://www.maizesequence.org). The distribution of NAC genes on the maize genome was visualized using MapChart software (version 2.2). Phylogenetic analysis of all tested NAC sequences were performed using PhyML software (version 2.4.3) using the maximum likelihood method [18]. The conserved sequence motifs present in NAC proteins were identified using MEME (http://meme.nbcr.net/meme3/meme.html).

2.2. Gene duplication

Complete genomic sequence data for maize, rice, and Arabidopsis were downloaded from the NCBI database. The putative NAC members were extracted based on the presence of the NAM domain (IPR003441). BLAST-based methods were used to identify orthologous and paralogous NAC gene pairs in maize, rice, and Arabidopsis, respectively. A comparison of the results was performed using the software Circos V0.22 [19].

2.3. Isolation and sequence analysis of seven OsNAC3 subgroup members

Specific primers were designed to amplify full-length cDNAs of NAC genes in the maize inbred line B73 using RT-PCR. Multiple sequence alignment was performed using the optimal alignment method of DNAMAN (version 5.0) and ClustalX software (version 1.83). Phylogenetic analyses were performed with the neighbor-joining (NJ) method with 1000 bootstrap replicates in the MEGA 4 program. GSDS (http://gsds.cbi.pku.edu.cn/) online software was used to confirm gene structures.

By searching the MaizeGDB database (http://www.maizegdb.org/), 1.5 kb of DNA sequence upstream of the initiation codon (ATG) were downloaded and considered to be the promoter region of the obtained NAC genes, as described previously. *Cis*-elements in the promoter region were analyzed using the PLACE web signal scan program (http://www.dna.affrc.go.jp/PLACE/signalup.html).

2.4. Plant growth and stress treatments

The seeds of the maize inbred line B73 were surface sterilized and grown in pots filled with vermiculite. Plants in the three-leaf stage were selected for various treatments.

Hoagland solution was prepared for the growth of hydroponic maize seedlings, as described previously. For salt, PEG, and hormone treatments, plants were grown in Hoagland solution supplemented with 200 mM NaCl, 20% PEG and 100 μM ABA, respectively. The roots and shoots of plants were selected and harvested at 0, 1, 3, 6, 12 and 24 h, respectively. All hydroponically grown plants were treated with ventilation pipes to provide sufficient oxygen for roots to inhibit the toxic stress caused by anaerobic respiration. For the control sample, maize seedlings without treatment were collected at the same time. For dehydration treatment, seedlings were air-dried on clean filter papers in a growth chamber at 28 °C and relative humidity of 30%. For cold treatment, the evaluated plants were transferred to a growth chamber at 4 °C. For soil drought treatment, seeds were sowed in pots and grown under normal irrigation conditions. Seedlings at the three-leaf stage were withheld water for 7 or 14 days. Watering was then resumed after 14 days of drought treatment.

2.5. Tissue-specific expression analysis

Tissue-specific transcript analysis was performed to investigate the spatial expression pattern of selected NAC members in maize. The root, stalk, and leaf of plants in the three-leaf stage, the aerial root, tassel, and ear in the pre-flowering stage, and silk in the flowering stage were collected and frozen in liquid nitrogen.

2.6. Expression analysis using quantitative real-time PCR

Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was an effective system for the synthesis of full-length first-strand cDNA from RNA templates. Quantitative real time-PCR (qRT-PCR) was performed in optical 96-well plates using the Bio-Rad IQ5 PCR detection system (Bio-Rad, CA, USA) and SYBR Premix Ex Taq™ (Takara, Dalian, China) according to the manufacturer's instructions. Transcripts of the *ZmGAPDH* gene (NM_001111943) were used as an internal control. Four biological replicates were used for each time point. Relative expression levels of selected NAC genes were calculated using the relative $2^{-\Delta\Delta Ct}$ method [20]. At least three replications were performed for each experiment.

3. Results

3.1. Identification and genome distribution of NAC transcription factor family genes in maize

A total of 191 putative proteins containing the NAC domain were identified from the Plant TFDB database. Multiple sequence alignment was performed to eliminate redundant sequences from the acquired NAC proteins. Consequently, 157 redundant NAC members constituted the NAC transcription factor gene family in maize.

To explore the genomic distribution of maize NAC genes, the genomic DNA sequences of maize NAC genes were used to search against the maize genome database. As shown in the physical position map, the 157 NAC members were distributed on 10 maize chromosomes (Fig. 1). Some ZmNAC genes were distributed individually or were present in clusters of physically linked genes. For example, chromosome 2 contained the largest number of maize NAC genes (22 of 157 members); chromosomes 4 and 3 contained 24 and 19 NAC members, respectively. In contrast, chromosomes 5 and 10 contained only 9 and 8 NAC members, respectively.

3.2. Phylogenetic analysis and gene duplication

A total of 151 NAC proteins from rice, 117 from *Arabidopsis*, and 157 from maize were collected to assess evolutionary relationships. As a result, all 425 sequences were classified into two large groups that could be divided into 18 subgroups. Phylogenetic analysis demonstrated that subgroups ATAF and OsNAC3 included numerous reported NAC genes closely related to the abiotic stress response in plants, including the reported rice SNAC1 and SNAC2 genes in the OsNAC3 subgroup and the ATAF1/2, OsNAC5, and OsNAC6 genes in the ATAF subgroup (Fig. 2).

Recurrent gene duplication and retention are important evolutionary mechanisms that can result in functional divergence in paralogs [21]. BLAST alignments were also performed to identify paralogs in maize and the relevant orthologs in important model plants such as rice and *Arabidopsis*. The comparative syntenic map showed 81 paralogous NAC gene pairs among the 10 maize chromosomes that may play an important role in maize evolutionary history. In addition, 62 and 29 NAC orthologous gene pairs were identified between maize and *Arabidopsis* and between maize and rice, respectively (Fig. 3).

3.3. Motif and membrane-bound ZmNAC analyses

As shown in Supplementary Fig. S1, three putative conserved motifs in the N-terminal region of maize NAC proteins were identified using MEME software. Some membrane-associated NAC transcription factors have been reported to play membrane-mediated regulatory roles in rapid transcriptional responses to external stimuli [22]. Of the 157 NAC proteins, seven NAC membrane-bound TFs were identified for the predicted single trans-membrane (TM) region at the C-terminal ends (Supplementary Fig. S2).

3.4. Cloning of the full-length cDNA of stress-related NAC genes and sequence analysis

Specific primers were designed to amplify full-length cDNAs of certain putative stress-related NAC members phylogenetically close to stress-responsive NAC genes characterized in *Arabidopsis*, rice, and soybean. Consequently, seven NAC genes, designated as *Zma000584*, *Zma006493*, *Zma054594*, *Zma001259*, *Zma003086*, *ZmSNAC052*, and *Zma029150* (GenBank accession numbers: KP283536, KM670443, KR010626, KM670444, KP900766, KM987612, and KP283537), were obtained from the maize inbred line B73. The length of the seven genes ranged from 705 bp to 1020 bp that encoded peptides of 234–339 amino acids (aa).

Multiple sequence alignment showed that these seven NAC members shared a typical conserved NAC domain: approximately 150 aa at the N-terminal region. Compared with other well-known NAC members, five comparative conserved subdomains A–E were located in the NAC domain. Sequences at the C-terminal region were quite divergent (Supplementary Fig. S3). An additional phylogenetic analysis was performed to explore the evolutionary relationship among NAC proteins, including the well-studied NAC members in other species. As shown in Supplementary Fig. S4 the seven selected NAC genes in maize and typical abiotic stress-responsive NAC proteins were clustered together. This result indicated that the seven NAC members selected in this study may be involved in plant responses to abiotic stresses.

3.5. Gene structure and phylogenetic analyses of the selected NAC genes

Gene structure analysis showed that *Zma003086*, *Zma001259*, *ZmSNAC052*, and *Zma029150* have three exons and two introns, whereas *Zma006493*, *Zma000584*, and *Zma054594* have two exons

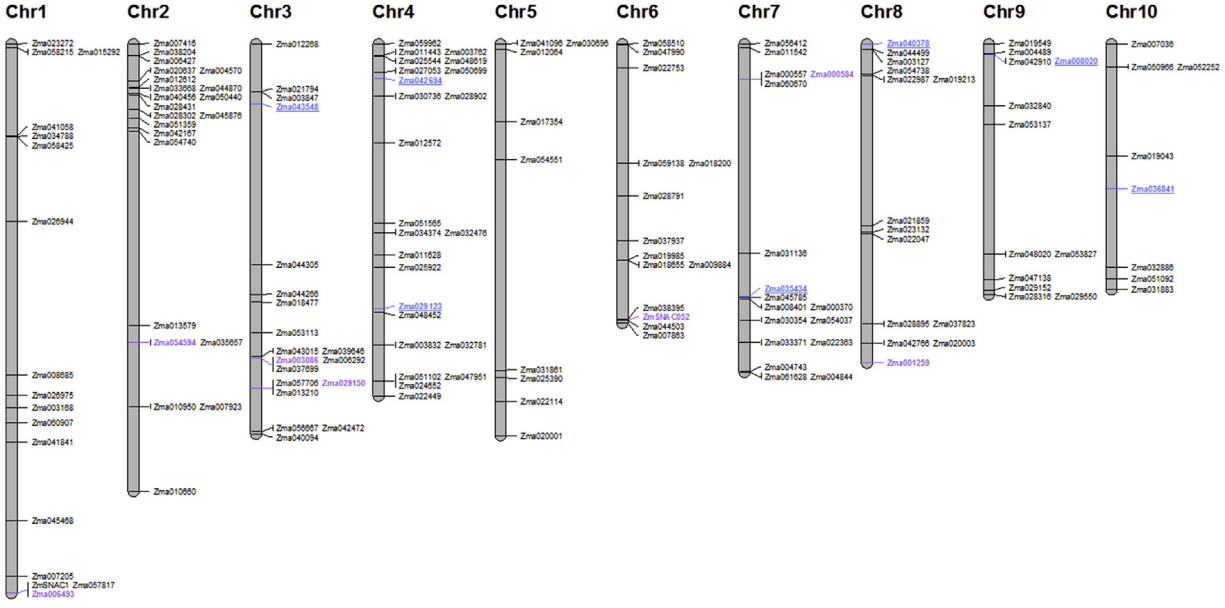

Fig. 1. Chromosomal mapping of the maize NAC transcription factor gene family. Presentation of the physical locations of the putative ZmNAC members on each maize chromosome. The names of the ZmNAC genes with trans-membrane motifs were marked with underlines. The putative stress-responsive NAC genes for expression analysis were marked in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

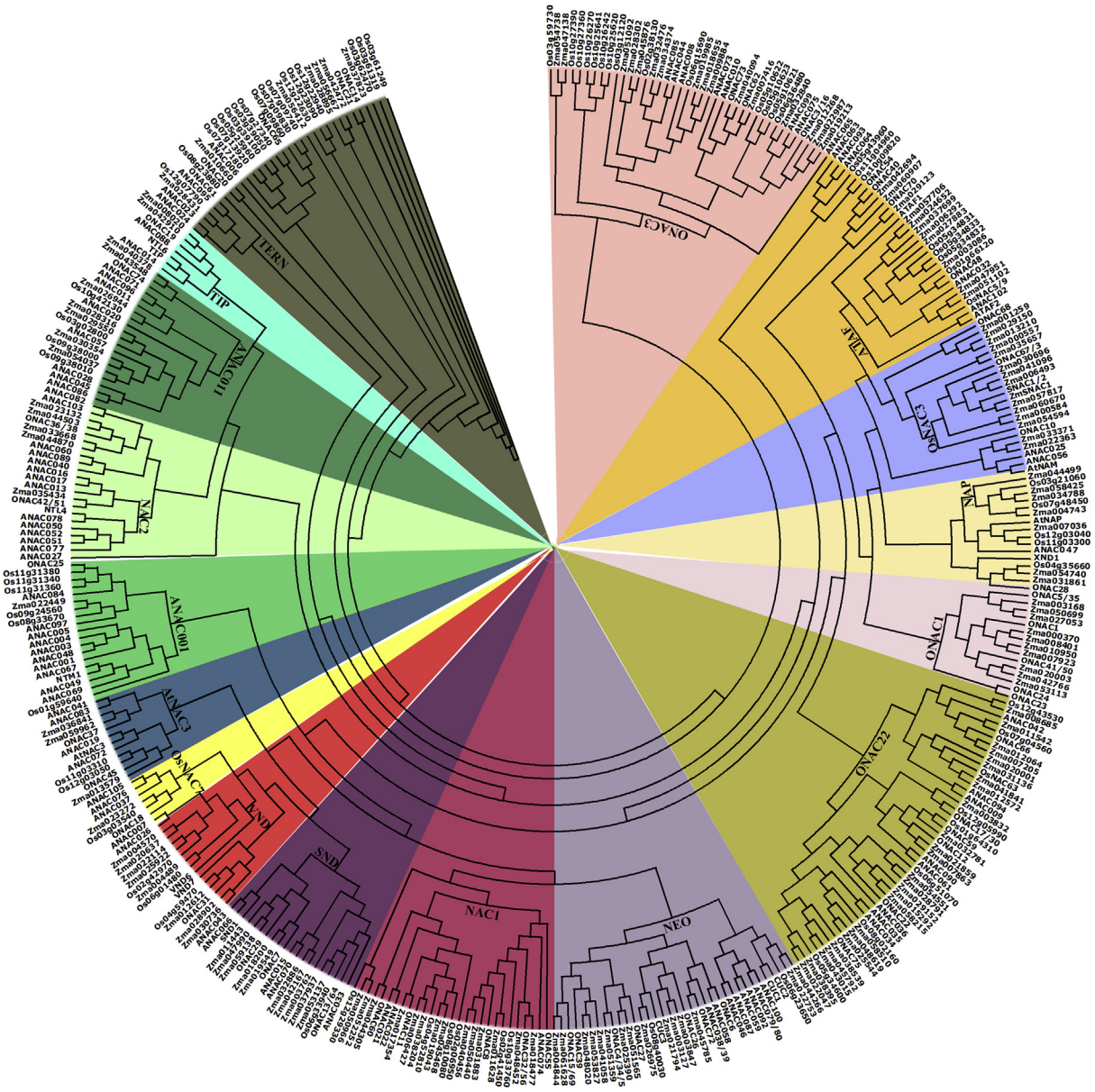

Fig. 2. Phylogenetic analysis of NAC transcription factors in maize, rice, and Arabidopsis. Full-length sequences of NAC proteins were aligned using ClustalW software. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates.

and one intron (Supplementary Fig. S5). In combination with the phylogenetic analysis, it is clear that most closely related members in the same subgroup share similar gene structures.

3.6. Promoter region analysis of selected ZmNAC genes

To investigate the putative *cis*-elements in the promoter region of selected ZmNAC genes, 1.5 kb of DNA sequence upstream of the translation start site (ATG) was used for analysis. A series of *cis*-acting elements including ABREs, GT1CONSENSUS, DRE/CRT, LTRE were identified (Supplementary Fig. S6). This suggested that these selected NAC genes respond to various abiotic and biotic stresses, and ABA, MYB, and WRKY transcription factorbinding sites identified in the promoter were suggestive of a potential regulatory mechanism under abiotic stresses in the ABA-dependent signaling pathway.

3.7. Expression profiles of selected ZmNAC genes using quantitative real-time PCR

To determine the effects of abiotic stresses on the expression of selected ZmNAC genes, quantitative real-time PCR was performed to identify temporal expression patterns at the seedling stage (Fig. 4). The seven *ZmNAC* genes in this study showed different expression patterns under abiotic stresses. As shown in Fig. 4A, *Zma001259*, *Zma000584*, *Zma003086*, Zma054594, and *ZmSNAC052* were strongly induced in both shoots and roots under dehydration treatment, whereas *Zma001259* and *Zma029150* showed higher transcript levels only in the roots. The upregulated expression levels of all selected NAC genes could also be detected in both the shoots and roots when maize seedlings were treated with Hoagland solutions supplemented with NaCl. In addition, *Zma029150* and *Zma001259* showed similar expression patterns under NaCl treatment. Under PEG treatment, *Zma003086*, *Zma000584*, and

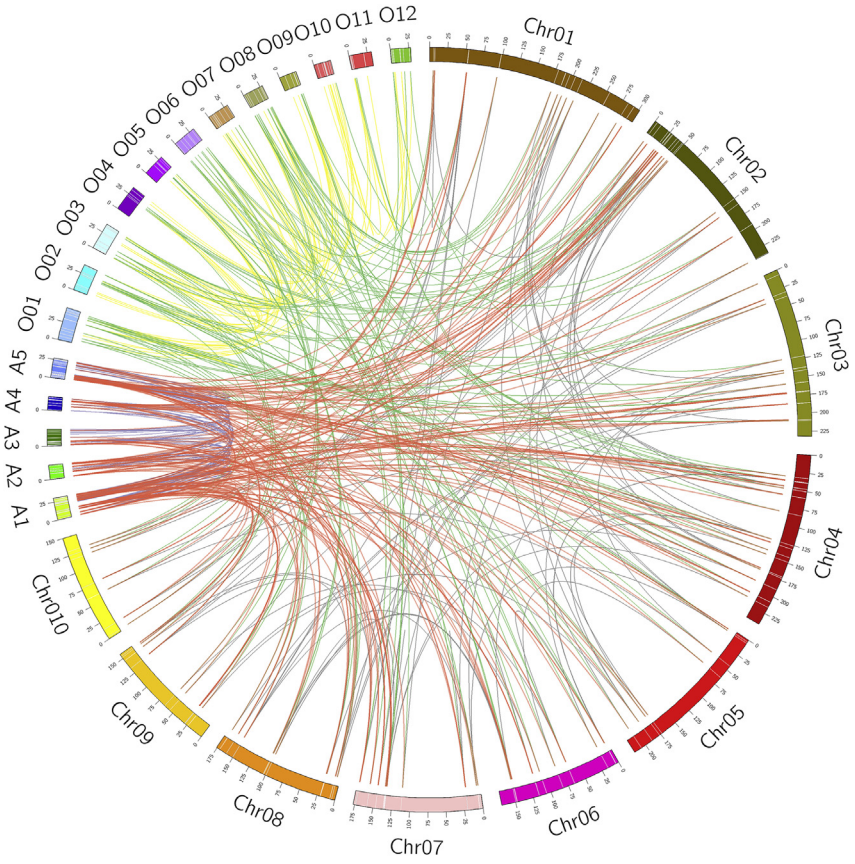

Fig. 3. Gene duplication and synteny analyses of NAC members among maize, rice and Arabidopsis. The red lines represent orthologous NAC genes between maize (Chr01–Chr10) and Arabidopsis (A1–A5), whereas the green lines demonstrate highly conserved synteny between maize and rice (O01–O12). The purple, yellow, and lavender lines represent paralogous NAC genes in Arabidopsis, rice, and maize, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Zma06493 were upregulated only in roots, whereas *Zma029150*, *Zma000584*, *Zma054594*, and *Zma001259* were significantly upregulated in both shoots and roots. The seven NAC genes may play a role in the ABA-dependent signaling pathway for their significantly enhanced transcript levels under ABA treatments, although *Zma000584*, Zma054594, and *Zma003086* showed higher expression levels than those of other tested NAC members. *ZmSNAC052*, *Zm000584*, *Zma003086*, *Zm054594*, and *Zma001259* (in varying degrees) responded to cold stress. Meanwhile, we also examined the transcript levels of these seven *ZmNAC* genes under water control treatment. The results showed no significant differences in shoots and roots of maize seedlings.

Under drought stress treatment, the transcript levels of *ZmNAC052*, *Zma000584*, *Zma006493*, and *Zma001259* increased with the degree of stress and then decreased to the control level after re-watering (Fig. 4B), suggesting that they play an important role in the response to drought stresses.

3.8. Expression patterns in different tissues

To investigate the spatial expression patterns of the selected *ZmNAC* genes in maize, different tissues including stalk, leaves, aerial roots, roots, ears, and tassels were detached from B73 at different developmental stages. As illustrated in Fig. 4C, transcripts of the seven *ZmNAC* genes were detected in all tissues evaluated. Higher transcript levels of all *ZmNAC* genes were observed in roots. Relatively higher expression levels of *ZmSNAC052*, *Zma001259*, and *Zma003086* were detected in both the leaves and tassels. However, the transcripts of these *ZmNAC* genes evaluated were detected at very low levels in ears and silks. Organ-specific expression patterns were indicative of functional divergence of these seven *ZmNAC* genes in different tissues at various development stages under normal or abiotic stress conditions.

4. Discussion

Abiotic stress triggers a series of plant responses including the alteration of gene expression patterns and cellular metabolism [27]. Transcription factors regulate the expression of genes that mediate multiple abiotic stresses responses and biological processes [23]. The NAC family encodes a large number of transcription factors. In recent years, NAC proteins have received much attention for their extraordinary functions in the plant response to different adverse environmental stresses. Numerous NAC members have been isolated and well-characterized in some typical model plants, whereas NAC genes have not been well-studied in maize. In this study, a total of 157 NAC members were identified after removing redundant sequences from 191 NAC proteins extracted from the Plant TFDB database. Chromosomal locations, phylogenetic relationships, gene duplication, and protein features of *ZmNAC* genes were analyzed in detail. Some putative ZmNAC genes were isolated and found to be responsive to various abiotic stresses and ABA. This information will provide a foundation for functional analysis of maize NAC genes in the future.

The maize genome has undergone several rounds of genome duplication that can distinguish maize from its close relative cereal

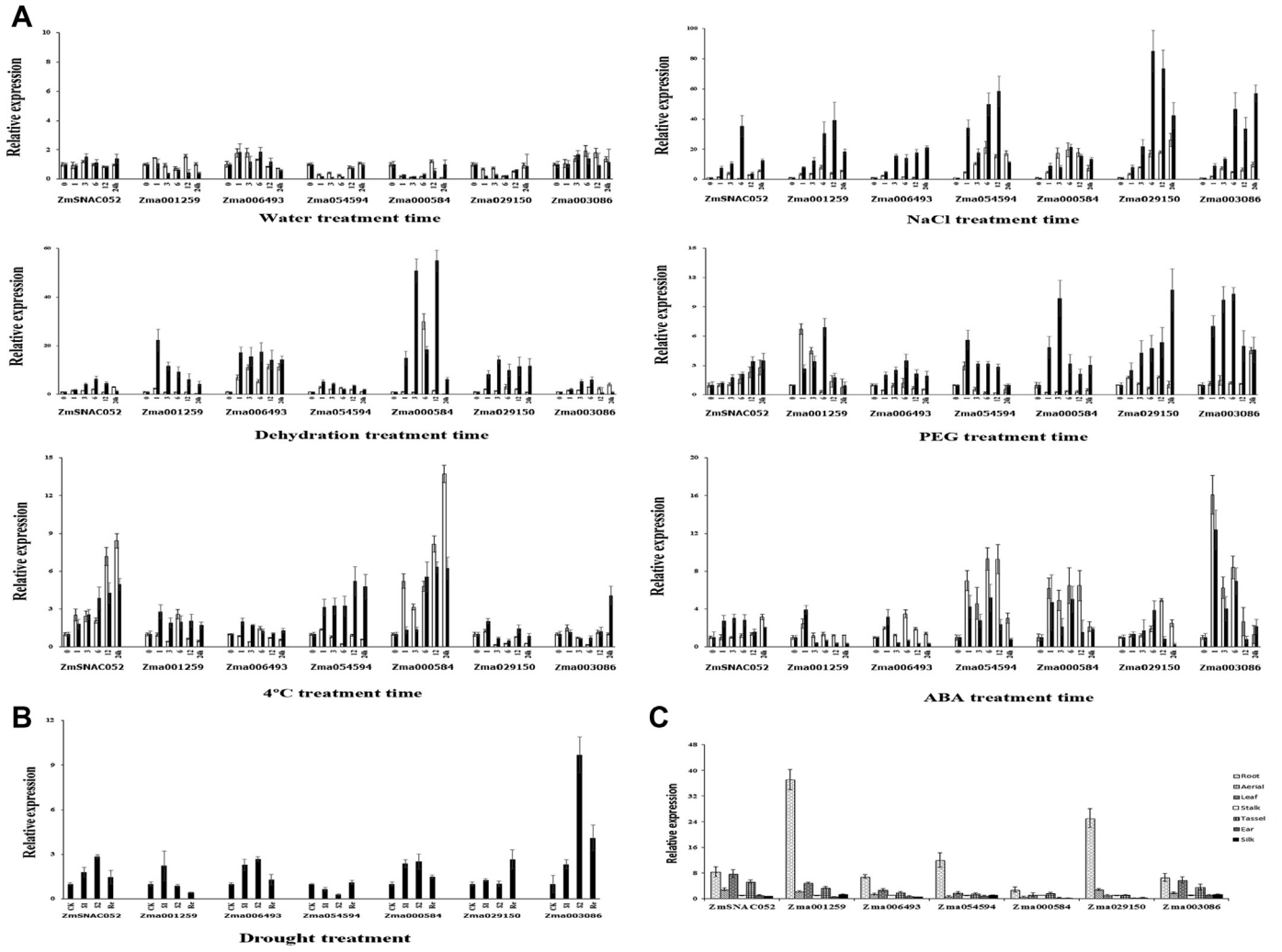

Fig. 4. A. Expression levels of the selected NAC genes in maize under various stress conditions, including NaCl, dehydration, PEG, 4 °C, ABA, and in the water control at given time points. The transcripts levels of these NAC genes in roots and shoots were indicated by the black and white bars, respectively. B. Relative transcript levels of the selected NAC genes in the mixture of shoots and roots under moderate (S1) and severe (S2) drought stress treatments and re-watering (Re) conditions. The transcript levels of these NAC genes in the control (CK) were used as calibrators. C. Organ specific expression analysis. The relative transcript expression levels in maize leaf, tassel, aerial root, and stalk were calculated relative to levels in the stalk. Each experiment was performed at least three times, and error bars represent standard deviation.

plants, such as Sorghum [24]. Gene duplication is believed to be the primary source of genetic novelty and progress evolution [21]. Moreover, the number of NAC genes in maize was two-fold less than that in Arabidopsis. This indicates that NAC gene duplication that occurred during maize evolution accompanied gene loss. According to the homologs annotated in *Arabidopsis* and rice, a series of NAC proteins in maize were identified for their putative functions based on comparative genomic analysis. This may assist functional studies of these maize NAC genes in the future.

NAC proteins constitute a large transcription factor gene family in higher plants. To date, at least 151 OsNAC genes in rice, 204 BrNAC genes in Chinese cabbage [21], and 152 GmNAC genes in soybean have been identified based on genome-wide analysis. In accordance with several typical NAC sequences that were reported in Arabidopsis and rice, the 157 ZmNAC family proteins shared three conserved motifs constituting a NAC domain that functions in the recognition and binding of a specific DNA sequence, whereas a divergent polypeptide located at the C-terminal region is considered the transactivation domain [25]. Based on protein sequence similarity, NAC proteins from Arabidopsis, rice, and maize could be classified into two large groups including 18 subgroups. ATAF, OsNAC3, and TIP subgroups were reported to play a vital role in abiotic and biotic stress tolerance [26]. For example, AFAF subgroup factor ATAF2 and tomato SISRN1 were identified as positive regulators of the defense response against pathogens and wounds, but negative regulators of the abiotic stress response. These results suggested that NAC genes with a close evolutionary relationship may have similar biological functions in plants. This information can be used to explore potential stress-related NAC genes in plants, especially in the staple crop plant maize.

Based on phylogenetic analyses of maize NAC genes and some well-known NAC genes in other plants, several predicted NAC proteins potentially involved in abiotic stress responses were isolated. Analysis of *cis*-elements in promoter regions provided background information for further analysis. The expression results indicated that, similar to their homologs in rice, sorghum, and wheat, these selected NAC members respond to abiotic stress. For example, *ZmSNAC1* and its homologs *SNAC1* in rice and *SbSNAC1* in sorghum were significantly induced by various abiotic stresses such as drought, cold, and salt. The different expression patterns under divergent stress treatments indicated that they may remain at the same level in multiple stress signal pathways. The spatial specific

expression profiles of the seven *ZmNAC* genes were suggestive of complicated transcriptional regulation in various tissues at different development stages. Thus, NAC transcription factors play important roles in resistance to abiotic stresses, but the regulatory mechanism is complex.

In conclusion, a comprehensive description regarding maize NAC transcription factor family genes was performed in this study. The phylogenetic relationship, distribution, and the conserved motifs of maize NAC proteins were analyzed systematically. Some predicted stress responsive NAC genes in the stress-related subgroup were isolated and shown to respond to various abiotic stresses in ABA-dependent pathways, although they showed specific temporal and spatial expression patterns. Although the functions of NAC genes in maize remain unclear, the data we present here increased our understanding on the involvement of maize NAC genes in the plant response to abiotic stresses and may be used to engineer stress tolerant crops.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.113.

Transparency document

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