Identification and analysis of *Chrysanthemum nankingense* NAC transcription factors and an expression analysis of OsNAC7 subfamily members

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NAC (NAM, ATAF1-2, and CUC2) transcription factors (TFs) play a vital role in plant growth and development, as well as in plant response to biotic and abiotic stressors [¹-²]. Chrysanthemum is a plant with strong stress resistance and adaptability; therefore, a systematic study of NAC TFs in chrysanthemum is of great significance for plant breeding. In this study, 153 putative NAC TFs were identified based on the *Chrysanthemum nankingense* genome. According to the NAC family in Arabidopsis and rice, a rootless phylogenetic tree was constructed, in which the 153 CnNAC TFs were divided into two groups and 19 subfamilies. Moreover, the expression levels of 12 CnNAC TFs belonging to the OsNAC7 subfamily were analyzed in *C. nankingense* under osmotic and salt stresses, and different tissues were tested during different growth periods. The results showed that these 12 OsNAC7 subfamily members were involved in the regulation of root and stem growth, as well as in the regulation of drought and salt stresses. Finally, we investigated the function of the *CHR00069684* gene, and the results showed that *CHR00069684* could confer improved salt and low temperature resistance, enhance ABA sensitivity, and lead to early flowering in tobacco. It was proved that members of the OsNAC7 subfamily have dual functions including the regulation of resistance and the mediation of plant growth and development. This study provides comprehensive information on analyzing the function of CnNAC TFs, and also reveals the important role of OsNAC7 subfamily genes in response to abiotic stress and the regulation of plant growth. These results provide new ideas for plant breeding to control stress resistance and growth simultaneously.
Identification and analysis of *Chrysanthemum nankingense*

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Abstract

NAC (NAM, ATAF1-2, and CUC2) transcription factors (TFs) play a vital role in plant growth and development, as well as in plant response to biotic and abiotic stressors\(^{[1-2]}\). Chrysanthemum is a plant with strong stress resistance and adaptability; therefore, a systematic study of NAC TFs in chrysanthemum is of great significance for plant breeding. In this study, 153 putative NAC TFs were identified based on the *Chrysanthemum nankingense* genome. According to the NAC family in Arabidopsis and rice, a rootless phylogenetic tree was constructed, in which the 153 CnNAC TFs were divided into two groups and 19 subfamilies. Moreover, the expression levels of 12 CnNAC TFs belonging to the OsNAC7 subfamily were analyzed in *C. nankingense* under osmotic and salt stresses, and different tissues were tested during different growth periods. The results showed that these 12 OsNAC7 subfamily members were involved in the regulation of root and stem growth, as well as in the regulation of drought and salt stresses. Finally, we investigated the function of the *CHR00069684* gene, and the results showed that *CHR00069684* could confer improved salt and low temperature resistance, enhance ABA sensitivity, and lead to early flowering in tobacco. It was proved that members of the OsNAC7 subfamily have dual functions including the regulation of resistance and the mediation of plant growth and development. This study provides comprehensive information on analyzing the function of CnNAC TFs, and also reveals the important role of OsNAC7 subfamily genes in response to abiotic stress and the regulation of plant growth. These results provide new ideas for plant breeding to control stress resistance and growth simultaneously.

Introduction
Transcription factors (TFs) are a class of important regulatory proteins, which inhibit or enhance gene expression by covalently binding to the DNA binding domain\[^3\]. NAC (NAM, ATAF1-2, and CUC2) TFs are a large family endemic to plants and are rarely found in the genomes of bacteria, fungi, or animals\[^4\]. NAC family proteins share a common NAC domain, which is identified by a consistent NAM sequence from Petunia and ATAF1/ATAF2 and CUC2 sequences from Arabidopsis. Arabidopsis ATAF is the first described member of NAC. NAM (NO APICAL MERISTEM) plays an important role determining the apical meristem and the location of primordia. Petunias with a NAM gene mutation cannot form apical meristem\[^5\]. The CUC2 gene plays a role in the development of the embryo and flower, and mutations in CUC1 and CUC2 cause the separation of cotyledons (embryonic organs), sepals, and stamens (floral organs), leading to defects in the formation of terminal bud meristematic tissues\[^6-8\]. NAC family genes have about 160 residues at the N-terminus, forming a typical highly conserved domain, which is divided into five subdomains. Among them, three subdomains are highly conserved, and two subdomains are diverse. These two subdomains are related to the multi-functions of the NAC family TFs\[^1, 9\].

NAC TFs are expressed during different development stages and in different tissues of plants, which are closely related to plant growth and development, as well as the regulation of plant response to abiotic and biotic stresses\[^10\]. Ooka et al. comprehensively analyzed 75 predicted NAC TFs in rice (Oryza sativa) and 105 predicted NAC TFs in Arabidopsis (Arabidopsis thaliana) and divided the NAC TFs into two groups (Group 1 and Group 2) according to similarities in their structural domains. Group 1 contains 14 subfamilies and Group 2 contains four subfamilies\[^9\]. Rushton et al. analyzed the evolutionary relationships among 450 NAC proteins in rice (O. sativa), Arabidopsis (A. thaliana), tobacco (Nicotiana tabacum), poplar (Populus trichocarpa), and other plants, and divided the NAC gene family into seven subfamilies. Among them, six subfamilies exist in all plants, while the other subfamily only occurs in tobacco, pepper, potato, and tomato\[^11\]. So far, 151 NAC family members have been found in O. sativa\[^4\], 117 in A. thaliana\[^4, 9\], 152 in tobacco (N. tabacum)\[^11\], 205 in soybean (Glycine max)\[^12\], 104 in tomato (Solanum lycopersicum)\[^13\], 163 in poplar (P. trichocarpa)\[^14\], 79 in grape (Vitis vinifera)\[^15\], 168 in durum wheat (Triticum turgidum)\[^16\], and 488 in bread wheat (Triticum aestivum)\[^2\].

Studies on NAC TFs have reported that the NAC genes respond to at least one biotic or abiotic stress, such as saline-alkali, drought, cold, or exogenous abscisic acid (ABA), which play important roles in plant drought tolerance, saline-alkali tolerance, virus resistance, and the cold resistance response\[^15, 17\]. In previous studies, most NAC TFs are closely related to salt and alkali tolerance, cold tolerance, and disease resistance of plants, whereas other NAC TFs improve plant resistance to multiple stressors at the same time\[^18-20\]. The A. thaliana NAC TFs ATAF1, ANAC016, ANAC019, ANAC055, and ANAC072\[^21-22\]; the O. sativa NAC TFs OsNAC045, OsNAC10, and OsNAC022\[^23\]; the G. max NAC TFs GmNAC085, GmNAC109, GmNAC30, GmNAC81, GmNAC003, GmNAC004, GmNAC11, and GmNAC20\[^24-26\]; and the T. turgidum NAC TF TaRNAC1\[^26-27\] enhance drought tolerance. The Lilium regale NAC TF LrNAC35, the
S. lycopersicum NAC TFs SlNAC1 and SlNAC61, and the A. thaliana NAC TFs ATAF2 and TIP are all related to virus resistance in plants\textsuperscript{[18, 28-31]}. The G. max NAC TF GmNAC20 significantly improves cold resistance in plants\textsuperscript{[19, 26, 32]}. The O. sativa NAC TFs OsNAC5 and OsNAC6 (the ATAF subfamily) improve drought resistance, salt tolerance, and freeze resistance of transgenic plants\textsuperscript{[33-34]} and the V. vinifera NAC TF VvNAC17 enhances the sensitivity of A. thaliana to ABA and improves salt tolerance, cold tolerance, and drought tolerance\textsuperscript{[15]}. Most genera in Anthemideae are distributed in southern Africa and the Mediterranean region, but are also widely distributed in Europe and Asia. The main economic crops in the family include Chrysanthemum carinatum Schousb., C. segetum L., C. nankingense, Dendranthema morifolium (Ramat.) Tzvel., Pyrethrum cinerariifolium Trev., and Artemisia annua. These are important vegetables, ornamental flowers, grasses, and herbs. Although Anthemideae plants are widely distributed, only some of the currently available plants can be cultivated in arid, saline, or cold regions, as their various traits are weakened and can only be planted as single-season cultivars. Under adverse circumstances, they lose the advantages of being an easy-to-manage perennial, leading to difficulties in popularity and application. Improving the growth characteristics and resistance to drought and saline-alkali stresses of Anthemideae plants would significantly improve their application range and enhance their cultural advantages. NAC TFs play an important role in abiotic stress in plants\textsuperscript{[19]}. Therefore, regulating the CnNAC TFs is an effective way to enhance the resistance of Anthemideae plants. However, most studies on the Chrysanthemum NAC genes are single-gene studies that focus on disease and insect resistance. Few comprehensive studies have been performed on CnNAC TFs and their abiotic stress responses. In this study, CnNAC TFs were analyzed based on published C. nankingense genome data. In addition, the expression levels of the CnNAC TFs belonging to the OsNAC7 subfamily were analyzed in different tissues at different growth stages in C. nankingense under osmotic and salt stresses. These results are expected to contribute to resistance breeding and growth and developmental regulation of Chrysanthemum.

**Materials & Methods**

**Gene retrieval of C. nankingense NAC TFs**

The A. thaliana NAC TF sequences were obtained from the Arabidopsis Information Resource (http://www.arabidopsis.org/). C. nankingense genomic data were downloaded from the website of the Institute of Chinese Medicine, Chinese Academy of Chinese Medical Sciences (http://www.amwayabrc.com/zh-cn/download.htm). Based on the A. thaliana NAC protein sequences, HMMER3.0 software was used to construct a multiple comparison HMM model, which was then used to search the C. nankingense genome protein data to obtain the CnNAC protein sequences. The CnNAC protein sequences were identified and screened using the SMART tool (http://smart.embl-heidelberg.de/) and Pfam (https://pfam.xfam.org/), and the predicted CnNAC TF members were obtained. The CnNAC TFs were used for subsequent analyses based on this comprehensive screening\textsuperscript{[35]}.  

**Bioinformatics analysis of C. nankingense NAC TFs**
The NAC TFs of *C. nankingense*, *A. thaliana*, and some of those of *O. sativa* were used to construct a rootless phylogenetic tree using the multiple comparison function in ClustalW (default parameters) and the neighbor-joining (NJ) method in MEGA-X software according to the existing *A. thaliana* and *O. sativa* NAC gene family classification system\[^1, 9, 36\]. The rootless phylogenetic tree was used to study the phylogenetic relationships of the CnNAC TFs\[^37\]. MEME online software (http://meme-suite.org/tools/meme) was used to predict the conserved motifs of the CnNAC TFs. The ProtParam online tool (https://web.expasy.org/protparam/) was used to analyze the physical and chemical properties of the CnNAC TF amino acid sequences and predict their subcellular locations\[^38\]. The TMHMM online tool (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to predict the protein transmembrane structure of the CnNAC TFs. The three-dimension (3D) models of the conserved NAC domains of the CnNAC proteins were predicted by Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index)\[^39\].

**Growth conditions and treatment of the plant materials**

*C. nankingense* seeds (from Beijing Forestry University) were sown on peat to produce seedlings under growing conditions of 25°C/23°C (day/night), 16 h/8 h (day/night), and 2,000 Lux. The roots, stems, and 3–5 new leaves on the top of the seedlings were collected and quickly flash frozen in liquid nitrogen as experimental materials at 28, 44, and 60 d after seeding, respectively, and stored at −80°C. Three replicates were taken for each period.

Rooted *C. nankingense* seedlings obtained from stem hydroponics were subjected to osmotic and salt stresses. The side shoots of *C. nankingense* seedlings grown for 44 d were cut out and inserted into cultivation medium containing MS medium and 0.1 mg/L IBA. The growing conditions were 25°C/23°C (day/night), 16 h/8 h (day/night), and 2,000 Lux. The cultivation medium was changed every 3–5 d, and rooted seedlings were obtained after 20 d. Then, the cultivation solution was changed to 20% PEG6000 or 200 mM NaCl solution for the osmotic and salt stress treatments, respectively. Samples were taken at 0, 1, 4, 8, 12, 24, and 36 h\[^40\]. The entire rooted seedling was taken as a sample and stored at −80°C after flash-freezing in liquid nitrogen. Three replicates were taken for each period.

**RNA isolation and real-time quantitative-polymerase chain reaction (RT-qPCR) analysis**

Total RNA was isolated using the FastPure Plant Total RNA Isolation Kit (Code No. RC401-01; Nanjing Vazyme Biotechnical Co., Ltd., Nanjing, China). The reverse transcription of RNA was carried out using the PrimeScript™ RT reagent Kit with the gDNA Eraser (Perfect Real Time, Code No. RR047A; Takara Bio., Shiga, Japan). The primers used to analyze the expression of the OsNAC7 subfamily members were designed based on the *C. nankingense* genome (Table 1). RT-qPCR was carried out with on the Applied Biosystems StepONEPlus Real Time PCR System (Foster City, CA, USA), using ChamQ Universal SYBR qPCR Master Mix (Code No. Q711-03; Nanjing Vazyme Biotechnical Co., Ltd., Nanjing, China). The reaction system was 40 cycles of 95°C for 20 s, 95°C for 3 s, 60°C for 30 s, 95°C for 15 s, and 60°C for 1 min\[^42\], with *actin7* from *C. nankingense* as the internal reference gene\[^41\]. The relative expression of genes was calculated by the 2−ΔΔCt method.
Subcellular localization and functional analysis

The super1300 vector was used as the overexpression vector (kindly provided by Professor Huitang Pan of Beijing Forestry University, Beijing, China. Supplementary Data 1). The vector uses super35S as the promoter. According to the open reading frame (ORF) of the \(CHR00069684\) gene (Supplementary Data 2) and the polyclonal sites of the super1300 vector, the endonucleases \(Pst\) I and \(Kpn\) I (NEB, Beijing) were selected to construct the binary expression vector according to the double enzyme method. The recombinant plasmid was transformed into Escherichia coli competent cells (DH5α), which were inoculated on solid LB medium containing 50 mg/L kanamycin; the resistance E. coli cells were validated by colony PCR. The obtained positive plaques were sent to the sequencing company (Beijing Ruibio BiotechCo.,Ltd) for sequencing and plasmid recovery. The super35S::GFP and super35S::\(CHR00069684\)::GFP vectors were introduced into Agrobacterium tumefaciens strain GV3101 (Weidi Biotechnology, Shanghai, China) by the freeze-thaw method and then inoculated on LB medium supplemented with 50 mg/L kanamycin, 50 mg/L gentamicin, and 50 mg/L rifampicin. The surviving plaques were validated by colony PCR, and the positive plaques were inoculated into LB liquid medium containing 50 mg/L kanamycin, 50 mg/L gentamicin, and 50 mg/L rifampicin for propagation. The transgenic tobacco (NT78) was obtained through A. tumefaciens-mediated transformation[43], screened by 30 mg/L hygromycin, and validated by PCR identification of the target gene. CHR000696884::GFP fusion protein and GFP protein signals were observed by a laser confocal microscopy (Leica TCS SP5) in roots, stems, and leaves of the transgenic tobacco plants. The super35S::GFP vector was used as control.

Results

Identification of the CnNAC TFs and the phylogenetic analysis

According to the \(A.\ thaliana\) NAC protein sequences, 153 predicted CnNAC TFs were identified from the \(C.\ nankingense\) genome data using HMMER3.0. Using ClustalW in MEGA, 153 \(C.\ nankingense\) NAC proteins, 105 \(A.\ thaliana\) NAC proteins, and 6 \(O.\ sativa\) NAC proteins were multi-sequence aligned, and a rootless phylogenetic tree was constructed using the NJ method by MEGA (Fig. 1, Supplementary Data 3). According to the existing \(A.\ thaliana\) and \(O.\ sativa\) NAC gene family classification system[1, 9, 36] and the topology of the rootless phylogenetic tree, the CnNAC TFs were divided into two groups of 19 subfamilies, including 17 known subfamilies and two unknown subfamilies. Group 1 contained 15 subfamilies, including OsNAC7, NAC1, NAM, ANAC077, ANAC011, OsNAC8, TIP, NAC2, ONAC022, TERN, SENU5, NAP, AtNAC3, ATAF, and unclassified 2, with a total of 111 members. Group 2 contained five subfamilies, which were ANAC063, ONAC003, ANAC007, unclassified 1, and ONAC001, with a total of 42 members. Among them, the ANAC077 subfamily occurred in both groups. The different branches of the phylogenetic tree testify to the functional diversity of this family. According to further analysis of the CnNAC TFs (Supplementary Fig. 1), 104 \(NAC\) genes were detected in the 17 known subfamilies, and 49 \(NAC\) genes were detected in the two unknown subfamilies. Three known subfamilies (ONAC022, NAC2, and OsNAC7) contained 13, 11, and
12 members respectively, with the greatest numbers of genes among all known subfamilies. However, the two unknown subfamilies, unclassified 1 and unclassified 2, contained 28 and 21 NAC genes, respectively.

Prediction of the protein structure is considered as a reliable analytical method to understand the molecular function of proteins. Therefore, the 3D models of one gene from each branch of the NAC phylogenetic trees (Fig. 1), and all 60 CnNAC family members were constructed using the Phyre2 server (Fig. 2). c3ulxA and d1ut7a were used as the reference model template, and the confidence level was set as 100%. The c3ulxA template is a DNA binding protein and annotated as stress-induced NAC1 according to the crystal structure of the conserved domain of rice stress responsive NAC1 in the PDB database. The d1ut7a template is annotated as a NAC domain. The 3D modeling results revealed that these CnNAC TFs possessed similar tertiary structures, implying that CnNAC TFs may evolved from the same ancestor sequence and/or under purifying selection to maintain stabilization during the long-term evolution after the initial divergence.

Analysis of the CnNAC TF conserved motifs

The online tool MEME-Motif Suite was used to analyze the motifs of the 153 predicted CnNAC TFs (Fig. 3) and the positions of the 15 motifs on different genes were determined (Fig. 4). As a result (Supplementary Fig. 2), five genes (CHR00045488-RA, CHR00007510-RA, CHR00036356-RA, CHR000079376-RA, and CHR000089260-RA) containing 11 motifs, respectively, constituted the most abundant motifs. These five genes all belonged to the unknown unclassified 1 subfamily. Nine genes (CHR00007511-RA, CHR00035414-RA, CHR00039887-RA, CHR00039888-RA, CHR00055754-RA, CHR00055752-RA, CHR00088453-RA, CHR00024060-RA, and CHR00058504-RA) containing 10 motifs, respectively, were also in the unknown subfamilies (six in unclassified 1 and three in unclassified 2). Sixty-two genes, which contained the highest number of 7 motifs, were distributed in 12 known subfamilies and two unknown subfamilies.

According to the comparative analysis of the motifs in different subfamilies (Fig. 4), the included motif bases were in motifs 1–7 in the known subfamilies of Group 1, but different subfamilies contained different numbers of motifs and different conserved motifs. For example, motifs 4, 6, 3, and 7 were in the NAM subfamily and motifs 1, 4, 2, 5, and 6 were in the OsNAC7 subfamily. However, some members in the unknown subfamily unclassified 2 had more motifs, such as motifs 10, 13, and 14, than in the other subfamilies. Each member in the known subfamilies of Group 2 contained far fewer motifs than those in the known subfamilies of Group 1. Members in the known subfamilies of Group 2 contained motifs 1, 3, 4, 5, 6, and 7, and lacked motif 2 compared to those in the known subfamilies of Group 1. However, the CnNAC TFs in unclassified 1 of Group 2 had the highest number of motifs, most of which were motifs 7–11.

Subcellular localization, protein transmembrane analysis, and physicochemical property analysis of CnNAC TFs

According to the subcellular localization and transmembrane analysis results (Supplementary Table 1), 106 of the 153 CnNAC TFs were located in the nucleus and 47 were located outside...
the cell. Of the 111 members in Group 1, 106 were nuclear and 15 were extracellular, of which 24 members within the unclassified 2 subfamily were all nuclear. Only 10 of the 42 members of Group 2 were located in the nucleus, the other 32 were located outside the cell, and 23 of the 28 members of the unclassified 1 subfamily of Group 2 were located outside the cell. However, the protein transmembrane analysis of the 153 NAC TFs revealed that only one member of Group 1 was expressed inside the membrane and eight members were expressed transmembrane, while the remaining 102 members were all expressed outside the membrane. There were no transmembrane-expressed Group 2 members, while two members were expressed inside the membrane and 40 members were expressed outside the membrane. The three genes expressed inside the membrane were all located in the nucleus, and the genes expressed transmembrane and outside the membrane all had members located in the nucleus and extracellularly, respectively.

The physicochemical properties of the CnNAC TFs were analyzed (Supplementary Table 2). The lengths of the CnNAC TF amino acids ranged from 68 to 693 (mean length = 307.99). The molecular weight range of the CnNAC TFs was 7,787.81–80,985.3 Da with an average molecular weight of 35,195.1 Da. The theoretical isoelectric point (pI) range of the CnNAC TFs was 4.43–10.2, and the average pI was 7.09. The grand average of hydropathy was −0.72896, while the lowest value was −1.37, and the highest value was −0.275. The aliphatic amino acid index was 44.15–94.84, with a mean value of 64.58.

Analysis of the OsNAC7 subfamily gene expression levels during different C. nankingense growth stages

The OsNAC7 subfamily included 12 genes in A. thaliana, including SND1, NST1, URP7, BRn1/2, and VND1/2/3/4/5/6/7, the functions of which are all related to regulation of the formation of secondary cell walls in plant stems, roots, and anthers. Here, we analyzed the expression levels of the OsNAC7 subfamily genes in the roots, stems, and leaves of C. nankingense during different growth stages (Figs. 5 and 6, Supplementary Table 3).

As a result, all the 12 members of the C. nankingense OsNAC7 subfamily played a role in roots, stems, and leaves. The expression levels of most of the OsNAC7 genes were generally low in the roots and leaves but high in the stems. However, differences in the expression levels were observed during different root, stem, and leaf developmental stages. Ten out of the 12 members in the OsNAC7 subfamily were expressed at higher levels in the stems at 44 d, including CHR00068684, CHR00042500, CHR00009966, CHR00043953, CHR00073261, CHR00032377, CHR00026420, CHR00003673, CHR00027311, and CHR00073261-RA. CHR00048081 was expressed at high levels in the stems at 60 d. CHR00020838 was expressed at high levels in the stems during the whole growth process, which were much higher than those in roots and leaves. Five out of the 12 members were expressed at higher levels in leaves at 60 d, such as CHR00043953-RA, CHR00032377-RA, CHR00026420-RA, CHR00003673-RA, and CHR00043175-RA.

Five out of the 12 members in the OsNAC7 subfamily were significantly related to root growth. Among the 12 members, CHR00048081-RA was expressed at a higher level at 28 d, CHR00020838-RA and CHR00043175-RA had higher levels at 44 d, and CHR00009966-RA and CHR000073261-RA had higher levels at 44 d and 60 d. Five out of the 12 genes were expressed at higher levels in leaves at 60 d, such as CHR00043953-RA, CHR00032377-RA, CHR00026420-RA, CHR00003673-RA, and CHR00043175-RA.
Expression analysis of the *C. nankingense* OsNAC7 subfamily genes under the osmotic and salt stress treatments

All *A. thaliana* OsNAC7 subfamily genes are associated with secondary metabolism\(^{[47]}\). However, among the *O. sativa* OsNAC7 subfamily genes, *ONAC106* inhibits leaf senescence and also increases salt damage and tillering angle\(^{[3, 48]}\); the expression levels of *ONAC052*, *ONAC056*, and *ONAC084* are downregulated under high salt and drought conditions\(^{[49]}\). Here, we analyzed the expression levels of the *C. nankingense* OsNAC7 subfamily genes under osmotic and salt stresses (Fig. 7, Fig. 8, Supplementary Table 3).

The results of gene expression under osmotic stress (Fig. 7) revealed that all of the 12 genes in the OsNAC7 subfamily were regulated by osmotic stress, but their expression patterns were not consistent. Among them, the expression levels of *CHR00003673-RA*, *CHR00027311-RA*, and *CHR00043175-RA* continued to increase after treatment, indicating that these three genes may play a key role in improving the resistance to osmotic stress in *C. nankingense*. The *CHR00026420-RA* expression fluctuated but was always higher than that of the control group, indicating that this gene is expressed during osmotic stress. *CHR00069684-RA* showed an obvious response to osmotic stress. The expression of *CHR00009966-RA* and *CHR00048081-RA* was inhibited at the initial stage of stress, and then gradually returned to normal expression levels. The response of *CHR00042500-RA* and *CHR00020838-RA* was not obvious, and *CHR00043953-RA* and *CHR00073261-RA* were severely inhibited by osmotic stress.

The gene expression results under salt stress (Fig. 8) revealed that all of the 12 genes in the OsNAC7 subfamily responded to salt stress, and were initially upregulated and then downregulated or were first downregulated and then upregulated; all decreased to the expression level of those of the control group or below by 36 h. The expression levels of *CHR000069684-RA*, *CHR00009966-RA*, *CHR00048081-RA*, *CHR00043953-RA*, *CHR00026420-RA*, *CHR0003673-RA*, *CHR00027311-RA*, and *CHR00043175-RA* increased first at different time points and then decreased. The significantly upregulated expression levels of these eight genes indicate that they may be important in the salt stress response. *CHR000042500-RA*, *CHR000020838-RA*, *CHR000073261-RA*, and *CHR000032377-RA* were slightly inhibited by salt stress, although their initial responses to salt stress were not obvious.

Subcellular localization and functional analysis

In this study, *CHR000069684* that was closely related to the NST protein was used to construct the super35S::*CHR000069684::GFP* vector, which was then transformed into large leaf tobacco for subcellular localization observation and functional validation. GFP signals were observed in the nucleus and cell membrane of leaves, stems, and roots of super35S::GFP transformed tobacco plants (Fig. 9). No fluorescence signal was detected in the super35S::*CHR000069684::GFP* transformed tobacco leaves (Supplementary Figure 3), whereas there were strong GFP signals in the cell membrane of their roots and stems (Fig 9 E F). The reason why the 35S promoter-guided *CHR000069684* expression showed tissue specificity need to be further studied in the future.
The super35S::CHR00069684::GFP transformed and control tobacco plants were treated with 200 mM NaCl, 200 mM ABA, or a low temperature of 4 °C (Fig. 10). The results showed that the transgenic tobacco plants were more resistant to salt and low temperature and more sensitive to ABA than the control plants. Moreover, the differences in the growth and development of tobacco plants were observed (Fig. 11); the flowering time of the transgenic tobacco was 10 d earlier than that of the control plants.

Discussion

According to the phylogenetic tree (Fig. 1) and motif analyses (Fig. 4) of CnNAC TFs, we found that the distinct NAC TF subfamilies each containing the most closely related members exhibited highly similar motif components. This included members in the OsNAC7 subfamily (Fig. 12), all of which possess motifs 1–6. The members clustering with ANAC012, ANAC066, ANAC043, ANAC033, ANAC070, and ANAC015, which were annotated as NST1–NST3, URP7, and BRN1/2, respectively, have the same motifs 1–7, and the remaining part was clustered with VND1–VND7, which lack the motif 6 or motif 7 (Fig. 4). These results indicate that the CnNAC TFs in the same subfamily have similar motifs and similar functions.

The NAC TFs are involved in various stages of plant growth and development and the responses to various stressors. It was demonstrated in O. sativa, A. thaliana, and Asteraceae (Table 2) that most NAC TFs regulate plant growth, secondary metabolism, and growth and development time by changing root or stem growth, and further regulate the resistance of plants to biotic and abiotic stressors, but each subfamily had specific characteristics. Compared to studies of Chrysanthemum and O. sativa, the ATAF subfamily member AaNAC1 (NAC1 in A. annua, KX082975.1), which was similar to CHR00002022-RA, CHR00045168-RA, and DgNAC1 (NAC1 in D. grandiform) has the same function as the O. sativa ATAF subfamily genes OsNAC5 and OsNAC6. All of these genes improve drought resistance, salt tolerance, and low temperature tolerance of plants. The ONAC022 subfamily member DINAC1 (NAC1 in D. nankingense), which was similar to CHR00056910-RA and OsNAC063, is involved in regulating drought-tolerance and salt-tolerance in plants. The functions of members belonging to the ATAF and ONAC022 subfamilies in chrysanthemum and rice are essentially the same, suggesting that closely related genes often have similar functions, so analyses of phylogenetic trees can be used to predict the functions of genes in the same subfamily. Through the NAC gene functions of O. sativa and A. thaliana, it has been speculated that the CnNAC genes in the NAC1 subfamily may improve the overall stress resistance of plants. The NAM subfamily genes improve tolerance to drought and high salt stress, which affect plant height and flowering time, and accelerate leaf senescence. The NAP subfamily genes enhance drought tolerance and salt tolerance in plants by influencing the size of the epidermis, cortex, and stellate cells in roots. The ONAC001 subfamily genes are induced by salt and drought stresses, the TERN subfamily genes may be associated with disease resistance, and the SENU5 subfamily genes enhance plant tolerance to salt, alkali, and drought stress.
Moreover, the two unknown subfamilies in CnNAC TFs (i.e., unclassified 1 and unclassified 2) contained more genes than the other subfamilies and had the most abundant numbers of motifs among the CnNAC TFs, indicating that these two unknown subfamilies may have diverse functions and may be involved in a wider range of growth and stress regulatory functions. The diversity of NAC family gene functions may be the reason why chrysanthemums, as a young and highly evolved large family, have strong adaptability to changing environments.

According to the subcellular localization and transmembrane analysis results (Supplementary Table 1), most CnNAC TFs were related to nuclear genes; however, most of them were mainly expressed outside the membrane. For example, CHR00069684 was a nuclear gene, but was expressed outside the membrane (Supplementary Table 1); CHR00069684 was expressed in the cell membrane of tobacco, and could only be observed in the stems and roots of transgenic tobacco plants (Fig. 9). These results are consistent with the finding that TFs found in poplar can be localized to the nucleus and cytoplasm at the same time, but they are transferred to and expressed in the nucleus when regulated by an external stress signal\[78\].

According to the phylogenetic tree (Fig. 1), the C. nankingense OsNAC7 subfamily members, including CHR00069684-RA, CHR00042500-RA, CHR00020838-RA, and CHR00009966-RA, were annotated as NST1–NST3 and have higher expression levels in the C. nankingense stems at 44 d (Fig. 6), which showed similar functions to the NST protein in A. thaliana\[50-52, 79-80\]. Moreover, CHR00009966-RA likely played a more important role in the root growth.

CHR00048081-RA, CHR00043953-RA, and CHR00073261-RA were annotated as URP7, BRN2, and BRN1 respectively, which are mainly responsible for the root cap. The expression of these three genes in the C. nankingense roots was similar to that of the URP7 and BRN1/2 genes in A. thaliana\[53-54\]. However, the expression of these three genes was higher in stems than in roots, indicating that CHR00048081-RA might play a regulatory role in the root growth and aging of the stem; CHR00043953-RA might play a regulatory role in the rapid growth of the stem and aging of the leaf; and CHR00073261-RA might play a regulatory role in the rapid growth of the stem. CHR00032377-RA, CHR00026420-RA, CHR00003673-RA, CHR00027311-RA, and CHR00043175-RA corresponded to the VND1, VND2, VND3, VND4, VND5, VND6, and VND7 genes, which co-participate in regulating the thickening of the secondary cell walls in A. thaliana\[55-58, 81\]. These genes were all involved in the stem growth and the aging process of leaves at the later growth stages (Fig. 6). Besides, CHR00043175-RA was also involved in the root growth.

All genes responded to the osmotic (Fig. 7) and salt stresses (Fig. 8), which has only been studied in the ONAC106 gene of the OsNAC7 subfamily in rice\[3, 48\]. Among them, CHR00026420-RA, CHR00003673-RA, CHR00027311-RA, and CHR00043175-RA may play a key role improving the resistance to osmotic stress in chrysanthemum. CHR00069684-RA, CHR00009966-RA, CHR00048081-RA, CHR00043953-RA, CHR00026420-RA, and CHR00003673-RA may be important genes in the salt stress response. To test this hypothesis, the super35S::CHR00069684::GFP transgenic tobacco lines were treated with salt stress, and the results confirmed the active function of CHR00069684 in the regulation of salt stress. In addition, the results obtained from ABA and low temperature treatments of the
super35S::CHR00069684::GFP transgenic tobacco plants indicated that CHR00069684 enhanced ABA sensitivity and low temperature stress resistance in the transgenic tobacco plants, leading to reduced growth potential and premature flowering. Functional analysis on CHR00069684 confirmed the dual roles of the OsNAC7 subfamily genes in the growth and stress regulation of plants. However, the function of the OsNAC7 subfamily members during abiotic stress remains speculative, and many other functions of the OsNAC7 subfamily genes remain to be further explored.

Conclusions

In this study, the CnNAC TFs were divided into two groups with 19 subfamilies according to the phylogenetic tree. These 19 subfamilies consisted of 17 known subfamilies and two unknown subfamilies. The conserved motifs, subcellular localization, transmembrane localization, and physicochemical properties of the CnNAC TFs were comprehensively analyzed. Combined with research on the NAC family genes of A. thaliana, O. sativa, and Asteraceae, the functions of the CnNAC TFs were investigated. Analyses on the expression of the 12 CnNAC genes in the OsNAC7 subfamily under osmotic stress, salt stress, and in different tissues at different time points showed that members of the OsNAC7 subfamily not only played a regulatory role in the growth and development of roots, stems, and leaves of C. nankingense, but also responded to osmotic and salt stresses. These findings may provide new ideas for regulating plant stress resistance and growth. The function of CHR00069684 was verified in transgenic tobacco plants, and it was found that CHR00069684 could confer enhanced resistance to salt and low temperature stresses, improve the ABA sensitivity, and lead to the early flowering of transgenic tobacco. Studies on the CHR00069684 function confirmed the dual roles of the OsNAC7 subfamily genes in stress regulation and plant growth and development. This study provides a theoretical basis for studying NAC TFs, the stress tolerance mechanism, and the plant growth process.

References

[1] Duan AQ, Yang XL, Feng K, Liu JX, Xu ZS, Xiong AS. 2019. Genome-wide analysis of NAC transcription factors and their response to abiotic stress in celery (Apium graveolens L.). *Computational Biology and Chemistry* 84: 107-186 DOI: 10.1016/j.compbiolchem.2019.107186.

[2] Guerin C, Roche J, Allard V, Ravel C, Mouzeyar S, Bouzidi MF. 2019. Genome-wide analysis, expansion and expression of the NAC family under drought and heat stresses in bread wheat (*T. aestivum* L.). *PLoS One* 14(3): 1932-6203 DOI: 10.1371/journal.pone.0213390

[3] Fang YJ, You J, Xie KB, Xie WB, Xiong L. 2008. Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. *Molecular Genetics and Genomics* 280(6): 547-563 DOI: 10.1007/s00438-008-0386-6

[4] Nuruzzaman M, Manimekalai R, Sharoni AM, Kouji Satoh K, Kondoh H, Ooka H, Kikuchi S. 2010. Genome-wide analysis of NAC transcription factor family in rice. *Genome* 465: 30-40 DOI: 10.1016/jgenome.2010.06.008.

[5] Souer E, van Houwelingen A, Kloos D, Mol J, Koes R. 1996. The no apical meristem gene of *Petunia* is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* 85: 159–170 DOI:10.1016/s0092-8674(00)81093-4.

[6] Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. 1997. Genes Involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. *The Plant Cell* 9: 841-857.

[7] Aida M, Ishida T, Tasaka M. 1999. Shoot apical meristem and cotyledon formation during Arabidopsis...
Ishida T, Aida M, Takada S, Tasaka M. 2000. Involvement of CUP-SHAPED COTYLEDON Genes in Gynoecium and Ovule Development in Arabidopsis thaliana. Plant and Cell Physiology 41(1): 60-67 DOI: 10.1093/pcp/41.1.60.

[9] Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Koijima K, Takahara Y, Yamamoto K, Kikuchi S. 2003. Comprehensive Analysis of NAC Family Genes in Oryza sativa and Arabidopsis thaliana. DNA Research 10 (6): 239-247 DOI: 10.1093/dnares/10.6.239.

[10] Liu H, Zhou Y, Li H, Wang T, Zhang J, Ouyang B, Ye Z. 2018. Molecular and functional characterization of ShNAC1, an NAC transcription factor from Solanum habrochaites. Plant Science 271: 9-19 DOI: 10.1016/j.plantsci.2018.03.005.

[11] Rushton PJ, Bokowiec MT, Han S, Zhang H, Brannock JF, Chen X, Laudeman TW, Timko MP. 2008. Tobacco transcription factors: novel insights into transcriptional regulation in the Solanaceae. DOI: 10.1016/j.plantsci.2018.03.005.

[12] Le DT, Nishiyama RIE, Watanabe Y, Mochida K, Yamaguchi-Shinozaki K, Shinozaki K, Tran L-SP. 2011. Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in Solanum lycopersicum. Journal of Plant Biochemistry and Biotechnology 24(2): 176-183 DOI:10.1007/s13562-014-0255-9.

[13] Su H, Zhang S, Yin Y, Zhu D, Han L. 2015. Genome-wide analysis of NAM-ATAF1,2-CUC2 transcription factor family in Solanum lycopersicum. DOI:10.1007/s13562-014-0255-9.

[14] Hu R, Qi G, Kong Y, Zhou G. 2010. Comprehensive Analysis of NAC Domain Transcription Factor Gene Family in Populus trichocarpa. BMC Plant Biology 145(10): 1471-2229 doi:10.1186/1471-2229-10-145.

[15] Ju YL, Yue XF, Min Z, Wang XH, Fang YL, Zhang JX. 2020. VvNAC17, a novel stress-responsive grapevine (Vitis vinifera L.) NAC transcription factor, increases sensitivity to abscisic acid and enhances salinity, freezing, and drought tolerance in transgenic Arabidopsis. Plant Physiology and Biochemistry 146: 98-111 DOI: 10.1016/j.plaphy.2019.11.002.

[16] Saidi MN, Mer gdy D, Brini F. 2017. Identification and expression analysis of the NAC transcription factor family in durum wheat (Triticum turgidum L. ssp. durum). Plant Physiology and Biochemistry 112: 117-128 DOI: 10.1016/j.plaphy.2016.12.028.

[17] Ying Gao Y, Wei W, Zhao X, Tan X, Fan Z, Zhang Y, Jing Y, Meng L, Zhu B, Zhu H, Chen J, Jiang CZ, Grierson D, Luo Y, Fu DQ. 2018. A NAC transcription factor, NOR-like1, is a new positive regulator of tomato fruit ripening. Physiological and Molecular Plant Pathology, 74, 394-402 DOI: 10.1016/j.pmpp.2010.06.005

[18] Sun D, Zhang X, Zhang Q, Ji X, Jia Y, Wang H, Niu L, Zhang Y. 2019. Comparative transcriptome profiling uncovers a Lilium regale NAC transcription factor, LrNAC35, contributing to defence response against cucumber mosaic virus and tobacco mosaic virus. Molecular Plant Pathology 20(12): 1662-1681 DOI: 10.1111/mpp.12868.

[19] Puranik S, Sahu PP, Srivastava PS, Prasad M. 2012. NAC proteins: regulation and role in stress tolerance Trends in plant science 17(6): 369-381 DOI: 10.1016/j.tplants.2012.02.004.

[20] Xia N, Zhang G, Sun YF, Zhu L, Xu LS, Chen XM, Liu B, Yu YT, Wang XJ, Huang LL, Kang ZS. (2010) TaNAC8, a novel NAC transcription factor gene in wheat, responds to stripe rust pathogen infection and abiotic stresses. Physiological and Molecular Plant Pathology, 74, 394-402 DOI: 10.1016/j.pmpp.2010.06.005

[21] Tran LSP, Nakashima K, Sakuma Y, Simpsonn SD, Fujita Y, 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. The Plant Cell 16: 2481-2498 DOI: 10.1105/tpc.104.022699.

[22] 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 Research 19(11): 1279-1290 DOI: 10.1038/cr.2009.108.

[23] Jeong JS, Kim YS, Baek KH, Jung H, Ha SH, Choi YD, Kim M, Reuzeau C, Kim JK. 2010. Root-specific expression of OsNAC10 improves drought tolerance and grain yield in rice under field drought conditions. Plant physiology 153(1): 185-197 DOI: 10.1104/pp.110.154773.

[24] Nguyen KH, Mostofa MG, Li W, Van Ha C. 2019. The soybean transcription factor GmNAC085 enhances drought tolerance in Arabidopsis. Environmental and Experimental Botany 151: 12-20 DOI: 10.1016/j.envexpbot.2018.03.017.

[25] Yang X, Kim MY, Ha J, Lee SH. 2019. Overexpression of the Soybean NAC gene GmNAC109 increases lateral root formation and abiotic stress tolerance in transgenic Arabidopsis plants. Frontiers in Plant Science 10: 1036 DOI: 10.3389/fpls.2019.01036.
Manuscript to be reviewed

Hao YJ, Wei W, Song QX, Chen HW, Zhang YQ, Wang F, Zou HF, Lei G, Tian AG, Zhang WK, Ma B, Zhang JS, Chen SY. 2011. Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. *Plant Journal* 68(2): 302-313 DOI: 10.1111/j.1365-313X.2011.04687.x.

Chen D, Chai S, McIntyre CL, Xue GP. 2018. Overexpression of a predominantly root-expressed NAC transcription factor in wheat roots enhances root length, biomass and drought tolerance. *Plant Cell Reports* 37(2): 225-237 DOI: 10.1007/s00299-017-2224-y.

Huang Y, Li T, Xu ZS, Wang F, Xiong AS. 2017. Six NAC transcription factors involved in response to TYLCV infection in resistant and susceptible tomato cultivars. *Plant Physiology and Biochemistry* 120: 61-74 DOI: 10.1016/j.plaphy.2017.09.020.

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA. 2005. A NAC Domain Protein Interacts with Tomato leaf curl virus Replication Accessory Protein and Enhances Viral Replication. *The Plant Cell* 17(1): 311-325 DOI: 10.1105/tpc.104.027235.

Wang X, Goregaoker SP, Culver JN. 2009. Interaction of the Tobacco mosaic virus replicase protein with a NAC domain transcription factor is associated with the suppression of systemic host defenses. *Journal of Virology* 83(19): 9720-9730 DOI:10.1128/jvi.00941-09.

Donze T, Qu F, Twigg P, Morris TJ. 2014. Turnip crinkle virus coat protein inhibits the basal immune response to virus invasion in Arabidopsis by binding to the NAC transcription factor TIP. *Virology* 449: 207-214 DOI: 10.1016/j.virol.2013.11.018.

Liu H, Ouyang B, Zhang J, Wang T, Li H, Zhang Y, Yu C, Ye Z. 2012. Differential modulation of photosynthesis, signaling, and transcriptional regulation between tolerant and sensitive tomato genotypes under cold stress. *Plant Physiology and Biochemistry* 68(2): 302-313 DOI:10.1016/j.plaphy.2011.03.016.

Yamaguchi-Shinozaki K. 2007. Functional analysis of a NAC-type transcription factor OsNAC6 involved in a biotic signaling, and transcriptional regulation between tolerant and sensitive tomato genotypes under cold stress. *Plant Journal* 51(4): 617-630 DOI: 10.1111/j.1365-313X.2007.03168.x.

Chen SY, Chen J, Dai XY, Zhang WH. 2011. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. *Planta* 234(2): 331-345 DOI: 10.1007/s00425-011-1403-2.

Li L, Zheng T, Zhuo X, Li S, Qiu L, Wang J, Cheng T, Zhang Q. 2019. Genome-wide identification, characterization and expression analysis of the HD-Zip gene family in the stem development of the woody plant *Prunus mume*. *PeerJ* 7: e7499 DOI: 10.7717/peerj.7499.

Hu W, Wei Y, Xia Z, Yan Y, Hou X, Zou M, Lu C, Wang W, Peng M. 2015. Genome-Wide Identification and Expression Analysis of the NAC Transcription Factor Family in Cassava. *PLoS One* 10(8): e0136993 DOI: 10.1371/journal.pone.0136993.

Zhang J, Yang E, He Q, Lin M, Zhou W, Pian R, Chen X. 2019. Genome-wide analysis of the WRKY gene family in drumstick (*Moringa oleifera* Lam.). *PeerJ* 7: e7063 DOI:10.7717/peerj.7063.

Li J, Gao K, Khan Wu, Yang X, Yang X, Zhao T, Chen Z, An X. 2019. Genome-wide analysis of the poplar NF-Y gene family and its expression in floral bud development of *Populus tomentosa*. *Trees* 34(1): 285-296 DOI: 10.1007/s00468-019-01917-3.

Kelley LA, Mezulis S, Yates CM, Wass MN and Sternberg MJ. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols* 10(6): 845-858 DOI: 10.1038/nprot.2015.053.

Fan Q, Song A, Jiang J, Zhang T, Sun H, Wang Y, Chen S, Chen F. 2016. CmWRKY1 Enhances the Dehydration Tolerance of Chrysanthemum through the Regulation of ABA-Associated Genes. *PLoS One* 11(3): e0150572 DOI: 10.1371/journal.pone.0150572.

Chen J, Shen CZ, Guo YP, Rao GY. 2018. Patterning the Asteraceae Capitulum: Duplications and Differential Expression of the Flower Symmetry CYC2-Like Genes. *Frontiers in Plant Science* 9:551 DOI: 10.3389/fpls.2018.00551.

Ewei Y, Esh H, Exia Z, Etie W, Eding Z, Eyan Y, Ewang W, Ehu W, Eli K. 2016. Genome-wide identification and expression analysis of the WRKY gene family in Cassava. *Frontiers in Plant Science* 7: 25 DOI: 10.3389/fpls.2016.00025.

R. B. Horsch, J. E. Fry, N. L. Hoffmann, D. Eichholtz, S. G. Rogers and R. T. Fraley (1985) A Simple and General Method for Transferring Genes into Plants. *Science, New Series* 227(4691): 1229-1231 DOI:10.1126/science.227.4691.1229.

Chen QF, Wang Q, Xiong LZ, Lou ZY. 2011. A structural view of the conserved domain of rice stress-responsive NAC1. *Protein Cell* 2(1): 55–63 DOI: 10.1007/s13238-011-1010-9

Heidi A.E, Addie NO, Karen S, Sine L, Leila LL. 2004. Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. *EMBO REPORTS* 5(3): 297-303 DOI:10.1038/sj.embor.7400939.
[46] Zhu YX, Yang L, Liu N, Yang J, Zhou XK, Xia YC, He Y, He YQ, Gong HG, Ma DF, Yin JL. 2019. Genome-wide identification, structure characterization, and expression pattern profiling of aquaporin gene family in cucumber. *BMC Plant Biology* 19(1): 345 DOI: 10.1186/s12870-019-1953-1.

[47] Masatoshi Y, Minoru K, Hiroom F, Taku D. 2008. VASCULAR-RELATED NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. *The Plant Journal* 55, 652–664 DOI: 10.1111/j.1365-313X.2008.03533.x.

[48] Sakuraba Y, Piao W, Lim JH, Han SH, Kim YS, An G, Paek NC. 2015. Rice ONAC106 inhibits leaf senescence and increases salt tolerance and tiller angle. *Plant Cell Physiology*. 56(12): 2325-2339 DOI: 10.1109/pcp/pcv144.

[49] Sun L, Huang L, Hong Y, Zhang H, Song F, Li D. 2015. Comprehensive analysis suggests overlapping expression of rice ONAC transcription factors in abiotic and biotic stress responses. *International Journal of Molecular Sciences* 16(2): 4306-4326 DOI:10.3390/ijms16024306.

[50] Zhong R, Demura T, Ye ZH. 2006. SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. *The Plant Cell* 18(11): 3158-3170. DOI:10.1105/tpc.106.047399.

[51] Mitsuda N, Iwase A, Yamamoto H, Yoshid M, Seki M, Shinozaki K, Ohme-Takagi M. 2007. NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. *Plant Cell*. 19(1): 270-280 DOI: 10.1105/tpc.106.047043.

[52] Zhong R, Ye ZH. 2015. The Arabidopsis NAC transcription factor NST2 functions together with SND1 and NST1 to regulate secondary wall biosynthesis in fibers of inflorescence stems. *Plant Signaling & Behavior* 10(2): e989746 DOI: 10.4161/15592324.2014.989746.

[53] Fendrych M, Van Hautegem T, Van Durme M, Olvera-Carrillo Y, Huysmans M, Karimi M, Lippens S, Guerin CJ, Krebs M, Schumacher K, Nowack MK. 2014. Programmed cell death controlled by ANAC033/SOMBRERO determines root cap organ size in Arabidopsis. *Current Biology* 24(9): 931-40 DOI: 10.1016/j.cub.2014.03.025.

[54] Bennett T, Van Den Toorn A, Sanchez-Perez GF, Campilho A, Willemsen V, Snel B, Scheres B. 2010. *VASCULAR-RELATED NAC-DOMAIN7* is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. *Current Biology* 20(6): 519-527 DOI: 10.10111/j.1365-313X.2009.03908.x.

[55] Zhou J, Zhong R, Ye ZH. 2014. Arabidopsis NAC Domain Proteins, VND1 to VND5, Are Transcriptional Regulators of Secondary Wall Biosynthesis in Vessels. *PLoS One*. 9(8): e105726 DOI: 10.1371/journal.pone.0105726.

[56] Endo H, Yamaguchi M, Tamura T. 2015. Multiple Classes of Transcription Factors Regulate the Expression of *VASCULARRELATED NAC-DOMAIN7*, a Master switch of xylem vessel differentiation. *Plant Physiology* 56 (2): 242-254 DOI: 10.1093/pcp/pcu134.

[57] Soyano T, Thitamadee S, Machida Y, Chua NH. 2008. *ASYMMETRIC LEAVES2-LIKE19/LATERAL ORGAN BOUNDARIES DOMAINS30* and ASL20/LBD18 regulate root cap element differentiation in Arabidopsis. *Plant Cell*. 20: 3359-3373 DOI: 10.1105/tpc.109.072272.

[58] Yamaguchi M, Ohtani M, Mitsuda N, Kubo M. 2010. VNDINTERACTING2, a NAC domain transcription factor, negatively regulates xylem vessel formation in Arabidopsis. *Plant Cell*. 22: 1249-1263 DOI: 10.1105/tpc.108.064048.

[59] Yang Y, Zhu K, Wu J, Liu L, Sun G, He Y, Chen F, Yu D. 2016. Identification and characterization of a novel NAC-like gene in chrysanthemum (*Dendranthema nankingense*). *Plant Cell Reports* 35(8): 1783-1798 DOI: 10.1007/s00299-016-1996-9.

[60] Liu QL, Xu KD, Zhao LJ, Pan YZ, Jiang BB, Zhang HQ, Liu GL. 2011. Overexpression of a novel transcription factor from Indonesia rice cultivar enhances drought and salt tolerance. *Emirates Journal of Food and Agriculture*. 11(1): 101-114 DOI: 10.1111/pbi.12011.

[61] Jeong JS, Kim YS, Redillas MC, Jung G, Jung H, Bang SW, Choi YD, Ha SH, Reuzeau C, Kim JK. 2009. The histone deacetylase OsHDAC1 epigenetically regulates the *OsNAC6* gene that controls seedling root growth in rice. *Plant Journal* 59(5): 764-776 DOI: 10.1111/j.1365-313X.2009.03908.x.

[62] Yokotani N, Ichikawa T, Kondou Y, Matsui M, Hirochika H, Iwabuchi M, Oda K. 2009. Tolerance to various environmental stresses conferred by the salt-responsive rice gene *ONAC065* in transgenic Arabidopsis. *Planta* 229(5): 1065-1075 DOI: 10.1007/s00425-009-0895-5.

[63] Hong Y, Zhang H, Huang L, Li D, Song F. 2016. Overexpression of a Stress-Responsive NAC Transcription Factor Gene ONAC022 Improves Drought and Salt Tolerance in Rice. *Frontiers in Plant Science* 7: 4 DOI:
Wang Z, Xia Y, Lin S, Wang Y, Guo B, Song X, Ding S, Zheng L, Feng R, Chen S, Bao Y, Sheng C, Zhang X, Wu J, Niu D, Jin H, Zhao H. 2018. Osa-miR164a targets OsNAC60 and negatively regulates rice immunity against the blast fungus Magnaporthe oryzae. *The Plant Journal* 95:584-597 DOI: 10.1111/tpj.13972.

Mao C, Lu S, Lv B, Zhang B, Shen J, He J, Luo L, Xi D, Chen X, Ming F. 2017. A Rice NAC Transcription Factor Promotes Leaf Senescence via ABA Biosynthesis. *Plant Physiology* 174(3): 1747-1763 DOI: 10.1104/pp.17.00542.

Shen J, Lv B, Luo L, He J, Mao C, Xi D, Ming F. 2017. The NAC-type transcription factor OsNAC2 regulates ABA-dependent genes and abiotic stress tolerance in rice. *Plant Physiology* 153(1): 185-197 DOI: 10.1104/pp.110.154773.

Chen X, Lu S, Wang Y, Zhang X, Lv B, Luo L, Xi D, Shen J, Ma H, Ming F. 2015. OsNAC2 encoding a NAC transcription factor that affects plant height through mediating the gibberellic acid pathway in rice. *The Plant Journal* 82(2): 302-314 DOI: 10.1111/tpj.12819.

Zheng X, Chen B, Lu G, Han B. 2009. Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. *Biochemical and Biophysical Research Communications* 379(4): 985-989 DOI: 10.1016/j.bbrc.2008.12.163.

Jeong JS, Kim YS, Baek KH, Jung H, Ha SH, Do Choi Y, Kim M, Reuzeau C, Kim JK. 2010. Root-specific expression of OsNAC10 improves drought tolerance and grain yield in rice under field drought conditions. *Plant Physiology* 153(1): 185-197 DOI: 10.1104/pp.110.154773.

Chen X, Wang Y, Lv B, Li J, Luo L, Lu S, Zhang X, Ma H, Ming F. 2014. The NAC family transcription factor OsNAP confers abiotic stress response through the ABA pathway. *Plant and Cell Physiology* 55(3): 604-619 DOI: 10.1093/pcp/pct204.

Bo W, Zhaohui Z, Huanhuan Z, Xia W, Binglin L, Lijia Y, Xiangyan H, Deshui Y, Xuelian Z, Chunguo W, Wenqin S, Chengbin C, Yong Z. 2019. Targeted Mutagenesis of NAC Transcription Factor Gene, OsNAC041, Leading to Salt Sensitivity in Rice. *Rice Science* 26(2): 98-108 DOI: 10.1016/j.rsci.2018.12.005.

Gao F, Xiong A, Peng R, Jin X, Xu J, Zhu B, Chen J, Yao Q. 2009. OsNAC52, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. *Plant Cell, Tissue and Organ Culture (PCTOC)* 100(3): 255-262 DOI: 10.1007/s11240-009-9640-9.

Sun L, Zhang H, Li D, Huang L, Hong Y, Ding XS, Nelson RS, Zhou X, Song F. 2013. Functions of rice NAC transcriptional factors, ONAC122 and ONAC131, in defense responses against Magnaporthe grisea. *Plant Molecular Biology* 81(1-2): 41-56 DOI: 10.1007/s11103-012-9981-3.

Dong F, Huang H, Liu J, Zhang M, Zhou Y, Dai S. 2018. Cloning and function analysis of CnNAC9 from Chrysanthemum nankingense. *Canadian Journal of Plant Science* 98(6): 1265-1279 DOI: 10.1139/cjps-2017-0349.

Liu Y, He M. 2019. The Chrysanthemum nankingense CnNAC9 gene positively regulates saline, alkaline, and drought stress in transgenic Chrysanthemum grandiflora. *Journal of the American Society for Horticultural Science* 144(4): 280-288 DOI: 10.21273/jashs04697-19.

Liu R, Wu M, Liu HL, Gao YM, Chen J, Yan HW, Xiang Y. 2020. Genome-wide identification and expression analysis of the NF-Y transcription factor family in *Populus*. *Physiologia Plantarum* 0:1-19 DOI: 10.1111/plp.13084.

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T. 2005. Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev.* 19(16): 1855-1860 DOI: 10.1101/gad.133105.

Ko JH, Yang SH, Park AH, Lerouxel O, Han KH. 2007. ANAC012, a member of the plant-specific NAC transcription factor family, negatively regulates xylary fiber development in *Arabidopsis thaliana*. *The Plant Journal* 50(6): 1035-1048 DOI: 10.1111/j.1365-313X.2007.03109.x.

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T. 2005. Transcription switches for protoxylem and metaxylem vessel formation. *Genes & Development*. 19(16): 1855-1860 DOI:10.1101/gad.133105.
Figure 1

Phylogenetic tree analysis of NAC transcription factors from *A. thaliana*, *O. sativa*, and *C. nankingense*.

The phylogenetic trees were derived using the neighbor-joining (NJ) method by 1000 bootstrap replications. The black circles on the outer ring represent Group1 and Group2. Each color inside represents a subfamily. The red dots represent *C. nankingense*. The blue dots represent rice. The unmarked represents *Arabidopsis thaliana*. Numbers on the branches indicate differences between different genes, and higher numbers indicate higher relatedness.
Figure 2

Predicted three-dimensional structure of NAC TFs from *C. nankingense*.

(A) Protein structure of different subfamilies in Group1. (B) Protein structure of different subfamilies in Group2. One gene from each branch of NAC phylogenetic trees is selected for prediction (Fig1). All genes of the OsNAC7 subfamily were predicted and compared with members of the OsNAC7 subfamily of *A. thaliana* NAC TFs.
Figure 3

Sequence logos of conserved domains in *C. nankingense*.

The overall height of the stack represents the level of sequence conservation. Heights of residues within a stack indicate the frequency of each residue at that site. E-value (Expect value) is an estimate of the expected number of motifs. Sites are the number of sites contributing to the construction of the motif. Width shows the motif, each motif describes a pattern of a fixed width.
Figure 4

Conserved motifs analyses of \textit{NAC} TFs in \textit{C. nankingense}.

Different colors represent different motifs. P-value is the probability value test. Motif Locations shows the location of motif sites.
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Figure 5

The morphology of *C. nankingense* at different growth stages.

(A) The seedling period 28 days after sowing. (B) The rapid growth period 44 days after sowing. (C) The stable growth and aging period 60 days after sowing.
Figure 6

Expression pattern analysis of 12 OsNAC7 subfamily genes in different tissues of *C. nankingense* at different growth stages.

The expression of root at 28d was used as a reference. The x-coordinate represents the different tissues. The y-coordinate represents the expression level. Different color columns indicate different growth periods. Each diagram represents a different gene. Different lower case letters indicate statistically significant differences at the P <0.05 level.
Figure 7

Expression pattern analysis of 12 OsNAC7 subfamily genes in \textit{C. nankingense} under different treatment times of osmotic stress induced by 20\% PEG6000.

The expression level of 0h was used as a reference. The x-coordinate represents the processing time. The y-coordinate represents the expression level. Each diagram represents a different gene. Different lower case letters indicate statistically significant differences at the P <0.05 level.
Figure 8

Expression pattern analysis of 12 OsNAC7 subfamily genes in C. nankingense under different treatment times of salt stress induced by 200mM NaCl.

The expression level of 0h was used as a reference. The x-coordinate represents the processing time. The y-coordinate represents the expression level. Each diagram represents a different gene. Different lower case letters indicate statistically significant differences at the P <0.05 level.
Figure 9

Subcellular localization of transgenic tobacco.

(A-D) Subcellular localization of super35S::GFP protein in root (A), leaf (B), root tip (C), and stem (D). (a-d) Enlarged image of GFP in root (a), leaf (b), root tip (c), and stem (d). (E, F) Subcellular localization of super3S::CHR00069684::GFP fusion protein in stem (E) and root (F). (e, f) Enlarged image of CHR00069684::GFP fusion protein in stem (e) and root (f). The red box area is the enlarged area.
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**A**

- root

**B**

- leaf

**C**

- root tip

**D**

- stem

**E**

- super35S::GFP

**F**

- stem

- root

**super35S::CHR00069684::GFP**
Figure 10

The growth status of tobacco seedlings treated with 200mmol/L NaCl stress, 4°C low temperature stress and 200µmol/L ABA treatment.

(A-C) Growth of super35S::CHR00069684::GFP transgenic tobacco seedlings under NaCl (A), ABA (B) and 4°C (C). (D-F) Growth of super35S::GFP transgenic tobacco seedlings under NaCl (D), ABA (E) and 4°C (F). All treatments were grown in stress medium for 30 days.
Figure 11

Growth and development of transgenic tobacco at 43 days.

(A) The growth and development status of super35S::CHR00069684::GFP transgenic tobacco. (B) The growth and development status of super35S::GFP transgenic tobacco. Both groups of tobacco grew in the same environment and underwent the same conservation management.
Figure 12

Sequence alignment and motif analysis of OsNAC7 subfamily genes.

Different color frames represent different motif regions. Different colored boxes show the positions of different motifs in genes.
**Table 1** (on next page)

RT-qPCR primers for the OsNAC7 subfamily genes of *C. nankingense*

Notes: actin7-F/R primer is an internal reference gene primer.
Table 1 RT-qPCR primers for the OsNAC7 subfamily genes of *C. lavandulifolium*

| Number | Gene name     | Primer sequence        | GC CONTENT (%) | TM(°C) |
|--------|---------------|------------------------|----------------|--------|
| 1      | actin7-F      | CGTTGCCCTGAGGTTCTTT    | 55             | 60.0   |
|        | actin7-R      | CTTGCTCATCCTGTACGA     | 55             | 60.0   |
| 2      | CHR00069684-F | CCAAGGCTGATGTCGCCA     | 60             | 59.9   |
| 3      | CHR00069684-R | AGGAAGTCAGTTGCATGGG    | 55             | 60     |
| 4      | CHR00042500-F | GACGCATCTGAGCCGAGA     | 55             | 60.3   |
| 5      | CHR00042500-R | CACGACATCCACCCACCA     | 55             | 60     |
| 6      | CHR00020838-F | AAAGGACGAAGCACCCTCAT   | 55             | 60     |
| 7      | CHR00020838-R | ACCACCCCTCTTCTGAGT     | 55             | 60.1   |
| 8      | CHR00009966-F | CACCTCCCACGGCTTGA     | 55             | 59.7   |
| 9      | CHR00009966-R | GTCAAGTGACACTACGGG     | 55             | 60     |
| 10     | CHR00048081-F | ACTGCTGAAGATGGTGGA     | 55             | 60.2   |
| 11     | CHR00048081-R | GCCTGCGAAAAGAACCAGC   | 55             | 60.7   |
| 12     | CHR00043953-F | ATCTACCGCGGAGAAGTC    | 55             | 60.4   |
| 13     | CHR00043953-R | AGACTGCTGACAGAGCTCA   | 55             | 59.7   |
| 14     | CHR00073261-F | CCGACGAGGAACGTCATC    | 55             | 60.1   |
| 15     | CHR00073261-R | GCCGTCGCCACTATCTACA   | 55             | 59     |
| 16     | CHR00032377-F | GGAAGGGACAGGCGAGT    | 55             | 60.2   |
| 17     | CHR00032377-R | CATGATGGAACGACTGGAG   | 60             | 59.9   |
| 18     | CHR00026420-F | GTTGCTGTCCTGTCATTCA   | 55             | 59.3   |
| 19     | CHR00026420-R | ATTTACCTCTGTGCTGGA   | 55             | 60     |
| 20     | CHR0003673-F  | ACGGGGAGAGATAAGGCCAG  | 55             | 60     |
| 21     | CHR0003673-R  | TCGGCTCTCTGTCCGTGTT   | 55             | 60     |
| 22     | CHR00027311-F | AAGAGTTGTCAGCGCTTGT   | 55             | 60.1   |
| 23     | CHR00027311-R | GTTTCCAAAATCCAGCTGCA  | 47.6           | 60     |
| 24     | CHR00043175-F | GCACCAACAGGCGCTACAA   | 55             | 59.4   |
| 25     | CHR00043175-R | CGTACGGAGCTCAGCGCTTTG | 60             | 60.2   |

Notes: actin7-F/R primer is an internal reference gene primer.
Table 2 (on next page)

Research status of NAC family of rice and chrysanthemum
Table 2: Research status of NAC family of rice and chrysanthemum

| Gene name | Subfamily | Function Annotation |
|-----------|-----------|---------------------|
| ONAC029/031 | NAC8 | Through alternative splicing, the thickening of fibrous cell walls during wood formation was regulated [78]. |
| OsNAC106 | NAC7 | A negatively regulated NAC transcription factor. Osna106 deletion mutants showed enhanced salt tolerance [48]. |
| OsSND2 | NAC7 | The AtSND2 homologous gene. The overexpressed transgenic plants showed leaf curl, increased cellulose content [79]. |
| OsNAC5/ONAC071/AK102475 | ATA | It can enhance the drought resistance, salt tolerance and cold tolerance of plants, and the phenotype is not affected [61]. |
| OsNAC6/ONAC048/AK068392 | ATA | The homologous gene of OsNAC5. The overexpression of OsNAC6 enhanced the tolerance of plants to high salt, drought and cold and its overexpression can also reduce the range of disease and improve the resistance of rice blast [34, 62-63]. |
| OsNAC60 | NAC8 | OsNAC60 overexpression enhanced the resistance of transgenic plants to rice blast [66]. |
| OsNAC2/ONAC004/AK061745 | NAM | Negatively regulated NAC TF. It can affect plant height and flowering time, reduce chlorophyll level, and accelerate leaf senescence [67-69]. |
| ONAC45 | NAM | Under drought and high salt stress, the survival rate of plants with overexpression of this gene was higher than that of wild-type [70]. |
| OsNAC10 | NAP | The root epidermis, cortex and column cells of overexpressed rice plants increased significantly, and the water absorption capacity was enhanced, which enhanced the drought and salt tolerance of the plants [70]. |
| OsNAC041 | ONAC001 | Induced expression under salt stress. The rice Osmac041 mutant had higher plant height and stronger salt stress sensitivity [73]. |
| OsNAC52 | ONAC001 | In A. thaliana overexpressed plants, transpiration of leaves decreased and drought resistance increased [74]. |
| ONAC063 | ONAC022 | Improved salt tolerance of transgenic A. thaliana. Its overexpression related to salt stress were up-regulated [64]. |
| OsNAC022 | ONAC022 | In overexpressed plants, the water loss rate and transpiration rate decreased, and drought and salt tolerance were enhanced [65]. |
| OsNAC122/OsNAC131 | TERN | These two are homologous genes. In rice plants silenced by VIGS method, the ability to infect rice blast was enhanced [75]. |
| OsNAP | NAP | Its overexpression could enhance the content of stress related genes, and improve the resistance of rice to different stress [72]. |
| AaNAC1 | ATA | Its overexpression can increase the content of artemisinin and improve resistance to drought and botrytis cinerea in A. annua [80]. |
| DINAC1 | ONAC022 | It can involve in regulating plant tolerance to drought and salinity, and can improve high temperature tolerance of tobacco [59]. |
| CINAC9 | SENU5 | It can enhance the tolerance of transgenic A thaliana to salt, alkali and drought stress [76]. Its transgenic plants chrysanthemum niU9717 can improve the resistance under salinization and drought stress [77]. |