

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

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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 (Duan *et al.*, 2019; Guerin *et al.*, 2019). *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.

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## 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 (Fang *et al.*, 2008). 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 (Nuruzzaman *et al.*, 2010). 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 (Souer *et al.*, 1996). 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 (Aida *et al.*, 1997; Aida, Ishida & Tasaka, 1999; Ishida *et al.*, 2000). 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 (Duan *et al.*, 2019; Ooka *et al.*, 2003).

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 (Liu *et al.*, 2018). Ooka *et al.* (2003) 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. Rushton *et al.* (2008) 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. So far, 151 NAC family members have been found in *O. sativa* (Nuruzzaman *et al.*, 2010), 117 in *A. thaliana* (Nuruzzaman *et al.*, 2010; Ooka *et al.*, 2003), 152 in tobacco (*N. tabacum*) (Rushton *et al.*, 2008), 205 in soybean (*Glycine max*) (Le *et al.*, 2011), 104 in tomato (*Solanum lycopersicum*) (Su *et al.*, 2015), 163 in poplar (*P. trichocarpa*) (Hu *et al.*, 2010), 79 in grape (*Vitis vinifera*) (Ju *et al.*, 2020), 168 in durum wheat (*Triticum turgidum*) (Saidi, Mergby & Brini, 2017), and 488 in bread wheat (*Triticum aestivum*) (Guerin *et al.*, 2019).

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 (Ju *et al.*, 2020; Gao *et al.*, 2018). 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 (Sun et al., 2019; Puranik et al., 2012; Xia et al., 2010). The *A. thaliana* NAC TFs ATAF1, ANAC016, ANAC019, ANAC055, and ANAC072 (Tran et al., 2004; Wu et al., 2009); the *O. sativa* NAC TFs OsNAC045, OsNAC10, and OsNAC022 (Jeong et al., 2010); the *G. max* NAC TFs GmNAC085, GmNAC109, GmNAC30, GmNAC81, GmNAC003, GmNAC004, GmNAC11, and GmNAC20 (Nguyen et al., 2018; Yang et al., 2019; Hao et al., 2011); and the *T. turgidum* NAC TF TaRNAC1 (Hao et al., 2011; Chen et al., 2018) 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 (Sun et al., 2019; Huang et al., 2017; Selth et al., 2005; Wang, Goregaoker & Culver, 2009; Donze et al., 2014). The *G. max* NAC TF GmNAC20 significantly improves cold resistance in plants (Puranik et al., 2012; Hao et al., 2011; Liu et al., 2012). The *O. sativa* NAC TFs OsNAC5 and OsNAC6 (the ATAF subfamily) improve drought resistance, salt tolerance, and freeze resistance of transgenic plants (Nakashima et al., 2007; Song et al., 2011) and the *V. vinifera* NAC TF VvNAC17 enhances the sensitivity of *A. thaliana* to ABA and improves salt tolerance, cold tolerance, and drought tolerance (Ju et al., 2020).

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 (Puranik et al., 2012). 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 (Li et al., 2019).

### 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 (Duan et al., 2019; Ooka et al., 2003; Hu et al., 2015). The rootless phylogenetic tree was used to study the phylogenetic relationships of the CnNAC TFs (Zhang et al., 2019). 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 (Li et al., 2019). 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>) (Kelley et al., 2015).

### 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 (Fan et al., 2016). 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 (Wei et al., 2016), with *actin7* from *C. nankingense* as the internal reference gene (Chen et al., 2018). The relative expression of genes was calculated by the  $2^{-\Delta C_t}$  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. Data S1). The vector uses super35S as the promoter. According to the open reading frame (ORF) of the *CHR00069684* gene (Data S2) and the polyclonal sites of the super1300 vector, the endonucleases *Pst*I and *Kpn*I (NEB, Beijing, China) were selected to construct the binary expression vector according to the double enzyme method. The recombinant plasmid was transformed into *Escherichia coli* competent cells (DH5 $\alpha$ ), 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, Amsterdam, the Netherlands) 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 (Horsch et al., 1985), screened by 30 mg/L hygromycin, and validated by PCR identification of the target gene. *CHR00069684*::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

**Table 1** RT-qPCR primers for the OsNAC7 subfamily genes of *C. nankingense*.

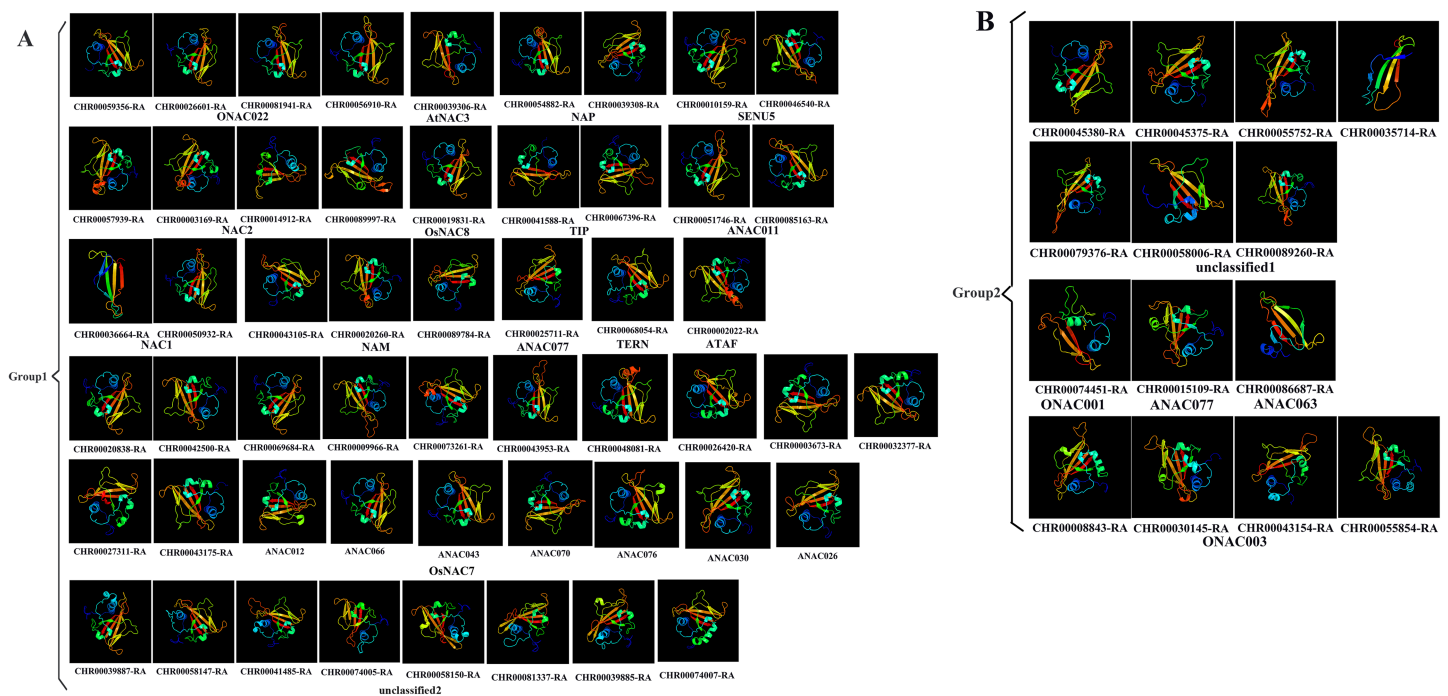
| Number | Gene name     | Primer sequence       | GC CONTENT(%) | TM (°C) |
|--------|---------------|-----------------------|---------------|---------|
| 1      | actin7-F      | CGTTGCCCTGAGGTTCTCTT  | 55            | 60.0    |
|        | actin7-R      | CCTTGCTCATCCTGTCAGCA  | 55            | 60.0    |
| 2      | CHR00069684-F | CCAAGGCTCGATAGTCCCAC  | 60            | 59.9    |
|        | CHR00069684-R | AGGAAGTCAGTTGCATGGGG  | 55            | 60      |
| 3      | CHR00042500-F | GAGCACCTCATGGCCAGAAA  | 55            | 60.3    |
|        | CHR00042500-R | CACGACATAACCACCAACCA  | 55            | 60      |
| 4      | CHR00020838-F | AAAGGACGAGCACCTCATGG  | 55            | 60      |
|        | CHR00020838-R | ACCCACCCCTCTTCTTGAGT  | 55            | 60.1    |
| 5      | CHR00009966-F | CACCTCCCACGGCTTGATAA  | 55            | 59.7    |
|        | CHR00009966-R | GTCAGCAAGTCACTCACGGA  | 55            | 60      |
| 6      | CHR00048081-F | ACTCCTGAAGATGGGTGGGT  | 55            | 60.2    |
|        | CHR00048081-R | GCCTGCGAAAAAGAACCAGC  | 55            | 60.7    |
| 7      | CHR00043953-F | ATCTACCCGCCGAAGAGTCA  | 55            | 60.4    |
|        | CHR00043953-R | GAGCTGCTTCAGAGGTGTCA  | 55            | 59.7    |
| 8      | CHR00073261-F | CCGACGAGGAACCTGCTTCAT | 55            | 60.1    |
|        | CHR00073261-R | GGCGTCGACCCTATCTTACA  | 55            | 59      |
| 9      | CHR00032377-F | GGAAGGGACAAGGCAGTGTT  | 55            | 60.2    |
|        | CHR00032377-R | CATGATGGGACGACTGGAGG  | 60            | 59.9    |
| 10     | CHR00026420-F | GTGGTGTGTCGTGCATTCAA  | 55            | 59.3    |
|        | CHR00026420-R | ATTTACCTCTGTGGCTGGGC  | 55            | 60      |
| 11     | CHR00003673-F | ACGGGGAGAGATAAGGCAGT  | 55            | 60      |
|        | CHR00003673-R | TCGGTCTTCTGTCCGTTTGG  | 55            | 60      |
| 12     | CHR00027311-F | AAGAGTTGTGCAGGCTTGGT  | 55            | 60.1    |
|        | CHR00027311-R | GCTTTCCAAAATCCAGCTGCA | 47.6          | 60      |
| 13     | CHR00043175-F | GCACCAACAGGGCTACAAAG  | 55            | 59.4    |
|        | CHR00043175-R | CGTTAGGAGCTCGACCCTTG  | 60            | 60.2    |

**Note:**

actin7-F/R primer is an internal reference gene primer.

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, Data S3). According to the existing *A. thaliana* and *O. sativa* NAC gene family classification system (Duan et al., 2019; Ooka et al., 2003; Hu et al., 2015) 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.





**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 (Fig. 1). All genes of the OsNAC7 subfamily were predicted and compared with members of the OsNAC7 subfamily of *A. thaliana* NAC TFs.

Full-size DOI: 10.7717/peerj.11505/fig-2

60 CnNAC family members were constructed using the Phyre2 server (Fig. 2). c3ulxA (Chen et al., 2011) and d1ut7a (Ernst et al., 2004) 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 (Zhu et al., 2019).

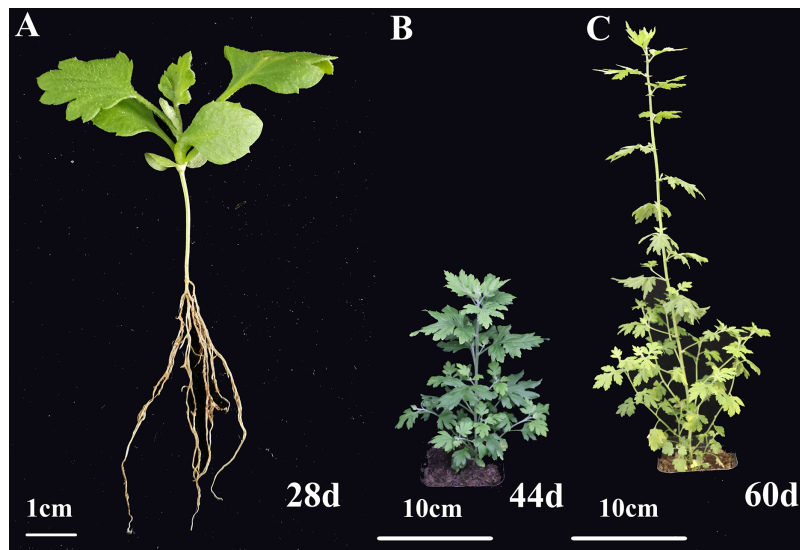
### Analysis of the CnNAC TF conserved motifs

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 (Fig. S2), five genes (*CHR00045488-RA*, *CHR00007510-RA*, *CHR00036356-RA*, *CHR00079376-RA*, and *CHR00089260-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







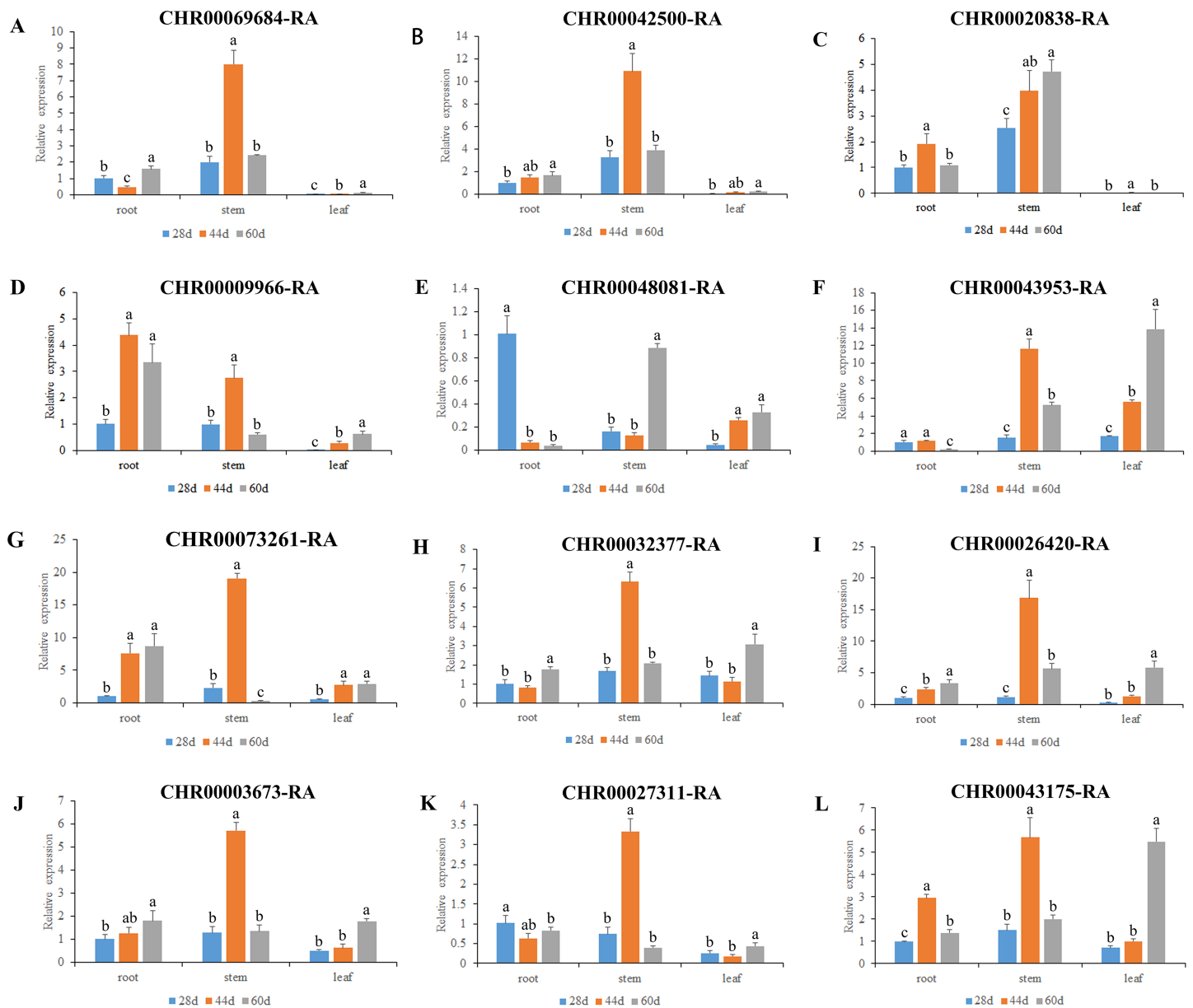


**Figure 5** The morphology of *C. nankingense* at different growth stages. (A) The seedling period 28 days after sowing. (B) The rapid growth period 44 days after sowing. (C) The stable growth and aging period 60 days after sowing. [Full-size](#) DOI: 10.7717/peerj.11505/fig-5

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 (Table S1), 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 the 21 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.



**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. (A-L) The letters represent the different genes in the OsNAC7 subfamily. [Full-size !\[\]\(5f471a71b78d7676bc356df190b88ab4\_img.jpg\) DOI: 10.7717/peerj.11505/fig-6](https://doi.org/10.7717/peerj.11505/fig-6)

The physicochemical properties of the CnNAC TFs were analyzed (Table S2). 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

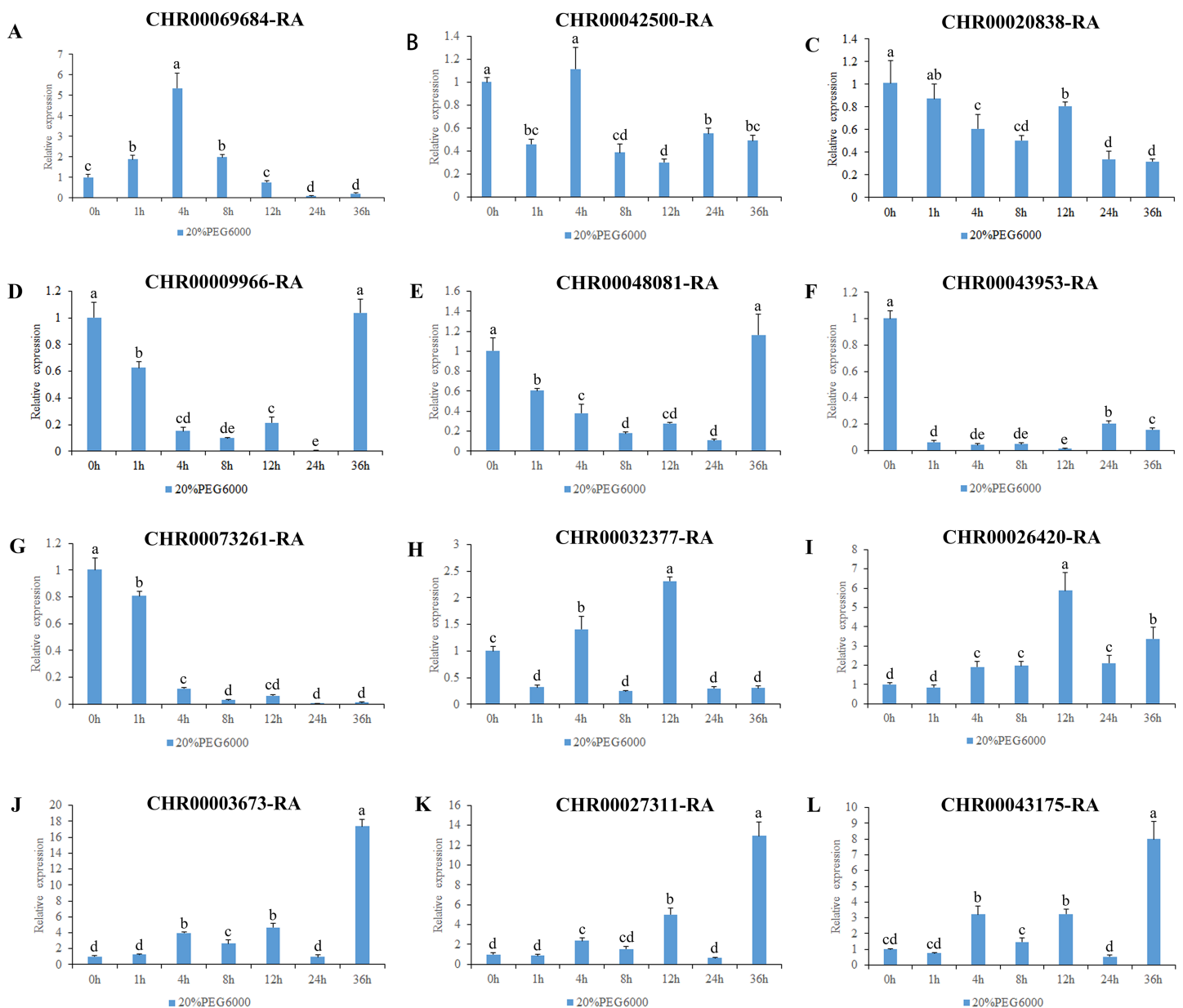
The OsNAC7 subfamily included 12 genes in *A. thaliana*, including *SND1*, *NST1*, *URP7*, *BRN1/2*, and *VND1/2/3/4/5/6/7*, the functions of which are all related to regulation of the formation of secondary cell walls in plant stems, roots, and anthers. Here, we analyzed the expression levels of the OsNAC7 subfamily genes in the roots, stems, and leaves of *C. nankingense* during different growth stages (Figs. 5 and 6, Table S3).

As a results, 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 *CHR00043175*. *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 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 *CHR00073261-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 (Yamaguchi et al., 2008). However, among the *O. sativa* OsNAC7 subfamily genes, *ONAC106* inhibits leaf senescence and also increases salt damage and tillering angle (Fang et al., 2008; Sakuraba et al., 2015); the expression levels of *ONAC052*, *ONAC056*, and *ONAC084* are downregulated under high salt and drought conditions (Sun et al., 2015). Here, we analyzed the expression levels of the *C. nankingense* OsNAC7 subfamily genes under osmotic and salt stresses (Fig. 7, Fig. 8, Table S3).

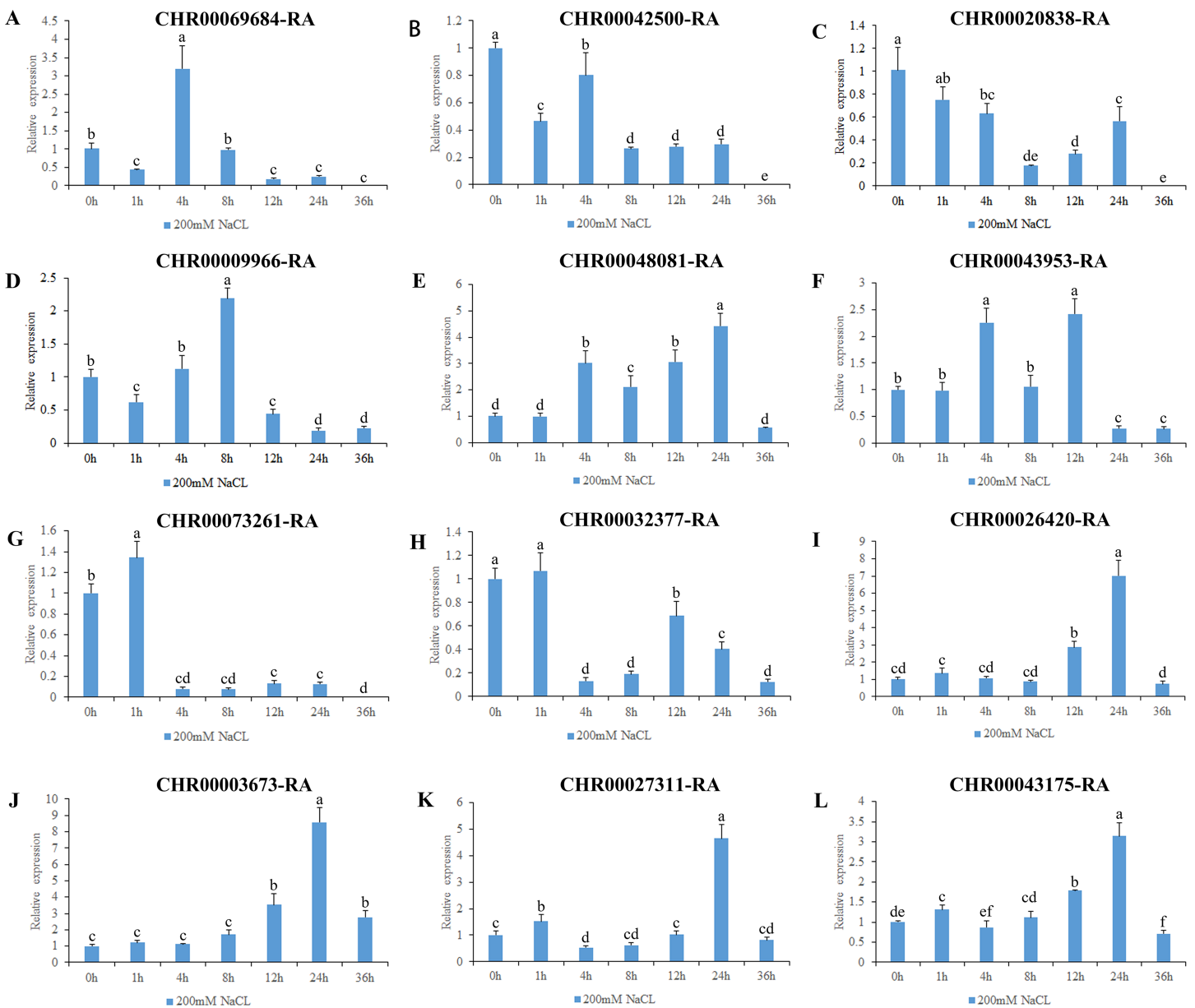
The results of gene expression under osmotic stress (Fig. 7) revealed that all of the 12 genes in the OsNAC7 subfamily were regulated by osmotic stress, but their expression patterns were not consistent. Among them, the expression levels of *CHR00003673-RA*, *CHR00027311-RA*, and *CHR00043175-RA* continued to increase after treatment, indicating that these three genes may play a key role 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*



**Figure 7** Expression pattern analysis of 12 OsNAC7 subfamily genes in *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. (A–L) The letters represent the different genes in the OsNAC7 subfamily. [Full-size !\[\]\(1679558f37f6db0dd8360a2a7e913e90\_img.jpg\) DOI: 10.7717/peerj.11505/fig-7](https://doi.org/10.7717/peerj.11505/fig-7)

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 *CHR00069684-RA*, *CHR00009966-RA*, *CHR00048081-RA*, *CHR00043953-RA*,



**Figure 8** Expression pattern analysis of 12 OsNAC7 subfamily genes in *C. nankingense* under different treatment times of salt stress induced by 200 mM 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. (A–L) The letters represent the different genes in the OsNAC7 subfamily. [Full-size !\[\]\(fd7fe780e8fd8eece60268c87d0c3e04\_img.jpg\) DOI: 10.7717/peerj.11505/fig-8](https://doi.org/10.7717/peerj.11505/fig-8)

*CHR00026420-RA*, *CHR00003673-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. *CHR00042500-RA*, *CHR00020838-RA*, *CHR00073261-RA*, and *CHR00032377-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, *CHR00069684* that was closely related to the NST protein was used to construct the super35S::*CHR00069684*::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::*CHR00069684*::GFP transformed tobacco plants (Fig. 9). No fluorescence signal was detected in the super35S::*CHR00069684*::GFP transformed tobacco leaves (Fig. S3), whereas there were strong GFP signals in the cell membrane of their roots and stems (Figs. 9E and 9F). The reason why the 35S promoter-guided *CHR00069684* expression showed tissue specificity need to be further studied in the future.

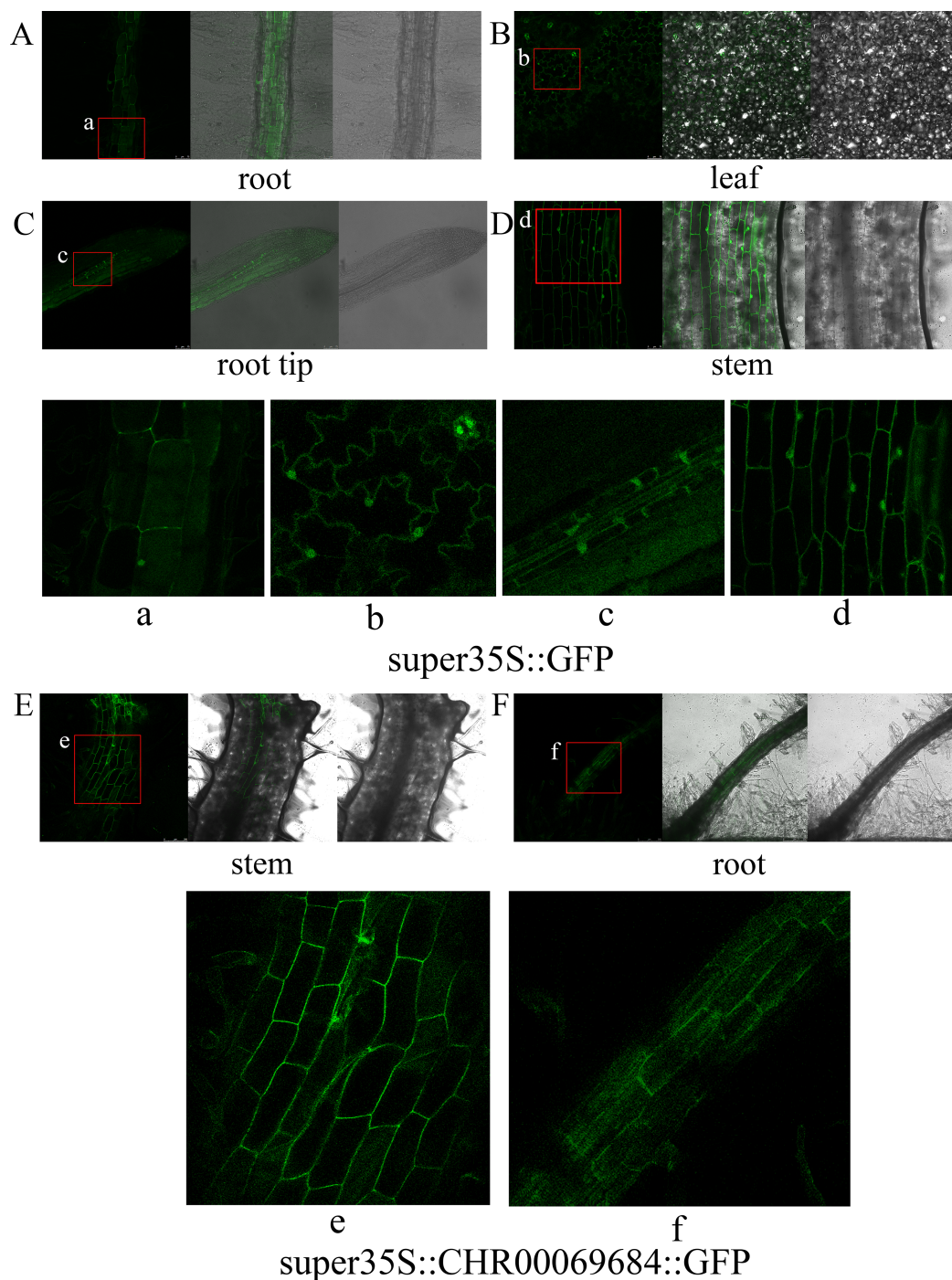
The super35S::*CHR00069684*::GFP transformed and control tobacco plants were treated with 200 mM NaCl, 200 mM ABA, or a low temperature of 4 °C (Fig. 10). The results showed that the transgenic tobacco plants were more resistant to salt and low temperature and more sensitive to ABA than the control plants. 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

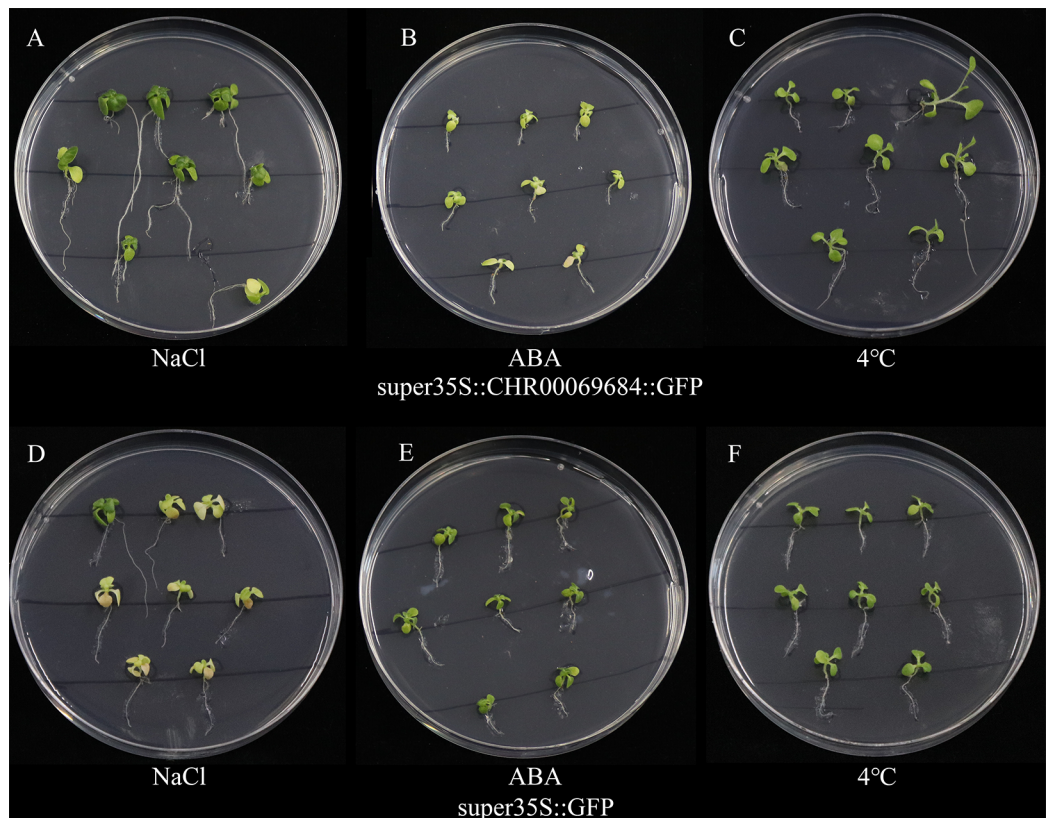
According to the phylogenetic tree (Fig. 1) and motif analyses (Fig. 4) of CnNAC TFs, we found that the distinct NAC TF subfamilies each containing the most closely related members exhibited highly similar motif components. This included members in the OsNAC7 subfamily (Fig. 12), all of which possess motifs 1–6. The members clustering with ANAC012, ANAC066, ANAC043 (Zhong, Demura & Ye, 2006; Mitsuda et al., 2007; Zhong & Ye, 2015), ANAC033, ANAC070, and ANAC015 (Fendrych et al., 2014; Bennett et al., 2010), 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* (Zhou, Zhong & Ye, 2014; Endo, Yamaguchi & Tamura, 2015; Soyano et al., 2008; Yamaguchi et al., 2010), 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 (Zhong, Demura & Ye, 2006; Mitsuda et al., 2007; Zhong & Ye, 2015; Fendrych et al., 2014; Bennett et al., 2010; Zhou, Zhong & Ye, 2014; Endo, Yamaguchi & Tamura, 2015; Soyano et al., 2008; Yamaguchi et al., 2010).

The NAC TFs are involved in various stages of plant growth and development and the responses to various stressors (Yang et al., 2019). 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 (Yang et al., 2019). Compared to studies of *Chrysanthemum* and *O. sativa*, the ATAF subfamily member *AaNAC1* (*NAC1* in *A. annua*, KX082975.1) (Yang et al., 2016), which was similar to *CHR00002022-RA*, *CHR00045168-RA*, and *DgNAC1* (*NAC1* in *D. grandiform*) (Liu et al., 2011), has the same function as the *O. sativa* ATAF subfamily genes *OsNAC5* and *OsNAC6* (Nakashima et al., 2007; Jeong et al., 2013; Rachmat et al., 2014; Chung et al., 2009). All of these genes improve drought resistance, salt



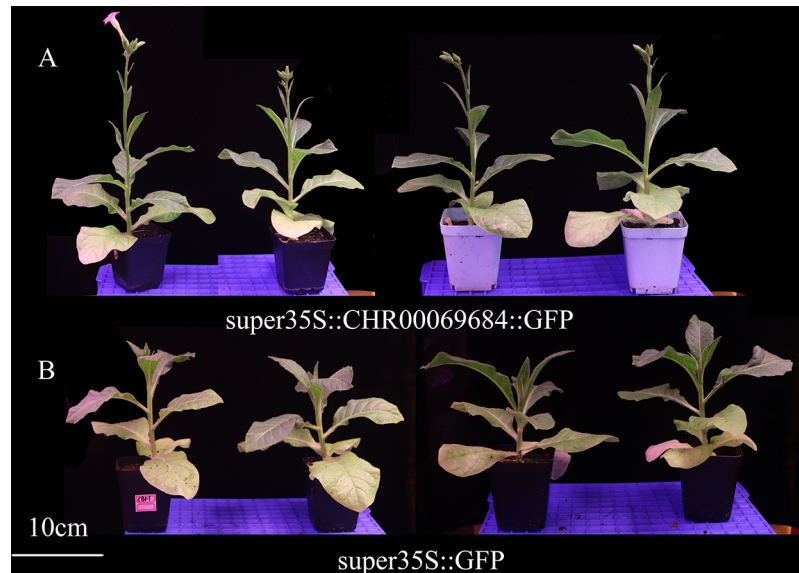


**Figure 9** Subcellular localization of transgenic tobacco. (A–D) Subcellular localization of super35S::GFP protein in root (A), leaf (B), root tip (C), and stem (D). (a–d) Enlarged image of GFP in root (a), leaf (b), root tip (c), and stem (d). (E, F) Subcellular localization of super35S::CHR00069684::GFP 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. [Full-size !\[\]\(fcc3264021d438d9732560e78099f674\_img.jpg\) DOI: 10.7717/peerj.11505/fig-9](https://doi.org/10.7717/peerj.11505/fig-9)

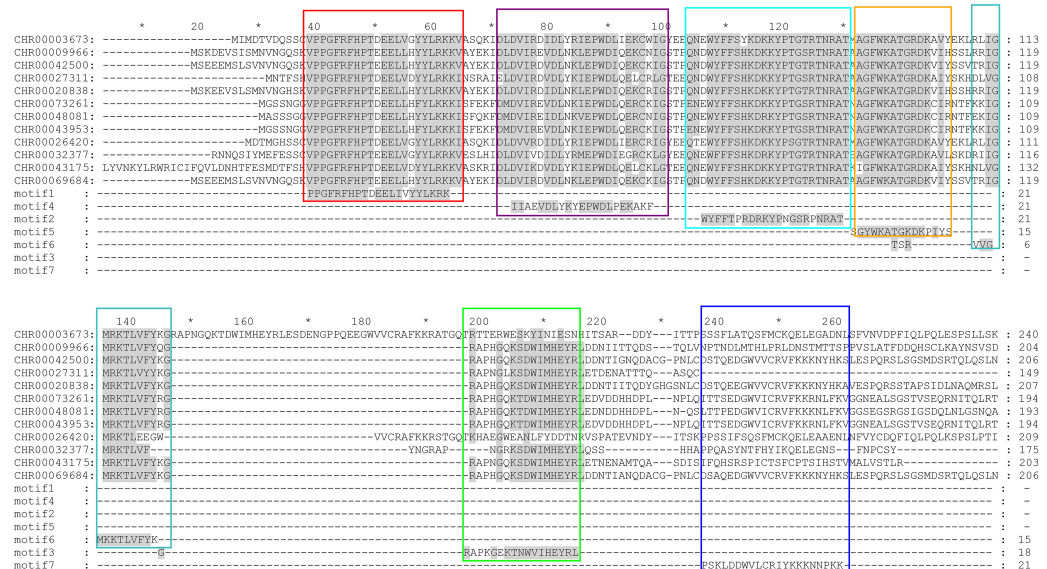


**Figure 10** The growth status of tobacco seedlings treated with 200 mmol/L NaCl stress, 4 °C low temperature stress and 200  $\mu$ mol/L ABA treatment. (A–C) Growth of super35S::CHR00069684::GFP transgenic tobacco seedlings under NaCl (A), ABA (B) and 4 °C (C). (D–F) Growth of super35S::GFP transgenic tobacco seedlings under NaCl (D), ABA (E) and 4 °C (F). All treatments were grown in stress medium for 30 days. Full-size [DOI: 10.7717/peerj.11505/fig-10](https://doi.org/10.7717/peerj.11505/fig-10)

tolerance, and low temperature tolerance of plants. The ONAC022 subfamily member *DlNAC1* (*NAC1* in *D. nankingense*) (Yang et al., 2016), which was similar to *CHR00056910-RA* and *OsNAC063* (Yang et al., 2016; Yokotani et al., 2009; Hong et al., 2016), 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 (Wang et al., 2018). The *NAM* subfamily genes improve tolerance to drought and high salt stress, which affect plant height and flowering time, and accelerate leaf senescence (Mao et al., 2017; Shen et al., 2017; Chen et al., 2015; Zheng et al., 2009). 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 (Jeong et al., 2010; Chen et al., 2014). The *ONAC001* subfamily genes are induced by salt and drought stresses (Bo et al., 2019; Gao et al., 2009), the *TERN* subfamily genes may be associated with



**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. Full-size [DOI: 10.7717/peerj.11505/fig-11](https://doi.org/10.7717/peerj.11505/fig-11)



**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. Full-size [DOI: 10.7717/peerj.11505/fig-12](https://doi.org/10.7717/peerj.11505/fig-12)

disease resistance (Sun et al., 2013), and the SENU5 subfamily genes enhance plant tolerance to salt, alkali, and drought stress (Dong et al., 2018; Liu & He, 2019).

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

**Table 2** Research status of NAC family of rice and chrysanthemum.

| Gene name                   | Subfamily | Function annotation  |
|-----------------------------|-----------|--|
| ONAC029/031                 | OsNAC7    | Through alternative splicing, the thickening of fibrous cell walls during wood formation was regulates ( <i>Liu et al., 2020</i> )   |
| OsNAC106                    | OsNAC7    | A negatively regulated NAC transcription factor. <i>Osnac106</i> deletion mutants showed enhanced salt tolerance ( <i>Sakuraba et al., 2015</i> )  |
| OsSND2                      | OsNAC7    | The <i>AtSND2</i> homologous gene. The overexpressed transgenic plants showed leaf curl, increased cellulose content ( <i>Kubo et al., 2005</i> )  |
| OsNAC5/ONAC071/<br>AK102475 | ATAF      | It can enhance the drought resistance, salt tolerance and cold tolerance of plants, and the phenotype is not affected ( <i>Jeong et al., 2013</i> )  |
| OsNAC6/ONAC048/<br>AK068392 | ATAF      | The homologous gene of <i>OsNAC5</i> . The overexpression of <i>OsNAC6</i> 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 ( <i>Song et al., 2011; Rachmat et al., 2014; Chung et al., 2009</i> ) |
| OsNAC60                     | NAC1      | <i>OsNAC60</i> overexpression enhanced the resistance of transgenic plants to rice blast ( <i>Wang et al., 2018</i> )  |
| OsNAC2/ONAC004/<br>AK061745 | NAM       | Negatively regulated NAC TF. It can affect plant height and flowering time, reduce chlorophyll level, and accelerate leaf senescence ( <i>Mao et al., 2017; Shen et al., 2017; Chen et al., 2015</i> )   |
| 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 ( <i>Zheng et al., 2009</i> )   |
| 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 ( <i>Zheng et al., 2009</i> )  |
| OsNAC041                    | ONAC001   | Induced expression under salt stress. The rice <i>Osnac041</i> mutant had higher plant height and stronger salt stress sensitivity ( <i>Bo et al., 2019</i> )  |
| OsNAC52                     | ONAC001   | In <i>A. thaliana</i> overexpressed plants, transpiration of leaves decreased and drought resistance increased ( <i>Gao et al., 2009</i> )   |
| ONAC063                     | ONAC022   | Improved salt tolerance of transgenic <i>A. thaliana</i> . Its overexpression related to salt stress were up-regulated ( <i>Yokotani et al., 2009</i> )  |
| OsNAC022                    | ONAC022   | In overexpressed plants, the water loss rate and transpiration rate decreased, and drought and salt tolerance were enhanced ( <i>Hong et al., 2016</i> )   |
| OsNAC122/<br>OsNAC131       | TERN      | These two are homologous genes. In rice plants silenced by VIGS method, the ability to infect rice blast was enhanced ( <i>Sun et al., 2013</i> )  |
| OsNAP                       | NAP       | Its overexpression could enhance the content of stress related genes, and improve the resistance of rice to different stress ( <i>Chen et al., 2014</i> )  |
| AaNAC1                      | ATAF      | Its overexpression can increase the content of artemisinin and improve resistance to drought and botrytis cinerea in <i>A. annua</i> ( <i>Ko et al., 2007</i> )  |
| DINAC1                      | ONAC022   | It can involve in regulating plant tolerance to drought and salinity, and can improve high temperature tolerance of tobacco ( <i>Yang et al., 2016</i> )   |
| CINAC9                      | SENU5     | It can enhance the tolerance of transgenic <i>A thaliana</i> to salt, alkali and drought stress ( <i>Dong et al., 2018</i> ). Its transgenic plants chrysanthemum niU9717 can improve the resistance under salinization and drought stress ( <i>Liu &amp; He, 2019</i> )   |

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 ([Table S1](#)), 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 (Table S1); *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 (Liu et al., 2020).

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* (Zhong, Demura & Ye, 2006; Mitsuda et al., 2007; Zhong & Ye, 2015; Kubo et al., 2005; Ko et al., 2007). 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* (Fendrych et al., 2014; Bennett et al., 2010). 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* (Zhou, Zhong & Ye, 2014; Endo, Yamaguchi & Tamura, 2015; Soyano et al., 2008; Yamaguchi et al., 2010; Kubo et al., 2005). 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 (Fang et al., 2008; Sakuraba et al., 2015). 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.

## ADDITIONAL INFORMATION AND DECLARATIONS

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### Competing Interests

The authors declare that they have no competing interests.

### Author Contributions

- Hai Wang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

- Tong Li performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Wei Li performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Wang Wang performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Huien Zhao conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

### Data Availability

The following information was supplied regarding data availability:

Sequence data are available at the Chrysanthemum Genome Database: <http://www.amwayabrc.com/index.html>. The raw experimental data are available as Supplemental Files.

### Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.11505#supplemental-information>.

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