Genome-Wide Analysis of NAC Proteins and Characterization of the ATAF Member RcNAC091 in Rose (*Rosa Chinensis*)

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Abstract

The NAC (NAM, ATAF, and CUC) transcription factors (TFs), as plant-specific transcription regulators, play a critical role in various developmental processes and stress responses. However, knowledge of the NAC TFs in rose (*Rosa chinensis*), one of the most important horticultural crops, is limited. In this study, 116 NAC genes were identified from the rose genome and classified into 16 subfamilies. The *RcNACs* were unevenly distributed on the chromosomes. Analysis of the stress-related *RcNACs* based on the transcriptome data showed differences in the expression patterns in different organs and flower developmental stages and during drought and salt stress in rose leaves and roots. Analysis of the *cis*-regulatory elements (CREs) in the promoter region revealed that stress-related *RcNACs* possessed three sets of CREs, corresponding to plant growth and development, plant hormonal response, and abiotic and biotic stresses. Reverse transcription-quantitative real-time PCR (RT-qPCR) analysis of 11 representative *RcNACs* revealed their diverse transcript expression in leaves and roots under abscisic acid (ABA), polyethylene glycol (PEG), and sodium chloride (NaCl) treatments. Furthermore, *RcNAC091* silencing verified its role in positively regulating the dehydration stress response. Overall, this study provides helpful insight into stress-related *RcNACs* and paves the way for stress tolerance via breeding in rose.

Key Message

The study identified 116 NAC genes in rose. Stress-related *RcNACs* showed different expression patterns. Silencing *RcNAC091*, a member of the subfamily ATAF, conferred decreased tolerance to dehydration stress.

Introduction

The NAC (NAM, ATAF, and CUC) transcription factors (TFs) are plant-specific regulatory proteins that have been extensively studied in the past few years. The NAC obtained the name from the first three members identified, the NAM of *Petunia hybrida*, the ATAF1/2, and the CUC2 of *Arabidopsis thaliana*. In general, NAC protein sequences contain two parts; the conserved NAC domain at the N-terminus, consisting of about 150 amino acids, and the highly differentiated transcriptional regulatory region at the C-terminus, regulating transcriptional and downstream gene expression (Mohanta et al. 2020). The conserved NAC domain is divided into five subdomains, named A, B, C, D, and E. Subdomains A, C, and D are highly conserved and correlates with DNA binding. In contrast, subdomains B and E are variable and endow NAC with different functions (Puranik et al. 2012).

In the past few years, NAC TFs have been discovered in various plant species such as *Arabidopsis* (Ooka et al. 2003), rice (*Oryza sativa*) (Fang et al. 2008), apple (*Malus domestica*) (Su et al. 2013), soybean (*Glycine max*) (Le et al. 2011), wheat (*Triticum aestivum*) (Guérin et al. 2019), corn (*Zea mays*) (Shiriga et al. 2014), and banana (*Musa acuminata*) (Cenci et al. 2014). Many studies have shown that NAC TFs regulate plant growth and development and abiotic stress (salt, drought, heat, mechanical damage) and defense (viruses) responses (Nakashima et al. 2012). Overexpression of *RESPONSIVE TO DESICCATION*

26 (*RD26*) in *Arabidopsis* increased the sensitivity of plants to abscisic acid (ABA), indicating the role of *RD26* in an ABA-dependent stress signaling pathway (Fujita et al. 2004). In rice, the *STRESS-RESPONSIVE NAC 1* (*SNAC1*) overexpression significantly promoted drought resistance and salt tolerance by enhancing ABA sensitivity (Hu et al. 2006). Furthermore, cold, salt, drought, and ABA had induced *OsNAC6* in rice, and overexpression of *OsNAC6* increased the tolerance of plants to multiple stresses (Ohnishi et al. 2005). Meanwhile, low temperature and water stress had induced *SsNAC23* in sugarcane (*Saccharum officinarum*), which is similar to rice *OsNAC6* (Fábio et al. 2005). The *MbNAC29* gene from *Malus baccata* improved the ability to remove reactive oxygen species (ROS) and subsequently enhanced cold and salt tolerance (Han et al. 2020).

Rose (*Rosa chinensis*), an ornamental perennial shrubs native to China, is widely used outdoors and indoors to improve the aesthetic value of landscapes and has important economic and nutritional values worldwide. The rose flowers also contains various nutrients and fragrances with health benefits. However, roses often encounter adverse environmental conditions that affect their growth characteristics and flower quality. TFs, such as NAC, WRKY, MYB etc have close relationship corresponding to these biological processes. For rose NACs, researchers have identified several key NAC genes, like *RhNAC100* (Pei et al. 2013), *RhNAC2* (Dai et al. 2012), *RhNAC3* (Jiang et al. 2014), and *RhNAC31* (Ding et al. 2019), associated with flower development or water deficit stress in rose. Besides, many rose TF families like AP2/ERF (Li et al. 2021) and WRKY (Liu et al. 2019) have been comprehensively studied. However, the comprehensive analysis of NAC family in rose has not been conducted. The genome-wide exploration and expression analysis of the stress-related NAC gene family will provide technical support for stress-resistant rose breeding and cultivation.

In this study, 116 *RcNAC* genes of rose were systematically identified, and the phylogeny, physicochemical properties, gene structure, conserved motifs, and the chromosomal location of these *RcNAC* genes were investigated. The collinearity relationship of NAC genes among five Rosaceae plant species (rose, apple, strawberry (*Fragaria vesca*), mei (*Prunus mume*), and almond (*P. dulcis*) was also analyzed. The *RcNACs* were categorized into 16 subfamilies, and 44 stress-related ones were selected for further analysis. The *cis*-regulatory element of these NAC gene members was analyzed. The expression patterns of these genes in different organs and under different situations, such as floral development, drought, and salt stress, were explored. In particular, 11 representative *RcNACs* were further selected and analyzed by quantitative reverse-transcription PCR (RT-qPCR). Additionally, the drought-induced *RcNAC091* was silenced in rose leaves to investigate its role in regulating tolerance to dehydration stress. Our results strengthen the understanding of *RcNACs* and provide candidate genes that help adapt to adverse conditions.

Materials And Methods

Identification of RcNACs

The complete genome sequence and gff3 files of rose were obtained from GDR (Genome Database for Rosaceae, <https://www.rosaceae.org>) (Jung et al. 2014). The corresponding data of the NAC family in Arabidopsis were obtained from PlantTFDB (Plant Transcription Factor Database, <http://planttfdb.gao-lab.org/>) (Jin et al. 2017). The Blast Wrapper in TBtools (Chen et al. 2020) was used to retrieve the possible rose NAC sequences based on the reference sequence of the gene family. The rose NAC sequences were compared with the NCBI database (<https://www.ncbi.nlm.nih.gov/>) to verify the existence of the NAM domain. Then, each RcNAC was checked through the Batch CD-Search tool in the NCBI database to confirm further the results (Aron et al. 2011). Finally, the Pfam database (PF02365) (<https://pfam.xfam.org/>) (Finn et al. 2014) detected the conserved NAM domains in the obtained sequences. The characteristics of RcNACs were analyzed using ExPASy (http://web.expasy.org/compute_pi/) (Panu et al. 2012) to predict the protein length, molecular weight (MW), and isoelectric point (pI).

Phylogenetic, gene structure, and motif analysis

NAC protein sequences of different species (Arabidopsis, rice, apple, and kiwifruit (*Actinidia chinensis*) were obtained from Ensembl Plants (<https://plants.ensembl.org/index.html>) (Kevin et al. 2020), and ten conserved motifs of these proteins were identified using the MEME suite (<http://meme-suite.org/tools/meme>) (Bailey et al. 2009). The exon-intron organization of the RcNAC genes was analyzed using the TBtools. The neighbor-joining method constructed a phylogenetic tree with 1000 bootstrap values using MEGA7 (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2016). Furthermore, the Batch CD-Search tool on the NCBI was used to analyze the domains in all NAC protein sequences.

RcNAC *cis*-regulatory element (CRE) analysis

The upstream 1.5 kb sequences of the selected 44 RcNACs were extracted by TBtools and submitted to the PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al. 2020) database to predict the CREs. According to their function, these CREs were categorized into three groups, corresponding to abiotic and biotic stress, phytohormone responsiveness, and plant growth and development. The graph, showing the type and number of *cis*-elements, was displayed by TBtools and modified by Adobe Illustrator CS5.

RcNAC chromosomal location and collinearity analysis

The chromosomal location of RcNACs was mapped according to the rose genome information obtained from the GDR and visualized by TBtools (Chen et al. 2020). The genome

data and gene annotation files of apples, strawberries, mei, and almond were downloaded from the JGI (Joint Genome Institute, <https://genome.jgi.doe.gov/portal/>) database to investigate the syntenic relationship among the NAC genes of rose and other plant species. Further, MCScanX (Wang et al. 2012) was used to identify the putative homologous chromosomal regions. To evaluate the evolution of protein-coding regions of the duplicated NAC genes, non-synonymous substitution (Ka), synonymous substitution (Ks), and Ka/Ks were calculated by TBtools.

Heatmap of RcNACs

The transcript levels of *RcNACs* in different rose organs (accession number GSE54486) (Yan et al. 2014), during the floral transition (accession number PRJCA000258) (Guo et al. 2017), and in the leaves and roots under drought (accession number PRJNA663119) (Li et al. 2021) or salt stress (accession number SRP128235) (Tian et al. 2018) were retrieved from the transcriptome data in GEO (Gene Expression Omnibus) database, GSA (Genome Sequence Archive) database and SRA (Sequence Read Archive) database. The expression profiles of *RcNACs* were determined using the FPKM (fragments per kilobase million) values. Expression levels of the *RcNACs* was illustrated based on the $\log_2$ normalized FPKM values using TBtools and visualized by ggplot2 package in R software.

RT-qPCR analysis

Further, 11 representative *RcNACs* were selected for RT-qPCR to validate the expression pattern under abiotic stress. Rose (*Rosa hybrida* 'sanmatha') tissue culture seedlings with eight compound leaves were transferred to MS medium or MS medium plus 50 μ M ABA, 10% PEG 6000, and 200 mM NaCl and allowed to grow for five days. The total RNA was extracted from rose leaves and roots using RNAprep Pure Plant Kit (TIANGEN, Beijing, China). Approximately 1 μ g of total RNA was used for cDNA synthesis using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China) to remove the genomic DNA. The SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China) was used to perform the RT-qPCR assay on a StepONE Plus system (Applied Biosystems, USA) in a 20 μ L reaction volume. The reaction mixture consisted of 10 μ L 2 $\times$ ChamQ Universal SYBR qPCR Master mix, 0.4 μ L of each primer, 2 μ L cDNA, and 7.2 μ L ddH₂O. The reaction program was as follows: 95 °C for 15 s, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and then 95 °C for 15 s. Ubiquitin (*RcUBI2*) gene was used as an internal control for normalizing all mRNA expression levels; three biological replicates were maintained per treatment, and the gene expression levels were analyzed by $2^{-\Delta\Delta CT}$ method (Livak et al. 2001). The primers used are listed in Table S1.

Silencing *RcNAC091* by VIGS

Virus-induced gene silencing (VIGS) approach was used to silence *RcNAC091* (genbank accession number MG878302) in rose leaves. A fragment of approximately 430 bp at the 3' end of *RcNAC091* was cloned into pTRV2 to obtain pTRV2-*RcNAC091*. The *Agrobacterium* resuspension cultures containing pTRV1 and pTRV2-*RcNAC091* (TRV-*RcNAC091*), as well as pTRV1 and pTRV2 (TRV, control) were mixed in equal proportions, respectively. The middle portion of the rose leaves with a punch (1 cm) of leaf disc was used. VIGS was carried out as described by Jiang et al. (2014). The TRV and TRV-*RcNAC091* samples were dehydrated for 0, 4, and 8 h and then rehydrated for 3 h under controlled conditions (23–25 °C, 40%–50% relative humidity, and 100 $\mu\text{mol}/\text{m}^2/\text{s}$ light intensity). The disc area was monitored at the indicated time points using Image J (<https://imagej.nih.gov/ij>). Ion leakage and chlorophyll content were measured after 3 h of rehydration. Cell death in TRV and TRV-*RcNAC091* discs after 3 h of rehydration was measured by trypan blue staining according to the method by Dai et al. (2018). The ROS in the leaves was stained using 3,3'-diaminobenzidine (DAB) and nitroretrozolium blue chloride (NBT) as described by Dai et al. (2018). The experiments were repeated at least three times, and each replicate contained five plants.

Results

Identification of NAC domain proteins in rose

The HMMER (Prakash et al. 2017) and the Pfam NAC domain (PF02365) were employed to identify the rose NAC family using the protein sequences obtained from the Arabidopsis NAC gene sequence. A total of 123 protein sequences were initially obtained by comparing and searching the rose genome database (<https://www.rosaceae.org>). A two-way comparison between TBtools (Chen et al. 2020) and the NCBI database was used to detect the NAC domain after removing the redundant sequences. A total of 116 *RcNACs* were identified and named from *RcNAC001* to *RcNAC116* according to their chromosome location. These 116 *RcNACs* were analyzed for their gene location, gene length, molecular weight (Mw), isoelectric point (pI), and subcellular localization (Table S2). The protein length of *RcNACs* ranged from 70 (*RcNAC076*) to 714 amino acids (aa, AA) (*RcNAC102*), with an average of 336 aa (Table S1). The pI ranged from 4.43 (*RcNAC107*) to 9.78 (*RcNAC98*), whereas the Mw ranged from 7.87 kDa (*RcNAC076*) to 79.99 kDa (*RcNAC102*). Subcellular localization analysis predicted nuclear localization for 49% of *RcNACs* and extracellular localization for 51%. These results suggest the crucial roles of *RcNACs* in the evolution of the rose genome.

Phylogenetic analysis of NAC proteins

A phylogenetic tree was constructed using 736 NAC protein sequences from five species, including rose (116), kiwifruit (156), Arabidopsis (105), apple (228), and *Oryza barthii* (131). Based on the classification of NAC family proteins in *Arabidopsis* by Ooka et al. (2003), the NACs were divided into 16 subfamilies, including ANAC011, NAM, NAC1, OSNAC7, OSNAC8, TIP, NAC2, ONAC22, ANAC001, SEUN5, ONAC003,

TERN, ANAC063, ATAF, NAP, and Unclassified. The ANAC001 subfamily was the largest with 23 members (~20% of the total RcNACs) in rose, followed by the NAM subfamily containing 11 RcNACs; OSNAC8 and ANAC063 subfamilies were the minor categories with only one member each. However, no plant species had all the subfamilies, and no OSNAC8 member was found in *Arabidopsis* (Table S3). Furthermore, the percentage of each subfamily was different among the plant species. For example, monocots like *O. barthii* (19%) showed a more significant percentage of the TIP subfamily than the dicots likeroses(4%), kiwifruit(6%), *Arabidopsis*(7%), and apple(4%) (Fig. 1). These findings indicate potential differences in levels of gene duplication or loss between monocots and dicots that might have occurred during evolution.

Gene structure and motif analysis of RcNACs

Further, the conserved motifs, exon-intron structure, and phylogeny were analyzed to understand better the structural diversity in rose NAC proteins (Fig. 2). A total of ten conserved motifs, mainly localized in the N-terminus, were identified for NAC proteins (Fig. S1). Motifs 1–7 were highly conserved and distributed in most rose NACs (~60%), while motifs 8–10 were distributed in a few subfamilies (ANAC001 and Unclassified). Most of the conserved motifs of rose NACs of the subfamilies, such as ANAC011, TIP, and NAC2, were similar, indicating a relatively close evolutionary relationship. However, the conserved motifs of RcNACs in subfamilies ANAC001, ONAC003, and ANAC063 were different, indicating a rather distant evolutionary relationship.

Meanwhile, the exon-intron organization showed that the intron number was diverse among *RcNACs*, ranging from one to nine (Fig. 2). All 116 *RcNACs* consisted of one or more exons. The maximum number of exons (seven) was found in *RcNAC102* of the TIP subfamily, and the average number of exons in the 116 *RcNACs* was four. Furthermore, the genes in the same subfamily had similar gene structures, with similar intron and exon arrangement and numbers, indicating highly conserved biological functions of these genes.

Chromosome localization and collinearity analysis of *RcNACs*

Chromosome mapping revealed that the *RcNACs* were unevenly distributed within the rose genome composed of seven chromosomes. Among them, chromosome 1 contained the maximum *RcNACs* (31, 26.7%), followed by chromosome 5 (21, 18.1%); the least number of *RcNACs* was on chromosome 4 (6, 5.2%; Fig. 3A).

Furthermore, a collinearity analysis was performed using NACs from rose and four other *Rosaceae* plants, including apple, strawberry, mei, and almond. As shown in Fig. 3B, the collinearity between the NACs in rose and apple (142) was significantly higher than the other three species (mei, 79; strawberry, 66; and

almond, 40). The *RcNACs* on chromosomes 5 and 7 had significantly more collinear gene pairs than the other chromosomes. Less than ten collinear gene pairs were identified between rose and almond, which may be related to the phylogenetic relationship between species (Fig. 3B). These results suggest that few *RcNACs* were possibly produced by gene and segmental duplication events, which probably played a major driving force for *RcNAC* gene evolution.

Relative transcript levels of stress-related *RcNACs* in different transcriptome datasets

NAC proteins play various roles in plant growth, development, and stress response. In this study, 44 stress-related *RcNACs* belonging to eight subfamilies (ATAF, NAC2, TIP, SNUE5, NAM, NAP, ONAC022, ONAC003) were selected for further analysis. Sequence homology of all these genes with already reported stress-related NACs indicated their close association with biotic or abiotic stresses (Shao et al. 2015). The expression levels of 44 *RcNACs* at the three stages of the rose flower transition (vegetative meristem, pre-floral meristem, floral meristem) and in the six organs (prickle, stamen, leaf, pistil and ovary, stem, and root) based on public RNA-seq data are shown in Fig. 4A. The transcript levels of *RcNACs* of the same or different subfamilies were highly variable among the organs or the transition stages, suggesting their multiple functions in growth and development. Interestingly, the expression levels of four *NAC2s* (*RcNAC008*, *RcNAC086*, *RcNAC009*, *RcNAC088*), three *ATAFs* (*RcNAC034*, *RcNAC061*, *RcNAC091*), two *TIPs* (*RcNAC031*, *RcNAC102*), and one *SNEU5* (*RcNAC056*) in the various organs and flower transition processes were relatively high, demonstrating their roles in rose growth and development.

To further gain insight into the putative functions of these 44 *RcNACs* in response to abiotic stress, their expression patterns under drought stress in leaves (no drought, NDL; mild drought stress, MDL; severe drought stress, SDL) and roots (NDR, MDR, SDR) and salt stress in leaves (TL0, TL2, TL4, TL7, TL9) and roots (TR0, TR2, TR4) were evaluated (Fig. 4B). Overall, the expression levels of the *RcNACs* from eight subfamilies were different. These genes showed dramatic changes under various degrees of drought or salt stress in rose leaves and roots, further confirming their roles in response to abiotic stress. Meanwhile, 11 *RcNACs* of the same clade showed induced expression under drought or salt stress in rose leaves and roots. Specifically, three *ATAFs* (*RcNAC034*, *RcNAC069*, *RcNAC091*) showed higher expression levels under drought or salt stress. These expression profiles demonstrated functional convergence and differences of the *RcNACs*, which probably occurred during evolution.

Analysis of the *cis*-regulatory element (CRE) in stress-related *RcNACs* promoter

To analyze the regulation of stress-related *RcNAC* gene expression, the CREs of its promoter, a region of approximately 1.5 kb upstream of the initial transcript site, were tested. Li et al. (2021) classified the

promoters into three categories based on functions, corresponding to plant growth and development (Box4, G-box, CAT-box, AE-box, GT1-motif, GATA-motif, I-box), plant hormone response (ABRE, CGTCA-motif, ERE, TGACG-motif, GARE-motif, TGA-element), and abiotic and biotic stress (TC-rich repeats, MBS, LTR, WUN-motif, W box, WRE3, STRE, MYB, MYC, ARE, Myb-binding site, TCT-motif). These CREs were randomly dispersed among the different *RcNAC*s promoters. *RcNAC034* had the most significant number of CREs (57), followed by *RcNAC091* (52), while *RcNAC081* had the least number of CREs (6). Among the different types of CREs, abiotic and biotic stress-related CREs, including MYB, MYC, Box4, G box, and ABRE, were significantly more than the other CREs (Fig. 5); 31 out of 44 *RcNAC*s contained ABRE elements, suggesting *RcNAC*s roles in regulating the response to abiotic stresses through ABA-dependent pathway.

Expression of selected *RcNAC*s

According to the transcriptome expression and CREs analysis, 11 representative genes (*RcNAC009*, *RcNAC031*, *RcNAC034*, *RcNAC056*, *RcNAC059*, *RcNAC062*, *RcNAC069*, *RcNAC086*, *RcNAC088*, *RcNAC091*, *RcNAC102*) belonging to six subfamilies of the same clade (Fig. 4B) were selected for further analysis using RT-qPCR under ABA, PEG, and NaCl treatments (abiotic stress) in rose leaves and roots. As shown in Fig. 6, members of the different subfamilies showed differential expression patterns. *RcNAC034* and *RcNAC069* showed upregulated expression levels when treated with ABA in leaves and roots, while *RcNAC059* and *RcNAC031* did not. PEG upregulated *RcNAC056* in leaves, and ABA upregulated in roots. The expression levels of both *RcNAC031* and *RcNAC102* showed no change under ABA, PEG, and NaCl treatments. However, *RcNAC091* showed upregulated expression levels when treated with ABA, PEG, and NaCl. These expression profiles of *RcNAC*s from different subfamilies indicated conserved or diverse roles in rose leaves and roots in response to external cues.

Silencing *RcNAC091* decreases tolerance to dehydration stress

RcNAC091 showed an strongly induced expression pattern in response to abiotic stress (Fig. 6). Therefore, VIGS was used to silence the selected *RcNAC091* for functional analysis. As shown in Fig.7, about 40% of *RcNAC091* was silenced in rose (Fig. 7A). No obvious difference in phenotype was observed between TRV and TRV-*RcNAC091* under dehydration 0 h. However, when dehydrated for 4 h and 8 h, discs of TRV-*RcNAC091* showed more wilting symptoms than TRV controls (Fig. 7B). The discs areas of TRV-*RcNAC091* under dehydration for 4 h and 8 h and rehydration for 3h were 510 mm², 395 mm², 556 mm², respectively, which were significantly lower than TRV controls at the indicated time points (574 mm², 459 mm², 615 mm²) (Fig. 7C). Further, TRV-*RcNAC091* samples after rehydration for 3 h showed lower chlorophyll content (Fig. 7D), with a higher ion leakage than TRV controls (Fig. 7E). Furthermore, TB, DAB, and NBT staining used to investigate the cell death rate and ROS accumulated levels revealed deeper

staining for TRV-*RcNAC091* than TRV controls under rehydration for 3 h (Fig. 7F), indicating severely damaged cell membranes. These results suggest that *RcNAC091* positively regulates dehydration stress response in rose plants.

Discussion

TFs regulate various important cellular processes in plants, especially when subjected to biotic and abiotic stresses. NAC family is one of the largest TFs that play critical roles in plant growth, development, and stress response (Wang et al. 2013). Genome-wide analysis of NAC has been carried out in *Arabidopsis* (Ooka et al. 2003), rice (Nuruzzaman et al. 2010), pearl millet (Dudhate et al. 2021), soybean (So and Lee, 2019), and various other species. Nevertheless, studies on the rose NACs are limited. The present study identified 116 NAC genes containing NAM domains from the rose genome. The number of NAC TFs varies between 30 and 228 among different plant species. Researchers have identified 33 TFs in *Physcomitrella patens* (Bo et al. 2014), 60 in rape (*Brassica napus*) (Wang et al. 2015), 105 in *Arabidopsis* (Ooka et al. 2003), and 228 in apple (Su et al. 2013). The genome sizes of these species are also different (rape, 630 Mb; *Arabidopsis*, 116 Mb; apple, 682 Mb); however, the number of NAC family members was not directly related to the size of the genome. These data suggest that NAC proteins numbers have extensively expanded with plant evolution.

Furthermore, the study found differences in the number of NAC subfamily members among rose and other species, indicating the evolutionary differences among plant species. The NAC proteins were categorized into 16 subfamilies; however, NAC genes of all plant species cannot be divided into 16 subfamilies (Fig. 1, Table S2). In *A. chinensis*, no members of the OSNAC8 subfamily were identified, indicating a loss of exons/introns during the evolutionary process.

Further analysis showed that although the pI, Mw, and length of RcNAC proteins were different, the gene composition was similar, indicating a relatively conserved gene structure. About 83% of the *RcNACs* contained more than six motifs, while the subfamily ONAC003 contained only four motifs (Fig. 2). Meanwhile, the subfamily ANAC001 contained motif 9 and 10 that others lacked. Besides, genes from the same subfamilies also had identical or similar exon-intron compositions. The differences in motifs distribution pattern between RcNAC subfamilies indicate that many genes have evolved. In addition, the composition and distribution of conserved motifs supported the phylogenetic relationship and classification of RcNACs. Previous research has shown that the conserved motifs play an important role (Singh et al. 2002). Most *RcNACs* contained motifs 1–7, while few contained motifs 8–10, indicating related functions of these NACs. Although the number of introns ranged from 1 to 8, about 74% of the 116 *RcNACs* had only two introns or even less, consistent with tobacco (Li et al. 2018), pepper (Diao et al. 2018), and *Coffea canephora* (Dong et al. 2019). Differences in intron number among three subfamilies suggest the occurrence of alternative splicing, which might help plants to adapt to environmental changes, especially abiotic stress (Laloum et al. 2018).

RcNACs were found unevenly distributed on seven rose chromosomes, and the numbers in each chromosome ranged from 7 to 31 (Fig. 3). The number of *RcNACs* on the shortest chromosome was not the least, and the number on the longest chromosome was not the highest, suggesting no positive correlation between the length of the chromosome and the number of *RcNACs*. Few genes such as *RcNAC003*, *RcNAC004*, *RcNAC005*, *RcNAC006*, and *RcNAC007* were located as clusters on chromosomes, which suggest that the genes may work together through protein isoforms. The collinearity analysis identified only seven homologous gene pairs between rose and almond, while more were identified between rose and strawberry, followed by plum. These observations suggest a close association of rose with apple and strawberry.

Based on the phylogenetic relationship (Fig. 2) and previous reports (Shao et al. 2015), 44 stress-related *RcNACs* from eight subfamilies were selected for further analysis. Investigation of 1.5 kb region upstream of stress-related *RcNACs* promoter revealed that these genes possessed different stress response CREs, such as ABRE, MBS, MYB, and MYC, which may play important roles in response to various stresses. In particular, three *RcNACs* (*RcNAC034*, *RcNAC069*, *RcNAC091*) of the ATAF subfamily contained more than 10 ABRE elements, proving their outstanding role in responding to environmental stimuli through the ABA-dependent pathway (Yoshihiro et al. 2003). Furthermore, 38 out of 44, and 37 out of 44, contained MYB and MYC CREs in their promoter regions, respectively. These two CREs are the binding sites of the MYB and MYC TFs, which can resist biotic and abiotic stresses and regulate plant growth and development (Dar et al. 2017).

Gene expression profiles are often correlated with their biological function. Analysis of the stress-related *RcNACs* based on transcriptome data revealed that not all *RcNACs* have a clear response to major abiotic stresses, such as drought and salt stress. The expression profiles of different *RcNACs* of the same subfamily were different (Fig. 4). The subfamily ATAF is the most studied NAC TFs in response to biotic and abiotic stresses in *Arabidopsis* (Hu et al. 2006) and *Populus euphratica* (Lu et al. 2018). Three rose ATAF genes (*RcNAC034*, *RcNAC069*, *RcNAC091*) were found induced under drought and salt (Fig. 5), consistent with RT-qPCR results (Fig. 6). In addition, the response of the other seven subfamily genes to drought and/or salt stress was not noticeable. Interestingly, these genes, induced under drought and/or salt, showed higher expression levels than other genes during flower differentiation stages or different organs. The analysis revealed that *RcNACs* (*RcNAC009*, *RcNAC056*, *RcNAC088*, *RcNAC091*) were highly expressed during different developmental stages and in various tissues, suggesting their role in growth and development in rose. To further verify the accuracy of the transcriptome data, 11 genes with high expression levels in response to drought and salt stress were analyzed by RT-qPCR. The RT-PCR results were slightly different from the transcriptome data, probably due to plant material size and processing method differences. The detailed role of NAC proteins in response to abiotic stresses remains to be elucidated.

Members of the ATAF subfamily have been shown to regulate plant responses during abiotic and biotic stresses. A few members from the ATAF subfamily have been well-characterized. For example, rice OsNAC6 (Ohnishi et al. 2005), *Haloxylon ammodendron* HaNAC1 (Gong et al. 2020), and *Miscanthus*

lutariopariis MINAC5 (Yang et al. 2005) enhanced drought tolerance. In contrast, certain other members of the ATAF subfamily negatively regulate the response to abiotic stress. For instance, *Arabidopsis* ATAF1 negatively regulated drought stress (Lu et al. 2007), while soybean GmNAC2 acted as a negative regulator during abiotic stress and participated in the ROS signaling pathway (Jin et al. 2013). In this study, RcNAC091, a member of subfamily ATAF, demonstrated a positive role in response to drought stress. Silencing of *RcNAC091* in rose leaves resulted in a decreased tolerance to dehydration, with higher ion leakage and lower chlorophyll content and disc area (Fig. 7). These findings indicate that the role of ATAF members in mediating stress tolerance is diverse and sophisticated among the various plant species.

Conclusions

In the present study, 116 *RcNAC* genes were identified from the rose genome and analyzed for their evolutionary relationship, gene structure, and conserved motifs. We have analyzed of the stress-related RcNACs CREs and their expression profiles. Further, the expression profiles of 11 representative *RcNACs* under ABA, PEG, and NaCl treatments were analyzed by RT-qPCR, and drought-induced *RcNAC091*, a member of the ATAF subfamily, was identified. Finally, the silencing of *RcNAC091* showed decreased of tolerance to dehydration. The present study improves the understanding of the role of NACs and provides a candidate stress-resistant NAC gene in rose.

Declarations

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Authors' contributions

JX conceived and designed the experiments; GL, SL, and FL carried out the experiments; GL and LS conducted the data analysis. JX and GL wrote the manuscript; ZJ and LQ revised the manuscript and contributed the plant material. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

Percentage of NAC proteins of each subfamily in five plant species. The proteins of rose, Arabidopsis, rice, apple, and kiwifruit were divided into 16 subfamilies, including ANAC011, NAM, NAC1, OSNAC7, OSNAC8, TIP, NAC2, ONAC22, ANAC001, SEUN5, ONAC003, TERN, ANAC063, ATAF, NAP, Unclassified. The percentage represents the proportion of members in each subfamily in the five plant species; different subfamilies are displayed in different colors.

Figure 2

Phylogenetic relationship, motif distribution, and gene structure of RcNACs. A phylogenetic tree was constructed using rose NAC proteins following the neighbor-joining method with 1000 replicates using MEGA 7.0. Colored boxes represent conserved amino acid motifs (1–10) in the RcNACs, and the black line indicates the non-conserved sequences. Exons and introns are indicated by TBtools. The green,

yellow, and pink boxes represent untranslated region (UTR), coding sequence (CDS), and NAM, respectively.

Figure 3

Chromosome location and collinearity analysis of RcNACs. A. RcNACs on the seven chromosomes of rose. The black line between the two gene names indicates tandemly repeated genes. The scale shows the gene position on. The black line indicates the position of the gene on each chromosome. B.

obtained during flower transition (Yu et al. 2019) and from the different organs (Dubois et al. 2011) were used. VM, TM, and FM indicate vegetative meristem, pre-floral meristem, and floral meristem, respectively. B. Relative expression profiles of RcNACs under drought and salt stress. The abscissas ND, MD, and SD represent control, moderate drought, and severe drought, respectively; T0, T2, T4, T7, and T9 represent salt treatment for 0, 2, 4, 7, and 9 days, respectively; L and R represent roots and leaves, respectively. The drought and salt stress treatments selected for this analysis were defined as described in Li et al. (2021) and Tian et al. (2018), respectively. The color scale on the right indicates expression values; blue indicates low transcript abundance, and red indicates high transcript abundance.

Figure 5

Analysis of cis-regulatory elements in the RcNAC promoter. The cis-regulatory elements (CREs) in the promoter region (1.5 kb upstream) of RcNACs were predicted by PlantCARE. Heat maps represent different CREs in different RcNACs promoter regions. Different colors indicate three different types of CREs (plant growth and development, plant hormone response, and abiotic and biotic stress). Box 4, Light-inducible promoter induced by ABA and gibberellin (GA) also; G-box, light-responsive; CAT-box, meristem expression; AE-box, light response; GT1-motif, light response; GATA-motif, light-responsive; I-box, light-responsive; ABRE, ABA- responsive; CGTCA-motif, Methyl jasmonat-responsivs; ERE, Light-inducible promoter also induced by ABA, GA; TGACG-motif, Methyl jasmonate-responsive; GARE-motif, GA-responsive; TGA-element, auxin-responsive; TC-rich repeats, defense and stress-responsive; MBS, MYB

binding site involved in drought-inducibility; LTR, low-temperature responsive; WUN-motif, wound responsive; W box, WRKY binding site; WRE3-element, high-temperature responsive; STRE-element, stress-responsive element; MYB, dehydration and ABA-responsive; MYC, dehydration and ABA-responsive; ARE, anaerobic induction; Myb-binding, MYB binding site; TCT-motif, part of a light-responsive element.

Figure 6

RT-qPCR analysis of selected stress-related RcNACs. RT-qPCR evaluated 11 stress-related RcNACs. Eight-week-old leaves and roots were treated with 50 μ M ABA, 10% PEG 6000, and 200 mM NaCl. Relative expression levels of different RcNACs was normalized to the RcUBI2 gene. Values are means $\pm$ SD of at least three biological experiments.

Figure 7

RcNAC091 gene silencing in rose leaves. A. Expression levels of RcNAC091 transcripts in TRV (Control) and TRV-RcNAC091. The expression levels of determined by RcNAC091 transcripts were normalized to RcUBI2. Data are represented as mean $\pm$ SE (standard error) of three biological replicates. Phenotypic changes (B), disc area (C), chlorophyll content (D), and ion leakage (E) in TRV and TRV-RcNAC091 under dehydration and rehydration conditions. Data are means $\pm$ SE of three biological replicates. Asterisk indicates a significant difference from the control (unpaired Student's t-test; *P < 0.05, ** P < 0.01) . (F) Trypan blue, diaminobenzidine, and nitroblue tetrazolium staining of TRV and TRV-RcNAC091 discs under rehydration 3 h in rose.

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