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GENOME-WIDE IDENTIFICATION, CHARACTERIZATION, AND EXPRESSION ANALYSIS OF NAC TRANSCRIPTION FACTOR FAMILY IN *TOONA SINENSIS*

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Abstract

The NAC transcription factor (TF) is among the most extensive and plant-specific gene families, playing a crucial role in plant growth, development, and reaction to abiotic stresses. Genome-wide identification of this gene family has not been conducted in Toona sinensis. In this study, we identified and analyzed 54 TsNAC genes in T. sinensis, examining gene structure, phylogenetic characteristics, chromosomal distribution, protein domains, cis-elements, synteny, and expression patterns. Subcellular localization analysis revealed predominant nuclear localization of the NAC protein in T. sinensis. A total of 6 tandem replication events and 21 fragment replication events were identified, which were speculated to promote the amplification of *TsNACs* gene family. According to their structural and phylogenetic characteristics, the 54 *TsNAC* genes were divided into 16 subfamilies. *TsNAC* genes were regulated by multiple cis-acting elements. The expression patterns of 13 *TsNAC* genes varied across four picking stages, with most genes showing significant changes in relative expression levels over time. This study provides new insights into the regulatory functions of *TsNAC* genes in the growth and development of *Toona sinensis*, thereby improving the understanding of characteristics and evolution of *TsNAC* gene family, and providing references for further studies on the functions of *TsNAC* genes in regulating terpenoid biosynthesis.

Key words: NAC family; Expression pattern; Gene evolution; Terpene synthesis; Genome-wide analysis.

Introduction

Toona sinensis (A. Juss) Roem*,* commonly referred to as "Xiang Chun," "Chinese toon," or "Chinese mahogany," is a deciduous perennial plant belonging to the Meliaceae family. (Dong *et al*., 2013). Indigenous to East and Southeast Asia, *T. sinensis* is extensively grown in various regions of China, including Northern, Southern, and Southwestern areas (Zhao *et al*., 2024). *T. sinensis* has been cultivated for more than 2 000 years in China. In our country, the tender buds of *T. sinensis* have been widely accepted as vegetables because of their unique aroma and rich nutritional value (Zhai & Granvogl, 2019). The young leaves of *T. sinensis* contains a variety of nutrients, while containing flavonoids, alkaloids and other secondary metabolites, with anticarcinogenic, antibacterial and prevention of diabetes, hypertension, cardiovascular and cerebrovascular diseases and other effects (Ji *et al*., 2021; Chen *et al*., 2022). Its root bark, bark, bud, leaf and fruit can be used as medicine (Liao *et al*., 2007). It is a well-known medicinal and edible woody plant. In addition, *T. sinensis* is not only used as an ornamental plant but also in furniture making. The planting of *T. sinensis* in Taihe County, Anhui province has a very long history, and it was used as a tribute in the Tang Dynasty, so the *T. sinensis* produced in Taihe County is also called 'tribute toon'(Ren *et al*., 2022). In Taihe County, T. sinensis is predominantly cultivated based on the color of the bud leaves, with the local designation of purple-hued leaves as 'Heiyouchun.' This cultivar is characterized by its robust, plump, and crisp sprouts, which are exceptionally tender and possess high oil content. 'Heiyouchun' is renowned for its superior taste, intense aroma, and exceptional quality. (Sui *et al*., 2019).

Transcription factors (TFs) are a class of specific protein molecules, which can bind specifically to cis-elements or interact with other proteins to regulate the expression intensity of target genes, and play important roles in life activities. According to different DNA binding domains (Nie *et al*., 2009), transcription factors can be divided into NAC (NAM, ATAF1/2, and CUC2), MYB (myeloblastosis related), bHLH (basic helix-loop-helix), SBP (squamosa promoter binding protein), bZIP (basic leucine zipper), and other families (Ng *et al*., 2018; Feng *et al*., 2020). The NAC gene family, prevalent across all plant species, stands out as one of the largest and most crucial transcription factor families in plants. It plays a significant role in regulating a diverse array of biological processes (Diao *et al*., 2020). The *NAC* gene family has been genomically identified in a variety of plants, such as Arabidopsis with 117 (Kim *et al*., 2006), *Dendrobium nobile* with 85 (Fu & Liu, 2023), rice with 151 (Nuruzzaman *et al*., 2010), sorghum with 131 (Sanjari *et al*., 2019), *Zanthoxylum bungeanum* with 109 (Hu *et al*., 2022), soybean with 101, (Pinheiro *et al*., 2009) and *Avena sativa* with 177 members (Ling *et al*., 2023).

The name of *NAC* gene family is derived from the initials NAM (apical meristem of petunia), ATAF1/2 (Arabidopsis transcriptional activator 1/2), and CUC2 (Arabidopsis cup cotyledon) (Souer *et al*., 1996). The N-terminal of NAC transcription factor protein has a conserved domain, which is made up of 5 subdomains (Kikuchi *et al*., 2000), about 160 amino acids in length, and the flanks contain β-fold andα-helix structures. The A, C, and D subdomains exhibit high conservation. The A subdomain potentially plays a role in NAC protein dimerization, while the C and D subdomains participate in DNA binding and harbor nuclear localization signals. The change of B and E subdomains is large, which may be closely associated with the functional multiformity of the *NAC* gene family (Jia *et al*., 2019). The C-terminal domains of NAC proteins are very different, have transcriptional inhibition or activation activity, and participate in the regulation of diverse networks (Puranik *et al*., 2012).

NAC transcription factor is a multifunctional protein that plays important biological functions in regulating plant development and growth, secondary metabolism and stress response, such as seed, embryo, stem apex meristem and lateral roots development (Aida *et al*., 1999; Xie *et al*., 2000; Sperotto *et al*., 2009), cell cycle regulation (Willemsen *et al*., 2008), leaf senescence (Lee *et al*., 2012), and hormone signaling pathways in various stress responses (Sperotto *et al*., 2009). In flower organ development, Arabidopsis AIF (*anther indehiscence factor*) is expressed ectopic during flowering, leading to anther indehiscence and sterile phenotype (Shih *et al*., 2014). The ectopic expression of sugarcane *ScNAC23* gene in *Arabidopsis thaliana* expedited leaf senescence and flowering of transgenic plants (Fang *et al*., 2021). The heterogeneously expressed cotton NAC transcription factor GhFSN5 in *Arabidopsis thaliana* led to smaller pods and severe sterility of Arabidopsis transgenic plants (Sun *et al*., 2020). Overexpression of *CcNAC1* gene in ephedra expedited plant growth and promoted early flowering (Zhang *et al*., 2021). Additionally, NACs can also act as a negative and positive regulator. Overexpression of *Fragaria vesca FvNAC29* gene enhances cold and salt Tolerance in *Arabidopsis thaliana* (Li *et al*., 2024). The *RcNAC091* gene heightens the drought tolerance of roses by an abscisic acid-dependent pathway (Geng *et al*., 2023). NAC proteins' regulation are closely related to various signaling pathways, all of which are critical in abiotic stress responses.

The scope of NAC's influence extends beyond coordinating defense mechanisms. NAC modulates the expression of biosynthetic genes responsible for terpenoid production through transcriptional inhibition or activation, as well as through interactions with other transcription factors, either independently or in conjunction with them. It was found that AaNAC1 transcription factor not only responds to the induction of jasmonic acid, drought and salicylic acid, but also activates the expression of *DBR2*, *ALDH1* and *ADS* genes in artemisinin synthesis pathway, promoting the accumulation of artemisinin, and enhancing the drought tolerance and *Botrytis cinerea* resistance in transgenic *Artemisia annua* (Lv *et al*., 2016). *AcNAC* from kiwifruit has been proved to bind specifically to the *AcTPS1* promoter and regulate the activity of the kiwifruit *TPS1* (*terpene synthase 1*) gene, indicating a significant role in monoterpene biosynthesis regulation (Nieuwenhuizen *et al*., 2015). Overexpression of *PgNAC72* gene contributed significantly to the accumulation of triterpenoid ginsenosides in *Panax ginseng* callus through activating the expression of the gene *PgDDS* (*dammarenediol synthase*) (Jiang *et al*., 2024). Even though the molecular mechanism of NAC regulating the synthesis of terpene-derived compounds is still unclear, it can be concluded that NAC Involves in Terpenoid Metabolism. In conclusion, the NAC genes research may shed light on the regulatory mechanism of terpene synthase in plants. Terpenoids are a kind of multifunctional natural compounds with unique value. With the exception of adding flavor to the vegetables, terpenes also have pharmacological functions, such as antiviral, anticancer, and cholesterol-lowering (Ren *et al*., 2022). In recent times, a research found 206 chemicals in *T. sinensis* tissues, but little is known about the concrete molecular processes that regulate fragrance production in the plant (Zhao *et al*., 2024). In reality, the gene family has not been significantly studied in *T. sinensis*, and how the gene regulates species is unknown.

The results of this study indicate that 54 TsNAC family members were identified and their biochemical characteristics, conserved motif, gene structure, gene promoter, phylogeny, chromosomal distribution, and evolutionary process were determined. In addition, the expression pattern analysis of partial *TsNACs* was completed in different leaf sampling stages. The research reveals the information of *TsNACs*, which helps to promote the discovery of its particular function.

Material and Methods

Identification of NAC transcription factors in *T. sinensis*: NAC protein sequences of *Arabidopsis* were downloaded from the TAIR database (http://www.arabidopsis.org/). The genomic data of *T. sinensis* reported in our research are available under Accession No. CNP0000958 in the CNGB Nucleotide Sequence Archive (CNSA: https://db.cngb.org/search/project/CNP0000958/). The HMM (Hidden Markov Model) file of NAC domain (Accession Number PF02365) was downloaded from the database Pfam (http://pfam.xfam.org/), and HMMER3.0 software was utilized for the identification of TsNAC proteins with a threshold of E-value $\leq 10^{-5}$ (Mistry *et al*., 2021). In addition, CCD (https://www.ncbi.nlm.nih.gov/cdd/) and SMARAT (http://smart.embl-heidelberg.de/) were utilized to confirm the conserved structural domain of potential TsNAC proteins. Structurally incomplete and repetitive sequences were deleted and members of the *TsNAC* gene family were identified. The basic physicochemical properties such as the grand average of hydropathicity (GRAVY), molecular weights (MW), isoelectric points (pI), aliphatic index (AI) and instability index (II) of TsNAC proteins were used the ProtParam online software. The subcellular location of TsNAC proteins was predicted by the website Plant-mPLoc (https://www.csbio.sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi/).

Phylogenetic analysis, multiple sequence alignment of *TsNAC* gene family: The conserved structural domains were aligned using the software ClustalW (Thompson *et al*., 2002), and then the result was modified using the Jalview 2.10.5 software. MEGA 7.0 software was applied to analyze the NAC protein sequences of *T. sinensis* and *A. thaliana*. A phylogenetic tree was constructed by utilizing the neighbor-joining (NJ) method, which was set to: 1000 bootstrap replicates, p-distance, and pairwise deletion (Kumar *et al*., 2016).

Gene structure, protein conserved motif, and Cis-regulatory element analysis of *TsNAC* genes: The GSDS (Gene Structure Display Server) online website (http://gsds.gao-lab.org/) was utilized to identify gene structures of *TsNACs*, acquiring the number and position of exon and intron (Hu *et al*., 2015a). The conserved motifs of TsNAC proteins were identified using the MEME program with specific parameters: a minimum motif width of 15, a maximum width of 50, and a maximum of 20 recognized motifs.(Bailey *et al*., 2015). For cis-regulatory element analysis, we obtained the 200 bp upstream sequence of each TsNAC gene and analyzed it using the PlantCARE online tool. (http://bioinformatics.psb.ugent. be/webtools/plantcare/html/) (Lescot *et al*., 2002).Subsequently, TBtools was employed for integrated visualization.

Gene duplication and chromosomal distribution of *TsNACs*: The genome annotation file was utilized for determining the chromosomal distribution of *TsNACs*. Using TBtools to identify and analyze the replication event in *TsNACs*. The MCScanX (Multiple Collinearity Scan tool kit) program was utilized to examine the segmental duplication event among *NACs* in the *T. sinensis* genome (Wang *et al*., 2012). The genomic data for Arabidopsis, tomato, citrus, pineapple, and rice were obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/) to assess the synteny relationship between the genomes of T. sinensis and these five plant species using TBtools and MCScanX (Chen *et al*., 2020).

Plant material of *TsNAC* genes: The variety 'Heiyouchun' used in this study was bred in Taihe County Forestry Nursery, Fuyang City, Anhui Province, China. From March 30 to April 20, young and healthy leaves were collected in four different stages. Plant sample RNA was obtained utilizing the Total RNA Extraction Kit. By gel electrophoresis and A260/A280 ratio detection, selecting qualified RNA to synthesize cDNA for subsequent experiments. The Primer Premmer5 was employed to design specific *TsNAC* gene primers, and the *TsActin* gene was used as a reference gene to normalize the expression level of four different sampling periods. The qRT-PCR was executed utilizing 2×SYBR Green qPCR Mix reagents and the CFX96 TouchTM RT-PCR system. In each set of experiments, triplicate biological replicates were conducted.

Results

Identification of the *NAC* gene family in *T. sinensis*: In order to comprehensively study NAC genes of *T. sinensis*, 54 TsNAC genes were finally identified by HMM search. Based on the position of these genes on the chromosome, they were named *TsNAC1-TsNAC54* in turn and used for subsequent analysis. TsNAC31 and TsNAC44 proteins were the smallest and largest among 54 TsNAC proteins, respectively, with 84 and 550 amino acids (aa). The proteins' molecular weights varied between 9.9 and 63.2 kDa, while their isoelectric points ranged from 4.20 to 10.64. Subcellular localization results showed that TsNAC protein was ubiquitous in the nucleus. All TsNAC proteins exhibit a GRAVY score below, indicating their hydrophilic nature.

Phylogenetic analysis, Multiple sequence alignment of *TsNAC* gene family: The NAC domain contains five subdomains (Singh *et al*., 2021). The software ClustalW was employed to compare the NAC protein sequences of *T. sinensis*. The results uncovered that the great mass of NAC proteins in *T. sinensis* have conserved 5 Subdomains in their amino terminus, as shown in (Fig. 1). This indicates that TsNAC transcription factors are highly conserved during evolution. It was found that subdomain A, C and D had high stability, followed by subdomain B, and subdomain E was the most unstable (Ernst *et al*., 2004). No color signifies that the similarity of amino acid residues at that site of the *TsNAC* gene family members is less than 35%, green is greater than 35%, pink is greater than 50%, and blue is greater than 75%.

The phylogenetic study using the Arabidopsis NAC gene family as a reference showed that the 54 TsNAC genes were more accurately classified into 16 subfamilies, namely SENU5, ATAF, NAP, ONAC022, TERN, ANAC011, NAC2, NAC1, AtNAC3, NAM, OsNAC7, OsNAC8, TIP, ONAC003, ANAC063, and ONAC1(Ooka *et al*., 2003). The *NAC* genes of *T. sinensis* and *A.thaliana* were found to be unevenly distributed in each subfamily. The ONAC003 group included the most TsNAC proteins (9), followed by the AtNAC 3 group (8) in TsNAC family (Fig. 2).

Motif composition and gene structure of *NAC* genes in *T. sinensis*: Sequencing of TsNAC proteins revealed the presence of 20 distinct motifs, each consisting of 15-50 amino acids (Fig. 3B). The majority of TsNACs exhibited motifs 1, 2, 7, 8, and 9, with additional class-specific motifs observed in other instances. For example, motif 3, 4, 15 is specific to group h, motif 5, 13, 14 is unique to group g, motif 17 is specific to group d, whereas motif 19 is discovered only in group a.

Fig. 3C presents the unique characteristics of the *T. sinensis NACs* gene structure. Except for *TsNAC22*, the number of introns with a range of 0~7 and an average of 2.17. Gene structure of the *TsNACs* is composed of two introns and three exons, accounting for more than 35% (19 of the 54). Gene structure of the *TsNACs* is composed of two exons and one intron, accounting for more than 27% (15 of the 54). Among *TsNAC*s, *TsNAC22* stands out with the highest count of exons and introns, boasting 11 exons and 10 introns, respectively. Similarly, TsNACs within the same cluster, such as d, f, g, and h, exhibit a notable degree of structural uniformity.

Analysis of *TsNAC* genes promoters: Cis-regulatory elements interact with motif-specific proteins to regulate downstream gene expression (Korkuc *et al*., 2014). The 2,000 bp upstream regulatory regions of all TsNACs were isolated, and visualization of the top 20 most prevalent regulatory elements was performed using TBtools (Fig. 4).

The results showed that *T. sinensis* contains many regulatory elements for various functions. We discovered many elements associated with abiotic stress responses, such as low-temperature responsive elements (WRE3 and LTR), drought-inducibility elements (MBS), wound-responsive elements (WUN-motif) and defense and stress responsive elements (TC-rich repeats). In addition, there are many hormonal response elements, circadian control elements (circadian) and anaerobic responsive elements (ARE).

All TsNACs examined in this study contained a light-responsive element and a stress response-related cis-acting element. Additionally, 22 TsNACs were found to harbor one or more W-boxes, indicating potential regulation through cross-regulation or autoregulation mechanisms (Rushton *et al*., 2010). A total of 42 *TsNAC* genes (77.8%) had one or more ABREs, which may signal that they have an ABA response in response to stress. Additionally, more than 75% of *TsNACs* contain AREs, which may aid *T. sinensis* in waterlogging adaptation (Olive *et al*., 1990). These TsNAC genes have the potential to positively regulate plant resistance to abiotic stress.

Fig. 1. Sequence alignment of NAC proteins of *T. sinensis.* The blue shade represents a similar amino acid with a highlight homology level > 75%, the pink shade represents a similar amino acid with a highlight homology level > 50%, and the green shade represents a similar amino acid with a highlight homology level > 35%. The underlines indicate the five subdomains of the NAC proteins.

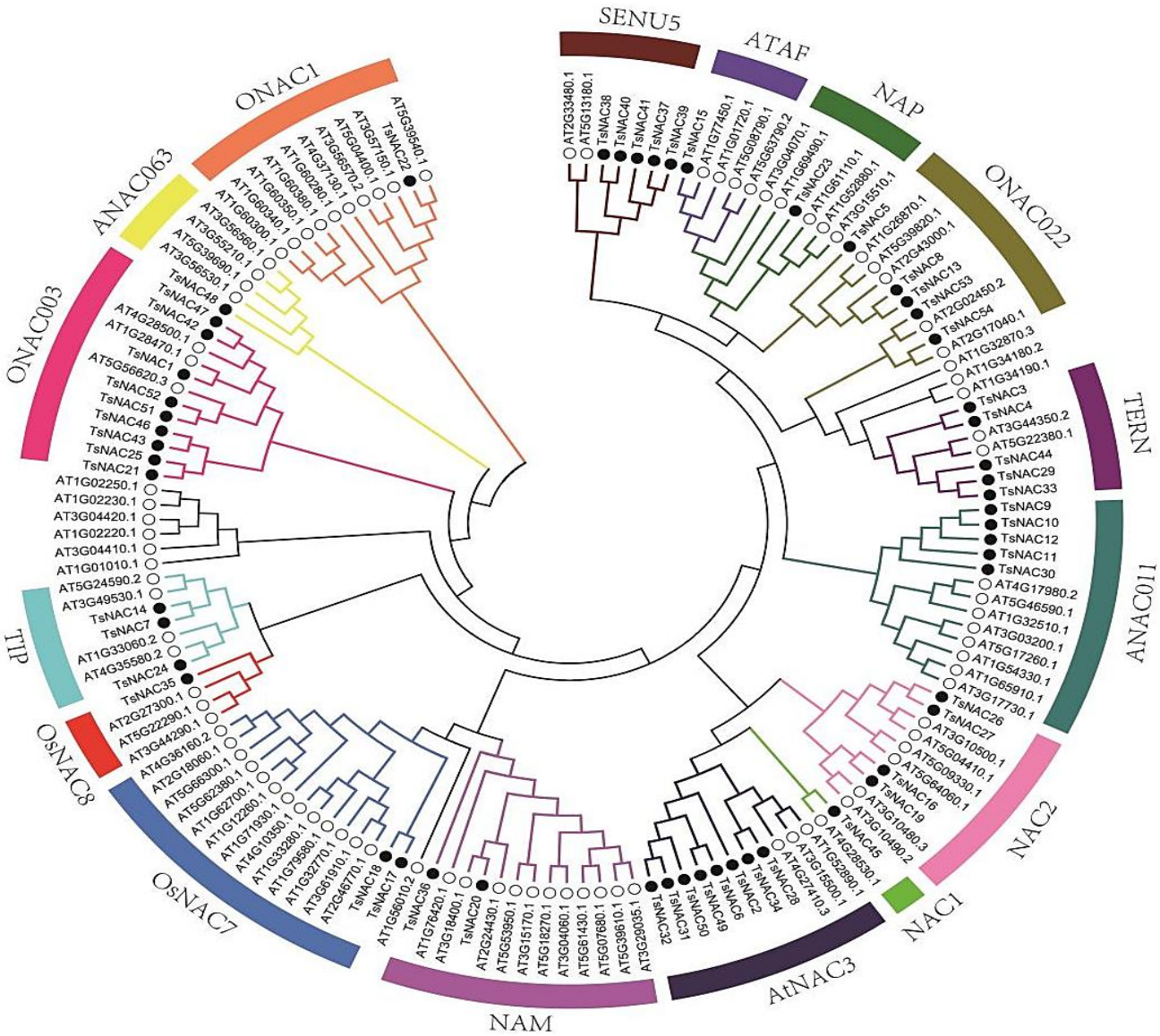

Fig. 2. A phylogenetic tree of NAC genes from *A. thaliana* and *T. sinensis*, respectively. The phylogenetic tree was built utilizing the neighbor-joining (NJ) techniqu. Different colored arcs represent various subgroups of NAC domains.

Synteny analysis of *TsNAC* genes: The 54 *TsNAC* genes were distributed on 28 *T. sinensis* chromosomes in a random manner (Fig. 5). Of all *TsNACs*, 6 were identified on Chr 07. In contrast, there is only one *TsNAC* gene on Chr 11, Chr 19, Chr 20, Chr 22, Chr 26, Chr 27, and Chr 28. Segmental and tandem duplications play a crucial role in fostering the expansion of gene families during evolution (Cannon *et al*., 2004). In this study, we explored duplication events involving TsNAC genes. Our analysis revealed that 12 TsNAC genes (22.2%) are tandem duplicates. There were 6 different pairs of tandemly duplicated genes on Chr 14, 16, 18, 21, and 25. In addition, 21 segmental duplication events were identified in this study, which occurred in 31 genes.

By constructing T. sinensis and several representative plants (Fig. 6), we can explore the volution of NACs in sinensis. The typical species are made up of two monocots, *Ananas comosus*, and *O. sativa*, and three dicots, *A. thaliana*, *Solanum lycopersicum*, and *Citrus clementina*. There were 46, 39, 37, 29, and 27 orthologous pairings between the five species (citrus, tomato, Arabidopsis, pineapple, and rice), respectively. In conclusion, TsNACs have more isobaric genes in dicotyledonous plants than in monocotyledonous plants. *C. clementina* presents greater synteny with *T. sinensis*, and both of them belong to the members of Sapindales.

Analysis of *TsNAC* genes expression level in four sprout stages: In this research, three major expression patterns of TsNAC gene were identified. Six *TsNAC* genes (*TsNAC21, TsNAC54, TsNAC48, TsNAC17, TsNAC15,* and *TsNAC25)* were significantly upregulated expressions. It is worth noting that the rising trend was manifested as first rising and then declining, and all reached the maximum value in the third stage (April 13). What is more surprising is that the expression level of *TsNAC48* gene reached more than 15 times in the third stage (April 13). Suppressed expression pattern was observed in three *TsNAC* genes (*TsNAC3, TsNAC33, TsNAC45*). The other *TsNAC* genes (*TsNAC34, TsNAC18, TsNAC28, TsNAC29)* were not significant variation (fold change ≥ 2) in four sprout stages.

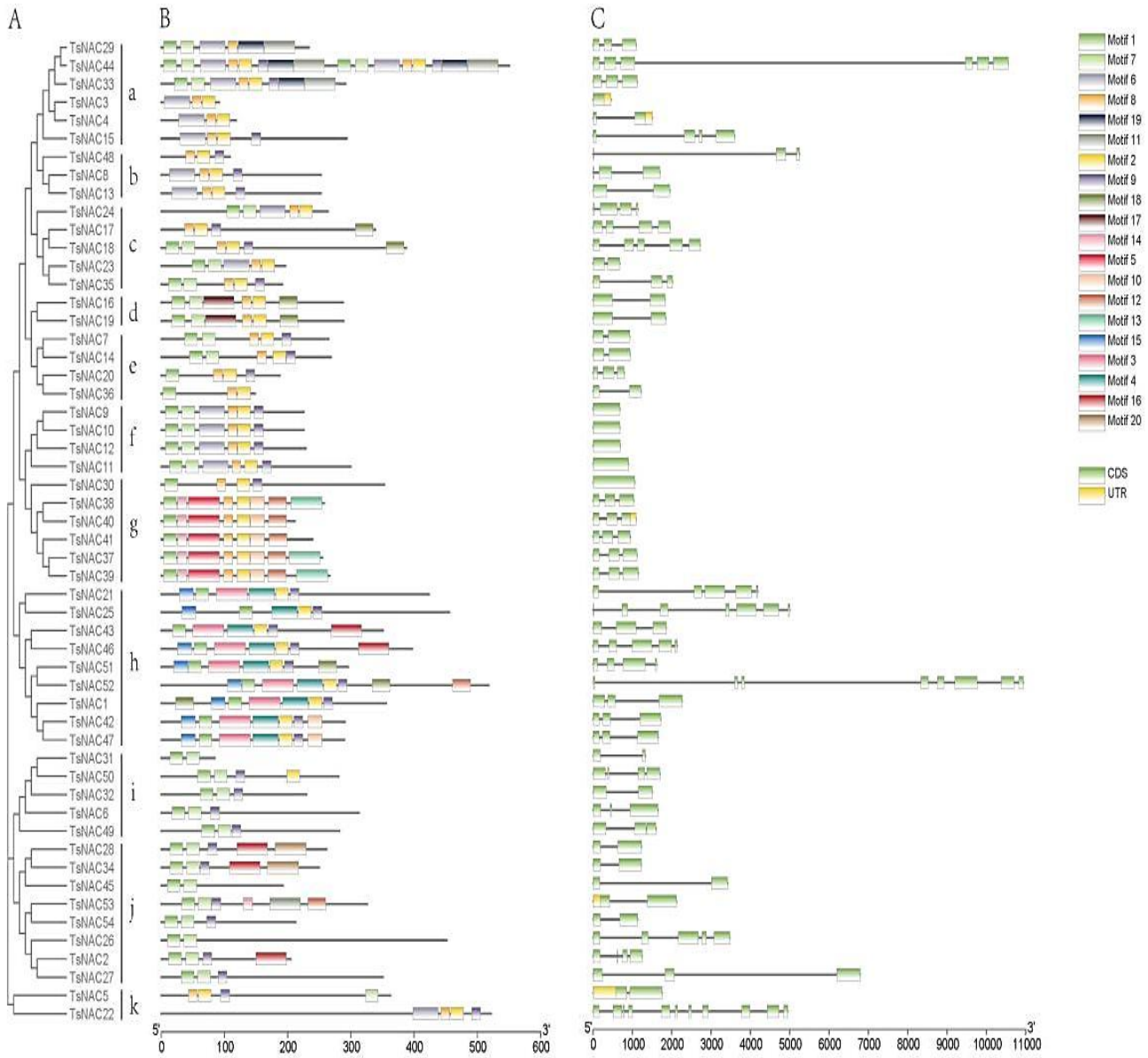

Fig. 3. Phylogenetic tree, conserved protein motifs, and intron-exon distribution of *TsNACs*. (A) The *TsNACs* are divided into several groups (a~k). (B) Each motif is indicated by a different color. (C) Black lines and green rectangles respectively represent introns and exons.

Discussion

NACs are a family of transcription factors specific to plants (Yang *et al*., 2021). It's widespread in plants (Pei *et al*., 2013; Mao *et al*., 2017), signal transduction (De Clercq *et al*., 2013; Ng *et al*., 2013; Gladman *et al*., 2016) and regulation under stress (Hu *et al*., 2006; Nakashima *et al*., 2007; Le *et al*., 2011). Because of their critical roles in adapting to stress and developing, the *NACs* have been discovered in the genomes of multiple plants. Published *T. sinensis* genome database advances *T. sinensis* gene family research and has great benefits for TsNAC transcription factor family research (Ji *et al*., 2021).

In total, we discovered 54 proper *TsNAC* genes in this investigation, which can be divided into 16 subfamilies. The number of presumed *TsNACs* is much lower than the 80 *NAC* genes identified in *Fagopyrum tataricum (Liu et al*., *2019)*, 152 *NAC* genes identified in soybean (Le *et al*., 2011), 152 *NAC* genes identified in *Zea mays* (Shiriga *et al*., 2014), and 110 *NAC* genes found in *Solanum tuberosum* (Singh *et al*., 2013). The difference in their numbers may be owing to the occurrence of whole-genome duplication (WGD) events during differentiation and evolution in each species.

Furthermore, we delved at the characteristics of TsNAC proteins. The physical and chemical property analysis of 54 TsNAC proteins showed that only 8 *TsNACs* were stable proteins, and the remaining 46 were unstable proteins. All NAC proteins were hydrophilic proteins, and most of the transcription factors in the NAC family were highly likely to have functional activities. Subcellular localization results showed that most TsNAC genes were localized in the nucleus, but a few TsNAC transcription factors were localized in the mitochondria, cytoplasm, and chloroplasts, suggesting that TsNAC transcription factors may be transmembrane transport or have multiple functions other than regulating downstream gene expression.

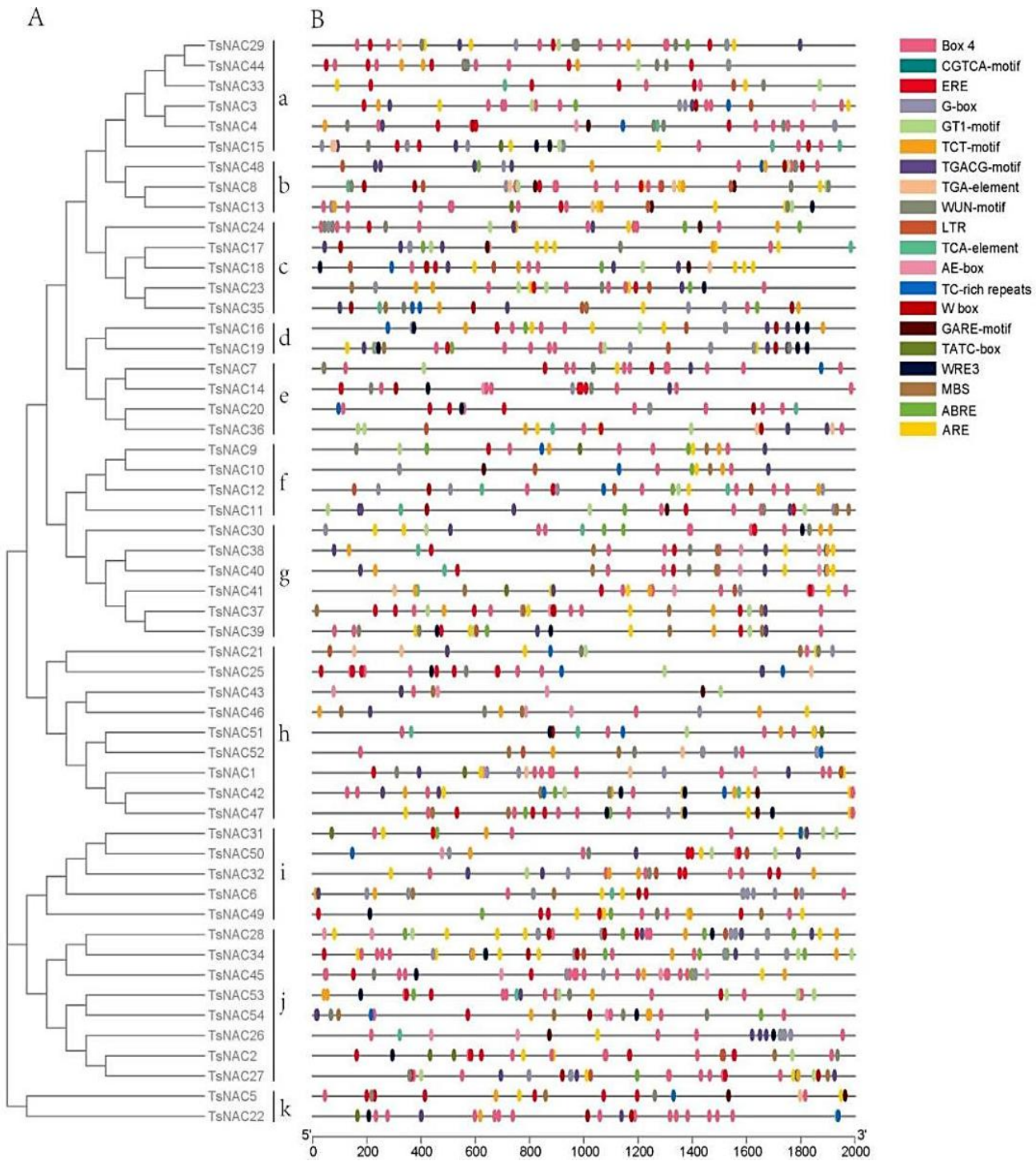

Fig. 4. Prediction of cis-acting elements in the 2000 bp promoter upstream of *TsNAC* genes.

Based on phylogenetic tree and sequence alignment analysis, the 54 TsNAC proteins were grouped into SENU5, ATAF, NAP, ONAC022, TERN, ANAC011, NAC2, NAC1, AtNAC3, NAM, OsNAC7, OsNAC8, TIP, ONAC003, ANAC063, and ONAC1, which is in line with how Arabidopsis categorizes its proteins (Ooka *et al*., 2003). In our study, the results of protein multiple sequence alignment revealed that the majority of the 54 *NAC* genes included conserved 5 subdomains (A ~ E), but a few proteins had incomplete subdomains. This difference may be owing to genetic differentiation due to changing environment during evolution.

The diversity in gene structures provides historical insights into gene family evolution and underpins phylogenetic classification (Xiao *et al*., 2017). Rice and cassava plants exhibit intron counts ranging from 0 to 16 and 0 to 5, respectively, while TsNACs display intron numbers ranging from 0 to 10 (Nuruzzaman *et al*., 2010; Hu *et al*., 2015b). Consistent exon-intron distribution patterns are observed within members of a given evolutionary cluster, serving as the basis for functional similarities among these members (Li *et al*., 2020). Almost all members of group g have two introns. Conserved motifs are important for proteins to perform their biological

functions. The motif composition of TsNAC proteins in the same group is nearly the same, but a few TsNAC proteins clustered in branches have different motif composition, which may be associated with the functional differentiation of TsNAC gene family members. A promoter is a specific DNA sequence that RNA polymerase recognizes, binds, and begins transcription. By analyzing the promoter elements, we can estimate their latent functions. The results revealed that *TsNAC* genes contain response elements associated with cold stress, drought stress, mechanical damage and endogenous hormone regulation, which is coincident with previous researches (Puranik *et al*., 2012).

Chromosome fragment duplication and single gene duplication may be related to the diversity of gene function (Yu *et al*., 2005). It is a driver of genomic evolution and essential for the adaptive evolution of plants (Moore & Purugganan, 2003; Kong *et al*., 2007). Analysis of it can help uncover the function of the *TsNAC* genes. According to the results of chromosome distribution and collinearity analysis of *TsNACs*, 21 segmental duplication events were observed in 31 *NAC* genes. In this family, we found that 12 out of the 54 genes (22.2%) in T. sinensis are tandem duplications, suggesting a potential association between a significant number of tandem duplications and the evolution of the TsNACs gene family. The inter-species collinearity analysis showed that nine genes of *TsNAC1, TsNAC3, TsNAC29, TsNAC33, TsNAC42, TsNAC45, TsNAC47, TsNAC52* and *TsNAC53* in *T. sinensis* were homologous in *Citrus clementina*, *Solanum lycopersicum*, *A. thaliana*, *O. sativa* and *Ananas comosus*. In addition, the homologous genes between *T. sinensis* and *Citrus clementina* were more than the other four plants, which may be due to the separate evolution of herbs and woody plants in the late stage of species evolution.

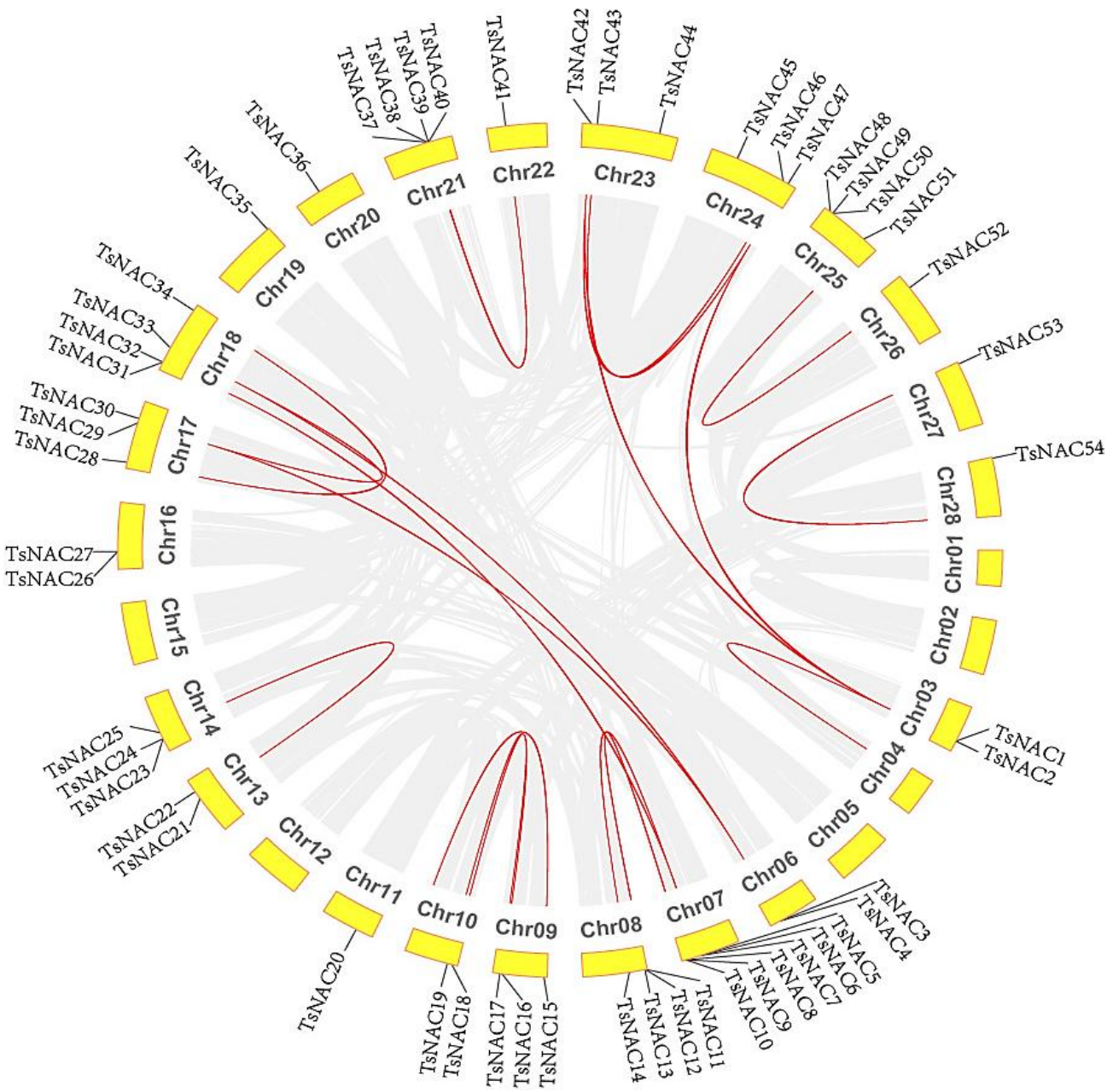

Fig. 5. Gene segmental duplication of *T. sinensis*. The gray lines indicate synteny blocks, while the red lines represent duplicated *TsNAC* gene pairs that have been found.

In *T. sinensis*, terpene has been shown to be an essential volatile compound. Aromatic substance is continuously synthesized and accumulated during the gradual growth. The results of qRT-PCR showed that 69.2 percent (9 of 13) of *TsNACs* had significant differences in transcription level during the four different periods of budding. Therefore, the expression pattern of *TsNACs* gene may be connected with the accumulation of aromatic compounds.

NAC gene is a significant regulator of plant secondary metabolite production, and the regulatory function varies greatly due to different binding mechanisms. At present, there have been some studies on the regulatory role of *NACs* in the synthesis of plant terpenoids. For example, in tomato, *SlNAC4* gene has the ability to interact with RIN (ripening inhibitor) and ROR (non-ripening). *NAC1* from *Artemisia annua* can bind to ADS promoter and up-regulate its expression to enhance drought stress tolerance and increase artemisinin content of plants (Lv *et al*., 2016). In conclusion, fruit development is related to NACs, and NACs are involved in this process and play a role.

Based on the results of differential gene analysis of *T. sinensis* obtained by our research group, 13 *TsNAC* genes with differential expression were screened and their relative expression levels were analyzed by fluorescence quantitative experiment. The expression pattern of 13 *TsNAC* genes was further analyzed in *T. sinensis* at different developmental stages combining qRT-PCR analysis (Fig. 6) and the transcriptome data. The qRT-PCR results showed that 69.2% (9 of 13) of the *TsNAC* genes had significant differences in expression levels at four different picking stages. A total of six *TsNAC* genes (*TsNAC21, TsNAC54, TsNAC48, TsNAC17, TsNAC15,* and *TsNAC25*) significantly presented higher expression level in the last three periods than that in the first period during growth. Three of these genes were suppressed (*TsNAC3, TsNAC33, TsNAC45*). Therefore, *TsNAC21, TsNAC54, TsNAC48, TsNAC17, TsNAC15,* and *TsNAC25* may positively regulate the biosynthesis of terpene, and conversely, *TsNAC3, TsNAC33, TsNAC45* may inhibit the synthesis of terpene. The unique aroma is one of the most appreciated qualities of *T. sinensis* buds. It is essential to improve key volatile flavor metabolites, which contribute to the fundamental sensory properties and distinctive flavor of *T. sinensis* buds. Studies on genes related to terpenoid synthesis of *T. sinensis*, as well as adjustments to planting and preservation processes, will be major future study areas.

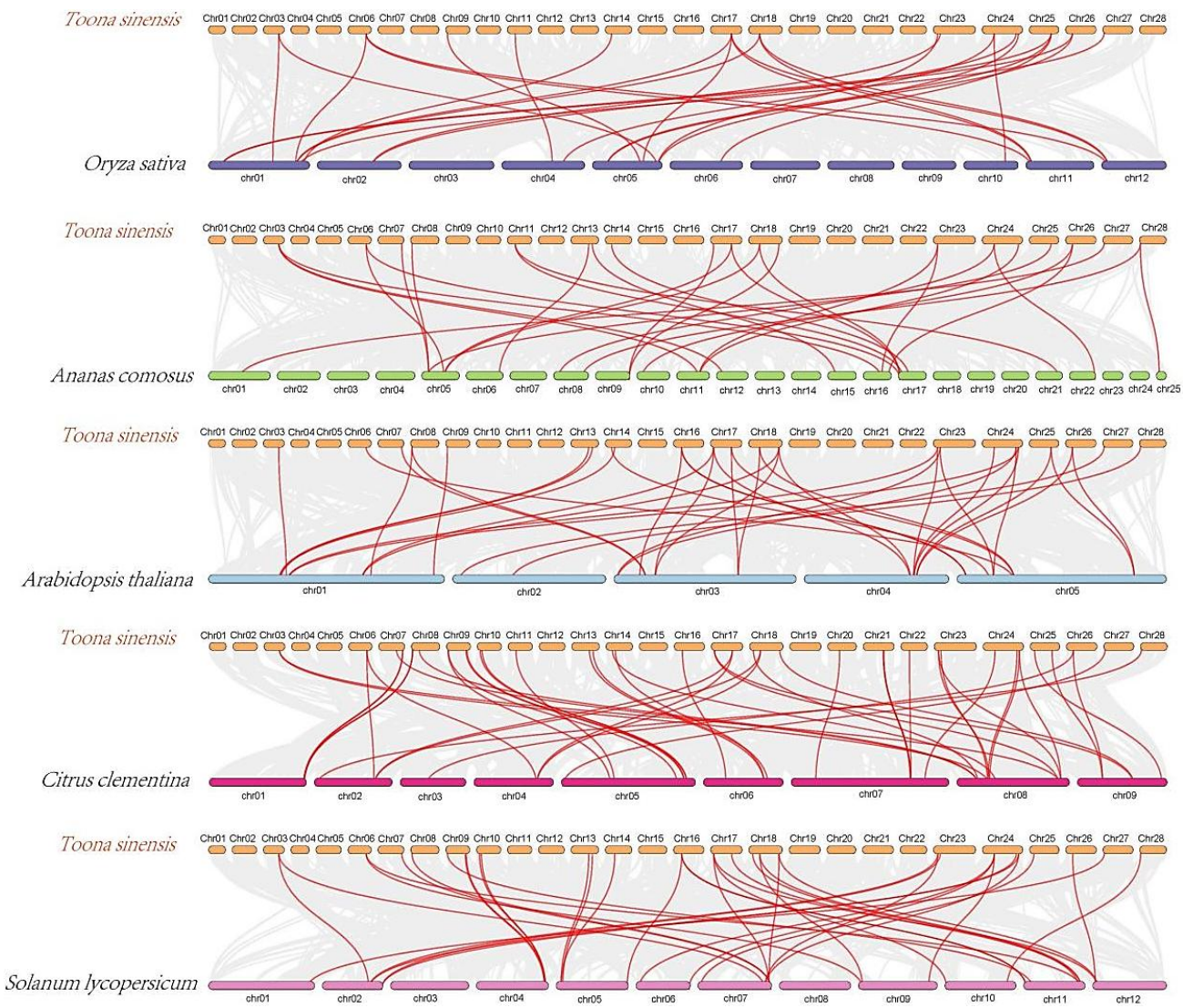

Fig. 6. Synteny analysis of *NAC* genes between *T. sinensis* and five typical plants: *Ananas comosus (A. comosus), Oryza sativa (O. sativa), Arabidopsis thaliana (A. thaliana)*, *Citrus clementina (C. clementina)*, and *Solanum lycopersicum (S. lycopersicum)*. The red curves indicate the syntenic *NAC* gene pairs, whereas the gray lines represent the collinear blocks of *T. sinensis* and other plants.

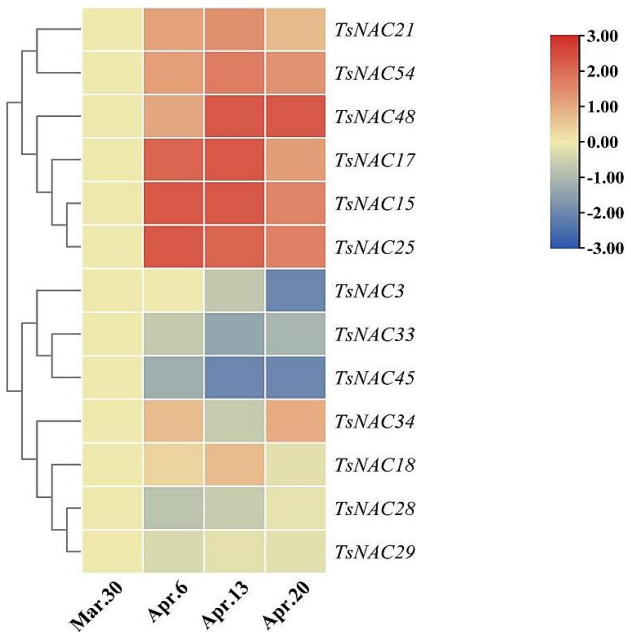

Fig. 7. Expression analysis of *TsNAC* genes in four different periods. Red and blue represent higher and lower transcript abundance severally.

Conclusion

In this study, NAC gene family in *T. sinensis* was identified and analyzed for the first time. 54 TsNAC gene family members were identified and divided into 16 subfamilies. The phylogenetic comparison and synteny analysis of *NAC* genes in several different plants provide valuable clues for comprehending the evolutionary characteristics of *TsNACs*. Phylogenetic and gene expression analyses reveal the function of the *TsNACs*. The expression patterns analysis prove *TsNACs* may participate in regulating terpenoid biosynthesis and plant growth and development processes. This research reveals comprehensive information on *TsNACs* and contributes to further study on the function of *TsNACs* in regulating the synthesis of volatile aromatic compounds.

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