

# Genome-wide analysis of NAM-ATAF1,2-CUC2 transcription factor family in *Solanum lycopersicum*

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**Abstract** NAM, ATAF1,2, and CUC2 (NAC) proteins constitute one of the largest families of plant-specific transcription factors. These proteins have diverse functions in biological processes. Particularly, NAC transcription factors have received considerable attention as regulators in stress signaling pathways. However, little is known about the *NAC* genes in tomato (*Solanum lycopersicum*). In this study, 104 *NAC* genes were identified in the tomato genome. The predicted *NAC* genes were distributed across all of 12 chromosomes at various densities and were phylogenetically clustered into six groups (I–VI), together with *NAC* genes from *Arabidopsis* and rice. The structure and motif compositions of the *NAC* genes in tomato were also analyzed. Analysis of available microarray data showed that most of the *NAC* genes in tomato had specific temporal and spatial expression patterns. Moreover, the expression profiles of eight selected *NAC* genes in tomato were analyzed in different tissues under different abiotic conditions by quantitative real-time RT-PCR. Except for one, all eight selected genes responded to one or more of the abiotic stress treatments. The results of this study provided

insights into the classification and putative functions of this family.

**Keywords** Genome-wide analysis · NAC transcription factor · Gene family · Tomato

## Abbreviations

NAC	NAM, ATAF, and transcription factor
CUC	
TF	transcription factor
TRR	transcriptional regulation region
BLAST	the Basic Local Alignment Search Tool
SMART	a Simple Modular Architecture Research Tool
HMM	Hidden Markov Model
MEGA	Molecular Evolutionary Genetics Analysis
MEME	Multiple Em for Motif Elicitation
NJ	the neighbor-joining
NCBI	National Center for Biotechnology Information

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## Introduction

Plant-specific NAM, ATAF1/2, and CUC2 (NAC) proteins constitute one of the largest transcription factor (TF) families. These proteins are characterized by a well-conserved N-terminal NAC domain (Olsen et al. 2005; Puranik et al. 2012). The NAC domain, which comprises nearly 160 amino acid residues, can be divided into five subdomains (A to E) based on its motif distribution (Aida et al. 1997; Ooka et al. 2003). The highly conserved subdomains C and D may be

responsible for binding to DNA, subdomain A may be involved in homo- and heterodimerization, and the divergent subdomains B and E may be implicated in the functional diversity of NAC proteins (Ooka et al. 2003; Jensen et al. 2010; Chen et al. 2011). The transcriptional regulation region (TRR) of NAC TFs is generally located in the highly divergent C terminus; this region confers regulation diversity in transcriptional activation activity. The TRR contains group-specific motifs that are rich in serine and threonine, proline and glutamine, or acidic residues. At least 10 motifs have been identified in the TRR of rice (*Oryza sativa*) NAC proteins. These motifs are conserved for a given subgroup of NAC subfamilies but vary across different subfamilies (Fang et al. 2008).

NAC TFs regulate many biological processes, including shoot apical meristem formation and maintenance (Aida et al. 1997; Hibara et al. 2003), floral development (Sablowski and Meyerowitz 1998), embryo development (Duval et al. 2002), hormone signaling (Fujita et al. 2004), and regulation of secondary cell wall synthesis (Zhong et al. 2010). Particularly, NAC TFs have received considerable attention as regulators in both biotic and abiotic stress signaling pathways. Some of these TFs have been considered potential targets for the engineering of plant tolerance (Jensen et al. 2010; Puranik et al. 2012). Although considerable knowledge has been gained about the physiological and molecular functions of NAC proteins, this area of research is still in its infancy.

Tomato (*Solanum lycopersicum*) is an important vegetable crop worldwide. Numerous studies on fruit development have used tomato as a model. Intensive research has been conducted on NAC TFs in many plants, such as *Arabidopsis*, rice, soybean, apple and potato (Nuruzzaman et al. 2010; Le et al. 2011; Singh et al. 2013; Su et al. 2013). However, only a few studies have characterized the NAC TFs in tomato. The genome sequence of tomato has been decoded, and this has provided an excellent opportunity for genome-wide analysis of all the genes belonging to specific gene families (Mueller et al. 2005). Kou et al. (2013) have recently detected 74 putative NAC genes in tomato. In the present study, we performed a genome-wide analysis of the NAC gene family and identified 104 NAC genes in tomato. The tissue-specific expression profiles of the NAC genes in tomato were analyzed based on available microarray data. Given the potential of NAC genes as targets for the engineering of plant tolerance to different stresses, we searched Genevestigator to gain insights into the differential expression profiles of the SINAC genes under various stress conditions. Furthermore, we selected eight tomato NAC genes and analyzed their expression profiles in different tissues under different abiotic stress conditions by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). The results of this study could serve as reference to select candidate NAC genes in tomato for further functional characterization.

## Materials and methods

### Identification and chromosomal location of the NAC genes in tomato

Two approaches were performed to identify the members of the NAC TFs in tomato. First, all known *Arabidopsis* and rice NAC gene sequences were used as queries in the multiple database searches that were performed against the proteome and genome files downloaded from the plantGDB database (<http://www.plantgdb.org/>). Stand-alone versions of Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov>) from the NCBI were used with an e-value cutoff of 1e-003. All protein sequences derived from the collected candidate NAC genes were examined using the Protein family (Pfam) programs (<http://pfam.sanger.ac.uk/>) for domain analysis and Simple Modular Architecture Research Tool (SMART, <http://smart.embl-heidelberg.de/>) with the default cutoff parameters. Second, the domains of all tomato peptide sequences were analyzed using a Hidden Markov Model with Pfam searching. We obtained the sequences using the PF02365 Pfam number containing a typical NAC domain from the tomato genome sequences using a Perl-based script. Finally, all protein sequences were compared with known NAC sequences using ClustalX (<http://www.clustal.org/>) to verify that the sequences were candidate tomato NAC genes.

The isoelectric points (pIs) and molecular weights of the proteins were obtained using the proteomics and sequence analysis tools on the ExPASy Proteomics Server (<http://expasy.org/>).

The chromosome locations were retrieved from the tomato genome data downloaded from the plantGDB database. The remaining genes were mapped to the chromosomes using MapDraw.

### Gene structure and motif analysis of the NAC genes in tomato

The gene structures of the NAC genes were generated using the Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). Multiple EM for Motif Elicitation (MEME, <http://meme.sdsc.edu>) version 4.8.1 was used to elucidate the motifs in the 104 deduced tomato NAC protein sequences. MEME was run locally with the following parameters: number of repetitions, any; maximum number of motifs, 20; and optimum motif widths, between 6 and 200 residues (Bailey et al. 2006).

### Sequence alignment and phylogenetic analysis of tomato NAC genes

The NAC TF sequences were aligned in ClustalX using BLOSUM 30 as protein weight matrix. The Multiple Sequence Comparison by Log-Expectation (MUSCLE)

program (version 3.52) was also used to perform multiple sequence alignments to confirm the ClustalX results (<http://www.clustal.org/>). A phylogenetic tree for the NAC TF protein sequences was constructed using the neighbor-joining method of Molecular Evolutionary Genetics Analysis (MEGA5, <http://www.megasoftware.net/>) and the p-distance for the complete deletion option parameters. The reliability of the obtained trees was tested using a bootstrapping method with 1,000 replicates. The image of the phylogenetic tree was drawn using MEGA5.

Data analysis from the microarray database

Microarray expression data from various datasets were obtained using Genevestigator (<https://www.genevestigator.com/gv/>) with the tomato Gene Chip platform. The whole-genome sequences for tomato were used as query sequences to blast against all gene probe sequences in the Affymetrix Gene Chip (<http://www.affymetrix.com/>). The best homologous probe was used to annotate the corresponding gene. The identified NAC-containing probe IDs were selected by a Perl-based program and used as query sequences to perform searches in the tomato Gene Chip platform of Genevestigator.

Plant growth, treatments, and collection of tissues

Tomato (*Solanum lycopersicum* cv. Zhongshu 6) plants were grown under normal conditions. Four-week-old tomato seedlings were subjected to various stress treatments. For drought and salinity stress treatments, the seedlings were irrigated with 15 % PEG 6,000 and 250 mM NaCl solution for the given time periods, respectively. For low temperature treatment, whole plants in pots were placed in illuminated incubation chambers at 4 °C. For each treatment, plant samples were rapidly frozen in liquid nitrogen and then stored at –80 °C. Each treatment was performed at least thrice. For tissue expression analysis, tissues of the root, stem, leaf, flower, and fruits were harvested and stored at –80 °C until use.

Quantitative real-time RT-PCR

Total RNA was extracted using the PureLink™ RNA Mini Kit (Invitrogen, USA) and treated with RNase-free DNase I. Approximately 2 µg of the extracted total RNA was used to synthesize first-strand cDNA using the PrimeScript First Strand cDNA Synthesis Kit (Takara, China).

Gene-specific primers for qRT-PCR were designed based on the nonconservative regions at the 3' end (Supplementary Table S1). Primer specificity was analyzed by blasting each primer sequence against the tomato genome and then confirmed when the corresponding melting curves yielded a single sharp peak or a single amplified fragment with the

correct predicted length. qRT-PCR was performed in 25 µL reaction volumes containing 10 µM of each primer, 50 ng of cDNA, and 12.5 µL of SYBR Premix Ex Taq II. The PCR amplification conditions included an initial heat-denaturing step at 95 °C for 3 min and then 40 cycles of 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s. Fluorescence was measured at the end of each cycle. A melting-curve analysis was performed by heating the PCR product from 55 °C to 95 °C. The expression data for the *NAC* genes were presented as relative units after their normalization to the tomato *Ubiquitin*, which served as the internal control, using the  $2^{-\Delta\Delta CT}$  method. The values for the mean expression and standard deviation were calculated from the results of three independent replicates.

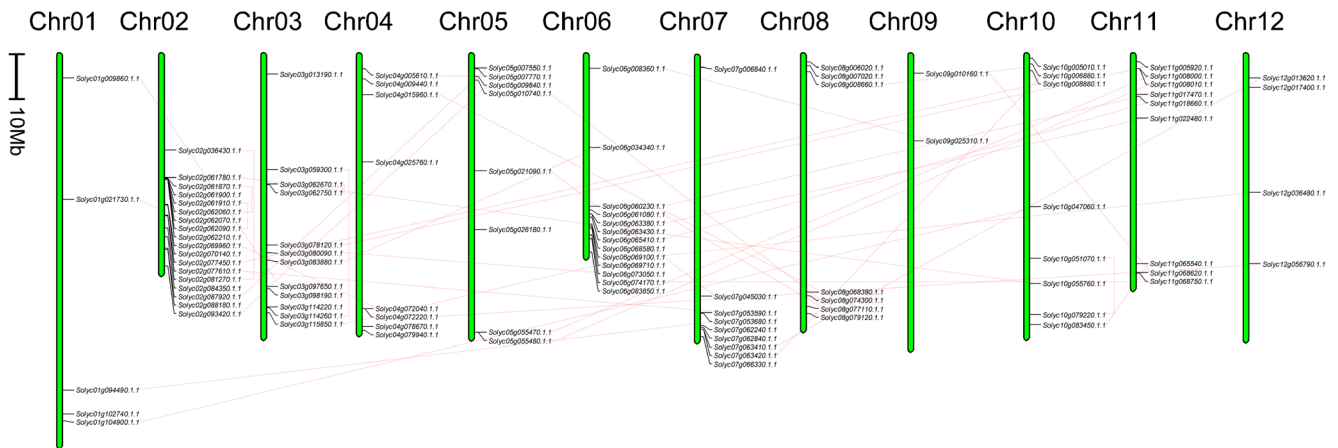
## Results and discussion

Identification and chromosomal localization of the tomato NAC gene family

We used bioinformatic methods to gather extensive information regarding the *NAC* gene family in tomato and identify its members. All putative *NAC* genes in the tomato genome were collected for sequence comparison. The presence of the NAM domain in these genes was confirmed by SMART and Pfam searches. Among the predicted *NAC* genes, 104 members contained full ORFs and named *SINAC1* to *SINAC104*. The size of the NAC family in tomato is similar to that in other plant species, such as *Arabidopsis* (~105), rice (~140), and soybean (~138) (Hu et al. 2010). Thus, the presence of a large number of *NAC* genes in the tomato genome suggests that these genes are crucial in the complicated transcriptional regulations in this species.

The identified *SINAC* genes encode proteins that range from 123 (*Solyc03g062750*) to 1,030 (*Solyc02g081270*) amino acids (aa) in length, with an average of 333 aa, and exhibit pIs that range from 4.58 (*Solyc06g063380*) to 9.9 (*Solyc03g062750*). The predicted *SINAC* TFs were further analyzed using a local BLAST alignment to identify the relevant orthologs in *Arabidopsis*. Gene ontology analysis was also performed (Supplementary Table S2).

Among these 104 *SINAC* genes, 103 (except for *Solyc00g255510*) were mapped onto the tomato chromosomes using MapDraw (Fig. 1). The *SINAC* genes were distributed on every chromosome (Chr) in tomato. Chr 2 contained the highest number of *SINAC* genes, with 18 out of 103 members (~17 %), whereas Chr 9 contained only one member (~1 %). Interestingly, many sister pairs of *SINAC* genes, such as *Solyc02g036430/Solyc02g081270*, *Solyc01g009860/Solyc03g097650*, *Solyc01g021730/Solyc04g072040*, and *Solyc01g094490/Solyc07g053680*, were located on the same or different chromosomes. These



**Fig. 1** Chromosomal mapping of the tomato NAC gene family. The scale bar represents a chromosomal distance of 10.0 Mb. The chromosome number (Chr01–Chr12) is indicated at the top of each chromosome. Sister paralogous pairs are indicated by light colored lines

results suggest that gene duplication participates in the expansion of the *NAC* gene family in tomato.

Gene structure and protein motif analyses of the NAC gene family in tomato

We analyzed the exon/intron organization in the coding sequences of individual *SINAC* genes in tomato to determine the structural diversity of these genes. The exon/intron structures of these genes are shown in Fig. 2b. Among the 104 *SINAC* genes, 18 had no intron, whereas the others had at least one intron. *Solyc02g081270.1.1*, which contained 16 introns, possessed the highest number of introns. In addition, a separate phylogenetic tree was generated from the complete protein sequences of all the *SINAC* genes. The *SINACs* were divided into 14 subfamilies (Fig. 2a). The most closely related members in the same subfamilies shared similar exon/intron structures in terms of intron number and exon length. For instance, most of the *SINAC* genes in subgroups A, B, and C harbored no intron, whereas all members in subgroups L and M possessed two introns. By contrast, the members of subgroups H, K, and N displayed a large variability in the number and distribution of introns.

Putative motifs were predicted by MEME and 20 distinct motifs were identified to further reveal the diversity of the *SINAC* genes. Most of the closely related members in the phylogenetic tree exhibited common motif compositions. This result suggests the presence of functional similarities among the NAC proteins within the same subgroup (Fig. 2c).

Phylogenetic analysis of the NAC TFs of *Arabidopsis*, rice, and tomato

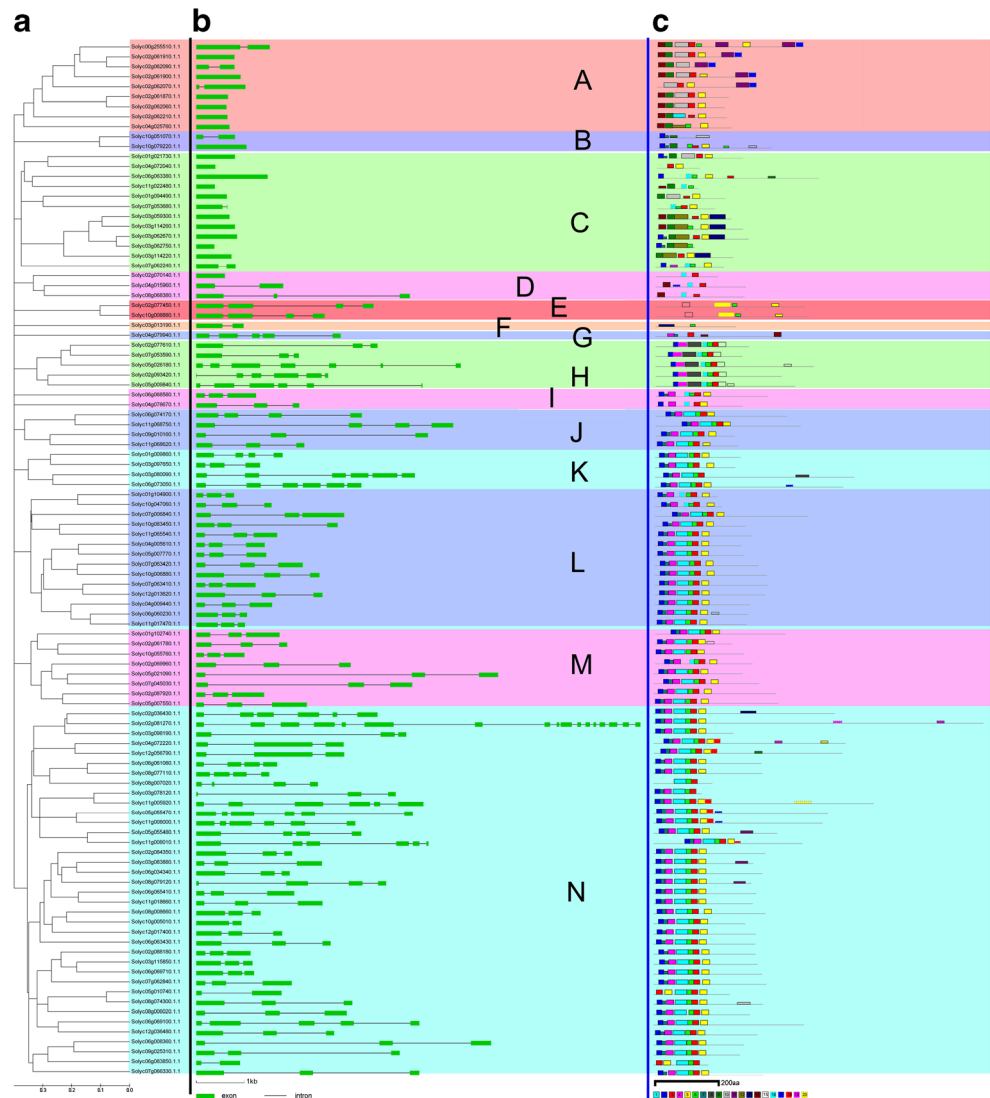
The full-length sequences of all *SINACs* and NACs from dicot (*Arabidopsis*) and monocot (rice) model systems were subjected to multiple sequence alignment using MUSCLE to study the evolutionary relationship among the NAC TFs of

different plant species. The multiple sequence alignment data were then used to construct an unrooted phylogenetic tree using MEGA5. As shown in Fig. 3, the phylogenetic tree divided the NACs into six groups (I to VI). Groups I, III, IV, and V were further divided into four, two, three, and two large subgroups, respectively. Group I constituted the largest clade with 35 *SINACs*, followed by group IV (33 *SINACs*), whereas group II had only one *SINAC* and one NAC from *Arabidopsis*. Most subgroups were shared among the NACs from *Arabidopsis*, rice, and tomato. Subgroup Id did not include any NACs from *Arabidopsis* and rice but included NACs from tomato. These results indicate that these genes are either lost in *Arabidopsis* and rice or acquired in tomato after their divergence from the last common ancestor. Therefore, these genes may have specialized functions in tomato.

The NAC genes with the same functions clustered into one subgroup, providing an excellent reference to explore the functions of the *SINAC* genes. For example, a subset of *SINAC* genes, including *Solyc10g047060*, *Solyc07g063410*, *Solyc04g009440*, and *Solyc12g013620*, were clustered with the well-known stress-related marker genes (e.g., *ATAF1/2*, *ANAC019*, *RD26*, *SNAC1/2*, and *OsNAC10*) in group V (Fujita et al. 2004; Bu et al. 2008; Hu et al. 2008; Jensen et al. 2010; Jeong et al. 2010). This result implies that these *SINAC* genes are involved in stress responses. This hypothesis was supported by the expression profile analysis discussed below. The results of the present study provided insights into functions of the *SINAC* genes.

Most *SINACs* were widely scattered across the phylogenetic tree. This finding indicates that these *SINACs* belong to the NAC subgroups that are also present in *Arabidopsis* and rice. Meanwhile, no NACs in *Arabidopsis* and rice but 26 *SINACs* clearly formed a clade in subgroup Id. This result suggests that the clade of these NACs is absent in the other two plant genomes but is present and may have special functions in tomato. A novel subgroup of NACs termed TNACs (tobacco NAC) was found in tobacco. This subgroup

**Fig. 2** Phylogenetic tree, gene structure, and protein motif analysis of *SINAC* transcription factors. **(a)** Phylogenetic tree. The amino acid sequences of the *SINAC* proteins were aligned using Clustal X, and the phylogenetic tree was constructed using the neighbor-joining method in the MEGA5 software. Each node is represented by a number that indicates the bootstrap value for 1,000 replicates. The scale bar represents 0.1 substitutions per sequence position. **(b)** Gene structure. The center illustrates the exon/intron organization of the corresponding *NAC* genes. The exons and introns are represented by the boxes and lines, respectively. The scale bar represents 1.0 kb (middle). **(c)** Protein motif. Schematic representation of the conserved motifs in the NAC proteins in tomato, which were elucidated using MEME. Each motif is represented by a number in the colored box. The black lines represent the non-conserved sequences. The scale bar represents 200 aa



contained the NAC domain at the N-terminal distinguished from other NACs and appeared unique to Solanaceae. Therefore, we hypothesized that some members of subgroup Id are the TNACs in tomato (Rushton et al. 2008). To test this hypothesis, we constructed an unrooted phylogenetic tree of the NAC domains of *SINAC*s (Supplementary Figure S1). As illustrated in Figure S1, 10 members of group Id and tobacco TNACs clustered into one group marked with red shadow. Furthermore, the partial NAC domain sequence (subdomains A to C) of the 10 candidate TNACs identified in tomato shared typical characteristics with the TNACs in tobacco. These results suggest that the 10 *SINAC*s are the TNACs in tomato.

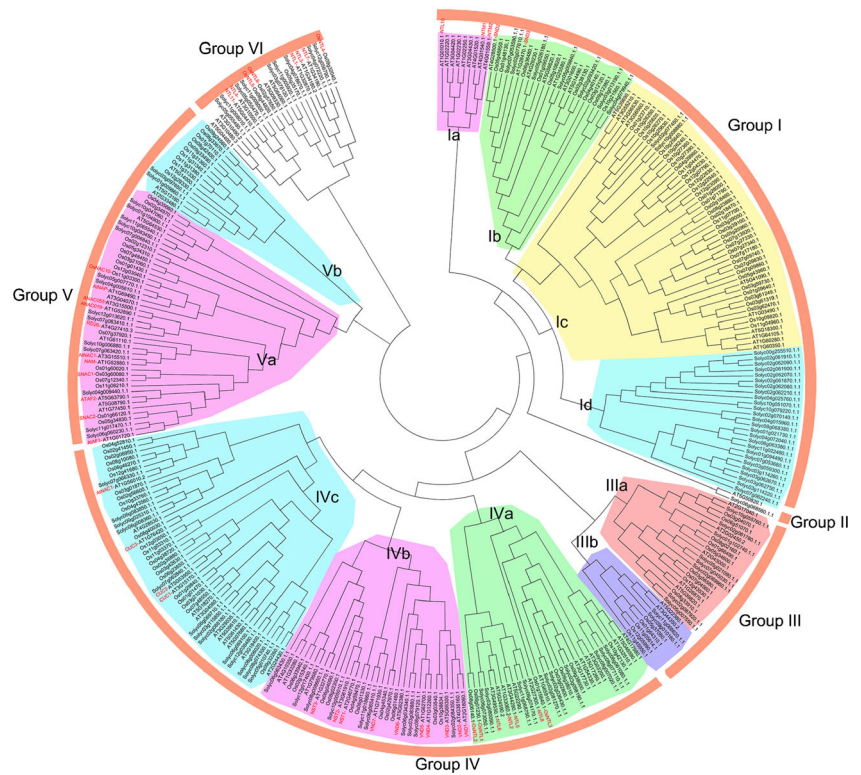
#### Differential expression profiles of the *SINAC* genes

We searched the literature for relevant tomato probes and available microarray data in the database to investigate the

potential functions of the *SINAC* TFs in tomato development. We then analyzed the temporal and spatial expression patterns of the *SINAC* genes under normal growth conditions using Genevestigator (Fig. 4). The expression profiles of 58 genes (55.8 %) were obtained, whereas those of the other 46 genes were not detected. Possibly, the 46 genes showed transcript levels that were too low to be detected, their special temporal and spatial expression patterns were not examined, or some of them are pseudogenes.

As illustrated in Fig. 4, the 25 *SINAC* genes denoted by stars showed distinct stage-specific expression profiles, whereas the others were expressed at all six stages tested from main shoot growth to fruit ripening, complete with different expression levels. The expression profiles of the 58 *SINAC* genes in 14 tissues were also analyzed. Most of the *SINAC* genes showed diverse tissue-specific expression profiles. This result indicates that *SINAC* TFs have multiple functions in the regulation of tomato growth and development.

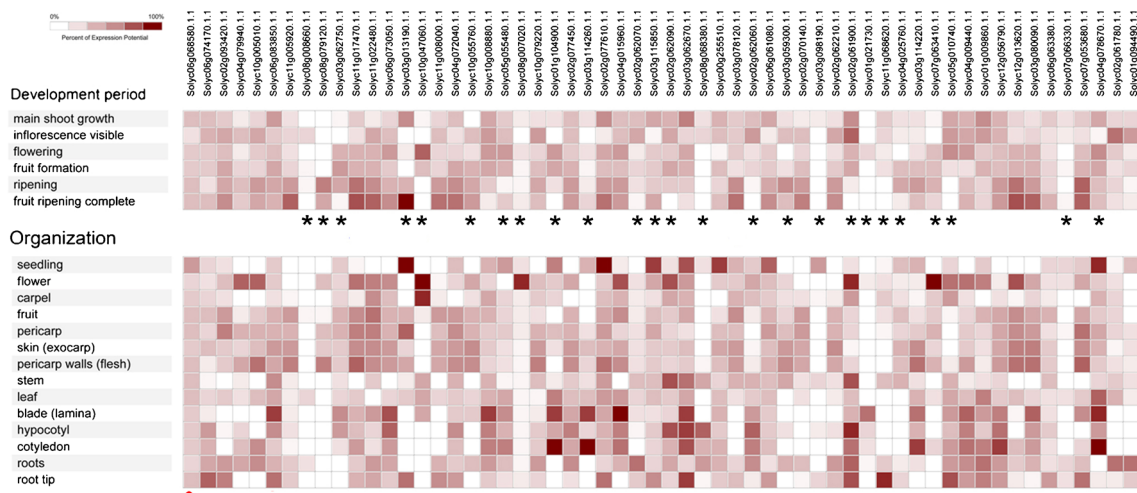
**Fig. 3** Phylogenetic analysis of NAC transcription factors of *Arabidopsis*, rice and tomato. The full-length sequences of the NAC proteins were aligned with Clustal X, and the phylogenetic tree was constructed using the neighbor-joining method in MEGA5 software. Each node is represented by a number that indicates the bootstrap value for 1,000 replicates



We also utilized Genevestigator to analyze the different expression profiles of the *SINAC* genes under various stress conditions. As predicted by Genevestigator analysis, most of the *SINAC* genes were downregulated or upregulated in response to biotic or abiotic stress treatments (Supplementary Figure S2). In addition, the different genotypes of the tomato strain had different expression profiles of the *SINAC* genes. The diverse expression profiles of the *SINAC* genes under stress conditions may reflect their multiple functions in regulating the stress signaling pathway.

Expression analysis of the selected *SINAC* genes by qRT-PCR

Basing on the results of Genevestigator analysis, we selected eight *SINAC* genes, namely, *Solyc10g005010*, *Solyc11g005920*, *Solyc08g079120*, *Solyc10g047060*, *Solyc07g063410*, *Solyc04g009440*, *Solyc12g013620*, and *Solyc01g094490*, for expression analysis. Consistent with the above data analysis from the microarray database, the eight *SINAC* genes showed distinct tissue-specific expression patterns, which represented the distinct functions of the



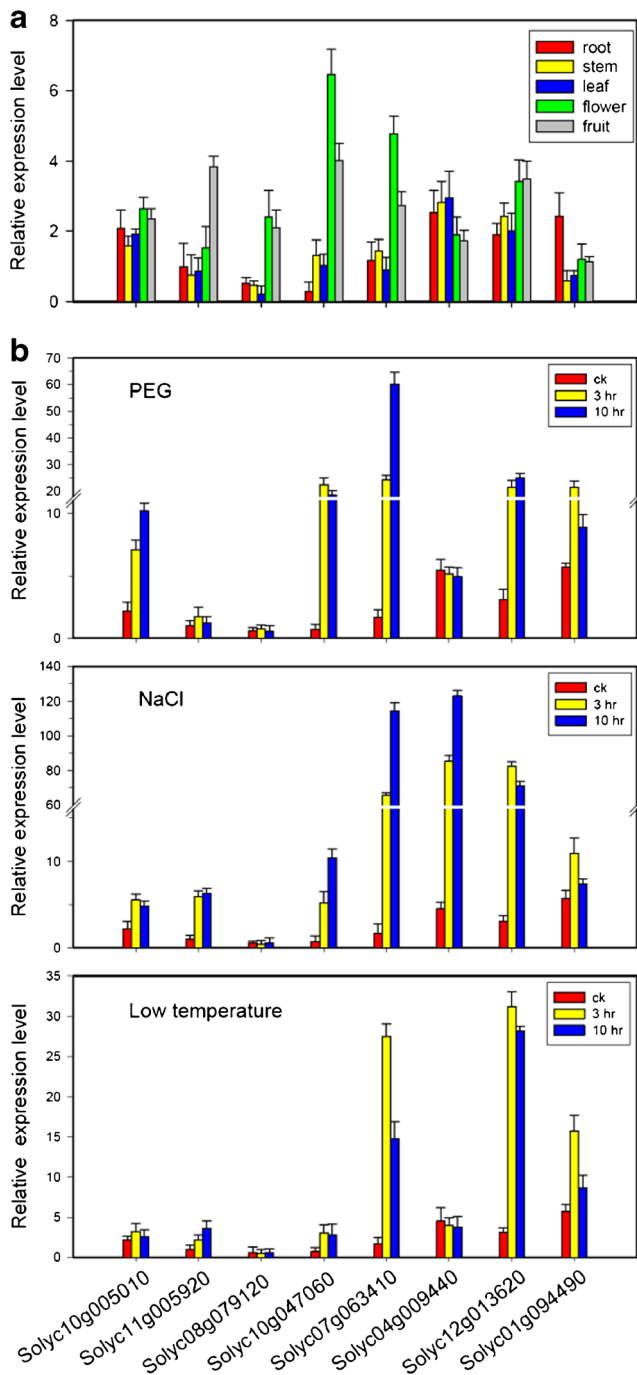
**Fig. 4** The temporal and spatial expression analyses of the *SINAC* genes. The dark and light shading represents the relative high or low expression levels of the *NAC* genes, respectively. The genes showing the distinct stage-specific expression profiles were denoted by stars

individual *SINAC* genes in tomato development (Fig. 5a). For instance, *Solyc01g094490* was highly expressed in the roots, whereas *Solyc04g009440* and *Solyc12g013620* were highly expressed in all tested tissues. *Solyc11g005920*, *Solyc08g079120*, *Solyc10g047060*, and *Solyc07g063410* were preferentially expressed in the flowers and fruits. This result indicates that these genes

have important functions in regulating the development of flowers and fruits. Substantial evidence has indicated that NAC TFs have important functions in plant growth, development, and stress responses. However, only a few studies have explored the functions of NAC TFs in fruit ripening (Olsen et al. 2005; Puranik et al. 2012). Six *NAC* genes, designated as *MaNAC1* to *MaNAC6*, have been recently characterized from banana fruit. These genes were suspected to participate in banana fruit ripening by interacting with ethylene signaling components (Shan et al. 2012). The results of this study provided insights into the possible functions of *SINAC* genes in the fruit development of tomato.

NAC TFs are involved in stress response and are potential targets in the development of improved stress-tolerant transgenic plants. Therefore, we utilized qRT-PCR to analyze the different expression patterns of the eight *SINAC* genes in the seedlings subjected to three abiotic stress treatments: low temperature (4 °C), drought (15 % PEG 6,000), and high salinity (250 mM NaCl). Except for one (i.e., *Solyc08g079120*), all the selected *SINAC* genes responded to one or more stress treatments (Fig. 5b). *Solyc10g047060*, *Solyc07g063410*, *Solyc04g009440*, and *Solyc01g094490* were significantly upregulated by all the three treatments, whereas *Solyc12g013620* was induced by high salinity and slightly repressed by the other two treatments. These results indicate that *SINAC*s have multiple functions in the regulation of abiotic stress signaling pathways and thus may be excellent candidates for the engineering of tomato plants with improved stress resistance.

Kou et al. (2013) have recently identified 74 putative *SINAC* genes in tomato using 10 reported NAC genes as queries for BLASTP search. They have also analyzed the expression profiles of six selected *SINAC*s in different tissues during fruit development and senescence, focusing on the relationship of these genes to hormone responsiveness. In this study, we performed a comprehensive genome-wide analysis of the *NAC* gene family in tomato using all known *Arabidopsis* and rice *NAC* gene sequences as queries for database search and identified 104 *NAC* genes. Furthermore, we identified candidate tissue-specific and/or stress-responsive *SINAC* genes. The results of this study could be used as reference to further characterize the functions of the *NAC* gene family in tomato. Moreover, these studies may facilitate the understanding of the molecular basis of many agronomically important traits of tomato, such as fruit development and defense against biotic and abiotic stresses.



**Fig. 5** The expression analyses of the selected *SINAC* genes by qRT-PCR. (a) The expression profiles of the selected *SINAC* genes in the different tissues. (b) The expression profiles of the *SINAC* genes under various abiotic conditions

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