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ORIGINAL PAPER

Overexpression of two *ATNAC3*-related genes improves drought and salt tolerance in tomato (*Solanum lycopersicum* L.)

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Abstract The NAC family is a multigene family that present uniquely in plants and whose members are involved in many important cellular processes including abiotic stress tolerance. In this study, sequences of two *ATNAC3*-related genes (*SlNAC3*) were identified in the tomato genome using different bioinformatics approaches. Phylogenetic analysis clustered 84 tomato identified NAC proteins into 19 different subfamilies that included 5 subfamilies for stress-related *NAC* genes with SlNAC3 members clustered with previously characterized ATNAC3 members from Arabidopsis. Gene expression analysis of *SlNAC3* genes indicated that both of them are expressed in response to drought and salinity stress conditions. The over-expression of two stress-related *SlNAC3* in tomato plants resulted in enhanced drought and salt tolerance when compared with wild type plants. The identified stress-related *NAC* genes could be a useful tool to improve tomato productivity under stress conditions.

Keywords Bioinformatics · Drought · Phylogenetics · Promoter · *Solanum lycopersicum* · Transcription factor

Introduction

Transcription factors (TFs) are highly important cellular proteins that regulate gene expression processes in living organisms. These proteins play important regulatory roles in every aspect of cellular activity in prokaryotic and eukaryotic organisms (Charoensawan et al. 2010). Transcription factors show sequence-specific DNA binding activity to regulatory elements commonly found in promoter regions of targeted genes (Agarwal et al. 2011). Therefore, they are capable of activating or repressing the transcription process of targeted genes involved in a range of different cellular functions. Plant genomes include a few thousands genes that encode TFs, and these make up, on average, ~ 2 % of their total genome size (Riechmann and Ratcliffe 2000). For instance, Arabidopsis and poplar genomes encode ~ 1,550 and 2,900 TFs, which constitute 6.2 and 6.4 % of their total encoding genes, respectively. Furthermore, TFs can be divided into families on the basis of their structural features. These features mainly include conserved DNA-binding domains, activation or repression domains and oligomerization domains (Agarwal et al. 2011). In plants, several TF families were characterized and found to play a major role in different aspects of plant growth and development.

One of the major plant specific TF families is the *NAC* gene family, which plays a major role in different aspects of plant growth and development (Puranik et al. 2012). It is

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widely distributed in the plant kingdom where it is found in monocotyledons, dicotyledons and gymnosperms (Olsen et al. 2005). For instance, in the genomes of rice, Arabidopsis, soybean and poplar more than one hundred genes were identified to encode *NAC* TFs in each plant species (Le et al. 2011; Pinheiro et al. 2009; Hu et al. 2010; Nuruzzaman et al. 2010; Ooka et al. 2003). The *NAC* gene name abbreviates the initial letters of the petunia *NAM* gene (*No Apical Meristem*), the *ATAF1/ATAF2* genes (*Arabidopsis Thaliana Activation Factor1/2*) and the *CUC* gene (*CUp-shaped Cotyledon*) from Arabidopsis (Ooka et al. 2003).

This family was originally characterized by the consensus sequence of the N-terminal DNA binding domain, commonly conserved in all family members (Aida et al. 1997). The C-terminal part of the NAC proteins however reveal diverse domain structure between the family members and are thought to be responsible for protein–protein interaction and transcription activation or repression (Grant et al. 2010; Guo and Gan 2006; Jensen et al. 2010; Kjaersgaard et al. 2011; Shan et al. 2012). In plants, *NAC* gene family members have divergent functions and they are involved in different developmental and physiological processes (Le et al. 2011; Pinheiro et al. 2009). The *NAC* family members are expressed in various tissues, as well as at different developmental stages and in response to different stimuli (Olsen et al. 2005; Hu et al. 2010; Grant et al. 2010). They are involved in shoot apical meristem development, lateral root development, xylogenesis, leaf senescence, embryo development, floral morphogenesis, grain nutrient remobilization, shoot branching determination and plant defense responses to both biotic and abiotic stresses (Hu et al. 2010; Grant et al. 2010; Mao et al. 2007; Hu et al. 2006; Hao et al. 2011; Ohnishi et al. 2005; Shen et al. 2009). Three Arabidopsis *NAC* genes (*AtNAC019, AtNAC055* and *AtNAC072*), which are induced in response to drought, high salinity and abscisic acid, were shown to bind promoter regions of several stress-responsive genes (Tran et al. 2004). The over-expression of these genes in Arabidopsis plant resulted in improved tolerance against drought stress. The drought-inducible rice *NAC* gene (*SNAC1*) was found to play a major role in drought tolerance and its over-expression resulted in drought tolerance of field grown rice plants (Hu et al. 2006). Likewise, the over-expression of another stress-related *NAC* gene, *OsNAC045,* in rice showed enhanced drought and salt tolerance (Zheng et al. 2009). In another study, the overexpression of SbSNAC1 from sorghum conferred drought tolerance in transgenic Arabidopsis (Lu et al. 2013). In chickpea, *Cicer arietinum*, the *CarNAC5* gene was induced by drought, heat, wounding, salicylic acid (SA) and indole-3-acetic acid (IAA) treatments (Peng et al. 2009). In soybean, *Glycine max*, *GmNAC20* and *GmNAC11* gene over-expression resulted in enhanced salt and freezing tolerance, and improved salt tolerance respectively (Hao et al. 2011). In barley, the overexpression of *HvSNAC1* gene was found to improve drought tolerance of field grown plants (Al Abdallat et al. 2014) and to confer resistance to Ramularia leaf spot (McGrann et al. 2014). For these reasons, such genes are considered promising targets for use in plant genetic improvement for the development of plants tolerant to adverse environmental conditions.

Recently, the *NAC* gene family has been analyzed comprehensively in different plant species including Arabidopsis, rice, soybean, poplar, potato and tomato (Le et al. 2011; Hu et al. 2010; Nuruzzaman et al. 2010; Ooka et al. 2003; Singh et al. 2013; Kou et al. 2014; Su et al. 2014). In tomato, few *NAC* genes have been characterized to date and the functions of most of them remain to be elucidated (Yang et al. 2011; Kou et al. 2014; Su et al. 2014). Since the *NAC* gene family encompasses members involved in both biotic and abiotic stress tolerance and in growth and developmental events, it is necessary to isolate and characterize stress-related *NAC* gene from tomato in order to decipher the potential for using them in tomato improvement with particular emphasis on tolerance against abiotic stresses.

In this study, the characterization of the two members of *ATNAC3*-related members in tomato (*SlNAC3*) was performed using modern bioinformatics and molecular tools. Twelve identified stress-related *NAC* members genes were subjected to multiple sequence alignment (MSA) and phylogenetic analysis to determine their relatedness and functional grouping with previously characterized members in other plants. Promoter analysis of the identified stress-related *NAC* members in tomato was carried out in order to identify conserved *cis*-elements responsible for their expression under stress conditions. Finally, the over-expression of *SlNAC3* genes in tomato plants resulted in enhanced tolerance against drought and high salt stresses.

Materials and methods

Plant material, growth conditions and stress treatments

Tomato cultivar “Moneymaker” was used for the drought and salinity gene expression analysis experiments. Transgenic tomato lines overexpressing *SlNAC3* members were used for performance analysis in comparison with Moneymaker plants. For gene expression experiments, tomato seeds were soaked in water for 2 days at 25 °C and then washed with sterilized water before sowing into small pots (10 cm diameter × 10 cm depth) filled with acid washed sand. After germination, tomato seedlings were placed under controlled conditions (continuous 25 °C temperature,

photoperiod of 16 h light/8 h dark with 80 µmol m^{-2} s^{-1} photon flux density) and irrigated daily with fixed volume of Hoagland solution. For drought treatment, 2-week-old tomato seedlings were subjected to water withholding for 3, 5 and 7 days. For salt treatment, tomato seedling roots were submerged in saline water (300 mM NaCl) for 1, 3, 5 and 10 h. Well-watered plants were included as a control for both stress conditions. For each treatment, three replicates were used and for each replicate young leaves were harvested from three plants. To evaluate transgenic lines performance under drought conditions, 2-week-old seedlings (20 plants) of wild type and transgenic lines grown under controlled conditions were subjected to water withholding conditions for 21 days and the wilting in the treated plants was monitored. For high salt stress tolerance test, the germination of tomato seeds in petri dishes containing 0.5 % NaCl was performed as described previously (Singh et al. 2012).

Identification of *NAC* genes in tomato

To identify *NAC* genes in the tomato genome, the SGN database (http://solgenomics.wur.nl) was the main source of retrieving the *NAC* genes as it is considered the main depository of tomato proteins and DNA sequences. Initially, the retrieval of tomato *NAC* genes was based on a similarity search using Basic Local Alignment Search Tool (BLAST) that relies on previously identified NAC proteins from model plants such as Arabidopsis, rice and poplar. For this purpose, the Arabidopsis and rice NAC protein sequences were downloaded from the Plant Transcription Factors Database (http://plntfdb.bio.uni-potsdam.de/v3.0/) while the poplar NAC proteins were obtained from Hu et al. (2010). The retrieved Arabidopsis, rice and poplar NAC proteins were blasted using different algorithms against different SGN databases: SGN tomato combined database, ITAG2.3 predicted cDNA and ITAG2.3 predicted protein sequences. The blast parameters were set with an e-value cut-off of $1e^{-10}$ and with 15 maximum hits to show. The retrieved tomato NAC DNA sequences were verified for the existence of a true NAC domain by performing a Blast search against the InterProScan database (Quevillon et al. 2005).

Phylogenetic analysis

Phylogenetic analysis of NAC proteins retrieved from tomato was performed to study the grouping and relatedness to other NAC proteins from different plant species. For this purpose, the retrieved NAC full length sequence or NAC domain amino acid sequences were subjected to multiple sequence alignment and phylogenetic analysis with previously characterized NAC family proteins from Arabidopsis, rice, poplar and soybean. Multiple sequence alignment (MSA) and phylogenetic analysis were carried out using the ClustalW algorithm (Thompson et al. 1997) embedded in the MEGA5 software (Tamura et al. 2011) by employing the neighbor-joining (NJ) method with a bootstrapping value of 10,000 as described previously (Pinheiro et al. 2009; Hu et al. 2010; Nuruzzaman et al. 2010). The tree branch repeatability in the phylogenetic tree is represented by the bootstrapping value that measures "confidence limits" on phylogenies.

Promoter analysis and identification of conserved motifs

For MSA analysis, the Clustal X program (version 2.0; Thompson et al. 1997) was used. The determination of five NAC sub-domains was based on Ooka et al. (2003) and the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/cdd). The complete downstream sequences from the NAC domain were investigated for potential putative motifs based on Ooka et al. (2003). The shading of consensus motif sequences was marked using the GeneDoc program (Version 2.7.000, NRBSC, USA).

For promoter analysis, the upstream sequences (~2,000 bp) of each identified tomato stress-related *NAC* gene were retrieved from the SGN web server. The upstream sequences were analyzed for the identification of regulatory *cis*-elements important for gene expression under stress conditions using the following databases: PLACE; Plant *cis*-acting regulatory elements (http://www.dna.affrc.go.jp/PLACE/; Higo et al. 1999), PLANTCARE; Plant *cis*-Acting regulatory elements (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/;(Lescot et al. 2002)).

Quantitative real-time RT-PCR analysis

For RT-qPCR analysis, total RNA was isolated from leaf samples taken from treated plants as described above. Gene-specific primers pairs for three selected stress-related *NAC* genes and *SlActin* (used as the reference internal control for relative gene expression analysis) were designed using Primer 3 Software (Rozen and Skaletsky 2000; Table 1). The amplification of the target genes was carried out using the GoTaq® qPCR Master Mix Kit (Promega, Madison, USA), and real-time detection of products was performed using Mini-Opticon Real Time PCR System (BioRad, Hercules, CA). All cDNA samples were analyzed in triplicate, and the cDNA was derived from at least two biological replicates. Thermal cycling conditions consisted of 40 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, plus a final extension at 72 °C

Table 1 List of primers used in this study

Primer name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
nptII	CTGGACGAAGAGCATCAGGG	AAAAGCGGCCATTTTCCACC
Solyc03g078400 (*SlActin*)	CCTGTTCTCCTGACTGAGGC	TGCTCCTAGCGGTTTCAAGT
Solyc10g075090 (*Le16*)	GGAAAAATTGCATGCTTTGTGG	TTGAGCAATCAGTGGAAGGG
Solyc04g005610	CCCTAAAGGTATCAAGACTG	TGAGATAGTGAACAAGTCCT
Solyc04g009440	CCCACACAATTAGCACCGAT	CCTACACCACCAAAGAACAAAG
Solyc05g007770	TGGGGAGAACTATAGAGATG	GTGGTTGTTGATAATTGAAG
Solyc06g060230	GCTCTCACCCGAATTCACAT	AGCGGCGACATCTGATAACT
Solyc07g006840	ACCTCAATGCCCTAACATGC	CCATAATCACCTCCGACACC
Solyc07g063410	GTAGAAAGCAGTGTTAGAGC	TACCCTTCCACTATTTACCC
Solyc07g063420	ACCTGCTAAGGCGACATTTG	CCAATGCCTTTTTGACACCT
Solyc10g006880	GTCAAAGAGAACGATGCATG	AGGAAACTGGCTCAGGAAAT
Solyc10g083450	CCTAACGGTCATAGGCCAAA	CAGCTGCATGCATTTTGATT
Solyc11g017470	AATGAACGGTGAAATGTCGC	TTTCCCACTCACTCGGTTTC
Solyc11g065540	TACCAATTCAGGGGCTATCG	AAATGGTGGTGGAAGGTTCA
Solyc12g013620	ATCAAGCTCCAGGCGTTCAG	ACGTCTGATTCGGACATTGG

for 5 min. The relative changes in gene expression were quantified as described in Vandesompele et al. (2002).

Isolation of *SlNAC3* members and plant transformation

Based on the obtained DNA sequence of two *SlNAC3* members, gene-specific primer pairs for *Solyc07g063410* (410Fwd: 5′-ATGGGTGTTCAAGAAATGGATC-3 and 410Rev: 5′-TTACCCAGTAAAACCCATATTTAC-3′) and *Solyc12g013620* (620Fwd: 5′-ATGGGTGTTCAAGAAAAGATCC-3′ and 620Rev: 5′-CTACTGCTTGAACCCGAGATTTA-3′) were designed. To isolate the full length cDNA of the corresponding stress-related *SlNAC3* genes, 2 week old tomato seedling cv. Moneymaker were subjected to drought stress by withholding water for 7 days. Total RNA was isolated from stressed leaf tissue using SV Total RNA Isolation System Kit (Promega, Madison, USA) following the manufacturer's instructions. The isolated RNA was used to synthesize a first strand cDNA library using the SuperScript® First-Strand Synthesis System (Invitrogen, USA) and oligo T(18) primer following the manufacturer's instructions. The full-length cDNAs of the two *SlNAC3* members were amplified using PCR in a 25 µl reaction mixture containing 5 µl of cDNA as a template, 2.5 µl of dNTPs (100 µM), 5 µl of 5 × PCR buffer, 0.5 µM of each primer and 0.25 µl of 5 U/µl GoTaq DNA polymerase (Promega, Madison, Wisconsin). The PCR conditions were 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, and a final 10 min extension at 72 °C. The amplified PCR products were separated in a 1 % agarose gel and stained with ethidium bromide. Positive PCR products were extracted from agarose gels using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin) and cloned into pGEM®-T Easy Vector System (Promega, Madison, Wisconsin) following manufacturer's instructions. Positive recombinant plasmids that contained the full-length cDNAs were fully sequenced using an ABI 3730XL machine by Macrogen (Seoul, Korea).

For transgenic plant generation, the full length cDNAs of *SlNAC3* were introduced into the binary plasmid pCABIMA1302 by replacing the *GFP* gene with the crossponding *SlNAC3* gene at the *Nco*I and *Bst*EII sites. The introduced *Solyc07g063410* and *Solyc12g013620* cDNAs were under the control of a *CaMV 35S* promoter. The binary plasmids harboring *Solyc07g063410* and *Solyc12g013620* cDNAs were used for *Agrobacterium tumefaciens*-mediated transformation of the Moneymaker cultivar at the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis (http://ucdptf.ucdavis.edu/). Transgenic seeds from T1 plants were selected on MS medium containing 50 mg/l kanamycin and lines showing 3:1 segregation for the antibiotic resistance were selected to get the T2 progeny plants. T2 plants seeds were further analyzed for transgene existence using PCR, segregation for antibiotic resistance plants and gene expression levels using RT-qPCR analysis. In addition, transgene copy number was estimated using RT-qPCR using *neomycin phosphoril-transferase II* (*nptII*) gene specific primers and the *SlActin* (*Solyc03g078400*) gene as internal control as described previously (Shepherd et al. 2009). Two T2 homozygous lines having single insertion event (reflected in *nptII* to *SlActin* ratios close to 1 as calculated in (Shepherd et al. 2009)) and showing high levels of transgene expression were selected and used for the stress experiments described above.

Results and discussion

Identification of stress-related *NAC* genes in tomato

A homology search was performed by using all previously identified NAC-protein members from Arabidopsis (126 AtNACs), rice (145 OsNACs) and poplar (163 PtNACs). The search resulted in the identification of 84 NAC-domain proteins in the tomato genome. All retrieved sequences were checked by thorough filtering and removal of redundant sequences manually. In addition, all unique protein sequences showing high similarity to NAC family members were subjected to InterProScan search using a cut-off e-value of $1e^{-10}$ to verify the presence of the NAM domain and to validate their identity (Quevillon et al. 2005). This step validated the existence of the NAC domain in the 84 *NAC* genes identified in tomato using the described approach. Detailed information on *NAC* gene family members in tomato is listed in Supplementary Table 1.

In this study, the followed methodology resulted in the identification of 84*NAC* gene family members in the tomato genome. Recently, 74 putative *NAC* genes were identified in tomato genome using a different approach based on BLAST search using 10 previously reported NACs sequences (Kou et al. 2014). In another study, 104 *NAC* genes were identified in the tomato genome using a similar approach described in this study with some differences (Su et al. 2014). The discrepancy in numbers of tomato NAC proteins could be attributed to databases sources and the e-value cutoff where Su et al. (2014) used the NCBI tomato protein database with a BLAST cutoff value of $1e^{-03}$ while the BLAST search in this study was against the SGN protein and cDNA sequences database using an e-value cutoff of $1e^{-10}$. In addition, all protein sequences identified by BLAST were considered in their study while the best 15 hits were selected here. In potato plant, Singh et al. (2013) used a Hidden Markov Model (HMM) search with a cut-off e-value of 1.0 using the NAM domain (PF02365) to retrieve 110 potato *NAC* genes. However, 17 potato NAC proteins were found to lack conserved NAC sub-domain regions and were described as NAC-like proteins (Singh et al. 2013). In rice, Nuruzzaman et al. (2010) used the NAM/NAC keywords and the Pfam (PF02365) domain amino acid sequence blast search against the NCBI and different rice databases. In poplar, Hu et al. (2010) also used the keyword search of Pfam NAC domain (PF02365) at the Phytozome database and the NAM keyword search against the NCBI nucleotide database. In Arabidopsis, Ooka et al. (2003) performed a sequence retrieval approach using the NAC InterPro domain. Therefore, the adoption of different approaches will result in variable numbers of identified *NAC* genes in different organisms including tomato.

Phylogenetic analysis of *NAC* gene family members in tomato

The phylogenetic relationships and the functional relatedness of the identified tomato NAC proteins were analyzed using two different approaches. In the first approach, combined phylogenetic trees of tomato NAC proteins were aligned with reference homologous NACs with known functions from other plant species as described previously (Hu et al. 2010; Nuruzzaman et al. 2010; Ooka et al. 2003; Fang et al. 2008; Yang et al. 2011; Rushton et al. 2008). The phylogenetic analysis was based on either full-length protein sequence or the NAC domain amino acid sequence. Using this approach, the phylogenetic trees resulted in the distribution of 55 tomato NAC proteins into 12 phylogenetic subfamilies (data not shown). This result was in general agreement with Kou et al. (2014) and Su et al. (2014) where the identified tomato NAC proteins were divided into 12 subfamilies. In the second approach, combined phylogenetic trees were constructed based on either full-length protein sequence or the NAC domain amino acid sequence aligned separately with the entire NAC family members of four different model plant species (Arabidopsis, rice, and poplar). The results of this analysis matched very well with the previous phylogenetic trees obtained using homologous NACs with some minor exceptions (data not shown). Moreover, additional tomato NAC proteins that were not clustered in phylogenetic trees after the first approach became affiliated to defined groups.

Based on the phylogenetic results produced using the two described approaches, a new phylogenetic tree was constructed using the full length amino acid sequences of tomato NAC proteins with newly selected reference homologous NACs from different plant species (Fig. 1). This approach resulted in the distribution of 83 tomato NAC proteins into 19 subfamilies with one tomato NAC (Solyc06g068580) protein remaining unlinked to any subfamily. The phylogenetic analysis of 136 potato NAC proteins with NAC proteins from Arabidopsis and rice identified 18 subfamilies (Singh et al. 2013), which is consistent with the results of the phylogenetic analysis in this study.

The distribution of the tomato NAC phylogenetic subfamilies was in general agreement with previous studies (Hu et al. 2010; Nuruzzaman et al. 2010; Ooka et al. 2003; Fang et al. 2008; Yang et al. 2011; Rushton et al. 2008). For instance, 11 subfamilies were related to Arabidopsis NAC phylogeny analysis that included the ANAC011, ATNAC3, NAC1, NAC2, NAP, ONAC022, OsNAC7, OsNAC8, SENU5, TERN and TIP (Ooka et al. 2003). Three subfamilies were named based on the rice NAC proteins phylogeny analysis that included CUC, ONAC4 and ONAC6 (Nuruzzaman et al. 2010; Fang et al. 2008). Two subfamilies were

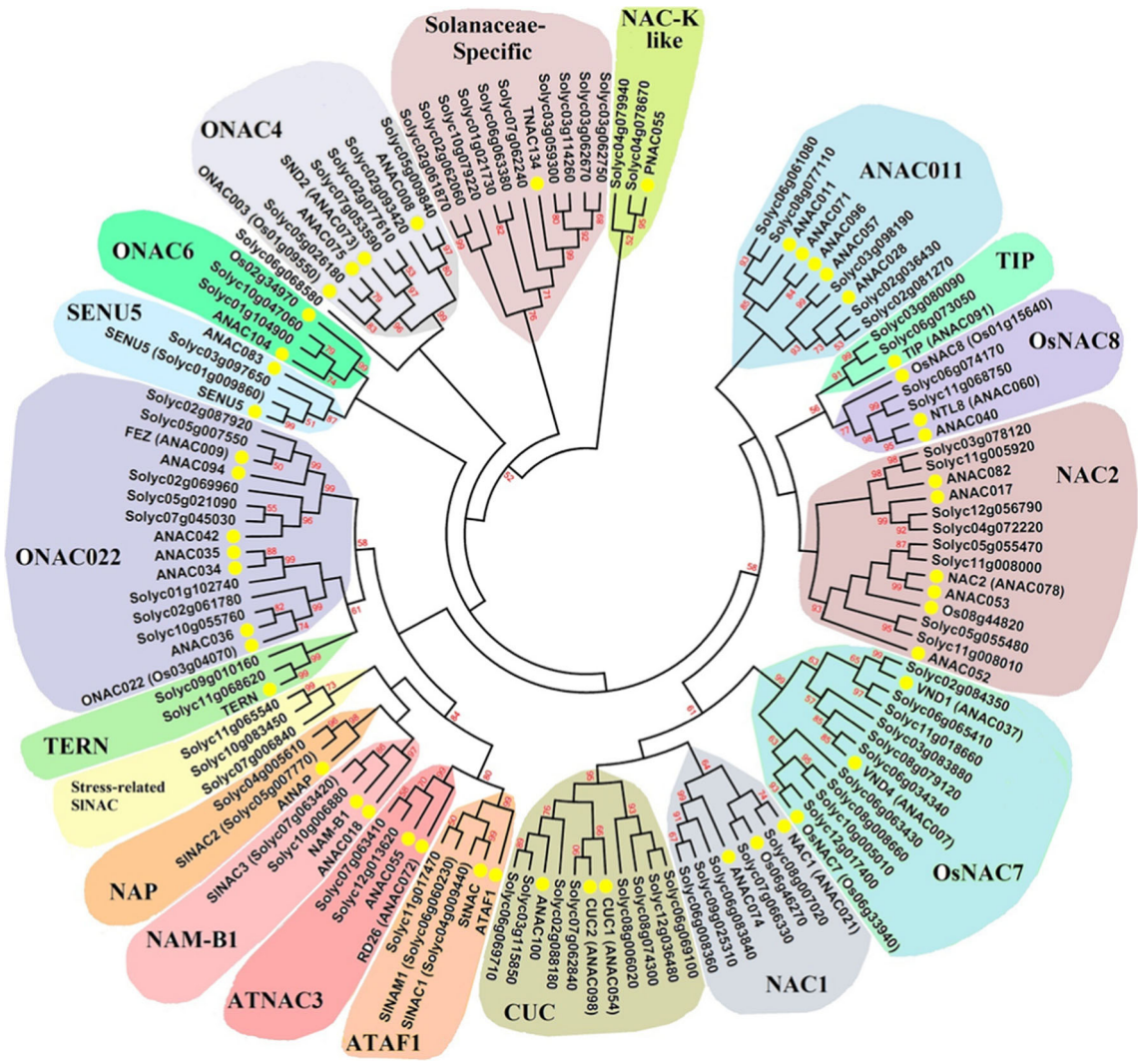

Fig. 1 Un-rooted phylogenetic tree of tomato NAC protein sequences with selected reference NAC proteins from other plant species. Each reference NAC protein is indicated with a *yellow color bullet* and each subfamily with a *specific color*. (Color figure online)

named based on soybean phylogeny that included ATAF1 and NAM-B1 (Nuruzzaman et al. 2010). One subfamily, NAC-K like, was named based on poplar NAC phylogeny analysis (Hu et al. 2010). Noticeably, a unique phylogenetic subfamily, called Solanaceae-specific, was identified that included 10 tomato NACs. The Solanaceae-specific NACs from tomato were clustered with the previously identified tobacco *TNAC134* gene (Fig. 1), which is a member of the previously described Solanaceae-specific *NAC* genes (Rushton et al. 2008). The Solanaceae specific NAC subfamily appears to be restricted to the Solanaceae family as their members were absent in Arabidopsis, rice, soybean and poplar genomes but present in tomato, pepper, and potato genomes (Singh et al. 2013;, Rushton et al. 2008). Interestingly, the Solanaceae-specific NACs from potato included 36 members and 50 members in tobacco compared to 26 members in Su et al. (2014) tomato NAC study and 10 members in the current study. Therefore, the major difference in tomato *NAC* genes number between the Su et al. (2014) analysis and the current study could be attributed to the Solanaceae-specific subfamily members.

Based on the phylogenetic analysis, the functional relatedness of several tomato NACs with reference homologous NACs from other plant species was revealed allowing fifteen subfamilies to be functionally predicted (Fig. 1). Based on the predicted functions of the reference homologous NACs from other plant species, five out of the nineteen subfamilies were predicted to include 12 stress-related tomato NACs. The five stress-related subfamilies included four subfamilies with previously characterized members from other plant species (ATAF1, ATNAC3, NAMB1 and NAP) while the remaining subfamily contains three putatively stress-related NACs that were specific to tomato (Fig. 1).. Further analysis of the tomato stress NAC subfamilies revealed that three NACs were grouped with the *ATAF1* gene, which is induced in Arabidopsis by

Table 2 Positions of stress-related CAREs in promoter regions of stress-related tomato *NAC* genes identified using PlantCare and Place databases

Subfamily	Gene name	*Cis*-elements distribution and position from TSS			
		ABRE	DRE	MYB	LTR
ATAF1	*Solyc04g009440*	1,273	–	310, 1,205, (−1,842[a])	(−328)
	Solyc06g060230	–	–	771	–
	Solyc11g017470	–	–	1,126	–
ATNAC3	*Solyc07g063410*	688,1,031	(−718)	–	–
	Solyc12g013620	106, 235, (−237), 1,621, (−1,631), 1,767	–	1,069	–
NAM-B1	*Solyc07g063420*	1,275	–	(−172), 611, (−728)	–
	Solyc10g006880	181, 1,210	–	(−118), 1,314, 1,157	–
NAP	*Solyc04g005610*	–	–	−(437), 1,174	–
	Solyc05g007770	–	–	661	–
Stress-putative NACs	*Solyc07g006840*	–	–	(−1,086), 1,809	(−789), 966, 1,876
	Solyc10g083450	–	–	(−717), 891, 1,651	120
	Solyc11g065540	1,396	–	–	–

[a] (−) indicates *cis*-elements found on the complementary DNA strand from TSS

drought, high-salinity, mechanical wounding and *Botrytis cinerea* infection (Lu et al. 2007; Wu et al. 2009). This subfamily contains two *NAC* genes (*Solyc04g009440* (also known as *SlNAC1*) and *Solyc06g060230* (also known as *SlNAM1*)) that were found to be induced by high salt stress (Yang et al. 2011). In another study, the *Solyc04g009440* (*SlNAC1*) gene was found to be induced by the *Tomato Leaf Curl Virus* and it was implicated in viral DNA replication (Selth et al. 2005). Moreover, *Solyc04g009440* (*SlNAC1*) was speculated to have important roles in mediating cross-talk between different stresses and hormonal signaling (Ma et al. 2013). The second stress NAC subfamily, ATNAC3, included two NACs that showed similarity to *ANAC055* and *ANAC072* (also known as *RD26* for *Responsive to Desiccation 26*) genes, which are induced by salt and drought stresses and confer drought tolerance in Arabidopsis (Tran et al. 2004) and tomato (Su et al. 2014). The third subfamily, NAM-B1 contain two NACs that clustered with the *NAM-B1* gene, which is known for its role in regulating grain protein content and in accelerating senescence in wheat (Uauy et al. 2006). The NAM-B1 subfamily contains a previously characterized *NAC* gene, *Solyc07g063420* that was found to be repressed by salt, drought stress and ABA treatments (Han et al. 2012). The NAP subfamily included two NACs that showed similarity to the *AtNAP* gene, which is known to regulate cell division and cell expansion in flower organs (Sablowski and Meyerowitz 1998). Moreover, *AtNAP* gene expression was associated with leaf senescence and programed cell death responses (Guo and Gan 2006). This subfamily contains one *NAC* gene, *Solyc05g007770* (also known as *SlNAC2*), that was previously studied and found to be induced by the virulence factor Conoratine produced by *Pseudomonas syringae* (Uppalapati et al. 2008). The 12 stress-related *NAC* genes identified in this study were selected and subjected to further analysis in order to clarify their roles in abiotic stress tolerance in tomato.

Promoter analysis and motifs identification

To support the phylogenetic findings and functional predictions of the stress-related *NAC* genes in tomato, a promoter and motif analysis were performed. For motif analysis, the 12 stress-related NAC genes were investigated for common motifs by performing MSA for each subfamily with their reference proteins from the phylogenetic analysis (Supplementary Figure 1). In all members, the NAC domain was located at the N-terminal region and was tightly conserved among all members with its defined sub-domains (A-E) structure, which is consistent with previous reports (Puranik et al. 2012; Ooka et al. 2003). The MSA results with reference NACs showed conserved motifs in C-terminal region, also known as the transcription activation region, which may indicate biological relevance (Supplementary Figure 1). The SlNAC3 proteins were the richest in number of identified motifs in C-terminal region that were previously characterized in ATNAC3 members of Arabidopsis and rice orthologous (Ooka et al. 2003).

For promoter analysis, *cis*-regulatory elements were identified in DNA sequences upstream of their putative start codons in the 12 stress-related *NAC* genes using different promoter analytical tools. Such analysis was carried out to identify *cis*-acting regulatory elements (CARE) in

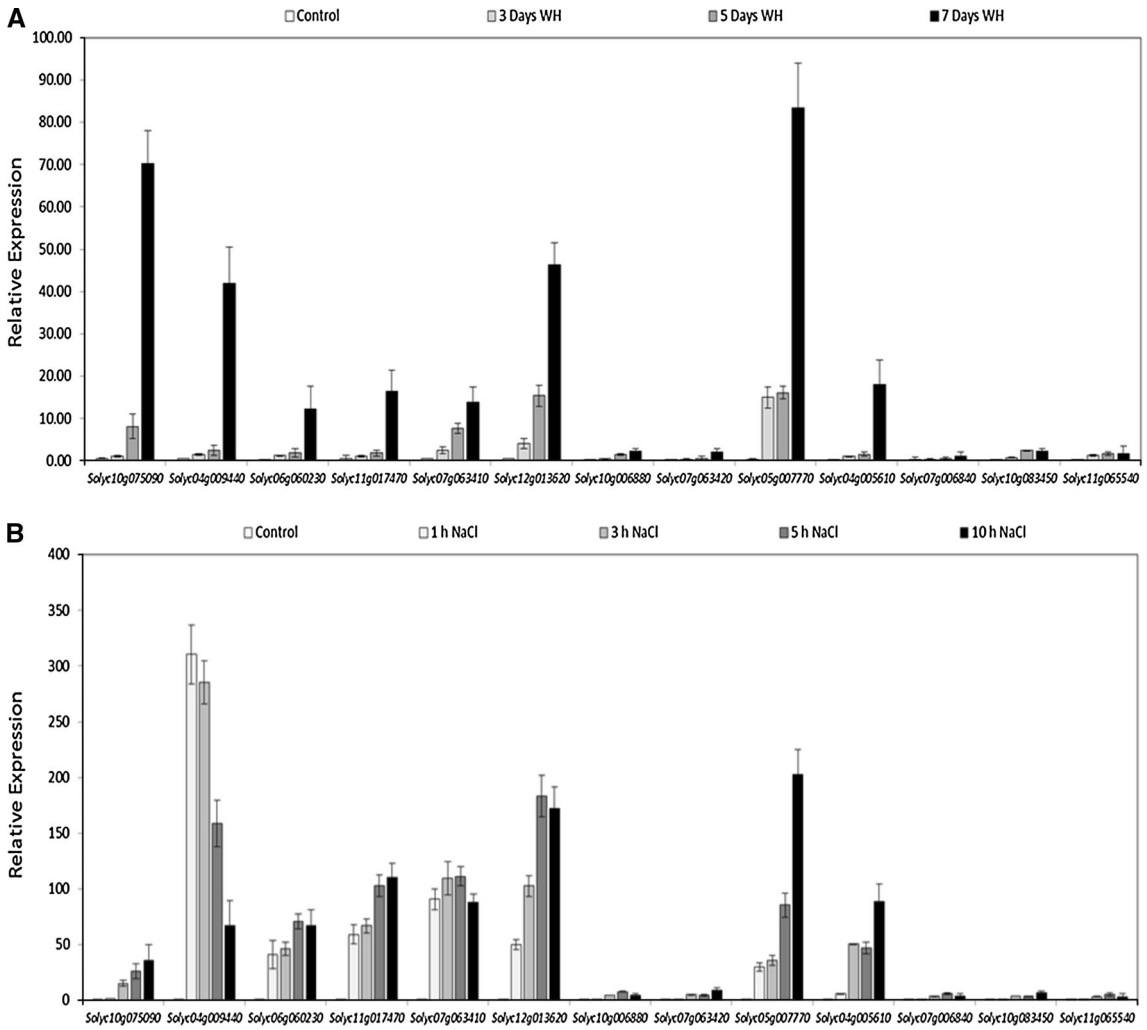

Fig. 2 Gene expression analysis of tomato stress-related *NAC* genes in response to different stress conditions. **a** Expression levels of 12 stress-related *NAC* genes in leaves of tomato plants subjected to water withholding (WH) conditions for 3, 5 and 7 days compared to well-watered plants (control). **b** Expression levels of 12 stress-related *NAC* genes in leaves of tomato plants subjected to 300 mM NaCl for 1, 3, 5 and 10 h compared to non-stressed plants (control). The *stress* responsive *Le16* (*Solyc10g075090*) gene was included as a control. The *bars* are standard deviations (SD) of three technical repeats

the upstream DNA sequences that are involved in regulation of gene expression under stress conditions. For this purpose, genomic DNA sequences, located 2 kb upstream of putative start codons were retrieved using the genome Browser tool at SOL Genomics Network (SGN; http://solgenomics.wur.nl/). Thereafter, transcription start sites (TSS) and key regulatory elements in the proximal or core promoter region including TATA box and CAAT box were identified (Supplementary Table 2) using the TSSP predication tool (www.Softberry.com, RegSite Plant DB, Softberry Inc. (Shahmuradov et al. 2003)).

To identify key regulatory elements or CARE involved in stress inducible expression, the distal promoter regions of the 12 stress-related *NAC* genes were subjected to a comprehensive promoter analysis using two CARE databases: Plant *cis*-acting regulatory elements [PLACE: http://www.dna.affrc.go.jp/PLACE/; (Higo et al. 1999)] and Plant *cis*-Acting regulatory elements [PLANTCARE: http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ ; (Lescot et al. 2002)]. Using this approach, different abiotic stress-related CAREs like *MYB*, *DRE/C-repeat*, *ABA-responsive* and *LTR* (*Low-Temperature Responsive* element) were identified in several stress-related tomato NAC promoter regions (Table 2). The data might indicate a major role for the identified stress-related *NAC* genes in regulating their gene expression in response to different stresses in tomato.

Expression patterns of tomato stress-related *NAC* genes

The expression patterns of tomato stress-related *NAC* genes in response to drought and salinity treatments were analyzed

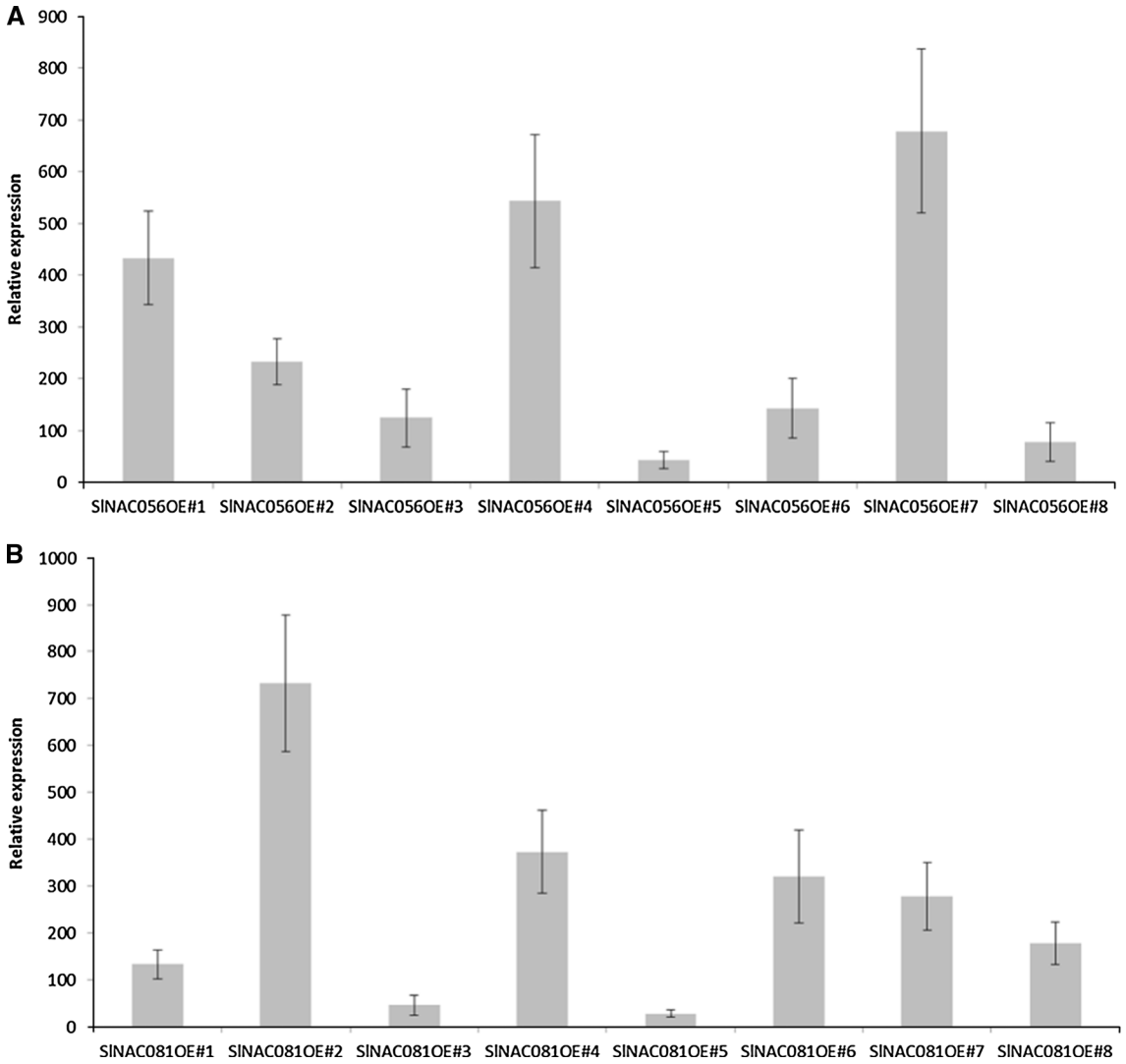

Fig. 3 Quantitative real-time PCR gene expression analysis of *Solyc07g063410* (*SlNAC056*) (**a**) and *Solyc12g013620* (*SlNAC081*) (**b**) genes in tomato cultivar "Money Maker" (Wt) and in transgenic overexpression lines (OE) used in this study. Values are mean ± SD of three technical replicates

using quantitative real-time PCR (RT-qPCR). In addition, the gene expression patterns of the stress-related *NAC* genes was compared with the patterns of the stress-inducible gene, *Solyc10g075090*, a phospholipid transfer protein from tomato also known as *Le16* [*Lycopersicon esculentum* protein 16; (Plant et al. 1991)]. The *Le16* gene is known to be highly induced by drought stress and to a lesser extent by high salt stress. The RT-qPCR was performed on all identified 12 stress-related *NAC* genes and *Le16* in response to drought and salinity treatments. No induction in response to drought and salinity treatment was observed for the NAM-B1 subfamily members, *Solyc07g063420* and *Solyc10g006880,* and for the three tomato *NAC* genes belonging to the stress-related putative subfamily (Fig. 2a, b). The observed pattern of *Solyc07g063420* was inconsistence with previous results where gene expression repression was observed after salt, drought stress and ABA treatments (Han et al. 2012). On the contrary, the *Solyc04g009440* (*SlNAC1*), *Solyc06g060230* and *Solyc11g017470,* all belong to ATAF1 subfamily showed induction in response to salinity and drought treatments (Fig. 2a, b). As shown in Fig. 2a, b, the induction patterns of *NAC* genes belonging to the ATAF1 subfamily varied in response to stress treatments where *Solyc11g017470* had the highest expression levels after drought and salinity stress followed by *Solyc04g009440* (*SlNAC1*). This was in general agreement with previous results where *Solyc04g009440* (*SlNAC1*) showed strong induction in response to different abiotic stresses that included drought and salinity (Yang et al. 2011; Ma et al. 2013; Su et al. 2014). Similarly, the NAP subfamily members, *Solyc04g005610* and *Solyc05g007770 (SlNAC2),* and the ATNAC3 subfamily, *Solyc07g063410* and

Fig. 4 Analysis of transgenic lines overexpressing tomato SlNAC3 subfamily members under drought conditions. **a** Performance of 2-week-old seedlings of transgenic lines overexpressing *Solyc07g063410* (*SlNAC056*) and *Solyc12g013620* (*SlNAC081*) and wild type under normal conditions. **b** Performance of transgenic lines overexpressing *Solyc07g063410* (*SlNAC056*) and *Solyc12g013620* (*SlNAC081*) and wild type after 21 days of water withholding. **c** Germination of transgenic lines overexpressing *Solyc07g063410* (*SlNAC056*) and *Solyc12g013620* (*SlNAC081*) and wild type after 14 days of sowing on 0.5 % NaCl

Solyc12g013620, were found to be responsive to the drought and salinity treatments (Fig. 2a, b). This is in general agreement with the induction patterns of both *SlNAC3* genes in response to PEG and NaCl treatments observed by Su et al. (2014).

Recently, two ATNAC3-related genes in potato (*PGSC0003DMG400019294* (*StNAC072*) and *PGSC0003 DMG400015342* (*StNAC101*)), was found to have high induction levels in response to different abiotic stresses including salt and mannitol treatments (Singh et al. 2013),

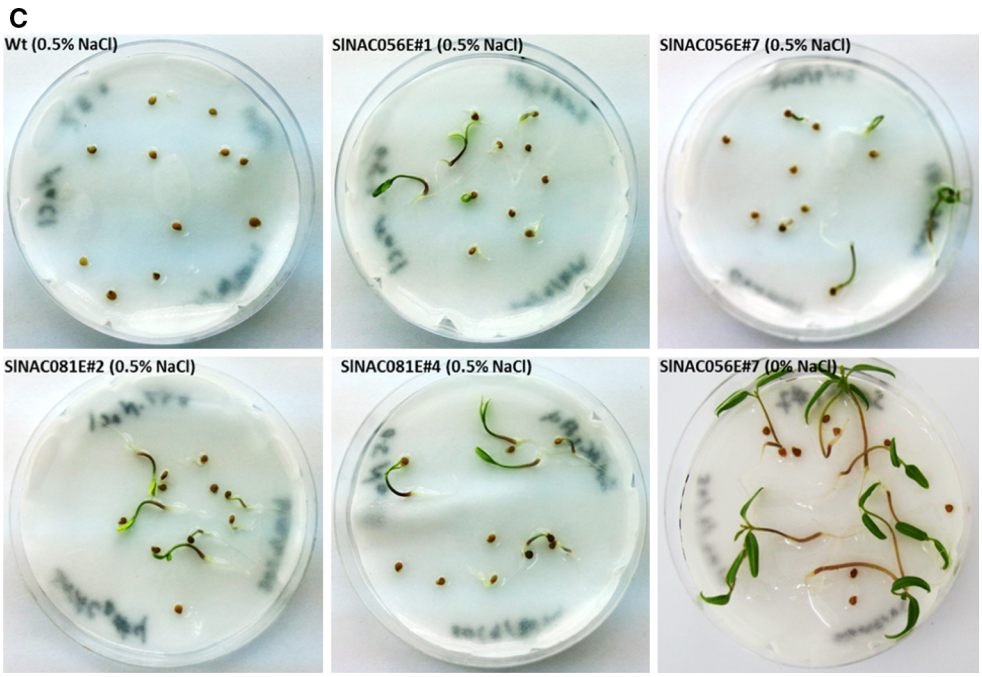

Fig. 4 continued

which indicate a conserved role of the ATNAC3 subfamily in stress tolerance in Solanaceae plants.

Overexpression of ATNAC3 subfamily members improves drought and salt tolerance in tomato

To validate the bioinformatics and gene expression results in predicting the stress-related *NAC* genes in tomato, members of the uncharacterized SlNAC3 subfamily, *Solyc07g063410* and *Solyc12g013620*, were selected to validate their role in mediating tolerance against drought stress. For this purpose, transgenic plants overexpressing *Solyc07g063410* and *Solyc12g013620* full length CDS were generated. Positive transgenic lines overexpressing the targeted genes were confirmed for the transgene copy number and gene expression levels by RT-qPCR analysis (Fig. 3). Positive transgenic lines with single transgene copy and overexpressing *Solyc07g063410* and *Solyc12g013620* showed growth retardation phenotypes where transgenic plants were shorter, with smaller leaf area and shorter internodes when compared with the wild type plants (Fig. 4a). These pleiotropic phenotypes were more pronounced in transgenic lines overexpressing *Solyc12g013620* when compared with lines overexpressing *Solyc07g063410*. Such variation in growth retardation phenotypes was also observed in transgenic Arabidopsis plants overexpressing three ATNAC3-related genes (*ANAC019*, *ANAC055* and *ANAC072*) (Tran et al. 2004).

To analyze stress tolerance in the transgenic tomato lines, 2-week-old seedlings growing in sand culture were subjected to drought conditions by withholding water for 21 days and salt stress by germinating seeds directly on 0.5 % NaCl. Wild type plants were included as controls and their performance under the same conditions were compared with that of the transgenic lines. Under drought conditions, nearly all non-transgenic plants had a wilted appearance indicating that they were suffering from water deficit (Fig. 4b). However, transgenic tomato plants overexpressing *Solyc07g063410* and *Solyc12g013620* genes showed delayed wilting and enhanced tolerance to drought conditions when compared with non-transgenic plants (Fig. 4b). Furthermore, transgenic tomato plants overexpressing *Solyc07g063410* and *Solyc12g013620* genes had higher germination percentages (after 14 days of sowing) on 0.5 % NaCl solution when compared with wild type plants (Fig. 4c). However, under such conditions, the transgenic plants showed slower growth rates and yellow cotyledons appearance when compared with non-stressed plants. The drought tolerance behavior of *SlNAC3* genes was similar to their counterparts in Arabidopsis where the overexpression of *ANAC019, ANAC055* and *ANAC072* conferred drought tolerance (Tran et al. 2004). Recently, the overexpression *Solyc04g009440* (*SlNAC1*) in tomato plants enhanced chilling tolerance through the maintenance of higher oxygen-evolving enzymatic activities that lowered ion leakage and malondialdehyde content in stressed transgenic plants when compared with non-transgenic plants (Ma et al. 2013). These results confirm that the bioinformatics approach used in this study was successful in predicting the functions of stress-related *NAC* genes in

tomato and uncovered their potential role in enhancing tomato growth under stress conditions.

In conclusion, 12 stress related *NAC* genes were identified in tomato using different bioinformatics and molecular approaches. The phylogenetic analysis clustered them into 5 different subfamilies with their functions predicated from their counterparts in other plants species. The phylogenetic analysis identified a new stress related subfamily specific to tomato with no clear role in stress tolerance till now. Promoter analysis of the stress-related *NACs* identified key regulatory elements involved in stress inducible expression. Furthermore gene expression analysis of stress-related *NACs* showed inducible expression patterns to different stresses including drought and salinity of different members. The overexpression of two selected stress-related *SlNAC3* genes in tomato resulted in enhanced drought tolerance when compared with non-transgenic plants. Finally, the comprehensive analysis described in this study provides valuable information on the putative functions of the identified stress-related tomato *NAC* genes, which will help in the future improvement of the tomato crop as an important commodity in the horticultural industry.

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