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Identification and expression pattern of one stress-responsive NAC gene from *Solanum lycopersicum*

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Abstract NAC (for NAM, ATAF1, 2, and CUC2) family genes have been found to play an important role in diversified developmental processes and environmental responses. A new NAC-type transcription factor *SlNAC3* was primarily identified and isolated from the cDNA libraries of tomato cultivar Ailsa Craig. It contains three exons and two introns within genomic DNA sequence and encodes a polypeptide of 329 amino acids. A plant-specific and conserved NAC domain is located in the N-terminus of SlNAC3. The protein SlNAC3 is subcellularly localized in the nucleus of onion epidemical cells and it has a transcriptional activation domain in the C-terminal region which shows extremely divergent among NACs. Phylogenetic analysis showed that *SlNAC3* belonged to the *OsNAC3* subgroup of the NAC protein family. Tissue expression profile analysis revealed that *SlNAC3* was expressed mainly in flower, fruit and root. The transcription expression of *SlNAC3* was inhibited by salt, drought stress and ABA treatment. These data demonstrate that SlNAC3 might interact with environmental and endogenous stimuli and probably function when plants response to salt and drought stresses through ABA signaling pathways as a transcriptional activator.

Keywords Expression profile · *SlNAC3* · Stress · Tomato · Transcriptional activity

Abbreviations

ABA	Abscisic acid
ABRE	ABA-responsive element
AC	Ailsa Craig
CaMV	Cauliflower mosaic virus
CUC	Cup-shaped cotyledon
DBD	DNA-binding domain
DRE/CRT	Dehydration responsive element/C-repeat
NAM	No apical meristem
NLS	Nuclear localization signal
SAM	Shoot apical meristem
UTR	Untranslated region
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

Introduction

Plants are exposed to kinds of environmental stresses such as cold, drought and high salinity. The mechanism of plants responses to different stresses has been investigated by studying the genes regulation pattern under stress conditions [1]. Till now, many transcription factors are reported to be involved in plant development and abiotic stress tolerance in tomato. Some function through binding with *cis*-acting elements of stress responsive gene promoters, or interacting with some other transcription factors, and then inducing or repressing relative gene expression [2]. Various transcription factors with functional domains such as

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AP2/EREBP, bZIP and NAC have been well studied in plants [3, 4].

The NAC is an acronym derived from the names of the first three genes that found to contain this domain, namely *NAM* from *Petunia* [5], *ATAF1*, *ATAF2* (GenBank accession numbers X74755 and X74756) and *CUC* from *Arabidopsis* [6]. It was reported that *NAM* might have important function in determining positions of meristems and primordia in *Petunia* [5]. *ATAF1* and *ATAF2* are negative regulators of defence response against kinds of pathogens in *Arabidopsis* [7, 8], while *CUC* genes are redundant that are involved in SAM formation and cotyledon separation during embryogenesis in *Arabidopsis* [9].

The NAC genes have been characterized with a highly divergent C-terminus and a conserved NAC domain in the N-terminus, which forms a twisted beta-sheet surrounded by a few helical elements and specifically binds with target DNA [10]. Normally the C-terminal region of NAC protein shows highly divergent in sequences and is considered to be a putative transcriptional activation domain [11]. The NAC domain has been shown to be a DBD and a dimerization domain [12, 13]. Putative NLS have been detected in subdomains C and D [14]. The DBD is contained within a 60 amino acid region located within subdomains D and E [13]. As a widespread and plant specific transcription factor, the *NAC* genes have been confirmed to play an important role in the process of plant growth and development [15]. Such as *NAP* gene which belongs to *NAC* gene family in *Arabidopsis* was reported to have function in flowering [16], and played a role in controlling cell division and cell expansion in stamens and petals [16]; Arabidopsis *NAC1* could transfer the auxin signal and promote lateral root development [12].

Increasing evidences also suggest that *NAC* family transcription factors play important roles in plant responses to pathogens, viral infections, and kinds of environmental stimuli [17, 18]. *SNAC1* was reported to play an important role in rice drought resistance and salt tolerance both at the vegetative stage and reproductive stage, and also have function in increasing the plants sensitivity to abscisic acid and decreasing water losing [19]. *AtNAC2* from *Arabidopsis* can be induced by salt stress and also several hormones including ABA, ACC and NAA, it can also mediate the environmental and endogenous stimuli and function in promoting plant lateral root development [11].

In this study, a new *NAC* gene *SlNAC3* isolated from tomato flower cDNA library was reported. The digital expression profile of *SlNAC3* showed abundance in tomato root tissue and fruit, whereas low in other tissues, revealed tissue-specifically expressed feature. Our results showed that *SlNAC3* encodes a transcription factor and has conserved NAC domain. The tissue expression profile and environmental responses were also investigated. Based on the data, we proposed that it may present a new *NAC* gene that regulates plant response to environmental stimuli.

Materials and methods

Plant materials and growth conditions

Plants of AC were grown in a climate-controlled greenhouse under sodium lights timed at the regime of 16-h days (24°C) and 8-h nights (18°C).

Methods

Bioinformatics analyses

GENESCAN (MIT, Cambridge, MA) and sequence alignment between cDNA and genomic DNA were used to analyze the exons and introns of gDNA. The theoretical molecular weight (Mw) was calculated with the ExPASy compute pI/Mw tool (http://expasy.org/tools/pi_tool.html) [20]. *cis*-Acting regulatory element in the promoter sequence was analyzed using PlantCARE (http://www.dna.affrc.go.jp/PLACE/index.html). Subcellular location of protein was predicted with ProComp v8.0 (http://linux1.softberry.com/berry). Multiple sequence alignment was performed using the ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Boxshade was produced by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). For phylogenetic tree analysis, peptide sequences of NAC genes were identified based on BLAST searches. Maximum parsimony trees were generated using PAUP 4.0 through heuristic searches of ten random stepwise additions. Tree support was assessed with bootstrap analysis.

RNA isolation, RT-PCR and real-time RT-PCR

Total RNA was isolated from root, stem, leaf, flower and fruits with the Trizol reagent (Invitrogen, USA). Dnase-I (Promega) treated RNA was reverse-transcribed using a high capacity cDNA reverse transcription kit (TOYOBO, Japan) and cDNA was used for RT-PCR and real-time RT-PCR. For RT-PCR detection of SlNAC3 transcript, the PCR reaction was performed with pre-incubation at 95°C for 4 min and followed by 25 or 27 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. Primers were designed with the Primer3 program (http://frodo.wi.mit.edu/primer3/input.htm). For real-time RT-PCR, the amplification was performed in a total reaction volume of 20 μl. Reactions included 5 μl of template, 10 μl of SYBR Green I Master (Roche, American), 1.0 μl of reverse primer (10 μM),

1.0 μl of forward primer (10 μM), and 3.0 μl of sterile molecular biology-grade water. All PCRs were performed with the same cycling conditions: 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 58°C for 15 s and 72°C for 20 s. The primers used are listed in Table S1.

Subcellular localization of SlNAC3

The *SlNAC3* coding region was amplified with primer 1 and primer 2 (*Sal*I site added at N-terminal and stop codon deleted), with plasmid pMD-SlNAC3 served as template. The ORF of *GFP* was amplified using primer 3 and primer 4 (added *Sac*I site), with plasmid pBIG (which contains *GFP*) served as template. Then fused gene *SlNAC3-GFP* was amplified using primer 1 and primer 4, those two PCR products together as template. Fused gene and pBI121 vector were digested with *Sac*I and *Sal*I, and then linked to get pBI-SlNAC3-GFP construct under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The resulting construct was then sequenced to confirm an intact in-frame fusion. The pBI-GFP vector, also controlled by the CaMV 35S promoter, was used as positive control. The two constructs were introduced into onion epidermal cells on solid plates for transient expression analysis with a Biolistic Particle Delivery System (Bio-Rad, Hercules, CA, USA). The bombarded onion cells were cultivated for 24 h at 25°C in the dark. After that, the epidermal layer was placed on a microscope slide for observation under Confocal microscope (Olympus FV500, Olympus, Tokyo, Japan). The relative parameters were as follows: emission at 488 nm, beam splitter at 545 nm, and excitation at 505–530 nm. All the images were processed with Adobe Photoshop 7.0. The primers used are listed in Table S1.

Transcriptional activation activity of the SlNAC3 protein

The yeast strain AH109 containing the *His3* and *lacZ* reporter genes was used as an assay system (Clontech). The coding sequence of SlNAC3, the fragment of SlNAC3 encoding N-terminal and C-terminal were obtained separately. The PCR primers names were as follows: SlNAC3-full for SlNAC3; SlNAC3-N for the N-terminal of SlNAC3; SlNAC3-C for the C-terminal of SlNAC3. According to the protocol of the manufacturer (BD Bioscinces Clontech), pGBKT7-SlNAC3 (the full length), pGBKT7-SlNAC3-N (N-terminal), pGBKT7-SlNAC3-C (C-terminal) and the negative control pGBKT7 vector (Clontech), were all transformed into the yeast strain AH109. The transformed strains were confirmed by PCR and then were streaked on SD/Trp^{-} and SD/Trp^{-}/His^{-} plates separately. The transcriptional activation activities of each protein were evaluated according to their growth status.

Gene expression analysis under stress and ABA treatment to seedlings

For tissue expression profile analysis, plants of AC were grown in soil under normal conditions; tissues of root, stem, leaf, flower and fruit that represent major tissues and the AC seedlings were frozen in liquid nitrogen immediately and used to extract RNA.

For stress and ABA treatment, AC seedlings were grown to 35 days (flower bud appearing stage). For drought stress, the seedlings were put on the surface of filter paper on the clean bench. For NaCl stress and ABA treatment, sprayed the whole seedlings with concentration of 200 mM and 100 μM separately. The seedlings sprayed with water were taken as control. For all the stress and ABA treatment, three individual seedlings as one sample were taken for 0, 1, 3, 6, 12 h, respectively.

Results

Identification and sequence analysis of *SlNAC3*

The full-length of a new *NAC* cDNA was obtained by RT-PCR. We named it *SlNAC3* followed with *SlNAC1* [21] and *SlNAC2* [22] that had been functionally identified in tomato. Sequence analysis showed that *SlNAC3* cDNA contained an open reading frame (ORF) of 990 bp, a 5′-UTR of 201 bp, and a 3′-UTR of 390 bp. The predicted SlNAC3 protein has 329 amino acids with an estimated molecular mass of 37 kD. There is an N-terminal module of 160 amino acids and a conserved acid region between amino acid 16 and 176 (Fig. S1). Additionally, we amplified *SlNAC3* from tomato genomic DNA and compared with its cDNA sequence. We found that *SlNAC3* gDNA sequence contained three exons and two introns (Fig. S2), the same as that of the other *NAC* genes [23].

To further analyze the NAC domain of SlNAC3, the overall amino acid sequences of NACs were analysed for alignment. Result showed that there are five conserved sub-domains (A–E) in the NAC domain region (Fig. S1). Each subdomain is distinguishing by blocks of heterogeneous amino acids or gaps.

A phylogenetic tree was conducted between the conserved domain protein of SlNAC3 and other known NAC-domain proteins. As shown in Fig. S3, SlNAC3 was highly homologous to NOR, AtNAC2 and OsNAC3, with similarity of 88, 88 and 70%, respectively (data not shown). Therefore, SlNAC3 belonged to OsNAC3 subgroup.

Promoter analysis

The 2,043 bp promoter region of the *SlNAC3* was evaluated for the presence of putative *cis*-acting regulatory

elements. A few stress-responsive related *cis*-acting elements were found in the *SlNAC3* promoter including ABRE (ACGTG), DRE/CRT complex (A/GCCGAC), pathogen and wound-responsive related (WRKY element, TGAC), drought-responsive related (MYB element, CNGTTR) and guard cell-specific related (DOF core element, AAAG) (Table 1). All of these elements display important stress tolerance function of genes. Two copies of ABRE in the *SlNAC3* promoter confirmed our conclusion, because repeated copies of ABRE can confer gene's responsiveness to ABA, while single copy of ABRE will not show responsive to ABA [24], and genes that contained both ABRE complex and DRE/CRT elements are activated by abiotic stress in the absence of ABA [25].

Table 1 Putative *cis*-acting element in the SlNAC3 promoter

cis-Element	Position (strand)	Sequence
ABRE element	1785 (+), 1792 (+)	ACGTG
WRKY element	1425 (+), 1916 (+)	TGAC
MYB core element	529 (+), 683 (+), 870 (+), 918 (+), 1147 (+), 1239 (+),	CNGTTR
DOF core element	248 (+), 465 (+), 559 (+), 590 (+), 637 (+), 799 (+), 822 (+), 878 (+), 1013 (+), 1093 (+), 1132 (+), 1192 (+), 1219 (+), 1301 (+)	AAAG

Positions of the *cis*-elements are related to the translation start sites. Sequences are indicated from 5′ to 3′

Expression pattern of *SlNAC3*

The expression pattern of *SlNAC3* in different tomato tissues were detected by RT-PCR and verified with real-time RT-PCR, operating with different primers. Both results seemed to be identical, the transcription of *SlNAC3* was abundant in flower, fruit and root, low in leaf and undetectable in stem (Fig. 1a, b).

The transcription expression of *SlNAC3* was further investigated by abiotic stresses and ABA treatment in seedlings. When exposed to drought, the transcripts of *SlNAC3* significantly decreased to 20–30% of control (Fig. 1c), means *SlNAC3* is highly responsive to drought stress. Results also showed that *SlNAC3* was significantly repressed when seedlings treated with NaCl for 1 and 3 h, however, the expression level came to normal after 6 h (Fig. 1d). During the process of seedlings treated with ABA for 1 and 12 h, *SlNAC3* expression showed distinctly inhibited to 20–35% of control (Fig. 1e). It means that SlNAC3 is involved in ABA-related stress responses.

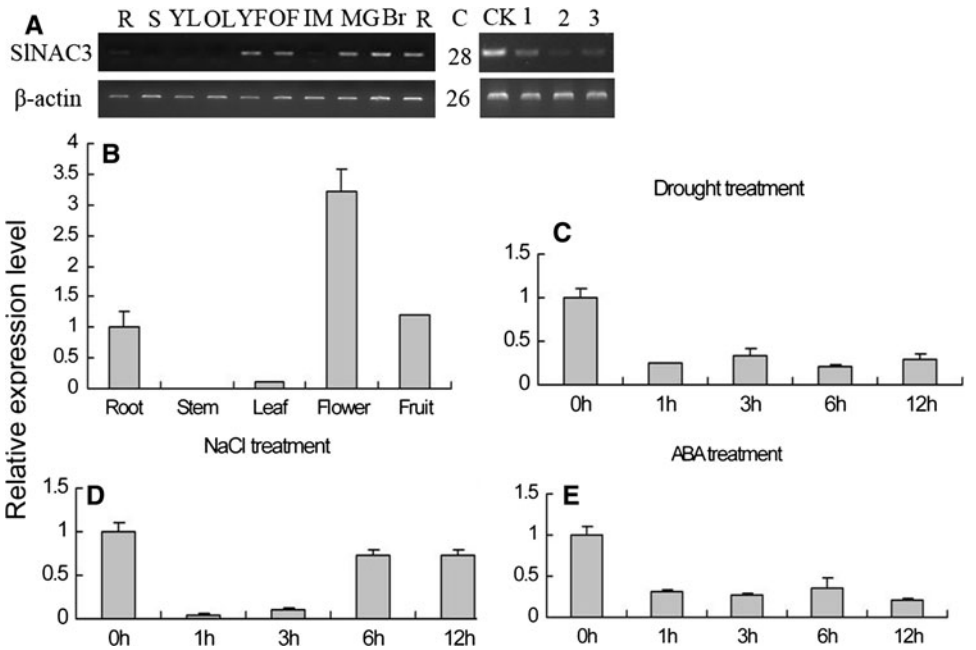

Fig. 1 Expression pattern of *SlNAC3*. **a** RT-PCR analysis of *SlNAC3* in wild-type tissues and seedlings treated with different stresses. *R* root, *S* stem, *YL* young leaf, *Ol* old leaf, *YF* young flower, *Ol* old flower, *IM* immature fruit, *MG* mature green fruit, *Br* breaker fruit, *R* red fruit, *C* PCR cycles, *CK* seedlings of control, *1* seedlings treated with drought for 1 h, *2* seedlings treated with NaCl (200 mM) for 1 h, *3* seedlings treated with ABA (100 μM) for 1 h. Tomato gene encoding *β*-actin was used as internal control. **b** Real-time RT-PCR analysis of *SlNAC3* in different tissues (root, stem, leaf, flower and immature fruit) of wild-type. **c–e** *SlNAC3* expression upon drought, NaCl (200 mM) and ABA (100 μM) treatments. The *error bar* indicates the SE of three independent replications. Compared Ct method was used for the data analysis

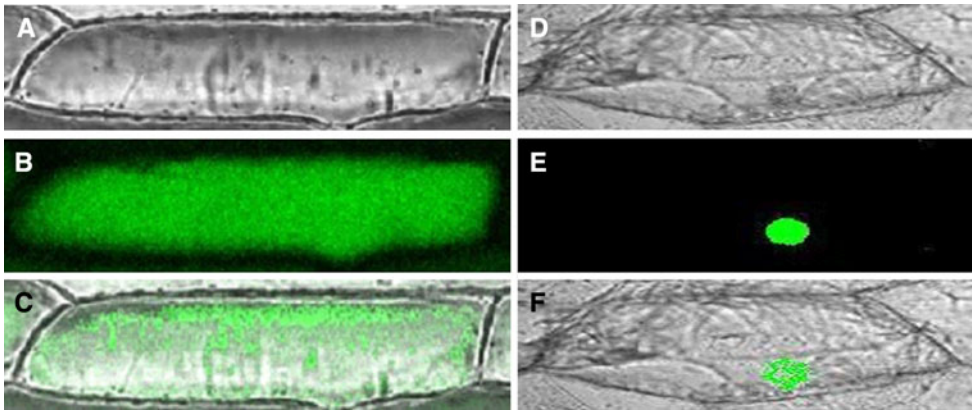

Fig. 2 Nuclear localization of SlNAC3 protein in onion epidermal cell. GFP alone or SlNAC3-GFP fusion protein was expressed transiently under the control of the CaMV 35S promoter in onion epidermal cells and then observed under a confocal microscope. The photographs were taken in the bright light for the morphology of the cell (**a**, **d**), in dark field for green fluorescence (**b**, **e**), and in combination (**c**, **f**). **a–c** Transformed cell expressing GFP control protein. **d–f** Transformed cell expressing the SlNAC3-GFP fusion protein

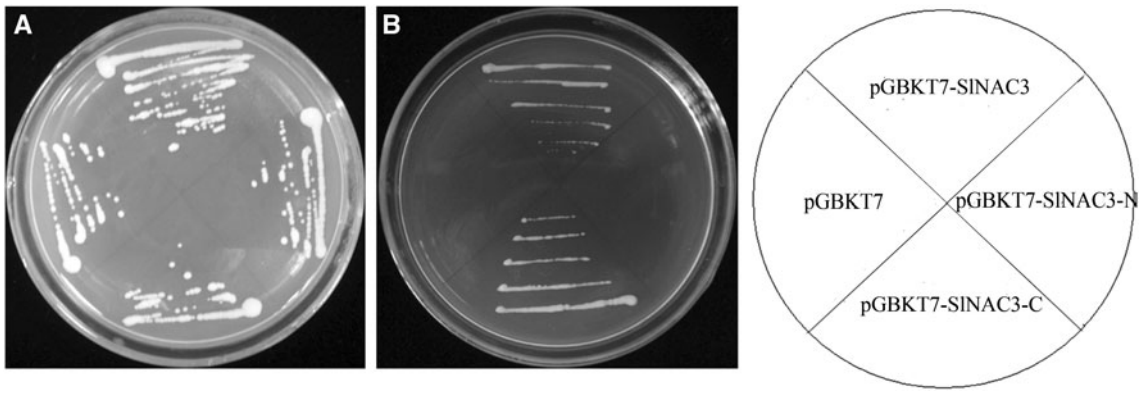

Fig. 3 Transcription activity of SlNAC3. Transactivation analysis of pGBKT7-SlNAC3, pGBKT7-SlNAC3-N and pGBKT7-SlNAC3-C, compared with negative control pGBKT7. The transformants were streaked on the SD/Trp$^-$ (**a**) and SD/Trp$^-$/His$^-$ medium (**b**), respectively, for examination of growth status

Subcellular localization of SlNAC3

Bioinformatics revealed that SlNAC3 protein was located in the nucleus. The subcellular localization of SlNAC3 in vivo was conducted by using a construct containing *SlNAC3-GFP* fusion gene in the plasmid pUC18. The *SlNAC3-GFP* fusion gene and *GFP* control in pUC18, both under the control of the CaMV 35S promoter, were transformed into onion epidermal cells by particle bombardment. Protein expression was observed under a confocal microscope. Our results showed that the SlNAC3-GFP fusion protein was targeted to the cell nuclei, while the control was observed in the whole cell (Fig. 2). It means that SlNAC*3* was a nuclear protein.

Transcriptional activation activity of SlNAC3

We examined the transcription activation activity of SlNAC3 protein using a yeast expression system. It could be seen that all of these yeast cells grew well on SD/Trp$^-$ medium (Fig. 3a). The yeast cells containing C-terminus of SlNAC3 (pGBKT7-SlNAC3-C) and the full length (pGBKT7-SlNAC3) grew well on SD/Trp$^-$/His$^-$ medium, while the cells containing N-terminus of SlNAC3 (pGBKT7-SlNAC3-N) and the negative control plasmid pGBKT7 could not grow (Fig. 3b). The results revealed that the C-terminal of SlNAC3 had transcriptional activation activity, while the N-terminal did not. In the presence of X-Gal, the yeast cells turned blue on the SD medium without histidine (data not shown), indicating the activation of another reporter gene *LacZ*.

Discussion

It was reported that there were at least 107 *NAC* genes in *Arabidopsis* [26], while 140 in rice have been identified [27]. There are usually more than 100 *NACs* in plant

genomes based on the information of decoded genome [28], and we speculate there may be over 100 *NAC* genes in tomato. Although quite a large number of *NAC* genes have been identified and evaluated for their functional significance in model plants *Arabidopsis* and rice, still there are many NAC members remained to be functionally unknown.

NAC-domain family members are defined by the highly conserved N-terminal region [29], and they encode a novel family of transcription factors that unique to plants [12]. The transcriptional activity of SlNAC3 protein in yeast existed in C-terminal region (pGBKT7-SlNAC3-C) and the NAC domain of SlNAC3 (position 16–176) did not activate reporter genes in yeast cells, indicating that SlNAC3 can function as a transcriptional activator in yeast, and also the transcriptional activation domain is located in the C-terminal part of the protein. This result was in accordance with several *NAC* members that were reported before, such as *OsNAC19* in rice and *CarNAC3* in *Cicer arietinum* [30, 31].

Transcription factors should locate in the nucleus to play a role in plants and are supposed to have NLS. For SlNAC3, the NLS was predicted to be in the subdomain D (Fig. S2) which is highly conserved in NAC proteins. Our result showed SlNAC3 was targeted to the nucleus in plant cell (Fig. 2), suggesting that SlNAC3 functioned as a transcriptional activator.

Based on the sequence similarity, *NAC* genes of rice and *Arabidopsis* could be classified into two groups (Group I and II) and 18 subgroups [32]. In this study, the overall amino acid sequences were used for phylogenetic analysis. Result revealed that SlNAC3 fell into the OsNAC3 subgroup containing NOR, AtNAC2 and OsNAC3. It is a general assumption that genes involved in the same subgroup have similar biochemical functions. *NOR* is related to fruit ripening in tomato, and loss-of-function mutant *nor* fruits cannot ripe normally, have no respiratory peak or ethylene emission during fruits maturation [33]. *AtNAC2* was involved in lateral root development and also be induced by NaCl, ABA, ACC and NAA in *Arabidopsis* [11], and *OsNAC3* was found to be induced by drought stress and the transgenic rice that over expressing this gene showed improved dehydration tolerance [34].

SlNAC3 was moderately expressed in flowers and fruits, revealing that *SlNAC3* was involved in flower/fruit development like *NOR* which showed high similarity with *SlNAC3*. Also in Arabidopsis flowers, *NAP* in NAC family which expressed in flower organ was identified to have function in stamen and petal formation [2]. Moreover, we found that *SlNAC3* also expressed highly in root, suggesting its function in root as well. Because *AtNAC2*, which is *OsNAC3* subgroup member, was found to be expressed mainly in root, and Arabidopsis NAC1 which chiefly expressed in root can promote lateral root development [12]. It can be assumed that *SlNAC3* is involved in root related stress responses.

Plants are confronted with kinds of stresses which induce or suppress the expression of a large number of genes. Majority of the *NAC* family members are known to be involved in various biotic and abiotic stresses as transcriptional regulators [5, 12]. ABA is reported to play an important role in plant abiotic stress tolerance [35], and drought resistance is regulated by both ABA-dependent and ABA-independent pattern. The gene reported here shows significantly repressed to drought, NaCl and also to ABA. So *SlNAC3* seems to have a potential role in the regulation of ABA-related drought stress. As many *NAC* family members have shown different responses to abiotic stresses. For example, *CsNAC* in "Navel" orange, was significantly induced by cold, wounding stress and ethylene [36]. Chickpea gene *CarNAC5* was also reported to be significantly induced by various stress responses including drought, heat, wounding, salicylic acid (SA), and indole-3-acetic acid (IAA) treatments [37]. The molecular analysis and expression pattern of *SlNAC1* and *SlNAM1* revealed that they are salt-responsive NAC-family members [28], and the tissue expression analysis and the defense response to environmental stimuli of *TaNAC4* demonstrated this wheat NAC transcription factor is a novel pathogen-inducible NAC gene [38].

Majority of drought-responsive genes function through ABA mediated pathway [39, 40]. Drought and salt stresses alter the accumulation level of ABA which plays important role in plants adaptation to abiotic stress. Many ABA-responsive genes contain ABRE in the promoter regions. Other transcription factors such as WRKY, MYC and MYB are also involved in ABA-dependent stress-signaling transduction pathway [41, 42]. Several *cis*-acting regulatory elements were found in *SlNAC3* promoter. Among of these regulatory elements, ABRE element were reported to participates in dehydration and salinity stress [43, 44]. Some genes such as NaCl and drought-induced gene *RD29A*, which contains ABRE and DRE/CRT complex in their promoter regions could be significantly activated by abiotic stress [45]. Six MYB recognition sites were found in the promoter region of *SlNAC3*, they were reported to be necessary in the drought and ABA-response for *RD22* [46–48], which functions as an important gene to salt stress tolerance [49]. The promoter analysis also showed that there were 14 DOF core elements, which can activate Dof transcription factors in guard cell-specific gene expression and function in plant defense [23, 50]. From all these analysis, we can deduce that *SlNAC3* is responsive to abiotic stresses such as salt and drought.

It was expected that *SlNAC3* might represent a gene that incorporate the stress response and/or endogenous factors

into plant development. Our data indicated that *SlNAC3*, as a transcription activator, is likely to be involved in various developmental processes and responses to drought stress. It would be interesting to identify the function of *SlNAC3* in transgenic plants under stress conditions. Therefore, we are currently investigating what effect *SlNAC3* would bring to transgenic tomato and how *SlNAC3* is integrated into ABA signaling pathways using over-expression and RNAi strategy.

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