Research Article

Le Jing, Jie Li, Yuzhu Song, Jinyang Zhang, Qiang Chen, Qinqin Han*

Characterization of a potential ripening regulator, *SlNAC3*, from *Solanum lycopersicum*

https://doi.org/10.1515/biol-2018-0062
Received May 22, 2018; accepted November 1, 2018

Abstract: NAC (for *NAM*, *ATAF1-2*, and *CUC2*) proteins are one of the largest transcription factor families in plants. They have various functions and are closely related to developmental processes of fruits. Tomato (*Solanum lycopersicum*) is a model plant for studies of fruit growth patterns. In this study, the functional characteristics and action mechanisms of a new NAC-type transcription factor, *SlNAC3* (SGN-U568609), were examined to determine its role in tomato development and ripening. The SlNAC3 protein was produced by prokaryotic expression and used to immunize New Zealand white rabbits to obtain a specific polyclonal antibody against SlNAC3. By co-immunoprecipitation and MALDI-TOF-MS assays, we showed that there was an interaction between the SlNAC3 protein and Polygalacturonase-2 (PG-2), which is related to the ripening and softening of fruit. Chromatin immunoprecipitation assays revealed the genome of the highly stress-tolerant *Solanum pennellii* chromosome 10 (sequence ID, HG975449.1), further demonstrating that *SlNAC3* is a negative regulator of drought and salinity stress resistance in tomato, consistent with previous reports. These results indicate that *SlNAC3* is not only involved in abiotic stress, but also plays a necessary role in mediating tomato ripening.

Keywords: Gene expression; protein polyclonal antibody; ripening; SlNAC3; stress tolerance; tomato developmental process

***Corresponding author: Qinqin Han,** Engineering Research Center for Molecular Diagnosis, Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, Yunnan, People's Republic of China, E-mail: qqhan10@kmust.edu.cn
Le Jing, Jie Li, Yuzhu Song, Jinyang Zhang, Qiang Chen, Engineering Research Center for Molecular Diagnosis, Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, Yunnan, People's Republic of China

1 Introduction

Fruits represent significant dietary sources of sugars, fiber, vitamins, and minerals, and therefore are important components of balanced diets, contributing to good health. Fruit developmental processes can generally be divided into five stages: organogenesis, expansion, maturation, ripening, and senescence [1]. Among these, fruit ripening is an irreversible process [2]. It is also a complex developmental program coordinating the color, texture, flavor, and aroma of fruit flesh. Studies have provided evidence about the genetic mechanisms that mediate fruit pigment synthesis, sugar metabolism, and cell wall metabolism [3, 4]. Tomato (*Solanum lycopersicum*) is an experimental model for investigations of fleshy fruit developmental processes owing to its favorable features, such as its well-characterized ripening mutants, efficient transient and stable transformation, short life cycle, and complete genome sequence [5, 6].

Ripening processes in plants that are regulated by ethylene occur at the physiological, biochemical, and molecular levels. Specifically, the regulatory process includes signal transduction and the regulation of numerous target genes, including those involved in ethylene biosynthesis and ethylene perception [7, 8]. Many genes involved in fleshy fruit ripening have been identified. These include genes related to ethylene synthesis and signal transduction, e.g., *Never-ripe* (*NR*), *ETHYLENE RESPONSE6* (*ETR6*), and *Green-ripe* (*GR*) [9, 10]. In tomato, in addition to hormones, ripening is mediated by numerous transcription factors (TFs), such as *Ripening inhibitor (RIN), Colorless Non-Ripening* (*CNR*), and *Non-ripening* (*NOR*), which have been confirmed to act upstream of ethylene biosynthesis [11-13]. In addition, ripening-related TFs are also targets of RIN, such as basic helix–loop–helix (bHLH), NAM, ATAF1/ATAF2, and CUC2 (NAC) [14-18], and a recently reported gene, *APETALA2a*, functions as a regulator of fruit ripening activity and carotenoid flux via the negative regulation of ethylene biosynthesis [19, 20]. The discovery of these genes has provided recent insight into the transcriptional control of fleshy fruit development and ripening via ethylene-dependent and independent processes.

The NAC gene family is among the largest families of plant-specific transcription factors. Notable members of the NAC gene family play important roles in diverse developmental processes. For example, ZmNAM1 in *Zea mays* inhibits the growth of specific cells, promotes the boundary formation of organization, and also enhances SAM [21]. NAC members are also reported to function in other processes; over 25% of NAC proteins are upregulated in dark-treated leaves of *Arabidopsis*, indicating potential roles in leaf senescence [22]. NAM-B1 in wheat can accelerate senescence and increase protein, iron, and zinc contents in wheat grain by altering nutrition remobilization [23]. Additionally, the expression of many NAC genes is induced by abiotic and biotic stresses [24-26]. ANAC016 has been reported as a positive regulator of the response to drought stress in *Arabidopsis* [27]. Overexpression of SNAC3 (ONAC003) or OsNAC6 in rice enhances the tolerance to drought and high-salt stress by up-regulating the expression of downstream stress-related genes [28]. Overexpression of SlNAC1 in tomato can improve chilling stress resistance by reducing the accumulation of ROS and up-regulating the expression of CBF1 [29].

In our previous study, we isolated a new NAC-type gene, *SlNAC3*, from a tomato flower cDNA library, and reported its cloning, characterization, expression, and subcellular localization. Proteins that interact with SlNAC3 were identified using a yeast two-hybrid (Y2H) system by screening the tomato cDNA library, using the SlNAC3 protein as the bait protein. The results revealed that 7 proteins interacting with SlNAC3 were filtered out, which include cell wall structural protein, transcription related proteins, and ethylene signal transduction pathway related proteins [30, 31]. In this study, interacting proteins of the SlNAC3 protein were fished using co-immunoprecipitation (Co-IP) and analyzed by MALDI-TOF-MS assays to further validate the yeast two-hybrid results. Interacting DNA was detected by chromatin immunoprecipitation (ChIP) assays. Ultimately, these studies clarified the role of SlNAC3 in the developmental processes of tomatoes.

2 Materials and methods

2.1 Plant materials and growth conditions

The tomato (*S. lycopersicum*) cultivar Ailsa Craig (National Clonal Germplasm Repository, USA) was grown in an environment-controlled glasshouse under sodium lights set to 16 h days (24°C) and 8 h nights (18°C) according to previous methods [18].

2.2 Expression and purification of the SlNAC3 protein

Based on the PCR-based Accurate Synthesis method, full-length splice primers were initially designed and then protective bases were designed at both ends of the primers to synthesize SlNAC3. By double digestion, SlNAC3 was ligated between the NdeI and XbaI sites of the pCzn1 vector. Next, the resulting recombinant plasmid pCzn1-SlNAC3 was transferred into the TOP10 clonal strain and ArcticExpress expression strain. IPTG was used to induce the expression of the target SlNAC3 protein and the induction conditions were adjusted to 11°C, with gentle shaking overnight. The expressed protein was identified by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the SlNAC3 protein was solved by the denaturation and refolding method and purified by affinity chromatography on a Ni column. Finally, the purified protein was analyzed by 12% SDS-PAGE and confirmed by a Western blot analysis. The gel was analyzed by western blotting with the mouse anti-His mAb and peroxidase-conjugated goat anti-mouse IgG as the primary and secondary antibodies, respectively.

2.3 Preparation of the SlNAC3 polyclonal antibody

To prepare polyclonal antibodies against the SlNAC3 protein, SlNAC3 protein was used as an antigen to immunize two New Zealand white rabbits (2–2.5 kg) (Zoonbio Biotechnology Co., Ltd., China) by subcutaneous administration of 400 μg, and immunized 4 times at 3 weeks interval. After the fourth immunization, blood sampling was performed to determine the titer of the antiserum against the SlNAC3 protein by indirect enzyme linked immunosorbent assay (ELISA). When the titer exceeded 1:50000, the antiserum was prepared and purified. Then the specific polyclonal antibodies (pAbs) against the SlNAC3 protein were obtained by antigen affinity purification. The SlNAC3 protein was conjugated to agarose medium to prepare an antigen-affinity chromatography column, and the resulting antiserum was mixed with an equal amount of phosphate-buffered saline (PBS) and slowly loaded into the column. After the antibody was bound to the column and eluted with glycine elution buffer, the purified SlNAC3 pAbs (anti-SlNAC3) was obtained. Ultimately, the purified antibody was identified by 12% SDS-PAGE and Coomassie brilliant blue staining.

2.4 Co-immunoprecipitation assay

Fresh tomato fruit (10 g) in the breaker stage was ground with liquid nitrogen into a powder and supplemented with 50 mL of extraction buffer [10 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM PMSF, 1 mM EDTA, 2 mM DTT, 10% glycerol, 0.2% TritonX-100, 0.1% SDS] [32], and placed on ice for 2 h for the complete lysis of proteins. The supernatant was obtained after centrifugation at 12000 × *g* for 15 min and the protein concentration of the supernatant was measured using the BCA assay. Anti-SlNAC3 (1 mL of 1 mg/mL solution was added to 1 μg of anti-SlNAC3) was added to the supernatant, and samples were incubated overnight at 4°C. The complex was recovered by incubating the samples with 60 μL of protein A/G Sepharose beads for 4 to 8 h at 4°C with gentle shaking. After centrifugation at 1000 × *g* for 3 minutes and removal of the supernatant, beads were collected, then washed five times with 1 mL of washing buffer (0.01 M PBS); the subsequent steps were as described above. After the last washing and centrifugation, the precipitate was added to 40 μL of 1× loading buffer for SDS-PAGE and silver stained. After obtaining an unknown protein that interacts with the SlNAC3 protein, it was analyzed by MALDI-TOF-MS to predict functionality.

2.5 Chromatin immunoprecipitation assay

Healthy young leaves were used for a chromatin immunoprecipitation assay by Ch-IP Assay Kit (Beyotime, China); the procedure can mainly be divided into the following steps. First, cross-linking was performed. Leaves (3 g) were harvested, submerged in cold extraction buffer 1 and 1% formaldehyde, vacuum infiltrated for 20 min, supplemented with glycine, and reverse crosslinked for 10 min. Second, nuclear extraction was performed. Leaves were ground in liquid nitrogen and added to extraction buffer 1. Samples were filtered through a nylon mesh and spun for 20 min at 2880 × *g* at 4°C. The supernatant was removed, and the pellet was resuspended in 10 mL of extraction buffer 2 and spun for 10 min at 12,000 × *g* at 4°C. After the supernatant was removed, the pellet was resuspended in 4 mL of nuclei resuspension buffer, spun for 10 min at 12,000 × *g* at 4°C, and the supernatant was still discarded. Third, sonication was performed. The pellet was resuspended in 1 mL of nuclei lysis buffer, and then lysed for 20 min on ice. Sonication was performed for 5 × 10 s at 15% power, followed by centrifugation for 5 min at 21,000 × *g* at 4°C. Fourth, immunoprecipitation was performed. Samples were treated with protein A/G agarose beads for at least 1 h at 4°C, with gentle shaking. The input control was removed and SlNAC3 pAbs was added to all samples, followed by incubation overnight at 4°C. The beads were pelleted and washed for 10 min sequentially with 1 mL of low-salt buffer, high-salt buffer, LiCl wash buffer, and TE buffer (two washes). Finally, DNA was eluted and purified. Complexes were eluted for 15 min at 65°C, reverse crosslinked for 6 h at 65°C, and treated with proteinase K for 1 h at 65°C. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol.

After the DNA fragments that interact with the SlNAC3 protein were obtained by Ch-IP assays, they were cloned, sequenced, and searched against the tomato NCBI database to determine the regulatory role of the DNA fragment.

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

3 Results

3.1 Expression and Purification of the SlNAC3 protein

After the *SlNAC3* gene was inserted into the prokaryotic expression vector pCzn1, the recombinant SlNAC3 protein was successfully expressed at a high level in *Escherichia coli*. It was predicted using Lasergene software to encode a recombinant protein with a molecular weight of approximately 17.514 kDa. To induce protein expression, 0.5 mM IPTG was added, followed by gentle shaking overnight at 11°C to detect expression by SDS–PAGE. These results showed that the target protein was mainly detected in the form of inclusion bodies, and the supernatant exhibited minor expression (Fig. 1A). After refolding and purification, the target SlNAC3 protein was enriched and no other proteins existed in the collection liquid (Fig. 1B). The Western blotting assay further confirmed that the recombinant protein was successfully expressed and the molecular weight of the target protein was approximately the same as that predicted by Lasergene software (Fig. 1C).

3.2 Preparation of the SlNAC3 Polyclonal Antibody

After the fourth immunization, ELISA was used to test the reactivity of serum from New Zealand white rabbits

immunized with the recombinant SlNAC3 protein, and the antiserum was tested at different dilutions from 1:500 to 1:512,000. The antibody titer was defined as the highest dilution of serum at which the A450 ratio (A450 of post-immunization serum/A450 of pre-immunization serum) was greater than 2.1, which is of biological significance. In accordance with the above criterion, the titer was greater than 512,000 (Table 1). Therefore, the New Zealand white rabbits could be used to produce the SlNAC3 pAb, and the antisera showed a high specificity against the SlNAC3 protein.

The SlNAC3 pAb was purified by affinity chromatography and confirmed by SDS-PAGE. For two different concentrations of the SlNAC3 pAb (Lane 1, Anti-SlNAC3, 0.3 mg/mL; Lane 2, Anti-SlNAC3, 0.6 mg/mL), a 55 kDa (heavy chain) band and a 28 kDa (light chain) band were observed on the membrane (Fig. 2) after SDS-PAGE electrophoresis. These results demonstrate that a pAb specific to the SlNAC3 protein was successfully obtained.

Table 1. The specific antibody titers in antiserum of the immunized rabbits

Dilution	OD450	
	0.6 mg/mL antiserum	0.3 mg/mL antiserum
500	3.723	3.052
1000	3.598	2.875
2000	3.422	2.839
4000	3.355	2.822
8000	3.246	2.790
16000	3.024	2.775
32000	2.954	2.542
64000	2.706	2.380
128000	2.556	2.175
256000	2.428	2.036
512000	1.914	1.526
Negative	0.094	0.103

*The antibody titer was defined as the highest dilution of serum at which the A450 ratio (A450 of post-immunization serum/A450 of pre-immunization serum) is greater than 2.1, and the pre-immunization serum as negative control.

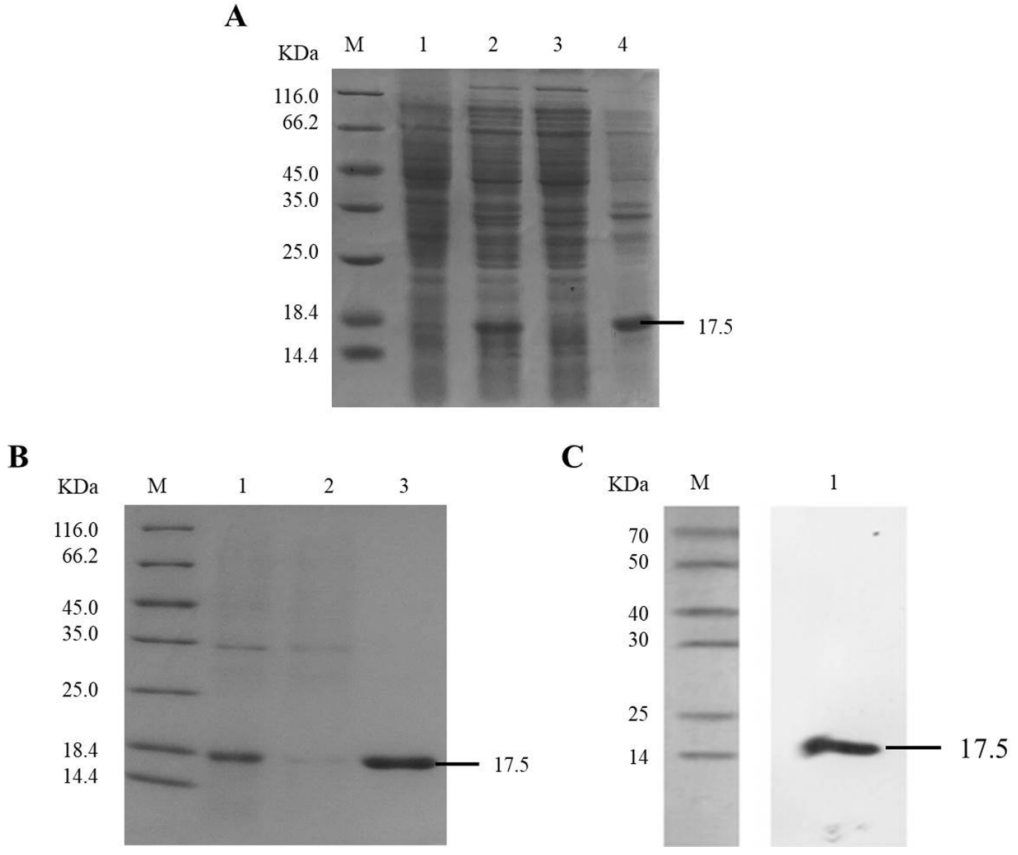

Fig. 1 Expression and Purification of the SlNAC3 protein. Expressions of the recombinant protein with IPTG induction were analyzed by 12% SDS–PAGE. Lane M, protein marker (Thermo); Lane 1, un-induced; Lane 2, induced; Lane 3, supernatant of 11° induction with 0.5 mM IPTG; Lane 4, precipitate of 11° induction with 0.5 mM IPTG. (B) Purification of the inclusion body protein by affinity chromatography on a Ni column. Lane 1, un-purified; Lane 2, flow through; Lane 3, elution. (C) Identification of the purified protein by western blotting. Lane 1, the purified protein.

3.3 Identification of proteins that interact with SlNAC3

Protein–protein interactions in plants are studied using Co-IP and MALDI-TOF-MS assays. In our experiments, under non-denatured conditions, the SlNAC3 polyclonal antibody was immobilized with protein A/G Sepharose beads for binding to the SlNAC3 protein, thereby indirectly yielding an unknown protein interacting with the SlNAC3 protein. Next, the immunoprecipitate was subjected to SDS–PAGE and silver staining. Compared to the other lanes, the bands appearing in the lane of the immunoprecipitates (Lane 4) were consistent with the SlNAC3 pAbs, the SlNAC3 protein, and an unknown protein that interacted with SlNAC3 (Fig. 3). This unknown protein was analyzed by MALDI-TOF-MS to predict its functionality and the result indicated that the unknown protein was Polygalacturonase-2 (PG-2) (Fig. S1). According to previous reports, PG is a major cell wall-degrading enzyme in tomato fruits and it is developmentally regulated and is synthesized *de novo* in ripening fruits [33]. Based on the above series of experiments, we successfully obtained a protein that interacts with the SlNAC3 protein and preliminarily found that it functions in the process of tomato ripening.

3.4 Genomic interactions with the SlNAC3 protein

The ChIP assay can be used to identify the DNA-binding sites in genes or genomic regions of the model species, tomato (*S. lycopersicum*). This method is based on the fixation of protein–DNA interactions *in vivo*, random fragmentation of chromatin, immunoprecipitation of protein–DNA complexes with specific antibodies, and quantification of the DNA associated with the protein of interest by PCR techniques. Here, we immunoprecipitated the SlNAC3 protein–DNA complexes with the specific SlNAC3 pAbs to obtain DNA fragments interacting with the SlNAC3 protein (Fig. S2). In order to identify the regulatory sites, the DNA fragment was cloned and sequenced (Table S1), and the sequence was searched against the tomato NCBI database. By BLAST analysis and sequence alignment, a sequence related to tomato was found on *Solanum pennellii* chromosome 10 (Sequence

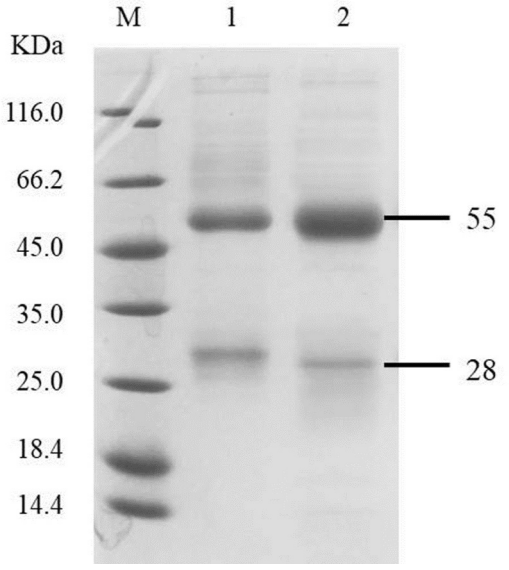

Fig. 2 Purification of the SlNAC3 polyclonal antibody. The purified SlNAC3 pAb was confirmed by 12% SDS-PAGE. Two different concentrations of anti-SlNAC3 in the gel: Lane 1, Anti-SlNAC3, 0.3 mg/mL; Lane 2, Anti-SlNAC3, 0.6 mg/mL; M, protein marker (Therno).

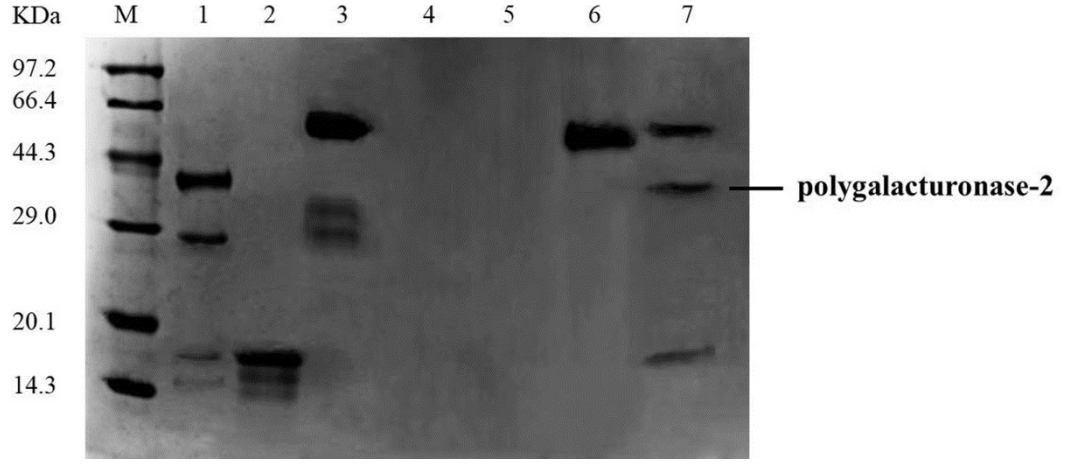

Fig. 3 Identification of proteins that interact with SlNAC3. The immunoprecipitates were subjected to SDS–PAGE and silver staining. M, protein marker (Thermo); Lane 1, input; Lane 2, SlNAC3 protein; Lane 3, Anti-SlNAC3; Lane 4, the protein A/G Sepharose beads; Lane 5, total input incubated with protein A/G Sepharose beads; Lane 6, anti-SlNAC3 incubated with protein A/G Sepharose beads. Lane 7, the immunoprecipitates (including the SlNAC3 pAbs, the SlNAC3 protein, and polygalacturonase-2, which interacted with the SlNAC3 protein).

ID, HG975449.1), which was related to differential stress resistance in the wild tomato species *Solanum pennellii* [34]. Thus, *SlNAC3* may be a negative regulator of tomato stress tolerance.

4 Discussion

In our study, an unknown protein interacting with the SlNAC3 protein was identified as polygalacturonase-2 (PG-2) by chromatin immunoprecipitation and MALDI-TOF-MS analysis. PG-2 is a structural component of cell walls, which is consistent with the result of the yeast two-hybrid assay. During the ripening of fruits, several polysaccharide-degrading enzymes are known to increase their activity, including cellulose [35], pectinesterase [36], and polygalacturonase (PG) [37, 38]. These last two enzymes are associated with cell walls and are involved in the substantial softening of the fruit during ripening. The identification and functional analysis of PG genes have shown that in the late stages of maturity of many climacteric fruits, PG genes mediate fruit softening by degrading pectin [39-41]. According to the results of the present study, PG is not detectable in green tomatoes and its activity appears at the onset of ripening and in ripe fruit and its protein levels tend to peak and then gradually decline as the fruit softens [42]. In our experiment, we focused on the tomato fruits at the color breaking stage based on the above studies. However, the activity of two isoenzymes (PG-1 and PG-2) only appears sequentially during ripening and they contain similar polypeptides, so it is probable that PG-1 is a dimer of PG-2 [33, 43]. In addition, the expression of cell wall metabolism-related genes such as PG is positively correlated with the degree of softening of the fruit and is regulated by ethylene. In the ethylene pathway, PG-2 is a receptor protein that mediates fruit ripening, such as NR, ETR, etc. As a transcription factor, *SlNAC3* is upstream of the ethylene pathway and regulates PG-2. But the mechanism of transcriptional regulation has not been reported, which is an attention-worthy point for further study.

Drought and high salt are the main stress factors affecting plant growth and development. When plants are under drought and high salt stress, plant cells sense the external stress and transmit signals to the transcription factor involved in the stress response via a series of complex signaling pathways, such as abscisic acid, salicylic acid, ethylene, and jasmonic acid signaling pathways. Various transcription factors are initiated in response to stress, resulting in changes in gene expression, thereby activating plant stress resistance and reducing or eliminating injury caused by drought and high salt stress. NAC transcription factors (Table 2) are directly involved in the plant response or regulate the expression of drought and high salt response genes, and play an important role in plant abiotic stress responses, such as drought resistance and high salt [7, 44]. Tomato, a vegetable crop

Table 2. NAC transcription factors known to be involved in plant abiotic stress responses.

Gene	Plants	Functions	Reference
SNAC1	Rice	Positive regulation of drought or salt stress response	[48]
SNAC3 / ONAC003	Rice		[28]
OsNAC5	Rice		[49]
ONAC045	Rice		[49]
ONAC131	Rice	Positive regulation of rice blast resistance	[50]
OsNAC6 / SNAC2	Rice		[51]
OsNAC10 / ONAC122	Rice		[50]
ANAC019	*Arabidopsis thaliana*	Exhibit an enhanced expression of stress-responsive genes and an improved tolerance to drought and salinity stress	[52]
ANAC055	*Arabidopsis thaliana*		[52]
ANAC016	*Arabidopsis thaliana*		[52]
ANAC072 / RD26	*Arabidopsis thaliana*	Improved tolerance to drought and salinity stress	[52]
ATAF1	*Arabidopsis thaliana*	Improve tolerance to drought, high salt, and chilling injury	[53]
SlNAC1	Tomato	Positive regulation of drought and temperature stress	[29]
SlNAC4	Tomato		[54]
TaNAC	Wheat	Resistance to drought, high salinity, low temperature	[55]
HvSNAC1	Barley	Improved drought tolerance	[56]
SlNAC35	Tobacco	Positive regulation of drought stress	[57]
SlNAC3	Tomato	Resistance to drought, high salinity	Our study

with important nutritional and economic value, has become an important model plant in botany research since the completion of tomato genome sequencing. The functional identification of *NAC* family genes has been mainly performed in *Arabidopsis thaliana* and rice, and the functions of tomato *NAC* family genes are less well-characterized. *SlNAC1*, *SlNAC3* and *SlNAM1* [18, 45] have been found to be involved in the tomato response to stress. In our study, the function of *SlNAC3* in tomato was predicted. ChIP assay showed that *SlNAC3* was involved in the highly stress-tolerant phenotype to improve tolerance to drought and salinity stress in the overexpress tomato cultivars, suggesting roles in alterations in cuticle composition and nonrandom associations of specific gene sets with transposable elements. In addition, recent studies have shown the potential of NAC transcription factors in transgenic breeding, e.g., transgenic rice plants that overexpress *SNAC1* and *SNAC2* also exhibit enhanced drought and salt tolerance [46, 47]. Therefore, it is possible to obtain new tomato cultivars with high resistance to drought and salinity stress by changing the expression level of *SlNAC3* using transgenic technology.

5 Conclusions

In conclusion, we investigated the functional characteristics and action mechanisms of *SlNAC3* in the growth of tomatoes. The mechanisms underlying the effects of *SlNAC3* were analyzed at the molecular level by Co-IP and ChIP assays. The discovery of PG provided evidence that *SlNAC3* is involved in the control of tomato fruit ripening, and the finding of a BLAST hit on *Solanum pennellii* chromosome 10 further proves that *SlNAC3* performs a specific function in the stress monitoring network. Thus, our work indicates that the new NAC-type transcription factor *SlNAC3* plays an important role in the developmental process of tomato and paves a theoretical basis for further tomato improvement.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (NSFC Grant No. 31560559), Yunnan Province Science and Technology Program (Project Nos. 2015FB121 and 2016FB146), and Yunnan Science and Technology Innovation Platform Construction Project (Project No. 2015DH10).

Conflicts of Interest: Authors state no conflict of interest.

References

[1] Farinati S, Rasori A, Varotto S, Bonghi C. Rosaceae Fruit Development, Ripening and Post-harvest: An Epigenetic Perspective. Front Plant Sci. 2017;8:1247.
[2] Bapat VA, Trivedi PK, Ghosh A, Sane VA, Ganapathi TR, Nath P. Ripening of fleshy fruit: molecular insight and the role of ethylene. Biotechnol Adv. 2010;28(1):94-107.
[3] Moing A, Aharoni A, Biais B, Rogachev I, Meir S, Brodsky L, et al. Extensive metabolic cross-talk in melon fruit revealed by spatial and developmental combinatorial metabolomics. New Phytol. 2011;190(3):683-96.
[4] Zhang J, Wang X, Yu O, Tang J, Gu X, Wan X, et al. Metabolic profiling of strawberry (*Fragaria x ananassa* Duch.) during fruit development and maturation. J Exp Bot. 2011;62(3):1103-18.
[5] Meng C, Yang D, Ma X, Zhao W, Liang X, Ma N, et al. Suppression of tomato SlNAC1 transcription factor delays fruit ripening. J Plant Physiol. 2016;193:88-96.
[6] Llorente B, D'Andrea L, Ruiz-Sola MA, Botterweg E, Pulido P, Andilla J, et al. Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. Plant J. 2016;85(1):107-19.
[7] Sun L, Sun Y, Zhang M, Wang L, Ren J, Cui M, et al. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. Plant Physiol. 2012;158(1):283-98.
[8] Yang Y, Wu Y, Pirrello J, Regad F, Bouzayen M, Deng W, et al. Silencing Sl-EBF1 and Sl-EBF2 expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. J Exp Bot. 2010;61(3):697-708.
[9] Gallusci P, Hodgman C, Teyssier E, Seymour GB. DNA Methylation and Chromatin Regulation during Fleshy Fruit Development and Ripening. Front Plant Sci. 2016;7:807.
[10] Hao PP, Wang GM, Cheng HY, Ke YQ, Qi KJ, Gu C, et al. Transcriptome analysis unravels an ethylene response factor involved in regulating fruit ripening in pear. Physiol Plant. 2018;16(1):124-35.
[11] Seymour GB, Granell A. Fruit development and ripening. Journal of Experimental Botany. 2013;64(16):219.
[12] Xu XX, Hu Q, Yang WN, Jin Y. The roles of call wall invertase inhibitor in regulating chilling tolerance in tomato. BMC Plant Biol. 2017;17(1):195.
[13] Huang Y, Li T, Xu ZS, Wang F, Xiong AS. Six NAC transcription factors involved in response to TYLCV infection in resistant and susceptible tomato cultivars. Plant Physiol Biochem. 2017;120:61-74.
[14] Fu J, Chu J, Sun X, Wang J, Yan C. Simple, rapid, and simultaneous assay of multiple carboxyl containing phytohormones in wounded tomatoes by UPLC-MS/MS using single SPE purification and isotope dilution. Anal Sci. 2012;28(11):1081-7.
[15] Fujisawa M, Nakano T, Ito Y. Identification of potential target genes for the tomato fruit-ripening regulator RIN by chromatin immunoprecipitation. BMC Plant Biol. 2011;11:26.
[16] Fujisawa M, Nakano T, Shima Y, Ito Y. A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. Plant Cell. 2013;25(2):371-86.

[17] Fujisawa M, Shima Y, Higuchi N, Nakano T, Koyama Y, Kasumi T, et al. Direct targets of the tomato-ripening regulator RIN identified by transcriptome and chromatin immunoprecipitation analyses. Planta. 2012;235(6):1107-22.
[18] Han Q, Zhang J, Li H, Luo Z, Ziaf K, Ouyang B, et al. Identification and expression pattern of one stress-responsive NAC gene from *Solanum lycopersicum*. Molecular Biology Reports. 2012;39(2):1713-20.
[19] Chung MY, Vrebalov J, Alba R, Lee J, McQuinn R, Chung JD, et al. A tomato (*Solanum lycopersicum*) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening. Plant J. 2010;64(6):936-47.
[20] Karlova R, Rosin FM, Busscher-Lange J, Parapunova V, Do PT, Fernie AR, et al. Transcriptome and metabolite profiling show that APETALA2a is a major regulator of tomato fruit ripening. Plant Cell. 2011;23(3):923-41.
[21] Shiriga K, Sharma R, Kumar K, Yadav SK, Hossain F, Thirunavukkarasu N. Genome-wide identification and expression pattern of drought-responsive members of the NAC family in maize. Meta Gene. 2014;2:407-17.
[22] Chen Y, Wang Y, Huang J, Zheng C, Cai C, Wang Q, et al. Salt and methyl jasmonate aggravate growth inhibition and senescence in *Arabidopsis* seedlings via the JA signaling pathway. Plant Sci. 2017;261:1-9.
[23] Asplund L, Bergkvist G, Leino MW, Westerbergh A, Weih M. Swedish spring wheat varieties with the rare high grain protein allele of NAM-B1 differ in leaf senescence and grain mineral content. PLoS One. 2013;8(3):e59704.
[24] Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. NAC transcription factors in plant abiotic stress responses. Biochim Biophys Acta. 2012;1819(2):97-103.
[25] Nuruzzaman M, Sharoni AM, Kikuchi S. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front Microbiol. 2013;4:248.
[26] Shao H, Wang H, Tang X. NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. Front Plant Sci. 2015;6:902.
[27] Sakuraba Y, Kim YS, Han SH, Lee BD, Paek NC. The Arabidopsis Transcription Factor NAC016 Promotes Drought Stress Responses by Repressing AREB1 Transcription through a Trifurcate Feed-Forward Regulatory Loop Involving NAP. Plant Cell. 2015;27(6):1771-87.
[28] Fang Y, Liao K, Du H, Xu Y, Song H, Li X, et al. A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. J Exp Bot. 2015;66(21):6803-17.
[29] Ma NN, Zuo YQ, Liang XQ, Yin B, Wang GD, Meng QW. The multiple stress-responsive transcription factor SlNAC1 improves the chilling tolerance of tomato. Physiol Plant. 2013;149(4):474-86.
[30] Han Q, Zhang J, Li H, Luo Z, Ziaf K, Ouyang B, et al. Identification and expression pattern of one stress-responsive NAC gene from *Solanum lycopersicum*. Mol Biol Rep. 2012;39(2):1713-20.
[31] Han QQ, Song YZ, Zhang JY, Liu LF. Studies on the role of the SlNAC3 gene in regulating seed development in tomato (*Solanum lycopersicum*). Journal of Pomology & Horticultural Science. 2014;89(4):423-429.
[32] Muinao T, Pal M, Boruah HPD. Cytosolic and Transmembrane Protein Extraction Methods of Breast and Ovarian Cancer Cells: A Comparative Study. J Biomol Tech. 2018;29(3):71-78.
[33] Samaranayake CP, Sastry SK. Effects of controlled-frequency moderate electric fields on pectin methylesterase and polygalacturonase activities in tomato homogenate. Food Chem. 2016;199:265-72.
[34] Bolger A, Scossa F, Bolger ME, Lanz C, Maumus F, Tohge T, et al. The genome of the stress-tolerant wild tomato species *Solanum pennellii*. Nat Genet. 2014;46(9):1034-8.
[35] Liu D, Zehfroosh N, Hancock BL, Hines K, Fang W, Kilfoil M, et al. Imaging cellulose synthase motility during primary cell wall synthesis in the grass Brachypodium distachyon. Sci Rep. 2017;7(1):15111.
[36] Omidvar V, Mohorianu I, Dalmay T, Zheng Y, Fei Z, Pucci A, et al. Transcriptional regulation of male-sterility in 7B-1 male-sterile tomato mutant. PLoS One. 2017;12(2):e0170715.
[37] Rui Y, Xiao C, Yi H, Kandemir B, Wang JZ, Puri VM, et al. POLYGALACTURONASE INVOLVED IN EXPANSION3 Functions in Seedling Development, Rosette Growth, and Stomatal Dynamics in *Arabidopsis thaliana*. Plant Cell. 2017;29(10):2413-2432.
[38] Joubert DA, de Lorenzo G, Vivier MA. Regulation of the grapevine polygalacturonase-inhibiting protein encoding gene: expression pattern, induction profile and promoter analysis. J Plant Res. 2013;126(2):267-81.
[39] Zhang W, Liu B, Lu Y, Liang G. Functional analysis of two polygalacturonase genes in *Apolygus lucorum* associated with eliciting plant injury using RNA interference. Arch Insect Biochem Physiol. 2017;94(4).
[40] Qian M, Zhang Y, Yan X, Han M, Li J, Li F, et al. Identification and Expression Analysis of Polygalacturonase Family Members during Peach Fruit Softening. Int J Mol Sci. 2016;17(11).
[41] Zhou HC, Li G, Zhao X, Li LJ. Comparative analysis of polygalacturonase in the fruit of strawberry cultivars. Genet Mol Res. 2015;14(4):12776-87.
[42] Liang Y, Yu Y, Shen X, Dong H, Lyu M, Xu L, et al. Dissecting the complex molecular evolution and expression of polygalacturonase gene family in *Brassica rapa ssp. chinensis*. Plant Mol Biol. 2015;89(6):629-46.
[43] Xie F, Yuan S, Pan H, Wang R, Cao J, Jiang W. Effect of yeast mannan treatments on ripening progress and modification of cell wall polysaccharides in tomato fruit. Food Chem. 2017;218:509-517.
[44] Narsai R, Wang C, Chen J, Wu J, Shou H, Whelan J. Antagonistic, overlapping and distinct responses to biotic stress in rice (*Oryza sativa*) and interactions with abiotic stress. BMC Genomics. 2013;14:93.
[45] Yang R, Deng C, Ouyang B, Ye Z. Molecular analysis of two salt-responsive NAC-family genes and their expression analysis in tomato. Mol Biol Rep. 2011;38(2):857-63.
[46] Songyikhangsuthor K, Guo Z, Wang N, Zhu X, Xie W, Mou T, et al. Natural variation in the sequence of SNAC1 and its expression level polymorphism in rice Germplasms under drought stress. J Genet Genomics. 2014;41(11):609-12.
[47] You J, Zhang L, Song B, Qi X, Chan Z. Systematic analysis and identification of stress-responsive genes of the NAC gene family in *Brachypodium distachyon*. PLoS One. 2015;10(3):e0122027.
[48] Liu G, Li X, Jin S, Liu X, Zhu L, Nie Y, et al. Overexpression of rice NAC gene SNAC1 improves drought and salt tolerance by enhancing root development and reducing transpiration rate in transgenic cotton. PLoS One. 2014;9(1):e86895.
[49] Escobar-Sepulveda HF, Trejo-Tellez LI, Garcia-Morales S, Gomez-Merino FC. Expression patterns and promoter analyses of aluminum-responsive NAC genes suggest a possible growth regulation of rice mediated by aluminum, hormones and NAC transcription factors. PLoS One. 2017;12(10):e0186084.
[50] Sun L, Zhang H, Li D, Huang L, Hong Y, Ding XS, et al. Functions of rice NAC transcriptional factors, ONAC122 and ONAC131, in defense responses against *Magnaporthe grisea*. Plant Mol Biol. 2013;81(1-2):41-56.

[51] Hu H, You J, Fang Y, Zhu X, Qi Z, Xiong L. Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. Plant Molecular Biology. 2008;67(1-2):169-181.
[52] Hickman R, Hill C, Penfold CA, Breeze E, Bowden L, Moore JD, et al. A local regulatory network around three NAC transcription factors in stress responses and senescence in *Arabidopsis* leaves. Plant J. 2013;75(1):26-39.
[53] Garapati P, Xue GP, Munne-Bosch S, Balazadeh S. Transcription Factor ATAF1 in *Arabidopsis* Promotes Senescence by Direct Regulation of Key Chloroplast Maintenance and Senescence Transcriptional Cascades. Plant Physiol. 2015;168(3):1122-39.
[54] Zhu M, Chen G, Zhang J, Zhang Y, Xie Q, Zhao Z, et al. The abiotic stress-responsive NAC-type transcription factor SlNAC4 regulates salt and drought tolerance and stress-related genes in tomato (*Solanum lycopersicum*). Plant Cell Rep. 2014;33(11):1851-63.
[55] Tang Y, Liu M, Gao S, Zhang Z, Zhao X, Zhao C, et al. Molecular characterization of novel TaNAC genes in wheat and overexpression of TaNAC2a confers drought tolerance in tobacco. Physiol Plant. 2012;144(3):210-24.
[56] McGrann GR, Steed A, Burt C, Goddard R, Lachaux C, Bansal A, et al. Contribution of the drought tolerance-related stress-responsive NAC1 transcription factor to resistance of barley to *Ramularia* leaf spot. Mol Plant Pathol. 2015;16(2):201-9.
[57] Wang G, Zhang S, Ma X, Wang Y, Kong F, Meng Q. A stress-associated NAC transcription factor (SlNAC35) from tomato plays a positive role in biotic and abiotic stresses. Physiol Plant. 2016;158(1):45-64.

Supplemental Material: The online version of this article (DOI: 10.1515/biol-2018-0062) offers supplementary material.