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Research article

Characterization of a NAC transcription factor involved in the regulation of pomegranate seed hardness (*Punica granatum* L.)

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ABSTRACT

The pomegranate, *Punica granatum* L., which has been cultivated since antiquity, is known to be a superfruit, possessing an array of functional anti-oxidants and various other health benefits. The hardness of pomegranate seeds is an important indicator of fruit quality, which in turn affects economic value and market demand. However, the molecular mechanism underlying pomegranate seed hardness remains to be fully understood. In this study, we found a positive correlation between seed hardness and lignin content in two pomegranate varieties: “Tunisia” and “Sanbai”. Specifically, genes associated with lignin biosynthesis were differentially expressed in soft-seed and hard-seed pomegranate varieties. Among these differential genes, we cloned and characterized the NAC transcription factor *PgSND1-like*. Sequence alignment found a single base replacement at the 166-bp position of CDS in the *PgSND1-like* gene from “Tunisia” and “Sanbai”. Both *PgSND1-like* (Sanbai) and *PgSND1-like* (Tunisia) proteins are localized in the cell nucleus and have a transcription activation domain in the C-terminus. Yeast two-hybrid analysis indicated that PgSND1-like protein interacts with itself to form a homodimer. Overexpression of *PgSND1-like* (Sanbai) in Arabidopsis showed a higher lignin content in inflorescence stem and mature seed compared with wild-type Arabidopsis. Accordingly, the expression levels of several lignin biosynthesis-associated genes were upregulated in stem cells and mature seeds of transgenic plants. However, *PgSND1-like* (Tunisia) transgenic Arabidopsis showed no phenotypic differences with wild-type Arabidopsis. Taken together, we suggest that *PgSND1-like* may regulate at least two different functions in two pomegranate varieties, promoting lignin biosynthesis and seed hardness of pomegranate.

1. Introduction

Pomegranate (*Punica granatum* L.), native to central Asia (Holland et al., 2009), has been cultivated widely in tropical and subtropical regions. It is known for its nutritional content, such as polyphenols, anthocyanins, tannins and vitamins (Miguel et al., 2010; Teixeira da Silva et al., 2013). Different parts of the plant are also used as a traditional medicine in many countries (Kim et al., 2002; Sanchez-Lamar et al., 2008; Li, 2013). Therefore, pomegranate is often called a super fruit (Teixeira da Silva et al., 2013). However, the pomegranate seeds are not perfect for consumption because they are too hard to chew and swallow. In 1986, a soft-seed pomegranate cultivar “Tunisia” was introduced in China and became popular.

Pomegranate seed coating contains a high level of lignin (Dalimov and Bhatt, 2003). Accordingly, Zhang and Cao found a positive correlation between the hardness of pomegranate seeds and their levels of lignin (Cao et al., 2015; Zhang et al., 2015). Lignin is an aromatic polymer predominantly deposited in the secondary cell walls (SCW) of vascular plants (Vanholme et al., 2010). Lignin, cellulose, hemicellulose, and other polysaccharide molecules are functionally bound to increase the mechanical strength of plant cells and tissue. At present, the biosynthesis pathway of lignin is relatively well understood. It begins with phenylalanine as the biosynthesis precursor, followed by deamination, hydroxylation, methylation, and reduction reaction to form a monomer. Finally, the monomers are further oxidized to form lignin (Van-Acker et al., 2013).

The lignin synthesis pathway is regulated by many genes. The key enzymes in the lignin synthesis process are Phenylalanine ammonia lyase (PAL), Cinnamic acid 4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), Cinnamoyl-CoA reductase (CCR), Caffeoyl-CoA O-

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methyl transferase (CC_OAOMT), Ferulate-5-hydroxylase (F5H), Caffeic acid-O-methyltransferase (COMT), and Cinnamyl alcohol dehydrogenase (CAD). PAL1 is the first rate-limiting enzyme in the pathway; its expression and abundance directly affect the entire process of lignin biosynthesis. Overexpression of *PAL1* significantly increases the content of lignin (Bate et al., 1994; Osakabe et al., 1995). *CC_OAOMT* is the original enzyme to regulate the synthesis of G lignin. Inhibition of *CC_OAOMT* can reduce the lignin content in transgenic tobacco plants (Sewalt et al., 1997).

Based on transcriptomic analysis, Xue and colleagues found that WRKY, MYB, and NAC transcription factors (TFs) expressed differently in soft- and hard-seed pomegranate varieties (Xue et al., 2017). Some NAC TFs in Arabidopsis were able to control lignin biosynthesis by regulating the cell wall synthesis-related genes (Zhong et al., 2007). Recently, novel functions of NAC TFs were found in several plants (Duval et al., 2002; Ooka et al., 2003; Grant et al., 2010; Su et al., 2014). The main feature of the NAC TF is its highly conserved NAC domain at the N-terminus. The NAC domain consists of approximately 150 amino acid residues that can bind to DNA and several proteins (Kubo et al., 2005). NAC members show differences in the transcriptional activation area, which is located at the C-terminus. The NAC TF family has many new members. In recent years, 117 NAC sequences have been found in *Arabidopsis thaliana* and 151 have been detected in rice (*Oryza sativa*) (Nuruzzaman et al., 2010). Some NAC TFs are upstream of the secondary wall synthesis network, and regulate lignin biosynthesis in combination with MYB TFs.

In Arabidopsis, NST1, NST2, and NST3/SND1 regulate secondary wall synthesis as NAC TFs. NST1 and NST2 act redundantly in the process of secondary wall thickening of anther cells. *NST1* overexpression in Arabidopsis thaliana can increase the expression levels of several lignin biosynthetic genes (Mitsuda et al., 2005). SND1 is located upstream of the secondary wall synthesis network. MYB46, SND3, MYB103, and KNAT7 are the direct target proteins of SND1 and its homologous proteins (NST1, NST2, VND6, and VND7) (Zhong et al., 2008). SND1 is a fiber-specific NAC TF that is specially expressed in interfascicular and xylem fibers. Zhong and colleagues found that dominant repression of SND1 causes a reduction in secondary wall thickening of fibers (Zhong et al., 2006). Recently, it was found that SND1 (NST3) and NST1 act redundantly to regulate secondary wall biosynthesis in fibers (Mitsuda et al., 2007; Zhong et al., 2007). As direct target proteins of SND1, MYB83 and MYB46 act redundantly in regulating the synthesis of secondary walls in fibers and vessels (Mc Carthy et al., 2009). MYB58 and MYB83, downstream transcription factors directly controlled by MYB46/MYB83, can activate the expression of lignin synthesis genes (Zhong and Ye, 2007). VND6 or VND7 dominant suppressor mutation results in a loss of metaxylem or protoxylem development (Kubo et al., 2005; Yamaguchi et al., 2010, 2011). Recently, the SND1 homologous proteins have been found in *Populus trichocarpa* L, rice and maize. In Arabidopsis, overexpression of *PtrWND2B* or *PtrWND6B* can prevent the secondary wall biosynthesis defect of *nst1-1nst3-1* and activate the expression of SCW synthesis genes, resulting in the ectopic deposition of cellulose, xylan, and lignin (Zhong et al., 2010).

Pomegranate seed hardness is an important economic trait. The molecular mechanism of regulating the hardness of pomegranate seeds is still not fully understood. In this study, we isolated a NAC-like gene (designated as *PgSND1-like*) from "Tunisia" and "Sanbai" 60 DAB (days after blooming) seed cDNA. Sequence analysis showed a base replacement. qRT-PCR results suggested that *PgSND1-like* is differently expressed in two pomegranate varieties. Overexpression of *PgSND1-like* (Sanbai) in Arabidopsis resulted in more lignin synthesis compared with wild type plants while overexpression of *PgSND1-like* (Tunisia) showed no difference. It was suggested that *PgSND1-like* (Sanbai) promotes the biosynthesis of lignin in transgenic Arabidopsis and may positively regulate seed hardness in pomegranates.

2. Materials and methods

2.1. Plant material and growth conditions

Pomegranate seeds were collected from "Tunisia" trees and "Sanbai" trees grown in the Zhengzhou Fruit Research Institute nursery at 30, 60, and 120 DAB.

Arabidopsis seeds (ecotype Columbia) were sterilized with 75% ethanol for 1 min and 10% NaClO for 3 min, followed by washing with sterile water several times. These seeds were vernalizated on MS medium at 4 °C for 48 h and germinated on MS medium for 7 days (16 h light/8 h dark, 22 ± 1 °C). The seedlings were then transferred into the soil.

2.2. Measurement of pomegranate seed hardness

Seed hardness was determined using a TA-XT Texture Analyzer. Twenty seeds of each variety were used in three replicates.

2.3. Measurement of pomegranate seed lignin content

The total lignin content of the pomegranate seed coat was determined using the UV spectrophotometer method and the average value of each sample was measured. The experiment was conducted three times using seeds from independent batches.

2.4. RNA extraction and qRT-PCR analysis

RNA was extracted from pomegranate seeds using the CTAB (Cetyltrimethyl Ammonium Bromide) method. The expression of *PgSND1-like* gene in pomegranate seeds was analyzed by quantitative RT-PCR using the fluorescent intercalating dye SYBR-Green in a Roche480 detection system. Pomegranate *ACTIN* gene was used as an internal control. *PgACTIN* primer sequences were as follows: forward 5′-AGTCCTCTTCCAGCCATCTC′-3′ and reverse 5′-CACTGAGCACAATGTTTCCA-3'.

PgSND1-like (RT) primer sequences: forward 5′-TCAACGCAGTCACTCACAGTT-3′ and reverse 5′-GATGAAGACTTGGTGAAGCTC-3'.

RT-PCR was used to analyze the expression of lignin synthesis-related and transcription factor genes. Each qRT-PCR analysis was performed in three replicates. The primers are listed in Table S1.

Six-week-old inflorescence stems of Arabidopsis and mature seeds were used for RNA extraction by Trizol reagent. RT-PCR was used to analyze lignin synthesis-related gene expression, with *AtACTIN2* as a normalization control. Arabidopsis *ACTIN2* primer sequences were as follows: forward 5′-GAAATCACAGCACTTGCACC-3′ and reverse 5′-AAGCCTTTGATCTTGAGAGC-3'. Each qRT-PCR analysis was performed in three replicates. The primers are listed in Table S2.

2.5. Isolation of PgSND1-like (Sanbai)/PgSND1-like (Tunisia) gene from pomegranate

We got *PgSND1-like* (Tunisia) sequence from "Tunisia" pomegranate genome (unpublished result). The full-length sequence of *PgSND1-like* gene was amplified from "Tunisia" and "Sanbai" seed cDNA by polymerase chain reaction (PCR). The *PgSND1-like* primer sequences are as follows: forward 5′-GGGTCTAGAATGCCACGAGACATGAAT-3′ and reverse 5′-CTTGGATCCTTATACCGATAAGTGGCG-3'.

2.6. Construction of overexpression vector and Arabidopsis genetic transformation

The coding sequence (CDS) of *PgSND1-like* gene, amplified from its cDNA by PCR with the proofreading pfu DNA polymerase, was cloned into the PEASY-Blunt-simple vector with *Bam*HI/*Xba*I sites to generate chimeric CaMV (cauliflower mosaic virus) 35S:PgSND1-like constructs.

The vectors were introduced into Arabidopsis by the floral dip method. Transformed seeds were selected on MS medium containing 50 mg/L kanamycin and transferred to soil for maturation and seed set. Homozygous lines of T3 generations were used for phenotypic analysis of transgenic plants.

2.7. Subcellular localization

The coding sequences of PgSND1-like (Sanbai) and PgSND1-like (Tunisia) were cloned into PBI121-eGFP vector. The vectors were transformed into Agrobacterium tumefaciens GV3101 strain, then introduced into Arabidopsis using the floral dip method. The 7-old-day roots of T1 seedings were observed for GFP fluorescence. Fluorescence microscopy was performed on a SP5 Meta confocal laser microscope (Leica, Germany).

2.8. Transcriptional activation activity analysis and yeast two-hybrid assay

The coding sequence of *PgSND1-like* (Sanbai) and *PgSND1-like* (Tunisia) was cloned into pGBKT7 vector. The fusion vectors were transferred into yeast strains AH109 and Y187 by using the high-efficiency lithium acetate transformation procedure (Gietz et al., 1992). The Y187 transformed yeast cells were confirmed with colour change on a β-galactosidase filter paper using the flash-freezing filter assay (James et al., 1996).

PgSND1-like-N was cloned into pGBKT7 vector and transformed into Y187 yeast cells. The full coding sequence of PgSND1-like was cloned into pGADT7 vector and transformed into AH109 yeast cells. Empty pGADT7 and pGBKT7 vectors containing the activation domain or binding domain were used as negative controls. The yeast two-hybrid was obtained by mating Y187 and AH109 (James et al., 1996). The transformates were further streaked on quadruple dropout medium (QDO medium, SD/–Trp/–Leu/–His/–Ade).

2.9. Measurement of lignin contents of Arabidopsis

The basal inflorescence stems of Arabidopsis grown for six weeks were used for lignin content measurement. The lignin contents were measured by Ultraviolet Spectrophotometric assay (Romualdo and Ronald, 2004). At least 10 seedlings in each line were chosen for the experiment. Every experiment was performed three times.

Lignin content was also measured in mature seeds of wild type and transgenic Arabidopsis. The lignin contents were measured using thioglycolic acid (TGA) assay (Campbell and Ellis, 1992) and a slight modification of the procedures (Liang et al., 2006). The experiment was conducted three times using seeds from independent batches.

3. Results

3.1. Seed hardness and lignin content

We measured the seed hardness and lignin content of two pomegranate cultivars, “Sanbai” and “Tunisia”. The result showed that with the extension of time after flowering and the development of the fruit, both seed hardness and lignin content gradually increased. In “Tunisia”, the seed hardness at 120 DAB was 2.03 kg and the lignin content was 10.606%. In “Sanbai”, the seed hardness at 120 DAB was 7.357 kg and the lignin content was 14.983%. The seed hardness of “Sanbai” was significantly greater than that of “Tunisia”, in proportion to the lignin content. According to statistic analysis, we found that there is a positive correlation between the seed hardness and the lignin content in two varieties, with a correlation coefficient at 0.974 (Fig. 1).

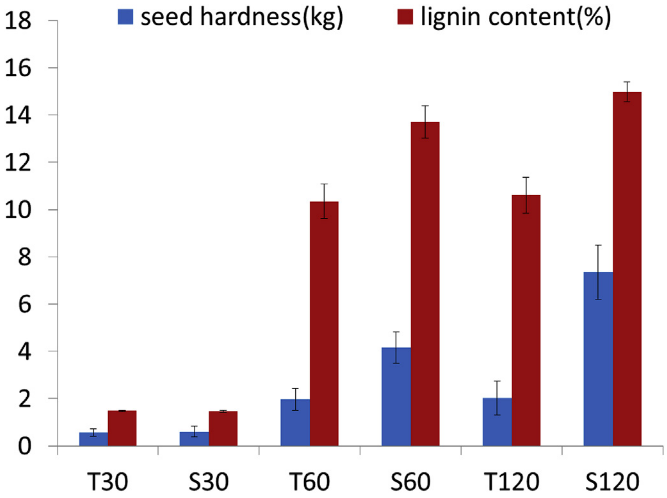

Fig. 1. Pomegranate Seed hardness and lignin content of at different developmental stages and in different cultivars. S30/60/120: 30/60/120 seed of “Sanbaiyingzi” T30/60/120: 30/60/120 seed of “Tunisiaruanzi”. Data shown is the mean values of 20 seeds. Mean values and standard errors were shown from three independent experiments with three biological replicates of plant materials.

3.2. Expression analysis of genes related to biosynthesis of lignin in pomegranate

According to our results, the hardness of the pomegranate seeds is positively correlated with the lignin content. Xue and colleagues performed a *de novo* assembly of the seed transcriptome in *P.granatum* L and found that a number of genes were differentially expressed between the soft- and hard-seed pomegranate varieties. These genes included key enzyme genes such as *CCR*, *CC*$_O$*AOMT*, *PAL* and *WRKY*, *MYB*, *NAC*, and other transcription factors (Xue et al., 2017). We then conducted a qRT-PCR experiment to confirm the expression levels of the differential genes and verify the reliability of the transcriptome results.

The biosynthesis of lignin is directly regulated by upstream structural genes. The lignin synthase genes are differentially expressed in “Sanbai” and “Tunisia” varieties during the critical period of lignin synthesis at 60 days after flowering. Differential expression of these structural genes is directly regulated by the upstream transcription factor MYB. The expression levels of MYBs (MYB46 and MYB63) were significantly higher in “Sanbai” than in “Tunisia” 30 and 60 days after flowering. MYBs are located downstream of SWNs (NST1, SND1,VND4, VND6, VND7) in the lignin synthesis network, and MYB46 is the direct target of SWNs. As shown in Fig. 2B, NAC TFs also show significant difference in expression in both pomegranate cultivars. Therefore, we suggested that NAC TFs control the synthesis of lignin by indirectly regulating the expression of downstream structural genes, which in turn affect the seed hardness.

3.3. Characterization of PgSND1-like

A NAC-like homolog with a complete open reading frame (ORF) of 1251bp and encoding a predicted 416 amino acids polypeptide was obtained from the “Tunisia” genome. *PgSND1-like* consists of 2 introns and 3 exons as observed in many other NAC-like genes (Fig. 3A). Analysis of phylogenetic relationships showed that the NAC-like homolog bore a high similarity to *AtSND1*, so it was named *PgSND1-like*.

We analyzed the expression level of *PgSND1-like* among the differential genes in the two pomegranate cultivars. No difference was observed in 30 DAB seed, but expression was higher in “Tunisia” seed at 60 DAB. The expression level of *PgSND1-like* in “Sanbai” was significantly higher than that of “Tunisia” at 120 DAB seed (Fig. 2B). We

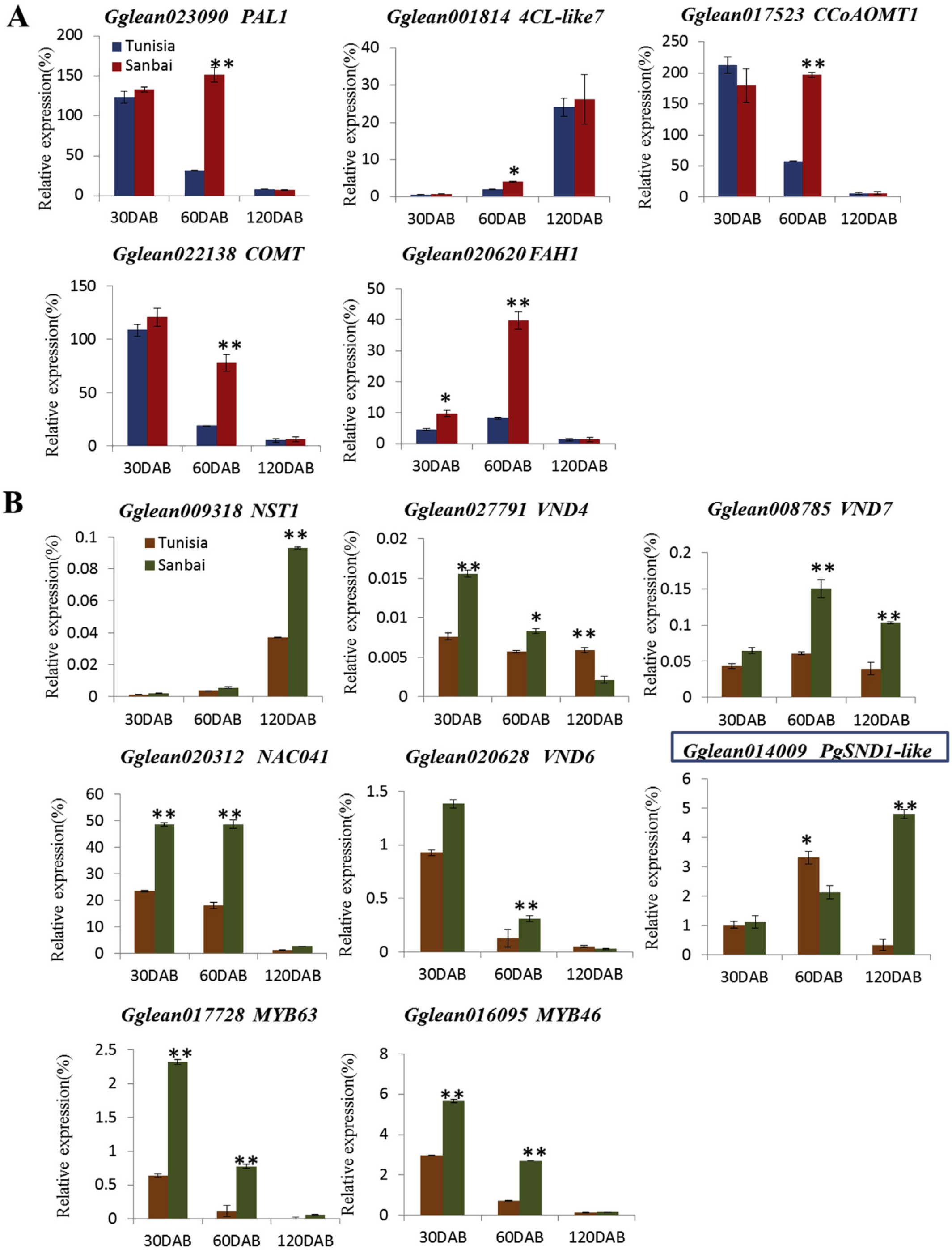

Fig. 2. Expression analysis of genes related to biosynthesis of lignin in pomegranate. A) Expression analysis of key enzyme genes in two pomegranate cultivars. B) Expression analysis of transcription factors related to lignin biosynthesis regulation. DAB: Day of booming. The relative values of gene expression are all shown as percentages of PgACTIN expression activity. Each RT-PCR analysis was reproduced for three independent experiments with three biological replicates of plant materials. Data is mean ± SE ($n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$ by *t*-test).

amplified *PgSND1-like* (Tunisia) and *PgSND1-like* (Sanbai) from 60 DAB seed cDNA, respectively. Sequence alignment displayed a single base replacement difference at the 166-bp position of the CDS sequence in *PgSND1-like* between "Tunisia" and "Sanbai" (Fig. 3A). The Lysine in PgSND1-like (T) is replaced by Glutamate in PgSND1-like (S) and other SND1 homologous proteins (Fig. 3C).

As shown in Fig. 3C, PgSND1-like (T) showed 99.75% match in amino acid identity with PgSND1-like (S). PgSND1-like (T) and PgSND1-like (S) exhibited 48.60% and 48.88% identity match with AtSND1, and 50.89% and 51.15% identity match with HbNAC43-like, respectively. Multiple Sequence alignment results also showed that PgSND1-like has a conserved NAC domain consisting of five conserved sub-domains (I,II,III,IV,V). Ooka et al. suggested that the sub-domains (I, III, IV) play an important role in the function of NAC proteins.

3.4. PgSND1-like proteins are localized in the cell nucleus

To investigate PgSND1-like protein localization in cells, the coding region of PgSND1-like was fused with an eGFP (enhanced green fluorescent protein) and introduced in Arabidopsis under the control of

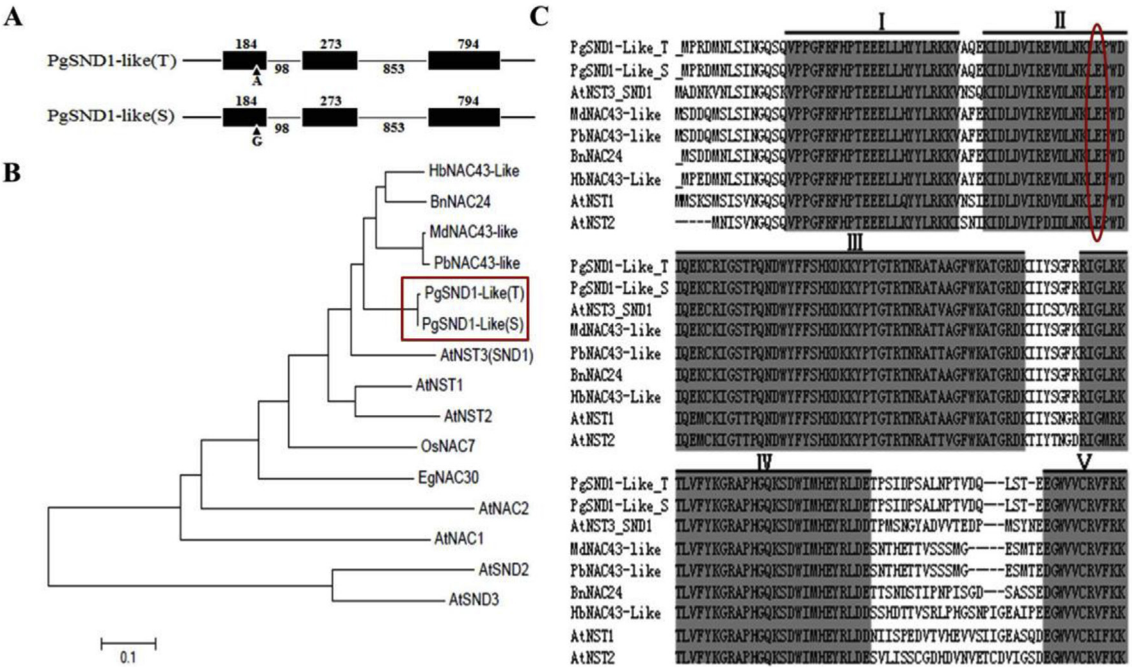

Fig. 3. Characterization of PgSND1-like. A) The structure of PgSND1-like gene. The black, bold line represents the 5-UTR or 3-UTR. Exons are denoted by black boxes. Introns are denoted by thin black lines. Triangles indicate base replacement. Red circle indicates amino acid mutation site. B) Phylogenetic analysis of PgSND1-like and other plants NAC-like homologs with Arabidopsis homologs. Phylogenetic trees were generated using MEGA 6.0 software. The accession numbers of these proteins in GenBank: Punica granatum: PgSND1-like (Gglean014009); Hevea brasiliensis: HbNAC43-like (XM_021804782.1); Boehmeria nivea: BnNAC24 (KF874848.1); Malusdomestica:MdNAC43-like (NM_001328763.1); Pyrusxbretschneideri:PbNAC43-like (XM_009361924.2); Arabidopsisthaliana:AtNST1 (AT2G46770),AtNST2 (AT3G61910),AtNST3 (AT1G32770). C) Sequence alignment of PgSND1-like protein with some known proteins. The alignment was generated using Clustal W software. The black line represents the conserved NAC domain (I,II, III, IV,V). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

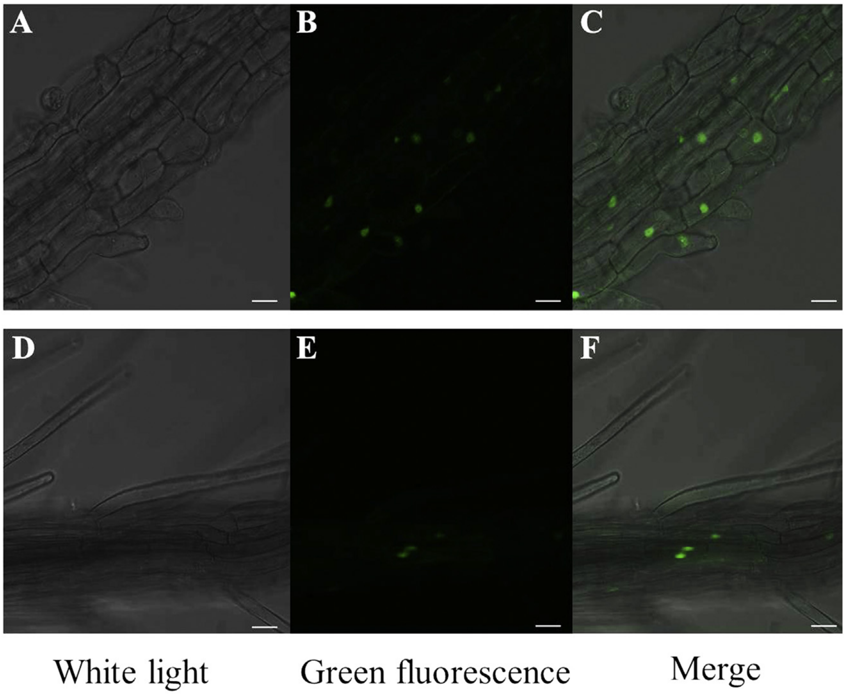

Fig. 4. Subcellular localization of PgSND1-like (S) and PgSND1-like (T). The fusion vector 35S:PgSND1-like(S)-eGFP and 35S:PgSND1-like(T)-eGFP were introduced into Arabidopsis. Green fluorescence signals were detected in the nuclei of the 7-day-old transgenic Arabidopsis root cells. (A/D) in the bright light for the morphology of the cell. (B/E) in the dark for the morphology of the cell. (C/F) The images of A and B were merged into one. (A–C):35S:PgSND1-like(S)-eGFP; (D–F): 35S:PgSND1-like(T)-eGFP. Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

35S CaMV promoter. As shown in Fig. 4, strong GFP fluorescence was detected in the cell nuclei of transgenic Arabidopsis root cells in 7-day-old seeding. These results showed that PgSND1-like (T) and PgSND1-like (S) proteins are located in the cell nuclei.

3.5. Analysis of PgSND1-like transcriptional activation and interaction with itself

To examine the transcriptional activity of PgSND1-like, pGBKT7-PgSND1-like vector was introduced into yeast strain AH109 and Y187, with PGBKT7 as the negative control. The AH109 yeast cells containing

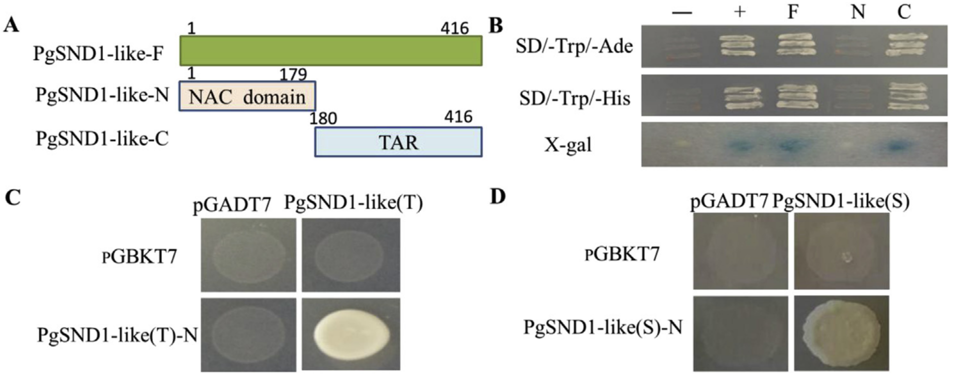

Fig. 5. Transactivation activity analysis and interaction of PgSND1-like in yeast. A) The diagrams display the conserved domain of PgSND1-like. PgSND1-like-F,PgSND1-like-N, and PgSND1-like-C indicate the 1th–416th aa, 1th–179th aa, and 180th–416th aa of PgSND1-like, respectively. B) Upper: the AH109 transformants were streaked on the SD/–Trp/–Ade/–His medium. Bottom: Y187 transformants were subjected to β-galactosidase activity assay. −: negative control; +: positive control. C) Yeast two-hybrid assay to test the interactions of PgSND1-like(T)−N and PgSND1-like(T)−F. D) Yeast two-hybrid assay to test the interactions of PgSND1-like(S)−N and PgSND1-like(S)−F. Yeast transformants were streaked on SD/-Ade/-His/-Leu/-Trp medium.

PgSND1-like-F grew well in the SD/–Trp/–Ade plate, suggesting that the reporter gene *Ade2* was activated. On the other hand, the Y187 yeast cells containing PgSND1-like-F turned blue, indicating that *LacZ*, another reporter gene, was also activated. Our results suggest that PgSND1-like gene has trans-activation activity. In order to locate the activation domain, we divided the full length of the gene into two parts: PgSND1-like-N (1-179AA) and PgSND1-like-C (180-416AA) and performed the assay. Our data showed that the activation domain is located in the C-terminal of the protein (Fig. 5B).

The NAC domain does not contain any known DNA binding motifs. However, NAC protein may form a functional homodimer or heterodimer to bind DNA (Ernst et al., 2004). We performed the yeast two-hybrid analysis to test whether the PgSND1-like protein forms a homodimer. As shown in Fig. 5C–D, the yeast zygote grew well on the SD/-Trp/-Leu/-His/-Ade. These results showed that PgSND1-like can interact with itself to form a homodimer.

3.6. Overexpression of PgSND1-like (S) in Arabidopsis increases the lignin biosynthesis in inflorescence stems

Pomegranate genetic transformation is still currently unexplored. Therefore, we constructed the fusion expression vectors of 35S-PgSND1-like (T) and 35S-PgSND1-like (S), and introduced them into Arabidopsis. We then constructed 16 *PgSND1-like(T)*-overexpression (OE) transgenic lines and 14 *PgSND1-like* (S)-overexpression (OE) transgenic lines. According to the data from the qRT-PCR experiment, we chose four plants that showed high expression levels of the gene for functional analysis (L3, L4, L3, L8). *PgSND1-like* (T)-OE transgenic plants exhibited a similar phenotype to WT Arabidopsis. However, the *PgSND1-like* (S)-OE transgenic plants displayed an upward curly leaf and a shorter capsule than wild type (Fig. 6). However, the phenotype is identical to the secondary wall-associated gene overexpression plants.

We examined the 6-week-old inflorescence stem cells of WT plant and transgenic lines using Safranine green staining. Histological examination of *PgSND1-like* (S) transgenic plants showed more lignin deposition than in wild-type in the xylary fibers. *PgSND1-like* (T) transgenic plants showed no difference compared with WT (Fig. 7A). There was some degree of thickening in the vessel cell walls of *PgSND1-like* (S) transgenic plants (Fig. 7B). Statistical analysis showed that the vessel cell wall thickness of *PgSND1-like* (S) transgenic plants is up to 1.04 μm, while that of WT is 0.63 μm (Fig. 7C). We also measured the lignin content of WT and transgenic plants. *PgSND1-like* (S) transgenic plants (L3, L4) had higher lignin content than the control, while *PgSND1-like* (T) (L3, L8) did not differ from wild type Arabidopsis (Fig. 7D). These results suggested that *PgSND1-like* (S) may positively regulate the lignin biosynthesis in Arabidopsis, while *PgSND1-like* (T) plays a different function in the process.

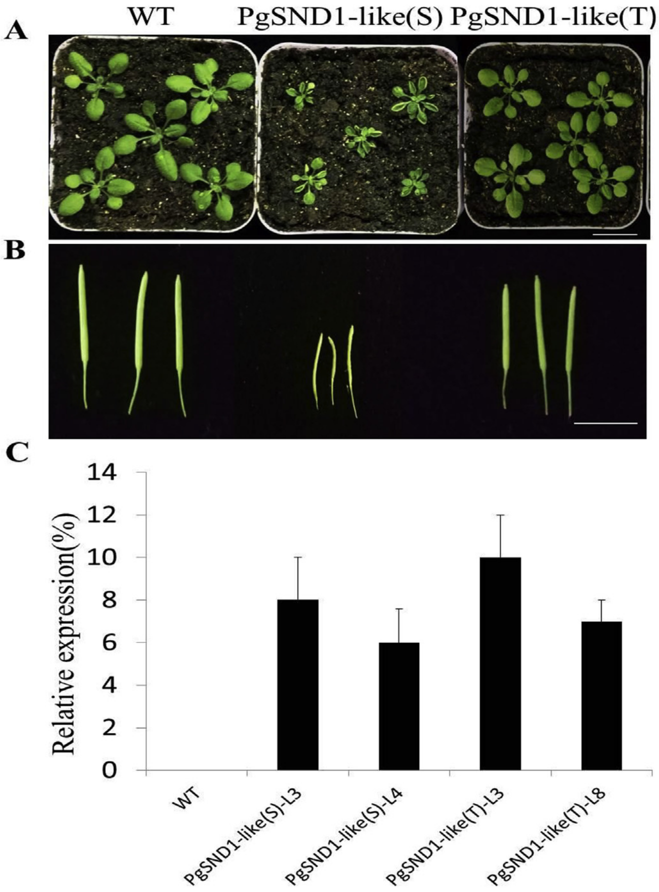

Fig. 6. Phenotype of wild-type Arabidopsis and PgSND1-like (S)/PgSND1-like (T) transgenic Arabidopsis. A) Two-week-old wild-type Arabidopsis and the PgSND1-like (S)/PgSND1-like (T) transgenic lines. Bar = 1 cm. B) Capsules of six-week-old wild-type Arabidopsis and the PgSND1-like (S)/PgSND1-like (T) transgenic lines. Bar = 1 cm. C) RT-PCR analysis of the expression levels of PgSND1-like in the six-week-old stems of wild-type Arabidopsis, PgSND1-like (S) transgenic Arabidopsis and PgSND1-like (T) transgenic Arabidopsis.

3.7. Expression of genes related to lignin formation in PgSND1-like transgenic Arabidopsis inflorescence stems

Total RNA was isolated from the inflorescence stems of *PgSND1-like* transgenic plants and WT plants and the expression of the lignin synthesis related genes was analyzed using quantitative RT-PCR. Additionally, *PAL1*, 4CL, *F5H*, *COMT*, *CCoAOMT*, *CCR*, and *CAD* are the key genes in lignin biosynthesis. As shown in Fig. 8, the expression levels of *PAL1*,4CL, *F5H*, *COMT*, *C4H*, *CCoAOMT*, *CCR*, *and CAD* in

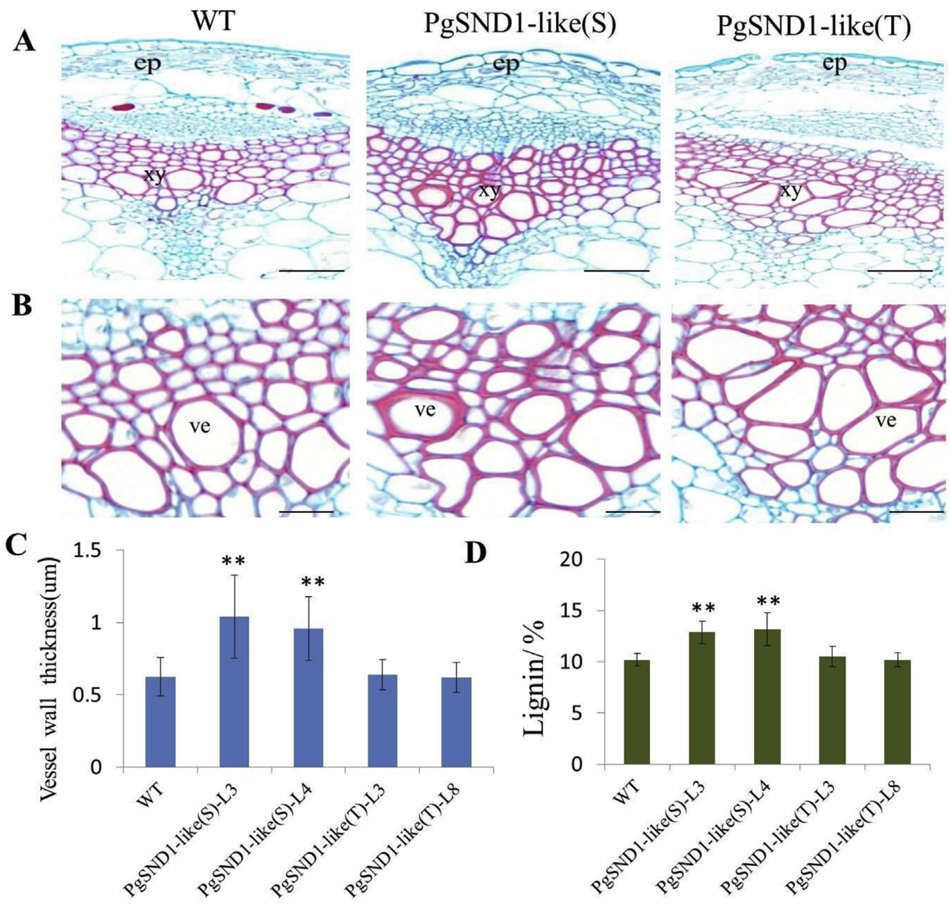

Fig. 7. Light micrographs of stem cross sections in wild-type and PgSND1-like(S)/(T) transgenic plant. A) Safranine green staining of the six-week-old stem cross sections of wild-type and PgSND1-like (S)/(T) transgenic plants. Bar = 50 μm. B) Thickened cell wall of vessels of wild-type and PgSND1-like (S)/(T) transgenic plant. C) Statistical analysis of the thickened cell wall of vessels. D) Lignin content in stems of wild type Arabidopsis and the PgSND1-like (S)/(T) transgenic Arabidopsis plants. Error bars represent SD. Mean values and standard errors were derived from three independent experiments with three biological replicates of plant materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PgSND1-like (S) transgenic plants (L3, L4) all showed a higher transcription level than in WT. In the *PgSND1-like* (T) transgenic plants (L3, L8), the expression levels of *PAL1*, *F5H*, *C4H*, *CCoAOMT, CCR, and CAD* did not differ from WT, while the expression levels of *COMT* and 4CL in transgenic plants L3 were slightly higher. These results suggested that *PgSND1-like (S)* may up-regulate the genes related to lignin biosynthesis, further regulating the lignin biosynthesis in Arabidopsis.

3.8. Overexpression of PgSND1-like(S) in Arabidopsis increases the lignin biosynthesis of seeds

Overexpression of *PgSND1-like* (S) in Arabidopsis can increase the lignin content of inflorescence stems. To find whether or not it affects the synthesis of lignin content in other Arabidopsis tissues, we used Safranine green staining to examine mature seed cells of WT and transgenic lines. Histological examination of *PgSND1-like* (S) transgenic plants showed that they had more lignin deposition than WT plants. Additionally, we analyzed the lignin content of WT and transgenic Arabidopsis plants. Our results showed that the lignin content of *PgSND1-like* (S) transgenic plants was up to 15.5%, and that of WT was 12.6%, similar to lignin content percentage in *PgSND1-like* (T) transgenic plants (Fig. S1).

Total RNA was isolated from mature seeds of WT and *PgSND1-like* transgenic plants. We examined the expression of genes involved in lignin biosynthesis. As shown in Fig. 9B, overexpression of *PgSND1-like* (S) resulted in an increase in the expression of lignin biosynthesis genes (*PAL1*, 4CL, *F5H*, *CCoAOMT, CCR*, and *CAD*). In the *PgSND1-like* (T) transgenic plants (L3, L8), the expression levels of lignin biosynthesis genes did not differ from WT, while the expression level of *CC_OAOMT* in transgenic plants L3 was slightly higher. Our results showed that *PgSND1-like* (S) can promote lignin synthesis in both inflorescence stems and seeds (Figs. 7–9). On the basis of these functional studies, we conclude that *PgSND1-like* (S) may positively impact pomegranate seed hardness, while *PgSND1-like* (T) not.

4. Discussion

Pomegranate contains rich natural substances with high health promoting value (Bi and Li, 2010). Seed hardness is one of the most important factors that affect fruit quality since lignified seeds significantly reduce pomegranate fruit taste. Previous researches showed that lignin is an important component that defines the pomegranate seed hardness (Cao et al., 2015; Dalimov and Bhatt, 2003; Zhang et al., 2015). It has been experimentally confirmed that the hardness of pomegranate seeds positively correlated with the lignin content. However, the molecular mechanism that emphasizes soft seed formation has rarely been reported. In our study, we found that a NAC-like transcription factor PgSND1-like, showed differential expression between soft- and hard-seed pomegranate varieties and followed seed hardness changes during the seed development stages. Furthermore, employing genetic transformation, we identified PgSND1-like (S) function and showed that this gene could control the synthesis of lignin in Arabidopsis thaliana. According to our data, we suggested that *PgSND1-like (S)* controls seed hardness in pomegranate. Our findings also indicate the cause of the pomegranate soft seed formation.

Lignin is one of the important components of vascular plant cell wall. Lignin plays an important role in the regulation of plant growth and development. The protein can enhance plant cell wall rigidity and hydrophobic properties (Schuetz et al., 2014). NAC transcription factors control lignin biosynthesis. The N-terminus of the NAC transcription factor has a highly conserved 150 amino acid NAC domain that binds to DNA and specific proteins (Ernst et al., 2004). The

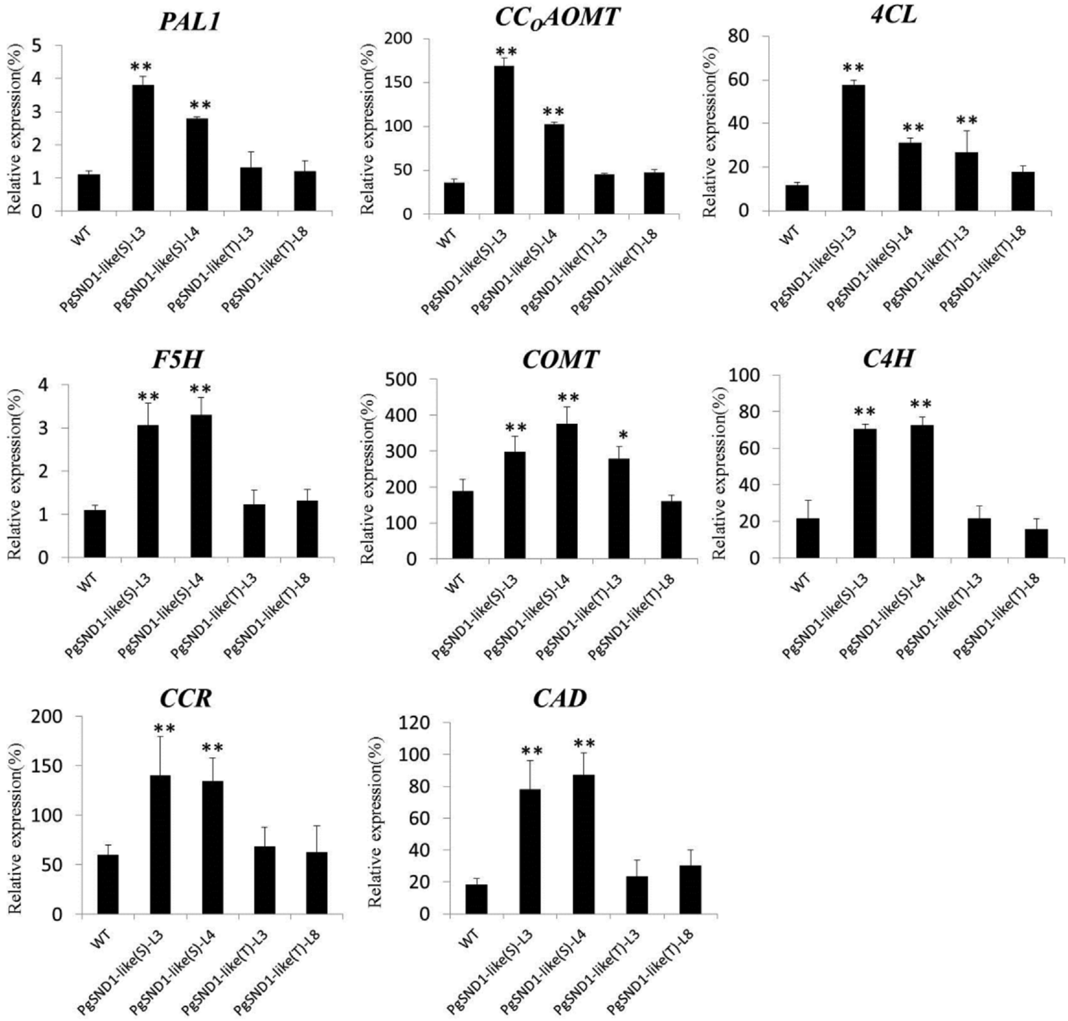

Fig. 8. Quantitative RT-PCR analysis of expression of the genes related to lignin formation in wild type and PgSND1-like (S)/(T) transgenic Arabidopsis. Total RNA was isolated from the inflorescence stems of 6-week-old wild-type and transgenic plants. Arabidopsis ACTIN2 was used as a control. RT-PCR analysis was generated from three independent experiments with three biological replicates of plant materials. Independent t-tests demonstrated that there was significant difference (*P value < 0.05) or very significant difference (**P value < 0.01) in gene expression levels between transgenic and wild-type plants.

transcriptional activation region of the NAC transcription factor is located at the C-terminus and there are significant differences among the various members of NAC family (Ernst et al., 2004).

In this study, we isolated a NAC-like gene from "Sanbai" seed cDNA, which was designated as *PgSND1-like* (S), and another NAC-like gene from "Tunisia" cDNA, which was designated *PgSND1-like* (T). The phylogenetic analysis revealed that *PgSND1-like* genes shared high similarity with *AtSND1*. The N-terminus of PgSND1-like protein also has a conserved NAC domain consisting of five conserved sub-domains (I,II,III,IV,V). PgSND1-like protein is located in the nucleus and its transcription activation domain is located at the C-terminus part of the protein molecule. Yeast two-hybrid analysis results suggested that PgSND1-like protein plays a transcriptional regulatory function by forming a homodimer. These results suggest that PgSND1-like is similar to NAC transcription factors and may have related functions.

Overexpression of *PgSND1-like*(S) resulted in formation of upward curly leaves and shorter capsule than WT. Histochemical staining and lignin content measurement in six-week inflorescence stems and mature seed of *PgSND1-like(S)* transgenic plants showed more lignin deposition compared to WT. qRT-PCR results showed that the expression level of lignin synthesis-related genes in the *PgSND1-like*(S) transgenic plants displayed a higher transcription activation level than WT. Our data showed that *PgSND1-like*(S) can promote the synthesis of lignin in Arabidopsis thaliana and may affect seed hardness.

However, overexpression of *PgSND1-like* (T) in the Arabidopsis showed a similar phenotype to WT. Sequence alignment displayed a single base replacement at the 166-bp position in the *PgSND1-like* CDS region between "Tunisia" and "Sanbai". Ooka et al. speculated that the sub-domains (I, III, IV) play an important role in the function of NAC protein (Ooka et al., 2003). The 166bp position was in the "I" subdomain of the NAC protein. Multiple sequence alignment results showed that the lysine in PgSND1-like (T) was replaced by Glutamate in PgSND1-like (S) and SND1-like proteins in other species. We cloned the PgSND1-like genes from four other soft seed and four hard seed varieties. The sequence alignment results showed that the 166bp position of PgSND1-like gene was base "A" in all soft seed varieties and base "G" in all hard seed varieties (Fig. S2). Above all, we suggested that the difference in protein function was due to one amino acid change. We also found that the location of the protein was not changed. The specific details of how this base affects protein function require further study and discussion.

Although pomegranate genetic transformation is still not well understood, our study is part of an ongoing effort to clarify the mechanisms of pomegranate genetic regulation. In future studies, we aim to transform *PgSND1-like* gene from pomegranate varieties, analyze the phenotype, and clarify the associated functions. These studies are

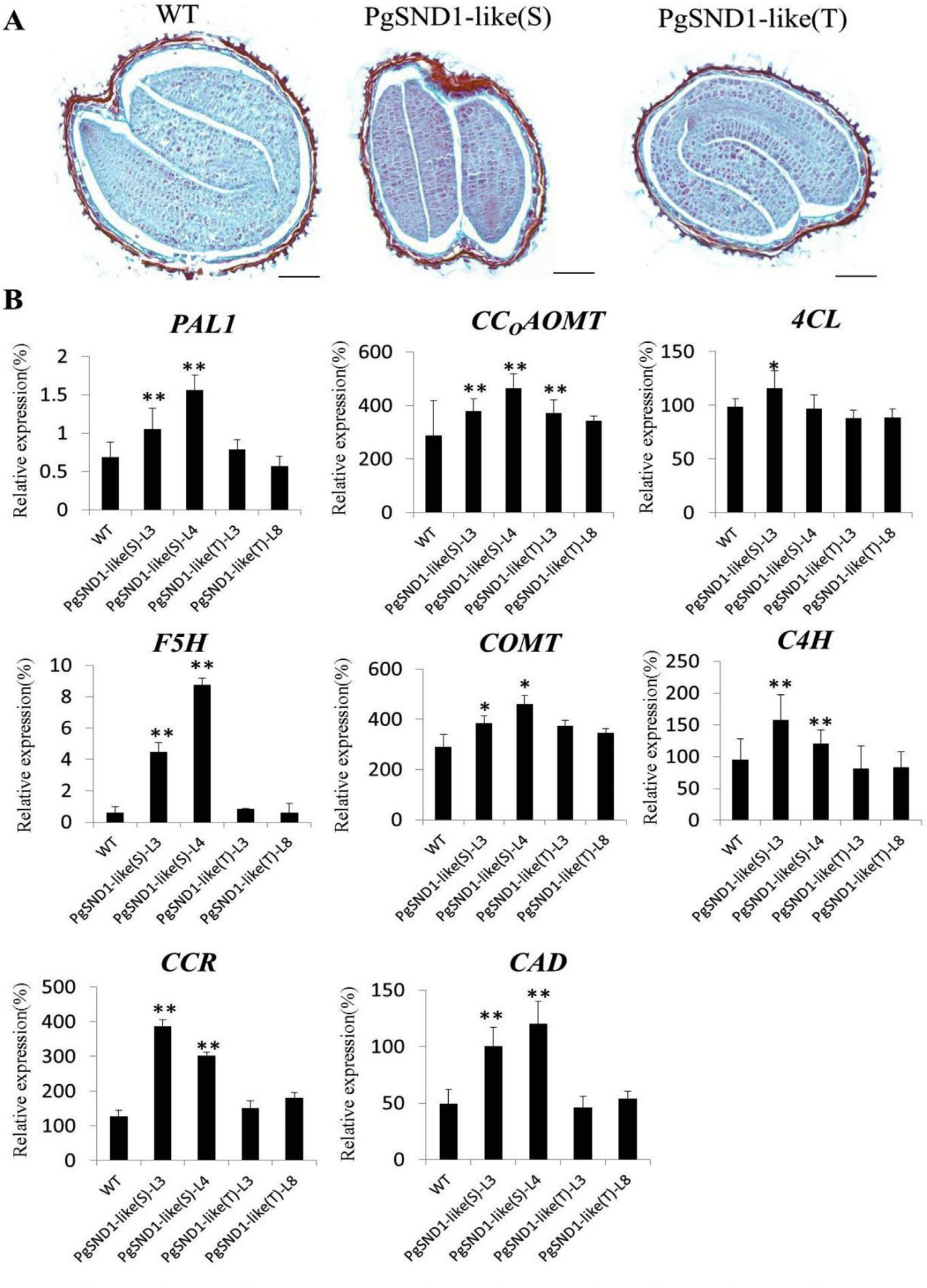

Fig. 9. Seed phenotype of wild-type and PgSND1-like (S)/(T) transgenic plant. A) The ripe seeds of wild-type and PgSND1-like (S)/(T) transgenic plants were subjected to Safranine green staining. Bar = 50 μm. B) RT-PCR analysis of expression of the genes related to lignin formation. Total RNA was isolated from the mature seeds. Arabidopsis *ACTIN2* was used as a control. RT-PCR analysis was generated from three independent experiments with three biological replicates of plant materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

currently in progress. In conclusion, our data indicated that PgSND1-like (S), as a NAC transcription factor, can promote lignin formation in transgenic Arabidopsis, further regulating the formation of pomegranate seed hardness. PgSND1-like (T), however, may play different functions in regulating seed hardness that requires further investigation. Previous studies showed that AtSND1 acts as a master switch, activating the biosynthetic genes of cellulose, xylem, and lignin. Whether the *PgSND1-like* is the main switch to control pomegranate seed hardness, should be clarified in future studies.

Conflicts of interest

The authors declare that they have no conflict of interest.

Authors' contributions

XXC and CSY designed the experiments. XXC, LHX, LH, CLN, LBB, WQ and JD performed the experiments. XXC analyzed the data. XXC and CSY wrote the paper. XXC and CD edited the manuscript. All of the authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.033.

References

Bate, N.J., Orr, J., Ni, W., Meromi, A., Nadler-Hassar, T., Doerner, P.W., Dixon, R.A., Lamb, C.J., Elkind, Y., 1994. Quantitative relationship between phenylalanine ammonia lyase level and phenylpropanoid accumulation in transgenic tobacco identifies a rate determining step in nature product synthesis. Proc. Natl. Acad. Sci. U.S.A. 91, 7608–7612.

Bi, X.F., Li, Y., 2010. Study on chemical composition and health functions of pomegranate. Mod Agri Sci Techn 22, 356–360.

Campbell, M.M., Ellis, B.E., 1992. Fungal elicitor-mediated responses in pine cell-cultures. Induction of phenylpropanoid metabolism. Planta 186, 409–417.

Cao, D.Q., Yang, J., Guan, X.W., Zhang, Y.J., Gong, L.Y., Zhang, Y.M., 2015. Cloning and expression of the transcription factor PgMYB related to lignin synthesis in pomegranate seed coat. J. Northwest Botanical Sci. 35, 0023–0029.

Dalimov, D.N., Bhatt, M., 2003. Chemical composition and lignins of tomato and pomegranate seeds. Chem. Nat. Comp. 39, 37–40.

Duval, M., Hsieh, T.F., Kim, S.Y., Thomas, T.L., 2002. Molecular characterization of AtNAM: a member of the Arabidopsis NAC domain superfamily. Plant Mol. Biol. 50, 237–248.

Ernst, H.A., Olsen, A.N., Larsen, S., LoLeggio, L., 2004. Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. EMBO Rep. 5, 297–303.

Gietz, D., Jean, A., Woods, R.A., Schiestl, R.H., 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20, 1425.

Grant, E.H., Fujino, T., Beers, E.P., Brunner, A.M., 2010. Characterization of NAC domain transcription factors implicated in control of vascular cell differentiation in Arabidopsis and Populus. Planta 232, 337–352.

Holland, D., Hatib, K., Bar-Yaakov, I., 2009. Pomegranate: botany, horticulture, breeding. Hortic. Rev. 35, 127–191.

James, P., Halladay, J., Craig, E.A., 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144, 1425–1436.

Kim, N.D., Mehta, R., Yu, W.P., 2002. Chemopreventive and adjuvant therapeutic potential of pomegranate (Punica granatum) for human breast cancer. Breast Canc. Res. Treat. 71, 203–217.

Kubo, M., Udagawa, M., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H., Demura, T., 2005. Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev. 19, 1855–1860.

Li, S.Z., 2013. Compendium of Materia Medica Essence. Science Press, Beijing, pp. 416–417.

Liang, M.X., Davis, E., Gardner, D., Cai, X.N., Wu, Y.J., 2006. Involvement of AtLAC15 in lignin synthesis in seeds and in root elongation of Arabidopsis. Planta 224, 1185–1196.

Mc Carthy, R.L., Zhong, R., Ye, Z.H., 2009. MYB83 is a direct target of SND1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in Arabidopsis. Plant Cell Physiol. 50, 1950–1964.

Miguel, M.G., Neves, M.A., Antunes, M.D., 2010. Pomegranate (Punica granatumL.): a medicinal plant with myriad biological properties - a short review. J. Med. Plants Res. 4, 2836–2847.

Mitsuda, N., Iwase, A., Yamamoto, H., Yoshida, M., Seki, M., Shinozak, i.K., Ohme-Takagi, M., 2007. NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. Plant Cell 19, 270–280.

Mitsuda, N., Seki, M., Shinozaki, K., Ohme-Takagi, M., 2005. The NAC transcription factors NST1 and NST2 of Arabidopsis regulates secondary wall thickening and are required for anther dehiscence. Plant Cell 17, 2993–3006.

Nuruzzaman, M., Manimekalai, R., Sharoni, A.M., Satoh, K., Kondoh, H., Ooka, H., Kikuchi, S., 2010. Genome-wide analysis of NAC transcription factor family in rice. Gene 465, 30–44.

Ooka, H., Satoh, K., Doi, K., Nagata, T., Otomo, Y., Murakami, K., Matsubara, K., Osato, N., Kawai, J., Carninci, P., Hayashizaki, Y., Suzuki, K., Kojima, K., Takahara, Y., Yamamoto, K., Kikuchi, S., 2003. Comprehensive analysis of NAC family genes in Oryza sativa and Arabidopsis thaliana. DNA Res. 10, 239–247.

Osakabe, Y., Ohtsubo, Y., Kawai, S., Katayama, Y., Morohoshi, N., 1995. Structure and tissue specific expression of genes for phenylalanine ammonia-lyase from ahybrid aspen, Pop-ulus Kitakamiensis. Plant Sci. 105, 217–226.

Romualdo, S.F., Ronald, D.H., 2004. Comparison of the acetyl Bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. Agric Food Chem 52, 3713–3720.

Sanchez-Lamar, A., Fonseca, G., Fuentes, J.L., 2008. Assessment of the genotoxic risk of Punica granatum L. (Punicaceae) whole fruit extracts. J. Ethnopharmacol. 115, 416–422.

Schuetz, M., Benske, A., Smith, R.A., Watanabe, Y., Tobimatsu, Y., Ralph, J., Demura, T., Ellis, B., Samuels, A.L., 2014. Laccases direct lignification in the discrete secondary cell wall domainsof protoxylem. Plant Physiol. 166, 798–807.

Sewalt, V., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C., Dixon, R.A., 1997. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of phenylalanine ammonialyase or cinnamate 4-hydroxylase. Plant Physiol. 115, 41–50.

Su, H., Zhang, S., Yuan, X., Chen, C., Wang, X.F., Hao, Y.J., 2014. Genome-wide analysis and identification of stress-responsive genes of the NAM-ATAF1, 2-CUC2 transcription factor family in apple. Plant Physiol. Biochem. 71, 11–21.

Teixeira da Silva, J.A., Rana, T.S., Narzary, D., Verma, N., Meshram, D.T., Ranade, S.A., 2013. Pomegranate biology and biotechnology: a review. Sci. Hortic. 160, 85–107.

Van-Acker, R., Vanholme, R., Storme, V., 2013. Lignin biosynthesis perturbations affect secondary cell wall composition and sacchari-fication yield in Arabidopsis thaliana. Biotechnol. Biofuels 6, 46–62.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W., 2010. Lignin biosynthesis and structure. Plant Physiol. 153, 895–905.

Xue, H., Cao, S.Y., Li, H.X., Zhang, J., Niu, J., Chen, L.N., Zhang, F.H., Zhao, D.G., 2017. De novo transcriptome assembly and quantification reveal differentially expressed genes between soft-seed and hard-seed pomegranate (Punica granatum L.). PLoS One 12, e178809.

Yamaguchi, M., Goué, N., Igarashi, H., Ohtani, M., Nakano, Y., Mortimer, J.C., Nishikubo, N., Kubo, M., Katayama, Y., Kakegawa, K., Dupree, P., Demura, T., 2010. VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR- RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system. Plant Physiol. 153, 906–914.

Yamaguchi, M., Mitsuda, N., Ohtani, M., Takagi, M.O., 2011. VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. Plant J. 66, 579–590.

Zhang, Y.M., Gong, L.Y., Cao, D.Q., Zhang, Y.J., Yang, J., 2015. Cloning and expression of PgCOMT and lignin content of PgCOMT in pomegranate seed coat. J. Trop. Subtropical Bot. 23, 65–73.

Zhong, R., Lee, C., Zhou, J., Ye, Z.H., 2008. A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in Arabidopsis. Plant Cell 20, 2763–2782.

Zhong, R., Demura, T., Ye, Z.H., 2006. SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. Plant Cell 18, 3158–3170.

Zhong, R., Lee, C., Ye, Z.H., 2010. Functional characterization of poplar wood-associated NAC domain transcription factors. Plant Physiol. 152, 1044–1055.

Zhong, R., Richardson, E.A., Ye, Z.H., 2007. The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. Plant Cell 19, 2776–2792.

Zhong, R., Ye, Z.H., 2007. Regulation of cell wall biosynthesis. Curr. Opin. Plant Biol. 10, 564–572.