Identification and Functional Analysis of the CgNAC043 Gene Involved in Lignin Synthesis from *Citrus grandis* “San Hong”

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Abstract: Overaccumulation of lignin (a physiological disorder known as granulation) often occurs during fruit ripening and postharvest storage in pomelo (*Citrus grandis*). It causes an unpleasant fruit texture and taste. Previous studies have shown that lignin metabolism is closely associated with the process of juice sacs granulation. At present, the underlying transcriptional regulatory mechanisms remain unclear. In this study, we identified and isolated a candidate NAC transcription factor, CgNAC043, that is involved in the regulation of lignin biosynthesis in *Citrus grandis*, which has homologs in *Arabidopsis* and other plants. We used the fruit juice sacs of ‘San hong’ as the material, the staining for lignin with HCl–phloroglucinol of fruit juice sacs became dark red from the various developmental stages at 172 to 212 days post anthesis (DPA). The RT-qPCR was used to analyze the gene expression of CgNAC043 and its target gene CgMYB46 in fruit sacs, it was found that the expression trend of CgNAC043 was basically same as CgMYB46, which increased gradually and peaked at 212 DPA. The expression level of CgNAC043 in juice sacs obtained away from the core was the lowest, while those near the core and granulated area were highly expressed. The transcriptional activation activity of CgNAC043 and CgMYB46 was analyzed by a yeast two-hybrid system, with only CgNAC043 showing transcriptional activation activity in Y2H Gold yeast. A transformation vector, p1301- CgNAC043, was transformed into the mesocarp of ‘San hong’ by *Agrobacterium*-mediated transformation. Results showed that the expression of transcription factors CgMYB58 and CgMYB46 are all upregulated. Further experiments proved that CgNAC043 not only can directly trans-activate the promoter of CgMYB46 but also trans-activate the promoters for the lignin biosynthesis-related genes CgCCoAOMT and CgC3H by dual luciferase assay. We isolated the CgNAC043 gene in pomelo and found CgNAC043 regulates target genes conferring the regulation of juice sacs granulation.

Keywords: NAC; MYB; gene expression; transcriptional activity; dual luciferase; pomelo

1. Introduction

Pomelo (*Citrus grandis* (L.) Osbeck.) is a distinctive citrus fruit characterized by its thick peel, large size, and high vitamin C content [1]. “Sanhongmiyou” (San hong) is a new variety bred from the mutants of “Guaxinmiyou”. It is sour, sweet, delicious, and nutritious. In citrus fruit juice sacs, the overaccumulation of lignin (a physiological disorder known as granulation) often occurs [2,3]. Granulation of citrus fruits also occurs in “San hong”, especially during fruit ripening and post-harvest storage, affecting the commercial properties greatly [4]; it causes a “gritty” texture and dryness of the juice sacs, and reduces consumer acceptance of the fruits [2]. Lignin, which normally accumulates in secondary cell walls, is one of the most important components of the plant cell wall [5]. Previous evidence has shown that granulation due to excessive lignin accumulation can greatly damage fruit sensory quality during the post-harvest process [6].
The NAC (no apical meristem (NAM), Arabidopsis transcriptional activator (ATAF), and cup-shaped cotyledon (CUC)) family is one of the largest classes of plant-specific transcription factors (TFs) discovered in the past decade [7]. The NAM (no apical meristem) gene was first isolated from petunia in 1996. Subsequently, ATAF1/2 and CUC2 genes with similar effects were found in Arabidopsis thaliana by Aida [8]. There is a highly conserved DNA binding domain in the N-terminal region, whereas the C-terminal region of NAC proteins is a diversified activation domain [8,9].

MYB and NAC TFs play important roles in the transcription and regulation of lignin synthesis, as both transcriptional activators and repressors [10–12]. NAC TFs have been shown to participate in diverse biological processes, including the development of root and shoot apical meristems [13,14], organogenesis [15], hormone signaling [16–18], fruit ripening [19,20], responses to biotic and abiotic stresses [21–24], and leaf senescence in various plant species [25–27]. In addition, NAC TFs regulate the fiber biosynthesis and development of the secondary cell wall [28–33]. In dicot species, including Arabidopsis, the overexpression of SND1 in Arabidopsis results in activation of the expression of secondary cell wall biosynthetic genes, leading to a massive deposition of the secondary walls [10]. Overexpression of GhFSN1 in gossypium thickens the fibrage of the second cell wall [33]. EjNAC1 is related to lignin synthesis and can activate the promoter that participates in the lignin synthesis pathway in loquat (Eriobotrya japonica) [34]. Overexpression of BpNAC012 activates the expression of the secondary cell wall associated genes, resulting in ectopic deposition in the stem epidermis [35]. Previous findings have revealed that AtMYB46/AtMYB83 is a direct target of AtSND1 and is another key player in the transcriptional network involved in the regulation of secondary cell wall biosynthesis in Arabidopsis [36,37]. Overexpression of AtMYB46 or AtMYB83 up-regulates lignin synthesis-related genes, resulting in abnormal secondary cell wall accumulation [38]. In orange (Citrus sinensis), CsMYB330 and CsMYB85, which are homologs of AtMYB58/AtMYB63 and AtMYB85, respectively, have been reported to interact with CsMYB308 through the binding of the Cs4CL1 promoter to regulate juice sac granulation [2,39]. Subsequently, in pomelo, Shi [40] revealed the expression of CgMYB58 led to lignin accumulation and the upregulation of 19 lignin biosynthetic genes. Among these, CgPAL1, CgPAL2, Cg4CL1, and CgC3H were directly modulated by CgMYB58 through interaction with their promoter regions. In loquat, several MYB TFs are associated with lignin synthesis; EjMYB1 and EjMYB2 have been found to regulate lignin metabolism by binding to the AC element of the Ej4CL1 promoter [41].

Zhong [42] reported that second wall NACs (SWNs) bind to a common cis-acting element, namely second cell wall binding element (SNBE), which is composed of an imperfect palindromic 19-bp consensus sequence, (T/A)NN(C/T)(T/C/G)TNNNNNNA (A/C)GN(A/C/T)(A/T). Subsequently, the studies of EMSA and transactivation assays have demonstrated that representative SNBE sequences from the promoters of these target genes are bound and activated by not only SND1 and VND7, but also by other SWNs, including NST1/2 and VND6 [42]. OsSWNs and ZmSWNs activate their target genes by directly binding to SNBE sites, leading to ectopic deposition of cellulose, xylan, and lignin [43].

The literature is rich in NAC TFs related to lignin metabolism, however, similar studies are lacking in citrus fruits juice sac lignification. In this report, we studied the functions of CgNAC043 in the transcriptional control of secondary wall biosynthesis. We show that CgNAC043 and CgMYB46 are expressed in secondary wall-forming cells and they are functional orthologs of NST1 and AtMYB46, respectively. When overexpressed, CgNAC043 in pomelo is able to activate the expression of CgMYB46 and CgMYB58. We further demonstrate that SNBEs are present in the promoters of CgMYB46 and the genes related to the lignin synthesis pathway, and they could be directly activated by CgNAC043. Our results demonstrate that the secondary wall-associated NAC and MYB46 genes are key transcriptional switches regulating secondary wall biosynthesis in pomelo.
2. Results

2.1. Microscopic Observation of Lignin Determination of Lignin Juice Sacs

Representative images of morphological changes in pomelo fruits are presented in Figure 1. Juice sacs at later maturation (212 DPA) are stained darker red with the lignin specific stain HCl−phloroglucinol relative to the juice sacs at earlier maturation (172 DPA). We observed that juice sacs in “San hong” at 212 DPA exhibited the most severe lignification, indicating a relatively high level of lignin accumulation.

2.2. Identification of CgNAC043 and Sequence Alignment

The CgNAC043 (Cg5g000340.1) cDNA was isolated from San hong, encoding a protein of 399 amino acids with a predicted molecular mass of 45.15 kDa and theoretical pI of 6.18. The total average hydrophilic coefficient was −0.867, indicating that this protein was hydrophilic. The instability coefficient of the protein was 57.62, indicating that the protein was unstable.

Multiple sequence alignments of the NAC proteins from Citrus clementina, Citrus sinensis, Pistacia vera, Durio zibethinus, Gossypium hirsutum, Populus trichocarpa, and Citrus grandis indicated that the CgNAC043 protein shares 99.25%, 99.25%, 76.43%, 72.21%, 68.75%, and 65.84% identity with these species, respectively (Supplementary Figure S1). The structure of the domain showed that the CgNAC043 protein contains a highly conserved NAM domain in the N-terminal portion. The conserved motif distribution displayed that motif3, motif2, and motif1 in the N-terminal region were highly conserved in all NAC proteins (Figure 2a).
Figure 2. (a) Distribution of the conserved motifs and structural domain of NAC genes; (b) The sequences of the 10 motifs are exhibited at the bottom. ANAC012 (AT1G32770.1), ANAC073 (AT4G28500.1), ANAC010 (AT1G28470.1), ANAC043 (AT2G46770.1), ANAC066 (AT3G61910.1), ANAC037 (AT2G18060.1), ANAC076 (AT4G36160.1), ANAC105 (AT5G66300.1), ANAC007 (AT1G12260.1), ANAC026 (AT1G62700.1), ANAC101 (AT5G62380.1), ANAC030 (AT1G71930.1), WND1A (HQ215847.1), WND1B (HQ215848.1), WND2A (HQ215849.1), WND2B (HQ215850.1), WND3A (HQ215851.1), WND3B (HQ215852.1), WND4A (HQ215853.1), WND4B (HQ215854.1), WND5A (HQ215855.1), WND5B (HQ215856.1), WND6A (HQ215857.1), WND6B (HQ215858.1), EJNAC1 (KJ919962.1), EJNAC2 (KJ919963.1), EJNAC3 (MG203936.1), OsSWN1 (JN634070.1), and PtoVNS11 (KU049786.1).

Furthermore, to analyze the phylogenetic relationship of the secondary cell wall NAC proteins, an unrooted neighbor-joining (NJ) phylogenetic tree was constructed according to the full-length protein sequences in Arabidopsis thaliana (https://www.arabidopsis.org/, accessed on 18 November 2021). The results showed that CgNAC043, ANAC043 (NST1), ANAC066, ANAC012 (SND1), ANAC043, ANAC033, ANAC015, ANAC070, ANAC037, ANAC076, ANAC0105, ANAC007, ANAC0101, and ANAC030 are located in the subfamily OsNAC7, suggesting that CgNAC043 may be similar to these species in function (Figure 3).

2.3. The Correlation Analysis of CgNACs and CgMYBs in the Process of Lignin Synthesis

We screened out two major types of transcription factors through RNA-seq data, namely CgNACs and CgMYBs. The FPKM values of these genes at 157 DPA, 180 DPA, and 212 DPA gradually increased with the synthesis of lignin (Supplementary Table S1). After further analysis of the correlation levels of these DEGs, we found that the correlation coefficient between CgNAC043 (Cg5g000340) and CgMYB46 (Cg2g030450) was 0.95, which is a highly positive correlation ($p < 0.01$) (Figure 4). This indicates that they may interact
with genes of the lignin synthesis pathway and participate in the granulation process of San hong juice sacs.

**Figure 3.** Phylogenetic analysis of NAC genes in *Arabidopsis thaliana*. The phylogenetic tree was constructed using the neighbor-joining method in the MEGA 6.0 program, and the bootstrap value was set to 1000. Each group is highlighted in a different color. Group I includes 14 sub-families, namely ONAC022, TERN, SENUS, OsNAC7, OsNAC3, ATAF, AtNAC3, NAP, NAC2, ANAC011, TIP, OsNAC8, NAC1, and NAM, while Group II includes four sub-families, namely ONAC003, ANAC001, ANAC063, and ONAC001.

**Figure 4.** The correlation heatmap of CgNACs and CgMYBs. This heatmap is marked with different colors; scale bars represent different correlation coefficients. The FPKM values of CgNACs and CgMYBs in different tissues and different developmental stages of San hong were obtained from RNA-seq data of our lab. *: 0.01 < p < 0.05; **: 0.001 < p < 0.01; ***: p < 0.001.
2.4. Protein-Protein Interaction Analysis of CgNAC043

Based on the *Arabidopsis* protein database, the protein–protein interactions of the six genes related to lignin biosynthesis were predicted using STRING software. CgNAC043 was identified to be a homologous protein of *Arabidopsis* NST1 (AT2G46770.1). CgMYB46 was identified to be homologous protein of *Arabidopsis* MYB46 (AT5G12870.1). CgMYB58 was identified to be homologous protein of *Arabidopsis* MYB58 (AT1G16490.1). CgC3H, CgC-CoAOMT, and Cg4CL were identified to be homologous proteins of *Arabidopsis* CYP98A3 (AT2G40890.1), CCoaOMT1 (AT4G34050.1), and 4CL1 (AT1G51680.1), respectively. These genes were predicted to have the ability to interact with the CgNAC043 protein (Figure 5).

![Interaction network of six proteins related to lignin biosynthesis on the basis of Arabidopsis orthologs. Medium confidence was set to 0.400.](image)

2.5. Expression Patterns of CgNAC043 Gene

The expression of CgNAC043 in the juice sac of San hong was analyzed to determine the regulatory role in the granulation of pomelo. The results showed that the expression level of CgNAC043 in juice sacs obtained away from the core was the lowest, while those near the core and granulated area were highly expressed. The expression in granulation increased from 157 to 212 DPA, its expression reached the highest level at 212 DPA (Figure 6a). Moreover, CgMYB46 (Figure 6b), CgC3H (Figure 6c), and CgCoAOMT (Figure 6d) exhibited a similar expression pattern to that of CgNAC043, suggesting their possible involvement in secondary wall biosynthesis.

![Expression patterns of CgNAC043 and other genes.](image)

Figure 6. Cont.
Figure 6. (a) RT-qPCR analysis of the expression level of \textit{CgNAC043} in different parts of pomelo at 157–212 DPA; (b) RT-qPCR analysis of the expression level of \textit{CgMYB46} in different parts of pomelo at 157–212 DPA; (c) RT-qPCR analysis of the expression level of \textit{CgC3H} in different parts of pomelo at 157–212 DPA; (d) RT-qPCR analysis of the expression level of \textit{CgCCoAOMT} in different parts of pomelo at 157–212 DPA. The expression level of near the core’s juice sacs at 157 DPA was used as a control (expression value = 1), the \(\beta\)-tublin gene was used as a reference gene for gene expression normalization. Significant difference between samples is indicated by different letters, which were found using the two-way ANOVA in the IBM SPSS Statistics 20. Error bars indicate SE \((n = 3)\).

2.6. Transcriptional Activation of \textit{CgNAC043} and \textit{CgMYB46} in Yeast

The full-length \textit{CgNAC043} and \textit{CgMYB46} sequences and their truncated N-terminal forms (1–429 nt and 1–378 nt of \textit{CgNAC043} and \textit{CgMYB46}) and C-terminal (430–1197 nt and 379–951 nt of \textit{CgNAC043} and \textit{CgMYB46}) were inserted into pGBK7-BD vector, generating fusion proteins with GAL4 BD. Yeast colonies containing the N-terminal region of \textit{CgNAC043} failed to grow on SD/–TDO and SD/–TDO (+X-a-Gal). By contrast, yeast colonies containing the full-length and C-terminal region of \textit{CgNAC043} not only grew on SD/–TDO, but also showed blue on SD/–TDO (+X-a-Gal), which indicated that the \textit{CgNAC043} had a transcriptional activation activity and the activation motif was located in the C-terminal region. However, yeast colonies containing the N-terminal and C-terminal region of \textit{CgMYB46} failed to grow on SD/–TDO and SD/–TDO (+X-a-Gal), indicating a lack of transcriptional activity of \textit{CgMYB46} in Y2H gold yeast (Figure 7).

Figure 7. The full-length N-terminal and C-terminal sequences of CDS (coding sequence) of \textit{CgNAC043} and \textit{CgMYB46} to the \textit{GAL4} DNA-binding domain in pGBK7 (BD) after transformation into Y2H gold yeast cells. The transformed cells were plated onto SD/–Trp (growth control), SD/–TDO, and SD/–TDO (+X-a-gal) medium.
2.7. Transient Expression in Mesocarp

To verify the biological function of CgNAC043 in lignin synthesis, we transiently expressed CgNAC043 in the mesocarp of pomelo fruits using Agrobacterium infiltration. The Gus staining of the mesocarp in the empty vector p1301 (SK) and p1301-CgNAC043 are shown as blue (Figure 8a,b). The RT-qPCR analysis showed that the expression level of CgNAC043 in transgenic plants was significantly higher than in the SK control plants (Figure 8c), indicating that the CgNAC043 gene was successfully expressed. Further RT-qPCR analysis showed that the expression levels of CgMYB46 and CgMYB58 were upregulated in the transgenic mesocarp, which were 1.38- and 1.67-fold higher than those of the SK control, respectively (Figure 8d).

![Figure 8](image-url)

2.8. Trans-Activation by CgNAC043 of Promoters of Lignin Transcription Factors and Biosynthesis Genes

According to the results derived from RT-qPCR and transient expression, CgNAC043 transcript levels are highly correlated with the lignification of the fruit juice sac. In order to further determine whether CgNAC043 is directly involved in the lignification process of pomelo, we constructed effector and reporter fusion protein for transient transformation via Agrobacterium injection methodology (Figure 9a). The results indicated that CgNAC043 could activate promoters of CgMYB46 by approximately 16.1-fold. Furthermore, CgNAC043 had effects on other promoters, such as CgCCoAOMT and CgC3H, by approximately 8.9- and 4.3-fold, respectively (Figure 9b). McCarthy [37] showed that AtMYB46 was specifically expressed in fibers and ducts with secondary wall thickening, and its expression is directly regulated by SND1 and its homologous genes, including NST1, NST2, VND6, and VND7. In this study, CgNAC043 was involved in the lignification of juice sacs by regulating the...
lignin biosynthesis genes, such as CgMYB46 and CgC3H, as well as other genes that may not have been studied. At the same time, this regulation may be related to the number of SNBEs elements involved in the lignin biosynthesis promoter (Table 1).

Figure 9. (a) The construct of effector and reporter. (b) CgNAC043 on the promoters of lignin biosynthesis genes as determined by dual-luciferase assays. The ratio of LUC/REN fluorescence obtained with the empty vector 62sk plus the promoters was used as a control (SK) (set as 1). Error bars indicate SE from three replicates. Student’s t-test: *: 0.01 < 𝑝 < 0.05; **: 0.001 < 𝑝 < 0.01.

Table 1. Position of SNBE elements in the promoters. The number shown at the left of each sequence is the position of the first nucleotide relative to the start codon. The critical nucleotides in the SNBE sequences are shaded.

| SNBE Element Position (Forward/Reverse) | Base Distribution |
|----------------------------------------|-------------------|
| CgMYB46-SNBE1 -1253+                  | T G A T T C T A A A C G G A A A |
| CgMYB46-SNBE2 -479+                  | T T A T G T G A A A G T G G A A G C A A |
| CgMYB46-SNBE3 -372+                  | T A C C T T G T A A A T G A A G G A A A |
| CgMYB46-SNBE4 -372-                  | T T C T C A T T A C A A A G G T A |
| CgMYB46-SNBE5 -454-                  | T G T T A A G T A A C C A A G G C T A |
| CgMYB46-SNBE6 -566-                  | A C A C G G T G T A T G T C A A G A T A |
| CgC3H-SNBE1 -1412+                  | T T A C C T A A A C A T C T A C G C C T T |
| CgC3H-SNBE2 -1378+                  | T A G C C T T A A G A A A A G A A G G C A |
| CgC3H-SNBE3 -142+                   | T A A T T T C T T A A C A A C G T A A |
| CgC3H-SNBE4 -34+                    | A A G T T T C A A G A A A A A A G G A A |
| CgC3H-SNBE -1378-                   | T G C C C T T C T T C T T A A G C C T A |
| CgCCoAOMT1-SNBE1 -974+               | A T A T G T G A G C C G T G A A G A C T |
| CgCCoAOMT1-SNBE2 -651+               | A C T C C T T A T T G T C A A A G A A A |
| CgCCoAOMT1-SNBE3 -1734-              | A T A T C T A A T C A T A A C G T T T |
| SNBE consensus                      | T N N C T T N N N N N N A A G N A A |
|                                       | A T C T G C |
|                                       | A C T T |
3. Discussion

Previous studies have shown that cell wall thickening and lignin deposition in fruit juice sacs occur during granulation [44]. The content of lignin, hemicellulose, and cellulose all increased in the cell wall of granulated juice sac [3]. A number of NAC TFs related to lignin metabolism have been widely reported, while their functions in citrus have never been researched. In this study, we isolated and identified a NAC gene CgNAC043 from San hong. Sequence analysis based on alignment and phylogenetic tree construction indicates that CgNAC043 is conserved with its orthologs, which have been associated with lignin biosynthesis in other plant species (Figure 2). Meanwhile, CgNAC043 is adjacent to PtrWND2A and PtrWND2B in the phylogenetic tree, and is in the same branch as ANAC043 and EjNAC2. Previous studies have shown that the overexpression of PtrWNDs in Arabidopsis snd1nst1 double mutants can effectively supplement for defects of the fiber secondary wall and activated the whole secondary wall biosynthesis process. Overexpressed PtrWND2B and PtrWND6B can induce the expression of secondary wall-related TFs and its biosynthesis genes [45]. In loquat, EjNAC1 can activate the promoters of the loquat lignin synthesis gene directly [34]. Therefore, we surmise that CgNAC043 may regulate lignin biosynthesis during fruit juice sac granulation in pomelo.

CgNAC043 expression was found to increase significantly during the progression of juice sac granulation, which is consistent with the expression of CgMYB46 (Figure 6). The results showed that the CgNAC043 had the lowest expression level in the tissues far away from the core, and the highest expression level in the tissues near the core and granulated. Juice sac granulation is usually prone to the tissues near the core, and then gradually develops to the tissues away from the core [44]. At the same time, we found the staining of fruit juice sacs in San hong became progressively stronger (Figure 1). So, we speculated that CgNAC043 may accelerate granulation during this process, which leads to the high level of lignin accumulation in juice sacs.

Some members in MYB and NAC TFs families are involved in the regulation of lignin biosynthesis in Arabidopsis. AtMYB85, AtMYB58, AtMYB63, AtMYB46, NST1, NST2, SND1, and SND2 are involved in the regulation of secondary cell wall biosynthesis [10,37,46–48]. Shi showed that the transient expression of CgMYB58 led to an increase in the lignin content in the pomelo fruit mesocarp, whereas its stable overexpression significantly promoted lignin accumulation and upregulated 19 lignin biosynthetic genes [40]. Zhong demonstrated that the AtMYB46 transcription factor is a direct target of SND1, which is a key transcriptional activator regulating the developmental program of secondary wall biosynthesis [36]. In this study, we transferred the CgNAC043 gene into the mesocarp of pomelo and found that it could up-regulate the expression of CgMYB46 and CgMYB58 (Figure 8). The transcriptional activation activities of CgNAC043 and CgMYB46 were analyzed using the yeast two-hybrid system—CgNAC043 showed transcriptional activation activity in Y2H gold yeast and the activation motif was located in the C-terminal domain (Figure 7). In this network, CgNAC043 may act as the master switch that activates the expression of many lignin biosynthetic genes.

In order to examine whether CgNAC043 interacts with the promoters of lignin biosynthetic genes, we isolated the promoters of the CgMYB46, CgC3H, CgCCoAOMT, Cg4CL, and CgCCR genes from pomelo juice sacs. Our results indicated that CgNAC043 not only activated the promoter of CgMYB46, but could also activate the promoter of CgC3H and CgCCoAOMT directly in vitro (Figure 9b), which is consistent with the regulation mode of SND1-MYB46 lignin synthesis in Arabidopsis. Zhong [42] reported the consensus SND1 binding sequence, (T/A)NN(C/T)(T/C/G)TNNNNNA(N/G)A(C/T)(A/T), and designated this 19-bp imperfect palindromic sequence as the SWN binding element (SNBE). SND1 together with other SWNs, including VND6, VND7, NST1, and NST2, bind to SNBE, designated as a secondary wall NAC binding element in the promoters of their direct targets. Genome-wide analysis of SND1 and VND7 revealed that they directly activate the expression of not only downstream TFs, but also a number of genes involved in secondary wall biosynthesis [42]. It was found that SNBEs are present in the promoters of all target
genes of CgMYB46, CgC3H, and CgCCoAOMT (Table 1). So, we speculated that CgNAC043 regulated juice sac granulation by recognizing the SNBE element of the gene promoter in the lignin synthesis pathway. Therefore, we believe that CgNAC043 plays a very important role in the granulation of pomelo. Further study is need to determine how CgNAC043 regulates juice sac granulation. In addition, our findings support the hypothesis that SWNs mediated the transcription regulation mechanism are conserved across others plants [49].

4. Materials and Methods

4.1. Plant Materials

The San hong used in this study was grown in the FAFU experimental base in Fuqing City. To better understand the expression of CgNAC043 at different developing stages and parts of fruits juice sacs, we collected samples at 157, 164, 172, 180, 188, 196, 204, and 212 days post anthesis (DPA). Every ninth fruit was picked from three different healthy trees and divided randomly into three replicates. The juice sacs from each sample were immediately separated, frozen in liquid nitrogen, and then stored at −80 °C.

4.2. Observation of Cell Walls of the Fruits Juice Sacs

The fruit juice sacs of San hong at 172 DPA and 212 DPA were sliced into thin sections. Thin sections were stained for lignin with a HCl–phloroglucinol solution [2% (w/v) phloroglucinol (dissolved in 95% (v/v) ethanol): 2M HCl = 1:1, fresh preparation] and observed under a Zeiss Axioscope A1 microscope (Leica) with a ×0.5 optical adapter.

4.3. Gene Isolation

The full-length coding DNA sequence (CDS) of CgNAC043 (Cg5g040070) and the promoters of CgMYB46 (Cg2g003450), CgCCoAOMT (Cg8g004310), CgC3H (Cg6g017470), Cg4CL (Cg4g022000), and CgCCR (Cg2g003700) were download from the Citrus genome website (http://citrus.hzau.edu.cn/orange/download/index.php, accessed on 30 May 2020). The protein sequences were analyzed using BLAST at the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 9 August 2021) to identify homologous sequences in other plant species.

The total RNA was isolated from pomelo fruit juice sacs using an RNA prep pure kit (BioFlux Biotech, Hangzhou, China). cDNA was synthesized with Transcript® One-step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China). The DNA was extracted using the CTAB method. The PCR amplification procedure was based on Phanta® Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China) and the vector construction on ClonExpressII One Step Cloning Kit (Vazyme, Nanjing, China). The primers used in this study are listed in Supplementary Table S2.

4.4. Bioinformatics Analysis

The amino acid sequences of the SWN genes were aligned using DNAMAN (8.0). The phylogenetic relationship of the second cell wall NAC proteins was constructed using MEGA7 software with 1000 bootstrap replications. The conserved domains of the NAC proteins were predicted used CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 4 December 2021). The conserved motif distribution was analyzed using the MEME Suite, and 10 motifs were identified (Figure 2b). The basic physical and chemical properties of the CDS sequence of CgNAC043 were predicted by EXPASY (http://web.expasy.org/protparam, accessed on 20 December 2021).

4.5. Construction of Interaction Networks of CgNAC043 Protein in Pomelo

STRING (https://string-db.org/, accessed on 20 December 2021) was used to construct the functional protein association networks for the CgNAC043 protein on the basis of Arabidopsis orthologs. The minimum required interaction score was set to medium confidence (0.400).
4.6. Transcriptional Activity Assay in Yeast

For the transactivation activity assays, the full-length C- and N-terminal sequences of CgNAC043 and CgMYB46 were amplified and inserted into the vector PGBK7 (BD) at the NdeI and SmaI sites. The recombinant plasmids were then transformed into the yeast strain Y2H gold according to the manufacturer’s instructions, and the transformed yeast cells were grown on SD/-Trp (growth control), SD/-TDO, and SD/-TDO (+X-a-gal) plates to check the transactivation activity. The primers are listed in Supplementary Table S3.

4.7. RNA Extraction and Real-Time PCR Analysis

The total RNA was extracted from the juice sacs of different tissues at different stages using an RNA prep pure Kit (BioFlux Biotech, Hangzhou China). First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). For relative transcript level analysis, we used a SYBR® Premix Ex TaqTM (Tli RNaseH Plus) (Takara, Dalian, China) and amplification was performed using the Jena qTOWER 2.2 real-time quantitative fluorescent PCR instrument. The normalized expression level of the gene in the tissues near the core at 157 DPA was used as a control value (expression set to 1), and the β-tublin gene was used as a reference gene for gene expression normalization [50]. All gene expression analyses were repeated using three biological replicates. The relative expression levels were calculated using the 2−ΔΔCt method [51]. The primers are listed in Supplementary Table S4.

4.8. Transient Expression in Pomelo Mesocarp

Primers were designed based on the vector sequence (p1301), gene sequence, and enzyme cutting sites to construct the overexpression vector, p1301-CgNAC043. The empty p1301 and p1301-CgNAC043 were transformed into Agrobacterium GV3101, then suspended in an infiltration buffer (10 mM MES, 10 mM MgCl₂, 150 µM acetosyringone, pH 5.6), and the OD₆₀₀ adjusted to 0.75. The p1301-CgNAC043 was infiltrated on one side pericarp of San hong, and p1301 was infiltrated on the other side in the same fruit as the control (SK). Three days after infiltration, the treated tissues were used for the gene transient expression analysis. Three individual fruit replicates were used and the gene expression was analyzed by the 2−ΔΔCT method. Gus stained referred to the X-gluc kit (Real-Times Biotechnology, Beijing, China), captured by the microscope (Leica).

4.9. Dual Luciferase Assay

Dual-luciferase assay is widely used for luciferase activity analysis [34,52,53]. The full-length sequence of CgNAC043 was integrated into the pGreen II 0029 62-SK vector (effector). The promoters of CgMYB46, CgC3H, CgCCoAOMT, Cg4CL, and CgCCR were inserted into the pGreen II 0800-LUC vector (Reporter). All constructs were transfected into Agrobacterium GV3101, then resuspended in an infiltration buffer to an optimal density (OD₆₀₀ = 0.75). Then, 1 mL of Agrobacterium cultures containing transcription factors were mixed with 250 µL of Agrobacterium containing promoters and the mixtures were injected into the leaves of Nicotiana benthamiana with needleless syringes. The pGreen II 0029 62-SK empty vector plus promoter was used as a control (set as 1). Three days after infiltration, the LUC and REN fluorescence intensities were measured using a Dual Luciferase Reporter Gene Assay Kit (Yeast, Shanghai, China). The analysis was carried out with three replicates for each plant. The primers are listed in Supplementary Table S5.

5. Conclusions

In this study, CgNAC043 was functionally analyzed, and it acts as a positive regulator of pomelo fruit lignification. CgNAC043 regulates the biosynthesis of lignin by activating the transcription of CgMYB46 and the lignin biosynthesis genes (CgC3H and CgCCoAOMT). We confirmed that CgNAC043 had a similar function to AtNST1 for secondary wall synthesis in Arabidopsis. Our findings support the hypothesis that the SWN-mediated transcription regulation mechanism is conserved across others plants.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11030403/s1. Supplementary Figure S1: Protein sequence alignment of CgNAC043 with homologs from other species. Supplementary Table S1: The FPKM values of CgNACs and CgMYBs in different tissues and different developmental stages of “San hong” were obtained from RNA-seq data of our lab. Supplementary Table S2: List of Primers used for gene cloning. Supplementary Table S3: List of Primers used for transcriptional activity assay. Supplementary Table S4: List of Primers used for RT-qPCR. Supplementary Table S5: List of Primers used for dual luciferase assays.

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