

PnNAC03 from *Panax notoginseng* Functions in Positively Regulating Saponins and Lignin Biosynthesis During Cell Wall Formation

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Research Article

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Abstract

NAC transcription factor family protein play an important role in modulation of secondary metabolites biosynthesis. Saponins are the major bioactive compounds for *Panax notoginseng* which is a world-famous medicinal plant, and possess multiple pharmacological activities. Secondary cell wall play crucial roles in *P. notoginseng* growth and stress resistance. However, the investigations on NAC transcription factors in regulation of saponin biosynthesis and secondary cell wall formation remain elusive. In this study, we cloned and characterized a NAC transcription factor, PnNAC03, which is nuclear-localized protein and exhibits transcriptional activation activity. Inhibition of *PnNAC03* with RNAi method in *P. notoginseng* calli resulted in a significant reduction in the content of saponin and the expression of the saponin biosynthetic genes, including *PnSS*, *PnSE*, and *PnDS*. Additionally, PnNAC03 was demonstrated to bind to the promoters of these genes and thereby enhancing their expression. Furthermore, overexpression of *PnNAC03* in *Arabidopsis thaliana* led to the increase of secondary cell wall thickness and lignin content, and upregulation of the expression of *AtPAL* and *AtC4H*. RNAi-mediated silencing of *PnNAC03* in *P. notoginseng* further confirmed its role in lignin biosynthesis, as lignin content and the expression levels of *PnPAL* and *PnC4H* were significantly lowered. Furthermore, PnNAC03 could directly bind to the promoters of *PAL* and *C4H* in both *A. thaliana* and *P. notoginseng*. Collectively, our results highlight the dual regulatory role of PnNAC03 in promoting both saponin biosynthesis and lignin accumulation, providing valuable insights for the molecular breeding of *P. notoginseng*.

Key message

PnNAC03 positively regulates saponin biosynthesis and secondary lignin accumulation during cell wall formation by directly binding to the promoters of *PnSS*, *PnSE*, *PnDS* and *PnPAL*, *PnC4H*, respectively.

Introduction

Panax notoginseng (Burkill), a member of the *Araliaceae* family, is globally recognized as a medicinal and functional herb, with pharmacological effects on the nervous system, immune-related disorders, and cardiovascular health (Wu et al. 2023; Xia et al. 2023; Yi et al. 2022). Saponins are the key compounds of *P. notoginseng* and exhibit remarkable therapeutic effects on Alzheimer's disease, cancer, thrombosis, and diabetic nephropathy (Jiang et al. 2021; Shen et al. 2024; Yang et al. 2022). About seventy kinds of saponin were identified and characterized from *P. nongensing* (Xie et al. 2018; Jasmine et al. 2022). Studies have shown that plant dammarane-type tetracyclic triterpenoid saponins are primarily synthesized through the acetic acid and mevalonate pathway, which can be generally divided into three stages (Hou et al. 2021): (1) the synthesis of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), with key enzymes including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), farnesyl pyrophosphate synthase (FPS); (2) the catalysis of IPP and DMAPP by prenyl transferase and terpenoid cyclase to form 2,3-oxidosqualene, with key enzymes including squalene synthase (SS), squalene epoxidase (SE); (3) the sequential modification of 2,3-oxidosqualene

through cyclization, hydroxylation, and glycosylation to produce triterpenoid saponins, such as saponins, with key enzymes including dammarenediol synthase (DS), cytochrome P450 monooxygenases (CYP450), and glycosyltransferases (GT) (Wang et al. 2018). Among the saponin biosynthetic genes, *PnSS*, *PnSE*, and *PnDS* are regarded as vital enzymes governing saponin content in *P. notoginseng* (Man et al. 2023). As research on the pharmacological properties of total saponins and individual monomer saponins in *P. notoginseng* deepens, the herb's range of applications continues to broaden, driving a growing market demand. Therefore, investigation on underlying the mechanism of regulation saponin biosynthesis has become a key focus in efforts to improve its quality and meet the increasing demand.

Plant secondary cell wall mainly occurs in vascular tissue, tubular cells, and fiber cells, providing the mechanical support necessary for plant growth (Colbert and Coleman 2023). Lignin, which typically accumulates in secondary cell walls, is a major structural element of plant cell walls (Choi et al. 2023). The hydrophobic properties of lignin reinforce tubular structures, allowing plants to efficiently transport water while resisting negative pressure (Xu et al. 2020). As a complex phenolic compound, lignin strengthens the cell wall, acting as a physical barrier against pathogen invasion. To date, more than 35 natural lignin monomers have been identified in *P. notoginseng*, which undergo oxidative polymerization to form lignin molecules (Vanholme et al. 2019). In the lignin biosynthetic pathway, phenylalanine ammonia-lyase (PAL) converts phenylalanine into cinnamic acid, which is further processed by 4-coumarate: CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD) to produce CoA-thioesters, aldehydes, and alcohols. The aromatic ring of lignin intermediates can be hydroxylated by cytochrome P450 enzymes, primarily catalyzed by cinnamic acid 4-hydroxylase (C4H), p-coumaric acid 3-hydroxylase (C3H), and ferulic acid 5-hydroxylase (F5H). O-methyltransferases, such as caffeoyl-CoA O-methyltransferase (CCOAMT) and caffeic acid O-methyltransferase (COMT), further methylate the product at 3-O and 5-O positions to produce various lignin monomers (Fang et al. 2020). Among these, *PAL* and *C4H* are key enzymes early in the lignin biosynthesis pathway (Ma 2024).

Transcription factors play an important role in plant growth and development. The NAC transcription factor family is one of the largest plant-specific transcription factor families, which play an important role in regulating plant secondary metabolism, including terpenoids biosynthesis, secondary cell wall formation. For example, SINAC4 from tomato can interact with ripening inhibitor and non-ripening to regulate carotenoid accumulation (Zhu et al. 2014). EsNAC in governing the impact of DNA methylation on saponin production in *Eleutherococcus senticosus* (Dong et al. 2024). In *Arabidopsis thaliana*, AtNST1 and AtNST2 (NAC transcription factors) are responsible for the thickening of the secondary wall of the anther intima, the secondary xylem and secondary wall of the hypocotyl (Liu et al. 2021; Tamadaddi et al. 2024; Zhong and Ye 2015). However, there are limited reports on NAC transcription factors that simultaneously regulate both saponin biosynthesis and the production of secondary cell walls.

In this study, we successfully isolated a nuclear-localized NAC transcription factor, PnNAC03, and investigated its functional roles in regulating saponin and secondary cell wall biosynthesis in *P. notoginseng*. Our findings demonstrated that PnNAC03 directly influences saponin content by targeting the promoters of key biosynthetic genes, including *PnSS*, *PnSE*, and *PnDS*. Overexpression of *PnNAC03*

positively regulated secondary cell wall formation and the lignin content, whereas suppressed the expression of *PnNAC03* in *P. notoginseng* leaves resulted in a decrease of lignin content. Furthermore, PnNAC03 specially binds the NAC-binding sites in the promoters of PAL and C4H gene in *A. thaliana* and *P. notoginseng*, respectively, and activates these genes transcription. These novel insights into the multifunctional role of PnNAC03 enhances our understanding of the molecular mechanisms governing both saponin biosynthesis and SCW-specific lignin formation, providing a promising strategy to improve the quality of *P. notoginseng*.

Materials and methods

Plant materials and growth conditions

Sterilized *A. thaliana* seeds were cultured on Murashige-Skoog (MS) medium supplemented with 30 g/L sucrose and 10 g/L agar for 15 days. After germination, the seedlings were trans-cultured in the soil and cultivated under controlled conditions in an incubator (20–23°C, 16/8 h light-dark cycle, 10,000 LUX light, 75% humidity). The roots, stems and leaves of two-year-old *P. notoginseng* were collected from Wenshan City, Yunnan Province, China. The seeds of *P. notoginseng* underwent two fermentations to induce budding. After storage in sand for several months, the buds were cut into 3–4 cm sections and surface-sterilized as described above. The treated buds were cultured in MS medium, with 2 µg/mL dichlorophenoxyacetic acid.

Determination of saponins and lignin content

The content of saponins in Radix Notoginseng was determined by Ultra-Performance Liquid Chromatography (UPLC) system with a Waters Acquity UPLC BEH C18 column (Agilent, 2.1 mm × 100 mm, 7 µm) at 40°C. The major components tested included notoginsenoside R1, ginsenoside Rg1, and ginsenoside Rb1. The mobile phase was acetonitrile (A)-water (B), and the flow rate was 0.22 mL/min. Chromatographic conditions was as follows: 0 min, 20% A, 20 min 40% A, 40 min 50% A, 50 min 100% A. The sample injection volume was 10µL, and detection was carried out at a wavelength of 203 nm

Lignin content of Arabidopsis and *P. notoginseng* were detected through Lignin Content Assay Kit (BC4200, Solarbio) according to the manufacturer's introduction. Those samples were measured using an ultraviolet spectrophotometer (752NPlus), with three biological replicates.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was extracted from *P. notoginseng* and *A. thaliana* using the Total RNA Purification Kit (Norgen). First-strand cDNA was generated from 2 µg total RNA with GoScript™ Reverse Transcriptase Kit (Promega). *PnACT2* and *AtACT* served as internal reference genes to normalize the relative transcription levels of genes in *P. notoginseng* and *A. thaliana*, respectively.

Primers for qRT-PCR were designed using Primer 5.0 program software with sequences provided in Table S1. SYBR Green PCR Master Mix was used for qRT-PCR. The 20 μ L PCR system consists of 10 ng template cDNA, 10 μ L Fast SYBR Green Master Mix, 0.2 μ M gene-specific primers and appropriate ddH₂O. Real-time PCR was initiated by incubation at 94°C for 30 s, followed by 40 cycles of incubation at 94°C for 5 seconds and 60°C for 30 seconds. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, based on three technical replicates and three independent biological replicates.

Cloning and identification of PnNAC03

The coding sequence of *PnNAC03* were amplified with gene-specific primers (Table S1) and cloned into the pMD19-T (Takara) vector, followed by transferred into the *Trans1-T1* competent cells. The sequence structure of *PnNAC03* was analyzed by the DNAMAN (v 6.0.3.99) software. The phylogenetic tree comparing PnNAC03 with other NAC family proteins from *A. thaliana* was constructed using the MEGA (v7.0) software, employing the Neighbor-Joining method for tree generation.

Subcellular localization of PnNAC03

The coding sequence of *PnNAC03* was cloned into the binary vector pCAMBIA1300-35S-GFP to yield pCAMBIA1300-35S-PnNAC03-GFP, where PnNAC03 was fused with a GFP reporter gene under the control of the 35S promoter. The recombinant vector was then infiltrated in to the *A. thaliana* mesophyll protoplasts isolated from leaves as previously described (Koop et al. 1996). pCAMBIA1300-35S:PnNAC03-GFP was co-expressed with pCAMBIA1300-35S: OsGhd7-RFP (nuclear marker) in *A. thaliana* protoplasts through the PEG-calcium-mediated method (Pečenková et al. 2017). After 18–24 h, florescence images were captured under an FV10 laser scanning confocal microscope.

Transcriptional activation

The ORF sequence of *PnNAC03* was inserted into the PGBKT7 vector to generate the pGBKT7-PnNAC03 plasmid. The recombinant plasmid was transformed into Y2HGold yeast competent cells, alongside the negative control pGBKT7 and the positive control pGBKT7-53. After 2 ~ 3 days of incubation at 30°C, PCR was performed to confirm the successful transformation of pGBKT7-PnNAC03 into yeast. The transformed yeast strains were streaked on SD / -Ade / -His / -Trp / X- α -gal selective medium to observe the plaque color changes.

Formation of PnNAC03-RNAi transgenic calli

A 200 bp fragment of *PnNAC03* was inserted into pBWA(V) HS-RNAi vector to construct the pBWA(V) HS-PnNAC03-RNAi vector (Table S2). The recombinant and empty vector were transformed into *GV3103 Agrobacterium tumefaciens* and then transformed into *P. notoginseng* calli. The calli were incubated on MS media with 50 μ g/mL hygromycin B and 300 μ g/mL cefotaxime in the dark at 25°C for 8 days.

Yeast One-Hybrid (Y1H) Assays

The coding sequence of *PnNAC03* was cloned and inserted into the pGADT7 vector, resulting in the construction of the pGADT7-*PnNAC03* plasmid (Table S1). The promoters of *PnSS* (1273 bp), *PnSE* (925 bp) and *PnDS* (882 bp) were amplified by specific primers and were transformed into pABAI vector to obtain pABAI-*PnSS*-Promoter, pABAI-*PnSE*-Promoter and pABAI-*PnDS*-Promoter bait vectors. The recombinant plasmids pABAI-*PnSS*-Promoter, pABAI-*PnSE*-Promoter and pABAI-*PnDS*-Promoter were placed in a constant temperature metal bath at 65°C for 15 min for linearization. The linearized product was purified and then transformed into yeast one-hybrid strain Y1H Gold competent cells. The promoter bait strains of *PAL* gene and *C4H* gene of *A. thaliana* and *P. notoginseng* were constructed by the same method. The prey plasmid was transferred into the bait yeast strains and cultured on SD plates lacking leucine with 0, 100*, 200*, 300*, 400* and 500* dilution series of aureobasidin A (AbA). The cells were cultivated on both types of media including prey proteins for the purpose of detecting the interaction of bait sequences.

Dual luciferase assay

The coding sequence of *PnNAC03* was cloned into pGreenII62-SK vector, while the promoter sequences of *PnSS*, *PnSE*, *PnDS*, *PnPAL* and *PnC4H* were constructed into pGreenII0800-LUC vector (Table S3). The recombinant plasmids were transformed into *EHA105* (pSOUP) competent cells and then infiltrated into leaf epidermal cells of *Nicotiana benthamiana*. After 24–48 hours of incubation, the relative activity of LUC was analyzed with the Dual-Luciferase Reporter Assay Kit (Vazyme, E1910).

Electrophoretic mobility shift assays (EMSAs)

The full length of *PnNAC03* was inserted into the pET28a(His-Tag)vector and then transferred into *BL21* (*DE3*) competent cells. The protein of *PnNAC03* was purified by nickel ion affinity chromatography. Probes of *PnSS*, *PnSE*, *PnDS*, *PnPAL*, *PnC4H*, *AtPAL*, *AtC4H* were designed respectively (Table S4), and the NAC binding sites (Table S3) in the promoters of *PnSS*, *PnSE*, *PnDS*, *PnPAL*, *PnC4H*, *AtPAL*, *AtC4H* were labeled with biotin probes using the Biyuntian EMSA probe biotin labeling kit (GS008), with unlabeled sequences serving as competitors. Gel shift assays (EMSA) were conducted to examine the interaction between *PnNAC03* and the biotin-labeled promoter probes of *PnSS*, *PnSE*, *PnDS*, *PnPAL*, *PnC4H*, *AtPAL*, and *AtC4H*, following the protocols of the Biyuntian Chemiluminescence EMSA Kit (GS009)

A. thaliana transformation

The coding sequence of *PnNAC03* was amplified using gene-specific primers and cloned into pCAMBIA1300-35S binary vector, resulting in the pCAMBIA1300-35S-*PnNAC03* plasmid. Subsequently, the pCAMBIA1300-35S-*PnNAC03* plasmid was transformed into wild-type *A. thaliana* via Agrobacterium-mediated floral dip transformation (Zhang et al. 2006). The positive transgenic *A. thaliana* plants were identified by RT-PCR. To investigate the function of *PnNAC03*, we cultured T3 seeds of *A. thaliana* and

formed three independent overexpression lines (OE) and wild type (WT) plants to observe their phenotypes.

Results

Identification and characterization of PnNAC03

Notoginsenoside R1, ginsenoside Rg1, and Rb1 are the major saponins in *P. notoginseng*, and predominantly store in its roots. Our previous analysis revealed that the content of these three saponins are significantly higher in the main root compared to that in the rootlet(Man et al. 2023). Our previous investigation indicated that PnNAC02 positively control the accumulation of saponins, suggesting that NAC transcription factor play an important role in regulation of saponin biosynthesis(Huang et al. 2024). We analyzed the expression of NAC transcription factors which have different expression in main root and rootlet according the transcriptome sequencing. We found another NAC transcription factor, *PnNAC03*, whose expression is similar to the trend of content of saponin in main root and rootlet, suggesting that PnNAC03 is associated with regulation of saponin biosynthesis. The *PnNAC03* cDNA sequence contains an open reading frame (ORF) of 1,050 nucleotides, encoding a protein of 349 amino acids with a calculated molecular mass (MW) of 38.8 kDa. We further compared the amino acid sequence of PnNAC03 with the amino acid sequences of *Arabidopsis thaliana* NAC family proteins including ANAC101 and ONAC004(Fig. S1), and found that PnNAC03 has a NAC transcription factor domain. To further investigate the function of PnNAC03, we constructed a phylogenetic tree using the sequences of PnNAC03 and NAC protein from *A. thaliana* (Fig. 1b). The results indicated that NAC family protein existed as 12 putative subfamilies and PnNAC03 was classified into the OSNAC7 isoforms and phylogenetically close to ANAC070 and ANAC015 (Fig. 1b), which was known as transcription activators for secondary cell wall formation and lignin biosynthesis, suggesting that PnNAC03 may involve in biosynthesis of secondary cell wall.

Subcellular localization and transcriptional activity of PnNAC03

We determined the subcellular localization of PnNAC03 by transiently co-expressing the PnNAC03: GFP fusion protein and the nuclear marker Osghd: RFP in *Arabidopsis* protoplasts. In protoplast transformed with GFP alone, the green fluorescent signal was distributed throughout the cell, whereas PnNAC03: GFP was predominantly accumulated in the nuclear and exclusively co-localized with Osghd7: RFP (Fig. 2a), indicating that PnNAC03 was a nuclear-localized transcription factor. To verified the transcriptional activities of PnNAC03, we fused it to the GAL4-DNA binding domain in pGBKT7 plasmid and transformed into yeast strain Y2HGold. Yeast cells with PnNAC03 was able to grow on SD-Trp / His / Ade medium and turned blue after X- α -Gal application (Fig. 2b), which proved that PnNAC03 had the transcriptional activation activity.

RNAi-mediated silencing of PnNAC03 led to a reduction in saponin content and decreased expression of key synthesis enzymes.

In order to explore the effect of *PnNAC03* on the synthesis of saponin, we inhibited the expression level of *PnNAC03* using RNAi method, and detected the content of saponins and the abundant of the key saponin biosynthetic genes including *PnSS*, *PnSE* and *PnDS* in *P. notogensing* calli. The results demonstrated that the content of saponins including notoginsenoside R1, ginsenoside Rg1 and ginsenoside Rb1 was significantly decreased, as well as the transcript level of *PnSS*, *PnSE* and *PnDS* was remarkably suppressed, suggesting that PnNAC03 functions as an activator for the biosynthesis of saponin.

PnNAC03 specially binds to the promoters of PnSS, PnSE, and PnDS and activates their transcription

Above results indicated that down-regulated PnNAC03 may remarkably inhibit the expression of *PnSS*, *PnSE* and *PnDS*. Furthermore, there were putative NAC binding motifs in the promoter of *PnSS* (GCTT), *PnSE* (GCTT and GCAC) and *PnDS* (CGTG and GTAC), respectively. We speculated that PnNAC03 could directly bind the promoters of *PnSS*, *PnSE* and *PnDS* to activate their expression. To elucidate this hypothesis, we carried out yeast one-hybrid assay, dual luciferase assay and electrophoretic mobility shift assay. In the yeast one-hybrid experiment, we used the pGADT7-PnNAC03 as the effector construct, pABAI-*PnSS* / *PnSE* / *PnDS* as the bait-specific reporter strain, and pGADT7 vector as the negative control, respectively. The results showed that the yeast strain with pGADT7-PnNAC03 could grow normally on SD/ -Leu (containing 400 ng/mL ABA) medium, whereas the yeast cells transforming with negative control could not grow (Fig. 4a-4c), indicating that PnNAC03 binds to the promoters of *PnSS*, *PnSE* and *PnDS*. To verify *PnSS*, *PnSE* and *PnDS* promoters transcriptionally activated by PnNAC03, a dual luciferase reporter assay was performed in *N. benthamiana* leaves. The results demonstrated that PnNAC03 significantly increased the transcriptional activities of the promoters of *PnSS*, *PnSE* and *PnDS* (Fig. 4d, 4e). In addition, we carried out EMSAs to verify whether PnNAC03 could directly bind to the NAC binding motifs in the promoters of *PnSS*, *PnSE* and *PnDS*. The probes containing NAC binding motif of the promoter of *PnSS*, *PnSE* and *PnDS*, which predicted by PlantCARE, were synthesized and labelled. The results showed that the purified recombinant PnNAC03 protein caused mobility shifts when bound to the probes, and the binding of PnNAC03 to the labeled fragments was partially abolished by increasing the concentration of the unlabeled competitor probes (100× and 500× excess) (Fig. 4f-4k), indicating that PnNAC03 can bind to two specific NAC binding motifs within the *PnSS*, *PnSE*, and *PnDS* promoters. In conclusion, PnNAC03 can bind to the promoters of *PnSS*, *PnSE* and *PnDS* and activate their transcriptional activities.

Overexpression of PnNAC03 induced SCW biosynthesis in A. thaliana

Based on the phylogenetic relationship, we speculated that PnNAC03 might influence the synthesis of plant secondary cell wall, in addition to regulate saponins. To validate this speculation, transgenic *A. thaliana* plants overexpressing *PnNAC03* were constructed. Homozygous transgenic lines were detected by RT-PCR, and three positive lines (OE1, OE2, OE3) were selected for functional analysis (Fig. S2a). As

shown in Fig. 5a, significant difference in the lignin content was observed in the *PnNAC03*-overexpressing group, compared to the wild-type (WT) group (Fig. 5b), despite of no visual variation. Additionally, stem segments of 43-day-old OE and WT *A. thaliana* plants were sectioned to study their anatomical characteristics. Transmission electron microscopy analysis clearly showed a significant increase in the SCW thickness in *PnNAC03* OE group compared to the wild type group (Fig. 5c, Figure S2c). Phloroglucinol staining showed that overexpression of *PnNAC03* resulted in higher xylem lignification in OE plants, compared to that in WT plants, consistent with the results of lignin content analysis (Fig. 5d, Figure S2d). Toluidine blue staining analysis showed that *PnNAC03* transgenic plants exhibited a significant increase in vessel cell wall thickness, compared with the wild-type (Fig. 5e, Fig. S2e). In summary of this part, these results collectively indicated that PnNAC03 involved in the positive regulation of SCW biosynthesis in transgenic *A. thaliana*.

Overexpression of PnNAC03 induced the expression of cell wall biosynthetic genes in *A. thaliana*

Transcriptome sequencing was performed on three *A. thaliana* lines (OE1, OE2, OE3) overexpressing *PnNAC03* for further exploring the regulatory mechanism of PnNAC03, along with a WT line. The number of differentially expressed genes (DEGs) in three OE and WT lines were 1827, 2886 and 2258, respectively (Fig. 6a). Compared with the WT line, 1065 DEGs were detected in the three OE lines. Those 1065 DEGs were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the gene ontology (GO) database. For the KEGG annotation, these DEGs were documented into 20 functional groups, with "phenylpropanoid biosynthesis" emerging as the dominant pathway. Specifically, 46 DEGs were found to be associated with secondary cell wall biosynthesis (Fig. S3a). For the GO annotation, these DEGs primarily belong to the "microtubule" category, followed by the "cell wall" category (Fig. S3b). It's worthy to note that the expression levels of *AtPAL* and *AtC4H* were significantly up-regulated in three OE lines (Fig. 6b, 6c), which correlated with the increased lignin content and cell wall thickness. These results suggested that PnNAC03 participants in regulating cell wall formation and lignin biosynthesis through promoting the expression of key cell wall biosynthesis genes *AtPAL* and *AtC4H*.

Based on the above, we subsequently investigate whether PnNAC03 directly binds to the promotor of *AtPAL* and *AtC4H*. Cis-element prediction by the online tool PlantCare showed the presence of two NAC binding sites (CGTG / CGTA) and a NAC binding motif (CGTG) on the promotor of *AtPAL* and *AtC4H*, respectively. Yeast one-hybrid experiments demonstrated that PnNAC03 binds to both the promoters of *AtPAL* and *AtC4H* (Fig. 7a, 7b). The dual luciferase analysis indicated that the relative activity of Luc derived from *AtPAL* and *AtC4H* promoters was significantly stimulated by PnNAC03 (Fig. 7c). Finally, the interaction of PnNAC03 with the *AtPAL* and *AtC4H* promoters were further confirmed by EMSA. The results showed that PnNAC03 could directly bind to the NAC binding element probes of 25 ~ 27 bp in the promoter of *AtPAL* and *AtC4H* (Fig. 7d ~ 7i). Collectively, these findings suggested that PnNAC03 activate cell wall biosynthesis in *Arabidopsis* by specially interacting with the promoters of *AtPAL* and *AtC4H*.

PnNAC03 promoted the accumulation of linin in *P. notoginseng* through directly binding the promoter of PnPAL and PnC4H

To evaluate the function of PnNAC03 in regulation of SW formation in *P. notoginseng*, we firstly investigate the correlation between PnNAC03 expression and lignin accumulation in different *P. notoginseng* tissues. The results indicated that *PnNAC03* expression was highest in main root, followed with stem, and then leaves, similar to the content trend of lignin in different tissues. (Fig. 8a, 8b). When *PnNAC03* expression was significantly reduced in the calli (Fig. 8e), the lignin content also decreased accordingly (Fig. 8f). The transcript levels of key lignin synthesized genes including *PnPAL* and *PnC4H* decreased significantly (Fig. 8g, 8h), suggesting that PnNAC03 may positively modulate the lignin accumulation through accelerating the abundant of *PnPAL* and *PnC4H*.

The above-mentioned DNA-protein interaction assays were performed to further explore the regulation mechanism of PnNAC03 on the lignin biosynthesis in *P. notoginseng*. The online tool plant CARE predicted results demonstrated that there were two putative NACRs (AAGC/GCTT) in the *PnPAL* promoter and two NACRs (TACG/CGTG) in the *PnC4H* promoter. In addition, the yeast one-hybrid experiments suggested that PnNAC03 binds to the promoters of *PnPAL* and *PnC4H* (Fig. 9a, 9b). At the same time, dual-LUC assays further demonstrated that PnNAC03 could significantly activate the transcriptional activity of *PnPAL* and *PnC4H* promoter (Fig. 9c). Meanwhile, the EMSA results showed that PnNAC03 binds specifically to two NACRs on the *PnPAL* and *PnC4H* promoter, respectively. These results indicated that PnNAC03 can specifically bind to the NAC binding sites of *PnPAL* and *PnC4H* promoters. Overall, these results confirmed that PnNAC03 directly interacts with the promoters of *PnPAL* and *PnC4H*, and play a key role in regulating lignin biosynthesis in *P. notoginseng*.

Discussion

P. notoginseng is a traditional Chinese medicine widely used in clinic, due to its significant therapeutic effects, particularly in treating cardiovascular diseases. Saponins, the vital active components in *P. notoginseng*, are known for their remarkable effects on curing tumors, cerebral ischemia-reperfusion injury, thrombosis, and other related conditions (Ji et al. 2022; Mancuso 2024). Lignification of cell wall is one of the main mechanisms of plant resistance. Lignin in the cell wall can increase the mechanical strength of plants and plays an important role in plant disease resistance (Vanholme et al. 2008). Therefore, understanding the regulated mechanism of saponins biosynthesis and SCW formation provides insights into potential biological modulation and thus helps in improving the quality of *P. notoginseng*. In this study, we cloned and characterized a NAC transcription factor, PnNAC03, which positively regulates saponins, thereby contributing to a comprehensive understanding of the regulatory network involved in the saponin production. In addition, the dual function of PnNAC03 on regulating both the formation of SCW and the content of lignin was further clarified through homologous and heterologous genetic engineering experiments in plants. This study offered a novel dimension to the functional of NAC transcription factors and highlighting its potential in multifaceted regulatory roles.

PnNAC03 positively regulate saponin biosynthesis

As a terpenoid compound, the biosynthetic pathway of notoginsenosides is usually mevalonic acid (MVA) pathway or methylerythritol phosphate (MEP) pathway. In the regulation of terpenoid metabolism, NAC transcription factors play a major role through a variety of mechanisms. Studies found that AaNAC1 promotes the accumulation of artemisinin, and enhances the drought tolerance and gray mold resistance of transgenic *Artemisia annua* (Lv et al. 2016). NAC transcription factors in kiwifruit can activate the transcription of monoterpene synthase TPS1 (Li et al. 2021). However, there are few studies on the regulation of saponin biosynthesis by NAC transcription factors. Saponins are important plant secondary metabolites, the content and composition of which primarily depend on key biosynthetic enzymes. In our study, we found that the content of saponins in *P. notoginseng* was significantly decreased after PnNAC03 gene silencing, and the expression pattern of *PnNAC03* in roots, stems and leaves of *P. notoginseng* was consistent with the distribution level trend of ginsenoside Rg₁, Rb₁ and notoginsenoside R₁. These findings indicated that PnNAC03 could positively regulate the saponin biosynthesis. NAC transcription factors always activate the expression of key biosynthetic genes to regulate the terpenoid biosynthesis. Recent reports have demonstrated that overexpression of *PnSS*, *PnSE*, and *PnDS* significantly enhances saponins production in *P. notoginseng* calli (Xia et al. 2022), highlighting their significance in saponin biosynthesis. Our research showed that PnNAC03 could directly bind to the promoters of *PnSS*, *PnSE* and *PnDS*. Liu et al. mapped the NAC gene family on 24 pairs of ginseng chromosomes and found numerous gene replications in the genome, among them, a NAC transcription factor *PgNAC41-2* was cloned and found that *PgNAC41-2* gene positively regulates the biosynthesis of saponins (Liu et al. 2023). Another report found a regulatory role of *PgNAC72* in MeJA-mediated ginsenoside biosynthesis (Jiang et al. 2024). However, the above studies are all in *P. ginseng*, and there are few studies on the regulation of saponins in *P. notoginseng*. Our previous study identified *PnNAC02* as a regulator of saponin content in *P. notoginseng*, through directly binding to the promoters of *PnSS*, *PnSE*, and *PnDS* and thereby regulating their expression (Huang et al. 2024). In this study, we found that another NAC transcription factor, *PnNAC03*, can also regulate notoginsenosides by binding to the promoters of *PnSS*, *PnSE* and *PnDS*. The results are of great significance for enriching the understanding of NAC transcription factors that regulate saponins.

PnNAC03 activate lignin accumulation during SW formation

Phylogenetic analysis of PnNAC03 revealed that it clusters with genes known to regulate SCW formation, suggesting its potential role. Further experiments showed that the SCW thickness and lignin content of *PnNAC03* overexpression (OE) lines were significantly greater than those of wild-type (WT) lines. This result was further validated through homologous genetic engineering in *P. notoginseng*, where lignin content was significantly reduced in *PnNAC03*-silenced calli. Further studies found that PnNAC03 binds to the promoters of *AtPAL* and *AtC4H* in *A. thaliana*. This finding was corroborated in *P. notoginseng*, where *PnNAC03* silencing led to a significant reduction in lignin content and decreased expression levels of *PnPAL* and *PnC4H*. Yeast one-hybrid, dual LUC, and EMSA assays further demonstrated that *PnNAC03* binds directly to the promoters of *PnPAL* and *PnC4H* in *P. notoginseng*.

Therefore, PnNAC03 regulates lignin accumulation during SCW formation by interacting *PnPAL* and *PnC4H*, respectively. A number of studies have shown that NAC transcription factors can affect the biosynthesis of lignin in a variety of ways. For example, Sun et al. demonstrated that a Novel NAC transcription factor from *Eucalyptus*, EgNAC141, positively regulates lignin biosynthesis and increases lignin deposition by binding the promoters of *CCOAMT1*, *COMT*, *CSE*, *CCR1*, and *CAD1*(Sun et al. 2021). Another experiments proved that *CgNAC043* from *Citrus grandis* not only can directly trans-activate the promoters for the lignin biosynthesis-related genes *CgCCoAOMT* and *CgC3H* to induce lignin synthesis (Li et al. 2022). In this study, PnNAC03 mainly influenced the lignin biosynthesis-related genes *PnPAL* and *PnC4H*. These two genes are located in the upstream of the shikimate pathway and play an important role in the formation of H-lignin, G-lignin, and S-lignin monomers. The shortcoming of this paper is that we only measured the total lignin content, in the follow-up study, we will continue to explore the function of PnNAC03 and clarify the type of monomer lignin which it affects.

PnNAC03 is a dual-functional transcription factor that regulates saponin biosynthesis and lignin accumulation during SCW formation

NAC transcription factor family is one of the large plant-specific transcription factor families, play various roles in plant growth and developmental processes, such as lateral root growth, secondary wall formation, leaf senescence, floral organ formation and fruit development. Additionally, NAC transcription factors are involved in plant responses to environmental stresses, including drought, cold, salinity, and pathogen invasion, which significantly enhance plant survival in diverse conditions (Ooka et al. 2003). In summary, NAC transcription factor is a multifunctional gene, but there is no report on the simultaneous regulation of saponins and lignin compounds by NAC transcription factors. In *P. notoginseng*, saponins and lignins are critical components that significantly contribute to its quality (Zaynab et al. 2021). Our results demonstrated that *PnNAC03* is multiple functional, regulating not only the biosynthesis of notoginsenosides, but also the formation of SCWs and lignin content during SCW development. For our future research, the apparent symptoms and mechanism of PnNAC03 on the quality regulation of *P. notoginseng* is worth explored, therefore broadening the understanding on the multifunctional roles of PnNAC03.

Conclusion

In summary, we identified a novel NAC transcription factor from *P. notoginseng*, designated as PnNAC03, which enhances the biosynthesis and accumulation of saponin in *P. notoginseng* through preferentially binding to the promoters of critical biosynthetic genes, including *PnSS*, *PnSE*, and *PnDS*. Additionally, PnNAC03 plays an important role in SCW formation and lignin accumulation by directly interacting with the promoters of the *PAL* and *C4H* genes. Collectively, these results provide valuable insights into the regulatory mechanisms of PnNAC03 in both saponin synthesis and SCW and lignin formation, offering a foundation for future studies focused on molecular breeding strategies aimed at improving the quality of *P. notoginseng*.

Declarations

Supplementary Information The online version contains supplementary material available.

Author contribution statement SW and X(Xiaohui)W designed the research. XZ and YH performed experiments and wrote the manuscript. YS amplified the genes of *PnNAC03* and conducted molecular biology experiments. X(Xin)W, BW and ZP analyzed the content of saponins data. WC and LAM analyzed the transcriptome.

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Data availability All data analyzed or generated in our study are included in this article and its supplementary information files.

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

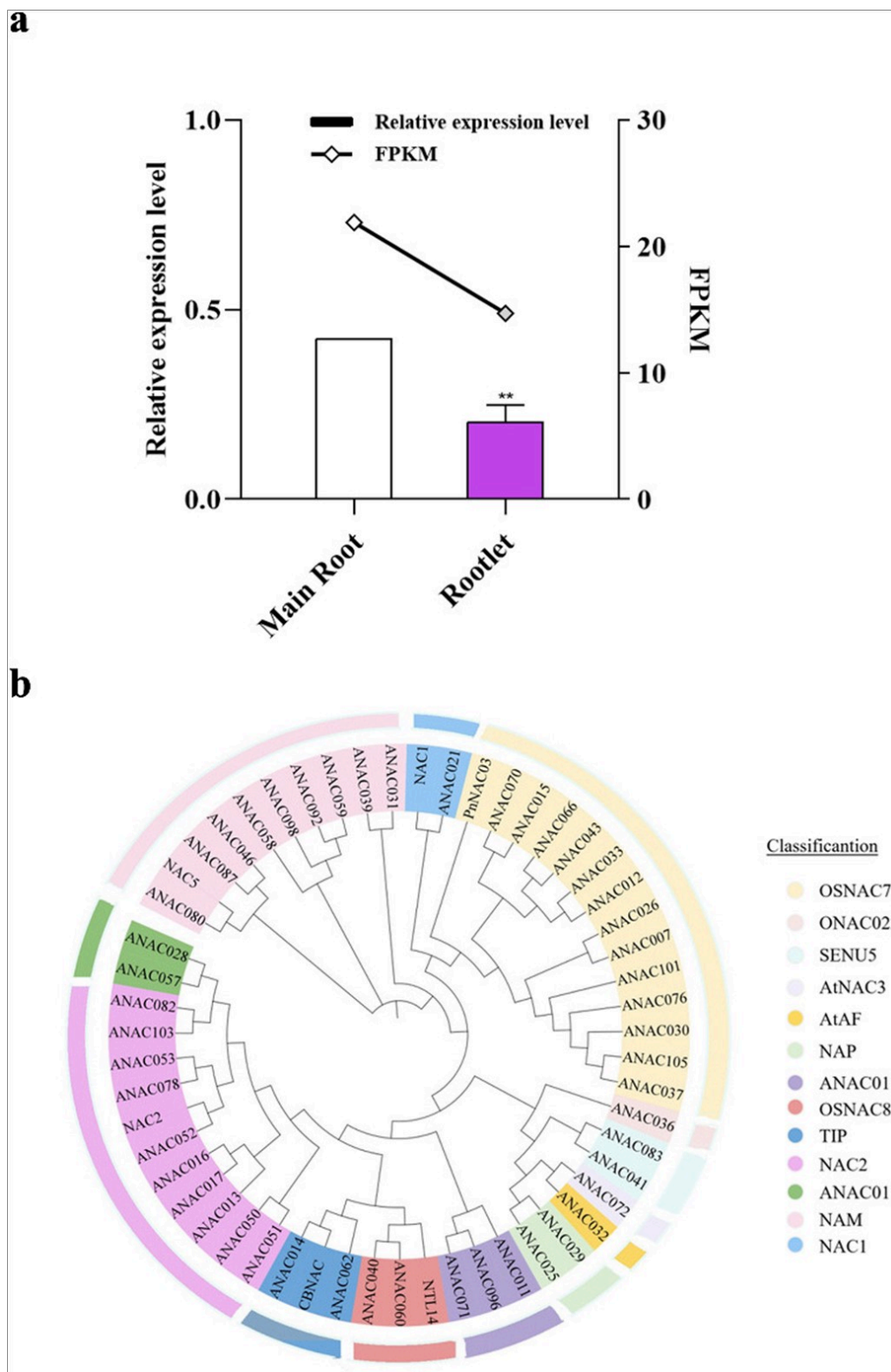
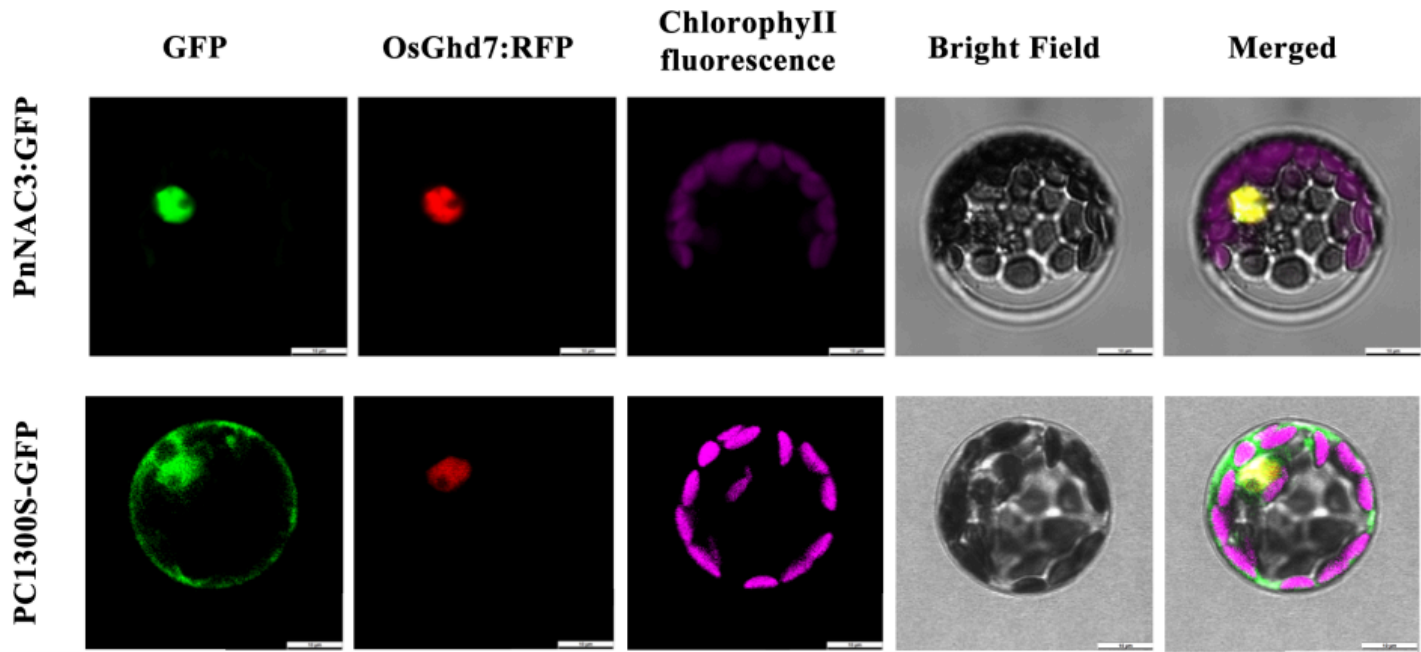


Figure 1

The expression of *PnNAC03* in the main root and Rootlet of *P. notoginseng* and the phylogenetic tree analysis of *PnNAC03* and the NAC protein from *A. thaliana*. (a) The expression of *PnNAC03* in the transcriptome of *P. notoginseng* main root and rootlet. Linear diagram represents the expression of *PnNAC03* in the transcriptome, and the histogram represents the real-time fluorescence quantitative results of *PnNAC03*. (b) Phylogenetic analysis of *PnNAC03* and the NAC protein from *A. thaliana*.

ClustalW was used to align the sequence of NAC sequences and the phylogenetic tree was performed using MEGA7.0 according to the neighbor-joining (NJ) method and 1000 bootstrap replicates.

a



b

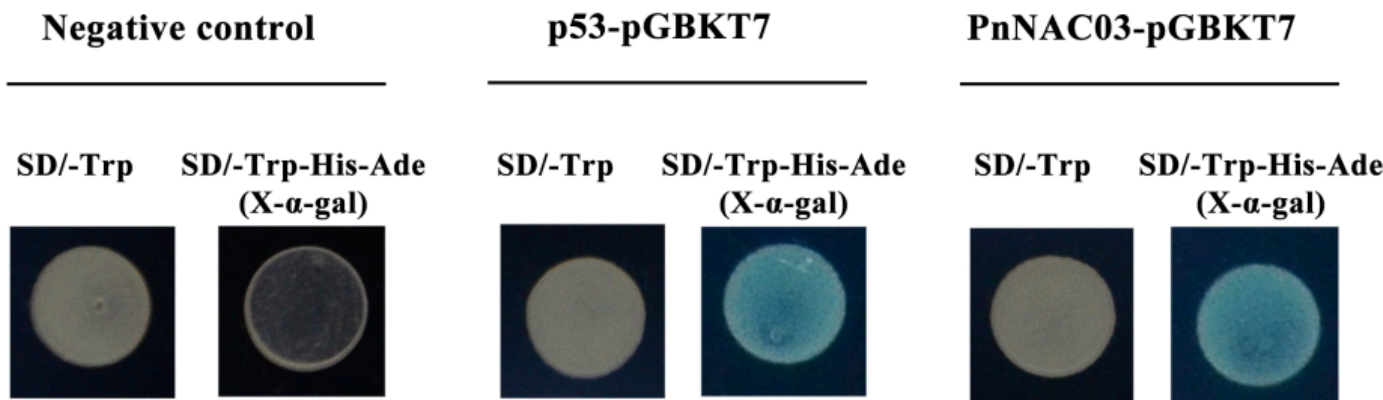


Figure 2

Subcellular Localization and Transcriptional Activity Assay of PnNAC03. (a) Subcellular Localization of PnNAC03 in *Arabidopsis thaliana* Protoplasts. The subcellular location of PnNAC03 within *A. thaliana* protoplasts was determined. Fluorescence signals from GFP and RFP were captured using a confocal microscope 24 hours post-transformation. Scale bars represent 10 μ m. (b) Transcriptional activity assay of PnNAC03 in Yeast. The transcriptional activity of PnNAC03 was assessed in yeast. Yeast strains containing the P53-pGBKT7 plasmid (positive control) and the PnNAC03-pGBKT7 vector were able to grow on medium lacking adenine, histidine, and tryptophan (SD/-Trp-His-Ade) and turned blue upon induction of X- α -gal activity, indicating transcriptional activity. In contrast, transformants harboring the pGBKT7 vector alone (negative control) were unable to grow under these conditions.

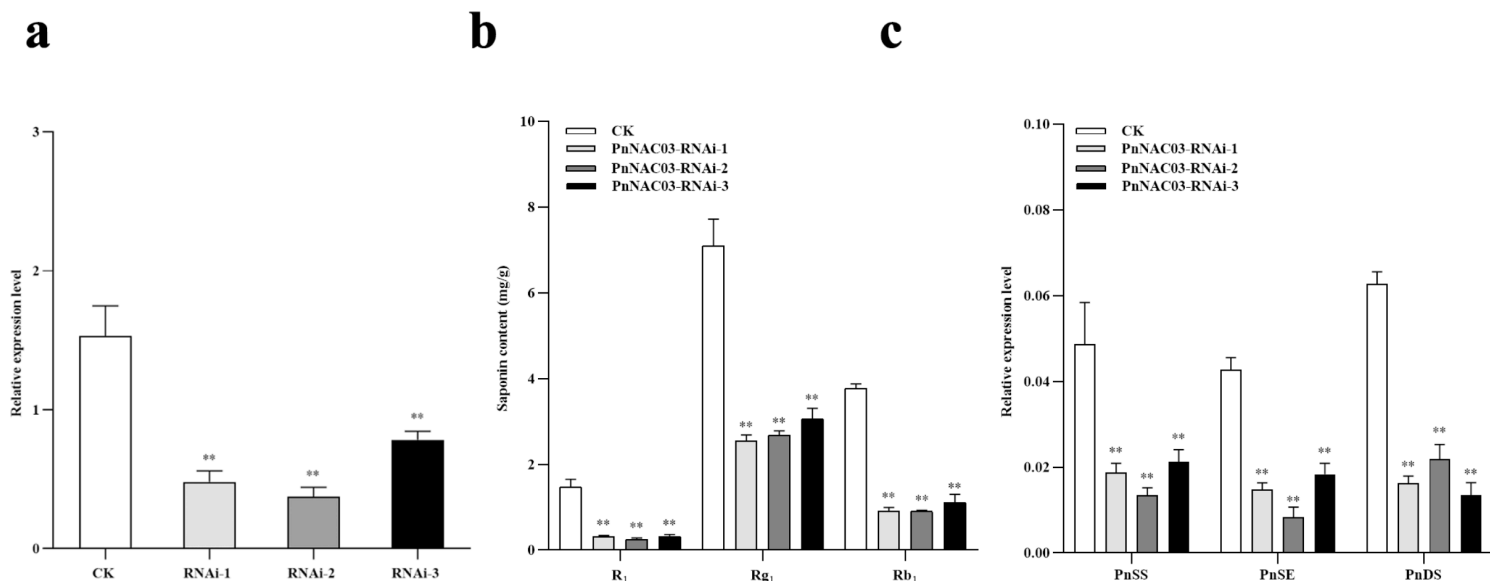


Figure 3

Gene expression and saponin content analysis in *PnNAC03*-RNAi transgenic calli. (a) Relative expression levels of *PnNAC03* in control and *PnNAC03*-RNAi transgenic Calli. The relative transcript level of the *PnNAC03* gene was determined in both control (CK) and *PnNAC03*-RNAi transgenic calli using quantitative real-time PCR (qRT-PCR). (b) Saponin content in control and *PnNAC03*-RNAi transgenic calli. The contents of saponins, including R₁, Rg₁, and Rb₁, were measured in both CK and *PnNAC03*-RNAi transgenic calli. (c) Transcript levels of saponin biosynthetic genes in control and *PnNAC03*-RNAi transgenic calli. The transcript levels of *PnSS*, *PnSE*, and *PnDS*, which are involved in saponin biosynthesis, were analyzed in CK and *PnNAC03*-RNAi transgenic calli. Statistical Significance: Asterisks indicate statistically significant differences relative to the control (CK). ** $P < 0.01$

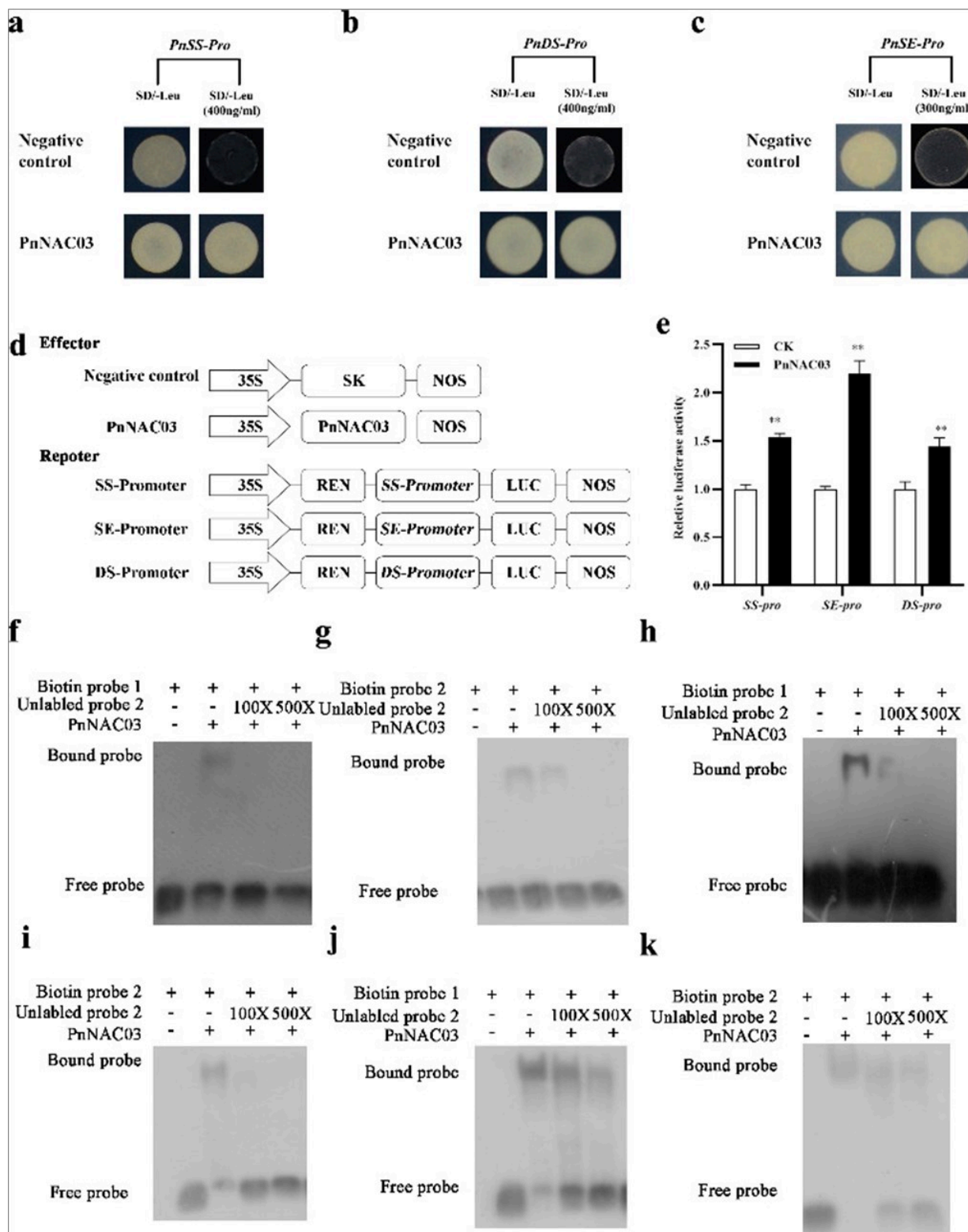


Figure 4

PnNAC03 binds to the promoters of *PnSS*, *PnSE*, and *PnDS* to activate their transcriptional activity. (a-c) Yeast One-Hybrid (Y1H) Assays: the binding of PnNAC03 to the promoters of *PnSS* (a), *PnSE* (b), and *PnDS* (c) was confirmed using Y1H assays. The pGADT7 vector served as a negative control. (d) Schematic Diagrams of effector and reporter vectors: the schematic diagrams illustrate the structure of the effector and reporter vectors utilized in the dual-luciferase assays. (e) Dual-Luciferase Assays: the

dual-LUC assays demonstrated that PnNAC03 significantly activated the transcriptional activity of the promoters for *PnSS*, *PnSE*, and *PnDS*. (f-k) Electrophoretic Mobility Shift Assay (EMSA): the specific binding between PnNAC03 and NACRS in the promoters of PnSS (f, g), PnSE (h, i), and PnDS (j, k) was validated by EMSA. Statistical Significance: Asterisks indicate a significant difference (** $P < 0.01$). The data represent the mean $\pm$ standard error (SE) of six biological replicates.

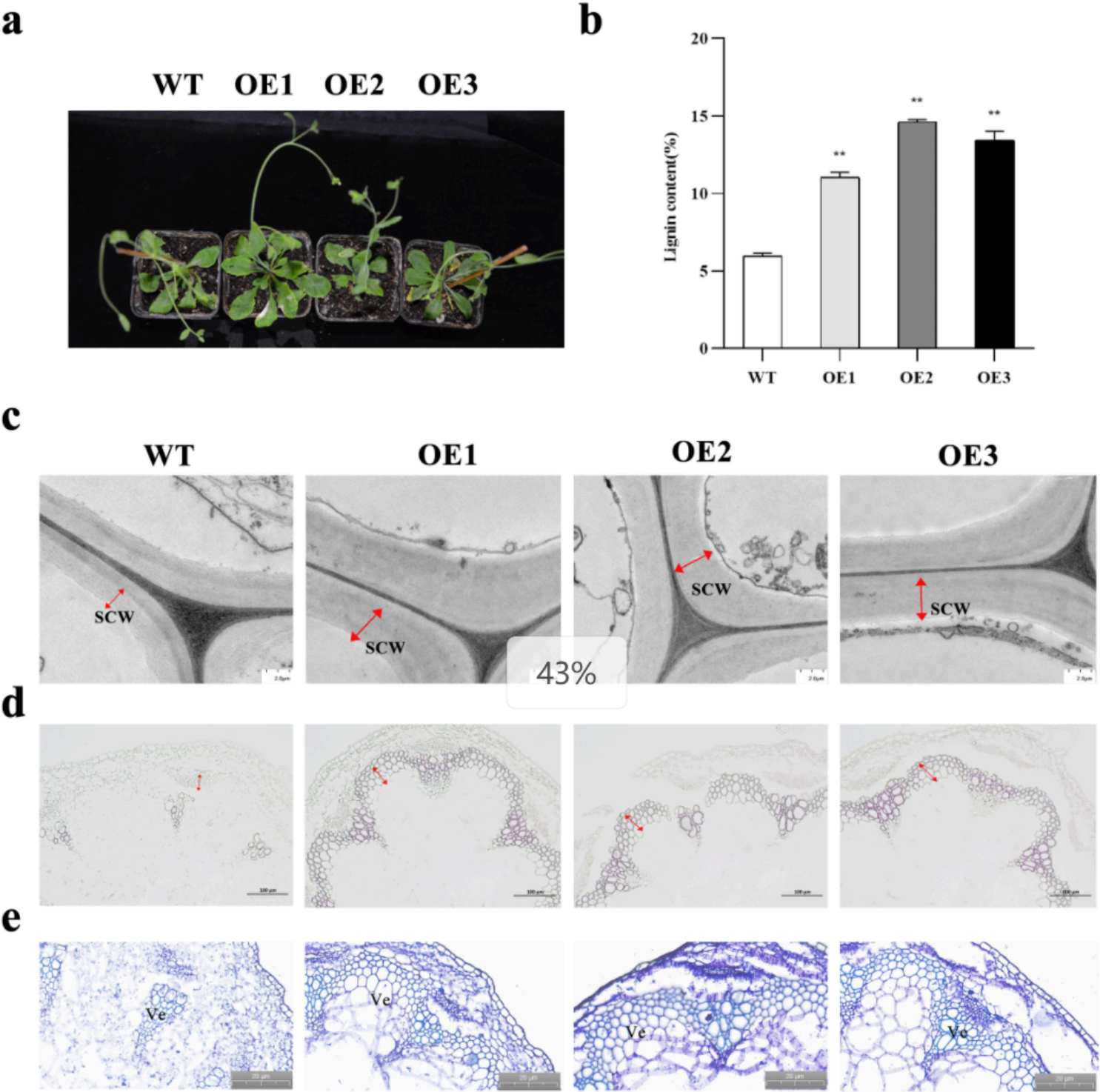


Figure 5

Phenotypes and microscopic characteristics of transgenic *A. thaliana* overexpressing *PnNAC03*. (a) Growth of 43-day-old *A. thaliana* wild-type and *PnNAC03* overexpression plants. (b) Lignin content in

PnNAC03 OE and WT plant stems. (c) Transmission electron microscopy analysis of PCW thickness in *PnNAC03*OE and WT plant stems. (d) Phloroglucinol staining in *PnNAC03* OE and WT plant stems. (e) Toluidine blue staining in *PnNAC03* OE and WT plant stems. Significance, ** $P < 0.01$. ve, vessel.

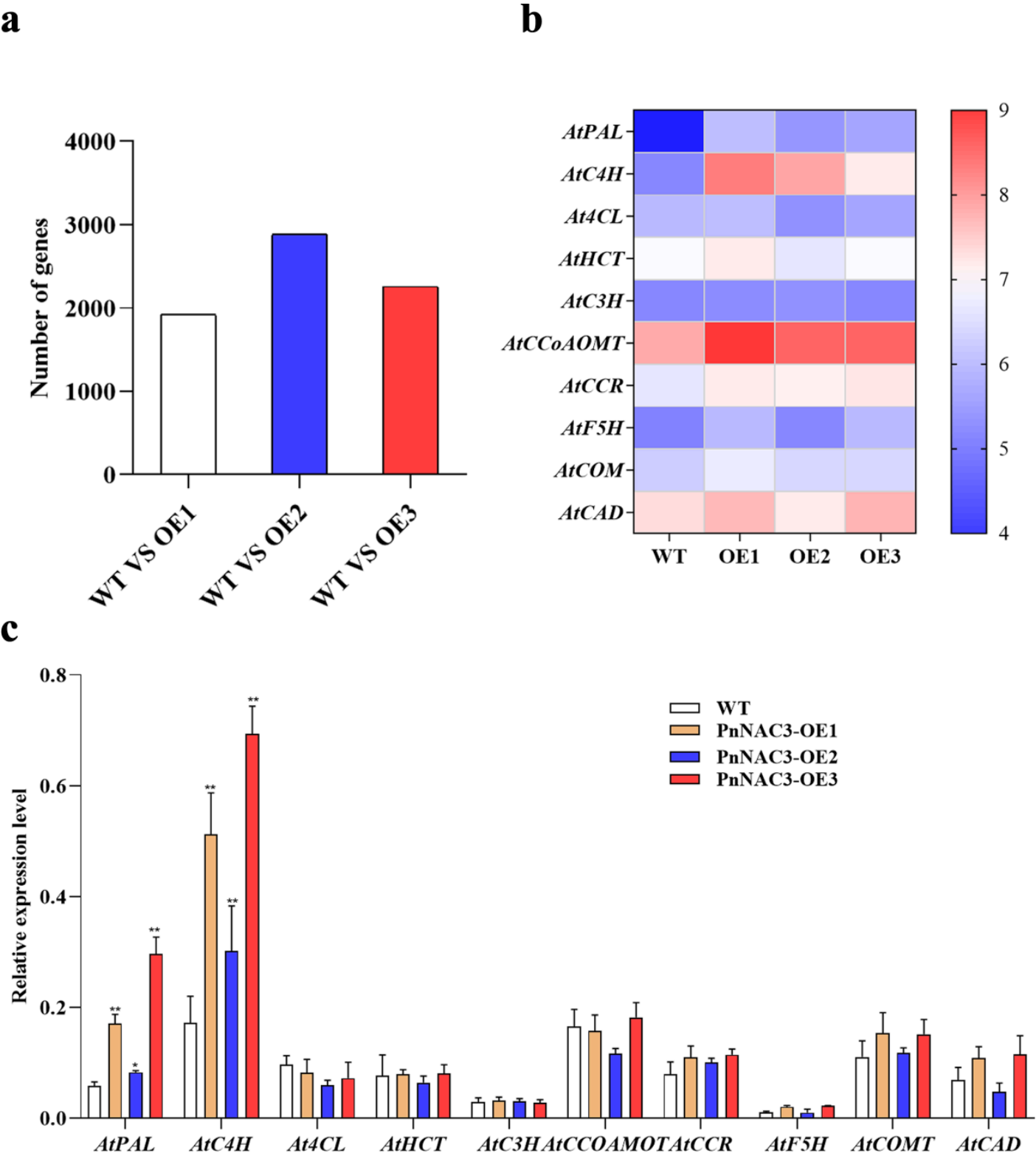


Figure 6

Transcriptome sequencing and qRT-PCR analysis of the expression of lignin biosynthetic genes after overexpressing *PnNAC03*. (a) Number of genes in different groups. (b) Heatmap of lignin biosynthetic

genes in the stems of WT and *PnNAC03*OE plants. (c) qRT-PCR analysis of expression levels of lignin biosynthetic genes in the stems of WT and *PnNAC03* OE plants. Statistical analysis was performed with one-way analyses of variance (ANOVA) with Dun-can's multiple range tests to separate means. Error bars (c) represent SD of three replicates; significance, * $P<0.05$, ** $P<0.01$.

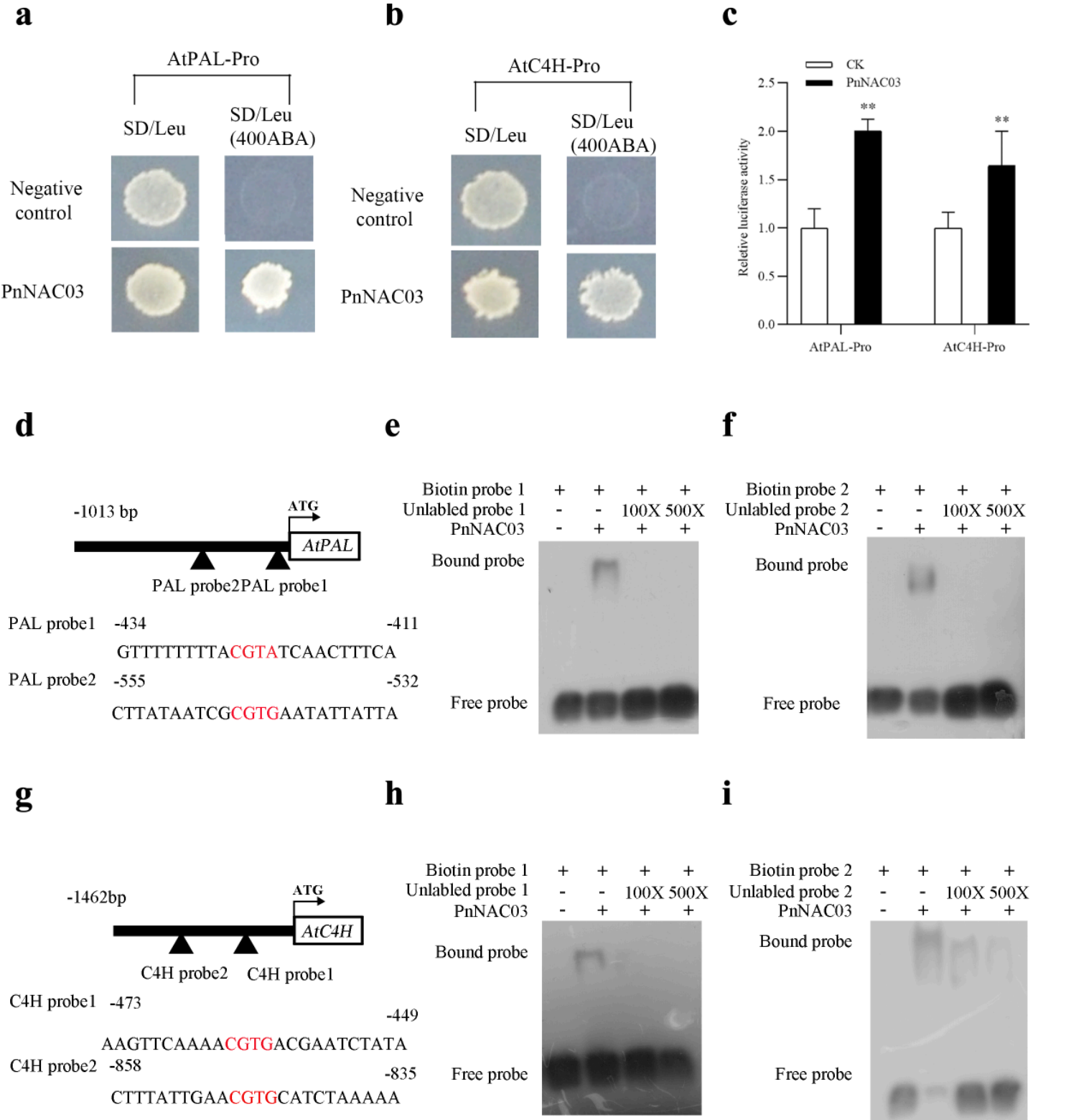


Figure 7

Interaction of PnNAC03 with the promoters of lignin biosynthetic genes *AtPAL* and *AtC4H*. (a,b) Y1H assays showing the binding of PnNAC03 to the *AtPAL* and *AtC4H* promoter. pGADT7 was used as the negative control. (c) The dual-luciferase assays showed that the *AtPAL* and *AtC4H* promoter can be activated by PnNAC03 in *N. benthamiana* leaf cells. (d-g) EMSA showing the specificity of in vitro binding between PnNAC03 and NAC binding sites in *AtPAL* and *AtC4H* promoter. The unlabeled probes used as competitors had the same sequence as the biotin-labeled probes. Statistical analysis was performed with one-way analyses of variance (ANOVA) with Duncan's multiple range tests to separate means. Error bars represent SD of three replicates; significance ** $P < 0.01$.

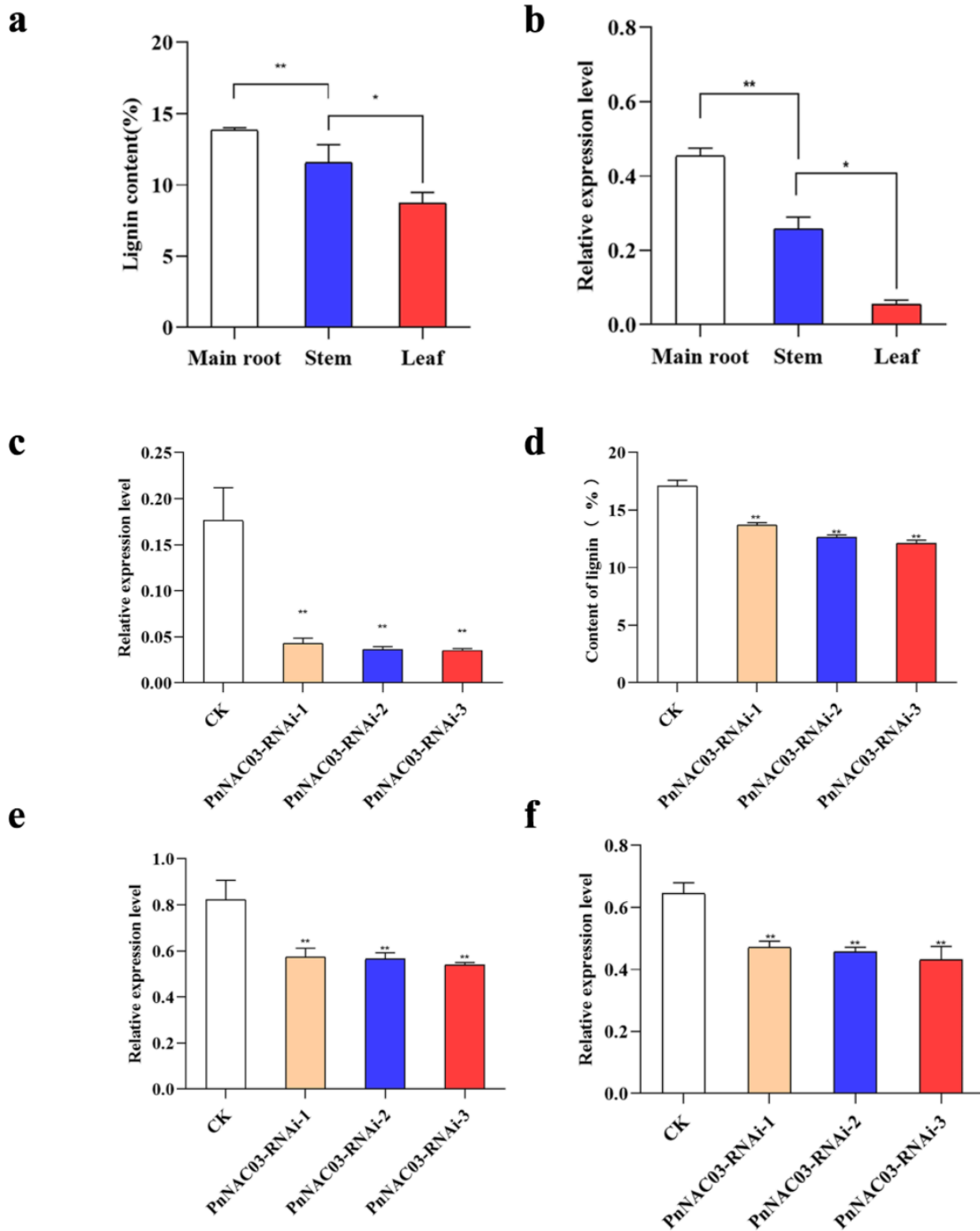


Figure 8

PnNAC03 positively regulates the content of lignin in *P. notoginseng* calli. (a) The content of lignin in the roots, stems and leaves of *P. notoginseng*. (b) Gene expression of *PnNAC03* in roots, stems and leaves of *P. notoginseng*. (c) Relative Expression Levels of *PnNAC03* in Control and *PnNAC03*-RNAi Transgenic Calli. The relative expression levels of the *PnNAC03* gene were determined in both control (CK) and *PnNAC03*-RNAi transgenic calli using quantitative real-time PCR (qRT-PCR). (d) Lignin content in control

and *PnNAC03*-RNAi transgenic Calli. The contents of lignin were measured in both CK and *PnNAC03*-RNAi transgenic calli. (e, f) Expression levels of lignin biosynthesis genes in control and *PnNAC03*-RNAi transgenic Calli. The expression levels of *PnPAL* (e) and *PnC4H* (f), which are involved in lignin biosynthesis, were analyzed in CK and *PnNAC03*-RNAi transgenic calli. Statistical Significance: Asterisks indicate statistically significant differences relative to the control (CK). ** $P<0.01$

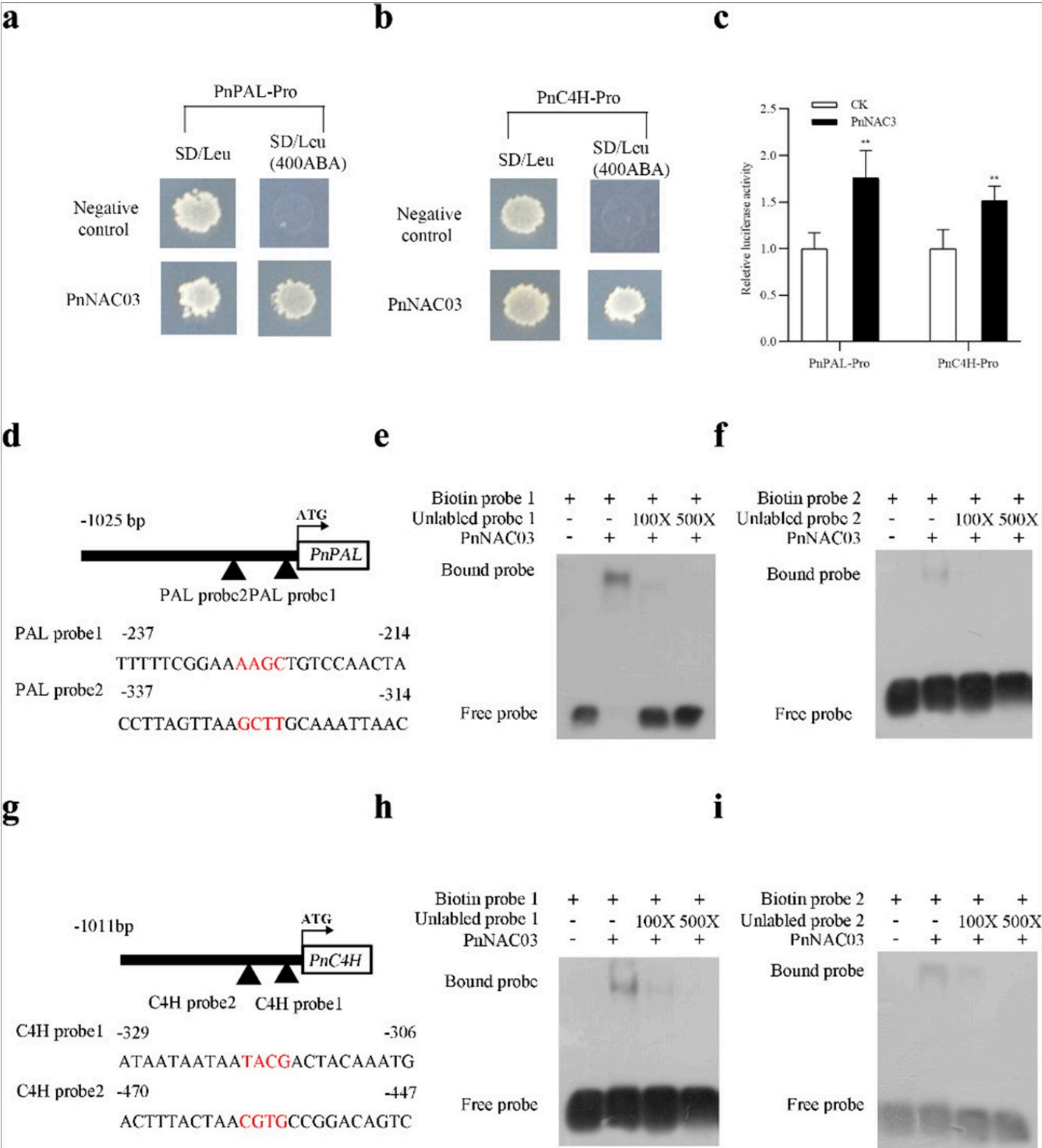


Figure 9

Interaction of *PnNAC03* with the promoters of lignin biosynthetic genes *PnPAL* and *PnC4H*. (a, b) Y1H assays showing the binding of PnNAC03 to the *PnPAL* and *PnC4H* promoter. pGADT7 was used as the negative control. (c) The dual-luciferase assays showed that the transcriptional activity of *PnPAL* and *PnC4H* promoter can be activated by PnNAC03 in *N. benthamiana* leaf cells. (d-g) EMSA showing the specificity of in vitro binding between PnNAC03 and NAC binding sites in *PnPAL* and *PnC4H* promoter. The unlabeled probes used as competitors had the same sequence as the biotin-labeled probes. Statistical analysis was performed with one-way analyses of variance (ANOVA) with Duncan's multiple range tests to separate means. Error bars represent SD of three replicates; significance ** $P < 0.01$.

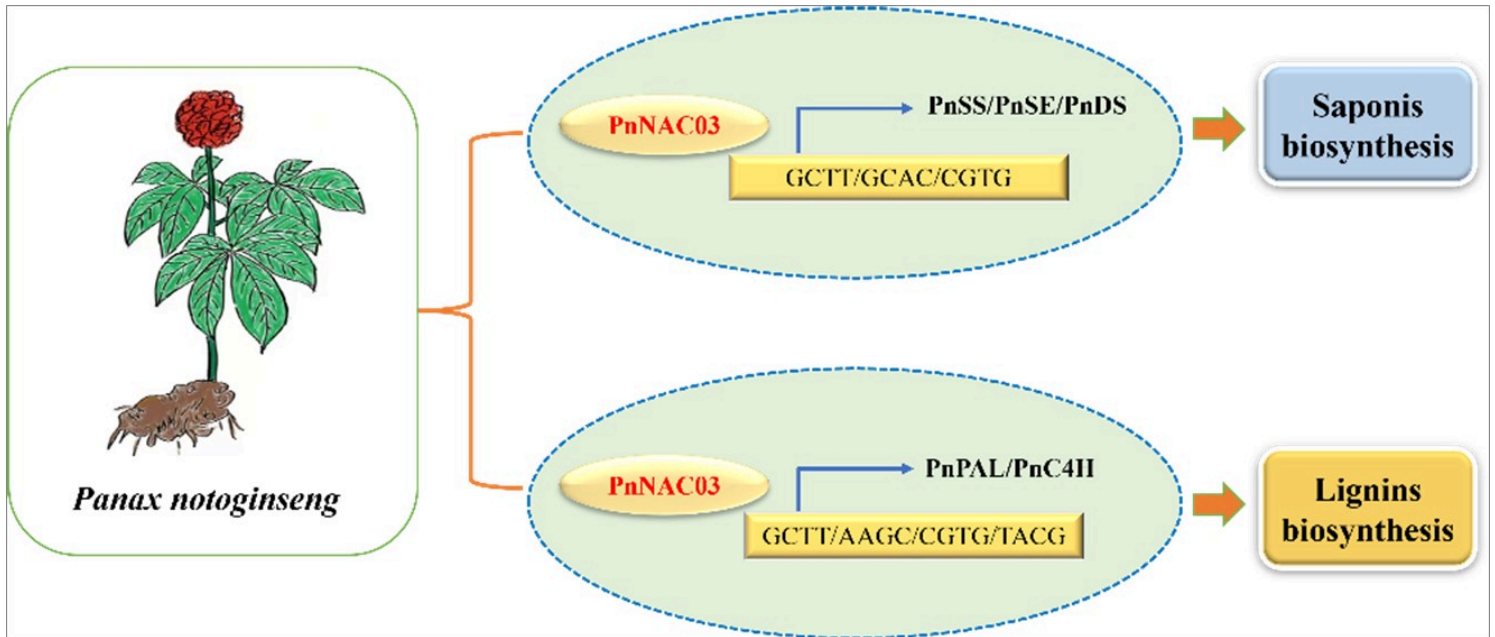


Figure 10

The dual function diagram of PnNAC03 in *P. notoginseng*.

Supplementary Files

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