

PpNAC187 enhances lignin synthesis in 'Whangkeumbae' pear (*Pyrus pyrifolia*) 'hard-end' fruit

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Research article

Keywords: Whangkeumbae', pear, hard-end, NAC, lignification

Posted Date: July 6th, 2019

DOI: <https://doi.org/10.21203/rs.2.11046/v1>

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Version of Record: A version of this preprint was published at Molecules on November 27th, 2019. See the published version at <https://doi.org/10.3390/molecules24234338>.

Abstract

Background: A disorder in pears known as ‘hard-end’ fruit affects the appearance, edible quality, and market value of pear fruit. To explore the mechanism underlying the formation of hard-end, RNA-Seq was carried out on the calyx end of ‘Whangkeumbae’ pear fruit with and without the hard-end symptom. **Result:** Results indicated that genes in the phenylpropanoid pathway affecting lignification were up-regulated in hard-end fruit. An analysis of differentially expressed genes (DEGs) identified three NAC transcription factors, and RT-qPCR analysis of PpNAC138, PpNAC186 and PpNAC187 confirmed that PpNAC187 gene expression was correlated with the hard-end disorder in pear fruit. A transient increase in PpNAC187 was observed in the calyx end of ‘Whangkeumbae’ fruit when they began to exhibit hard-end symptom. Concomitantly, the higher level of PpCCR, Pp4CL and PpCOMT transcripts was observed; which are the key genes in lignin biosynthesis. Notably, lignin content in the stem and leaf tissues of transgenic tobacco overexpressing PpNAC187 was significantly higher than in control plants transformed with an empty vector. Furthermore, transgenic tobacco overexpressing PpNAC187 had a larger number of xylem vessel elements. **Conclusion:** The results of this study confirmed that PpNAC187 functions in inducing lignification in pear fruit during the development of the hard-end disorder.

Background

The hard-end disorder in pear fruit occurs in many pear-growing regions [1, 2]. The disease is frequently found in the USA in pear varieties such as ‘Anjou’ (*Pyrus communis* L), ‘Winter Nelis’ (*P. dimorphophylla*) and ‘Comice’ (*P. communis*) [3, 4]. In recent years, the hard-end disorder also appeared in some Asian pear varieties, including ‘Whangkeumbae’ (*P. pyrifolia*) and ‘Xueqing’ (*P. nivalis*), and other varieties may also be affected [5, 6]. Hard-end of pear is a physiological disorder. Pear varieties grafted on Japanese pear (*P. serotina* Rehd) rootstocks often exhibit this disorder due to scion-rootstock compatibility problems that cause water imbalance problems and low Ca content and a low Ca/K ratio [6, 7, 2]. Fruits with hard-end are culled out during grading and packing, and severely misshaped fruits are deemed as unmarketable. The first symptom of hard-end disorder is observed as an abnormally green or yellow color at the blossom end when fruits have grown to one third or half of their full size. A readily apparent protrusion of the calyx forms due to the delayed development of the surrounding tissues. The epidermis over the calyx-end of the fruit appears tight and shiny, and the flesh near the calyx turns dry and hard due to an accumulation of lignin [5].

We previously reported that lignin content and the number of stone cells increases significantly during hard-end development in ‘Whangkeumbae’ fruit [5]. These hard-end pear fruit also contain high levels of enzymes that are involved in lignin synthesis; including phenylalanine ammonia lyase (PAL), 4-coumarate: coenzyme A ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), and peroxidase (POD). As a result, the development of hard-end in pear fruit is correlated with lignin accumulation. Lignin is a phenylpropanoid-derived polymer that is deposited in secondary cell walls to increase the mechanical strength of xylem tissues in vascular plants and to provide defense against attacks from pathogens [8]. Several genes and transcription factors are known to be involved in lignin biosynthesis, including PAL,

4CL, CAD, cinnamoyl CoA reductase (CCR), and caffeic acid 3-O-methyltransferase (COMT) [9, 10]. In pear fruit, transcript levels of *Pp4CL1*, *PpCAD1*, and *PpCAD2* were elevated in hard-end pear, relative to normal fruit [5].

Several transcription factors involved in lignin biosynthesis, such as MYB (myeloblastosis), NAC (NAM, ATAF, and CUC), bHLH (basic helix-loop-helix), and others, have been identified and characterized in *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), and loquat (*Eriobotrya japonica*) fruit [11-14]. The NAC transcription factor family is one of the largest families of plant-specific transcription factors and they participate in several physiological processes [15]. Most proteins containing a NAC domain are located at the upstream end of a regulatory network. In *Arabidopsis thaliana*, *AtNST1* and *AtNST2* promote secondary wall thickening in the endothecium of anthers, and knock-out mutants of NST1 and NST3 lose secondary-wall deposition in stems [16]. Several studies have reported that NAC genes function as master switches in the biosynthetic pathways for cellulose, xylan, and lignin by initiating a transcriptional signaling network that either affects MYB transcription factors or regulates the expression of structural genes [17, 18]. A study on loquat chilling-induced lignification demonstrated that *EjNAC3*-regulated expression of the *EjCAD-like* gene; which is a key gene in lignification [19].

'Whangkeumbae' pear fruit was previously reported to accumulate lignin during the development of hard-end fruit. In the present study, DEGs regulated by NAC transcription factors were identified in hard-end and normal 'Whangkeumbae' fruit using RNA-seq. The association between NAC and lignin accumulation in hard-end tissues was evaluated using transient expression of the identified genes in 'Whangkeumbae' fruit and the analysis of transgenic tobacco overexpressing the respective genes.

Results

RNA-Seq analysis

Total of 545 DEGs were identified in the comparison of hard-end and normal fruit in samples collected on the day of harvest (120 d after anthesis). A KEGG pathway enrichment analysis placed these DEGs into six pathways which include protein processing in endoplasmic reticulum; ribosome; glycine, serine and threonine metabolism; phenylalanine metabolism; phenylpropanoid biosynthesis; and starch and sucrose metabolism (Fig. 1). The phenylpropanoid biosynthesis and ribosome were enriched in 7 genes; which was the highest among all of the identified pathways. The DEGs in the phenylpropanoid biosynthesis pathway were annotated as *PpCCR*, *PpC3H*, *PpF5H*, *Pp4CL*, *PpCOMT*, *PpPOD* (GDR accession No., PCP040222, PCP022543, PCP016311, PCP044725, PCP024172, PCP007841, PCP013947, and PCP030808). Lignin-related genes, *PpCCR*, *Pp4CL*, *PpCOMT*, *PpCAD1*, and *PpCAD2* also exhibited significantly higher levels of expression in hard-end fruit, relative to normal pear fruit (Fig. 2).

The phylogenetic analysis of *PpNACs* and their expression pattern

Transcription factor family genes were among the genes represented in the transcriptome data. NAC transcription factor genes were selected from the identified transcription factors for further analysis.

Three *PpNAC* genes were found to be DEGs, and PCP044783 was the same sequence with *PpNAC138*. The other two genes of PCP044783 and PCP012487 were named as *PpNAC186* and *PpNAC187* according to the reference (Fig. 3) [38]. The three *NAC* genes that were identified as DEGs were either up- or down-regulated ($\text{Log}_2\text{FC} > 2$ or $\text{Log}_2\text{FC} < -2$) in hard-end fruit, relative to normal pear fruit.

The expression patterns of *PpNAC138*, *PpNAC186* and *PpNAC187* in hard-end and normal fruit during fruit development and postharvest storage were analyzed by RT-qPCR. Results indicated that the relative transcript abundance of *PpNAC187* exhibited a significant increase of expression in hard-end fruit at 90 and 120 d after anthesis, while *PpNAC138* and *PpNAC186* were all down-regulated in hard-end fruits during fruit development (Fig. 4). The relative transcript abundance of *PpNAC187* increased gradually in hard-end fruit during postharvest storage, while no significant changes in expression were detected in normal fruit. The transcript abundance of *PpNAC187* was consistently higher in hard-end fruit than in normal fruit. The relative abundance of *PpNAC138* exhibited some greater level of expression in hard-end fruit than in normal fruit at 60 d, while *PpNAC186* only exhibited a higher level of expression at 120 d after harvest in hard-end pear fruits (Fig. 4). Since *PpNAC187* exhibited the greatest difference in expression in hard-end vs. normal fruit, it was selected for further analysis.

Subcellular localization of *PpNAC187*

A pCambia1300-*PpNAC187* vector, carrying a Green Fluorescent Protein (GFP) reporter protein, was constructed and subsequently inoculated into onion scales to determine the subcellular localization of *PpNAC187*. An empty vector was used as a control. When viewed under a fluorescent microscope, the cytomembrane and nucleus in living onion epidermal cells infected with the pCambia1300 empty vector exhibited green fluorescence. In contrast, only the nucleus exhibited green fluorescence in onion epidermal cells infected with the pCambia1300-*PpNAC187*. These results indicate that the *PpNAC187* transcription factor is expressed and localized in the nucleus of onion epidermal cells (Fig. 5). Transient expression of *PpNAC187* in 'Wangkeumbae' pear flesh

Transient expression analysis of *PpNAC187* was conducted in 'Wangkeumbae' pear flesh by injecting pCambia1300-*PpNAC187* into fruit flesh, while the injection of an empty vector served as a control (Fig. 6a) with each 50 fruit. No obvious changes in the pear fruit surface were observed over a three-day period following injection with either vector of all the fruit. Subsequently, however, the color around the inoculation site of fruit injected with *Agrobacterium* harboring the pCambia1300-*PpNAC187* vector changed to dark green by day 5 of 16 fruit among 20 fruit, and the green color progressively deepened by the 10th d after inoculation. In contrast, the control fruit inoculated with the empty vector exhibited no significant change in color over the ten-day post-injection period. Results of the lignin staining indicated no obvious differences between fruit inoculated with empty vector vs. pCambia1300-*PpNAC187* after 3 d, but the level of staining was noticeably higher in pCambia1300-*PpNAC187* inoculated fruit than control fruit at 5 d and 10 d post-injection (Fig. 6b). The results on the transient expression of *PpNAC187* are in agreement with increased lignin accumulation observed in 'Wangkeumbae' hard-end fruit.

The expression of *PpNAC187* and lignin-synthesis-related genes was also analyzed in fruit tissues surrounding the injection site. In comparison to fruit injected with the empty vector control, the relative expression level of *PpNAC187* was higher in fruit injected with the pCambia1300-*PpNAC187* vector. There was a significant increase in both *PpCAD1* and *PpCAD2* expression of fruit injected with the pCambia1300-*PpNAC187* vector compared to empty vector after 3 d post-injection. The expression pattern of *PpCCR* was analogous to *PpNAC187*, which exhibited an increase in expression at 3 d post-injection. The expression of *Pp4CL* and *PpCOMT* increased after 10 d and 5 d post-injection respectively (Fig. 7). Thus, it was concluded that the expression of *PpNAC187* (TF) and *PpCCR*, *Pp4CL* and *PpCOMT* (lignin biosynthesis genes) was correlated with the lignification of flesh tissues in pears and reflected what occurred during the normal development of hard-end pear fruit.

Functional verification of *PpNAC187* in transgenic tobacco

Transgenic tobacco plants overexpressing *PpNAC187* were generated using an *Agrobacterium*-mediated transformation method. Insertion of *PpNAC187* into the tobacco genome was confirmed by PCR analysis. Results revealed that a band in the size-range of *PpNAC187* was amplified in *PpNAC187* transgenic tobacco but not in plants transformed with the empty vector (Additional File 2: Fig. S1). Two independent *PpNAC187* transgenic lines (#1 and #3) were selected. *PpNAC187* was highly-expressed in #1 and #3 *PpNAC187*-overexpressing transgenic plants and not expressed at the empty vector line (Fig. 8 a-b). The level of stem's lignin staining was higher in *PpNAC187*-overexpressing lines than that in empty vector plants (Fig. 8 c). Lignin content in stem tissues of *PpNAC187*-overexpressing lines was notably higher than in empty vector line (Fig. 8 d). When autofluorescence was examined under a fluorescence microscope, more cell layers of xylem elements were observed in the stem in *PpNAC187*-overexpressing plants compared to the control plants (Fig. 8 e). The autofluorescence within the stem sections in *PpNAC187*-overexpressing plants was also more pronounced compared to the control tobacco plants transformed with an empty vector (Fig. 8 e). Meanwhile, lignin content in leaf tissues of *PpNAC187*-overexpressing lines also higher than in empty vector line. And the leaf vein of *PpNAC187*-overexpressing plants have more cell layers of xylem elements were observed than the control plants, The autofluorescence within the leaf veins in *PpNAC187*-overexpressing plants was more pronounced than the empty vector line (Fig. 9 a). The expression patterns of the tobacco endogenous lignin-related genes were also conducted, the genes of *NtCCR2*, *Nt4CL* and *NtCAD1* were upregulated in #1 and #3 lines separately when compared with empty vector line (Fig. 10). *PpNAC187*-overexpressing plants also grew more fibrous roots relative to the S1 line (Additional File 3: Fig. S2 a). There was no apparent difference in lignin content in root tissues between *PpNAC187*-overexpressing and empty vector plants (Additional File 4: Fig. S2 b).

Discussion

The hard-end disorder of 'Whangkeumbae' fruit is a major problem in the pear industry. Hard-end fruits contain significantly more and larger sclerotic cells in the calyx-end of the fruit compared to normal pear fruit; as well as a higher level of synthesis and deposition of lignin [5]. Several genes, including *PAL*,

4CL, *CCR*, *COMT*, *CAD*, are components of the phenylpropanoid pathway are associated with lignin synthesis [27, 28, 29, 30, 25]. Among these genes, *PpCAD1* and *PpCAD2* are continuously expressed at high levels during fruit development in fruit exhibiting hard-end symptoms [5]. *CCR* and *CAD* family genes are also responsible for the regulation of lignin synthesis and stone cell development in pear fruit [31]. In the present study, KEGG analysis of transcriptome data from hard-end and normal 'Whangkeumbae' pears revealed several DEGs that are part of the phenylpropanoid biosynthesis pathway. Based on RPKM values, the expression level of these genes was notably higher in hard-end fruit than in normal fruit. These results indicate that the lignin synthesis pathway is more highly-activated in hard-end fruit than in normal 'Whangkeumbae' pear fruit and are in accordance with our previous research showing lignin accumulation during the development of hard-end symptoms [5].

Many physiological activities of plants are regulated by the activity of transcription factors [32]. Previous studies have reported that NAC transcription factors are involved in lignin synthesis in fruits. In fruits, *EjNAC1* expression was induced in response to low temperature but inhibited by a heat treatment (HT); the latter of which also inhibited lignification [33]. In the present study, the conducted RNA-seq analysis revealed that three pear *NAC* genes are expressed at significantly different levels in hard-end 'Whangkeumbae' pear fruit, relative to normal pear fruit. In particular, the expression level of *PpNAC187* was significantly higher during the development of hard-end fruit than in normal fruit, as well as during postharvest storage. *PpNAC187* was localized in nuclei, confirming that *PpNAC187* is a functional transcription factor. When a vector containing *PpNAC187* was injected into pear flesh tissues, the relative expression level of *PpNAC187* was significantly enhanced, concurrent with the lignin biosynthesis-related genes (*PpCCR*, *Pp4CL*, and *PpCOMT*). And in *PpNAC187*-overexpressing transgenic tobacco, the expression level of lignin biosynthesis-related genes (*NtCCR2*, *Nt4CL* and *NtCAD1*) was also dramatically increased (Fig. 7). We suggest that lignin synthesis is potentially influenced by the NAC transcription factor. Several NAC genes, including *AtVND* and *AtNST*, have been previously reported to be involved in the regulation of phenylpropanoid biosynthesis and these NAC TFs also play a role in secondary xylem development and/or secondary wall formation in *A. thaliana* [34-37]. A greater number of xylem vessel elements were observed in our present study in transverse sections of stems and leaf veins in *PpNAC187*-overexpressing transgenic tobacco lines, relative to tobacco plants transformed with an empty vector (positive control). Extra cell layers of vessel elements were also observed in the *PpNAC187*-overexpressing transgenic tobacco and individual elements were larger in diameter, relative to xylem elements in tobacco plants transformed with empty vector. Our previous studies demonstrated that *PpCAD2*-overexpressing tobacco plants also produced a greater number of xylem vessel elements in stem and leaf veins than in WT tobacco plants [25]. In the current study, lignin content was significantly higher in stem and leaf tissues of *PpNAC187*-overexpressing transgenic tobacco. Collectively, the data indicate that *PpNAC187* plays a role in enhancing lignin accumulation by inducing the expression of *PpCCR*, *Pp4CL* and *PpCOMT* in 'Whangkeumbae' pear fruit during the development of hard-end symptoms. The ectopic expression of NACs in *PpNAC187*-overexpressing transgenic tobacco activated biochemical and metabolic processes resulting in a greater number of vessel elements, sclerotic cells, and a higher level of lignin accumulation.

Conclusion

In the present study, we demonstrated that the expression of lignin biosynthesis-related genes including *4CL*, *CCR*, *COMT*, *CAD* exhibited significantly increased in 'Wangkeumbae' pear 'hard-end' fruit. Furthermore, transient overexpression of *PpNAC187* in 'Wangkeumbae' pear flesh induced the expression of lignin synthesis related genes *PpCCR*, *Pp4CL*, and *PpCOMT* and the degree of lignification. In *PpNAC187*-overexpressing transgenic tobacco, lignin biosynthesis-related genes (*NtCCR2*, *Nt4CL* and *NtCAD1*) also exhibited increased. And the lignin content in both stem and leaf of *PpNAC187*-overexpressing transgenic tobacco was increased. These results suggest that *PpNAC187* enhances lignin synthesis by regulating the expression of lignin synthesis related genes in 'Wangkeumbae' pear 'hard-end' fruit.

Materials And Methods

Plant material

'Wangkeumbae' pear fruit were picked in orchards located in Wulong and Laiyang, Shandong province, People's Republic of China. Hard-end fruit were picked from ten-year-old 'Wangkeumbae' pear trees in one orchard, and normal pears were harvested from healthy trees in another orchard. Normal and hard-end fruit were sampled at 60, 90 and 120 days after anthesis, and sampled again at 0, 60 and 120 days after harvest when stored under 0 °C. Three biological replicates comprised of ten fruits each were used for each condition (normal vs. hard-end) and at each sampling timepoint. The fruit tissues near the bottom third of the calyx end were taken. After removal of the peel and/or seed, fleshy tissues were sliced into small pieces (approximately 1 cm³) and immediately frozen in liquid nitrogen. Samples were stored at -70 °C until further analysis.

RNA-seq analysis

The calyx pulp of normal and hard-end fruit at 120 days after anthesis were RNA-seq analysis. Total RNA was extracted using an RNA extraction kit (Omega, Georgia, USA) according to the manufacturer's instructions. The integrity and quality of the total RNA was evaluated using a 2100 Bioanalyzer RNA Nano chip device (Agilent, Santa Clara, CA, USA). The poly A - mRNA fraction was enriched by treatment of the extracted RNA with oligo (dT) beads and was then reverse-transcribed into first strand cDNA for use in preparation of the sequencing libraries.

The cDNA libraries were sequenced using an Illumina HiSeq 2500 system at the Biomarker Technologies Corporation (Beijing, China). Raw reads were first filtered to remove adaptors and low quality sequences, and then mapped to the pear reference genome

(https://www.rosaceae.org/species/pyrus/pyrus_communis/genome_v1.0) using TopHat software. A false discovery rate (FDR) < 0.01 and a fold change of ≥ 2 were used to identify differentially expressed genes (DEGs). The predicted product of each unigene sequence was aligned to a set of proteins retrieved from the NCBI Nr, Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Cluster of

Orthologous Groups of proteins (COG) databases. The Reads Per Kb per Million Fragments (RPKM) was used to determine the expression level of genes. The total number of reads for each unigene and gene length were normalized by RPKM. The formula used to calculate was as follows: $RPKM = \frac{\text{total exon reads}}{(\text{mapped reads (millions)} \times \text{exon length (KB)})}$. KEGG pathway enrichment analysis was performed using KOBAS software and utilized an adjusted P-value of <0.05. Transcription factors were identified and classified into different families by reference to the NCBI Nr, Swiss-Prot, and COG databases. The raw sequences generated for 'Whangkeumbae' in this study were deposited in NCBI (NCBI BioProject Accession: SRP063324, <http://www.ncbi.nlm.nih.gov/bioproject/PRJNA294723>).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from pear flesh tissue using RNAlplant Reagent (TianGen, Shanghai, China) according to the manufacturer's instructions. Tobacco leaf RNA was extracted using an EASYspin Plant RNA Kit (Yuanpinghao, China) and genomic DNA was removed by treatments with DNase (Fermentas, Vilnius, Lithuania). The cDNA was synthesized by reverse transcription using the Prime Script™ RT reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions and was subsequently used as template in the RT-qPCR analyses. RT-qPCR was performed on a Light Cycler® 480 instrument (Roche, Switzerland). The protocol included annealing at 94 °C for 5 min, followed by 40 cycles of 94 °C for 15 s, and 60 °C for 1 min. Actin genes from pear and tobacco were used for the normalization of transcript levels. Gene-specific primers used in the RT-qPCR analyses were designed with Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) software and are listed in Additional File 1: Table S1. Mean expression level was calculated using the $2^{-\Delta\Delta C_t}$ method [39]. The expression level in normal fruit at 60 days after anthesis was set as 1 in the RT-qPCR analyses conducted on samples collected during fruit development, and the day of harvest was set as 1 in the post-harvest analyses. Three biological and three technical replicates were used in the RT-qPCR analysis of each gene at each timepoint.

Cloning of *PpNAC187*

Total RNA isolation and cDNA synthesis followed the same protocol used in the RT-qPCR analyses. The PCR primers used to clone *PpNAC187* are shown in Additional File 1: Table S2. The PCR program was: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, followed by an extension cycle at 72 °C for 10 min and a final cycle at 4 °C. PCR products were cloned into PMD19-T vectors (Takara, Dalian, China). The open reading frame (ORF) of *PpNAC187* was amplified and cloned using Phusion® High-Fidelity DNA Polymerase (Thermo scientific, Lithuania, EU).

Sequence alignment and phylogenetic analysis

The amino acid sequence alignment analysis of NACs was conducted using DNAMAN software. A phylogenetic tree was reconstructed with Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>) online software. The amino acid sequence alignment analysis of pear NACs were referred to Ahmad [20].

Construction of the expression vector

The ORF of *PpNAC187* was ligated into the expression vector, pCambia1300, under the control of a 35S promoter. The ORF fragment isolated by digestion with KpnI and HindIII was inserted into the expression vector, pSuper1300, under the control of a 35S promoter. The vectors, pCambia1300-PpNAC187 and pSuper1300-PpNAC187, were transferred into *Agrobacterium tumefaciens* EHA105 using the freeze-thaw method [21]. The sequences of primers used to construct the expression vector are listed in Additional File 1: Table S2.

Subcellular localization of the PpNAC187 transcription factor

The subcellular localization of gene expression was determined using the method described by Sun with some modifications [22]. After incubation for 24 h at 28 °C in the dark, fresh onion scales (1.5×1 cm) were placed on a 9 cm plate with their inner surface submerged in a 10 mL *Agrobacterium* solution (OD₆₀₀ = 0.6-0.8) supplemented with 20 mg acetosyringone/L for 15-20 min. The onion scales were then transferred to a 1/2 MS solid medium amended with 20 mg acetosyringone/L and cultured for 16-24 days at 28 °C. The onion scales were subsequently rinsed with water and epidermal cell layers were peeled and directly transferred to glass slides. *Agrobacteria* harboring the pCambia1300-PpNAC187 or the empty pCambia1300 vector were used in the analysis of subcellular localization. The GFP of onion scales inoculated with these vectors were observed under a confocal laser scanning microscope (TCSSP5, Leica, Germany).

Transient expression of *PpNAC187* in ‘Wangkeumbae’ pear

The method of transient expression of *PpNAC187* in pear ‘Wangkeumbae’ followed the method described by Spolaore with some modifications [23]. Holes were punched on the calyx end of hard-end fruits on the harvest day using a sterile syringe needle. One ml of *Agrobacterium* solution (OD₆₀₀ = 0.6-0.8) was then injected into the fruit via the holes using a syringe without a needle and the injected fruit was stored in the dark. Fruits inoculated with pSuper1300-PpNAC187 (treated) or the empty pSuper1300 vector (control) were photographed at the sampled timepoints. Samples were taken at 1, 3, 5 and 10 days after the injection, and were immediately frozen in liquid nitrogen and stored at -70 °C until further processing.

Agrobacterium*-mediated transformation of tobacco with *PpNAC187

The empty pSuper1300 vector and pSuper1300-PpNAC187 were independently transformed into tobacco plants using the *Agrobacterium*-mediated transformation method as described by Zheng with some modifications [24]. Portions of tobacco leaves without veins were cut into discs (1×1 cm) and pre-cultured on MS solid medium for 2 days at 28 °C in the dark. The leaf discs were then submerged in 15 mL of *Agrobacterium* solution (OD₆₀₀ = 0.6-0.8) supplemented with 20 mg acetosyringone/L for 15-20 min. Transgenic tobacco plants were generated on selection media after a 24 h light treatment following the method of Wang [25].

PCR verification of transformed *PpNAC187* tobacco

DNA was extracted from tobacco leaf tissue using DNAplant Reagent (TianGen, Shanghai, China) according to the manufacturer's instructions. *PpNAC187* primers listed in Additional File 1: Table S2 were used to verify the presence of *PpNAC187*. The PCR program utilized was: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, followed by a 10 min extension at 72°C and a final cycle at 4 °C.

Wiesner staining and microscopy

Wiesner reagent (phloroglucinol/HCl) staining of plant tissue for 5 min was used to visualize lignification [5]. Two grams of phloroglucinol were dissolved in 100 ml of 95% alcohol and then filtered into 40 ml of concentrated hydrochloric acid. A razor blade was used to dissect leaf tissue prior to observation. Lignified structures appeared pink or fuchsia in color. Auto-fluorescence within stem sections was observed with the aid of an EVOS smart fluorescence microscope (Thermo Fisher, America).

Lignin assay

Lignin content was assayed using the method described by Dyckmans with some modifications [26]. Samples were washed three times in a 10 ml solution (100 mM K₂HPO₄/KH₂PO₄, 0.5% Triton X-100, 0.5% PVP, PH 7.8), followed by an additional three washes in 100% methanol. The samples of fruit tissues were then dried overnight and tissue samples were then transferred into 1ml of solution composed of 2 M HCl and 0.1ml thioglycolic acid. Lignin was extracted in this solution by placing samples in a boiling water bath for 4 h. Pellets obtained by centrifugation were resuspended 2 ml 1M NaOH followed by agitation for 18 h. After the addition of 0.2 ml HCl, the mixture was incubated for 4 h at 4 °C. The end product was dissolved in 1 ml 1M NaOH and absorbance at 280 nm was recorded to estimate lignin content. All measurements were performed in triplicate.

Statistical analyses

Two-tailed *t-test* were performed to determine the statistical significance of differences between samples. Figures were drawn using Origin 6.0 (Microcal Software Inc. Northampton, MA, USA).

Abbreviations

PAL, Phenylalanine ammonia lyase; 4CL, 4-Coumarate: coenzyme A ligase; CAD, Cinnamyl alcohol dehydrogenase; POD, Peroxidase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid 3-O-methyltransferase; NAC, NAM, ATAF and CUC; MYB_n, bHLH_n; FDR, false discovery rate; DEG, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; COG, Cluster of Orthologous Groups of proteins; RPKM, Read Per Kb per Million Fragments; MS, Murashige and Skoog; PVP, Polyvinyl Pyrrolidone; PH, pondus hydrogenii; LSD, Least significant differences; GC, guanine and cytosine; EV, empty vector; RACE, rapid amplification of cDNA ends; ORFs, open reading frames.

Declarations

Availability of data and material

All data generated or analyzed during this study are included within the article and its additional files.

Competing interests

None of the authors have any competing interests.

Funding

This work was funded by the National Key Research and Development Program of China (2016YFD0400100), the Project of Shandong Natural Science Foundation (ZR2017MC006, ZR2014CL026), National Natural Science Foundation of China (31201608), the Project of Shandong Modern Fruit Technology Industry System (SDAIT-06-06).

Authors' contributions

Shaolan Yang and Xinfu Zhang conceived and designed the experiments, Mingtong Li performed the experiments and analyzed the data, Suping Zhou, Caihong Wang, Chunhui Ma, and Shaolan Yang wrote the manuscript. All authors read and approved the manuscript.

References

1. Charles FP, Michael JC, Lacy PM, Dean HR. Market diseases of apples, pears, and quince. Agricultural Research Service. 1971.
2. Yamamoto T, Watanabe S. Initial time of development of hard end disorder in 'Bartlett' pear. J Japan Soc Hort Sci. 1982;51:42-151.
3. Rose DH, Mccolloch LP, Fisher DF. Market Diseases of Fruits and Vegetables: Apples, Pears, Quinces. US Department of Agriculture, Washington. 1951.
4. Raese J, Drake S. Calcium foliar sprays for control of alfalfa greening, cork spot, and hard end in 'anjou' pears. J Plant Nutr. 2006;29:543-552.
5. Lu GL, Li ZJ, Zhang XF, Wang R, Yang SL. Expression analysis of lignin associated genes in hard end pear (*Pyrus pyrifolia* Whangkeumbae) and its response to calcium chloride treatment conditions. J Plant Growth Regul. 2015;34:251-262.
6. Wang YL, Zhang XF, Wang YZ, Yang SL, Qu HY. The changes of intracellular calcium concentration and distribution in the hard end pear (*Pyrus pyrifolia* 'Whangkeumbae') fruit. Cell Calcium. 2018;15-23.
7. Fumio T. Recent advances in Research on Japanese Pear Rootstocks. J Japan Soc Hort Sci. 2012;81:1-10.
8. Zhao Q, Dixon RA. Transcriptional networks for lignin biosynthesis: more complex than we thought. Trends Plant Sci. 2011;16:227-233.

9. Cheng X, Li ML, Li DH, Zhang JY, Jin Q, Sheng LL, Cai YP, Lin Y. Characterization and analysis of CCR and CAD gene families at the whole-genome level for lignin synthesis of stone cells in pear (*Pyrus bretschneideri*) fruit. *Biol Open*. 2017;6:1602-1613.
10. Vermerris W, Abril A. Enhancing cellulose utilization for fuels and chemicals by genetic modification of plant cell wall architecture. *Curr Opin Biotechnol*. 2015;32:104-112.
11. Wang WQ, Zhang J, Ge H, Li SJ, Li X, Yin XR, Grierson D, Chen KS. *EjMYB8* transcriptionally regulates flesh lignification in loquat fruit. *PLoS One*. 2016;11:e0154399.
12. Akiyoshi K, Pulla K, Kazuya Y, Saori E, Keiko Y, Hiroyasu E. Functional analysis of tobacco LIM protein Ntlm1 involved in lignin biosynthesis. *The Plant Journal*. 2000;22:289-301.
13. Cassan-Wang H, Goué N, Saidi MN, Legay S, Sivadon P, Goffner D, Grima-Pettenati J. Identification of novel transcription factors regulating secondary cell wall formation in Arabidopsis. *Frontiers in plant science*. 2013;4:
14. Xu Q, Wang WQ, Zeng JK, Zhang J, Donald G, Li X, Yin XR, Chen KS. A NAC transcription factor, *EjNAC1*, affects lignification of loquat fruit by regulating lignin. *Postharvest Biology and Technology*. 2015;25-31.
15. Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MA, de Vries SC. The CUP-SHARPED COTYLEDON3 gene is required for boundary and shoots meristem formation in Arabidopsis. *Plant Cell*. 2003;15:1563-
16. Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M. NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. *Plant Cell*. 2007;19:270-
17. Zhong RQ, Ye ZH. Regulation of cell wall biosynthesis. *Current Opinion in Plant Biology*. 2007;10:564-572.
18. Zhong RQ, Ye ZH. Transcriptional regulation of lignin biosynthesis. *Plant Signal Behav*. 2009;4:1028-
19. Ge H, Zhang J, Zhang YJ, Li X, Yin XR, Grierson D, Chen KS. *EjNAC3* transcriptionally regulates chilling-induced lignification of loquat fruit via physical interaction with an atypical CAD-like gene. *J Exp Bot*. 2017;68:5129-
20. Ahmad M, Yan XH, Li JZ, Yang QS, Jamil W, Teng YW, Bai SL. Genome wide identification and predicted functional analyses of NAC transcription factors in Asian pears. *BMC Plant Biology*. 2018;18:214.
21. Weigel D, Glazebrook J. Transformation of agrobacterium using the freeze-thaw method. *CSH Protoc*. 2006;
22. Sun W, Cao Z, Li Y, Zhao YX, Zhang H. A simple and effective method for protein subcellular localization using Agrobacterium-mediated transformation of onion epidermal cells. *BIOLOGIA*. 2007;62:529-
23. Spolaore S, Casadoro G, Trainotti L. A simple protocol for transient gene expression in ripe fleshy fruit mediated by Agrobacterium. *Journal of Experimental Botany*. 2001;52:845-

24. Zheng L, Liu GF, Meng, XN, Li YB, Wang YC. A versatile agrobacterium-mediated transient gene expression system for herbaceous plants and trees. *Biochemical Genetics*. 2012;50:761-
25. Wang YL, Zhang XF, Yang SL, Wang CH, Lu GL, Wang R, Yang YJ, Li DL. Heterogenous expression of *Pyrus pyrifolia* PpCAD2 and PpEXP2 in tobacco impacts lignin accumulation in transgenic plants. *Gene*. 2017;181-
26. Dyckmans J, Flessa H, Brinkmann K, Mai C, Polle A. Carbon and nitrogen dynamics in acid detergent fibre lignins of beech (*Fagus sylvatica* L.) during the growth phase. *Plant Cell Environ*. 2002;25:469-
27. Olsen KM, Lea US, Slimestad R, Verheul M, Lillo C. Differential expression of four *Arabidopsis* PAL genes; PAL1 and PAL2 have functional specialization in abiotic environmental-triggered flavonoid synthesis. *Journal of Plant Physiology*. 2008;165:1491-1499.
28. Lee D, Meyer K, Chapple C, Douglas CJ. Antisense suppression of 4-coumarate: coenzyme A ligase activity in *Arabidopsis* leads to altered lignin subunit composition. *The Plant Cell*. 1997;9:1985-1998.
29. Jean CL, Rebecca D, Kris M, Vronique S, Catherine L, Brigitte P, Annette N et al. Downregulation of Cinnamoyl-Coenzyme A Reductase in Poplar: Multiple-Level Phenotyping Reveals Effects on Cell Wall Polymer Metabolism and Structure. *The Plant Cell*. 2007;19:3669-3691.
30. Guo D, Chen F, Inoue K, Blount JW, Dixon RA. Downregulation of Caffeic Acid 3-O-Methyltransferase and Caffeoyl CoA 3-O-Methyltransferase in Transgenic Alfalfa: Impacts on Lignin Structure and Implications for the Biosynthesis of G and S Lignin. *Plant Cell*. 2001;13:73-88.
31. Cheng X, Li ML, Li DH, Zhang JY, Jin Q, Sheng LL, Cai YP, Lin Y. Characterization and analysis of CCR and CAD gene families at the whole-genome level for lignin synthesis of stone cells in pear (*Pyrus bretschneideri*) fruit. *Biol Open*. 2017;6:1602-
32. Liu WS, Stewart CN. Plant synthetic promoters and transcription factors. *Current Opinion in Biotechnology*. 2016;36-
33. Xu Q, Wang WQ, Zeng JK, Zhang J, Donald G, Li X, Yin XR, Chen KS. A NAC transcription factor, EjNAC1, affects lignification of loquat fruit by regulating lignin. *Postharvest Biology and Technology*. 2015;25-31.
34. Zhong R, Demura T, Ye ZH. SND1 a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of *Arabidopsis*. *Plant Cell*. 2006;18:3158-3170.
35. Zhong R, Demura T, Ye ZH. SND1 a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of *Arabidopsis*. *Plant Cell*. 2006;18:3158-3170.
36. Yamaguchi M, Kubo M, Fukuda H, Demura T. Vascular-related NACDOMAIN7 is involved in the differentiation of all types of xylem vessels in *Arabidopsis* roots and shoots. *Plant J*. 2008;55: 652-664.
37. Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T. Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev*. 2005;19:1855-1860.
38. Ahmad M, Yan XH, Li JZ, Yang QS, Jamil W, Teng YW, Bai SL. Genome wide identification and predicted functional analyses of NAC transcription factors in Asian pears. *BMC Plant Biology*.

2018;18:

39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. 2001;25:402-408.

Additional File Legend

Additional File 1: Table S1 Gene-specific primer sequences used in the RT-qPCR analysis of gene expression. Table S2 Primer sequences used in the cloning of *PpNAC187* and transgenic plant validation.

Additional File 2: Fig. S1 Validation of transgenic tobacco plants. The first row represents the PCR products generated using *PpNAC187*-specific primers while the second row represents PCR products generated using NPT^{II}-PCR primers. In the figure; 1,2,3,4 and 5 represent the S1, S2, S3, S4, and S5 transgenic lines transformed with empty vector. Additionally, 8, 9, 10, 11, 12, 13, and 14 represent the N1, N2, N3, N4, N5, N6, and N7 transgenic lines overexpressing *PpNAC187*.

Additional File 3: Fig. S2 The morphology and lignin content of roots in *PpNAC187*-overexpressing transgenic tobacco plants.

Figures

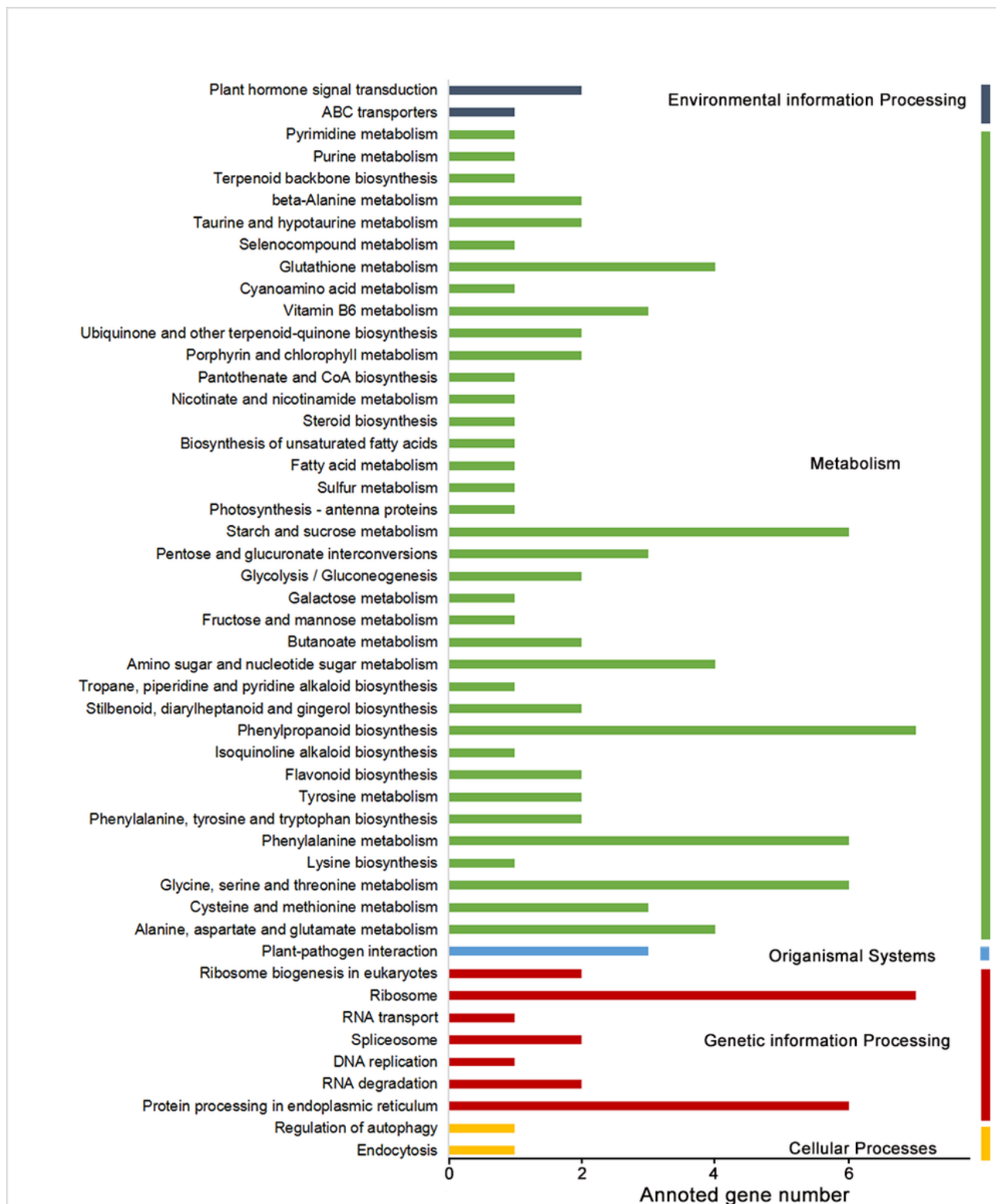


Figure 1

KEGG enrichment analysis and the annotation of DEGs in control vs. hard-end fruit. The yellow columns indicate cellular processes, the red columns indicate genetic information processing, the blue columns indicate organismal systems, the green columns indicate metabolism, and the purple columns indicate environmental information processing. The figure displays the number of annotated genes in each category.

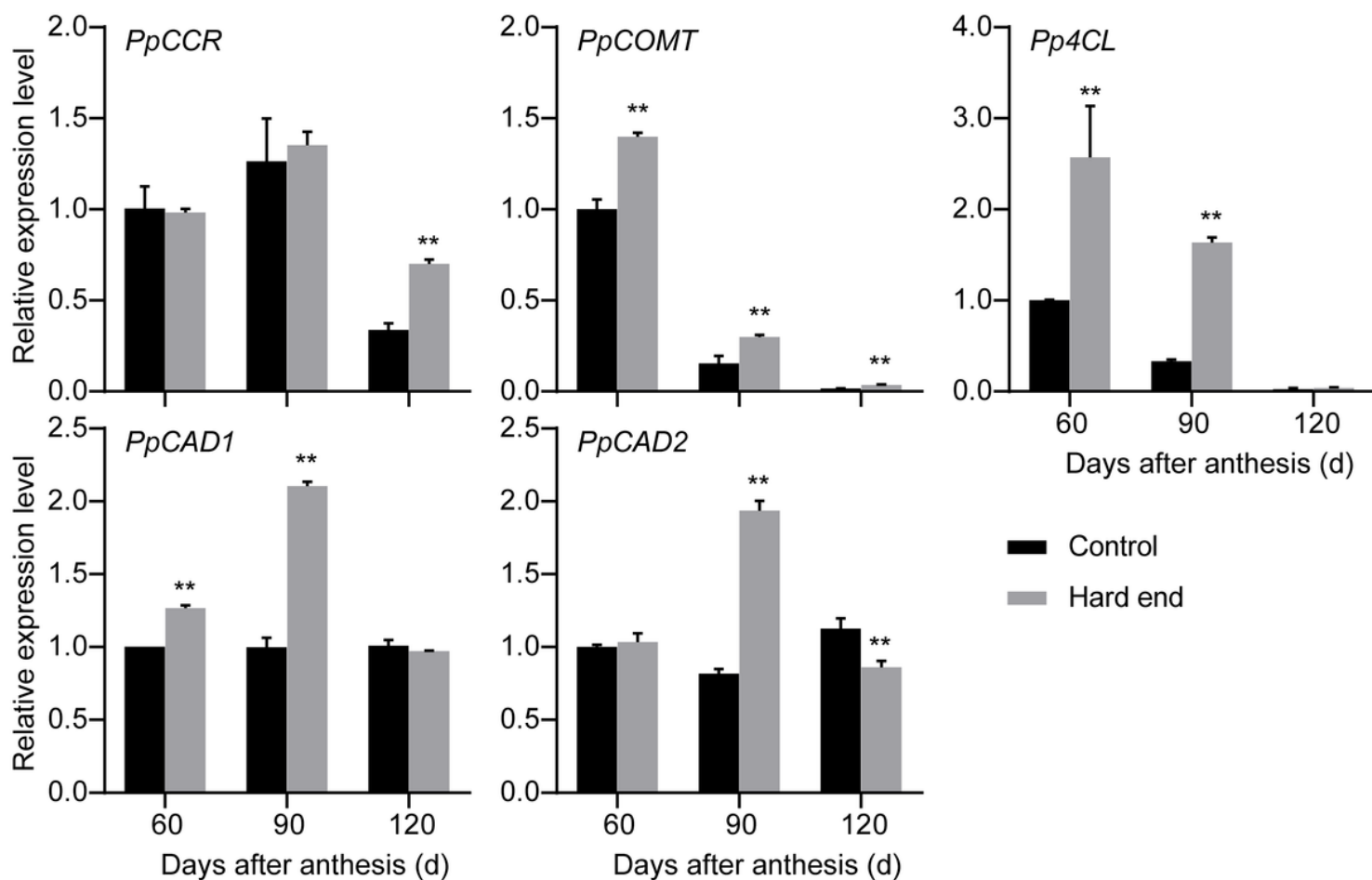


Figure 2

The relative expression of lignification-related genes during the period of fruit development. Asterisks indicate significant differences between control and hard end fruit (**, $P < 0.01$; two-tailed t-test).

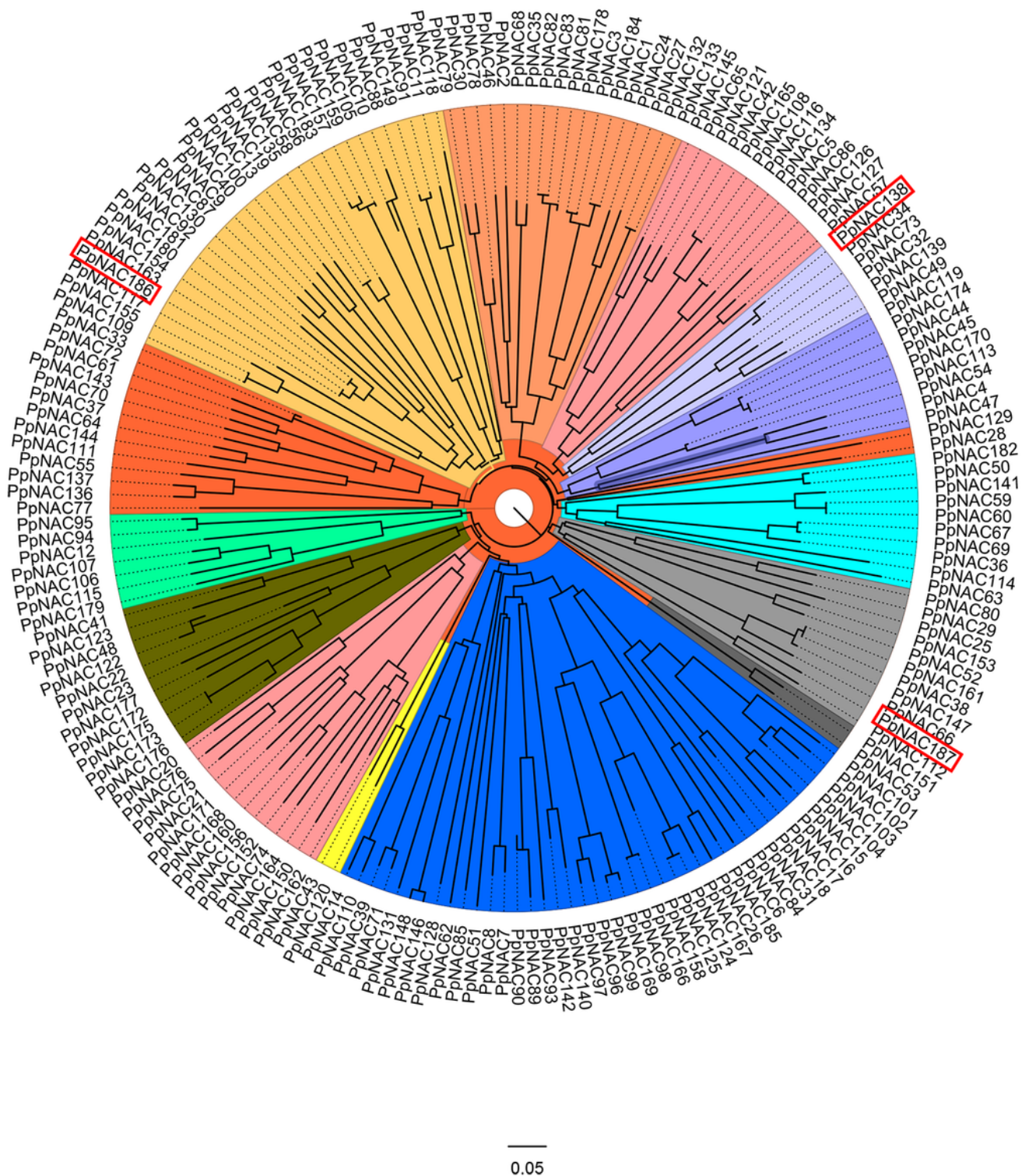


Figure 3

Phylogenetic analysis of pear NAC genes based on deduced amino acid sequences. PpNAC138, PpNAC186, and PpNAC187 are framed in a red box. Genes listed with the same color indicate genes that are highly homologous.

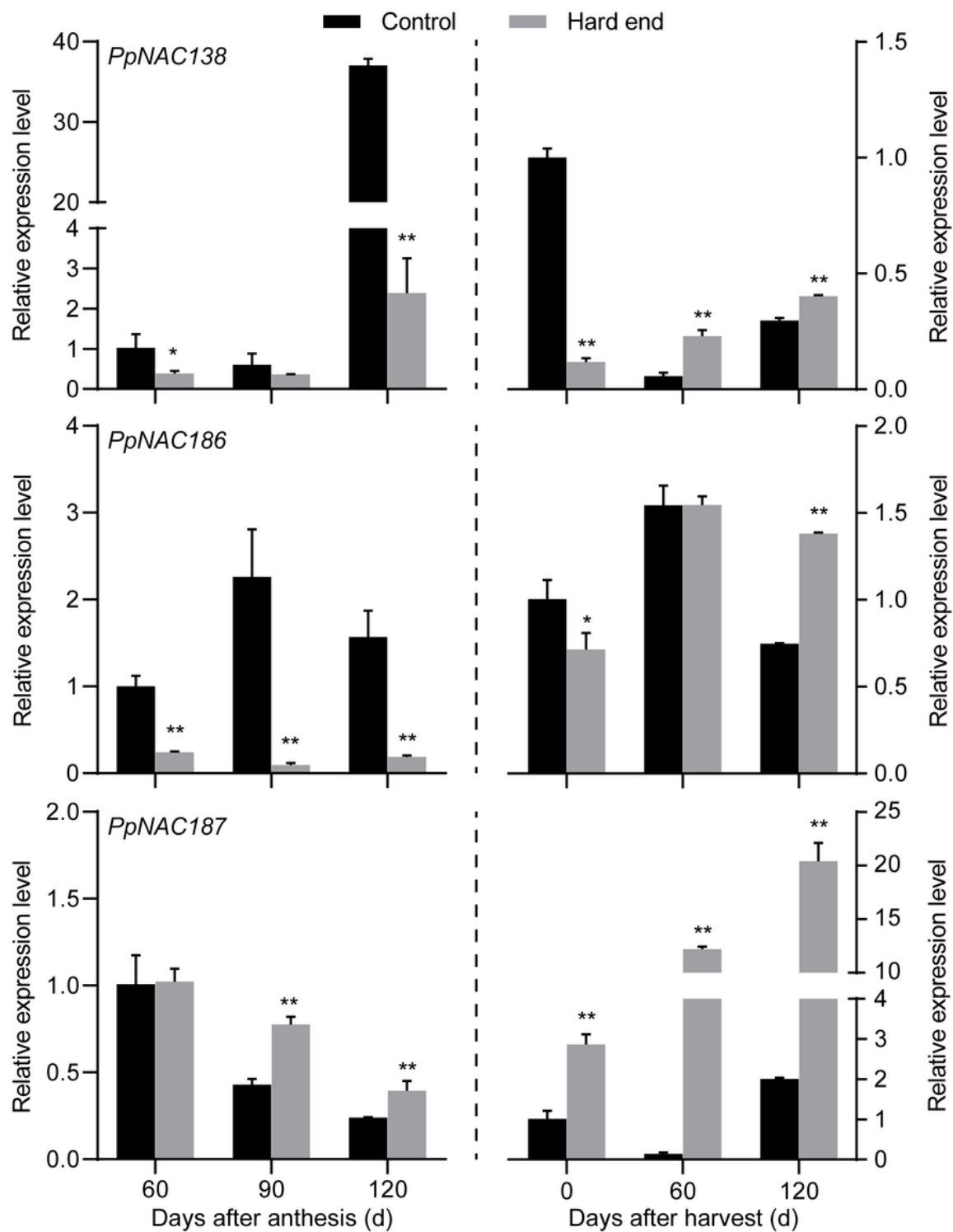


Figure 4

The relative expression of NAC transcription factors during fruit development and postharvest storage. Asterisks indicate significant differences between control and hard end fruit (*, $P < 0.05$; **, $P < 0.01$; two-tailed t-test).



Figure 5

Subcellular localization of a PpNAC187-GFP fusion protein in onion epidermal cells. The laser-scanning confocal microscopy was used to obtain light, fluorescent, and merged images. Dark images represent fluorescent images, bright represent light images, and merged represent merged dark and light images.



Figure 6

Transient expression of PpNAC187 in 'Whangkeumbaë' pear fruit. (a) Infiltration of the pCAMBIA1300-PpNAC187 or pCAMBIA1300 empty vector into 'Whangkeumbaë' pear fruit. (b) Fruit phenotype (left) and Wiesner staining of fruit sections (right) at 1 d, 3 d, 5 d and 10 d after infiltration of the vectors. EV represents images of fruit infiltrated with the pCAMBIA1300 empty vector, and PpNAC187 represents images of fruit infiltrated with the pCAMBIA1300-PpNAC187 vector.

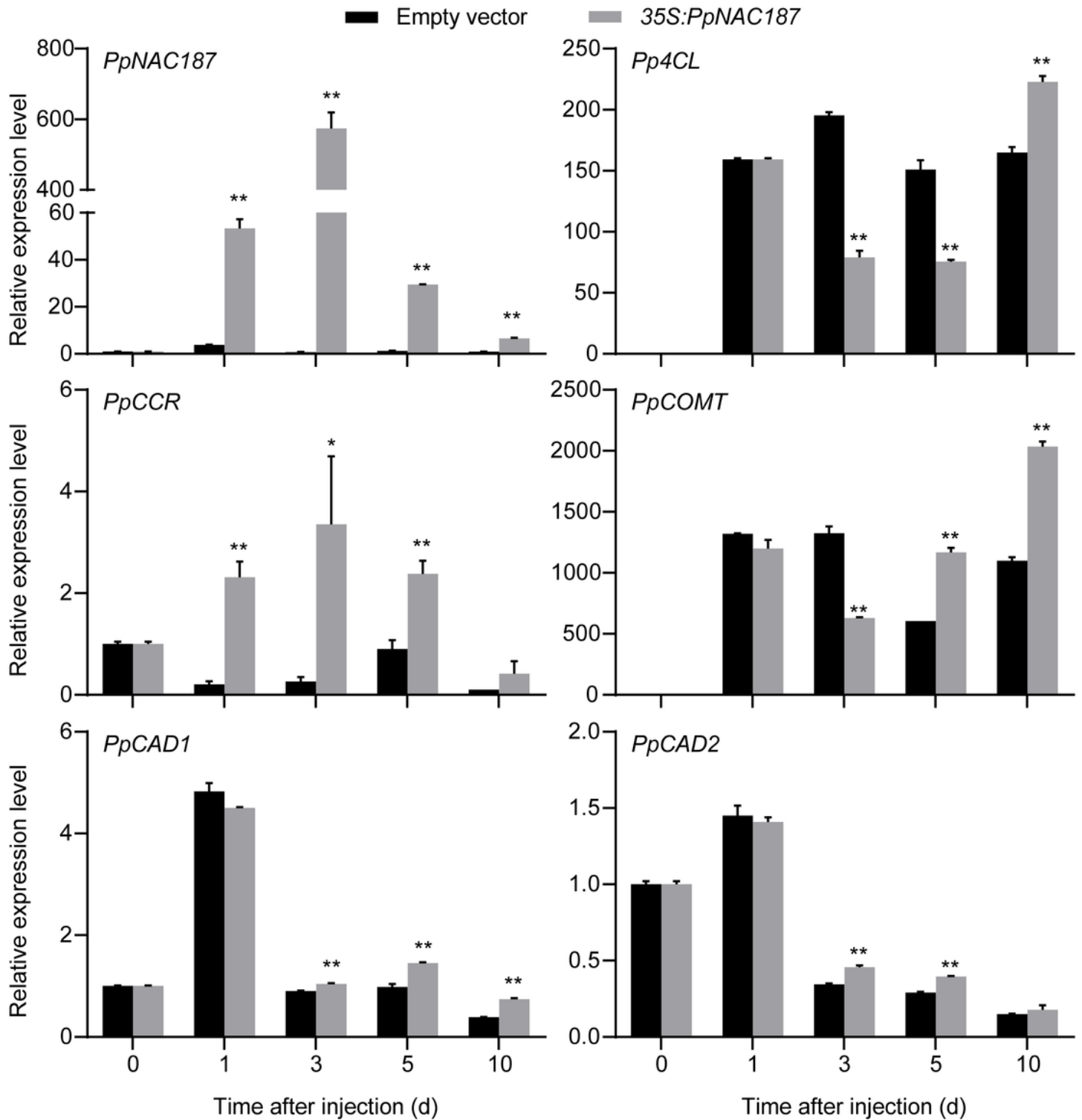


Figure 7

The relative expression of lignification-related genes in pear fruit surrounding the site of infiltration. 35S:PpNAC187 represent 'Whangkeumbae' pear fruit in which PpNAC187 was transiently expressed. Empty vector represent fruit that were infiltrated with the pCAMBIA1300 empty vector. The x-axis represents time after infiltration, and the y-axis represents relative expression. Asterisks indicate

significant differences between empty vector and 35S:PpNAC187 fruit (*, $P<0.05$; **, $P<0.01$; two-tailed t-test).

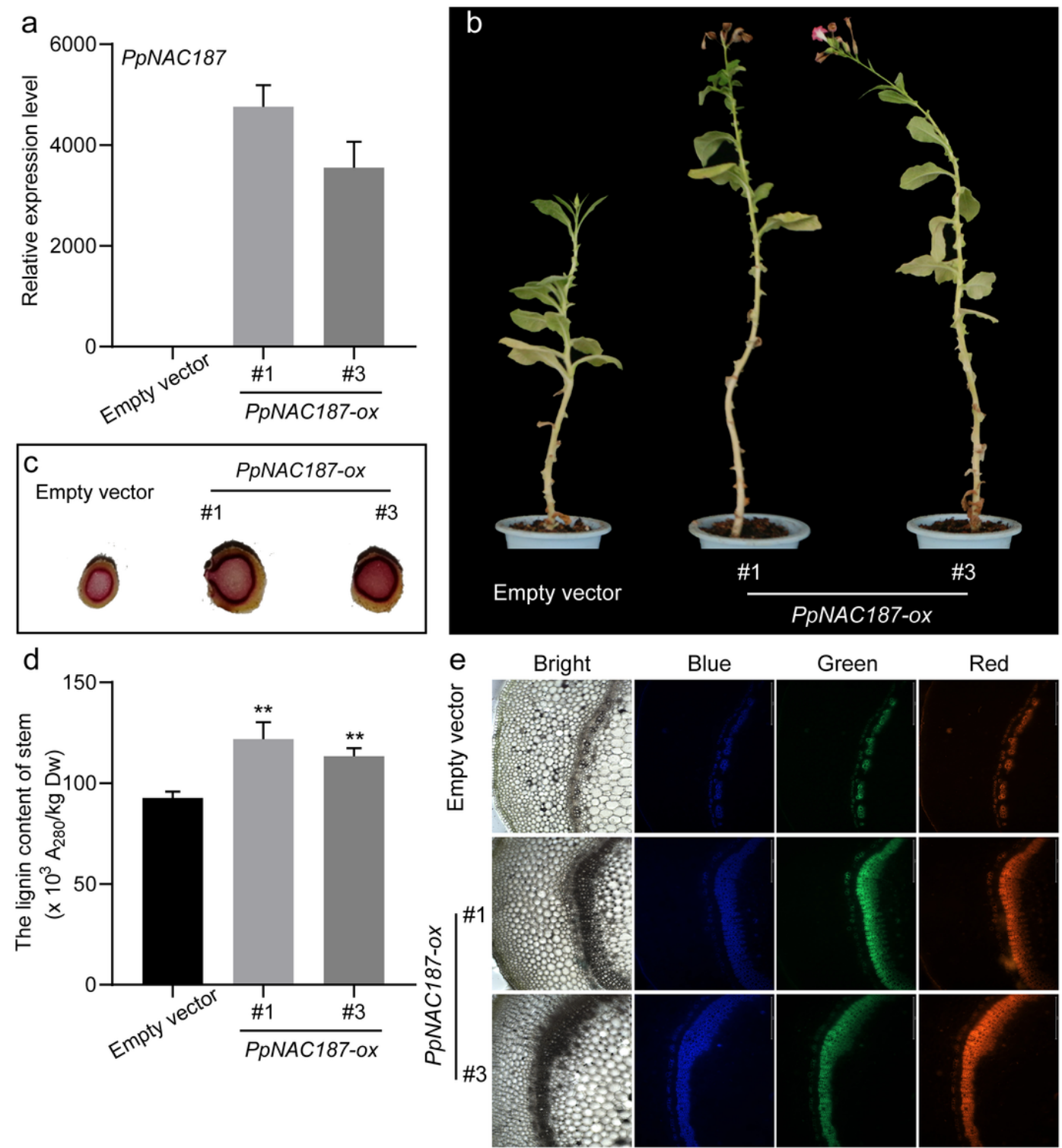


Figure 8

PpNAC187 increase the lignin content in the stem of transgenic tobacco plant. (a) The relative expression level of PpNAC187 in transgenic tobacco. (b) Phenotype of empty vector and PpNAC187-overexpressing (PpNAC187-ox) transgenic tobacco lines. (c) Transverse sections of stem were stained with

phloroglucinol–HCl for detection of lignin. (d) Lignin content in transgenic tobacco stem tissues. (e) Autofluorescence of the stem transverse slice. Bright: bright field images, Blue: blue autofluorescence, Green: green autofluorescence, Red: red autofluorescence. Significant differences between the empty vector and PpNAC187-ox plants are indicated (**, $P<0.01$; two-tailed t-test).

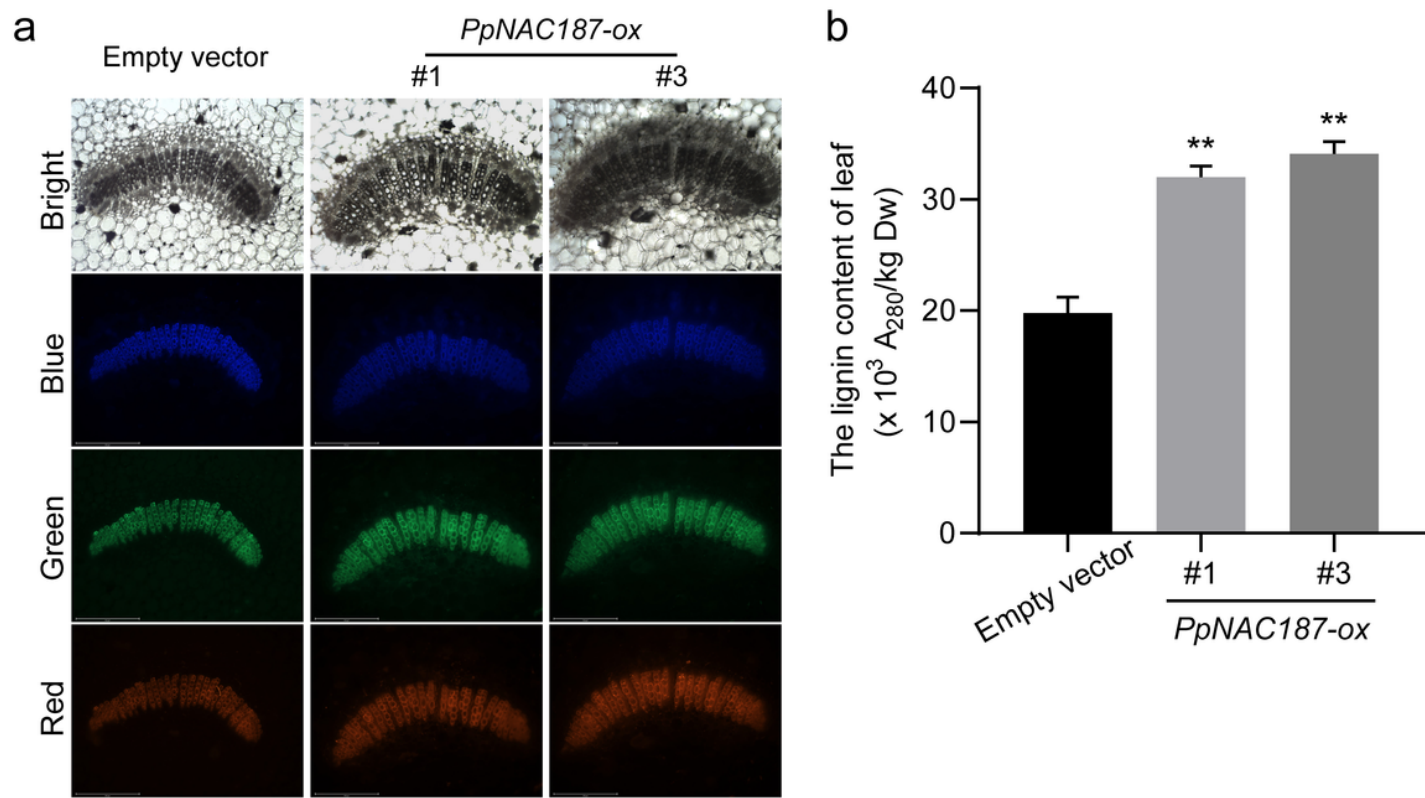


Figure 9

PpNAC187 increase the lignin content in the leaves of transgenic tobacco plants. (a) Autofluorescence of transverse slice in the leaf veins. Bright: bright field images, Blue: blue autofluorescence, Green: green autofluorescence, Red: red autofluorescence. (b) Lignin content of leaves in the empty vector and PpNAC187-ox plants. Significant differences between the empty vector and PpNAC187-ox plants are indicated (**, $P<0.01$; two-tailed t-test).

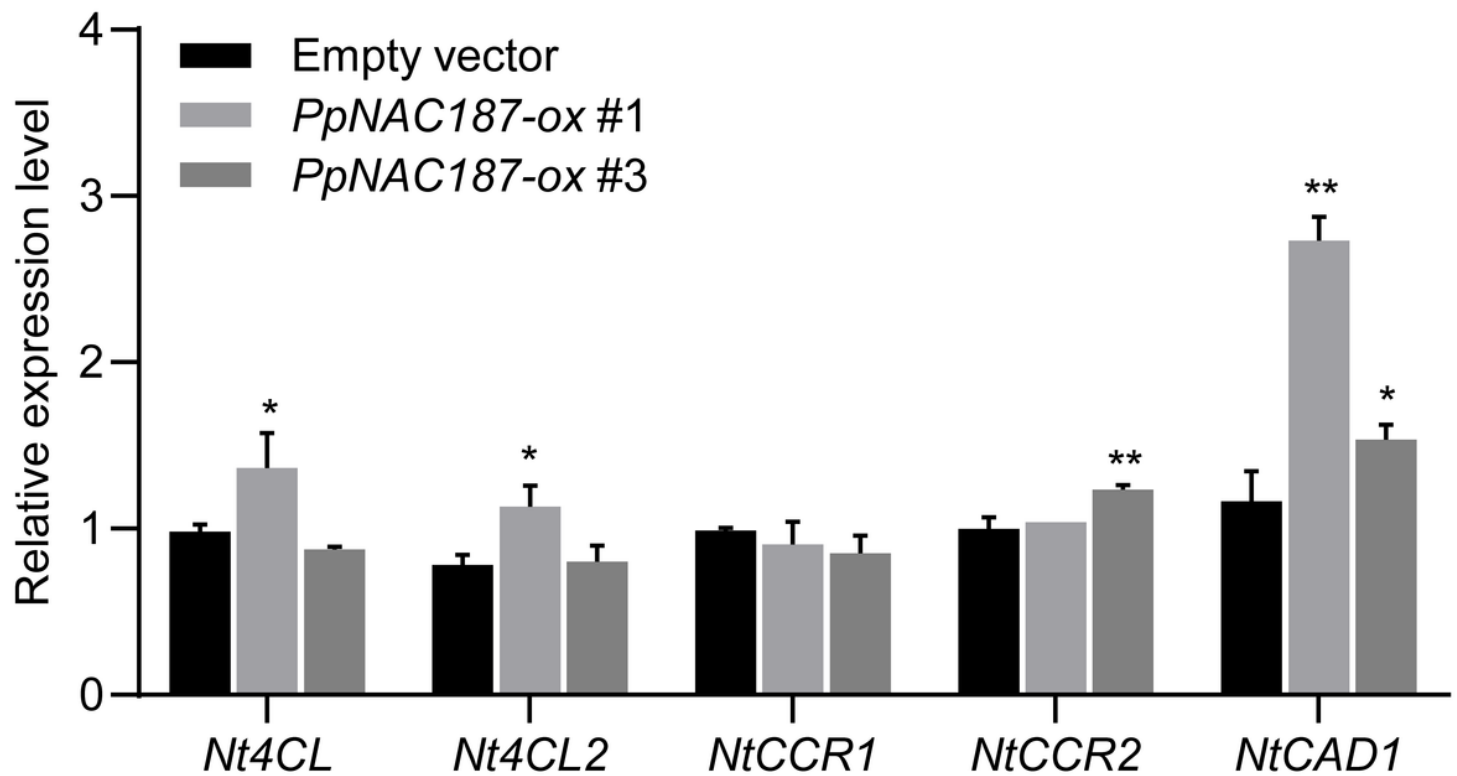


Figure 10

The relative expression of lignification-related genes in the transgenic tobacco plants. The presented data represent the mean $\pm$ SD ($n = 3$). Asterisks indicate significant differences between the empty vector and *PpNAC187-ox* plants (*, $P < 0.05$; **, $P < 0.01$; two-tailed t-test).

Supplementary Files

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