RESEARCH ARTICLE

**RcPAL, a key gene in lignin biosynthesis in Ricinus communis L.**

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Abstract

**Background:** Castor (Ricinus communis L.) is an important seed oil crop. Castor oil is a highly demanded oil for several industrial uses. Current castor bean varieties suffer from low productivity and high risk of insect pests and diseases. High productive and pest/disease resistance varieties are needed. Lignin has been associated to the resistance for pest, disease and lodging. Lignin is produced from several metabolites of the phenylpropanoid pathway. PAL is the key enzyme of the phenylpropanoid pathway. The gene PAL may assist in the improvement of resistance of castor bean.

**Results:** The RcPAL CDs was amplified and its function was examined by transgenic overexpression and antisense expression, lignin histochemical staining, real-time PCR, lignin content measurement and morphological investigation. Its full length was 2145 bp, encoding 714 amino acids. The overexpression of RcPAL (7.2 times) increased significantly the PAL activity, dyeing depth of xylem cells and lignin content (14.44%), resulting in a significantly lower plant height, deeper and thicker blade, more green leaves, shorter internode, thicker stem diameter, and opposite in antisense expression plants (lignin content lowered by 27.1%), demonstrated that the gene RcPAL was a key gene in castor lignin biosynthesis.

**Conclusions:** The gene RcPAL is a key gene in castor lignin biosynthesis and can be induced to express under mechanical damage stress. When up-regulated, it increased the lignin content significantly and dwarfed the plant height, and opposite when down-regulated. The gene RcPAL may assist in the improvement of resistance and plant type of castor bean.

**Keywords:** Ricinus communis L., RcPAL, Amplification, Transformation, Overexpression, Antisense expression, Lignin

Background

Castor (Ricinus communis L.), belonging to Ricinus family, Euphorbiaceae, is an important industrial oil crops in the world. Due to its unique chemical properties, castor oil is widely used in aviation, aerospace, machinery manufacturing, pharmaceutical, chemical and other industries with more than 700 industrial uses [1]. The demand for castor oil in the world is rising at 3–5% per annum [1], but current castor varieties suffer from low productivity and high risk of insect pests and diseases [2, 3]. Castor bean is prone to lodging due to its tall plant type and hollow stem [4]. High productive varieties with resistance to pest, disease and lodging are needed.

Lignin has been associated to pest/disease resistance [5–7] and lodging resistance [8–10]. The resistant varieties generally have the characters such as stem tenacity, wax layer, thick leaf, deep leaf color and developed sclerenchyma, most of which are related to cell wall development and the accumulation of lignin [11, 12]. Lignin is produced from several metabolites of the phenylpropanoid pathway [13, 14]. Phenylalanine ammonia-lyase (PAL) is the key enzyme of the phenylpropanoid pathway [5, 15–17]. Under stress of exogenous signal compounds, mechanical damage, bacteria, viruses, pests, etc., the expression of gene PAL can be induced at the transcriptional level and the PAL activity will be increased rapidly to activate phenylpropanoid metabolism in defense system [18–22].

Most of the reported studies has focused on phenylalanine ammonia-lyase, however, limited work was done on the expression of the gene PAL itself. Transferring the soybean gene PAL into tobacco resulted in the down
regulation of gene PAL expression, along with stunted growth, scab, abnormality in leaf shape and flower development, decreased pollen fertility [23]. Reducing expression of gene PAL by antisense oligonucleotide technique delayed the growth of Medicago sativa L. [24]. The total flavonoids content was increased obviously after transferring the gene PAL cloned from particularly high content rice into low content rice [25]. In this study, the full length cDNA of gene PAL was obtained by RT-PCR method from HY1, a special castor resistant accession, the overexpression and antisense plant expression vector were constructed and the transgenic plants were obtained by acupuncture-vacuum infiltration assisted Agrobacterium tumefaciens mediated method to analyze the expression of gene PAL and the relationship between the expression of gene PAL and accumulation of lignin in castor.

Results and discussion
Amplification of RcPAL CDs
The extracted total RNA was detected with 1% agarose gel electrophoresis and no degradation was found (Fig. 1a), with OD_{260}/OD_{280} ratios between 1.8~2.0 and OD_{260}/OD_{230} ratios over 2, indicating that the purity and integrity of extracted RNA satisfied the experimental requirements. A specific band of ~ 2150 bp was amplified from cDNA template (Fig. 1b), consistent with the size of RcPAL CDs released by NCBI, with a homology of 99, 92 and 91% with Ricinus communis (XM_002519475.1), Jatropha curcas (DQ883805.1) and Manihot esculenta (AF383152.1) respectively (Table 1). It also had a homology of 87% with the gene PAL1 (HQ331118), one of the three PAL genes in Epimedium brevicornu Maxim., which was involved in lignin synthesis [26].

Construction of expression vectors
The overexpression vector pYLRNAi-PAL+ and antisense expression vector pYLRNAi-PAL− were constructed. Firstly, the RcPAL CDs were amplified from the cDNA template, the PCR products had the same size as the expected maximum RcPAL ORF (open read frame) (Fig. 2a). Secondly, a band of 2.2 kb was amplified from the cloning vectors of pMD-PAL (Fig. 2b, Fig. 2c), proving that the coding sequence has been inserted the vector. Thirdly, the band of 2.2 kb was also obtained from the digested products of recombinant vectors pYLRNAi-PAL+ and pYLRNAi-PAL− (Fig. 2c, Fig. 2f), which were constructed by double digesting the pMD-PAL and pYLRNAi2.0 by BglIIand MluI and connecting the target fragments with pYLRNAi 2.0, demonstrating that the expression vectors has been constructed successfully. Finally, a bright band of 2.2 kb was amplified from the transformed Agrobacterium tumefaciens EHA105 (Fig. 2d, Fig. 2g), proving the successful transformation of pYLRNAi-PAL+ and pYLRNAi-PAL−.

Identification of transgenic plants
Overall, 79, 85 and 93 positive plants transformed by pYLRNAi-PAL+, pYLRNAi-PAL− and pYLRNAi0.2 respectively were screened out by both hygromycin detection (Fig. 3) and PCR identification (Hyg-F/Hyg-R) in which a target band of 520 bp was amplified (Fig. 4), with a transformation rate of 82.3, 81.0 and 85.3% respectively, which showed that the exogenous DNA has been integrated into the castor genome.

Histochemical staining of lignin in transgenic plants
The xylem cell of petiole and stalk top in overexpression plants (OPs) (35S+-18) were stained deeper than wild type plants (WTs) (35s 0–5) and antisense expression plants (APs) (35 s−9), just WTs were a little deeper than APs (Fig. 5, Fig. 6). The dyeing depth reflected the lignin content in tissue, which was consistent with the expression of gene RcPAL.

Morphological differences between OPs, APs and WTs
As showed in Fig. 7, compared with WP (35S 0–8), the OPs (35S+-49, 35S+-43, 35S+-63) was significantly changed on plant type, exhibited a lower plant height, deeper and thicker blade, more green leaves, shorter internode, thicker stem diameter and extended mature period, but opposite in APs (35S−38, 35S−4, 35S−14) except for mature period (Table 2).
Up-regulation or down-regulation of \textit{RcPAL} expression can accelerate or inhibit the production of intermediate products in phenylpropane pathway such as trans-cinnamic acid, coumaric acid, ferulic acid, erucic acid and so on [27]. These products can be transformed into coumarin and chlorogenic acid or trans-coumarin-CoA ester which will be further transformed into lignin, flavonoids and other substances through several ways to increase or decrease the content of lignin and flavonoids, and ultimately influence the phenotype of transformants [28, 29]. The insertion of overexpression and antisense genes changed the content of lignin and endogenous auxin [30], which lead to a series of phenotypic variation finally. On one hand, the increase of lignin content made OPs possessed darker and thicker leaves and greener leaves; on the other hand, the polar transport inhibition of endogenous auxin caused the leaf margin of OPs became sharper. Table 1 shows the results of nucleotide sequence blastn.

### Table 1 \textit{PAL} gene CDs nucleotide sequence blastn

| Description                                  | Max score | Total score | Query cover | E value | Ident  | Accession      |
|----------------------------------------------|-----------|-------------|-------------|---------|--------|----------------|
| Phenylalanine ammonia-lyase [\textit{Ricinus communis}] | 1482      | 1482        | 99%         | 0.0     | 100%   | AGY49231.1     |
| Phenylalanine ammonia-lyase [\textit{Ricinus communis}] | 1476      | 1476        | 99%         | 0.0     | 99%    | XP002519521.1  |
| Phenylalanine ammonia-lyase [\textit{Jatropha curcas}]     | 1368      | 1368        | 99%         | 0.0     | 92%    | XP012082374.1  |
| Phenylalanine ammonia-lyase [\textit{Manihot esculenta}]  | 1358      | 1358        | 99%         | 0.0     | 92%    | XP021660472.1  |

\textbf{Fig. 2} Amplification profiles of \textit{RcPAL} CDs, digested vectors and transformed EHA105. a Amplification of \textit{RcPAL} CDs. Lane M: Marker DL2000; lanes 1~2: overexpression CDs; lanes 3~4: antisense CDs. b Amplification of pYLRNAi-PAL\textsuperscript{+}. Lane M: Marker DL2000; lane 1: negative control pYLRNAi.02; lane 2: positive control pMD-PAL\textsuperscript{+}; lanes 3~10: recombinant vectors, among which 4, 5, 7, 9, 10 were positive. c Double digestion of pYLRNAi-PAL\textsuperscript{+}. Lane M: Marker DL2000; lane 1: double digestion products. d Amplification of pYLRNAi-PAL\textsuperscript{+}. Lane M: Marker DL2000; lane 1: positive control pYLRNAi-PAL\textsuperscript{+}; lane 2~7: transformed Agrobacterium EHA105, among which 3, 5, 6 and 7 were positive. e Amplification of pYLRNAi-PAL\textsuperscript{−}. Lane M: Marker DL2000; lane 1: negative control pYLRNAi.02; lane 2: positive control pMD-PAL; lanes 3~10: recombinant vectors, among which 4, 5, 8, 9, 10 were positive. f Double digestion of pYLRNAi-PAL\textsuperscript{−}. Lane M: Marker DL2000; lane 1: double digestion products. g Amplification of pYLRNAi-PAL\textsuperscript{−}. Lane M: Marker DL2000; lane 1: positive control pYLRNAi-PAL\textsuperscript{−}; lane 2~7: transformed Agrobacterium EHA105, all were positive.
auxin and its accumulation in growing point caused the multiple variations on OPs such as reduced plant height, shortened internode, thickened stem and extended maturation period. Because the molecular structure of flavonoids is very similar to that of auxin polar transport inhibitors, it had been considered as endogenous auxin polar transport inhibitors for a long time [30]. The significant difference between OPs probably resulted from the different copy number or insertion site. As for APs, the decrease of lignin content and flavonoid synthesis caused the opposite variations such as higher plant height, lighter and thinner leaf, less green leaves, longer internode and thinner stem, no maturation period changed.

As it can dwarf the plant height by means of shorter main stem height and internode length (Table 2), OPs are expected to facilitate the plant type breeding in castor.

**Determination of PAL activity**

Mechanical damage can result in the increase of PAL activity in plant, it is the response of plant to external stress, which was presumed that the damage signal activated the expression of the defense enzyme genes which furtherly induced the PAL synthesis [30–32]. In this study, the PAL activity in OPs and APs was significantly lower than control before stab treatment (Fig. 8). After stab treatment, it increased firstly, then decreased slightly, and finally reached a peak at 6~48 h, whether in OPs or APs or control. The OPs and APs could respond to mechanical damage stress more rapidly by increasing the PAL activity, the increase was 42.06%~79.38 and
12.34%~ 44.41% respectively at 6 h, and 78.18%~ 127.16 and 30.65%~ 71.50% at 48 h respectively, much more than in control. In addition, the increase in OPs was much greater than in APs.

The RcPAL can express both constitutively and inductively. Its overexpression or silence can reduce the PAL background activity but can raise it under mechanical damage stress, especially in the case of over expression. The increase was much greater in OPs than in APs and WT, which coincided with the expected results.

Expression of gene PAL under mechanical damage
The RcPAL expression significantly increased (up to 14.82 times) in OPs and decreased (down to 0.11 times) in APs in comparison with WT (Fig. 9). There was also significant difference between OPs, which was presumed due to the different sites or copy numbers of inserted gene, but no between APs. As expected, OPs up-regulated the expression of gene RcPAL, while opposite in APs.

Lignin content in transgenic plants
The leaf lignin content was extremely significantly increased in OPs (13.05 and 15.83%) and extremely significantly decreased (9.53 and 44.67%) in APs in comparison with WT (Fig. 10). The overexpression and silencing of gene RcPAL resulted in the increase and decrease of lignin content respectively which supported the conclusion that the gene RcPAL played a key role in lignin biosynthesis in castor.

Conclusions
The RcPAL is a key gene in castor lignin biosynthesis and can be induced to express under mechanical damage stress. When up-regulated, it can increase the lignin content significantly and dwarf the plant height, and opposite when down-regulated. The RcPAL gene may assist the plant breeding for resistance and architecture in castor bean.

Methods

Plant and bacterial material
The castor material was an inbred line HY1, developed by the laboratory of molecular breeding for energy crops in Guangdong Ocean University. The plant expression vector pYLRNAi.02 (Fig. 11) was provided by Prof. Liu Yaoguang of South China Agricultural University. Escherichia coli TOP10 and Agrobacterium EHA105 were provided by Prof. Jie Xinning of South China Agricultural University. 2 × Taq Master Mix, DNA Marker, LA Taq, Ex Taq, 10 × Loading buffer, reverse transcription kits were all bought from TaKaRa.
DNA, RNA extraction and cDNA synthesis
Genomic DNA of transgenic plants was extracted with modified CTAB method. The RNA of transgenic receptor was extracted by Trizol method with extract RNAiso Plus and adjuvant RANiso-mate for Plant Tissue (TaKaRa) according to the manual of TRIzol kit. After testing for purity and integrity, genomic DNA residue in RNA was eliminated with DNase I to guarantee RNA purity. The first strand of cDNA was synthesized in accordance with the reverse transcription kit instructions.

Primer design
Upstream and downstream primers were designed at the start and stop codon regions of RePAL according to the

| Individual | Plant height (mm) | Main stem height (mm) | Stem diameter (mm) | Node number | Internode length (mm) | Green leaf number | Leaf thickness (mm) | Mature period (d) |
|------------|------------------|----------------------|-------------------|-------------|----------------------|------------------|-------------------|-----------------|
| 35S-0     | 660 –            | 415 –                | 17.54 –           | 25 –        | 16.6 –               | 7 –               | 0.62 –            | 155 –           |
| 35S-43    | 370 –39.94       | 240 –                | –41.17            | 25 0        | 9.6 –42.17           | 9 –28.57          | 0.88 14.94        | 187 20.65       |
| 35S-49    | 540 –18.18       | 240 –                | –41.17            | 25 0        | 9.6 –42.17           | 7 0.00            | 0.72 16.13        | 187 20.65       |
| 35S-63    | 520 –21.21       | 310 –                | –25.30            | 25 0        | 12.4 –25.30           | 9 28.57           | 0.71 14.52        | 187 20.65       |
| 35S-0     | 830 25.76        | 570 37.35            | 17.82 1.60        | 25 0        | 22.8 37.35           | 6 –14.29 0.6     | –3.23 0.51        | 155 0.00        |
| 35S-14    | 860 30.3         | 510 22.89            | 16.3 –7.07        | 25 0        | 20.4 22.89           | 6 –14.29 0.56    | –9.68 155 0.00    | 155 0.00        |
| 35S-38    | 1000 51.52       | 710 71.08            | 14.7 –16.19       | 25 0        | 28.4 71.08           | 6 –14.29 0.51    | –17.74 155 0.00   | 155 0.00        |

Fig. 7 Morphological characteristics of different transformants. a Control and OPs; b Control and APs; c Control, OP and APs
ORF sequence released in GenBank (XM_002519475.1) to amplify RcPAL CDs, named RcPAL-F-1 and RcPAL-R-1. The Actin was used as reference gene and its forward and reverse primers were designed, named as RcACTINs and RcACTINa. The forward and reverse primers of target gene were also designed according to primer design principle of real-time PCR, named RcPAL-F-2 and RcPAL-R-2. A pair of specific primers were designed on hygromycin gene sequence to detect transgenic plants, named Hyg-F and Hyg-R. The forward and reverse primers for overexpression vector/antisense expression vector (pYLRNAi-PAL+/ pYLRNAi-PAL−, after the same) were designed at the start and stop codon regions of RcPAL CDs, adding the corresponding restriction site, removing the stop codon (TTA) from the reverse primer for antisense expression vector, named as RcPAL+-F/RcPAL+-R and RcPAL−-F/RcPAL−-R respectively. Primer sequences were as follows.

- **RcPAL-F-1**: 5′-ATGGCAGCAATGGCAGAAAATGGC-3′.
- **RcPAL-R-1**: 5′-TTAGCAAATTGGAAGAGGGGC-3′.
- **RcACTINs**: 5′-CCCAGCACACACAGCAGCAA-3′.
- **RcACTINa**: 5′-AGGACTTGAAGAGGAAGAGAGAAACC-3′.
- **Hyg-F**: 5′-GGCGAAGAATCTCGTGCTTTCA-3′.
- **Hyg-R**: 5′-CAGGACATTGTTGGAGCCGAAA-3′.
- **RcPAL+-F** (Adding BglII site): 5′-ATCTGAGGCATCTGGAGGAA-3′.
- **RcPAL-R-2**: 5′-CAGCATAAGGCAAAGACATACTC-3′.
- **Hyg-F**: 5′-GGCGAAGAATCTCGTGCTTTCA-3′.
- **Hyg-R**: 5′-CAGGACATTGTTGGAGCGCCAA-3′.
- **RcPAL−-F** (Adding MluI site): 5′-ATCTGAGGCATCTGGAGGAA-3′.
- **RcPAL-R-2**: 5′-CAGCATAAGGCAAAGACATACTC-3′.
- **Hyg-F**: 5′-GGCGAAGAATCTCGTGCTTTCA-3′.
- **Hyg-R**: 5′-CAGGACATTGTTGGAGCGCCAA-3′.

Fig. 8 Effect of stab on PAL activity in transformants. a OPs; b APs. Capital letters indicate the significance (P < 0.05) between different times in the same plant; Lower-case letters indicate the significance (P < 0.05) between different plants at the same time.
Target fragment amplification

Maximum ORF sequence of RcPAL was amplified with primers RcPAL-F-1 and RcPAL-R-1 using HY1 cDNA as template. The PCR reaction procedure was 95 °C 5 min for 1 cycle, 94 °C 35 s, 62 °C 35 s and 72 °C 3 min for 35 cycles; extension for 10 min at 72 °C. PCR products were checked with 1% agarose gel electrophoresis.

Construction of overexpression and antisense expression vector of RcPAL

The overexpression vector pYLRNAi-PAL+ and antisense expression vector pYLRNAi-PAL− were constructed by digesting plant expression vector pYLRNAi.02 (with bacterial screening marker Kanr, and plant screening marker Hygr) and the target CDs sequences of RcPAL with BglII and MluI, recycling vector and target fragments and ligating them with T4 ligase. The CDs sequences of RcPAL was amplified from pMD-RcPAL with the upstream and downstream primers for overexpression and antisense expression respectively. The recombinant vectors were used to transform Escherichia coli TOP10.

Transformation

Imbibing seeds were transformed with vectors pYLRNAi.02 (wild type), pYLRNAi-PAL+ and pYLRNAi-PAL− respectively by acupuncture-vacuum infiltration assisted Agrobacterium tumefaciens mediated method. Firstly, the dry plump castor seeds were sterilized with 70% ethanol for 1 min and then with 10% sodium hypochlorite for 30 min. After being rinsed thoroughly, the seed were dipped in water of 40 °C for 30 min and transferred onto filter papers previously moistened with distilled water for imbibing at 28 °C for 24 h. Secondly, the seed coat was cracked with an anatomical needle and the seed was pierced once with a disposable syringe of 1 mL (the syringe needle diameter was 0.45 mm) to a depth of ~ 1 mm at the site near the caruncle on the longitudinal midline of seed back, exactly at the the junction of the inclined plane and the plane, beneath which the epicotyl was located. Note that before piercing, the syringe needle had been dipped in the Agrobacterium inoculum. In order to inoculate Agrobacterium into the embryonic meristem effectively and avoid damaging the growing point, the acupuncture point should be behind the plumule which lay beneath the seed coat where a shoot would emerge later (Fig. 12a). Thirdly, the pierced seeds were then soaked into the Agrobacterium inoculum within a air-permeable conical flask (Fig. 12b) and the conical flask was placed into a vacuum compartment, pumped at a pressure of 50 kPa for 20 min, released for 2 min and then vacuum pumped again at same pressure for 5 min (Fig. 12c). Fourthly, the inoculated seeds were transferred without rinsing again onto filter paper moistened with Agrobacterium inoculum and further incubated in the dark at 28 °C for 3 days and until beginning of germination after ~ 9 days (Fig. 12d). Finally, the seedlings were immersed into a 250 mg/L carbenicillin solution for 1 h to remove the remnant Agrobacterium, and after being rinsed thoroughly with sterile water, they were transplanted to a seedling tray with NOVARBO substrate (Finland) and grown in greenhouse (Fig. 12e). Each vector transformed 150 seeds [33].

Identification of transgenic plants

Hygromycin identification

The third leaf from the top of the transgenic plants with 3–4 leaf was cut into rectangular pieces of 1.5 cm × 1.0 cm and soaked in 16 mg/L hygromycin solution containing
0.5 mg/L 6-BA (screening system established in laboratory). At 4 days later, the individuals with dark stripes or necrotic plaques were negative ones, while those remaining green were primarily judged as positive ones.

**PCR identification**

PCR identification was performed with the primers Hyg-F / Hyg-R using the leaf DNA of transgenic plant as template. The PCR reaction procedure was 95 °C 5 min, one cycle; 94 °C 35 s, 55 °C 35S, 72 °C 1 min, 35 cycles and 72 °C 10 min. PCR products were detected by 1% agarose gel electrophoresis.

**Phenotype investigation of transgenic plants**

When the transgenic plants were grown in plastic barrels for 80 days, the plant height, stem diameter (base, middle and upper part), stem length, leaf thickness, number of nodes per stem and number of green leaves per plant were investigated.

**Determination of PAL activity in transgenic plants**

A total of 3 positive transgenic plants with pYLRNAi-PAL+, 3 positive transgenic plants with pYLRNAi-PAL− and 1 transgenic plant with pYLRNAi.02 were selected at 5-leaf stage for PAL activity analysis. Firstly, each blade of these plants was for 6 times with insect needle 5#. At 0, 6, 12, 24 and 48 h after stabbing respectively, 200 mg of fresh leaf tissue was taken from the third leaf from the top on the main stem of each plant, repeated 3 times. The samples were quickly frozen in liquid nitrogen and stored at −80 °C. Secondly, each sample was ground into homogenate in an ice-cooled mortar with 1 ml of enzyme extraction buffer (0.05 mol/L boric acid, 5.0 mmol/L β-mercaptoethanol, 1.0 mmol/L EDTA-Na2, 5% glycerinum and 5% PVP). The homogenate was transferred into a 2 mL centrifuge tube, setting volume to 2 mL with enzyme extraction buffer, vibrating for 1 min, and centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant was collected as sample solution for enzyme assay. Thirdly, PAL activity was determined based on the rate of cinnamic acid production as described by Ochoa-Alejo [34]. Briefly, 1 mL 0.02 mol/L L-phenylalanine and 2 mL 0.1 mol/L H3BO3 buffer were added into a 4 mL centrifugal tube, besides, 0.5 mL sample solution was added into the measuring tube and 0.5 mL enzyme extraction buffer was added into the control tube. After water bathing at 30 °C for 60 min, the reaction was terminated by adding 0.2 mL 6 mol/L HCl. With the control tube adjusting zero, the absorbance A290 of the reaction liquid in measuring tube was measured at the wavelength of 290 nm, 1 U of enzyme activity equals to 0.01 of A290 value increased per min.
PAL activity \[
\text{[U/(gFW h)]} = \frac{A_{290} \times V_t \times V}{0.01 \times V_s \times F_w \times t}
\]

\(A_{290}\): absorbance; \(V_t\): Total volume of the enzyme fluid; \(V_s\): The quantity of the enzyme fluid taken for measurement; \(V\): Total volume of reaction liquid; \(t\): Reaction time; \(F_w\): Fresh weight of sample) [35].

**Histochemical staining of lignin**

3 positive transgenetic plants with pYLRNAi.02, pYLRNAi-PAL\(^+\) and pYLRNAi-PAL\(^-\) respectively were selected for histochemical staining of lignin with Wiesner staining method. Wiesner staining method was as follows: prepared freehand tissue slice (50~100 \(\mu\)m) and soaked them in 2% (v/v) phloroglucin (dissolved in absolute ethanol) for 5 min, then immersed in 12% (v/v) hydrochloric acid for 5 min, finally, fixed on the slide to be observed and photographed by microscope.

**Determination of gene expression**

After 24 h of mechanical stab treatment in transgenic plants, the total RNA of leaves with pYLRNAi.02, pYLRNAi-PAL\(^+\) and pYLRNAi-PAL\(^-\) were extracted respectively. The synthesis of cDNA was performed according to the instructions of PrimeScript\(^*\) RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara) with the castor gene Actin as internal reference. Quantitative PCR program was 95°C for 30 s (20°C/s) of 1 cycle; 95°C for 5 s (20°C/s), 60°C for 30 s (20°C/s) of 40 cycles; 95°C for 0 s (20°C/s), 60°C for 15 s (20°C/s), 95°C for 0 s (0.1°C/s) of 1 cycle. The melting curve was checked after completion and relative expression was calculated with \(2^{-\Delta\Delta\text{CT}}\) method.

**Determination of total lignin**

The determination of total lignin content was carried out in accordance with acetyl bromide method [35].

**Acknowledgements**

Not applicable.

**Funding**

This paper is supported by the National natural science foundation of China (31271759), Guangdong provincial science and technology projects (2013B060400024, 2014A020208116, 2016A020208015) (China) and Project of enhancing school with innovation of Guangdong ocean university (GDOU2013050206) (China).

**Availability of data and materials**

The raw data supporting the findings are provided in the figures and tables included in this published article.

**Authors’ contributions**

XY and JN conceived and designed the studies and wrote the manuscript. JN completed the gene amplification, primer design and transformation. YS performed the PAL activity and lignin content measurement and histochemical staining of lignin. WL performed the identification of transgenic plants. SC performed DNA, RNA extraction and cDNA synthesis. YW and XH conducted PCR amplification and phenotype investigation of transgenic plants. All authors have read and approved the manuscript, and we ensure that this is the case.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
Prunus davidiana

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