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Functional and kinetics of two efficient phenylalanine ammonia lyase from *Pyrus bretschneideri*

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Abstract

Background The enzyme phenylalanine ammonia lyase (PAL) controls the transition from primary to secondary metabolism by converting L-phenylalanine (L-Phe) to cinnamic acid. However, the function of PAL in pear plants (*Pyrus bretschneideri*) has not yet been fully elucidated.

Results We identified three PAL genes (*PbPAL1*, *PbPAL2* and *PbPAL3*) from the pear genome by exploring pear genome databases. The evolutionary tree revealed that three *PbPALs* were classified into one group. We expressed *PbPAL1* and *PbPAL2* recombinant proteins, and the purified *PbPAL1* and *PbPAL2* proteins showed strict substrate specificity for L-Phe, no activity toward L-Tyr in vitro, and modest changes in kinetics and enzyme characteristics. Furthermore, overexpression of *PbPAL1* and *PbPAL1*-RNAi, respectively, and resulted in significant changes in stone cell and lignin contents in pear fruits. The results of yeast one-hybrid (Y1H) assays that *PbWLM1* could bind to the conserved PAL box in the *PbPAL* promoter and regulate the transcription level of *PbPAL2*.

Conclusions Our findings not only showed *PbPALs* potential role in lignin biosynthesis but also laid the foundation for future studies on the regulation of lignin synthesis and stone cell development in pear fruit utilizing molecular biology approaches.

Keywords Phenylalanine ammonia lyase (PAL), Lignin, Kinetics, *Pyrus bretschneideri*

Background

PAL is the first key enzyme in the biosynthesis phenylpropanoid pathway, and has been extensively investigated. It is the rate-limiting step in phenylpropanoid metabolism, catalyzing the conversion of L-phenylalanine to cinnamic acid to connect the primary and secondary metabolism [1]. PAL does not have a single Km value, and its active center has an electrophilic center consisting of a dehydroalanyl group [2]. In general, PAL coding genes contain one to five members [3, 4]. The PAL gene family contains more than five members in several plants, including *Eucalyptus grandis* [5] and watermelon (*Citrus lanatus*) [6].

The expression and activity of the PAL enzyme determine the flux through the phenylpropane pathway and the production rate of phenylpropane compounds [7].

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Previous studies have shown that PAL is the key gene for lignin production [8]. Consequently, a better understanding of plant PAL expression and activity will aid in controlling the synthesis of phenylpropanes via molecular mechanisms, hence regulating the synthesis of lignin. For example, overexpression of *AtPAL1* and *AtPAL2* led to an increase in lignin content in *Arabidopsis*. In addition, the lignin content of *Arabidopsis* with *pal1* and *pal2* double mutants was dramatically reduced [9]. *RcPAL* is a crucial gene in *Ricinus communis* lignin production that can be induced under mechanical damage stress. When *RcPAL* is overexpressed, it significantly increases lignin content while reducing plant height. However, this the gene is silenced, it significantly reduces lignin synthesis [10].

PAL expression is regulated by the transcription factors MYB, LIM and NTS [11, 12]. In *Arabidopsis*, *PAL* activity is targeted for degradation to through the ubiquitination of Kelch F-box (KFB) protein after transcription, decreasing lignin biosynthesis [13]. The LIM transcription factor family has been widely studied in animals, and it has been gradually found to have many important functions in plants in recent years [14]. Research on the overexpression of found that four LIMs have been confirmed to regulate lignin synthesis or secondary wall lignification, they are respectively tobacco *NtLIM1*, upland cotton *GhWLM1a*, poplar *PtaGLIM1a* and *Eucalyptus camaldulensis* *EcLIM1* [15–17]. Recently, GhXLIM6 isolated from upland cotton was shown to affect cotton fiber development by regulating cellulose synthesis genes, which may be due to the functional differentiation of LIM domain sequence changes [18]. Cheng et al. [19] identified 14 nonredundant PbLIMs in the pear genome, of which 2LIMs are only distributed in the WLIM1, WLIM2 and PLIM2 subclasses, and PbWLIM1a and PbWLIM1b belong to the WLIM1 subclass. The expression levels of PbWLIM in different developmental stages of pear fruit showed that two members of PbWLIM1, PbWLIM1a and PbWLIM1b, were probably closely related to the lignin synthesis of pear fruit [19].

Pear is grown worldwide as a commercial fruit crop pear. Cai et al. [20] reported that the ‘Dangshan Su’ pear (*Pyrus bretschneideri* cv. Dangshan Su) is a commercial fruit crop species in China (Dangshan County, Anhui Province, China). The size and content of the stone cell mass are major factors for determining the quality of pear fruit [21]. Lignin is one of the primary components of pear stone cells [22], and the activities of key enzymes in the lignin biosynthesis pathway are regulated via the expression of corresponding genes [23].

Phenylpropane, the first substrate in lignin synthesis in pear fruit, is produced by the shikimate pathway, and cinnamic acid is formed by phenylpropane catalyzed by the PAL enzyme [24, 25]. However, the PALs

in pear plants remain unclear despite their crucial roles in the formation of procyanidins, flavonols, and their phenolic derivatives. In this study, three genes encoding PALs were identified in the pear genome. Previous research results showed that *PbPAL1* and *PbPAL2* are key genes involved in lignification in pear fruits [8]. Consequently, *PbPAL1* and *PbPAL2* were intensively characterized. Enzymatic activities were examined via heterologous expression in vitro, and the effects of overexpression and RNAi of *PbPAL1* on lignin content were examined in pear fruits. We hoped to clarify its role in lignin synthesis in pear stone cells by in vivo and in vitro experiments.

Results

Phylogenetic tree analysis among members of the PbPAL family

A recent study showed that *NnPAL1*, an ancient member of the PAL gene family, may be the origin of PAL diversity in angiosperm evolution [26]. To explore the evolutionary relationships of *PbPAL*, a phylogenetic tree was created based on the protein sequences of other species. These PALs are divided into three categories, including ferns/bryophytes, monocotyledons and dicotyledons (Fig. 1). The evolutionary tree can be divided into dicotyledons, monocotyledons, gymnosperms and angiosperms, which are similar to those previously reported [27]. The cladistic structure of the phylogenetic tree is usually matched with traditional taxonomic classification. In this study, the PALs of monocotyledons and dicotyledons are found in each subfamily of the PAL family of angiosperms, whereas other evolutionary branches are divided into angiosperms and gymnosperms. This analysis clearly showed that *PbPAL* is phylogenetically into the angiosperm-type PAL family and the dicotyledon subfamily.

Functional prediction of PbPAL proteins in each phylogenetic group

In our study, a phylogenetic tree was built based on the PALs whose functions (in lignin biology, flavonoid synthesis, and coumarin synthesis) have been confirmed, including the members *PbPAL* gene family in pear and 64 other species, to further analyze the potential roles of the *PbPAL* gene family in the phenylpropane pathway (Fig. 2). According to the results of the phylogenetic tree, PALs are divided into four phylogenetic groups (Groups I–IV). Three *PbPALs* exist in the group II, including *AtPAL1*, *EbPAL1*, *RcPAL* and other genes closely related to lignin synthesis. It is speculated that *PbPAL1*, 2 and 3 may have similar biological functions and participate in the synthesis of lignin in pear fruit.

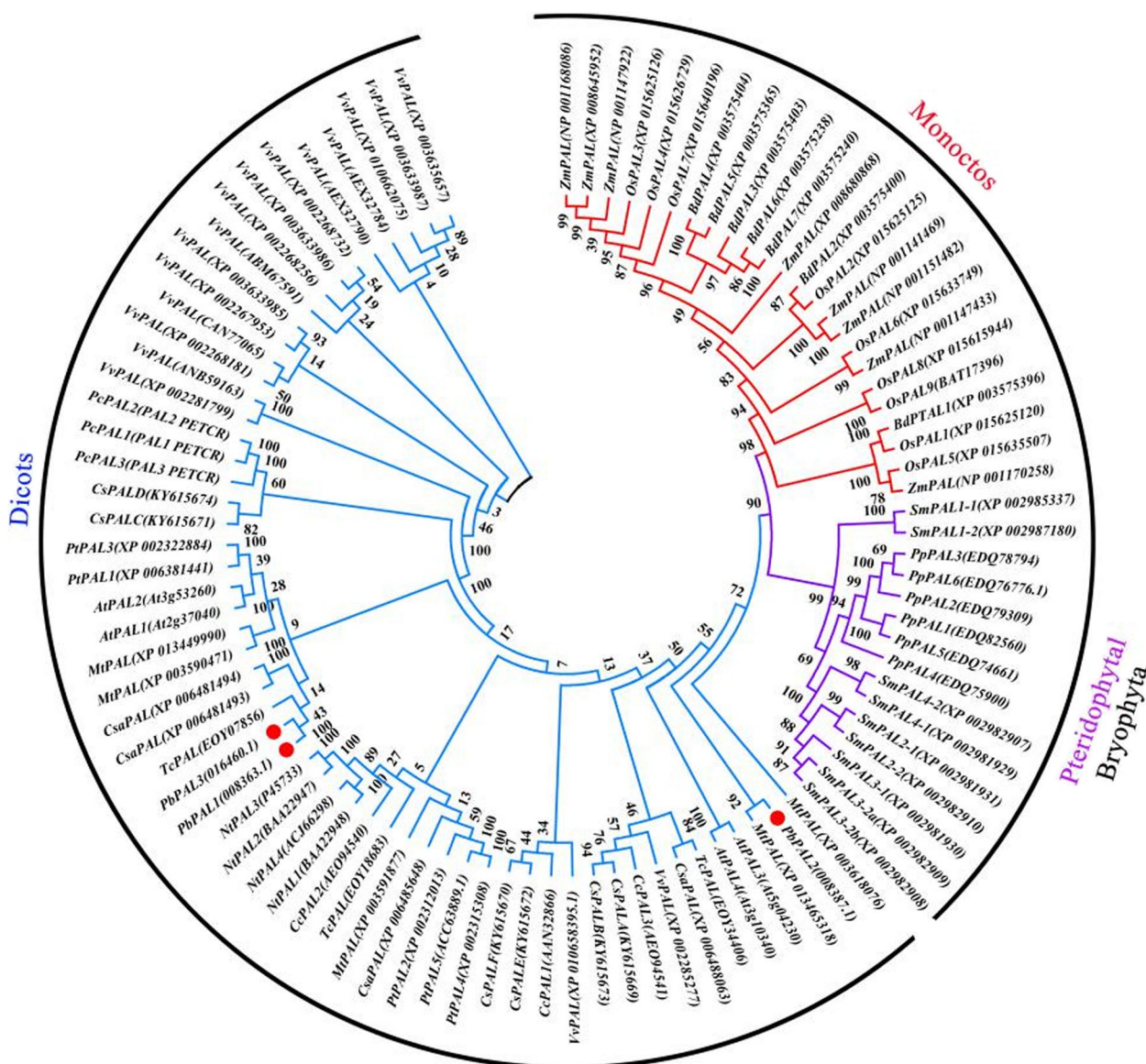


Fig. 1 Phylogenetic analysis of PALs. Note: A total of 96 PAL genes were divided into three clades from *Selaginella lamariscina*, *Vitis vinifera*, *Cucumis sativus*, *Zea mas*, *Nicotiana tabacum*, *Arabidopsis thaliana* etc.

Expression and enzyme properties of PbPAL proteins in vitro

Recombinant histidine-labeled PbPAL1 and PAL2 proteins were successfully expressed in the pET-22b vector. We determined that the fusion protein was expressed after inoculation and induction of the successfully transformed strain by 12% SDS-PAGE (Additional file 1: Fig. S1A). The molecular weights of the recombinant proteins PbPAL1 and PbPAL2 were approximately 79.0 kDa (including the label), which was close to the molecular weight of the target protein (Additional file 1: Fig. S1B).

Native *E. coli* produces a basic level of major metabolites to maintain its survival. Therefore, in the expression

strain, even if no foreign substrate is added to the crude extract, the recombinant PAL protein can also use the internal L-Phe to produce some cinnamic acid [28]. Therefore, this study first measured PAL activity in the absence of L-Phe (Fig. 3). Recombinant PAL protein can create cinnamic acid without the addition of exogenous L-Phe, although the amount produced is negligible compared to the amount produced with the addition of exogenous L-Phe.

The deamination of aromatic amino acids were catalyzed by aromatic amino acid lyase (AAAL). In this experiment, the amino acid alignment showed that PbPAL1 and PbPAL2 are AAAL, possibly HAL or PAL (Additional

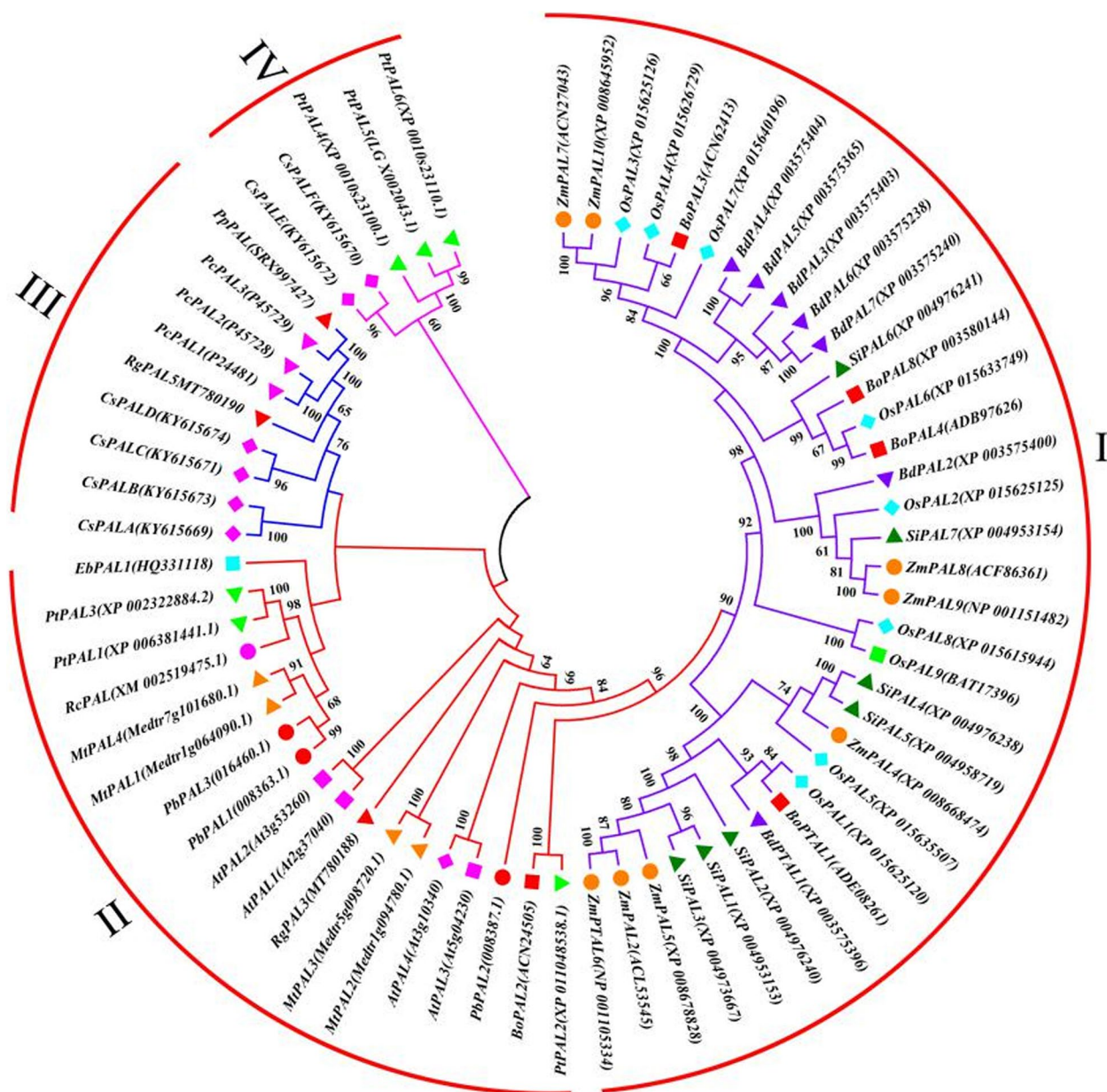


Fig. 2 Phylogenetic analysis of *PbPAL* and characterized PALs from other plant species. Note: A total of 67 PAL genes were divided into four clades from *Brachypodium distachyon*, *Vitis vinifera*, *Cucumis sativus*, *Zea mays*, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Populus euphratica*, *Ricinus communis* etc.

file 1: Fig. S2). Therefore, *PbPAL1* and *PbPAL2* were first reacted with L-Tyr, and the deamination of L-Tyr directly resulted in the generation of *p*-coumaric acid. Unfortunately, the HPLC failed to reveal the appropriate product (Fig. 4A), proving that neither *PbPAL1* nor *PbPAL2* possess the HAL function. The background interference is eliminated when L-Phe is used as the substrate, as shown in Fig. 4B, since the broken cell carrying the empty plasmid's crude enzyme solution cannot catalyze L-Phe. In addition, when L-Phe was used as the substrate,

the HPLC results showed that both *PbPAL1* and *PbPAL2* recombinant protein molecules could transform L-Phe into less polar products in approximately 29.00 min. The retention time was almost identical to the cinnamic acid standard value.

Enzyme kinetic analysis of *PbPAL1* and *PbPAL2* proteins

To understand the biochemical characterizations of PAL1 and PAL2, the enzyme assays were carried out with L-Phe as the substrate. The optimal pH (8.5 ~ 9.0) and

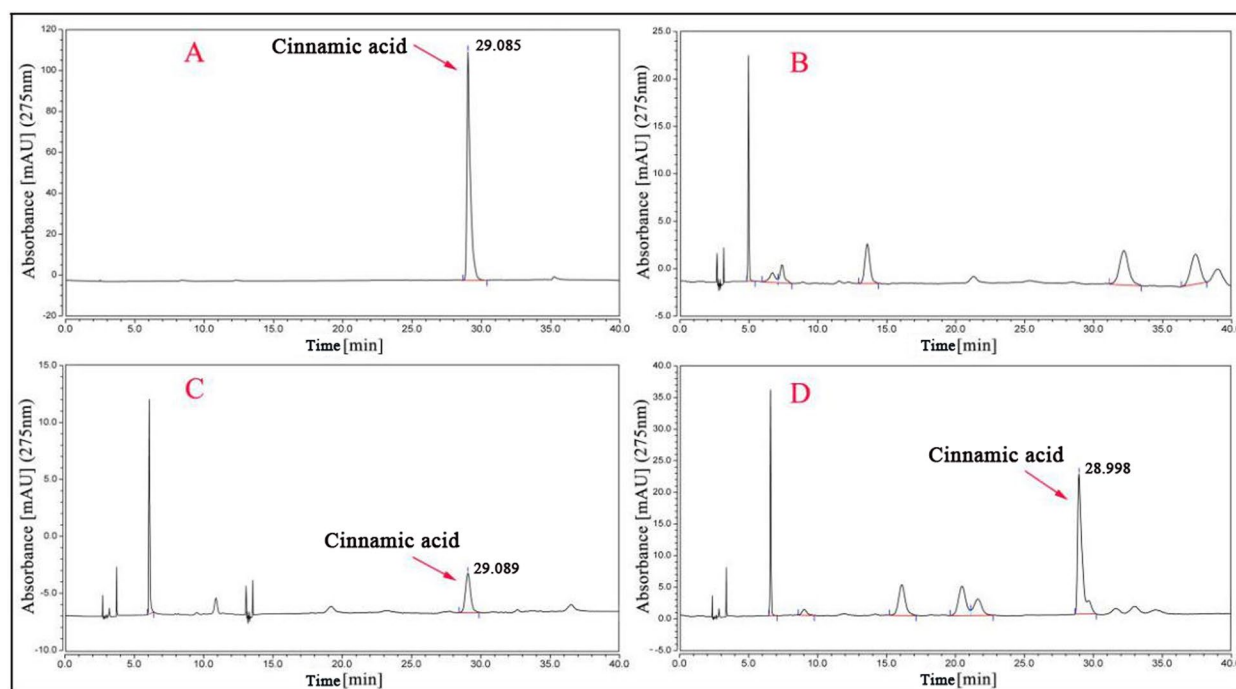


Fig. 3 HPLC chromatograms for the initial assay of PbPAL enzymatic activity. **A** Cinnamic acid standard. **B** The crude extracts of the naïve strain without supplementation with L-Phe. **C** The crude extracts of the expression strain without L-Phe. **D** The crude extracts of the expression strain with 0.6 mM L-Phe

temperature (45 °C ~ 55 °C) of the two purified PAL proteins were roughly the same (Additional file 1: Fig. S3), which was in good agreement with the results in *Arabidopsis* [29] and *Camellia sinensis* [28]. Moreover, the apparent kinetic parameters of two purified proteins, PbPAL1 and PbPAL2, were determined using L-Phe as substrate. Compared the PbPAL2 protein, the PbPAL1 protein showed a higher K_{cat}/K_m (higher catalytic efficiency) for L-Phe (Table 1). Based on the above results, this study found that the two PbPAL proteins showed strict substrate specificity for L-Phe but had no catalytic activity for L-Tyr toward PbPAL1 had higher catalytic efficiency.

Functional verification of *PbPAL1* in pear fruit

To further explore the role of *PbPAL* in the development of pear fruit stone cells transiently overexpressing *PbPAL1* (*PbPAL1*-OE) and RNAi (*PbPAL1*-RNAi), and each treatment was used to transform 40 pear fruits. Pear fruits were collected immediately after, and detect the expression level of *PbPAL1* in the fruits was detected (Additional file 1: Fig. S4). In pear *PbPAL1*-OE fruit, the transcript level of *PbPAL1* was 1.592 times higher than that of the control fruit. However, the expression level of *PbPAL1* in *PbPAL1*-RNAi fruit was only 53.82% of that in the control fruit. Therefore, transient overexpression and

RNAi of *PbPAL1* in pear fruits can effectively increase or inhibit the expression of *PbPAL1*.

As shown in Fig. 5, the content of stone cells in pear *PbPAL1*-OE fruit (4.98%) was higher than that of the control group; In *PbPAL1*-RNAi fruit, the content of stone cells was only 4.21%, which was lower than that of the control fruit (4.67%). At the same time, the lignin content of pear fruit overexpressing *PbPAL1* was 12.1% higher than that of the control fruit. The lignin content of *PbPAL1*-RNAi pear fruit was only 75.36% of that of the control fruit (the lignin content of the control fruit was 1.99%). In general, the contents of stone cells and lignin in pear fruits overexpressing *PbPAL1* were higher than those in the control group, but they did not reach statistical significance. However, the content of lignin and stone cells was reduced in the *PbPAL1*-RNAi pear fruit, and the level of lignin reached a substantial difference.

To further understand the role of *PbPAL* in responses to the development of stone cells in pear fruit, we performed the histochemical staining to observe the stone cell differences in the transverse section of the pear fruit. The safranin fixation green staining results of *PbPAL1*-OE and *PbPAL1*-RNAi pear fruits and phloroglucinol staining were similar (Fig. 6). Compared with the fruits of the control group, more stone cells in *PbPAL1*-OE pear fruits were stained red and were not

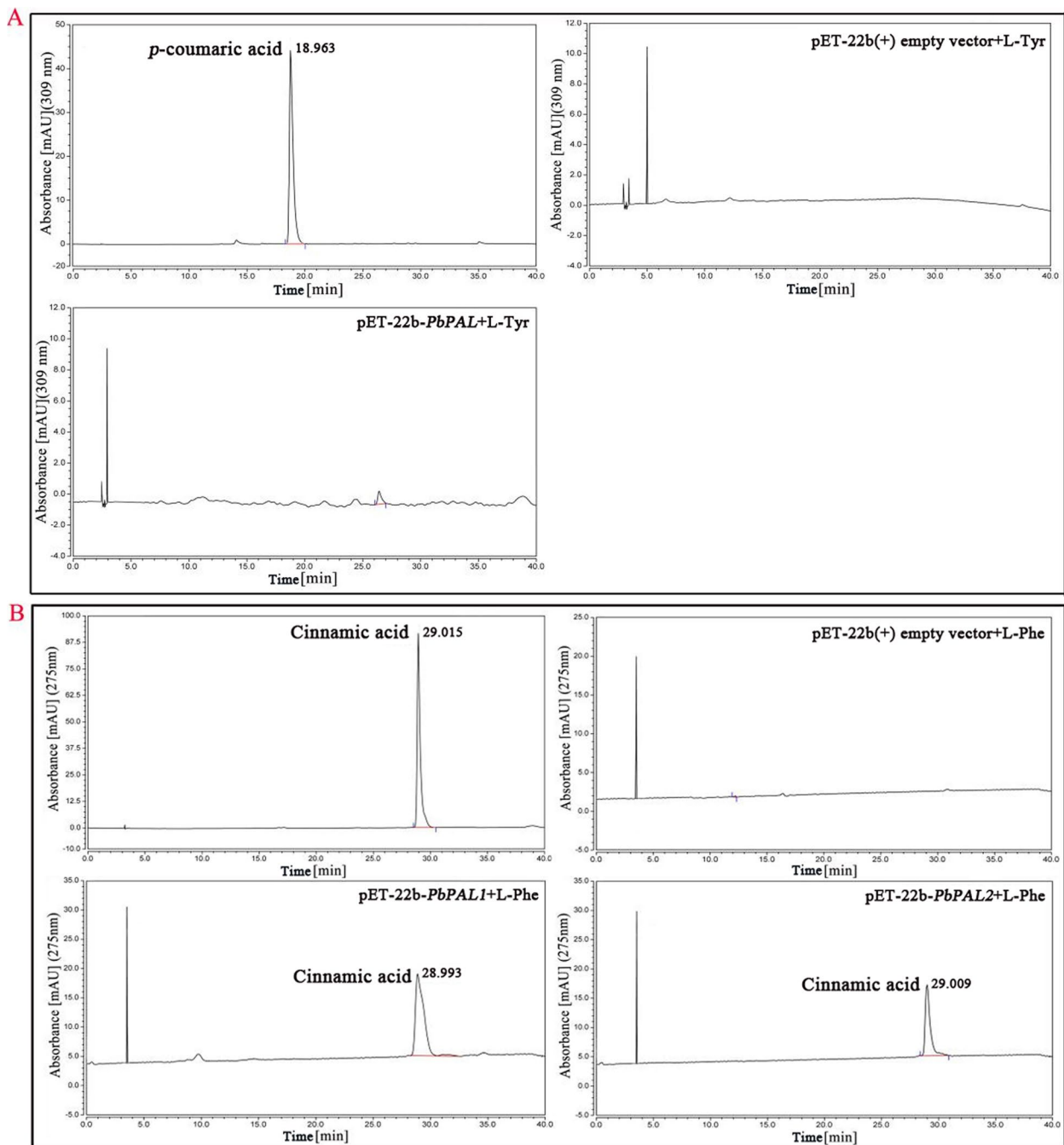


Fig. 4 HPLC analysis of enzyme reaction products from the incubation of PbPAL1 and PbPAL2 infusion protein. Note: **A** Analysis of protein products of PbPAL1 and PbPAL2 using L-Tyr as substrate, **B** Analysis of protein products of PbPAL1 and PbPAL2 using L-Phe as substrate

only accumulated near the fruit core but were also distributed in the pulp area, and the number and size of stone cell clusters in the fruit increased. The staining intensity of stone cells in *PbPAL1*-RNAi pear fruit was

low in *PbPAL1*-RNAi pear fruit, the location of stone cells around the fruit core and pulp area was greatly reduced compared to the control, and the quantity and size of stone cell clusters were also significantly reduced.

Table 1 Biochemical characterization of PAL1 and PAL2, including the pH optima, temperature optima, and kinetic parameters

Enzyme	<i>K_m</i> (M)	<i>V_{max}</i> (nKat/mg ⁻¹)	<i>K_{cat}</i> (S ⁻¹)	<i>K_{cat}/K_m</i> (S ⁻¹ /M ⁻¹)
PbPAL1	89.4	2.3	0.15	596.33
PbPAL2	116.3	3.6	0.28	415.36

Metabolite accumulation analyses in transgenic pear fruit

Cinnamic acid is an important intermediate in lignin synthesis in pear fruit and is closely related to lignin synthesis and the formation of stone cells [30]. The retention time of cinnamic acid in pear control fruit was 28.992 min. The retention time of cinnamic acid in *PbPAL1*-OE and *PbPAL1*-RNAi fruits was 29.036 min and 28.965 min, respectively (Fig. 7A). The content of cinnamic acid in *PbPAL1*-OE pear fruit increased significantly, reaching 2.791 µg/mL. Compared with the control pear fruit, the content of cinnamic acid in *PbPAL1*-RNAi fruit decreased significantly by 0.817 µg/mL (Fig. 7B). This showed that PbPAL1 can promote the production of cinnamic acid in the synthesis of lignin in pear fruit to improve the accumulation level of lignin.

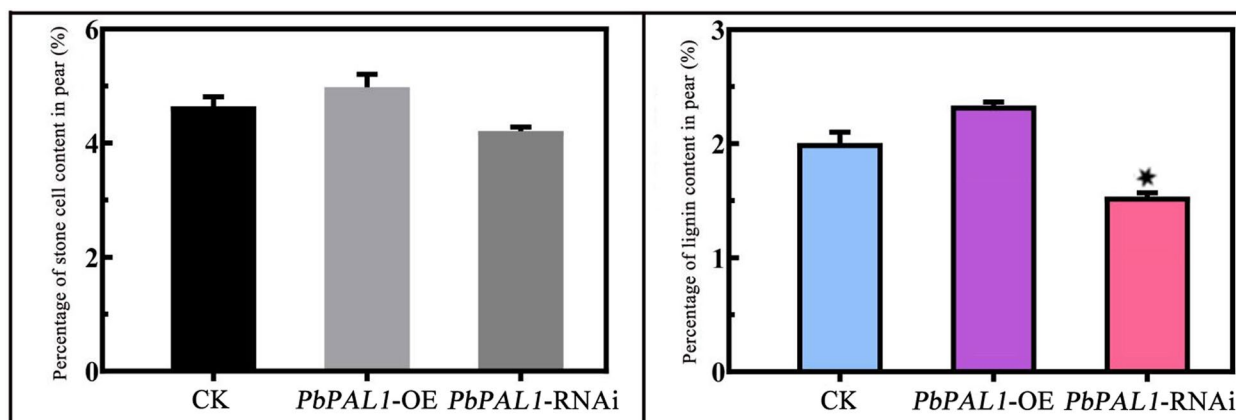
Expression profile of key genes in lignin synthesis in pear fruit

Overexpression of *PbPAL1* (*PbPAL1*-OE) in pear fruit can activate the expression of most of the key enzyme coding genes for lignin synthesis (such as *PbC4H1*, 3, *Pb4CL1*, *PbHCT17*), making its expression level 1.7~2.3 times higher than that of the control (Fig. 8). However, in *PbPAL1*-RNAi pear fruit, the expression of most genes, including *PbPOD2* and *PbLAC1*, was significantly

inhibited (the expression amount was only 48.1%~91.3% of the control group), while the expression level of *PbPAL3* was increased. The change in the *PbPAL1* expression level had little effect on the *PbC3H*, *PbCCR2* and *PbHCT49* expression levels, and there was no substantial change in the expression levels of these three genes. In addition, *PbPOD2* and *PbLAC1*, two genes related to lignin polymerization, were significantly affected by changes in the expression level of *PbPAL1*. The expression of *PbPOD2* and *PbLAC1* was inhibited when the expression of *PbPAL1* decreased and the lignin content decreased.

***PbWLM1* specifically binds PAL-box elements to regulate *PbPAL* gene expression**

At present, research on *NtLIM1* and *GhWLM1a* is relatively in depth, revealing that LIM transcription factors can selectively bind PAL-box elements in the promoter of lignin metabolism-related genes [CCAC (A/C) AN (A/C) N (C/T) (A/C)], thereby activating their transcription [16]. To explore whether *PbWLM1* can regulate the transcription level of *PbPAL* by binding with the PAL box, our study screened the potential binding sites of *PbWLM1* in the *PbPAL* family gene promoter (Additional file 2: Table S1). Our results showed that there were conserved PAL-box elements in the promoters of some members of the *PbPAL* family. To further prove that *PbPAL* is a target gene regulated by *PbWLM1*, yeast single hybridization (Y1H) was performed, and the results (Fig. 9) showed that *PbWLM1* could combine with the conserved PAL box in the *PbPAL2* promoter (Fig. 9A). However, pGBKT7-*PbWLM1a* and pGBKT7-*PbWLM1b* did not show transactivation activity in yeast (Fig. 9B). It is speculated that *PbWLM1a/1b* may play a role in the nucleus by combining with other transcription regulators to form a complex, thus realizing the function of regulating gene expression.

**Fig. 5** Determination of stone cells and lignin content in *PbPAL1* overexpression and RNAi pear fruit. Note: Error bars represent the mean \pm SD ($n = 3$), *indicates a significant difference ($P < 0.05$)

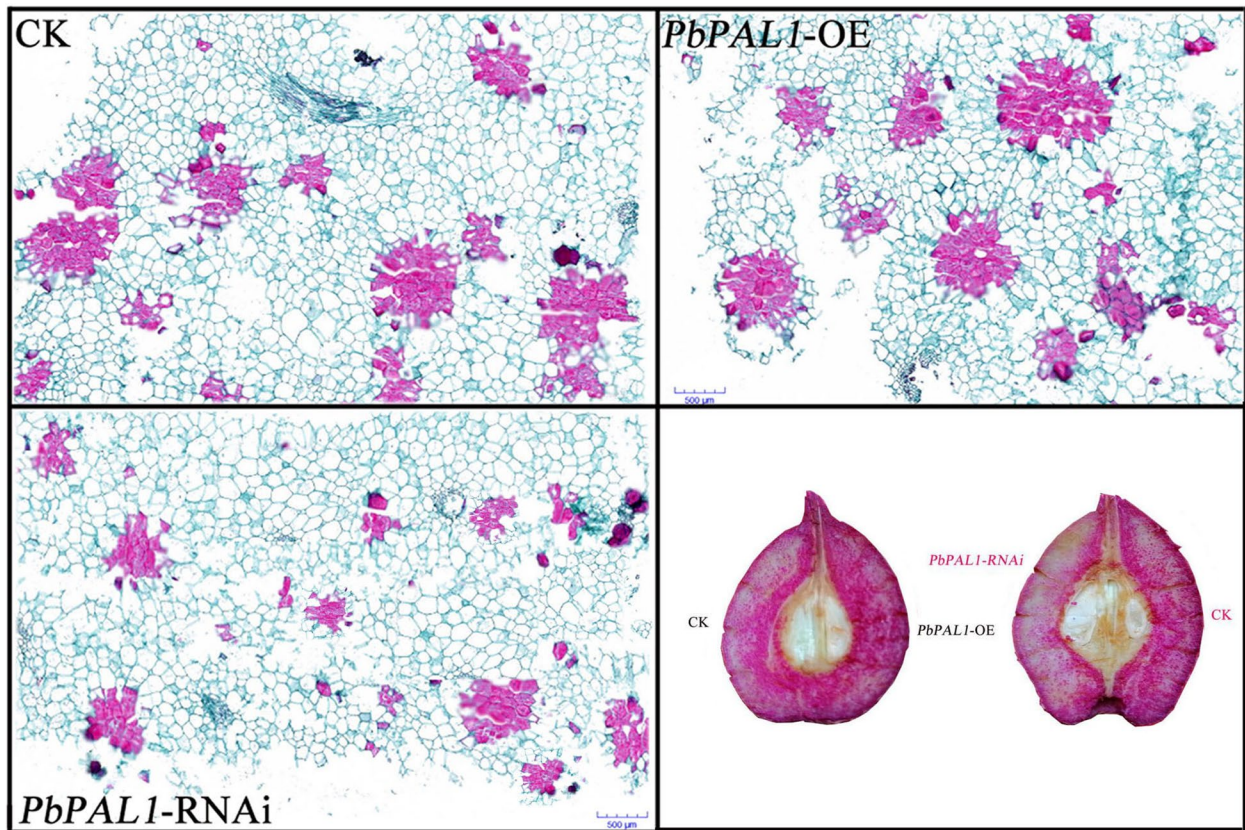


Fig. 6 Observation of saffron fixation green and phloroglucinol staining of transient transformation pear fruit

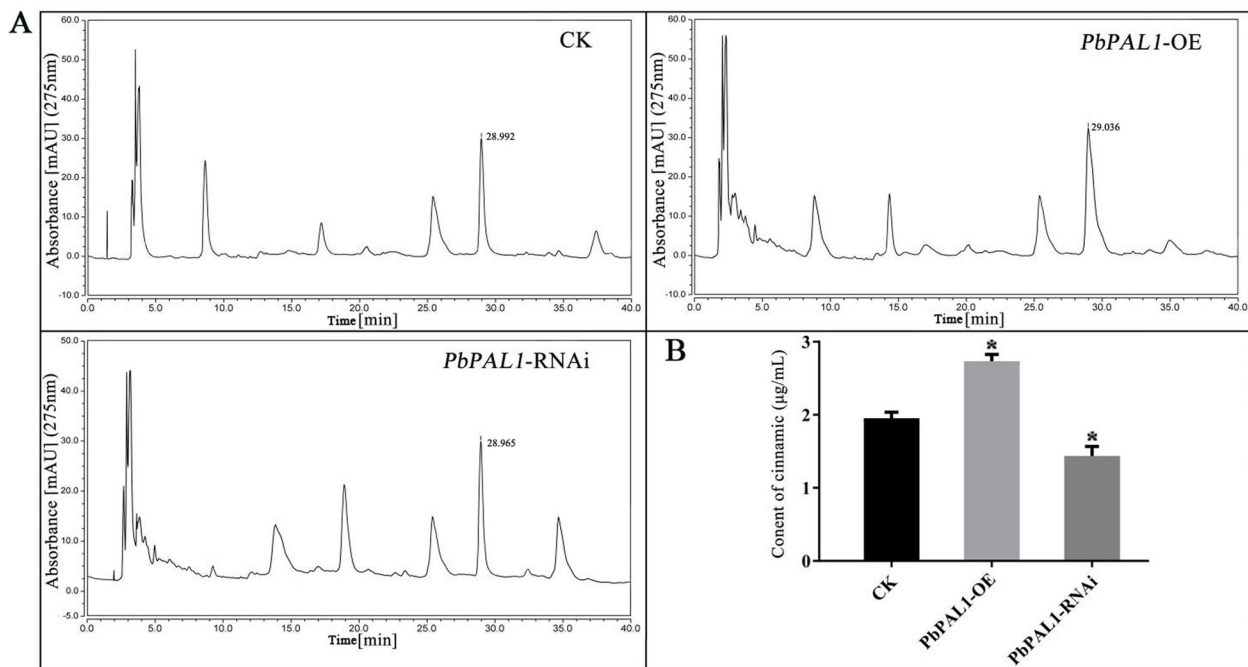


Fig. 7 Determination of cinnamic acid content in *PbPAL1* overexpression and RNAi of pear fruit. Note: **A** HPLC chromatograms for cinnamic acid content; **B** determination of cinnamic acid content, *indicates a significant difference ($P < 0.05$)

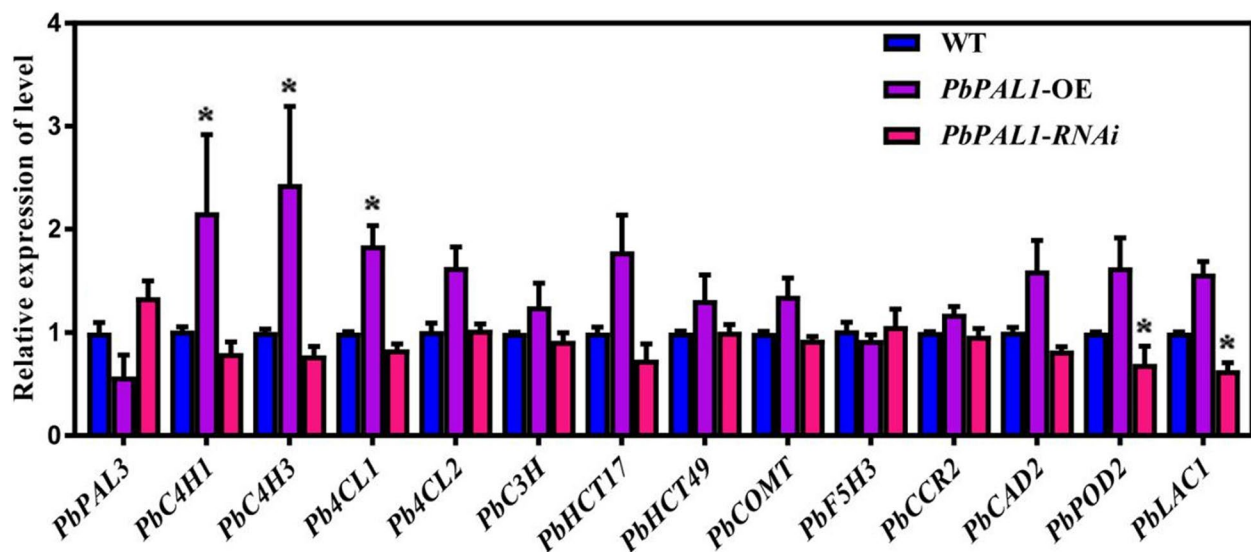


Fig. 8 Transient overexpression of *PbPAL1* and RNAi expression pattern analysis of key genes for lignin synthesis in pear fruit. Note: *indicates a significant difference ($P < 0.05$)

Discussion

Phenylpropane compounds, including lignin, anthocyanins, plant antitoxins, and condensed tannins, play important roles in plant development. All of these secondary metabolites are produced by plants through the phenylpropane route. Phenylalanine ammonia lyase (PAL) plays an important role in the general phenylpropanoid pathway. It catalyzes the conversion of L-phenylalanine to cinnamic acid, which is the rate-limiting step of the phenylpropane pathway. Therefore, PAL controls the metabolic flux in the phenylpropanoid pathway [7]. In recent years, although PAL family members have been identified in some angiosperms, research on the PAL family in fruit trees, especially in pears, has not been reported. Here, three *PALs* were identified in the pear genome, and to clarify their functions in lignin synthesis in pear fruit, bioinformatics and functional analyses were carried out systematically.

In several plants, PAL proteins are encoded via a multigene family, which has different numbers of members in different plants. For example, there are 3 members in *Nelumbo nucifera*, 4 members in *Arabidopsis*, 8 *PALs* in *Brachypodium distachyon*, and 9 *PALs* in the eucalyptus genome [26, 31, 32]. Because PAL exists in a polygene family, it has been proposed that different PAL isozymes may be responsible for producing different metabolites. During the evolutionary process of PAL, lineage-specific duplication took place in *Arabidopsis*, *Populus euphratica* and *Selaginella lamariscina*, which is a common phenomenon promoting the diversity of polygenic families. *PALs* were classified into three categories after

the monocotyledon and dicotyledon split, with no gene expansion events, such as grapes (*Vitis vinifera*) and cucumbers (*Cucumis sativus*) [33]. *PbPAL1*, *PbPAL2*, and *PbPAL3* were all found in the same subgroup (Fig. 1), indicating that *PbPALs* were created via gene duplication.

The 67 *PAL* members of pear can be divided into four phylogenetic groups (Fig. 2). In Group I, there are a large number of *PALs* of other species, including multiple *PTALs* with dual functions, such as *BdPTAL1*, *ZmPTAL* and *BoPTAL3*, which seem to have dual functions and can effectively utilize L-Phe and L-Tyr [31]. In group III, *PpPAL* participates in the biosynthesis of coumarin in *Peucedanum* [34], indicating that this subfamily may contain *PAL* members involved in coumarin biosynthesis. Group IV contains *CsPALE* and *CsPALF* which may be involved in the synthesis of flavonoids and lignin [28], indicating that *PAL* members of this family may be involved in multiple pathways of phenylpropanoid metabolism.

In plants, *PALs* are aggregated ferns/bryophytes and monocotyledons, such as *ZmPAL*, *NnPAL1* and *BdPAL1*, which seem to have dual functions and can effectively utilize L-Phe and L-Tyr [26, 31]. In tobacco, L-Phe has been proven to be a real physiological substrate, and the three isoforms of *PAL* proteins showed significant dynamic differences [6]. In this study, we used a prokaryotic expression system to verify the function of *PbPAL1* and *PbPAL2* (Fig. 4). The results showed that the two recombinant proteins expressed in vitro both possessed enzymatic activity, and they showed slight differences in enzyme kinetics. The catalytic efficiency of *PbPAL1* was

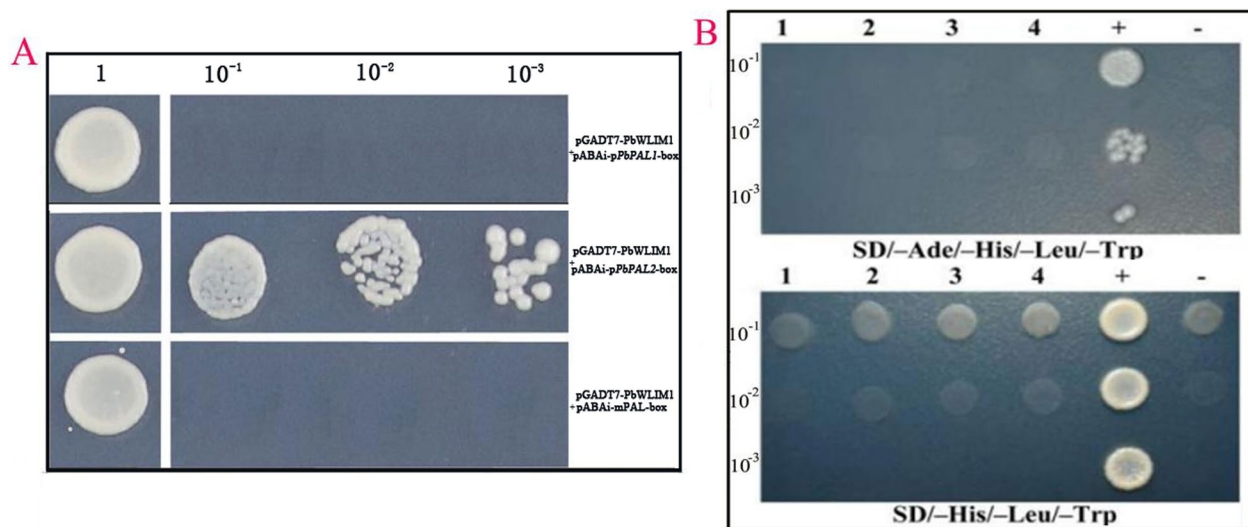


Fig. 9 Yeast one-hybrid assay to verify the binding activity of PbWLM1 and PAL-box element. Note: **A** PbWLM1 can bind to the conserved PAL box in the PbPAL promoter, 1: Stock solution, 10^{-1} , 10^{-2} and 10^{-3} ; the bacterial solution is diluted 10, 100 and 1000 times; **B** Detection of the activation activity of pGBT7-PbWLM1a/b in yeast, 1,2,3,4 represents the adjustment of 4 bacterial plaques, + and—represent positive and negative controls respectively, 1, 10^{-2} and 10^{-3} ; the bacterial solution is diluted 10, 100 and 1000 times

relatively high. These critical proteins were functional, and PbPAL1 may play a more vital role in the biology of pear solid lignin, laying the groundwork for increasing pear fruit quality at the molecular level in the future.

Some enzymes have a very clear function in the manufacture of lignin. It has occasionally been possible to alter the genetic makeup of plant lignin or decrease lignin content by genetic manipulation [35]. Previous studies found that although *pal1* and *pal2* single mutants did not have obvious phenotypes during growth and development, the phenotypes of *pal1* and *pal2* double mutants changed, including sterility, significant reduction of lignin accumulation, and changes in the ultrastructure of secondary cell walls [36]. In addition, recent studies have found that *RcPAL1* is a key gene in ricin lignin biosynthesis. When it is upregulated, it will significantly increase lignin content and plant height [10]. Unfortunately, little is known about PAL in lignin synthesis in pear fruit. To verify the actual function of pear PALs, *PbPAL1* was instantaneously overexpressed and subjected to RNAi in pear fruit to further analyze its role in pear fruit lignin synthesis. Our results showed that the instantaneous overexpression of *PbPAL1* and RNAi led to changes in stone cells and lignin content of pear fruit (Figs. 5 and 6). However, *PAL* is the most upstream gene of the phenylpropanoid pathway, and it has little inhibitory effect lignin synthesis. In the future, it is hoped that RNAi of multiple phenylpropanoid pathway genes (*PAL*, *C4H*, *4CL*, etc.) can effectively inhibit the synthesis of lignin in pear fruit and improve the quality of pear fruit.

In recent years, the integration of metabolomics studies has been used as an important means to identify crucial genes involved in the synthesis of target compounds [37, 38]. The identification of phenolic compounds involved in lignin biosynthesis has been performed mainly through liquid chromatography [35]. Cinnamic acid is essential to the production of lignin, similar to any other intermediate [20]. In this paper, based on cinnamic acid as the product of the PAL target enzyme, we analyzed the metabolites resulting from *PbPAL1* overexpression and RNAi in pear fruit via HPLC. Our results showed that increased expression of *PbPAL* could lead to increased conversion of phenylalanine to cinnamic acid (Fig. 7), which may be used for the downstream lignin synthesis pathway. This finding implies that researchers can change the content of cinnamic acid to regulate the synthesis of lignin and the development of stone cells in pear fruit by regulating the expression level of *PbPAL* in pear fruit, ultimately improving the quality of pear fruit.

Although poplar *PtaGLIM1a* and *Eucalyptus camaldulensis* *EcLIM1* have been indicated to affect plant lignin synthesis, their specific regulatory mechanisms have not been reported [15, 17]. In this study, *PbWLM1* can combine with the conserved PAL-box element in the *PbPAL* promoter to regulate the expression of the *PbPAL* gene (Fig. 9A). Interestingly, these two genes have no transactivation activity in yeast (Fig. 9B). Coincidentally, millet *SiWLM2b* cannot activate the reporter gene in yeast, but overexpression of this gene can significantly enhance the transcription level of phenylpropanoid

metabolism-related genes in plants [39]. Recent studies have also found that the transcription factor of sand pear PpHY5 (ELONGATEDHYPOCOTYL 5) has no transactivation activity in yeast, but dual-LUC can activate the expression of the target gene PpMYB10. When PpHY5 is cotransformed with its interacting protein PpBBX18 (B-box protein), the activity of the PpMYB10 promoter can be greatly improved [40]. It can be inferred that since the dual-LUC experiment is carried out in plants, LIM can ‘recruit’ certain interacting proteins to form a complex in plants to regulate gene expression or have a more efficient regulatory effect. Because there is no homologous gene to that encoding this interacting protein in yeast, *PbWLIM1* does not show transactivation activity. *PbWLIM1a/1b* acts as a ‘bridge’ connecting promoters and other proteins in the regulatory complex, or both DNA binding function and trans activation activity. This series of problems needs to be further studied.

Conclusions

In summary, a total of three full-length *PAL* genes were identified in pear, designated PbPAL1-PAL3, and two *PAL* genes have been cloned. The resulting proteins both showed strict substrate specificity toward L-Phe and subtle differences in kinetics and enzymatic properties. The analysis of enzyme kinetics showed that *PbPAL1* may be the key gene for lignin synthesis in pear fruit. Furthermore, the instantaneous overexpression and RNAi of *PbPAL1* in pear fruit provided evidence for the involvement of this gene in lignin metabolism. The results of Y1H showed that *PbWLIM1* may regulate the transcription level of *PbPAL*. Our research provides a new strategy for reducing the lignin content and stone cell size in pear fruit.

Materials and methods

Plant materials

Fruit samples (39 days after flowering (DAF)) were collected from 40-year-old *Pyrus bretschneideri* cv. Dangshan Su, which was grown in an orchard in Dangshan, Anhui, China. All samples were stored at -80 °C for subsequent use.

Identification of PAL family members

In this study, the genome data of Chinese white pear were obtained from the pear genome (<http://gigadb.org/dataset/100083>) [41]. The obtained amino acid sequence of the whole genome of pear used the ‘BioEdit’ software to establish a local database and used the *AtPAL1* (At2G37040) and *AtPAL2* (At3G53260) sequences as query sequences. Then, all candidate *PbPALs* were verified using Pfam and SMART3 to confirm that they contained the core domains [42, 43]. Finally, all potentially

redundant *PAL* sequences were discarded according to the sequence alignments.

Construction of PAL phylogenetic tree

Sequence analysis of *PbPAL* gene family members of pear by was performed with the ClusterW tool in MEGA 11 with bootstrap analysis (1000 replicates) and compared with *PAL* sequences of other species collected through the NCBI database. After the sequence alignment was completed, the neighbor-joining (N-J) method was used to construct the phylogenetic tree to obtain the evolutionary analysis of the *PAL* gene family of pear.

Construction of the plant expression vector

The *PbPAL1* and *PbPAL2* genes were PCR amplified from pear fruit. The primers are listed in Table S2 and contain restriction sites (*Bgl* II and *Spe* I). Subsequently, the amplicon was inserted into the vector pCAMBIA1304 (GenBank: AF234300.1) by T4 DNA ligase. Finally, the resulting vectors were integrated into *Agrobacterium tumefaciens* strain EHA105.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the collected samples using TRIzol reagent (Invitrogen). Then, we used a one-step RT-PCR kit (Takara, China) to reverse transcribe RNA into first-strand cDNA according to the manufacturer’s instructions. The *tubulin* gene was used as an internal control [41], and the gene-specific primers (Additional file 2: Table S3) of each *PbPAL* gene were designed using Beacon Designer 7.9 software. qRT-PCR was performed with a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method [44].

Instantaneous expression of PbPAL1 in pear fruit

Fresh pear fruits at 39 DAF were selected for transient expression. A syringe was used to take up the *Agrobacterium* culture liquid and slowly and evenly inject it into the pear fruit, fully inject 50% (50 μ L) of culture liquid. A total of 35 ~ 45 pear fruits were injected with each kind of *Agrobacterium* infection solution. After 7 DAF of living culture on fruit trees, the fruits were removed and stored.

Determination of stone cell and lignin content in instantaneously transformed pear fruit

The stone cell content of pear fruit was determined according to the method described by Cheng [45]: the flesh (5.0 g) was frozen at -20 °C for 24 h and then centrifuged at 20 000 rpm/min for 3 min. The homogeneous pulp was incubated in water, and then the upper suspension was discarded. This process was repeated 4–5 times, and the collected stone cells were dried and weighed in

the oven. The content of stone cells was calculated as follows: (weight of stone cells/weight of pulp \times 100% = stone cell content (%)).

According to the method reported by Yan [46], the lignin content of pear fruit was determined with some adjustments. The instantaneously transformed pear fruit grinding powder (0.02 g) was collected, filtered using a 20 mesh sieve and placed into a 10 mL frosted glass tube. Subsequently, 2 mL of 25% bromoacetyl and glacial acetic acid was added, and the tube was sealed with a glass stopper. The mixture was allowed to react in a 70 °C water bath for approximately 30 min, and then 2 mL of 2 M NaOH was added to terminate the reaction. The mixture was transferred to a volumetric flask and diluted to 100 mL with glacial acetic acid. Finally, the absorbance (ABS) of the solution was measured at 280 nm.

Observation of pear fruit tissue section staining

The pear fruit was peeled immediately after transformation. A fruit tissue sample from near the fruit core was washed and placed in FAA fixative solution. The sample was placed under vacuum and allowed to stand for 12 h. After fixation, the samples were embedded in wax blocks, placed on an automatic microtome for sectioning, and then stained with safranin and green for observation. Then, 1.0 mol/L hydrochloric acid in 1.0% phloroglucinol solution was added. The results were observed and photographed.

Determination of cinnamic acid content in pear fruit

Samples were obtained by quartering. An electronic balance was used to weigh 100 mg of pear flesh, which was then frozen with liquid nitrogen. Next, 80% ethanol solution was added at a material:liquid ratio of 1:15. It was ground with a ball mill, ultrasonicated at 45 °C for 50 min, and centrifuged at 12 000 r/min for 10 min. The supernatant was removed, and 1 mL of 100% chromatographic grade methanol was added for redissolution. The solution was passed through 0.22 μ M organic phase membrane filtration, with three biological replicates for each group of samples. Metabolites were analyzed according to the Proestos and Komaitis methods [47].

Heterologous expression and purification of recombinant PbPALs

The full-length PbPAL1 and PbPAL2 cDNAs were cloned into plasmid pET-22b encoding water-soluble maltose-binding protein (MBP) upstream of the inserted site, followed by the heat shock method, and transfected into *Escherichia coli* BL21(DE3) cells. The purity of the His-tag-fused PbPALs was assessed by

analyzing the total protein on 12% SDS-PAGE gels. The purified proteins were used for further enzymatic assays.

Enzymatic assays of PbPALs

The activity of PbPAL1 and 2 was analyzed according to the method of Wu et al. [28] with minor modifications. The purified recombinant PAL proteins (6 μ g) were incubated with 100 μ L of reaction buffer consisting of 0.6 mM L-Phe (100%, Aladdin) or L-Tyr (100%, Aladdin) and 100 mM Tris HCl buffer (pH 8.0) (or boiling for 5 min as a control). After incubation at 50 °C for 30 min, the reaction was terminated with an equal volume of 100% methanol.

To determine the optimal reaction pH, assays were performed at 50 °C, and 2 μ g each of NaAc-HAc (100 mM, pH 5.0~6.0), Tris HCl (100 mM, pH 7.0~8.0), and Na₂CO₃-NaHCO₃ (100 mM, pH 9.0~11.0) was added. The purified recombinant PbPAL protein was reacted for 15 min. Then, to determine the most appropriate temperature, the mixture was incubated at pH 8.0 for 15 min with the temperature varying from 20 °C to 80 °C. To measure the K_m and V_{max} of the recombinant protein, the concentration of L-Phe in Tris HCl (pH 7.5) was changed from 2 μ M to 250 μ M, and the mixture was incubated at 30 °C for 15 min.

Products were subjected to the HPLC elution (150 \times 2.1 mm, 1.7 μ m) procedure as follows: starting from 10% eluent B (100% methanol), the linear gradient was 15%–30% B for 0 min~5 min, 30%-45% B for 5 min~35 min, 45%-10% B for 35 min~39 min, and then stopped at 40 min.

Yeast one-hybrid assay

For the Y1H assay, by cloning the target (*PbPAL1* and *PbPAL2*) gene into the vector pAbAi, the pBait AbAi bait carrier was obtained, pBait AbAi was loaded into yeast, and the self-activation of the bait carrier was detected on the AbA plate. The total RNA of pear fruit was extracted by TIANGEN's RNAPrep Pure plant Total RNA Extraction Kit, the poly A + mRNA was enriched by the NucleoTrap mRNA Kit, and the first strand was synthesized by oligo dT (CDS III). Long-distance PCR (LD-PCR) was used to amplify and synthesize double-stranded (ds) cDNA, and ds cDNA was purified by a CHROMA SPIN+ TE-400 column. The ORF of *PbWLIM1* was fused in-frame with the GAL4 activation domain encoded by the sequence in the pGADT7 vector to generate pGADT7-PbWLIM1. The purified ds cDNA and pGADT7 vector were cotransferred into yeast with the pBait AbAi bait vector and screened with SD medium supplemented with AbA.

Statistical analysis

Samples were analyzed in biological triplicate, and the data are presented as mean standard deviation. Statistical significance was determined using one-way ANOVA and Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.01$). Bars with different letters represent significantly different means ($P < 0.05$).

Abbreviations

ABS	Absorbance
PCR	Polymerase chain reaction
qRT-PCR	Real Time PCR
RT-PCR	Reverse transcription PCR
RNAi	RNA interference

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-023-04586-0>.

Additional file 1: Fig. S1. A: SDS-PAGE of protein induction (M: Mark, 1: a uninduced sample, 2-3 are samples induced by PbPAL1 proteins, 4: a uninduced sample, 5-6: are samples induced by PbPAL1 PbPAL2 proteins); B: is the purification of PbPAL1 and PbPAL2 proteins (M: Mark, 1: a uninduced sample, 2-3 are samples induced by PbPAL1 proteins, 4: a uninduced sample, 5-6: are samples induced by PbPAL1 PbPAL2 proteins).

Fig. S2. Sequence alignment of PAL amino acids in different species. **Fig. S3.** Biochemical characterization of PALa-PALf, including the pH optima and temperature optima. **Fig. S4.** Analysis of PbPAL1 expression level in overexpression and RNAi pear fruit.

Additional file 2: Table S1. Potential PbWLIM1 binding sites in the PbPAL promoter. **Table S2.** Primer sequences contained artificial restriction enzyme sites for *Bgl* II and *Spe* I. **Table S3.** Primer sequences used for qRT-PCR.

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Authors' contributions

Conceptualization: GHL, CS, MAM and DYL; Software: GHL, CS, MAM and YPC (1); Writing-review & editing: GHL, MAM, CS and YPC (2); Funding acquisition: GHL.

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Availability of data and materials

The *PbPAL1* and *PbPAL2* genes sequence used in this study were available in GenBank with accession numbers of MF346686 and MF346687.

Declarations

Ethics approval and consent to participate

The experiments did not involve endangered or protected species. No specific permits were required for these locations/activities because the fresh pear fruits used in this study were obtained an orchard in Dangshan, Anhui, China. All methods were carried out in accordance with relevant guidelines and regulations, under ethical approval and consent to participate.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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