

Transcriptome analysis provides insights into the non-methylated lignin synthesis in *Paphiopedilum armeniacum* seed

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Abstract

Backgrounds: *Paphiopedilum* is an important genus of orchid family (Orchidaceae) with high horticultural value. The wild populations are under the threat of extinction because of over collection and habitat destruction. Mature seeds of most *Paphiopedilum* species are difficult to germinate, which severely restricts the germplasm resources protection and commercial production. The germination inhibition factors are largely unknown.

Results: In this study, we found large amounts of non-methylated lignin were accumulated during seed maturation of *Paphiopedilum armeniacum* (*P. armeniacum*), which negatively correlates with the germination rate. We then further compared the transcriptome profiles of *P. armeniacum* seed at different development stages to explore molecular clues for the non-methylated lignin synthesis. KEGG enrichment analysis showed that a large number of genes associated with phenylpropanoid biosynthesis and phenylalanine metabolism as the seed maturation were differentially expressed. Several key genes in the lignin biosynthetic pathways displayed different expression patterns during the lignification process. *PAL*, *4CL*, *HCT* and *CSE* were up-regulated to accelerate the C and H lignin accumulation. The expression of *CCoAOMT*, *F5H* and *COMT* were maintained at a low level or down-regulated to inhibit the conversion to the typical G and S lignin. Quantitative real-time RT-PCR analysis confirmed the altered expression levels of these genes among seeds and vegetative tissues.

Conclusions: This work demonstrated the plasticity of natural lignin polymer assembly in seed, and provided a better understanding of the molecular mechanism of seed-specific lignification process.

Background

Paphiopedilum Pftzer (Orchidaceae) are commonly known as Lady's slipper orchids because of their slipper-shaped pouch. Members of this genus have high ornamental value because the unique flowers are available in a wide variety of colors, sizes and shapes. Besides the horticultural value, wild populations of *Paphiopedilum* are under the threat of extinction due to over-collection and habitat destruction, and all species are listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I and their trade is prohibited¹.

Many terrestrial orchid seeds including *Paphiopedilum* are tiny and contain no endosperm, in contrast to well-developed embryos in most angiosperm seeds. In the natural habitat, *Paphiopedilum* seeds are generally unable to germinate on their own. They need to engage in a mycorrhizal relationship with fungus that help to feed the emerging seedling^{2,3}. Even offering enough nutrient supply in asymbiotic germination, mature seeds of most *Paphiopedilum* species are still difficult to germinate, which severely restricts their conservation and large-scale production. However, the factors inhibiting germination are still unknown. *Paphiopedilum* seeds developed heavily lignified secondary cell walls to reinforce mechanic support and to make it impermeable to water and nutrients^{4,5}. In the natural habitat, this feature will

enhance the survival of orchid seeds in harsh conditions. However, several studies have implied that the accumulation of lignin contributed to the germination inhibition during aseptic germination^{6,7}. Immature orchid seeds with a low degree of lignification, exhibit higher germination percentages than mature seed^{2,8}. Enhance germination of mature seeds was documented after seed lignin degradation^{2,6,9}. Based on these findings, some researchers have proposed that the high amounts of lignin accumulation contributed to the germination inhibition.

Lignin is a phenolic polymer formed by oxidative polymerization of the primary hydroxycinnamyl alcohols (monolignols), p-coumaryl, caffeyl, coniferyl, sinapyl and 5-hydroxycinnamyl alcohols, to give rise to p-hydroxyphenyl (H), catechyl (C), guaiacyl (G), syringyl (S) and 5-hydroxyguaiacyl (5H/5-OH-G) lignin. The five monolignols differ by their degree and position of methoxylation. The H and C units are non-methoxylated, whereas the G and 5H units are singly methylated on the 3-hydroxyl group and the S subunit is methylated on both the 3- and 5-hydroxyl moieties¹⁰. Lignin composition varies among plant species and tissue types. Lignins found in the vascular tissues are mainly composed of G and S units. While seed lignins have a significant variation in monolignol compositions. In addition to the typical H, G and S units, C unit is found in the seed of certain species belonging to *Orchidaceae*, *Cactaceae*, *Cleomaceae*, and *Euphorbiaceae*¹¹⁻¹³. The significance of this variation remains unknown. The non-methylated monolignols are incorporated into lignin polymers via benzodioxane bonds forming a linear structure without side chains^{14,15}. The linear lignin has less cross-linking with other cell wall components, and is capable of enhancing the hydrophobicity and stability of the plant tissue¹⁶. Lignin biosynthesis pathway is well established, involving functionalization of intermediated by O-methyltransferase (CCoAOMT and COMT) to generate monolignols differing in their degree of methylation¹⁰. Disruption or downregulation of those genes can lead to the lignin composition changed, and therefore change the cell wall integrity and mechanical properties of the tissue^{17,18}.

Here, we describe the presence of non-methoxylated H and C lignin in the seed of *Paphiopedilum armeniacum* (*P. armeniacum*). The deposition of the non-methylated lignin started at the early stage of the seed development of *P. armeniacum*, and eventually made up approximately 39% of the total seed dry mass. Despite the unique structure and potentially important function, the biosynthesis of lignins in plant seeds has received little attention, relative to that in vascular tissues. In this study, a detailed gene expression profiling was performed at five key development stages of *P. armeniacum* seeds to help us understand the seed-specific regulation of lignin biosynthesis.

Results

Lignin accumulation and embryo developmental features during seed development

Several studies suggested the heavily lignified seed coat contributed to the germination inhibition in several species of orchids^{3,6,7}. The germination was scored along with the lignin content during the seed development (Figure 1A). At the early stage of the seed development (45-95 DAP), the germination rate gradually increased as the embryo formation. At 95 DAP when the globular embryo has been fully developed, the germination rate reached 96.2%. During this stage, lignin was maintained at a relatively low level (5-9% of the dry mass). After that, even though the starch and lipid globules continue to accumulate as described in our previous study³, the germination rate dropped sharply from 96% to 5%. Meanwhile, the amounts of lignin started to increase rapidly, as the seed coat turning from white to brown (Figure 1B). Lignin accumulation reached a plateau (~39% of the dry mass) at 150 DAP, by which time the seed coats became dark and hard.

Zygotic embryos of *P. armeniacum* began to develop at 45 DAP as we previously discovered³. At 66 DAP, the T-shaped pre-embryo with four cells was formed (Table1). At 87 DAP, the globular embryo was formed along with the rapid lignification of seed coat. Around 108 DAP, the inner testa disappears, starch and lipid globule accumulated. At 122 DAP, the embryo is fully mature with a compact testa and no further morphological changes were observed. Five developmental stages (66, 87, 108, 122 and 150 DAP) of *P. armeniacum* embryos were selected for further lignin structure characterization and transcriptome profiles on the basis of lignin accumulation pattern and embryo developmental characteristics.

Identification of non-methylated lignin in seeds

To analyze the monolignol composition from various tissue types, the samples were subjected to pyrolysis-gas chromatography (Py-GC/MS). The identities and relative molar abundances of the released compounds are listed in Table 1. The results indicated the lignin in the seeds contain high levels of C units and H units without any trace of G and S units. In contrast, lignins present in other tissues (stems, leaves and pods residues after seed isolation) were composed mainly of G and S units with essentially no C units.

Previous work has established the assignments for C lignin and G/S lignin in FTIR and NMR spectra in orchid seeds¹¹⁻¹³. Next, the composition of monolignol structure in seeds was further characterized by those two techniques. FTIR spectra from the seeds at five developmental stages indicated C lignin deposited in the early stage with distinct C lignin bands at 1154 cm⁻¹ and 823 cm⁻¹ (Figure 2A). The rapid development of those two bands was observed at 108 DAP. In agreement with Py-GC analysis, no typical G/S lignin was detected in *P. armeniacum* seeds of all five developmental stages. Analysis of the aromatic region of the 2D NMR spectra of mature seeds confirmed the presence of H and C lignin (Figure 2B). The results indicated that the monolignol composition varied among tissue types in *P. armeniacum*, and the non-methylated C lignin and H lignin specifically deposited in the seed.

RNAseq analysis, *de novo* assembly and functional annotation

To characterize the transcriptome dynamics of *P. armeniacum* seed development and to identify genes involved in the seed-specific lignification, we performed an RNA-seq analysis of *P. armeniacum* seeds at five developmental stages (66, 87, 108, 122 and 150 DAP). Three biological replicate sequencing libraries were prepared from each stage. A total of 104.12 Gb clean data were originated from each library after filtering out low-quality data. The transcriptome details for each sample are given in Table S1. Q30 of the raw data ranged from 92.10 to 94.11% indicating a high-quality reads worthy of further analysis. Since no reference genome is available for *Paphiopedilum*, we *de novo* assembled the total 347,075,856 reads into 433,854 transcripts with an N50 length of 1180 bp and 183,737 unigenes with an average length of 860 bp, respectively. The transcripts and unigenes length distribution are shown in Table S2. The biological reproducibility was assessed using the Pearson's correlation coefficient, and the correlation between samples among the same biological replicates was high (Figure S1A). Principle component analysis (PCA) of all samples during seed development was also performed (Figure S2B). Consistent with their distinct developmental stages, samples from different biological replicates were clustered separately

For annotation, 183,737 unigenes were subjected to BLASTX searches against the sequences in the NCBI non-redundant protein sequences (NR), Swissprot, Gene Ontology (GO), the Clusters of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. As a result, a total of 92,235 unigenes (50.20% of all unigenes) could be assigned at least one putative function from one of these databases (Table S3). A total of 89,285 unigenes were annotated to the NR database. In a further analysis of the matched sequences, *P. armeniacum* transcript was highly similar to those *Dendrobium catenatum* (29.86%), *Apostasia shenzhenica* (8.38%) and *Phalaenopsis equestris* (5.45%), as shown in Figure 3A. For GO annotation, a total of 29,301 unigenes from *P. armeniacum* seeds were annotated into three GO pathways, as shown in Figure 3B. The functions of unigenes in biological process classifications contained cellular process, metabolic process and biological regulation. Cell part, cell and organelle were the most abundant functions in terms of cellular component classifications. The most abundant biological process functions are metabolic process and cellular process. In the molecular function classification, binding and catalytic activity were more abundant. The main GO entries were consistent with the facts that the cells divided frequently during seed development, and some catalytic, metabolic and binding activities were relatively high.

Differential gene expression and KEGG enrichment analysis

With the restrictive conditions of False Discovery Rate (FDR) < 0.05 and log₂ ratio ≥ 1.0, unigenes that were differentially expressed in the seeds at five stages were identified. In total 8,722 differentially

expressed genes (DEGs) were identified among all the libraries. The number of DEGs increased with seed development, and peaked among 122 DAP and 66 DAP seeds (Figure 3A). A total of 576 common DEGs were identified in 87 DAP, 108 DAP, 122 DAP and 150 DAP among all developmental stages, implying that these DEGs might be associated with seed development (Figure 3B).

To explore more insight into the DEGs regulating the lignin deposition at the later stage of seed development, we further performed KEGG enrichment analysis with the DEGs in 87 DAP vs 66 DAP, 108 DAP vs 66 DAP, 122 DAP vs 66 DAP and 150 vs 66 DAP (Figure 4 and Table S4). The top-enriched KEGG pathways of these DEGs were secondary metabolite biosynthesis pathways, including flavonoid, phenylpropanoid and flavone and flavonol biosynthesis pathways, etc. Among them, flavone and flavonol biosynthesis (ko00944) and flavonoid biosynthesis (ko00941) are highly enriched throughout the five key development stages, implying those pathways are generally important for seed development such as pigment accumulation, but not specifically related to lignin deposition. Our premise is that genes involved in non-methylated lignin accumulation would be induced primarily during the lignin rapid accumulation stage (from 101 DAP to 150 DAP). The analysis showed that phenylpropanoid biosynthesis (ko00940) and phenylalanine metabolism (ko00360) are indeed highly enriched in 108 DAP vs 66DAP, 122DAP vs 66DAP and 150 vs 66DAP. Other highly enriched pathways were DNA replication (ko03030) in 87DAP vs 66DAP, starch and sucrose metabolism (ko00500) in 87DAP vs 66DAP and 108DAP vs 66DAP, plant hormone signal transduction (ko04075) in 150DAP vs 66DAP, etc.

Identification of genes potentially involved in non-methylated lignin biosynthesis

The monolignol biosynthesis pathway is well established, and a series of enzymatic reactions catalyzed by specific enzymes have been identified, including phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT), 4-coumarate 3-hydroxylase (C3H), caffeoyl shikimate esterase (CSE), ferulic acid 5-hydroxylase (F5H), caffeic acid O-methyltransferase (COMT), and caffeoyl-CoA 3-O-methyltransferase (CCoAOMT)^{10,15} (Figure 5). We used the annotated lignin pathway gene in KEGG database, and identified 20 *Paphiopedilum* genes that are homologous to genes potentially involved in monolignol synthesis (Table S5). Phylogenetic trees for the individual protein families of Arabidopsis, Dendrobium and Phalaenopsis were constructed using the CDS sequenced listed in Table S10. The results showed that the identified genes in lignin biosynthetic pathways had a close genetic relationship with those reported in other plant species (Figure 6).

Most lignin-related genes displayed a very similar transcript level between 66 DAP and 87 DAP, when the rapid accumulation of lignin has not started. Several monolignol synthesis related genes *PAL*, *4CL*, *HCT* and *CSE* demonstrated increased expression levels from 87 DAP to 150 DAP. Despite the high expression

level of these genes, most *CCoAOMT*, *COMT* and *F5H* were absent or down-regulated from developing seeds, and a few of them displayed a significant decrease in the expression level. *CCoAOMT* expression level is relatively high at 66 DAP. But the low expression level of *F5H* made the conversion of caffeoyl moieties to feruloyl moieties inefficient, which resulted in no G and S lignin production at the early stage of the seed development. Later, the decrease in the expression level of *CCoAOMT* and upregulation of upstream genes in monolignol pathway caused the accumulation of H and C lignins instead of G and C lignins.

qRT-PCR validation of candidate genes involved in lignin biosynthesis

To verify the accuracy and reproducibility of the RNA-seq results, we selected contigs corresponding to *PAL*, *4CL*, *HCT*, *C4H*, *CCoAOMT*, *F5H* and *COMT* in five development stages of seed development for qRT-PCR validation. Methylated lignin rich tissue stems were also included to verify our predicted monolignol pathway. The sequences of the primers used are given in Table S6. Reliable estimation of gene expression by qRT-PCR requires stable reference genes for data normalization. Six housekeeping genes *ACTIN1*, *ACTIN2*, *TUBLIN*, *UBIQUITIN*, *RPS3A1* and *RPS3A2* were tested for suitability as reference gene in five development stages of seeds, leaves and stems. qRT-PCR was performed using the primers listed in Table S8 to evaluate the expression variation of these candidates across seeds and vegetative tissues. The average threshold (C_T) values of the candidate genes ranged from 17.25 to 23.66 in seeds and vegetative tissues (Table S9). The coefficient of variation (CV) and ΔC_T are parameters used to identify suitable reference genes. The lower the values, the more stable the gene expression. *ACTIN1* and *ACTIN2* were the most stable genes among the six candidate reference genes. Therefore, we chose to use *ACTIN1* as a reference gene for this study. The additional experimental details on qRT-PCR were listed in Fig S3 to show that our assay was reliable.

In agreement with the transcriptomic data, *PAL*, *4CL* and *HCT* showed increased expression level as the seed approach maturity (Figure 7). *CCoAOMT* displayed relatively high transcript levels at the early stage of the seed development, and low transcript level during the later stage. *F5H* and *COMT* were also down-regulated during seed development. The results confirm the reliability of the transcriptomic data. Stems displayed relatively high transcript levels of *CCoAOMT*, in contrast to the mature seeds, which suggested that down-regulation of the first lignin methylation gene *CCoAOMT* was crucial for non-methylated lignin production.

Discussion

In contrast to well-developed embryo and endosperm present in most angiosperm seeds, the structure of orchid seeds is quite simple with a balloon like seed coat and a globular embryo. There is no endosperm. Orchids typically have tiny wind dispersed seeds, often called “dust seeds”¹⁹. Most species are difficult to germinate on their own under natural conditions. *P. armeniacum* is one of the most difficult species for

propagation, and the difficulty in germination severely restricted its large-scale production. The factors that inhibit germination are still unknown. Plant hormones play essential roles in seed development and germination performance. Abscisic acid (ABA) is necessary for inducing the synthesis of reserve proteins and lipids, as well as for the acquisition of desiccation in the developing seeds^{20,21}. In *Cypripedium formosanum*, a hard-to-germinate orchid species, ABA was proposed to be the key germination inhibitor²². In *P. armeniacum* seed development, ABA content was increased to 14.4 ng/g at 122 DAP from 5.6 ng/g at 66 DAP (Table1). It is possible that increase level of ABA inhibits the germination and promote desiccation before the seeds are fully mature. After that, the ABA level dropped to 2.3 ng/g at 150 DAP with continued decrease on germination percentage. Our exogenous application of ABA and ABA inhibitor fluridone can change the ABA levels within the seeds, but no significant difference on germination percentage was observed (data not shown). Therefore, we believe that other factors may contribute to the germination inhibition.

Mature orchid seeds generally have a heavily lignified seed coat developed offering protection to the embryo. Some studies have attributed the accumulation of lignin to germination inhibition of mature seeds^{2,6,9}. In this study, we first measured the lignin content during the seed development process to check whether the lignin content correlates with the germination rate. During the early stage of the development, the germination rate gradually increased as the embryo became fully developed with lignin maintained at a low level. The germination rate abruptly dropped when the seed lignin started rapid accumulating around 95 DAP. The results implied that the level of lignin negatively correlated with the germination rate. The lignin-rich seed coat provided mechanical restraint by preventing water and nutrients uptake⁴.

Besides the content, the structure and composition of lignin have been shown to impact the cell wall mechanical properties²³. Here, we discovered that lignins in the *Paphiopedilum* seeds are composed of H and C units, as clearly evident from Py-GC, two-dimensional NMR and FTIR analysis (Table 2 and Figure 2). While the lignins in other parts of the *Paphiopedilum* plant are composed of typical G and S units. This discovery raised an important question: what's the mechanism for regulating the seed specific deposition of non-methylated lignin. For non-model species lacking a well-studied genetic background, transcriptome analysis is a powerful approach to seeking answers for this question. Based on the dynamics of lignin accumulation and key anatomical features in embryo development, we selected five key stages for transcriptome analysis. A total of 104.12 Gb clean data were obtained from 15 RNA-seq libraries of 66, 87, 108, 122 and 150 DAP. The assembled data had an average N50 length of 1180 bp, which is similar to that in other orchid species, such as *Dendrobium catenatum* and *Apostasia shenzhenica*^{24,25}.

Phenylpropanoid biosynthesis and phenylalanine metabolism are highly enriched during lignin rapid accumulation stages. Further DEGs analysis of phenylpropanoid biosynthesis pathway showed that major structural genes were upregulated with the exception of *CCoAOMT*, *F5H* and *COMT*. Both CCoAOMT and COMT catalyze the O-methylation of the C3 and C5 hydroxyl groups of lignin precursors

during G and S lignin formation. Earlier studies demonstrated that low O-methyltransferase (both CCoAOMT and COMT) enzyme activities in the C-lignin containing seed of *Cleome hasslerianan* (*C. hasslerianan*)¹⁷. CCoAOMT is the first OMT in monolignol pathway. Caffey alcohol would be formed if CCoAOMT became depleted or lost its function. Down regulation of *CCoAOMT* in *Pinus radiata* introduced caffey alcohol into lignification, resulting in low levels of C units present in the G lignin dominant tissues¹⁸. In our data, the low expression level of *CCoAOMT*, *COMT* and *F5H* is likely the biochemical basis for the accumulation of non-methylated lignin in seeds. In stem tissues where G and S lignins are abundant, the expression levels of *CCoAOMT2* and *COMT2* are significantly higher than those in the seeds (Figure 7). However, suppression or loss of function of *CCoAOMT* and *COMT* in several angiosperm species such as Arabidopsis, alfalfa, poplar and tobacco did not result in the incorporation of caffey alcohol²⁶⁻²⁸. Other than the methylation genes (*CCoAOMT*, *COMT* and *F5H*), biosynthesis of C lignin requires the expression of CAD with a preference for the caffeyaldehyde in the seed coat of *Cleome hassleriana*²⁹. Cinnamyl alcohol dehydrogenase (CAD) catalyzes the conversion of the corresponding cinnamyl aldehydes to cinnamyl alcohols, which is the last step in the synthesis of monolignols before their polymerization in cell walls. However, none of the CAD genes was expressed in a manner consistent with specific involvement in C-lignin formation in *P. armeniacum*.

Other factors might also determine the lignin composition, such as transcriptional regulation or availability of the specific monolignol transporters. NAC and MYB families have been identified as regulators of lignin depositions^{10,30,31}. In our RNA-seq dataset, a total of 944 unigenes that encode TFs associated with 54 families were identified from our assembled transcripts. 40, 188, 274 and 247 differentially expressed TFs were found in 87 DAP vs 66 DAP, 108 DAP vs 66 DAP, 122 DAP vs 66 DAP and 150 vs 66 DAP (Table S6). Instead of the master switches, we are interested in the TFs that can specifically regulate the methylation genes, since these TFs can control the non-methylated lignin accumulation. We identified a few MYBs that are potential regulators of *CCoAOMT*, such as MYB4 and MYB21 (Figure S4). *MYB4* was down-regulated as the seed approach maturity. *MYB4* is a positive regulator of *CCoAOMT* and a negative regulator of *PAL*, *C4H*, *4CL* in Arabidopsis and *Pinus tadedda*^{32,33}. *MYB21* is a negative regulator of *CCoAOMT*, the expression level of other structural genes remain unchanged in Poplar³¹. The expression of these TFs agreed with the expression of major structural genes involved in monolignol pathways, implying a potential role in lignin synthesis regulation.

Conclusions

In summary, the presence of H and C lignin in *Paphiopedilum* seeds exemplifies the flexibility in the lignin monomers assembly in nature. Transcriptome profiling revealed the lignin related candidate genes expression pattern, which clarified the seed specific monolignol pathways. However, the seed monolignol composition is not consistent even within the same families of *Cypripedium*, *Euphorbiaceae* and *Cleomaceae* plants, suggesting the feature is not conservative. The difference in the degree of methylation and molecular structure of C and H lignin strongly suggests a functional difference

compared to typical G and S lignin. Future work should focus on linking these unique non-methylated lignin structure to the mechanistic role for seed development and germination.

Methods

Plant materials and Growth Conditions: *P. armeniacum* S. C. Chen et F. Y. Liu plants were maintained in a glass greenhouse in the South China Botanical Garden, Guangzhou, China. The plants were potted in a substrate of Zhijing stone for orchids under $800 \mu \text{mol m}^{-2} \text{s}^{-1}$ natural light maintained by a sunshade net. The average temperature and relative humidity ranged from 10–32 °C and 70–98%, respectively. The flowers from three-year-old adult plants were labeled and artificially self-pollinated. Capsules of different developmental stages from 45 DAP to 180 DAP were collected at 7-day intervals from grown plants. Seeds were removed from capsules, and immediately frozen in liquid nitrogen.

Seed germination assay: Seed capsules were surface sterilized by the method described previously³⁴. More than 100 seeds were sown on Hyponex N026 medium supplemented with 1.5 g/l activated charcoal, 2 g/L peptone, 15g/L sucrose and 5% coconut water. Germination was scored after embryo swollen and testa rupture.

ABA content: ABA content was measured as described previously³⁵. About 100 mg of seeds were used for ABA quantification by high-performance liquid chromatography–mass spectrometry.

Water content: Seeds after capsule removal were dried at 100°C until constant dry

weight was achieved (40–48 h) for determination of water content, expressed on a percentage fresh weight basis.

Lignin content and composition: Lignin content was estimated using the acetyl bromide method with slight modification³⁶. Five (5) mg of finely ground seed was thoroughly extracted with ethanol/toluene (1:1) until the extracts no longer absorbed UV light at 280 nm. The dried samples were placed into loosely capped glass tubes containing 1 mL of acetyl bromide/ acetic acid (1:3) and incubated at 70°C for 30 min. The sample was then cooled in an ice bath and then mixed with 0.9 mL of 2 M NaOH, 5 mL glacial acetic acid, and 0.1 mL 7.5 M hydroxylamine hydrochloride. The final volume was adjusted to 10 mL with glacial acetic acid and the absorbance measured at 280 nm with a microplate reader (Tecan Infinity). A standard curve was generated with alkali lignin (Sigma-Aldrich, 471003) for lignin content calculation.

The chemical composition of lignin was analyzed by pyrolysis-gas chromatography (GC)/ mass spectrometry (MS) using a previously described method with some modifications³⁷. The pyrolysis was performed on Pyroprobe 5000 (CDS Analytical Inc.) with direct connection to Shimadzu GCMS-QP2010A equipped with HP-5MS column (30 m×0.25 mm×0.25 mm). The pyrolysis was carried out at 550 °C. The chromatograph was programmed from 50 °C to 250 °C at a rate of 15 °C/min, and the final temperature was held for 10 min. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The mass spectrometer was operated in scan mode and the ion source was maintained at 300 °C. The compounds

were identified by comparing their mass spectra with those of the NIST library and those previously reported^{38,39}. Peak molar areas were used to calculate the lignin degradation products, and the summed areas were normalized. The relative abundance was calculated from the summed peak areas of each unit.

Fourier transform infrared spectroscopy (FTIR): The dried samples were embedded in KBr pellets in the concentration of about 1 mg/100 mg KBr. FTIR spectra in the range of 4000–400 cm^{-1} were recorded by using a Shimadzu IRAffinity-1S FTIR spectrophotometer. The spectra were recorded in the absorption mode at 64 scans per sample with a resolution of 4 cm^{-1} .

2D ^{13}C - ^1H heteronuclear single-quantum coherence (HSQC) NMR spectroscopy: Seeds from 122 DAP was extracted and ball-milled as previously described⁴⁰. The gels were formed using DMSO- d_6 /pyridine- d_5 (4:1) and sonicated until homogenous. The homogeneous solutions were transferred to NMR tubes. HSQC spectra were acquired at 25 °C using a Bruker Avance-500 MHz instrument. The detailed running conditions and assignment of the spectra was described elsewhere¹³.

RNA extraction, library construction and RNA sequencing: The five different development stages analyzed were 66, 87, 108, 122 and 150 days after pollination (DAP), with three biological replicates per developmental stage. The total RNA was extracted using Column Plant RNAout2.0 (Tiandz Inc., Beijing, China) according to the manufacturer's protocol, which was specifically designed for materials rich in polysaccharides and polyphenolics. Extracted RNA was treated with DNase (Tiandz Inc., Beijing, China) to remove genomic DNA. The RNA quality was validated using agarose gel electrophoresis, Nanodrop One (Nanodrop Technologies Inc., DE, USA), and Agilent 2100 (Agilent Technologies Inc., CA, USA) to confirm the purity, concentration, and integrity, respectively. Library construction and sequencing were performed using Illumina HiSeq4000 platform (Illumina Inc., CA, USA) by Genepioneer Technologies Corporation (Nanjing, China).

De novo assembly, functional annotation of unigenes and DEGs analysis: The clean data was generated by removing adaptor sequences, ambiguous reads ('N' > 10%), and low-quality reads (that is, where more than 50% of bases in a read had a quality value $Q \leq 5$) using perl script. All sequence data was uploaded into the BioProject databased hosted by the National Center for Biotechnology Information (NCBI) under the BioProject PRJNA550294. The transcripts were assembled using Trinity v2.4.0 program with default parameters⁴¹, and gene expression was estimated by applying the fragments per kilobase per million mapped reads (FPKM). Functional annotations were performed using the public databases, including the NR⁴², Swiss-Prot⁴³, COG⁴⁴ and GO⁴⁵. Differentially expressed genes (DEGs) between libraries were identified as those with the fold change (FC) of the expression level ($FC \geq 2$ or $FC \leq 0.5$ under P-value ≤ 0.05), FDR ≤ 0.05). GO enrichment and KEGG enrichment were performed using the obtained DEGs. To identify genes encoding for TFs, all DEGs were compared with protein sequences downloaded from a plant TF database, PlantTFDB with the E-values threshold of 10^{-10} .

Verification of gene expression using qRT-PCR: Samples of RNA-seq were reversed transcribed into cDNA for real-time qPCR validation using GoScript Reverse Transcription System (Promega, CA, USA). The qRT-PCR reactions were performed on ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA) and the SYBR Premix ExTaq Kit (Takara, Dalian, China). Primer sequences are listed in Table S7. The expression level was calculated as $2^{-\Delta\Delta C_t}$, and normalized to the Ct values of *P. armeniacum Actin* (TRINITY_DN40678_c2_g1).

Phylogenetic analysis: The phylogenetic trees were constructed using Neighbour-Joining of MEGA 7 software (Molecular Evolutionary Genetics Analysis version 7.0) with 1000 bootstrap replicates. Analysis of C4H, C3H, F5H-COMT-HCT and CSE was processed together, because at least four taxa are needed for bootstrapping.

Statistical analysis: Statistical analysis was performed with Students t-test as indicated in the figure legends. All experiments were performed on at least independently grown biological replicates. g All values represent the mean \pm SD.

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Supplemental Information

Figure S1. (A) Correlation indices between different samples. (B) Principal component analysis performed on the 15 samples. A:65 DAP, B: 87 DAP, C:108 DAP, D: 122 DAP, E:150 DAP.

Figure S2. Functional annotations of the unigenes of *P. armeniacum* seed transcriptome. (A) NR annotated species distribution map similar to the *Paphiopedilum armeniacum* transcriptome. *Dendrobium catenatum* shows the highest similarity. (B) GO function annotation. The most abundant

functions are binding and catalytic activity in terms of molecular function, and metabolic process and cellular process in terms of biological process.

Figure S3. Additional data for qPCR assays. (A) The quality of RNA used for qPCR assessed by agarose gel electrophoresis. 1-4: 66 DAP, 5-8 :87 DAP, 9-12: 108 DAP, 13-16: 122 DAP, 17-20: 150 DAP. Lanes 1 and 2 are the 28S and 18S RNA bands. (B) Quantification of RNA used for qPCR estimated by Nanodrop One and Agilent 2100 Bioanalyzer. (C) Melt curve from qPCR of PAL1, PAL2, C4H, 4CL, HCT, CCoAOMT1, CCoAOMT2, F5H, COMT1 and COMT2 genes. The single peak observed presented the pure and single amplicon resulted from the assay.

Figure S4. (A) Distribution of differentially expressed transcription factors (TFs); (B) Expression profiles of MYBs potentially related to lignin synthesis.

Table S1. Overview of transcriptome sequencing and *de novo* assembly results.

Table S2. Overview of transcriptome assembly showing length distribution of transcript and unigene.

Table S3. Summary of functional annotation of contigs from BLAST searches against public databases.

Table S4. KEGG enrichment analysis list.

Table S5. Lignin related genes with RPKM values.

Table S6. Differentially expressed transcription factors (TFs) list.

Table S7. Primers used for qRT-PCR.

Table S8. Primers used in the reference genes selection.

Table S9. Average Ct , ΔC_T and coefficient of variation (CV) values used for the reference genes selection. The average values are shown for at least three replicates per gene and tissue type.

Table S10. CDS sequences for monolignol biosynthetic genes.

Abbreviations

P. armeniacum: *Paphiopedilum armeniacum*; Py-GC/MS: pyrolysis-gas chromatography; DAP: Days after pollination; FTIR: Fourier transform infrared spectroscopy; DEG: Differentially expressed genes; NR: non-redundant protein sequences; GO: Gene Ontology; COG: the Clusters of Orthologous Groups; KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: False Discovery Rate; PAL: Phenylalanine ammonia lyase; COMT: Caffeic acid O-methyltransferase; CCoAOMT: Caffeoyl-CoA 3-O-methyltransferase; TFs: Transcription factors.

Declarations

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Consent for publication

The authors all agree to publish this manuscript.

Ethics approval and consent to participate

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and the supplementary information files. The sequence data was deposited at NCBI database

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Authors' contributions

LF, KW and SZ conceived the research program and designed research. LF, XX, JL, FZ, JY and YL performed research, KW, ML, XZ, GM, FL and SZ analyzed the data. LF, XX, KW and SZ wrote the manuscript, AZ revised this manuscript. All authors read and approved the final manuscript.

Tables

Table 1. Characteristics of the developing seeds of *P.armeniicum* after pollination.

DAP	Developmental status	Seed color	Germination percentage (%)	Lignin content (%)	ABA content ng/g FW	Water content (%)
66	T-shaped pre-embryo with four cells	Yellowish white	5.0±0.2	6.0±0.3	5.6±2.7	87.0±4.3
87	The globular embryo forms, and the outside-layer cells of the outer integument begin to dehydrate and the suspensor starts to degenerate	Yellowish white	44.0±5.5	5.0±0.9	6.5±2.1	79.8±2.1
108	The inner testa disappears, and starch and lipid globules accumulate rapidly	A mixture of yellow and light brown	66.0±5.2	13.0±3.5	9.0±2.2	72.2±1.1
122	The embryo is fully mature with a compact testa and no further morphological changes	Brown dark	37.0±7.2	22.0±4.1	14.4±3.1	68.6±2.6
150	Dry and mature seeds	Dark	10.0±1.7	39.0±5.45	1.4±0.2	51.5±0.6
180	Dry and mature seeds	Dark	5.0±0.75	37.0±3.56	2.3±1.2	49.2±0.7

Table 2. Relative molar abundances (%) of the compounds released after pyro-GC/MS of seeds, stems, leaves and pods of *P. armeniicum*. Values in brackets are the standard deviations from three biological replicates.

Compound name	Origin	Formula	Molecular Mass	Main Mass fragments	Seeds (%)	Stems (%)	Leaves (%)	Pods (%)
Phenol	H	C ₆ H ₆ O	94	65,66,94	16.2 (0.1)	-	23.0 (6.9)	5.7 (1.2)
2-Methylphenol	H	C ₇ H ₈ O	108	77,107,108	18.7 (0.4)	-	-	-
3-Methylphenol	H	C ₇ H ₈ O	108	77,107,108	18.3 (0.4)	-	-	-
2,5-Dimethylphenol	H	C ₈ H ₁₀ O	122	77,107,122	7.2 (1.3)	-	-	-
Catechol	C	C ₆ H ₆ O ₂	110	53,64,110	19.6 (2.7)	-	-	-
4-Methylcatechol	C	C ₇ H ₈ O ₂	124	51,97, 125	13.4 (0.7)	-	-	-
4-Ethylcatechol	C	C ₈ H ₁₀ O ₂	138	51,78,124	6.5 (0.1)	-	-	-
2-Methoxyphenol	G	C ₇ H ₈ O ₂	124	81,109,124	-	14.9 (0.8)	16.1 (2.1)	19.0 (2.1)
2-Methoxy-5-methylphenol	G	C ₈ H ₁₀ O ₂	138	95,123,138	-	5.6 (0.2)	-	13.4 (2.7)
4-Ethenyl-2-methoxyphenol	G	C ₉ H ₁₀ O ₂	150	107,135,150	-	31.6 (4.6)	23.1 (3.2)	31.7 (3.2)
4-Hydroxy-3-methoxybenzaldehyde	G	C ₈ H ₈ O ₃	152	109,151,152	-	4.5 (0.1)	-	5.5 (0.3)
2-Methoxy-4-propenylphenol	G	C ₁₀ H ₁₂ O ₂	164	131,149,164	-	7.9 (0.1)	-	11.2 (0.6)
4-Hydroxy-3-methoxyphenyl acetone	G	C ₁₀ H ₁₂ O ₃	180	122,137,180	-	4.1 (0.3)	18.1 (0.7)	-
2,6-Dimethoxyphenol	S	C ₈ H ₁₀ O ₃	154	111,139,154	-	-	19.8 (4.7)	13.6 (2.6)
4-Hydroxy-3,5-dimethoxystyrene	S	C ₁₀ H ₁₂ O ₃	180	137,165,180	-	31.6 (2.7)	-	-
% H units					60.5 (2.0)	-	23.0 (4.3)	5.7 (0.9)
% C units					39.5 (2.0)	-	-	-
% G units					-	68.4 (6.9)	57.2 (3.1)	80.7 (2.5)
% S units					-	31.6 (2.1)	19.8 (2.9)	13.6 (1.9)

Figures

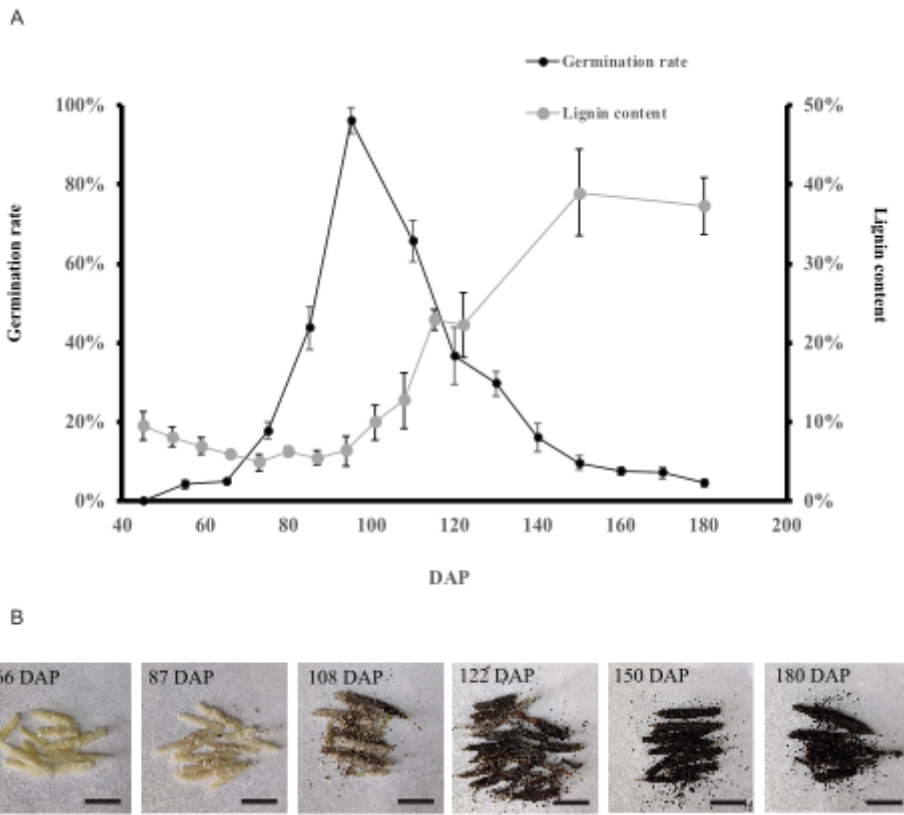


Figure 1

Dynamic changes in seed morphology, lignin accumulation and germination rate during *P. armeniacum* seed development. (A) Lignin content and germination rate during *P. armeniacum* seed development. DAP, days after pollination. The lignin content was estimated using the acetyl bromide method. (B) Phenotypes of *P. armeniacum* seeds. Scale bar = 1 cm.

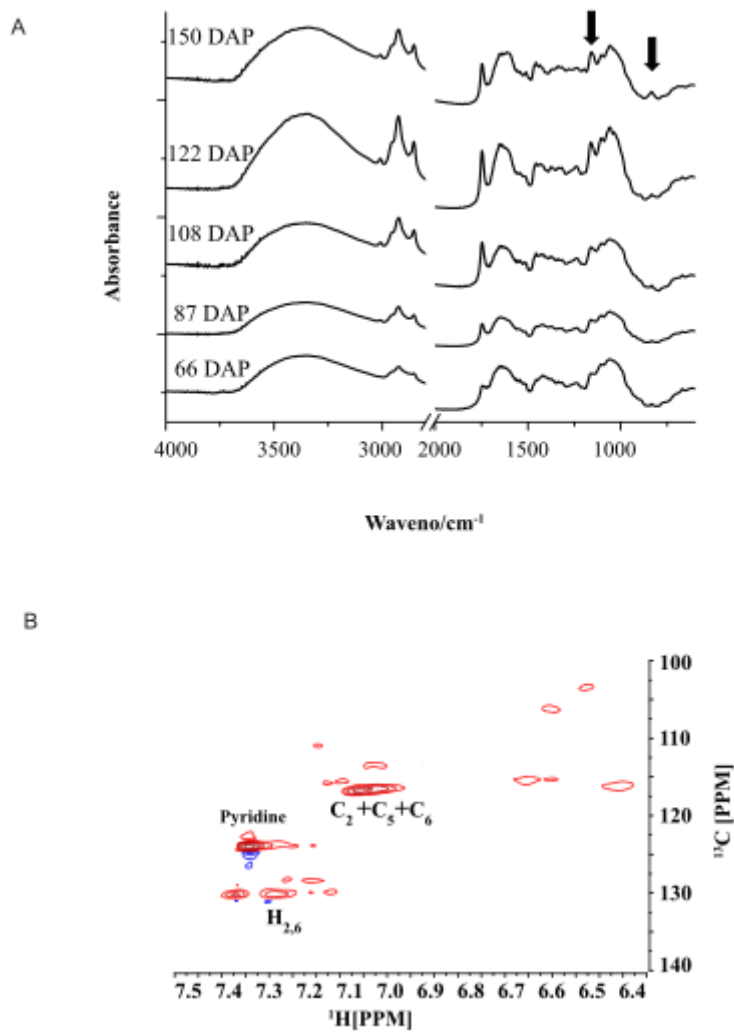
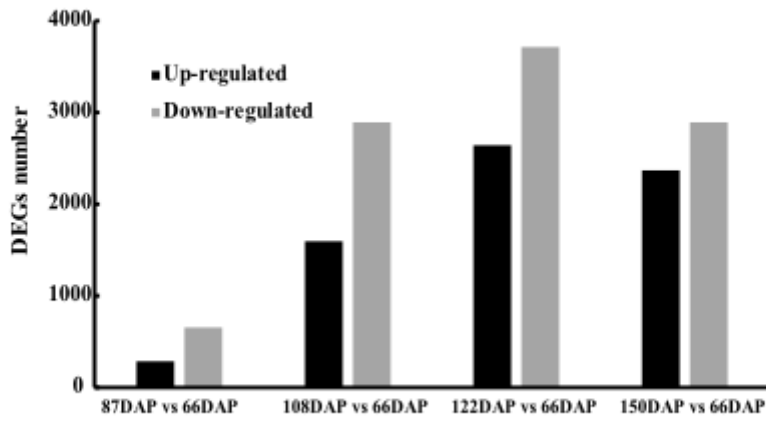


Figure 2

Lignin structure characterization. (A) FTIR spectra of *P. armeniacum* seeds at five developmental stages. Positions of C lignin bands are marked by arrows. (B) Partial short-range ¹³C–¹H (HSQC) spectra (aromatic region) of 122 DAP mature seeds.

A



B

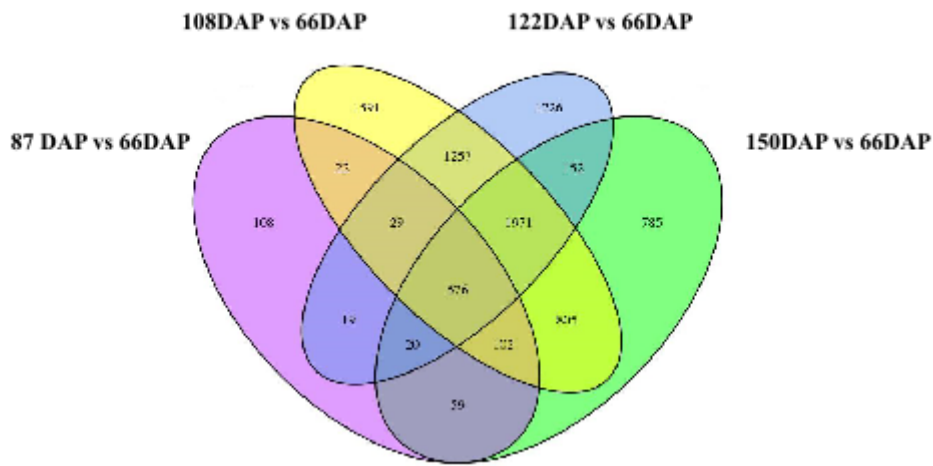


Figure 3

Statistical analysis of differentially expressed unigenes (DEGs) during *P. armeniacum* seed development. (A) up/down-regulated unigenes in all development stages. The total DEG number peaked between 122 DAP and 66 DAP. (B) Venn diagram of all DEGs.

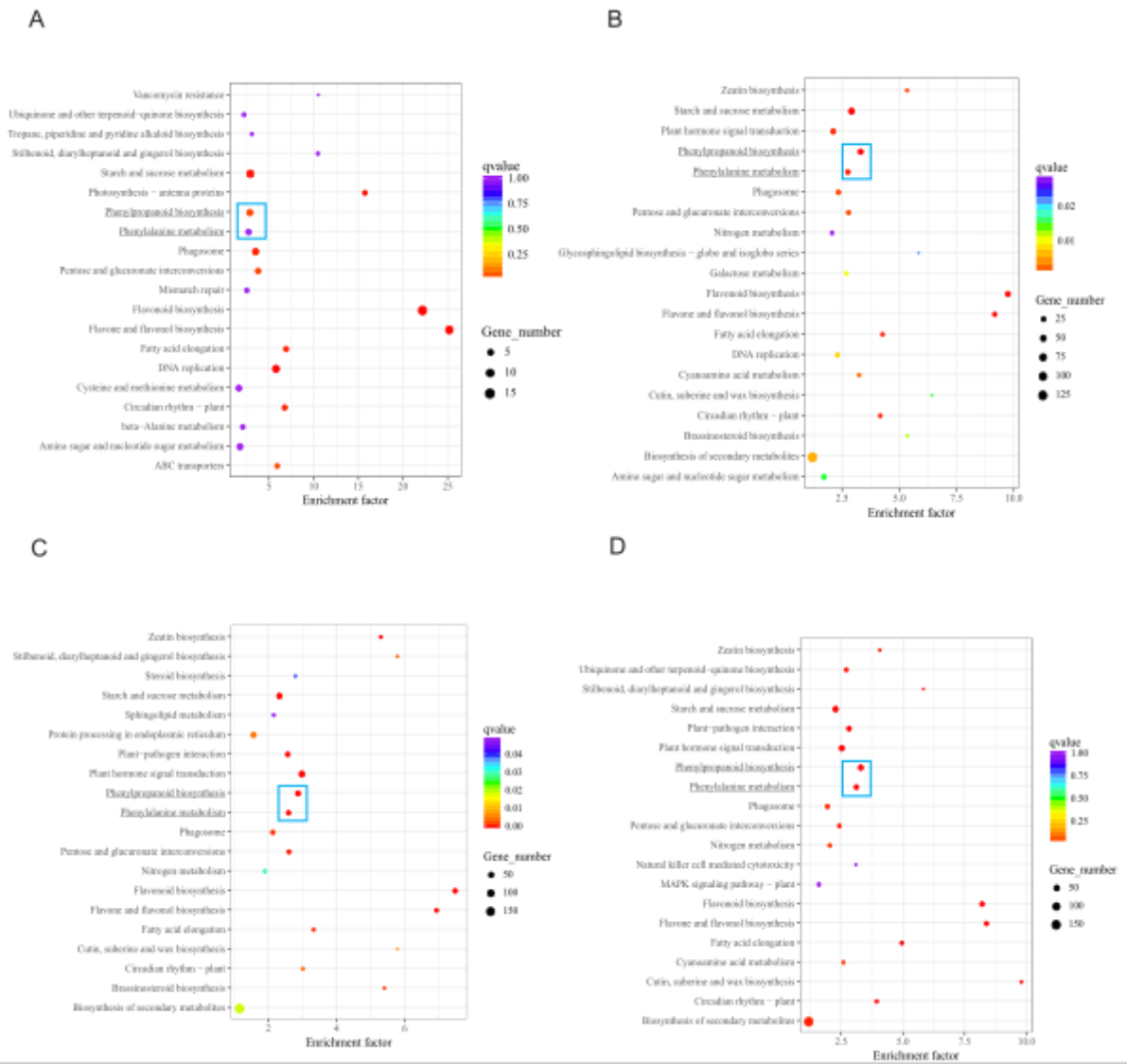


Figure 4

KEGG enrichment analysis with the DEGs. (A) 87 DAP vs 66 DAP, (B) 108 DAP vs 66 DAP, (C) 122 DAP vs 66 DAP, (D) 150 DAP vs 66 DAP. Flavone and flavonol biosynthesis (ko00944) and flavonoid biosynthesis (ko00941) are highly enriched throughout the five key development stages. Phenylpropanoid biosynthesis (ko00940) and phenylalanine metabolism (ko00360) were enriched during the seed lignification.

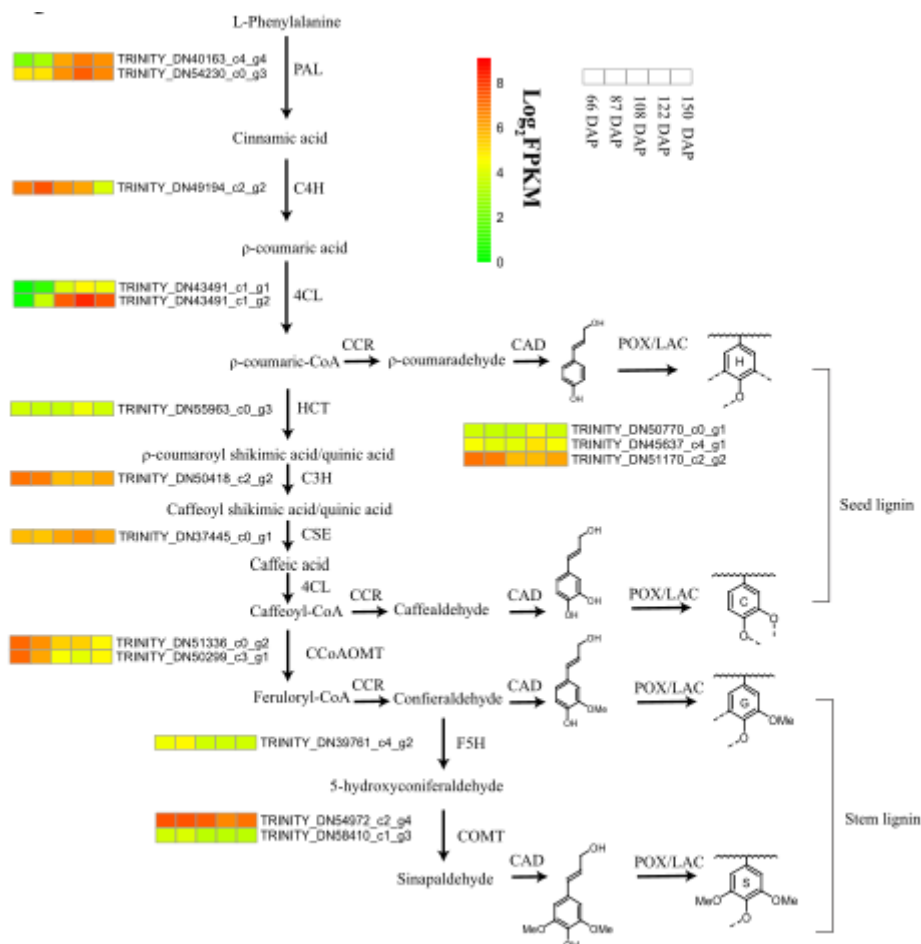


Figure 5

Lignin biosynthesis pathway and monolignol biosynthesis related gene expression in *P. armeniacum*. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; C3H, 4-coumarate 3-hydroxylase; HCT, hydroxycinnamoyl-CoA:shikimate/quinic acid hydroxycinnamoyl transferase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA 3-O-methyltransferase; COMT, caffeic acid O-methyltransferase; F5H, ferulate-5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; POX/LAC, peroxidases/laccase. Gene expression was scaled using the Z-score of FPKM (mean value of three biological replicates) in the heatmap. For each heatmap, the key is located at right side with FPKM values increasing from green to red.

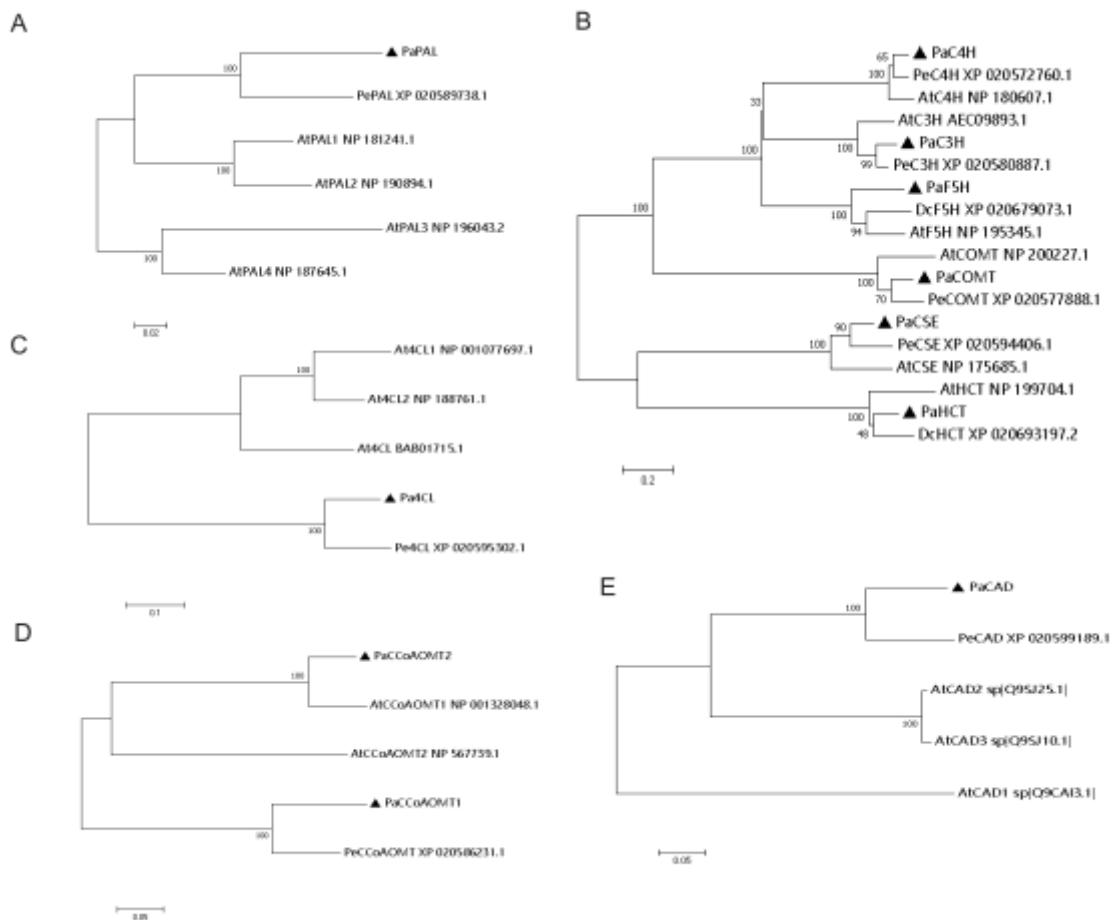


Figure 6

Phylogenetic analysis of monolignol pathway gene candidates among different plant species. The phylogenetic trees were constructed using Neighbour-Joining of MEGA 7 software (Molecular Evolutionary Genetics Analysis version 7.0) with 1000 bootstrap replicates. Analysis of C4H, C3H, F5H, COMT, HCT and CSE was processed together, because at least four taxa are needed for bootstrapping. (A) PAL, phenylalanine ammonia lyase; (B) C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; F5H, ferulate-5-hydroxylase; COMT, caffeic acid O-methyltransferase; CSE, caffeoyl shikimate esterase; HCT, hydroxycinnamoyl-CoA:shikimate/quinic acid hydroxycinnamoyl transferase; (C) 4CL, 4-coumarate:CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; (D) CCoAOMT, caffeoyl-CoA 3-O-methyltransferase; (E) CAD, cinnamyl alcohol dehydrogenase.

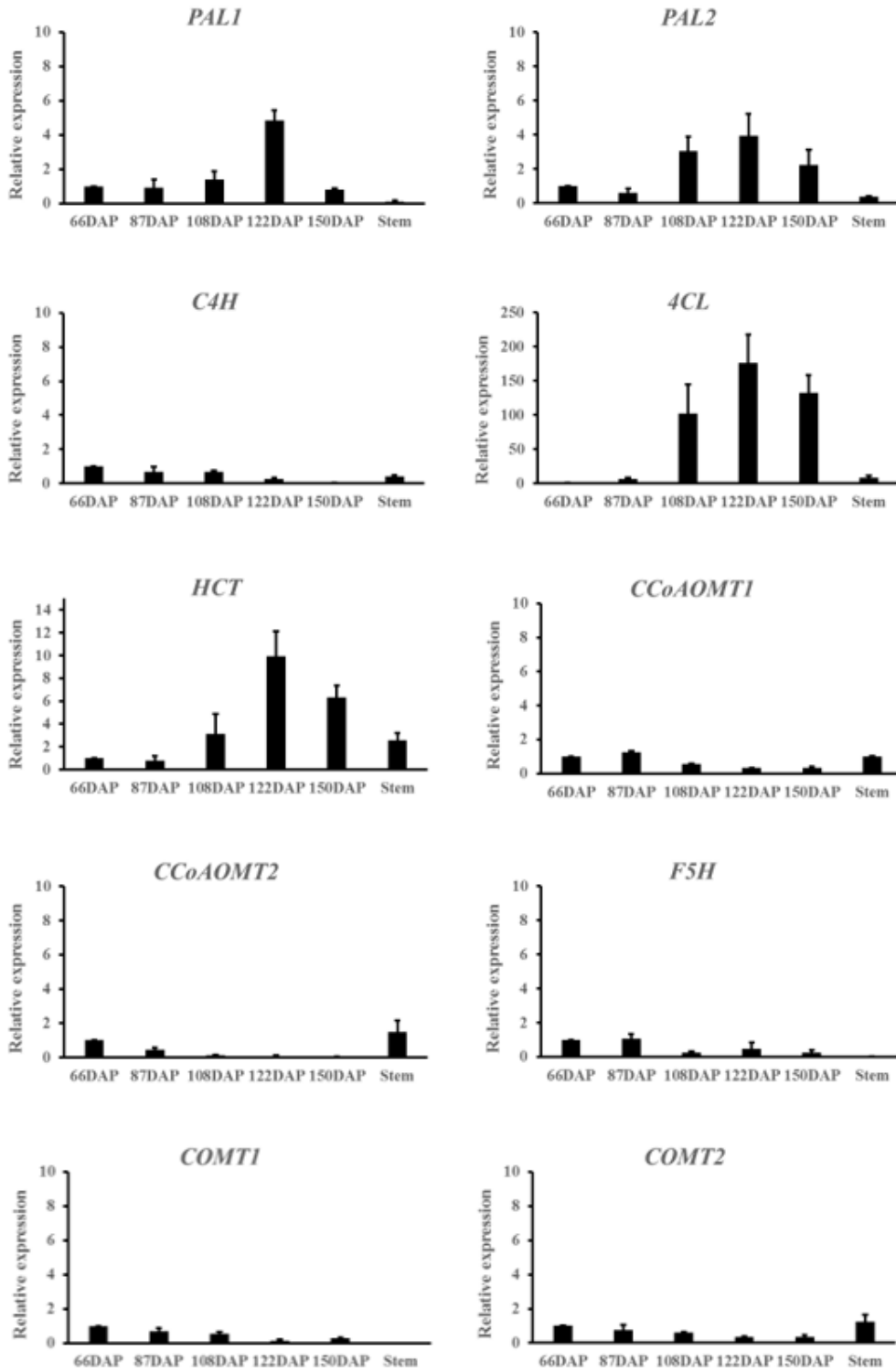


Figure 7

Validation of lignin-related gene expression in five stages of seed development, and stems. Expression levels were normalized to expression levels of actin.

Supplementary Files

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