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Effects of Metaxenia on Stone Cell Formation in Pear (*Pyrus bretschneideri*) Based on Transcriptomic Analysis and Functional Characterization of the Lignin-Related Gene *PbC4H2*

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Abstract: The deposition of lignin in flesh parenchyma cells for pear stone cells, and excessive stone cells reduce the taste and quality of the fruit. The effect of metaxenia on the quality of fruit has been heavily studied, but the effect of metaxenia on stone cell formation has not been fully elucidated to date. This study used *P. bretschneideri* (Chinese white pear) cv. 'Yali' (high-stone cell content) and *P. pyrifolia* (Sand pear) cv. 'Cuiguan' (low-stone cell content) as pollination trees to pollinate *P. bretschneideri* cv. 'Lianglizaosu' separately to fill this gap in the literature. The results of quantitative determination, histochemical staining and electron microscopy indicated that the content of stone cells and lignin in YL fruit ('Yali' (pollen parent) × 'Lianglizaosu' (seed parent)) was significantly higher than that in CL fruit ('Cuiguan' (pollen parent) × 'Lianglizaosu' (seed parent)). The transcriptome sequencing results that were obtained from the three developmental stages of the two types of hybrid fruits indicated that a large number of differentially expressed genes (DEGs) related to auxin signal transduction (*AUX/IAA*s and *ARF*s), lignin biosynthesis, and lignin metabolism regulation (*MYB*s, *LIM*s, and *KNOX*s) between the CL and YL fruits at the early stage of fruit development. Therefore, metaxenia might change the signal transduction process of auxin in pear fruit, thereby regulating the expression of transcription factors (TFs) related to lignin metabolism, and ultimately affecting lignin deposition and stone cell development. In addition, we performed functional verification of a differentially expressed gene, *PbC4H2* (cinnamate 4-hydroxylase). Heterologous expression of *PbC4H2* in the *c4h* mutant not only restored its collapsed cell wall, but also significantly increased the lignin content in the inflorescence stem. The results of our research help to elucidate the metaxenia-mediated regulation of pear stone cell development and clarify the function of PbC4H2 in cell wall development and lignin synthesis, which establishes a foundation for subsequent molecular breeding.

Keywords: pear; stone cell; lignin biosynthesis; metaxenia; cinnamate 4-hydroxylase; transcriptome analysis; functional verification

1. Introduction

Pear (*Pyrus* spp.) is not only one of the world's most important *Rosaceae* deciduous trees, but it is also the third most abundant fruit in China [1]. The currently cultivated pear species can be divided into two categories: Asian pears and European pears (*P. communis*). Asian pears are mainly grown in Asia, including *P. bretschneideri*, *P. pyrifolia*, *P. sinkiangensis* (Xinjiang pear), and *P. ussuriensis* (Ussurian pear) [2]. Among these varieties, 'Dangshan Su' (*Pyrus bretschneideri* cv. 'Dangshan Su'), which belongs to Chinese white pear (*P. bretschneideri*), is the most abundant pear variety that is cultivated in China and it is one of the main exported varieties. However, the large diameter of stone cell clusters in the fruit of this variety is excessive, which causes a gritty texture and coarse taste [3–5]. 'Lianglizaosu' (*P. bretschneideri* cv. 'Lianglizaosu') is the natural bud sport of 'Dangshan Su', which has now been selected as a new variety and approved. The characteristic of 'Lianglizaosu' is that the size and content of the stone cell clusters are significantly lower than 'Dangshan Su', and other traits are largely consistent with 'Dangshan Su' [6,7]. Therefore, 'Lianglizaosu' is also known as the low stone cell content bud sport of 'Dangshan Su'. This new variety of pear compensates for the defects of 'Dangshan Su'.

A large number of previous reports have shown that excessive stone cells can have a significant negative impact on the texture and flavour of pear fruit, such as fruit hardness, adhesiveness, chewiness, and sucrose content. Therefore, the content and size of stone cell clusters are the key factors that determine the quality of pears [3,8,9]. The stone cells of pears are highly lignified sclerenchyma cells with approximately 30% to 39% lignin content [10]. Electron microscopy revealed that a large amount of lignin was deposited in the secondary cell wall (SCW) of the stone cells. Lignin is a major component of stone cells and it is essential for the formation of stone cells [8,11,12].

Plant lignin metabolism can be divided into three parts: the general phenylpropanoid pathway, the ester intermediary pathway, and the monolignol-specific biosynthesis pathway [6]. Previous studies have shown that the lignin metabolic pathway in pear fruit begins with the conversion of cinnamic acid to *p*-coumaric acid, and the *p*-coumaric acid content was positively correlated with the lignin synthesis of pear fruit [4,13]. Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) are the key enzymes responsible for the synthesis of cinnamic acid and *p*-coumaric acid, respectively [14,15]. Although in silico analysis of some gene families that are related to pear lignin synthesis has been reported, it is unclear which families play a major role in stone cell lignification, and the function of most lignin metabolism-related members has not been validated [10,11,16–20].

Recent studies have demonstrated that the content and size of stone cells are not only determined by the genotype of pear varieties, but also by the external environment and cultivation methods, such as pollination, water, bagging, and exogenous mineral elements [7,21–24]. Pears are gametophytic self-incompatible plants and they cannot be inseminated by self-pollination [2]. Therefore, cross-pollination is essential in the normal insemination and bearing of pear trees. However, pollination with different pollen parents will have an impact on the fruit traits of the maternal plant, including the skin colour, weight, and fruit diameter, due to the pollen effect (metaxenia) [25,26]. The use of metaxenia is an effective means of regulating the development of pear stone cells and improving the quality of fruits.

'Yali' and 'Cuiguan' belong to *P. bretschneideri* and *P. pyrifolia*, respectively [27–29]. These varieties are often used as pollen parents in Dangshan County, Anhui Province, China. Previous studies have shown that the 'Yali' has a high stone cell content, but the 'Cuiguan' has a low stone cell content [28,30]. However, the effect that the pollen parents with different stone cell levels have on the formation of stone cells in the fruit of 'Lianglizaosu' remains unknown. This study used *P. bretschneideri* cv. 'Yali' and *P. pyrifolia* cv. 'Cuiguan' as the pollen parent, used 'Lianglizaosu' as the seed parent, and analysed the difference in stone cell development between the two pollination combinations to address this problem. In addition, we used transcriptome sequencing to explore the effects of metaxenia on gene transcription in fruits and identify key differentially expressed genes. On this basis, we performed a functional characterization of one (C4H, cinnamate 4-hydroxylase) of the differentially expressed

genes (DEGs) that are involved in lignin synthesis. These findings may establish a foundation for revealing the mechanism of the cross-pollination regulation of pear stone cell development and will create conditions for further elucidation of the relationship between lignin and stone cell formation.

2. Methods and Materials

2.1. Plant Materials and Cultivation Conditions

Thirty-year-old 'Lianglizaosu' trees in the Center of Pear Germplasm Resources, Dangshan County, Anhui Province, China, were selected as seed parents. 'Cuiguan' (*P. pyrifolia*) (low-stone cell-content variety) and 'Yali' (*P. bretschneideri*) (high-stone cell-content variety) were used as the pollen parents (Supplementary Figure S1). All of the varieties were maintained under the same water-fertilizer regimen and management during the years of cultivation. Artificial pollination was performed by placing pollen on the stigmas of flowers on the branches with 3rd, 4th, and 5th order flowers during the pear blooming period after the manual removal of the stamens. After pollination, the stigmas were immediately covered with bags for seven days [6]. We named 'Cuiguan' (pollen parent) × 'Lianglizaosu' (seed parent) as CL and 'Yali' (pollen parent) × 'Lianglizaosu' (seed parent) as YL. We collected fruits at eight developmental stages: 23 days after flowering (DAF), 39 DAF, 47 DAF, 55 DAF, 63 DAF, 71 DAF, 87 DAF, and maturity. 40 fruits with uniform size and without disease were collected for each period.

Fresh fruit was used for histochemical staining of stone cells. The remaining fruits were frozen in liquid nitrogen and then stored at −80 °C for RNA extraction, stone cell, and lignin content determination.

2.2. Determination of Stone Cell and Lignin Content in Pear Fruit

The method for measuring the levels of stone cells and lignin was used as described previously [4]. The stone cell and lignin levels are shown as percentages (calculated stone cell content/calculated flesh weight of test sample × 100%; calculated lignin content/calculated dry weight of test sample × 100%). Three biological replicates were performed for each sample.

The Wiesner method carried out the histochemical staining of the stone cells of the pear fruit [18]. The transmission electron microscopy (TEM) observation of the SCW of stone cells was performed in accordance with the method that was described by Jin et al. [8].

2.3. RNA Extraction and Illumina Sequencing

The samples (pear fruits) that were obtained for transcriptomic analysis in this experiment were collected in equal amounts from parts of the tree facing four different directions (East, South, West, and North). Subsequently, RNA from these fruits (collected in the same period) was extracted and mixed in equal amounts for transcriptomic analysis to avoid error.

The total RNA from different fruits of pear was extracted according to the instructions of the RNAprep Pure Plant Kit (polysaccharides and polyphenolics-rich) (Tiangen, Beijing, China). The construction of the cDNA library and high-throughput sequencing (Illumina HiSeq™ 2500 sequencing platform) were commissioned by the Biomarker Technology Company (Beijing, China). Raw data (raw reads) in fastq format were first processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adaptor sequences, reads containing poly-N sequences, and low-quality reads from the raw data. At the same time, the Q20, Q30, GC content, and sequence duplication level of the clean data were calculated. All of the downstream analyses were based on clean data with high quality.

2.4. Screening and Functional Annotation of Differentially Expressed Genes (DEGs)

The adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were then

mapped to the reference genome sequence. Only reads with a perfect match or one mismatch were further analysed and annotated based on the reference genome. Tophat2 software v. 2.1.1 (Center for Bioinformatics and Computational Biology, University of Maryland, College Park, USA) was used for mapping with a reference genome. The reference genome of the pear (*P. bretschneideri*) was downloaded from the GigaDB Dataset (http://gigadb.org/dataset/100083).

The annotation of genes was performed while using the following method: Swiss-Port protein databases, Cluster of Orthologous Groups (COG) database, Gene Ontology (GO) database, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database [6]. For the detection of DEGs, fold change (FC) ≥ 1 and false discovery rate (FDR) < 0.05 were used as screening criteria. For differential expression analysis, the well-known Benjamini-Hochberg correction method was used to correct the *p*-value of the original hypothesis test, and FDR was finally used as the key indicator for DEG screening [6].

The absolute transcript abundance values that were obtained for the 30 lignin biosynthetic genes were computed from fragments per kilobase of exon per million fragments mapped (FPKM) values. The FPKM values were obtained from the RNA-sequencing (RNA-seq) data. The expression level of each gene was visualized in the form of a heatmap while using TBtools software v. 0.66836 (https://github.com/CJ-Chen/TBtools/releases). The RNA-seq data have been deposited in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) with the access numbers SUB6384421 and SUB2967341.

2.5. Quantitative Real-Time PCR (qRT-PCR) Analysis

30 lignin biosynthetic genes were analysed using qRT-PCR to validate the expression patterns revealed by the DEG results. qRT-PCR analysis was performed while using SYBR Green Master Mix (Takara, Shiga, Japan) and detected by CFX96 Touch™ Real-Time PCR Detection System (Singapore). Three biological replicates were performed for each sample. Relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method [31]. The pear *tubulin* gene (GenBank accession no. AB239680.1) was used as the reference gene [32]. Supplementary Table S1 lists all primers.

2.6. Genetic Transformation of Arabidopsis thaliana

We designed specific primers (Supplementary Table S1) for the *C4H* gene (Pbr017290.1) based on information in the genomic database to amplify the coding sequence. The overexpression vector pCAMBIA1304-*PbC4H* was constructed with the eukaryotic expression vector pCAMBIA1304 (GenBank: AF234300.1) as the backbone. Electroporation introduced the constructed vector into *Agrobacterium tumefaciens* EHA105.

The seeds of wild-type *Arabidopsis thaliana* (Columbia-0) and *c4h* mutants (At2g30490, SALK_070079) were purchased from the Nottingham Arabidopsis Stock Centre, UK [33]. The genetic transformation of Arabidopsis was carried out according to the floral dip method [34]. The method for the identification of Arabidopsis transgenic plants was consistent with that described by Cheng et al. [35]. *β*-Glucuronidase (GUS) staining was examined while using a GUS histochemical assays kit (Real-Times, Beijing, China).

2.7. Microscopic and Ultramicroscopic Observation of the Cell Wall of Arabidopsis

The inflorescence stems from *Arabidopsis* plants (T_3 generation transgenic plants, WT plants, *c4h* mutant plants) were sectioned while using a skiving machine (Leica RM2016, Wetzlar, Germany) and then stained with phloroglucinol-HCl or toluidine blue as described by Pradhan et al. [36].

The TEM observations and cell wall thickness measurements were performed using the previously described method [35,37]. Microsoft Excel 2010 (Student's t test) was used for the statistical analyses.

2.8. Analysis of Lignin Content in the Inflorescence Stem of Arabidopsis

The acetyl bromide method (acetyl bromide soluble lignin content) determined the lignin content of the inflorescence stem of *Arabidopsis*. The specific operation follows the method that was described by Anderson et al. [37]. Three biological replicates were performed for each sample.

3. Results

3.1. Differences in Stone Cell and Lignin Content of Pear Fruit Obtained from Two Pollination Combinations

We compared the differences in the content of stone cells in the eight developmental stages of the two pollination combinations to clarify the effects of different pollen parents on the content of stone cells in the fruit (Figure 1a). We used 'Yali' and 'Cuiguan' to pollinate 'Lianglizaosu' separately and collected pollinated pear fruits at eight developmental stages (23 DAF, 39 DAF, 47 DAF, 55 DAF, 63 DAF, 71 DAF, 87 DAF, and the mature period). It is well-known that lignin is one of the key components of stone cells. Therefore, we also measured the lignin content in the 'Yali'♂ × 'Lianglizaosu'♀(YL) and 'Cuiguan'♂× 'Lianglizaosu'♀(CL) fruits, in addition to determining the content of stone cells (Figure 1b).

Notably, the different pollen parents did not change the trends of stone cell and lignin content during fruit development, and they both showed a rise-fall tendency (Figure 1). The stone cell and lignin content in YL and CL fruits peaked at 55 DAF. However, it can be observed that the content of lignin and stone cells in the CL fruit are lower than the YL fruit in the selected eight developmental stages. In particular, the stone cells and lignin content of YL fruit were significantly higher than that of CL fruit from 39 DAF to fruit ripening. Therefore, different pollen parents have an effect on the stone cell content and lignin synthesis in the pear fruit.

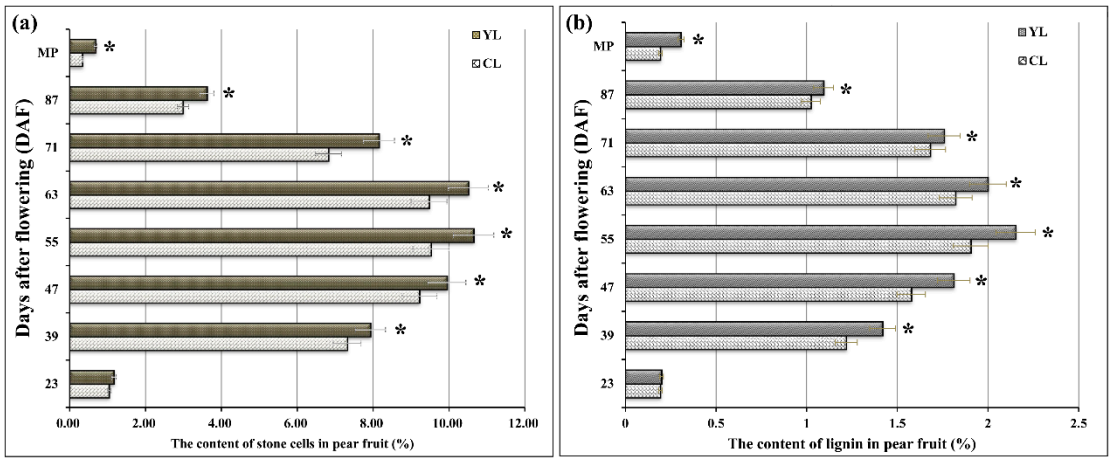


Figure 1. Differences in stone cell and lignin content of pear fruit obtained from two pollination combinations: (**a**) The content of stone cells in YL and CL fruits; (**b**) The content of lignin in YL and CL fruits. YL: 'Yali'♂ × 'Lianglizaosu'♀; CL: 'Cuiguan'♂ × 'Lianglizaosu'♀. Student's t test was used for the statistical analyses. Four fruits were selected for analysis at each period. * indicates a significant difference ($p < 0.05$).

3.2. Characterization of Stone Cells in Pear Fruit after Pollination by Two Pollen Parents

We selected mature fruit for Wiesner staining (phloroglucinol-HCl) to explore the effects of different pollen parents on the distribution of stone cells in pear fruits. As shown in Figure 2, we can observe that, regardless of whether the 'Yali' or 'Cuiguan' was used as the pollen parent, the stone cell density in the inner flesh region of the fruit was relatively high. However, there is a large difference in the distribution of stone cells in the middle flesh region. The distribution density and range of the stone cell clusters in the mature fruit from 'Yali', as the pollen parent are larger than those in the mature fruit from 'Cuiguan' as the pollen parent. This finding indicates that metaxenia plays a regulatory role in the development of stone cells in fruits. This further suggests that the stone cell content of the pollen parents might determine the stone cell content of the hybrid fruits.

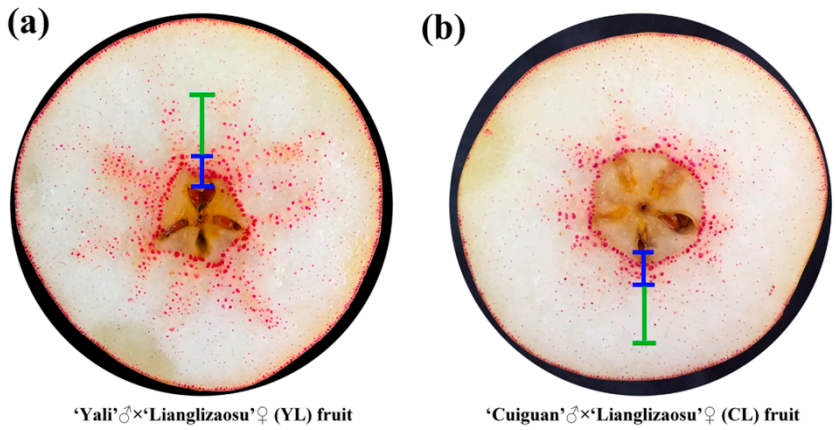


Figure 2. Distribution of stone cell clusters of 'Yali'♂ × 'Lianglizaosu'♀ (YL) and 'Cuiguan'♂ × 'Lianglizaosu'♀ (CL) fruit: (**a**) YL fruit; and, (**b**) CL fruit. The blue marked area is the inner flesh region, and the green marked area is the middle flesh region.

We further observed differences in the ultramicrostructure of the SCW of stone cells in YL and CL fruits (Figure 3). The SCW of stone cells in the CL and YL fruits are composed of light (cellulose microfibrils) and dark stripes (lignin) (Figure 3). Notably, the dark stripes of the SCWs of the stone cells in the YL fruit are significantly darker than the CL fruit. This finding indicates that the degree of lignification of stone cells in YL fruit is higher than that of CL fruit (Figure 3c,f). In addition, we also found that the stripes that were composed of lignin and cellulose microfibrils in the SCW of stone cells in CL fruit were loose at three developmental stages (Figure 3a–c). The light and dark stripes in the stone cells in the YL fruit are clearly separated and tightly bound, which results in a high density (Figure 3d–f). We hypothesize that the higher degree of lignification and density of the SCW structure may give the stone cells greater rigidity. This finding also reflects the effect of different pollination on the deposition of lignin in the SCW of stone cells.

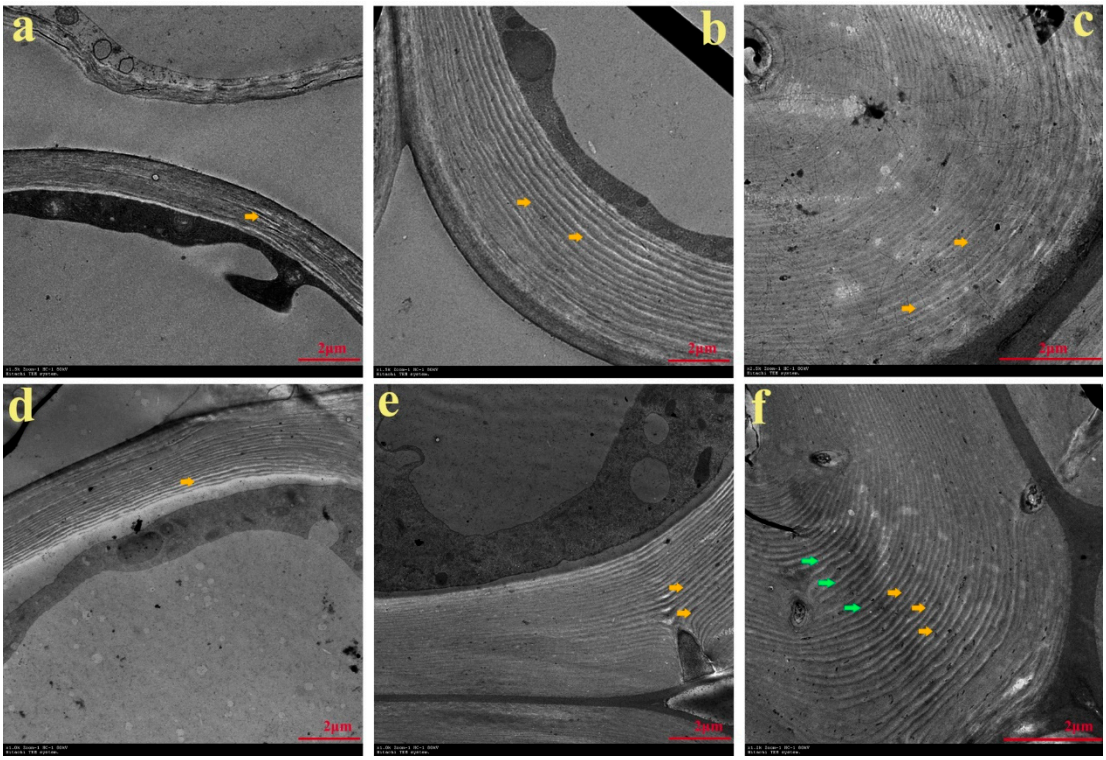


Figure 3. Structure of the secondary cell wall of stone cells in CL and YL fruits: (**a**–**c**): The SCW structure of stone cells in CL fruits at the early, middle and mature stages; (**d**–**f**): The SCW structure of stone cells in YL fruits at the early, middle and mature stages. Dyeing with potassium permanganate can make lignin appear dark under electron microscopy. The orange arrows indicate the dark streaks formed by the deposition of lignin. The green arrows indicate the bright streaks formed by the deposition of cellulose microfibrils.

3.3. Analysis of Transcriptomic Data and Mapping to Reference Genome Sequences

We performed transcriptome sequencing of the two types of pear fruit to reveal the molecular mechanism underlying the differences in stone cell development in CL and YL fruits. The SCW of the stone cells began to thicken and lignify at 23 DAF [11]. The stone cell and lignin content peaked at 55 DAF and then reached a steady level as the fruit matured (Figure 1). Therefore, we selected the initial phase (23 DAF), the vigorous phase (55 DAF), and the stable phase (mature period) of stone cell formation for comparative transcriptome analysis. We named the YL fruits that were collected at 23 DAF, 55 DAF, and the mature period (MP) as YL-23, YL-55, and YL-MP, respectively. Similarly, CL-23, CL-55, and CL-MP represent the CL fruits that were collected at 23 DAF, 55 DAF, and MP, respectively. Whole-genome sequencing of Chinese white pear (*P. bretschneideri* Rehd.) has been completed as a reference genome for this study [1].

It can be observed from Supplementary Table S2 that a total of 47.67 Gb of Clean Data was obtained after sequencing quality control, the Q30 base percentage of each sample was not less than 96.60%, and the GC content was not less than 48.09%. This result indicates that the sequencing yield and sequence quality were high. Mapping efficiency refers to the percentage of Mapped Reads in Clean Reads. The mapping efficiency between the reads and the reference genome of each sample ranged from 71.40% to 72.99%. These results indicate that we obtained transcriptome data that could be used for subsequent analysis and that the selected reference genome assembly was sufficient for information analysis.

3.4. Identification of DEGs in Pear Fruits Pollinated by Two Pollen Parents Based on Transcriptome Sequencing

We identified a total of 9018 DEGs between the fruits of YL and CL at three different developmental stages, according to the results of transcriptome sequencing (Figure 4 and Supplementary Table S3).

Among these DEGs, as compared with YL fruit, there were 4245 upregulated genes and 4773 downregulated genes in CL fruits. As shown in Figure 4a, YL and CL fruits had the highest number of DEGs at 23 DAF, followed by 55 DAF, with the lowest number of DEGs between the two types of pear fruits being observed at maturity. In each library, YL-23 vs_CL-23, YL-55 vs_CL-55, and YL-MP vs_CL-MP had 5454, 2583, and 981 DEGs, respectively (Figure 4b). These results indicate that pollination with different pollen parents resulted in significant changes in the transcriptional levels of a large number of genes in early fruit development. However, the effect on gene expression in fruits in the middle and late stages of development was relatively small. These results indicate that early fruit development in pears is a highly active process, while the metabolic processes in mature fruits are slowed.

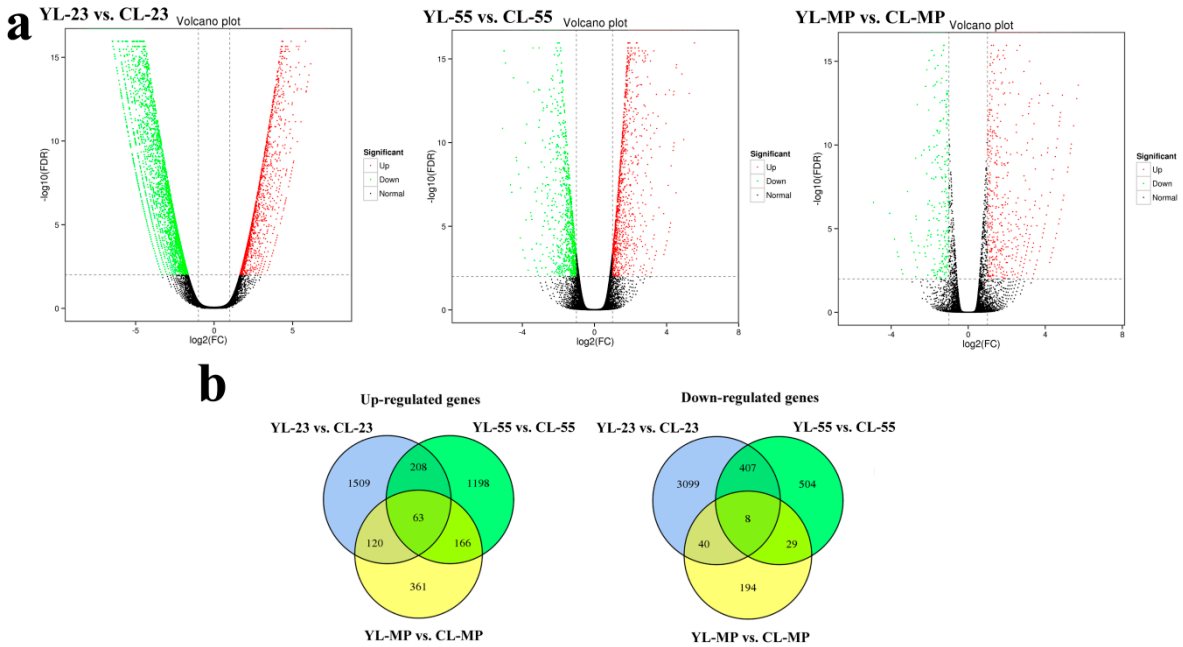
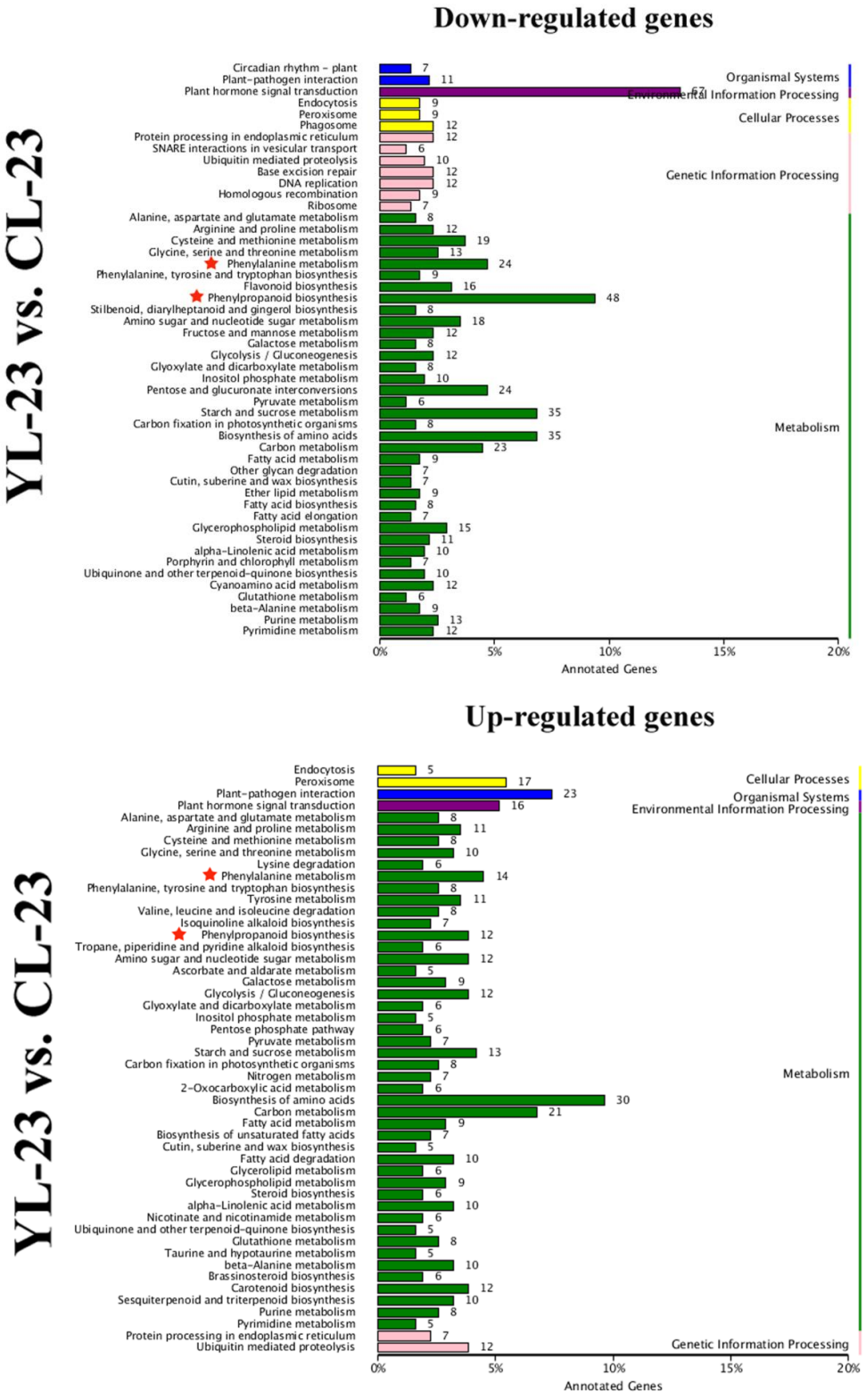


Figure 4. Statistics on the number of differentially expressed genes: (**a**) Volcano plot of differentially expressed genes (DEGs) between different samples; (**b**) Venn diagram of DEGs between different samples. Up-regulated genes in A_vs._B: genes whose expression level in sample B are higher than that in sample A; otherwise they are down-regulated genes.

We used multiple databases to annotate the DEGs to predict the functions of the DEGs (Supplementary Table S4 and Figure S2). According to the KEGG annotation, most of the DEGs were related to plant hormone signal transduction, phenylpropanoid biosynthesis, phenylalanine metabolism, starch and sucrose metabolism, biosynthesis of amino acids, and carbon metabolism (Figure 5). Notably, at 23 DAF, there were 98 DEGs that were associated with phenylalanine metabolism and phenylpropanoid metabolism between YL and CL fruits. However, the number of these DEGs between YL and CL fruits was only 30 and 13 at 55 DAF and at maturity. In particular, we used the KEGG method to annotate upregulated genes and downregulated genes at different developmental stages between YL and CL fruits. Most genes that were related to phenylpropane metabolism in CL fruits showed a downward trend during the early stages of fruit development (Figure 5).



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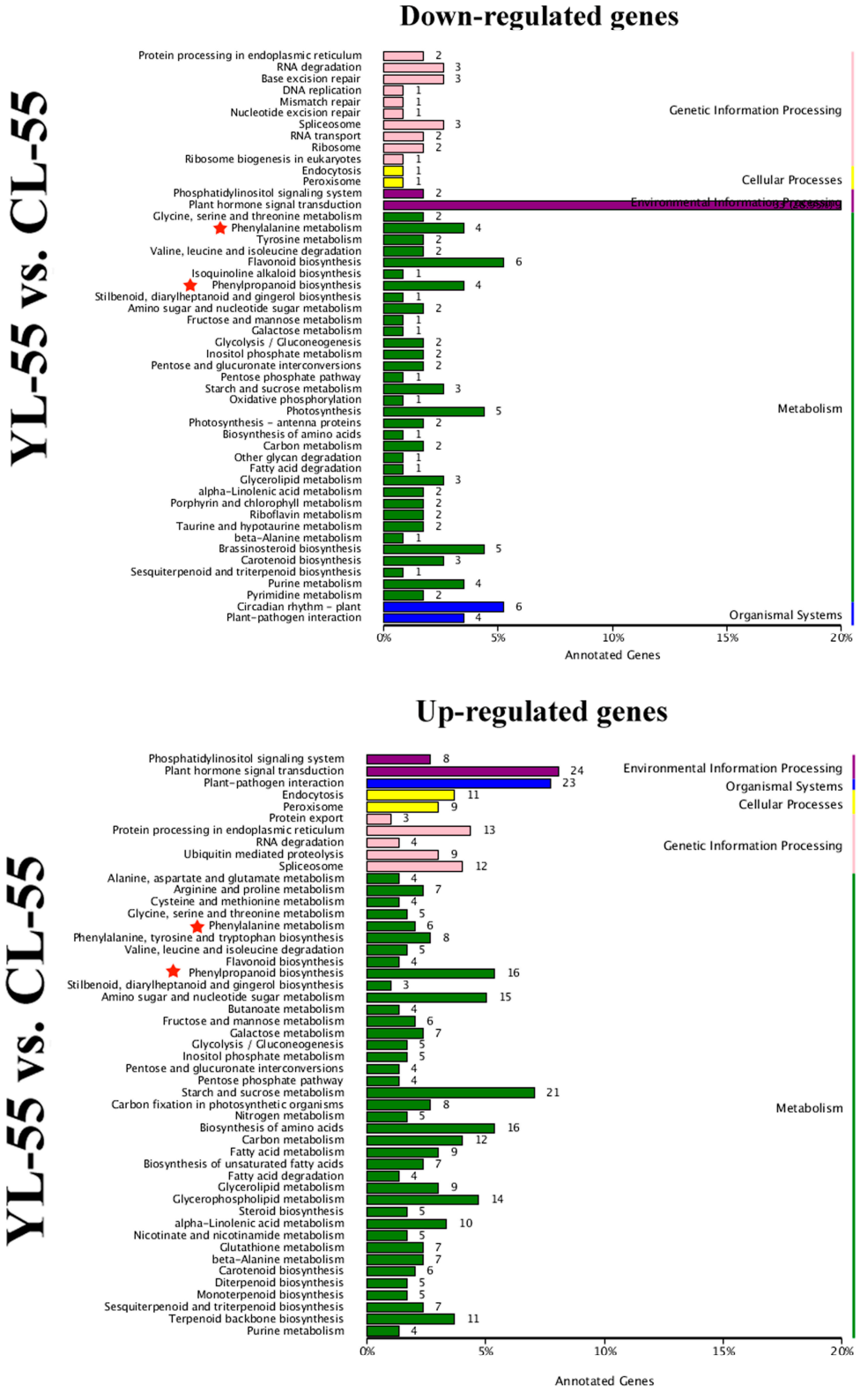

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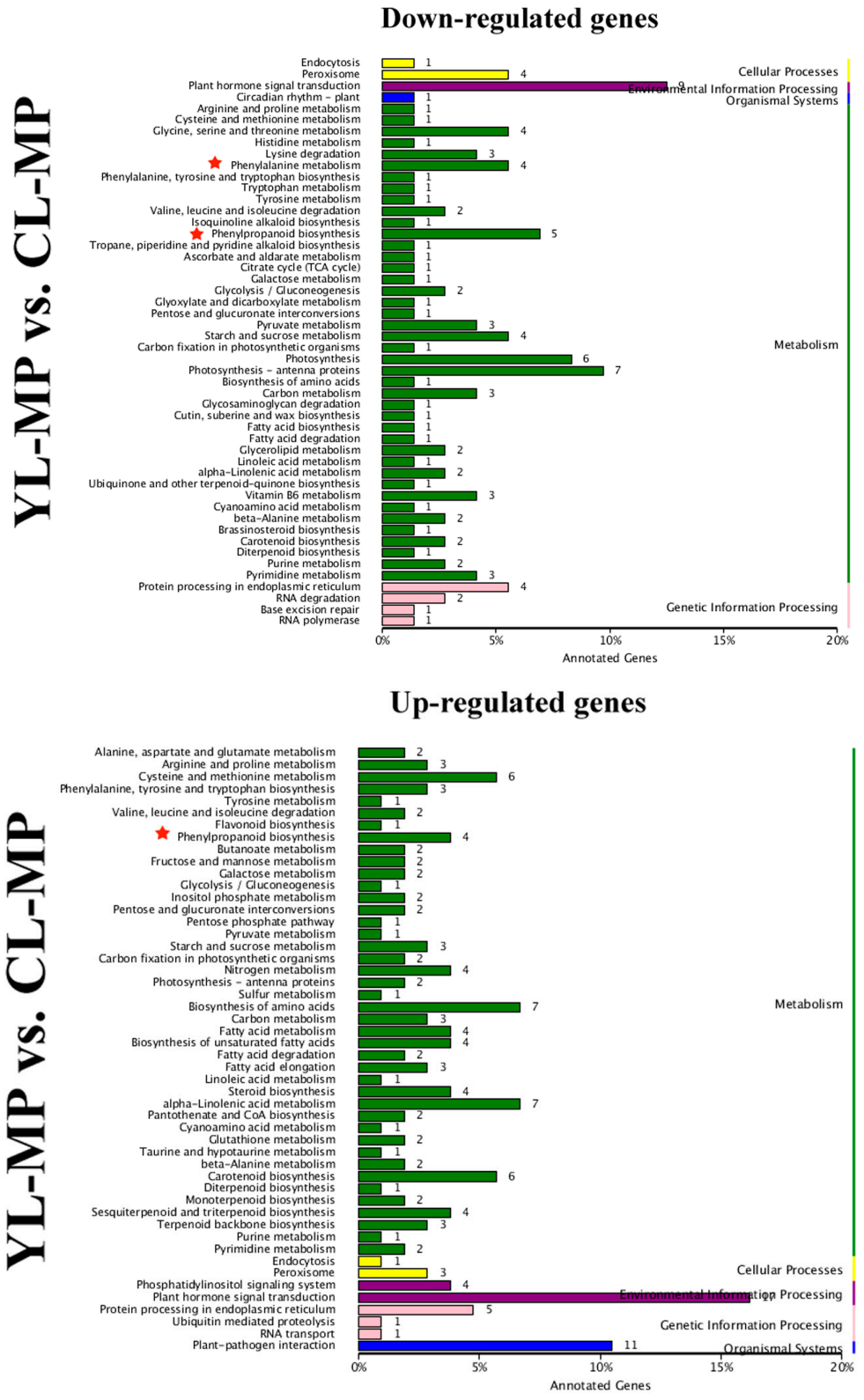

Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) classification maps of differentially expressed genes. The ordinate represents the name of the KEGG metabolic pathway. The abscissa represents the proportion of genes in a metabolic pathway to the total number of annotated genes. Phenylpropanoid biosynthesis and phenylalanine metabolism are marked with an asterisk because they are closely related to lignin metabolism. Up-regulated genes in A_vs._B: genes whose expression level in sample B is higher than that in sample A; otherwise they are down-regulated genes.

We further performed KEGG pathway enrichment analysis with the DEGs in YL-23 vs_CL-23, YL-55 vs_CL-55, and YL-MP vs_CL-MP (Figure 6). The results indicate that plant hormone signal transduction was highly enriched throughout the three key developmental stages (23 DAF, 55 DAF, and mature period). This result suggests that metaxenia has a significant effect on hormone metabolism in fruits. In addition, phenylpropanoid biosynthesis and phenylalanine metabolism were enriched during the early stages of fruit development. When combined with the results of KEGG annotation, it was shown that metaxenia significantly affected the phenylpropanoid metabolic pathway (including lignin biosynthesis) in the early stage of fruit development. At 55 DAF and at maturity, the number of DEGs that were associated with phenylpropanoid metabolism between YL and CL fruits gradually decreased. We speculate that the difference in transcription of phenylpropanoid/phenylalanine metabolism-related genes in the early stage of fruit development might be the cause of the difference in the content of stone cell clusters in YL and CL fruits.

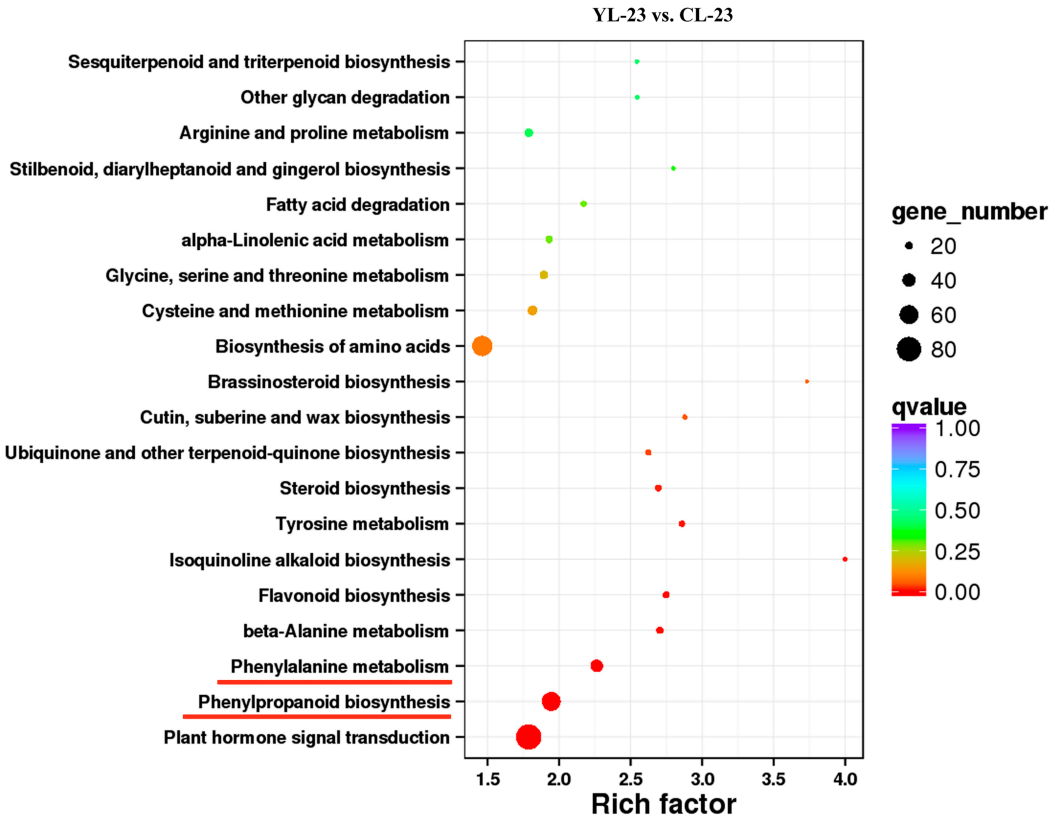

Figure 6. *Cont.*

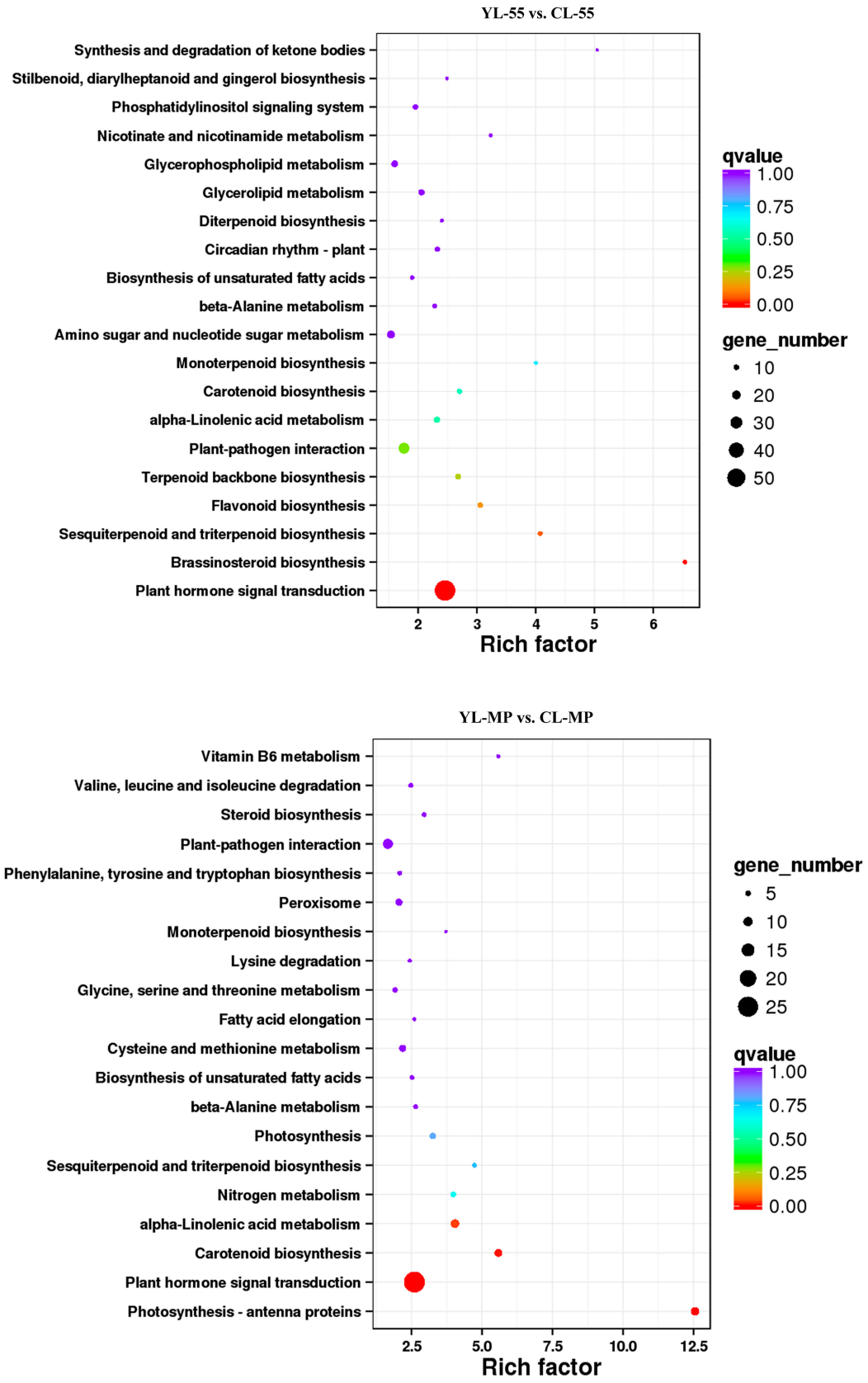

Figure 6. KEGG pathway enrichment analysis of differentially expressed genes. Each circle in the figure represents a metabolic pathway of KEGG. The ordinate indicates the name of the metabolic pathway. The abscissa represents the enrichment factor. Phenylpropanoid biosynthesis (ko00940) and phenylalanine metabolism (ko00360) were enriched in the early stage (23 DAF) of pear fruit development.

3.5. Differentially Expressed Transcription Factors (TFs) between YL and CL fruits

Lignin is one of the main components of stone cells, and lignin metabolism is an important branch of phenylpropanoid metabolism. Therefore, we screened for differentially expressed phenylpropanoid pathway-related TFs between YL and CL fruits (Supplementary Tables S5 and S6). As shown in Supplementary Table S5, several *MYB*s (V-myb myeloblastosis viral oncogene homolog proteins), *WRKY*s, and *KNOX*s (KNOTTED1-like homeobox) were differentially expressed between the two types of pear fruits.

The KEGG annotation showed that the number of DEGs associated with phenylpropanoid metabolism was the highest in the fruit at 23 DAF. Therefore, we focused on the types of TFs that were differentially expressed at 23 DAF. Previous studies have shown that *PbMYB169* (Pbr012624, LOC103959908) and *PbKNOX1* (Pbr019805, LOC103951709) positively and negatively regulate the lignification of pear stone cells, respectively [35,38]. The expression level of *PbMYB169* in YL fruits of 23 DAF was significantly higher than that of CL fruits, while *PbKNOX1* showed the opposite pattern (Figure 7). The differential expression of these genes might result in altered transcriptional levels of the regulated target genes (structural genes for lignin synthesis).

In addition, we analysed the expression profiles of five putative positive regulatory TFs (*PbWLIM1a*, *PbWLIM1b*, *PbMYB4*, *PbMYB30*, and *PbMYB171*) that were related to lignin metabolism in pear fruit [32,39]. The expression levels of these TFs in YL fruits were higher than those in the CL fruits (Figure 7), which suggested that the differential expression of these TFs might be one of the reasons for the difference in phenylpropanoid metabolism between the two types of pear fruits.

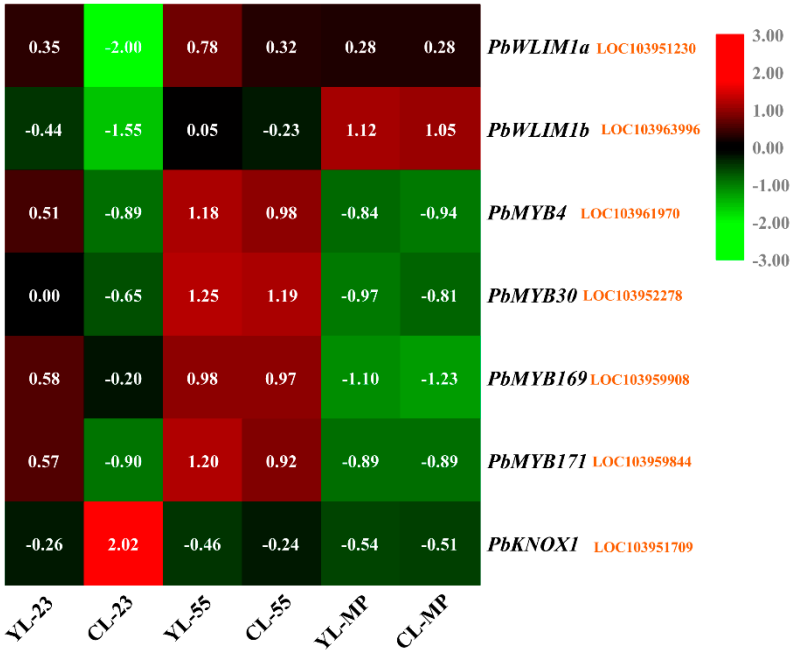

Figure 7. Expression profiles of lignin synthesis-related TFs at different developmental stages of YL and CL fruits. Fragments per kilobase of exon per million fragments mapped (FPKM) values were obtained by RNA-seq analysis and are presented as a heatmap. The heatmap was drawn while using TBTools v. 0.66836 software (College of Horticulture, South China Agricultural University, Guangzhou, China). CL-23: Fruit samples from CL at 23 DAF; CL-55: Fruit samples from CL at 55 DAF; CL-MP: Fruit samples from CL at mature period; YL-23: Fruit samples from YL at 23 DAF; YL-55: Fruit samples from YL at 55 DAF; YL-MP: Fruit samples from YL at mature period. YL: 'Yali'♂ × 'Lianglizaosu'♀; CL: 'Cuiguan'♂ × 'Lianglizaosu'♀.

3.6. Differences in Monolignol Metabolism between YL and CL Fruits

We found that multiple TFs that regulate phenylpropanoid or lignin metabolism had significant differences in the transcription levels between the two types of pear fruit, especially at 23 DAF. Therefore, we systematically compared the structural gene expression profiles in the lignin biosynthesis, transport, and polymerization pathways of pear fruit (Supplementary Table S7 and Figure S3). We previously analysed members of the *4CL* (4-coumarate: CoA ligase), *OMT* (*O*-methyltransferase), *CCR* (cinnamoyl CoA reductase), *CAD* (cinnamyl alcohol dehydrogenase), *UGT* (family-1 uridine diphosphate-glycosyltransferases), and *DIR* (dirigent) gene families [11,17,18,40]. Therefore, members of these gene families have a fixed nomenclature. The naming of members of the shikimate *O*-hydroxycinnamoyltransferase (HCT) and laccase (LAC) gene family was performed according to Xue et al. [19] and Ma et al. [41].

It can be observed from Figure 8 that the transcript abundance of other lignin synthesis genes in YL-23 (YL fruit at 23 DAF) was higher than that in CL-23 (CL fruit at 23 DAF), except for *PbC4H1* and *PbCAD3*. The transcriptional levels of TFs or structural genes were significantly different at 23 DAF, which further indicated that stone cell formation and lignin deposition occurred during the early stages of pear fruit development.

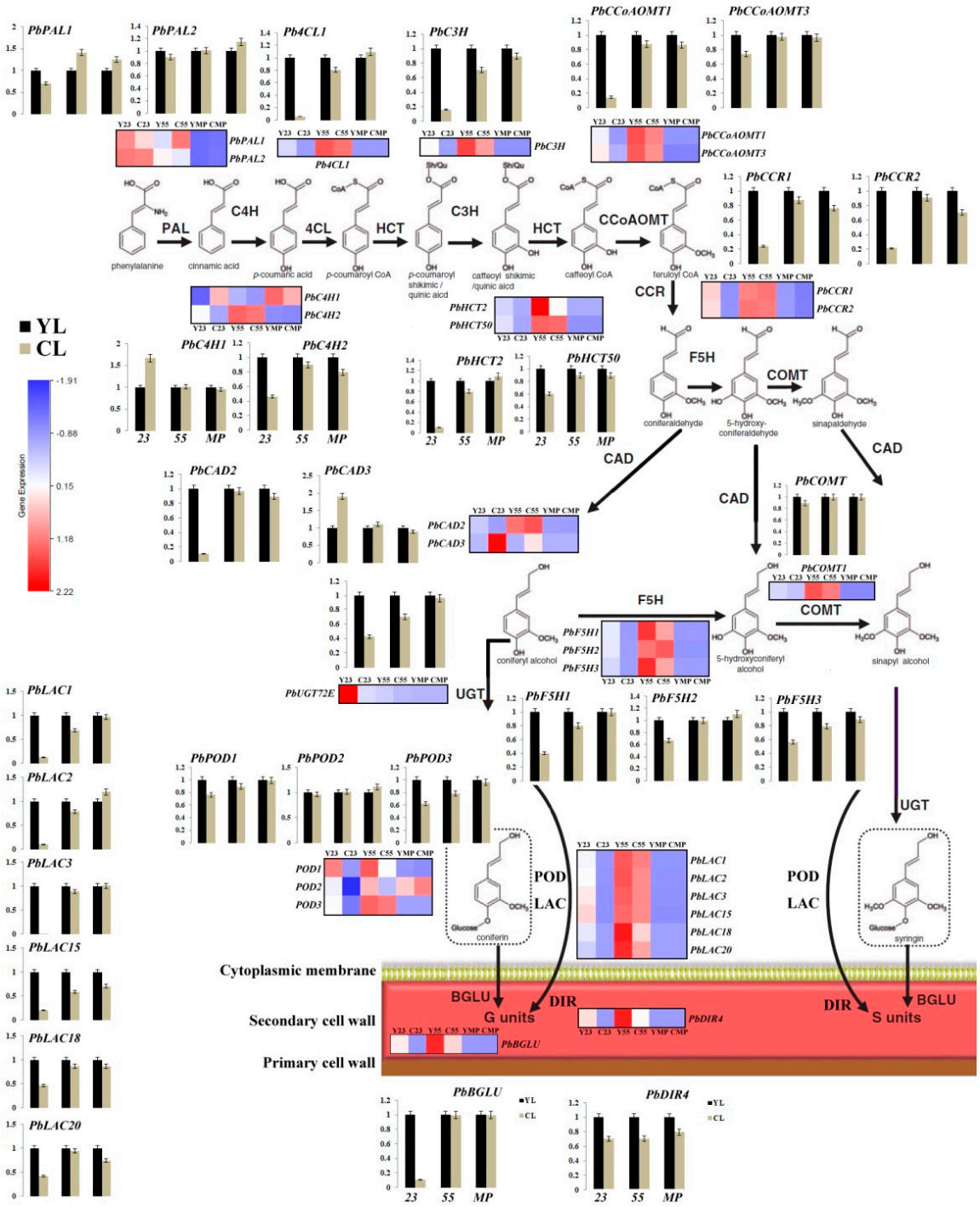

Figure 8. Expression profiling of structural genes involved in monolignol biosynthesis, transport and polymerization. YL: 'Yali'♂ × 'Lianglizaosu'♀; CL: 'Cuiguan'♂ × 'Lianglizaosu'♀; C23: Fruit samples from CL at 23 DAF; C55: Fruit samples from CL at 55 DAF; CMP: Fruit samples from CL at mature period; Y23: Fruit samples from YL at 23 DAF; Y55: Fruit samples from YL at 55 DAF; YMP: Fruit samples from YL at mature period. A colour scale is provided with the heat map to indicate differential pattern of expression. Red indicates a high level of expression, white signifies a medium level of expression, and blue denotes a low level of expression. FPKM values were obtained by RNA-seq analysis and presented as a heatmap. Histograms represent qRT-PCR analyses of selected candidate genes examined in this study. The ordinate of the histogram indicates the relative gene expression level. The abscissa indicates the three developmental stages (23 DAF, 55 DAF, mature period) of pear fruit.

In addition, the transcript abundance of most genes in YL-55 was still higher than that in CL-55, such as *Pb4CL1*, *PbC3H* (*p*-coumarate 3-hydroxylase), *PbC4H2*, *PbHCT2*, *PbHCT50*, *PbCCoAOMT1* (caffeoyl CoA 3-*O*-methyltransferase), *PbCCoAOMT2*, *PbCCR2*, *PbF5H1* (ferulate 5-hydroxylase), *PbF5H3*, *PbCOMT* (caffeate/5-hydroxyferulate *O*-methyltransferase), *PbUGT72E*, *PbBGLU* (*β*-glucosidase), *PbLAC18*, *PbLAC20*, *PbPOD1* (peroxidase), *PbPOD2*, *PbPOD3*, and *PbDIR4* (Figure 8). These results indicate that 'Yali' as the pollen parent promotes the transcription of lignin synthesis-related genes in 'Lianglizaosu' fruit, which results in a large amount of stone cell lignification.

We selected 30 key genes involved in lignin synthesis for qRT-PCR validation to verify the accuracy and reproducibility of the RNA-seq results (Figure 8). The results showed that the transcript abundance and FPKM values of most genes were largely consistent during the three developmental stages of pear fruit, which indicated that the results of transcriptome sequencing were reliable.

3.7. Cloning and Phylogenetic Analysis of Putative Lignin-Related PbC4H2

The results of transcriptome sequencing and qRT-PCR showed that the transcript abundance of *PbC4H2* was significantly different between the YL and CL fruits, and the expression pattern of *PbC4H2* at the three developmental stages of the fruit was consistent with the changes in stone cells and lignin content (Figure 1 and Supplementary Figure S3). In addition, the function of *C4H* in pears, as a second key enzyme in lignin synthesis [42], has not been studied. Therefore, we selected this gene for functional verification to demonstrate whether *PbC4H2* plays a role in stone cell lignification at different stages of pear fruit development.

We designed specific primers to clone the full-length sequences of the coding regions (CDS) of *PbC4H2* (Pbr017290.1) based on information from the pear genome database. The results of maximum likelihood (ML) phylogenetic tree clustering indicated that PbC4H2 and dicotyledon-derived C4Hs clustered into a clade and had close genetic relationships with known lignin-related C4Hs, such as AtC4H, AaC4H, LrC4H, GbC4H, CsC4Ha, CsC4Hb, and CsC4Hc (Figure 9).

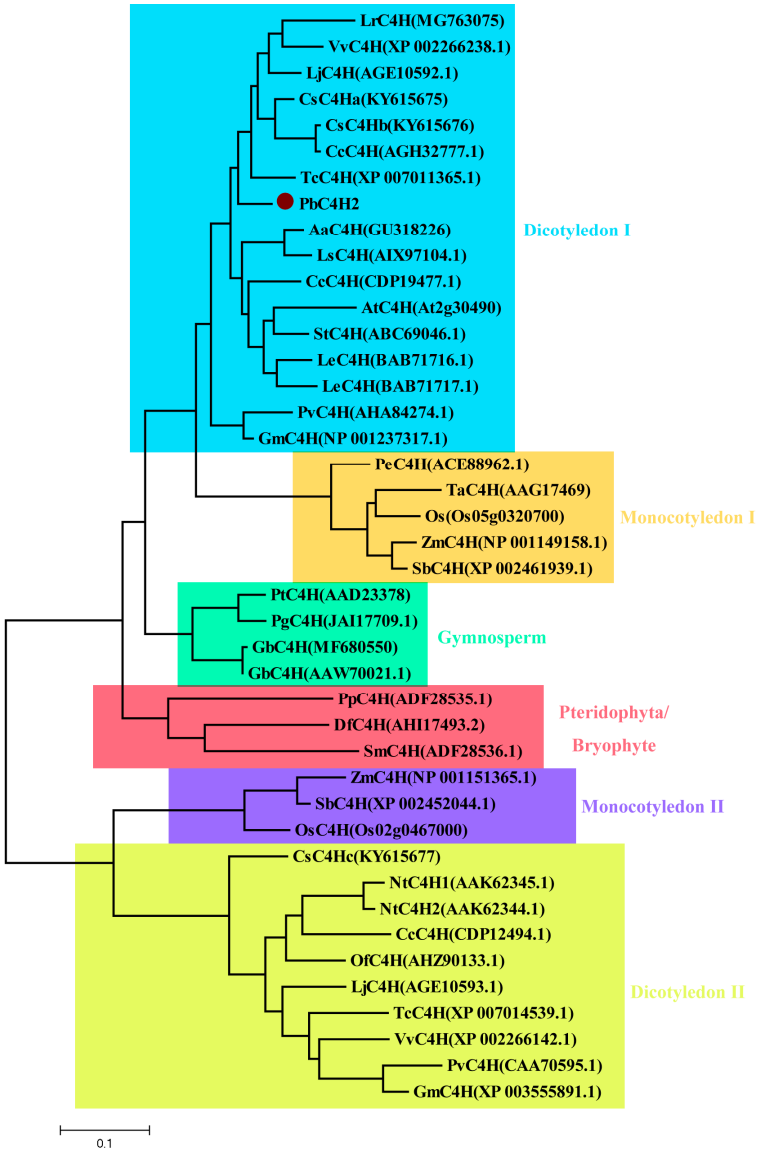

Figure 9. Phylogenetic tree of C4H protein sequences from various plants. Protein sequences were aligned, and a phylogenetic tree was constructed by the maximum likelihood (ML) method using MEGA 5.1 software (Arizona State University, Phoenix, USA).

3.8. Complementation Analysis of the c4h Mutant with PbC4H2

A total of five rescued lines were obtained from the transformation of the Arabidopsis *c4h* mutant. We performed further analysis by selecting three transgenic lines (rescued line-1, rescued line-4, and rescued line-5) with higher transcript levels of *PbC4H2* (Supplementary Figure S4). The cross-sections of the inflorescence stems of the three genotypes (WT, *c4h* mutants, and rescued plants) of Arabidopsis were used for toluidine blue staining, Wiesner staining, and TEM observation (Figure 10). The results of Wiesner and toluidine blue staining showed that the degree of staining of the xylem of the *c4h* mutant was weaker than that of WT and rescued plants, which suggested that the lignin deposition in the cell wall of the *c4h* mutant xylem cells was decreased. In addition, the xylem cells of the *c4h* mutant show some wall irregularities and even collapsed, which is consistent with previous report [43]. However, the heterologous expression of *PbC4H2* in the *c4h* mutant resulted in xylem cells recovering the phenotype of WT plants.

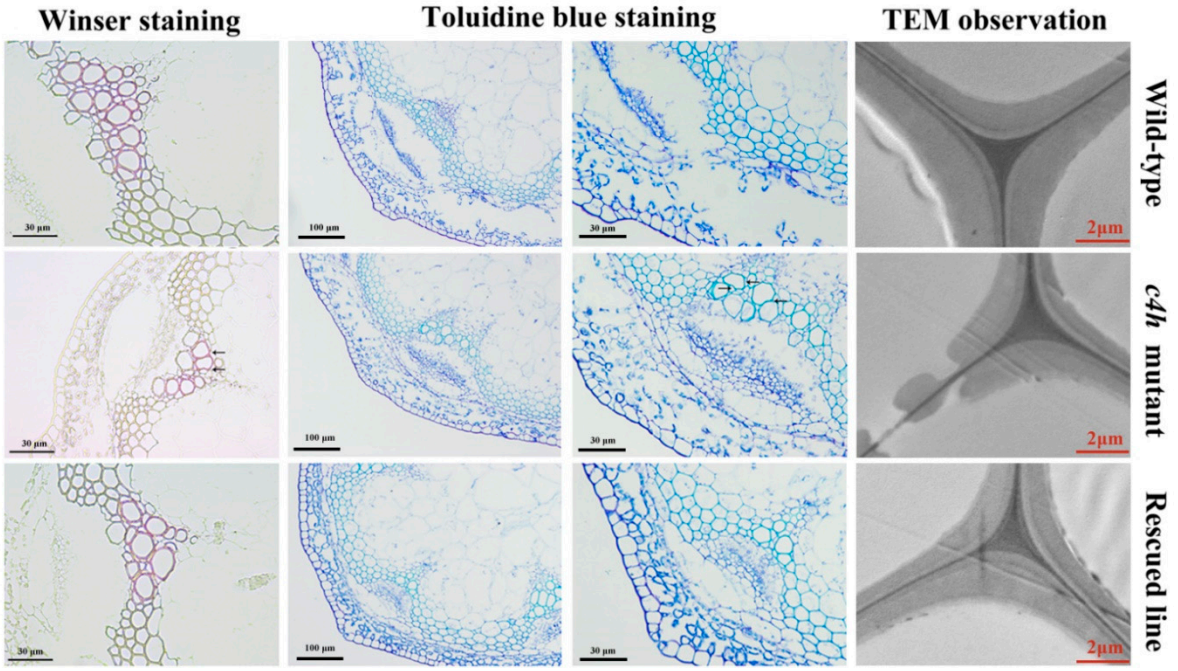

Figure 10. Histochemical staining and ultramicroscopic observation of cell walls in the inflorescence stems of *c4h* mutants, WT, and rescued lines. Some cell wall irregularities or collapses are indicated by the arrows. Three plants of each genotype were selected for observation.

We found that the AcBr lignin content in the inflorescence stem of the *c4h* mutant was reduced by approximately 24% s compared with that of the WT plants, and the difference reached a significant level, through the detection of acetyl bromide (AcBr) lignin (Figure 11a). It is worth noting that the heterologous expression of *PbC4H2* in the *c4h* mutant can almost restore the AcBr lignin content of the *c4h* mutant to the level of WT plants.

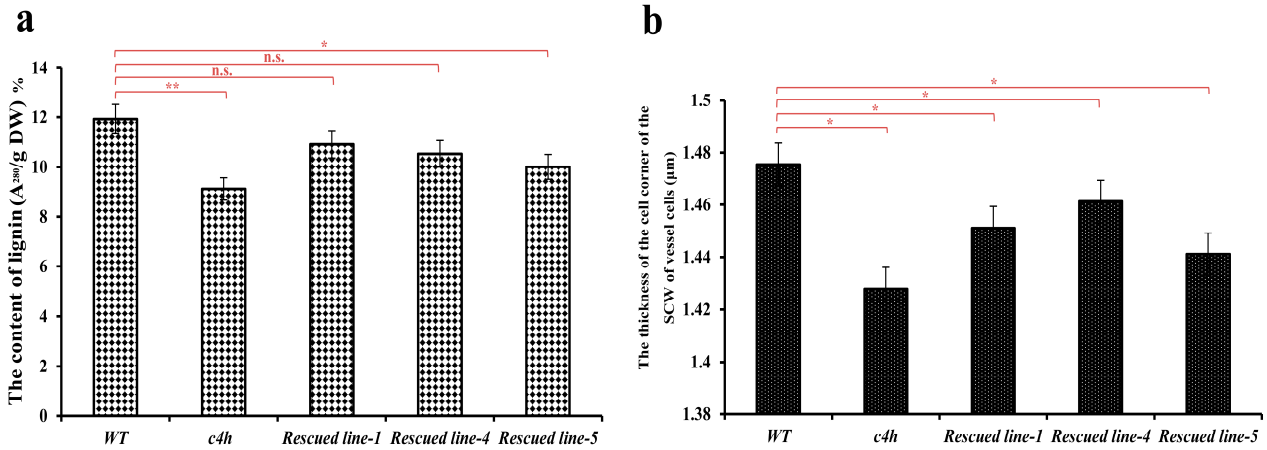

Figure 11. Statistical analysis of lignin content: (**a**) and thickness of SCW at the cell corner of vessel cells; (**b**) in WT, *c4h* mutants and rescued plants. WT: wild-type Arabidopsis; *c4h*: *c4h* mutants. DW: dry weight; gDW: g dry weight. Three biological replicates were performed for each sample. Student's t test was used for the statistical analyses. ** $p < 0.01$; * $p < 0.05$; n.s. no significance.

In addition, the TEM observations revealed that the *PbC4H2* gene also promoted the development of SCW in vessel cells (Figure 10). Although the heterologous expression of *PbC4H2* did not completely restore the cell wall thickness of the mutant to the level of WT plants, it still significantly promoted

the SCW formation of xylem cells of mutant plants (Figures 10 and 11b). These results indicate that *PbC4H2* plays an important role in the development and lignification of SCWs.

4. Discussion

The deposition of lignin in fruits has a certain positive effect [44], but it also has some negative effects, such as lignification in stone cells of pears and in citrus juice sacs [19,45]. At present, there are few studies regarding the regulation of lignification of pear stone cells by metaxenia, and the related molecular mechanisms have not been elucidated. In this study, varieties with high and low stone cell content were used as the pollen parent to analyse its effect on stone cell formation in the pear fruit after pollination.

Through transcriptome sequencing, we found that *PbPAL2*, *PbC4H2*, and *Pb4CL1*, which were involved in the general phenylpropanoid pathway, were upregulated in YL fruits (especially at 23 and 55 DAF) (Figure 8). This upregulation not only significantly affects the monolignol metabolic flux, but also provides sufficient anabolic intermediates for monolignol synthesis. In addition, the transcript abundance of *PbC3H*, *PbHCT2*, *PbHCT50*, *PbCCoAOMT1*, and *PbCCoAOMT2* in YL fruit was also significantly higher than that of CL fruit, which promoted the conversion of hydroxycinnamic acid to hydroxy-CoA thioester (Supplementary Table S7 and Figure S3). Previous studies have shown that the PbCCoAOMT-catalysed reaction is a rate-limiting step in the metabolism of pear lignin [1]. Therefore, high levels of *PbCCoAOMT* expression may significantly increase the accumulation of the lignin monomer.

Our analysis revealed that the key genes in the monolignol-specific biosynthesis pathway (*PbCCR1*, *PbCCR2*, *PbF5H1*, *PbF5H2*, *PbF5H3*, and *PbCOMT*), the transport pathway (*PbUGT72E* and *PbBGLU*), and the polymerization pathway (*PbPOD1*, *PbPOD2*, *PbPOD3*, *PbLAC1*, *PbLAC2*, *PbLAC3*, *PbLAC15*, *PbLAC18*, *PbLAC20*, and *PbDIR4*) were significantly higher in the YL fruit of 23 DAF than in the CL fruit of the same developmental stage (Supplementary Figure S3). This finding suggests that the biosynthesis of lignin monomer in YL fruit is stronger than that of CL fruit during the development of pear fruit, especially in the early stage of fruit development, which might be one of the main reasons for the high content of stone cells in YL fruit. Interestingly, we noticed that the transcript abundance of lignin-specific *LAC*s in YL fruit was higher than that of CL fruit. Xue et al. [19] demonstrated that salicylic acid (SA) can induce *PbrmiR397a* expression and inhibit *LAC* transcription [19]. It has been clarified that the catalytic reaction of C4H and synthesis of SA compete for the same precursor (cinnamic acid) [14]. The high level of transcription of *PbC4H2* in YL fruit causes an increase in the amount of cinnamic acid participating in lignin synthesis, which might reduce the SA level in the fruit to some extent. This results in a decrease in the transcriptional level of *PbrmiR397a*, resulting in an up-regulation of lignin-specific *LAC*s (Figure 12).

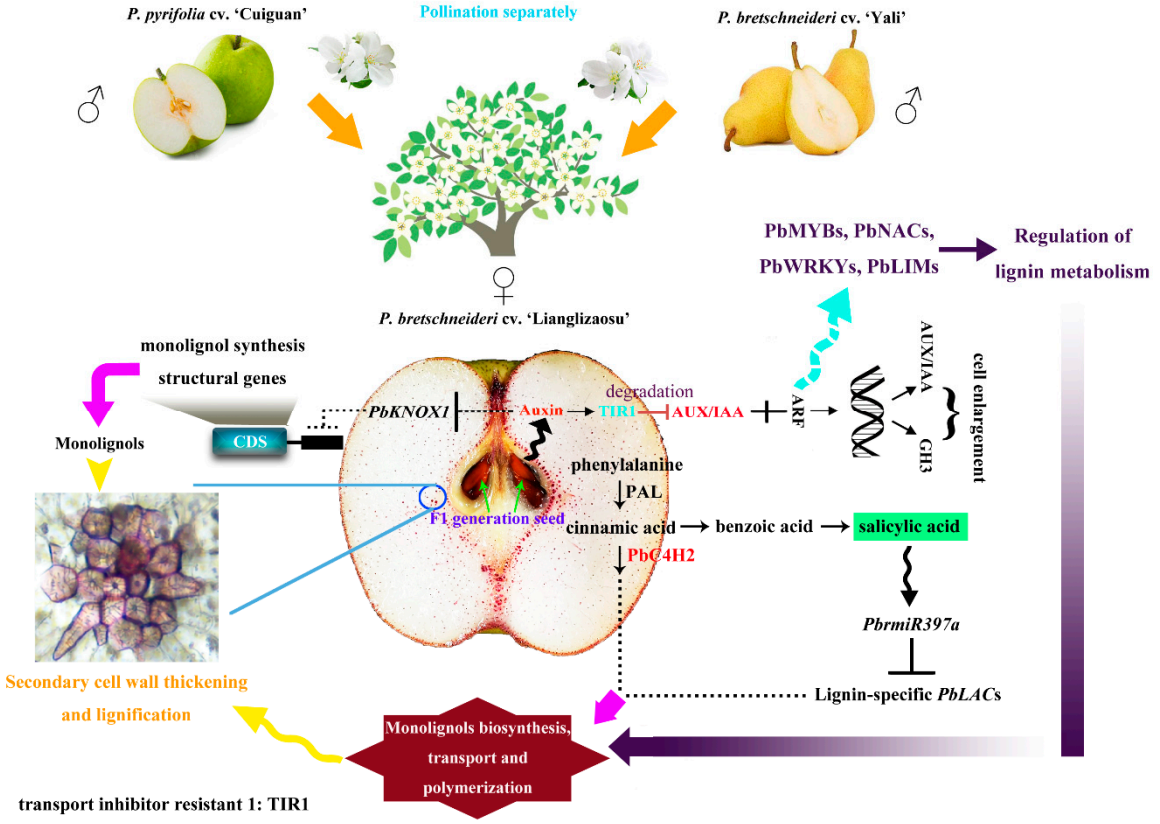

Figure 12. Hypothetical model of the effect of metaxenia on stone cell development and lignin metabolism in pear fruit.

Notably, the difference in lignin and stone cell contents between YL and CL fruits was not significant, although the expression levels of most lignin synthesis-related structural genes and TFs in YL fruits were higher than those in CL fruits at 23 DAF (Figure 1). This result might be due to the formation of stone cells and lignin deposition with hysteresis when compared to gene expression. Xue et al. [19] also found that most lignin-specific LACs reached the peak of transcription level at 35 DAF, but the stone cell content in the flesh reached a high level at 49 DAF [19].

Importantly, the results of transcriptome sequencing and qRT-PCR suggest that metaxenia has a significant effect on lignin metabolism in the early stage of pear fruit development (Supplementary Figure S3). It has been clarified that the parenchyma cells of the flesh first differentiate into sclereid primordium cells in the early stage of pear fruit development, and then further lignified into stone cells [7,11,28]. Therefore, we speculate that lignin synthesis in the early stage of pear fruit development determines the number of sclereid primordium cells. As stone cells and lignin are not degraded after they are formed, they remain in the flesh; therefore, the absolute content of stone cells and lignin in the fruit remains constant, even if the stone cell development and lignification process stop in the late stage of fruit development. It is possible that the growth of stone cell clusters represents mainly volume expansion and the number of newly formed sclereid primordium cells is limited in the middle and late stages of pear fruit development. Therefore, the number of sclereid primordium cells in the early stage of fruit development directly determines the content of stone cells in mature pear fruit.

More strikingly, we found that plant hormone signal transduction-related genes are differentially expressed in YL and CL fruits (Figure 6 and Supplementary Table S8), which suggests that metaxenia has a significant effect on hormone levels in hybrid fruits. There is a hypothesis that seeds in the fruit are heterozygous for the parental genotype. Metaxenia might alter the synthesis of hormones in heterozygous seeds, thereby affecting the levels of hormones secreted into the fruit and ultimately regulating the relevant metabolic processes [26,46]. Therefore, we speculate that the effect of metaxenia

on lignin biosynthesis in pear fruit might be achieved by regulating hormone metabolism. In the early stage of fruit development, a large number of auxin signal transduction-related genes have significant differences in transcript abundance between YL and CL fruits, such as Aux/IAA (auxin/indoleacetic acids) family genes, auxin response factors (ARFs), auxin-responsive proteins, and indole-3-acetic acid-amido synthetase GH3s (Supplementary Table S8) [47,48]. This phenomenon might have a large effect on the content of auxin and its signal transduction in pear fruit. Previous studies have shown that auxin can inhibit the transcription of the lignin biosynthesis negative regulator *BREVIPEDICELLUS* (a member of the *KNOX* gene family) [35], thereby regulating the synthesis of lignin. In addition, Aux/IAAs and ARFs have also been shown to function to regulate lignin synthesis and SCW development [47]. Therefore, metaxenia might change the signal transduction process of auxin in pear fruit, thereby regulating the expression of TFs that are related to lignin metabolism and ultimately affecting lignin deposition and stone cell development (Figure 12).

Cinnamate 4-hydroxylase (EC 1.14.13.11) belongs to the CYP73A subfamily of the cytochrome monooxygenase superfamily [49]. This protein is not only the second key enzyme in the general phenylpropanoid pathway but it also plays a highly important role in the entire pathway [14,42]. Previous studies have shown that the product of C4H, *p*-coumaric acid, is a key substance affecting the metabolism of pear lignin and the development of stone cells [4,13]. Therefore, we investigated the function of the DEG *PbC4H2* in SCW development and lignification in this study. After the heterologous expression of *PbC4H2* in the *c4h* mutant, the lignin content of the inflorescence stems of the rescued plants increased by approximately 15% and the SCW thickness increased by approximately 2% (Figure 11). In particular, *PbC4H2* can restore the collapsed phenotype of the cell wall of the *c4h* mutant (Figure 10). This result strongly indicates that *PbC4H2* plays an important role in pear cell wall development and lignin synthesis. Interestingly, we found that *PbC4H1* and *PbC4H2* showed distinct expression patterns, which are similar to those that were observed in tea plants [49]. *PbC4H1* exhibits high transcript abundance during fruit ripening (Figure 8). The synthesis of lignin and the development of stone cells have largely stopped in this stage [7]. Therefore, *PbC4H1* might not be a lignin-specific *C4H* and it may be responsible for the biosynthesis of other phenylpropanoids. Since studies have shown that phenylpropanoid metabolism plays an important role in fruit ripening, its function in pear fruit warrants further research.

5. Conclusions

In summary, this study used 'Cuiguan' (pollen parent) × 'Lianglizaosu' (seed parent) and 'Yali' (pollen parent) × 'Lianglizaosu' (seed parent) as the materials to demonstrate that metaxenia can significantly affect stone cell development and lignin metabolism in pear fruit. RNA-seq showed that the pollen parents with different stone cell contents had a significant effect on the expression of structural genes (*PbC4H*, *Pb4CL*, *PbC3H*, *PbHCT*, *PbCCoAOMT*, *PbCCR*, *PbF5H*, *PbCOMT*, *PbUGT72E*, *PbBGLU*, *PbPOD*, *PbLAC*, and *PbDIR*) and TFs (*PbKNOX1*, *PbWLIM1a*, *PbWLIM1b*, *PbMYB4*, *PbMYB30*, *PbMYB169*, and *PbMYB171*) that were related to lignin metabolism in the early stage of fruit development. By heterologous expression of *PbC4H2* in the *c4h* mutant, it was demonstrated that this gene has functions responsible for cell wall development and lignin biosynthesis. Our findings not only establish a foundation for regulating the intrinsic quality of pears through metaxenia in the future, but also provide a target gene for the molecular regulation of pear stone cell development.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/1/53/s1, Figure S1: Comparison of stone cell contents in mature fruits of two pollen parents, Figure S2: GO (top line) and COG (bottom line) classification of putative functions of differentially expressed genes, Figure S3: Comparison of transcription levels of lignin synthesis-related genes between YL and CL fruits, Figure S4: Detection of positive plants from transgenic Arabidopsis seedlings, Table S1: Primers used in this study, Table S2: Summary statistics for pear genes based on RNA-seq data, Table S3: Statistics on the number of differentially expressed genes, Table S4: Number of differentially expressed genes annotated, Table S5: Changes of phenylpropanoid metabolism-related TFs in YL and CL fruits, Table S6: Changes of phenylpropanoid pathway-related TFs in YL and CL fruits at 23 DAF, Table S7: DEGs related to lignin metabolism in YL and CL fruits, Table S8: Changes of plant hormone signal transduction-related TFs in YL and CL fruits at 23 DAF.

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Conflicts of Interest: The authors declare that they have no conflict of interest. The experiments were performed in accordance with all relevant Chinese laws.

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