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Genome-Wide analysis of phenylalanine ammonia-lyase (*PAL*) gene family in five Rosaceae plants and expression analysis and functional identification of Chinese white pear

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Phenylalanine ammonia lyase (PAL) plays an important role in the biosynthesis of secondary metabolites regulating plant growth response. To date, the evolutionary history of the *PAL* family in Rosaceae plants remains unclear. In this study, we identified 16 *PAL* homologous genes in five Rosaceae plants (*Pyrus bretschneideri*, *Fragaria vesca*, *Prunus mume*, *Prunus persica*, and *Malus* × *domestica*). We classified these *PAL* genes into three categories based on phylogenetic analysis and all *PAL* genes were distributed on 13 chromosomes. Subsequently, we track gene replication events and perform sliding window analysis. These results revealed the evolution of *PAL* genes in five Rosaceae plants. We predicted the promoter of the *PbPAL* genes by PLANTR CARE online software, and found that the 5'regulatory region of both *PbPAL1* and *PbPAL3* has at least one AC element motif. The results of qRT-PCR analysis found that *PbPAL1* and *PbPAL2* were highly expressed in the stem and root , while expression level of *PbPAL3* was relatively low in different tissues. The expression of *PbPAL1* and *PbPAL2* genes increased firstly and then decreased at different developmental periods of pear fruit. Among them, the expression of *PbPAL1* reached the highest level 55 days after flower. Three *PbPAL* genes were induced by abiotic stress to varying degrees. We transfected *PbPAL1* and *PbPAL2* genes into *Arabidopsis thaliana*, resulting in lignin content increased significantly and thick cell wall of intervascular fibers and xylem cells. In summary, This study laid a foundation for better understanding the molecular evolution of *PAL* genes in five Rosaceae plants. Furthermore, the present study revealed the role of *PbPAL* genes in lignin synthesis, and provided basic data for regulating lignin synthesis and stone cell development of pear.

Genome-Wide analysis of phenylalanine ammonia-lyase (*PAL*) gene family in five Rosaceae plants and expression analysis and functional identification of Chinese white pear

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ABSTRACT

Phenylalanine ammonia lyase (PAL) plays an important role in the biosynthesis of secondary metabolites regulating plant growth response. To date, the evolutionary history of the *PAL* family in Rosaceae plants remains unclear. In this study, we identified 16 *PAL* homologous genes in five Rosaceae plants (*Pyrus bretschneideri*, *Fragaria vesca*, *Prunus mume*, *Prunus persica*, and *Malus* × *domestica*). We classified these *PAL* genes into three categories based on phylogenetic analysis, and all *PAL* genes were distributed on 13 chromosomes. Subsequently, we track gene replication events and perform sliding window analysis. These results revealed the evolution of *PAL* genes in five Rosaceae plants. We predicted the promoter of the *PbPAL* genes by PLANTR CARE online software, and found that the 5'regulatory region of both *PbPAL1* and *PbPAL3* has at least one AC element motif. The results of qRT-PCR analysis found that *PbPAL1* and *PbPAL2* were highly expressed in the stem and root , while expression level of *PbPAL3* was relatively low in different tissues. The expression of *PbPAL1* and *PbPAL2* genes increased firstly and then decreased at different developmental periods of pear fruit. Among them, the expression of *PbPAL1* reached the highest level 55 days after flower. Three *PbPAL* genes were induced by abiotic stress to varying degrees. We transfected *PbPAL1* and *PbPAL2* genes into *Arabidopsis thaliana*, resulting in lignin content increased significantly and thick cell wall of intervascular fibers and xylem cells. In summary, This study laid a foundation for better understanding the molecular evolution of *PAL* genes in five Rosaceae plants. Furthermore, the present study revealed the role of *PbPAL* genes in lignin synthesis, and provided basic data for regulating lignin synthesis and stone cell development of pear.

Keywords: Phenylalanine ammonia lyase (PAL); Rosaceae plants; *P. bretschneideri*; expression analysis; lignin;

INTRODUCTION

Pear, a major fruit variety of the Rosaceae, one of the most important deciduous fruit trees in the world. 'Dangshan Su' pear (*Pyrus bretschneideri* cv. Dangshan Su), originating in Dangshan County, Anhui Province, China, which is the most widely cultivated pear variety at present in China (*Konarska 2013*). But there is a defect in the variety: the content of stone cell mass in its fruit is high and its diameter is large, which restricts the development of 'Dangshan Su' pear industry.

The content and size of the stone cell mass is one of the key factors determining the quality of pear fruit.

The content and diameter of stone cell group significantly affected the meat quality, and the size of stone cell group was highly negatively correlated with the fruit's fine degree (*Jin et al., 2013; Cheng et al., 2018*). In the development of 'Dangshan Su' pear fruit, the two peak of lignin content appeared before the peak of the stone cell content and the maximum diameter of the stone cell mass (*Cheng et al., 2017*). A large amount of lignin synthesis may be material preparation for the development of stone cells. The development of stone cells is closely related to lignin biosynthesis, deposition, and polymerization (*Wu et al., 2013; Yan et al., 2014*). Therefore, by regulating the metabolism and polymerization of lignin in pear fruit. It will affect the development process of the stone cell group, so as to achieve the purpose of changing the content and size of the stone cell mass in the pear fruit.

Phenylalanine ammonia lyase (PAL) plays significant role in phenylpropanol metabolism pathway. PAL, as the first key enzyme in phenylpropanol biosynthesis, catalyzes the conversion of L-phenylalanine to cinnamic acid, linking primary metabolism with secondary metabolism, which is a speed-limiting step in phenylpropanol metabolism (*Wang et al., 2014*). PAL is widely found in various plants. Since the discovery of the first *PAL* gene in barley, more and more PAL genes have been cloned from many higher plants, such as *Rhus chinensis* (*Ma 2013*), *Dendrobium* (*Jin et al., 2013*), *Lycoris radiata* (*Jiang et al., 2013*). Interestingly, *PAL* genes also have been successfully cloned, expressed in some liverworts (*Yu et al., 2014*) and fungi (*Yun et al., 2015*). PAL is the fulcrum enzyme controlling primary metabolism to secondary metabolism in the phenylpropanol metabolic pathway. This metabolic pathway not only produces well-studied flavonoids, concentrated tannins and lignin, but also produces less-studied benzene compounds and phenolic glycosides.

PAL is encoded by a polygenic family and has different numbers of members in different plants, for example, *Brachypodium distachyon* (8 PALs), *Populus trichocarpa* (5 PALs), and *Eucalyptus grandis* (9 PALs) (*Jaime et al., 2016*; *Shi et al., 2016*; *Chong et al., 2018*). In a previous study, the importance of *PAL* genes in plant development and defense has been confirmed. Recently, four *PAL* genes were identified, expressed and characterized in *Arabidopsis thaliana.* Among them, *AtPAL1* and *AtPAL2* are mainly expressed in most tissues, while *AtPAL3* and *4* are relatively low in different tissues (*Cochrane et al., 2014*). Previous studies have shown that there is redundancy in the role of AtPAL protein in PAL double mutants, and the lignin content of *A. thaliana* plants with *pal1 pal2* double mutant decreased significantly, tannicacid in seed coat was lack of concentration (*Chong et al., 2018*). *AtPAL1* and *AtPAL2* sensitive strongly to abiotic environmental factors, such as, temperature and UV-B, and play a redundant role in the synthesis of flavonoids and lignin (*Huang et al., 2010*). In contrast, the expression level of *PAL* genes showed significant difference in poplar. For example, *PtPAL1* and *3* are expressed in most tissues, which they are mainly responsible for the production of concentrated tannins, flavonoids and other phenolic metabolites. Whereas *PtPAL2*, *4* and *5* were found to be mainly expressed in xylem tissues. It is speculated that they may be mainly responsible for lignin synthesis in poplar trees *(Kao et al., 2002; Shi et al., 2010*). Therefore, it can be seen that *PAL* is indispensable in the lignin synthesis.

At present, although the *PAL* family is screened and identified in *A. thaliana*, *Camellia sinensis* and other plants, and their cirtical roles in the formation of catechins, flavonols and their derivatives have also been clarified (*Cass et al., 2015*). However, genome-wide analysis of the phenylalanine ammonia lyase (PAL) gene family in Rosaceae plants is rarely reported. The function of *PAL* family in lignin polymerization is also rarely studied, and there is no report in the study of the pear. We know nothing about which members of the pear *PAL* family are involved in lignin polymerization. To fill this gap, we screened 3 *PAL* members from pear

genome and analyzed them systematically. It includes amino acid property, gene structure, conservative motif, phylogenetic relationship, *cis*-acting elements. Combined with lignin content determination and spatiotemporal expression pattern analysis, the candidate *PAL* members associated with lignin polymerization were identified. In order to lay a solid foundation for the mechanism of lignin polymerization and control the development of stone cells.

MATERIALS AND MEHODS

Plant Materials and Treatments

The bud, stem, leaf, flower, root and fruit were collected from 60 years old pear trees, which managed on a farm in Dangshan, Anhui, China. Fruits with the uniform size were collected at eight time points: 15 DAF (day after flowering), 39 DAF, 47 DAF, 55 DAF 63 DAF, 79 DAF, 102 DAF and 145 DAF. All fruit were stored at-80°C until further use.

To investigate the effect of hormone treatment on gene expression of lignin biosynthesis pathway in pear fruit, we seected pests-free of pear trees at the same age and plant height. The concentration of the hormone treatment [the 0.5 mmol/L abscisic acid (ABA), 0.5 mmol/L methyl jasmonate (MeJA), or 0.2 mmol/L salicylic acid (SA)] was sprayed onto fruits at 39 DAF (*Cheng et al., 2019*). All samples were treated for 3 hours under the same conditions. According to Cai et al.(2010). The pear flesh was weighed about 100 g and frozen at -20°C for 24 hours. Centrifugation at rotational speed of 2000 rpm/min for 5 minutes with distilled water. Then the upper suspended solids are poured out. Subsequently, the sediment was then suspended in 0.5 $moll^{-1}$ HCl for 30 minutes and washed with distilled water. We repeated this operationrseveral times until the upper layer is clear. Finally, stone cells were obtained by filtration and drying. The procedure was repeated three times.

Collection and identification of *PAL* genes

In this study, we have identified the number of *PAL* gene members in five Rosaceae plants. Pear genome database was obtained from (http://gigadb.org/dataset/100083) (*Wu et al., 2013*). The sequence information of *Prunus mummer* (mei), *Malus domestica* (apple), *Prunus persica* (peach) and *Fragaria vesca* (strawberry) gene were obtained from the Phytozomes database (https://phytozome.jgi.doe gov/pz/portal.html) (*Jung et al., 2014*). Initially, we acquired the Hidden Markov Model (HMM) profile of PAL proteins from the Pfam database (http://pfam.sanger.ac.uk/). Subsequently, utilize the HMM profile as a query to identify all PAL-containing sequences by searching against the three of Rosaceae species genome (E-value=0.001). Then, all candidate *PAL*s are validated using Pfam (http://pfam.xfam.org/) (Punta 2011) and SMART database (http://smart.embl-heidelberg.de/) (*Letunic et al., 2012*) to confirm that they contain core domains. Finally, we removed all potentially redundant PAL sequences according to the results of the sequence align-ments.

Conserved motif, *cis*-element and feature analyses of the *PAL* genes

Online analysis tool ExPASy (http://web.expasy.org/compute_pi/) is used to predict the isoelectric point (pI) and protein molecular weight of (kDa) of each PAL the amino acid sequence encoded. Prediction of subcellular localization using online tool MBC (http://cello.life.nctu.edu.tw/). Phylogenetic trees were constructed by the N-J method (bootstrap=1000) in MEGA6.0 software (*Tamura et al., 2011*). Analysis of exons and introns was carried out using the gene structure display server (GSDS) program (*Liu et al., 2016*).

Conserved protein motifs were confirmed by MEME (http://meme-suite.org/) (*Bailey et al., 2015*), which following parameters: the maximum number of motifs is 20, and the base length is between 6-200.

The 2000 bp promoter sequence of the *PbPAL*s family members were obtained from the genome database of 'Dangshan Su' and then the online software PLANTR CARE database was employed to analyze the *cis*-acting elements in the promoter regions (*Lescot et al., 2002*).

Chromosomal locations and Ka (nonsynonymous)/Ks (synonymous) analysis

The chromosomal locations of the *PAL* genes in five Rosaceae plants were obtained from genome annotation documents. The data were then plotted using the Circos software (*Krzywinski et al., 2009*). The duplicated events were categorized into whole genome duplication (WGD)/segmental, and tandem duplicates (*Cao et al., 2018*). Ka and Ks were calculated by DnaSPv5.0 software with the Nei-Gojobori (NG) (*Wang et al., 2010*). Sliding window analysis was also carried out using this software.

RNA extraction and qRT-PCR analysis for *PbPAL* genes

Extraction of total RNA from tissues of pear and pear fruit by plant RNA Isolation Kit (Tiangen, China) for qRT-PCR analysis. Then, the DNA is trans-synthesized from 1 μgRNA transcriptase M-MLV system (Tiangen, Beijing, China), according to the manufacturer instructions. Primers (Table S1) were designed for real-time quantitative PCR (qRT-PCR) using the Beacon Designer 7 software. Tubulin (GenBank accession no. AB239680.1) (*Wu et al., 2013*). Transcript levels were determined using a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD). The total volume of the reaction mixture was 20 μL: 10 μL SYBR Premix Ex Taq II (2x), 2 μL template cDNA, 0.8 μL forward and reverse primers, and ddH_2O to 20 μL. Relative expression of genes was calculated using $2^{-\Delta\Delta CT}$ method.

Arabidopsis transformation

The full-length CDS of *PbPAL1* (GenBank: MF346686) and *PbPAL2* (GenBank: MF346687) were cloned from pear. The correct pMD18-T-*PbPAL* plasmid and pCAMBIA1304 (GenBank: AF234300.1) vector plasmid were digested by restriction endonuclease *Bgl* II and *Spe* I (Takara, Japan) (Table S2), respectively. Subsequently, the recombinant eukaryotic expression plasmid pCAMBIA1304-*PbCPAL* was constructed and successfully obtained by ligation with T4 DNA ligase (Takara, Japan). Transformation of recombinant plasmid pCAMBIA1304-*PbPAL* into *Agrobacterium* tumefaciens EHA105. The *A. tumefacien* culture at 28℃medium with recombinant plasmid pCAMBIA1304-*PbPAL.* Suspension of bacteria with infection buffer (0.02% Silwet L-77, 1/2 MS, 5% Sucrose). The OD_{600} value of the infection solution is about 0.7-0.8, which can be used for subsequent infection.

The seeds of *A*. thaliana were sterilized (75% ethanol for 1 minute, 10% sodium hypochlorite for 13 minutes). After 4 times of sterile water cleaning, the seeds were evenly sown on MS solid medium plate containing hygromycin. After about 15 days, seedlings with 4 true leaves were transplanted into nutrient soil for further cultivation.

Selected pCAMBIA1304-*PbPAL* plants and wild type plants of some lotus leaves growing for about 20 days. Leaf of DNA was extracted and detected by PCR with gusA nonspecific primers at the same time (Table S3).

Lignin staining analysis

Stem segments of 50-day-old transgenic T_3 generation and wild type *A. thaliana* in the same position

155 were taken respectively. The sections were stained with toluidine blue and Wiesner respectively, and directly
156 observed with a microscope. Photographs were taken under a binocular microscope.

157 **RESULTS**

158 **Collection and identification of *PAL* genes in five Rosaceae plants**

159 Based on the HMM sequence on Pfam website (http://pfam xfam org/) and BLASTP strategies, PAL
160 family members were identified from five Rosaceae species. The target sequence was compared with the
161 DNATOOLS software in the genome database, then remove repetitive redundant sequences. Finally, in our
162 study, we identified 16 non-redundant and complete PAL genes in five Rosaceae species (Table 1). The
163 correspondent proteins displayed that their lengths, molecular weights, isoelectric points (pI), were within the
164 ranges of 414-753 amino acids, 44.42-87.75 kDa, 5.79-8.79, respectively (Table 1).

165 **Conserved motifs and gene structure of *PAL* genes of five of Rosaceae species**

166 To investigate the evolutionary relationships of *PAL* family of five Rosaceae species, we constructed a
167 phylogenetic tree using MEGA6.0 (Fig. 1A). Phylogenetic analysis of *PbPAL*s revealed that the existence of
168 highly differentiated *PAL* genes in *P. bretschneideri* and some other Rosaceae plants, which the 16 *PAL* genes
169 were clustered into three major clades. Conservative gene structures may provide a record of key events in the
170 evolution of genes. Furthermore, *PAL* genes structure analysis also supported clustering of occurrence groups.
171 We found that in the same subfamily, the structure of *PAL* is usually very similar (Fig. 1B). But sometimes
172 there are special phenomena, for example, in Cluster II members, the results shown that *FvPAL2* gene structure
173 is longer and contains more than one exon and intron, while *FvPAL1* only contains three exons. Besides, the
174 number, length and location of exons and introns are also different in *PAL* gene. In this study, we found that
175 most members of *PAL* genes in five Rosaceae species contain two or three exons, which means that these
176 genes are highly conserved during evolution.

177 To better understand the structural diversity of PALs, we captured twenty conversed motifs in PAL with
178 the PAL protein sequences using MEME software (Fig. 1C). The conserved motif analysis of PALs proved the
179 reliability of the phylogenetic relationship. Moreover, our results also suggested that most PAL proteins have
180 similar motifs in the same subfamily. Besides, the number of motifs involved in PAL protein sequence was
181 quite uncertain. Coincidentally motifs 1, 2 , 3, 7and 19 were existed all PAL protein sequences of five
182 Rosaceae species. However, some of the motifs were found to be unique to a subfamily. For example, motif 20
183 only was found in Cluster Ⅰ. PbPAL3 had fewer motifs, indicating the PAL domain may be incomplete.

184 **Chromosome location and gene replication event analysis of *PAL* gene family in five Rosaceae plants**

185 To clarify the distribution of *PAL* family members on the chromosomes of five Rosaceae species.
186 According to the genome information of each species, and we constructed a chromosomal location map (Fig.
187 2). The *PAL* genes are randomly distributed on 13 chromosomes. Two genes each are located on one
188 chromosome in strawberry and plum blossom. Three genes each are located on one chromosome in *P.*
189 *brestschneideri.* Two chromosomes containing three genes in *P. persica.* Four out of the 13 chromosomes
190 harbored *MdPAL*s, with 2 (chromosomes 1and 8) possessing one *MdPAL* and 2 (chromosomes 4 and 12)
191 possessing two *MdPAL*s.

192 Segmental or tandem replication is the main way to increase the number of family members in plants. In
193 order to further explore the driving forces of *PAL* gene evolution, we calculated the rate of

194 nonsynonymous/synonymous substitution (Ka/Ks) among five gene. Five pairs of gene replication events were
195 found in sixteen *PAL* genes of five Rosaceae species (Fig. S1). Generally, Ka/Ks>1 indicates positive selection
196 and accelerates evolution; Ka/Ks<1 indicates functional constraints of negative selection. Our results showed
197 that all Ka/Ks pairs of *PAL* genes were less than 1 (Table 2), which illustrates that they have undergone strong
198 evolutionary selection, and their functions have not been seriously differentiated. Except *MdPAL3/MdPAL6*
199 belonged to tandem replication, the others were fragment replication, which indicated that the expansion of
200 PAL family of five Rosaceae species was mainly due to fragment replication events.

201 **Promoter analysis of *PAL* genes in pear**

202 To further understand the regulation mechanism of *PbPAL* genes expression, we predicted possible *cis*-
203 acting elements using PLANTR CARE online software (Table S4 and Fig. 3). It was found that the promoter
204 of *PbPAL* genes contained two types of stress response regulatory elements, such as MBS and LTR repetitive
205 sequences, which responds to drought induction, and cold stress, respectively. Among which four kinds of
206 hormone regulatory elements: ERE, ABRE, CGTAC-motif and TCA-element were associated with ethylene,
207 ABA, MeJA and SA responses respectively. In addition, two members of the *PbPAL*s families contain the
208 MRE light-responsive element, which hinted that expression of *PbPAL*s were closely related to light.
209 Furthermore, we found that *PbPAL1* and *3* gene contains at least one AC element, AC element can activate
210 lignin monomer synthesis gene by binding with MYB transcription factor (*Patzlaff et al., 2010*). Therefore, we
211 proposed that expression of *PbPAL* genes are closely related to lignin formation.

212 **Phylogenetic analysis of *PAL* genes in pear and other plants**

213 In a recent study shown that *NnPAL1* as an ancient member of the *PAL* gene family, and was found to be
214 a polybasic origin in the evolution of PAL in angiosperms (*Wu et al., 2014; Wu et al., 2017*). To investigate
215 the phylogenetic relationships of *PbPAL* genes with other plants *PAL* genes, which a neighbor-joining tree was
216 created. The phylogenetic tree clustering results showed that *PAL* genes of fifteen species could be divided into
217 three well-supported families (Fig. 4). Formely studies have shown that the *PAL* genes family was divided into
218 a subfamily of *A. thaliana*, which was consistent with our classification results (*Jaime et al., 2016*). During the
219 evolution of *PAL*, the recurrence of specific pedigrees occurred in *A. thaliana*, *P. trichocarpa* and *Selaginella*
220 *moellendorffii*. This is supposed to be a universal phenomenon that promotes the diversity of polygenic
221 families. In this study, the *PbPAL*s were intimately related to dicotyledon plant PAL and belongs to the group.
222 However, the three *PbPAL*s were aggregated with each other and form a different subgroup. Interestingly, just
223 as the results of *PbPAL*s classification are resemble, most of plant *PAL* genes are clustered by species, and
224 *PAL* genes are in one species are closer to each other than their homologues in another. Based on this evidence,
225 PAL diversity occurs independently in each species.

226 **Expression profiles of pear *PAL* genes in different tissues and developmental stages of fruits**

227 As everyone knows, it is possible to probe the potential functions of gene families by means of gene
228 expression analysis (*Cao et al., 2016*). In order to further describe the function of pear *PAL* genes, and
229 comparative gene expression analysis was carried out in different tissues or organs (leaf, stem, flower, root and
230 bud) (Fig. 5). Transcript levels for *PbPAL1* and *2* were higher in lignified tissues (roots and stems) than in less
231 lignified tissues (leavess, buds and flowes) (Fig. 5A). Therefore, *PbPAL1* and *2* are highly expressed in stems
232 and roots, and we conjectured that they may be involved in lignin biosynthesis in pear. While expression level
233 of *PbPAL3* was relatively low in different tissues. These results suggested that different *PbPAL* genes may

234 play key roles in the development of specific tissues.

235 Stone cell content is an important factor affecting the quality of pear fruit. As one of the main components
236 of stone cell wall, lignin synthesis directly affects the formation of stone cells rich in pear fruits (*Cai et al.,*
237 *2010; Jin et al., 2013*). Moreover, the change of lignin content is also related to the change of stone cell
238 content.Subsequently, the expression profiles of these *PbPAL* genes at different the stages of fruit development
239 were also surveyed by using qRT-PCR (Fig. 5B). Formely, studies have shown that the content of stone cell
240 and lignin in pear fruit first increased and then decreased during fruit development, reaching the peak at 47 and
241 55 DAF (*Cai et al., 2013*). It is notewory that the expression levels of *PbPAL1* and *2* were similar to the
242 content of stone cell and lignin in pear fruits, indicating that these genes might be related to lignin aggregation
243 and stone cell formation in pear fruits. This study implying that *PbPAL1* and *2* are closely related to lignin
244 synthesis and stone cell development. While *PbPAL3* was hightly expressioned in the 79, 102 and 145 DAF,
245 indicating that this gene might play important roles in the mature stage of pear fruit development.

246 **Differentially expressed *PbPAL* genes under hormonal treatment**

247 Previous studies have shown that the expression of *PAL*s are subjected to abiotic stress *(Chong et al.,*
248 *2015*). However, information on *PAL*s involvement in pear hormone response is limited. Previous studies have
249 found that spraying exogenous hormones on pear fruits can regulate stone cell development and lignin
250 synthesis in pear fruits to a certain extent (*Yang et al., 2014*). We through the analysis of *cis*-acting elements in
251 promoters of *PbPAL* family members, and found that most of the promoters of *PbPAL* genes contain a variety
252 of biological or abiotic stress-related elements (Table S4). Consequently, we hope to further study whether the
253 hormones involved in these stress responses (SA, MeJA and ABA) could alter the expression of these genes
254 (Fig. 6). After ABA treatment, the expression of *PbPAL1* was obviously induced, while the expression of
255 *PbPAL2* was reversed, and the expression level was significantly inhibited. Interestingly, the expression of
256 *PbPAL3* was induced at 1 and 3 hours of treatment, but inhibited at 2 hours, which the lowest expression level
257 was found in 2 hours of treatment (Fig. 6A).

258 In the MeJA-treated pear fruit, *PbPAL2* and *PbPAL3* showed the same trend, and were inhibited in 1 hour
259 and 3 hours of treatment. After 2 hours of treatment, they were significantly induced and the expression level
260 reached peak. However, the expression level of *PbPAL1* showed an obvious opposite trend. The expression of
261 *PbPAL1* was induced at 1 and 3 hours of treatment, and the expression level reached peak at 3 hours after
262 treatment. After 2 hours of treatment, the expression level was significantly inhibited (Fig. 6B).

263 The response patterns of *PbPAL*s to SA can be divided into two categories, including inhibiting gene
264 expression and inducing gene expression. SA inhibited the expression of *PbPAL1* and *PbPAL2*, which was the
265 lowest at 1 h. The other *PbPAL3* was induced by SA and peaked at 1 h with the prolongation of treatment time
266 and the induction degree decreased (Fig. 6C).

267

268 **Determination of lignin content in transgenic *A. thaliana* of *PbPAL*s**

269 To further determine the role of candidate *PbPAL* genes in plant lignin synthesis, and we obtained
270 transgenic *A. thaliana* plants with candidate genes. Firstly, we constructed an eukaryotic expression vector
271 (Fig. 7A). The DNA of the transgenic strain was amplified by GFP specific primers on pcambiA1304 vector
272 (Fig. 7B). The successful cloning of the target fragment of about 700 bp indicated that the foreign gene has
273 been successfully integrated into the *A. thaliana* genome (Fig. 7C). Subsequently, we successfully obtained

three T_3 generation transgenic lines of *PbPAL1* and *PbPAL2.* We determined the lignin amount of *A. thaliana* inflorescence stem and leaf by acetyl bromide method (Fig. 8). The results made clear that the lignin content in stems of transgenic plants of *PbPAL1* (12.42%) and *PbPAL2* (12.17%) was significantly higher compared to that of wild type plants (10.47%) (Fig. 8A). In addition, we determined that the lignin content in the leaves of transgenic *PbPAL1* (7.15%) and *PbPAL2* (7.01%) plants was also higher than that in wild *A. thaliana* (6.18%) (Fig. 8B). Our work demonstrated that both *PbPAL1* and *2* genes may are involved in plant lignin synthesis.

Lignin staining analysis

To observe the distribution of lignin in the inflorescence stem of transgenic *A. thaliana* intuitively. Hand cross-sections of stems of wild-type, transgenic plants was stained with phloroglucinol to identify possible changes in the content and/or distribution of lignified tissues. The Wiesner staining results showed that the strongest staining of xylem and intervascular fibers were observed in the stem of *PbPAL1* and *PbPAL2* transgenic *A. thaliana* than in wild type plants (Fig. 9). Furthermore, toluidine blue staining showed the cell wall of cross-sectional area of pedicels in *A. thaliana* (Fig. 10). The cell wall thickness of *PbPAL1* and *PbPAL2* transgenic plants increased significantly. Two dyeing results showed that *PbPAL1* and *PbPAL2* could increase lignin synthesis. This is consistent with many previous studies, which *PAL* gene is related to the degree of lignification of plants (*Chong et al., 2018*).

DISCUSSION

The content and size of stone cells are the critical factors affecting fruit quality (*Jin et al., 2013; Li et al., 2017*). It has been found that lignin plays a key role in the formation of stone cells. Lignin deposits on the cell wall of pear fruits, making the secondary cell wall thicker (*Cai et al., 2010; Tao et al., 2015*). The present study found that there is a strong correlation between the formation of stone cells and lignin biosynthesis, which supported the view that lignin plays a vital function in stone cell biosynthesis (*Jin et al., 2013*). Therefore, the study of lignin metabolism in pear fruits is of great significance to the regulation of stone cell development. Lignin is produced by several metabolites of phenylpropanol *(Rao et al., 2018; Wang et al., 2017*). Phenylalanine ammonia lyase (PAL) is one of the key enzymes in lignin metabolism pathway (*Starr et al., 2014*). Therefore, screening and identifying *PbPAL* genes related to lignin synthesis are of great significance for regulating lignin synthesis and stone cell development in pear.

In addition, PAL is also one of the branching enzymes linking primary and secondary metabolism (*Ma et al., 2016*). The first step in catalyzing cinnamic acid (a precursor) to form various phenylpropanol derivatives. In the present study, we identifified 16 *PAL* genes from five Rosaceae species (Table 1). The number of *PAL* genes in apple are nearly twice than that in pear, while the chromosome numbers of pear and apple were the same. Previous studies have revealed that genome-wide replication of pear and apple offspring is based on WGD event learning in recent genome evolution processes (*Xu et al., 2018*). At the initial stage of evolution, the common ancestor of Rosaceae plants had nine chromosomes (*Chong et al., 2018*). Pear (*P. bretschneideri*) and apple (*M. domestica*) both experienced WGDs (Mya) and 30-45 Mya twice 130 million years ago, but only 17 chromosomes were found (*Guo et al., 2013*). This discovery indicatesd that the ancestors of the nine chromosomes of Rosaceae plants experienced doublingand breaking. After a long period of fusion, 17 chromosomes of pear and apple were finally formed. In this evolutionary process, the genome of a species may become very unstable, and it is ease to chromosome rearrangement, gene replacement and gene loss. In this

process, the *PAL* genes in pear may be lost, which also explains why the number of *PAL* genes in pear is much lower than that in apple.

Gene structure and conserved sequence construction may be intimately interrelated to the diversity of gene function (*Cao et al., 2018*). As anticipated, conserved domain analysis using these PAL protein sequences showed that genes of the same subfamily often had very similar genetic structures, suggesting that these genes might have similar functions (Fig. 1B). For example, *PmPAL2* and *PbPAL2* in Cluster III have the same genetic structure (two exons and one introns) and almost the same exon length. In addition, basing on the results of MEME analysis (Fig. 1C), we found that members of the same subfamily tend to have approximately the same conserved protein motif, but there are some differences in the motif composition among members of different subfamilies. We also found that some families contain specific conservative motifs, which means that these specific conservative motifs may be necessary for the specific functions of the subgroup, such as motifs 20 to Cluster Ⅰ family.

Promoters regulate gene expression mainly at the transcriptional level and are coordinated by a variety of *cis*-acting elements and trans-acting factors (*Soliman et al., 2019*). We discovered a great deal of hormone responsive *cis*-acting components in the upper reaches regulatory sequences of *PbPAL* genes family members (Table S4). Especially, *PbPAL1* only contains abscisic acid (ABA)-responsive elements (ABREs) and *PbPAL2* only contains salicylic acid (SA)-responsive element. While abscisic acid (ABA)-responsive elements (ABREs), the methyl jasmonate (MeJA)-responsive element (CGTCA motif) and salicylic acid (SA)-responsive element (TCA element) were all found in *PbPAL3*. In addition, ethylene responsive elements (EREs) was only identified in *PbAL1* gene. These exogenous hormones are extensively participated in signaling pathways of mature aging or stress response *(Betz et al., 2001*), which suggests that *PbPAL* family members might be involved in pear maturation and stress response.

In addition, we also found some cis-acting elements related to biological and abiotic stress in the upstream regulatory sequences of the *PbPAL* genes, such as the TC-rich repeat element (related to defence) and microtherm stress-related (LTR), and drought stress-related (MBS) elements (Table S4). These results suggested that *PbPAL* gene family members may play a role in response to various abiotic and biological stresses. Interestingly, we found that the 5'regulatory region of *PbPAL1* and *PbPAL3* has at least one AC element motif. AC element is a *cis*-acting element extensive consisting in the 5'regulatory region of lignin biosynthesis genes such as *PAL*, *C4H* and *CAD* (*Xu et al., 2014*). It can activate lignin monomer synthesis gene by binding with MYB transcription factor (*Cao et al., 2016*). In addition, AC is in charge of the xylem-specific expression of lignin biosynthetic genes (*Chong et al., 2018*). Therefore, we founde that the AC ements in the 5'regulatory region of *PbPAL1* and *PbPAL3,* which hinted that they may be participated in the biosynthesis of pear lignin.

Gene expression patterns can provide important clues for exploring gene function (*Budak et al., 2017; Thomas et al., 2018*). Previous researches have shown confirmed that the expression of the *PAL* genes were affected by exogenous hormone and salt in *C. sinensis* or drought stress in oil palm (*Chong et al., 2018; Cao et al., 2016*). To date, the role of *PAL* gene in fruit development is still unknown. Stone cell is one of the crucial factors affecting character of pear fruit and lignin is the essential contituent of stone cell (*Yang et al., 2015*). The stone cells in ‘Dangshan Su’ pear was increased first and then decreased from between 39-63 DAF and the highest content was 47 DAF (*Cai et al., 2010; Chen et al., 2014*). In this study,the qRT-PCR results shown that the *PbPAL1* and *PbPAL2* genes expression pattern showed a change tendency similar to that of the conent

of lignin at different stages of pear fruit development. More importantly, we found that the expression of *PbPAL1* increased significantly at 55 DAF and showed a similar expression pattern to that of key genes participated in the regulation of lignin biosynthesis pathway (*Xie et al., 2013*). These results strongly hated that the *PbPAL1* and *PbPAL2* genes may regulate lignin synthesis in pear fruit. In addition, we found that the expression level of *PbPAL3* gene was low at early stage of pear fruit development, but higher in the late stages of fruit development. This is basically consistent with the expression level of *RiPAL2* gene in Raspberry (*Ellis et al., 2001*), which implies that *PbPAL3* gene plays an important role in the later stage of pear fruit development. These results suggested that the genetic diversity and functional differentiation of *PbPAL* genes are necessary for plants to adapt to the environment.

Not only can gene replication events promote the functional differentiation of *PAL* family during plant growth and development, but also PAL gene family under abiotic stress (*Wu et al., 2017*). For example, only *AtPAL1* and *AtPAL2* have functional specificity for nitrogen deficiency and low temperature in *A. thaliana* (*Olsen et al., 2008*). To understand the effect of abiotic stress on the expression level of *PbPAL* genes, We analyzed *cis*-molecules in the 5'upstream region and discovered that *PbPAL* genes comprise a great deal of elements responsive to ABA, SA and MeJA (Table S4) and studied the hormonal response pattern of *PbPAL* genes. *PbPAL*s were induced or inhibited to varying degrees under several exogenous hormones treatments. MeJA can enhance disease resistance by stimulating plant defense mechanisms. Previous studies have reported that exogenous MeJA therapy enhances the induction of resistance, including the improvement of PAL activity in the phenylpropanol pathway (*Wang et al., 2014*). In present study, the expression levels of three *PbPAL* were all up-regulated after MeJA treatment. Therefore, the application of MeJA in pear fruit production can improve the disease resistance and content of phenylpropanoid compounds. The same gene expressed differently in different hormone treatments. Treating different genes with the same exogenous hormone results in similar or opposite expression trends of different genes. This indicated that the response pattern of *PbPAL* gene to hormones is very complex. We speculated that different *PbPAL* genes play a role in different periods of time in adverse situation.

We have clearly known that some enzymes are involved in lignin synthesis. In some cases, appropriate genetic manipulation has altered the composition of lignin or reduced the content of lignin (*Weng et al., 2010*). In many studies, *PAL* genes have been found to be associated with lignification in plants. So far, very little has been reported about on lignin synthesis of pear *PAL* genes. Our results suggested that *PbPAL1* and *PbPAL2* may be involved in lignin biosynthesis in pears. Our hypothesis is further supported by the study of *PbPAL1* and *PbPAL2* in transgenic *A. thaliana*. The results showed that overexpression of *PbPAL1* and *PbPAL2* genes in *A*. thaliana could aggrandize the lignin content and cell wall thickness of plants. In future studies, we will transform the *PbPAL* genes mutant into *A*. thaliana to further analyze its role in lignin synthesis.

CONCLUSIONS

In the present study, we screened and identified members of the PAL family from five Rosaceae genomes. In the aggregate, 16 *PAL* genes were identified and three of them are from Chinese white pear. All *PAL* genes are divided into three subfamilies on basis of phylogenetic analysis and structural characteristics of protein sequences. All *PAL* genes were evenly distributed on 13 chromosomes. Gene replication event analysis showed that tandem or fragment replication played an important role in the expansion of PAL gene in Rosaceae species. Finally, qRT-PCR expression analysis showed that *PbPAL1* and *PbPAL2* might be involved

394 in the formation of lignin and stone cells in pear fruits and transgenic experiments confirm the above
395 conclusions.
396 PAL genes has many functions, our research focuses on the relationship between *PAL* gene and lignin and
397 stone cell formation, which is a complete analysis of pear fruit. Heterologous expression of *PbPAL1* and
398 *PbPAL2* genes in *A*. thaliana indicated that it was involved in lignin metabolism and cell wall growth. All in all,
399 our observations can a provied basis understood of the five Rosaceae species' *PAL* genes. Moreover, this
400 research not only revealed the role of *PbPAL* genes in lignin synthesis, and provided basic data for regulating
401 lignin synthesis and stone cell development of pear by molecular biology technology.

402 **Patents**

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557 **Figure legends**

558 **Figure 1 Phylogenetic relationships, and gene structures and domain of *PAL*.** (**A**) Phylogenetic tree of *PAL*
559 genes was conserved using MEGA 6.0 by the N-J method. (**B**) Exon-intron organization of *PAL* genes using GSDS
560 program. The exons and introns are indicated by arrows and thin lines, respectively. (**C**) Conserved domains in
561 PAL proteins using the MEME program.

562 **Figure 2 *PAL* genes chromosomal location of five Rosaceae species**. The chromosome number is indicated at the top of each
563 chromosome. Different chromosome colours represent different species.

564 **Figure 3 Distribution of main *cis*-elements and putative regulating factors in the promoter regions of pear antioxidant**
565 **enzyme genes.** Different *cis*-elements with the same or similar functions are shown in the same color.

566 **Figure 4 Phylogenetic analysis of *PAL*s constructed by the Neighbor-Joining method.** These PAL sequences were clustered
567 into three groups, purple, green and red lines indicate the three subfamilies of the PAL proteins.

568 **Figure 5 Expression analysis of *PbPAL* genes in various tissues of pear.** (**A**) and in different stages of fruit development. (**B**)
569 15 days after flowering (DAF), 39 DAF, 47 DAF, 55 DAF, 63 DAF, 79 DAF, 102 DAF and mature stage (145 DAF),
570 respectively. Y- axes on the left indicates the relative gene expression levels; (X-axis) by bar charts, and the Y- axes on right
571 showed the content of stone cells during fruit development with line charts. Each histogram represents the mean value and the bar
572 ± standard error of three biological replicates.

573 **Figure 6 Hormone response pattern analysis of *PbPAL*s. *PbPAL*s expression in pear fruits in response to exogenous**
574 **hormone (A: ABA; B: MeJA; C: SA;) treatment for 0, 1, 2, and 3 h.** As shown in the bar at the lower right corner, gene
575 transcription abundance is expressed in different colors on the map.

576 **Figure 7 Vector Construction and Overexpression**. (**A**) pCAMBIA1304-*PbPAL*; (**B**) *PbPAL* gene was cloned and then
577 inserted into the expression plasmid pCAMBIA1304-PbPAL; (**C**) The PCR analysis used specific primers to amplify the 700 bp
578 internal fragment of gus, M: DL2000 DNA Marker;1-2: pure water; 3-4: pCAMBIA1304; 5-7: *PbPAL1* transgenic lines; 8-10:
579 *PbPAL2* transgenic lines.

580 **Figure 8 Determination of lignin content in *A. thaliana* stem and leaf .** The lignin content of transgenic and
581 wide plants were *significantly different from that of wild plants (P<0.05). (**A**) The lignin content of stem. (**B**) The
582 lignin content of leaf. WT: wide Arabidopsis; OE-PbPAL1: Overexpression of *PbPAL1* Arabidopsis; OE-PbPAL2:
583 Overexpression of *PbPAL2* Arabidopsis. Error bar represents the standard error of three bioreplication.

584 **Figure 9 Wiesner cross section staining of *A. thaliana* stem**. All Arabidopsis plants were planted in the same environment;
585 inflorescence stems were taken from Arabidopsis thaliana plants and grew for 50 days. (**A**) WT plants. (B) *PbPAL1*-
586 overexpressing transgenic plants. (**C**) *PbPAL2*-overexpressing transgenic plants; F: interfascicular cells; X: xylem; bar = 51 μm.

587 **Figure 10 Toluidine blue ross section staining of the inflorescence stems from WT and transgenic lines.** (**A**)WT plants. (B)
588 *PbPAL1*-overexpressing transgenic plants. (**C**) *PbPAL2*-overexpressing transgenic plants. F: interfascicular fibre; X: xylem; bar
589 = 100 μm.

590 **Supplementary materian**

591 **Table S1.** Primer sequences used for qRT-PCR and vector construction
592 **Table S2.** Primer sequences contained artificial restriction enzyme sites for *Bgl* II and *Spe* I
593 **Table S3.** GusA nonspecific primers
594 **Table S4.** Numbers of *cis*-elements in promoter region of *PbPAL*s
595 **Figure S1.** Sliding window analysis of *PAL* duplicated genes
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Table 1(on next page)

Table 1 Sequence information of the PAL family genes of five Rosaceae plants

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1 **Table 1 Sequence information of the *PAL* family genes of five Rosaceae plants**

Species	Gene name	Gene ID	Length (aa)	Mw (kDa)	pI	chromosome	Strand
Apple	*MdPAL1*	MDP0000668828	720	78.55	6.09	Chr1	cyto
	MdPAL2	MDP0000787168	643	69.90	6.39	Chr8	cyto
	MdPAL3	MDP0000261492	720	78.15	6.29	Chr4	cyto
	MdPAL4	MDP0000388769	753	87.75	6.21	Chr12	cyto
	MdPAL5	MDP0000139075	589	63.41	6.31	Chr12	cyto
	MdPAL6	MDP0000191304	702	76.16	6.18	Chr4	cyto
Mei	*PmPAL1*	Pm030127	717	77.92	6.10	Chr8	cyto
	PmPAL2	Pm018524	719	78.18	6.19	Chr5	cyto
Strawberry	*FvPAL1*	Fv23261	718	77.98	6.00	Chr7	cyto
	FvPAL2	Fv09753	724	78.98	6.10	Chr6	cyto
Peach	*PpPAL1*	Ppa002328m	686	74.63	6.28	Chr2	cyto
	PpPAL2	Ppa002099m	716	78.00	6.10	Chr6	cyto
	PpPAL3	Ppa002878m	625	67.87	6.39	Chr2	oute
Pear	*PbPAL1*	Pbr008363	720	78.15	6.29	Chr12	cyto
	PbPAL2	Pbr008387	715	77.83	5.79	Chr3	cyto
	PbPAL3	Pbr016460	414	44.42	8.79	Chr5	cyto

2

Table 2 (on next page)

Table 1 Sequence information of the PAL family genes of five Rosaceae plants

1 **Table 2 Analysis of gene replication events of PAL family members in Rosaceae species**

Paralogous pairs	Ks	Ka	Ka/Ks	Purifing selection	Duplicate type
MdPAL1/MdPAL2	0.2676	0.0250	0.0903	No	Segmental
MdPAL4/MdPAL5	0.1034	0.0961	0.9294	No	Segmental
MdPAL3/MdPAL6	0.1368	0.0532	0.3889	No	Tandem
PbPAL2/PmPAL2	1.9819	0.2017	0.1017	No	Segmental
PmPAL1/PmPAL2	0.0577	0.0037	0.0641	No	Segmental

2

Figure 1

Figure 1 Phylogenetic relationships, and gene structures and domain of *PAL*

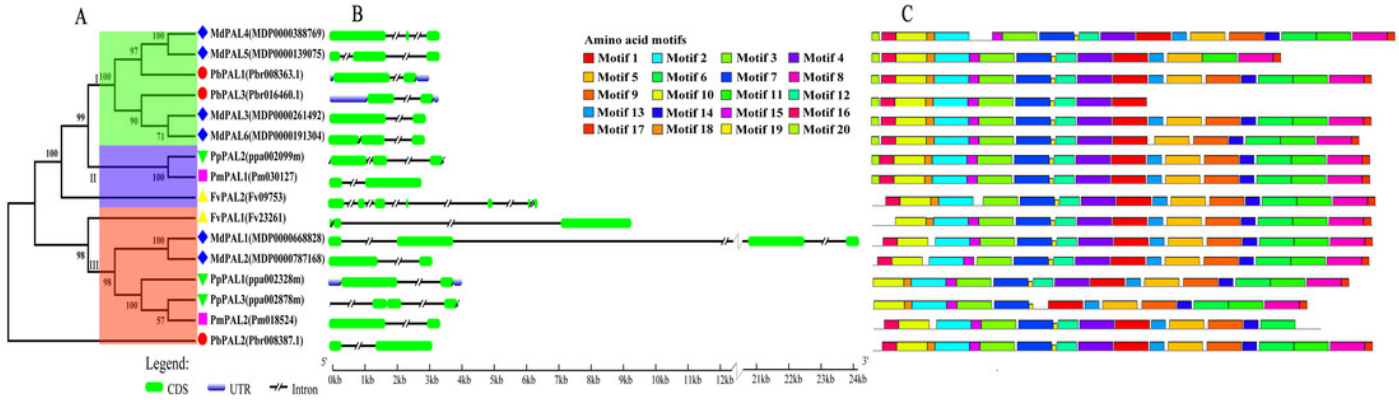

Figure 2

Figure 2 *PAL* genes chromosomal location of five Rosaceae species.

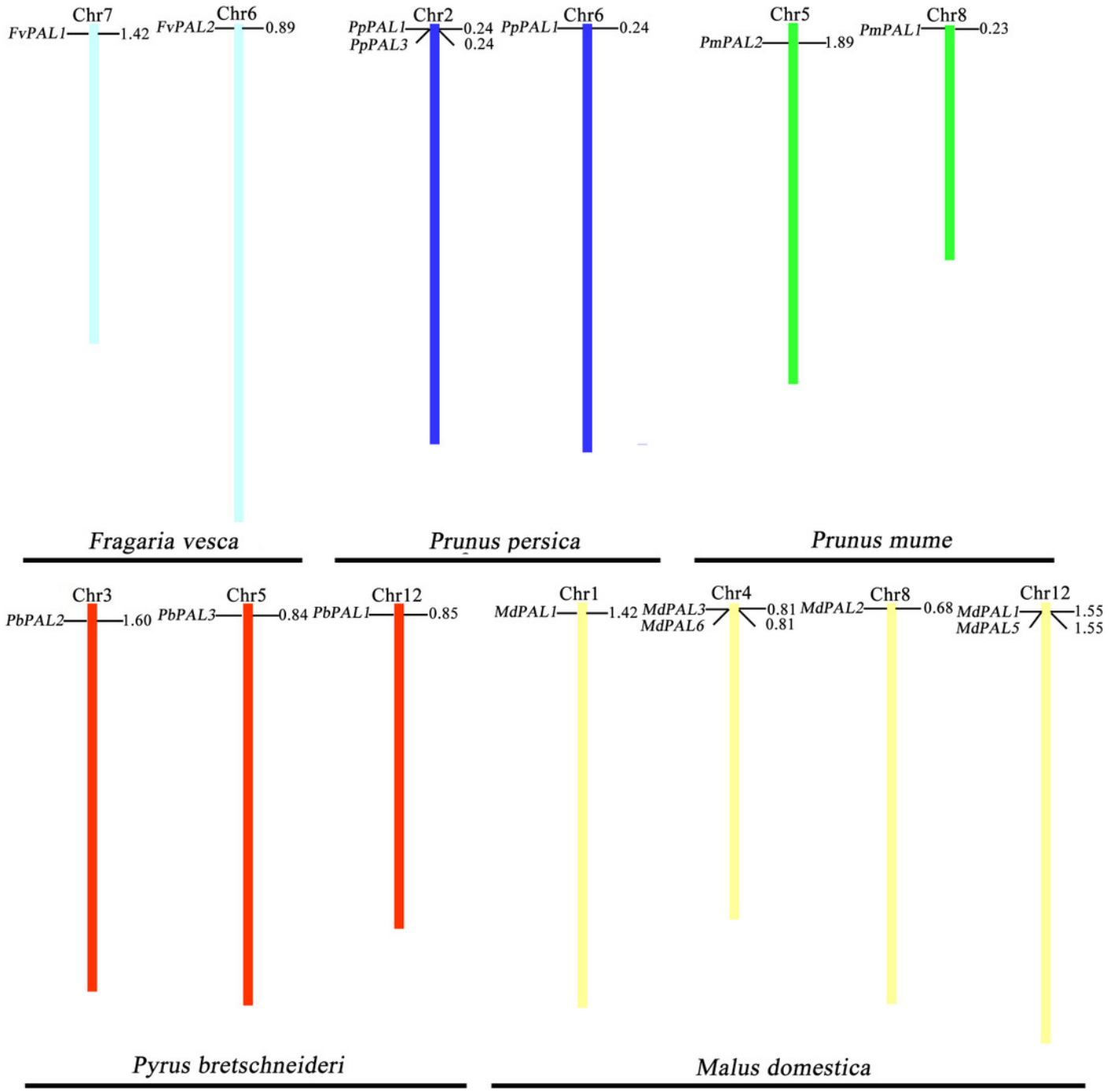

Figure 3

Figure 3 Distribution of main *cis*-elements and putative regulating factors in the promoter regions of pear antioxidant enzyme genes

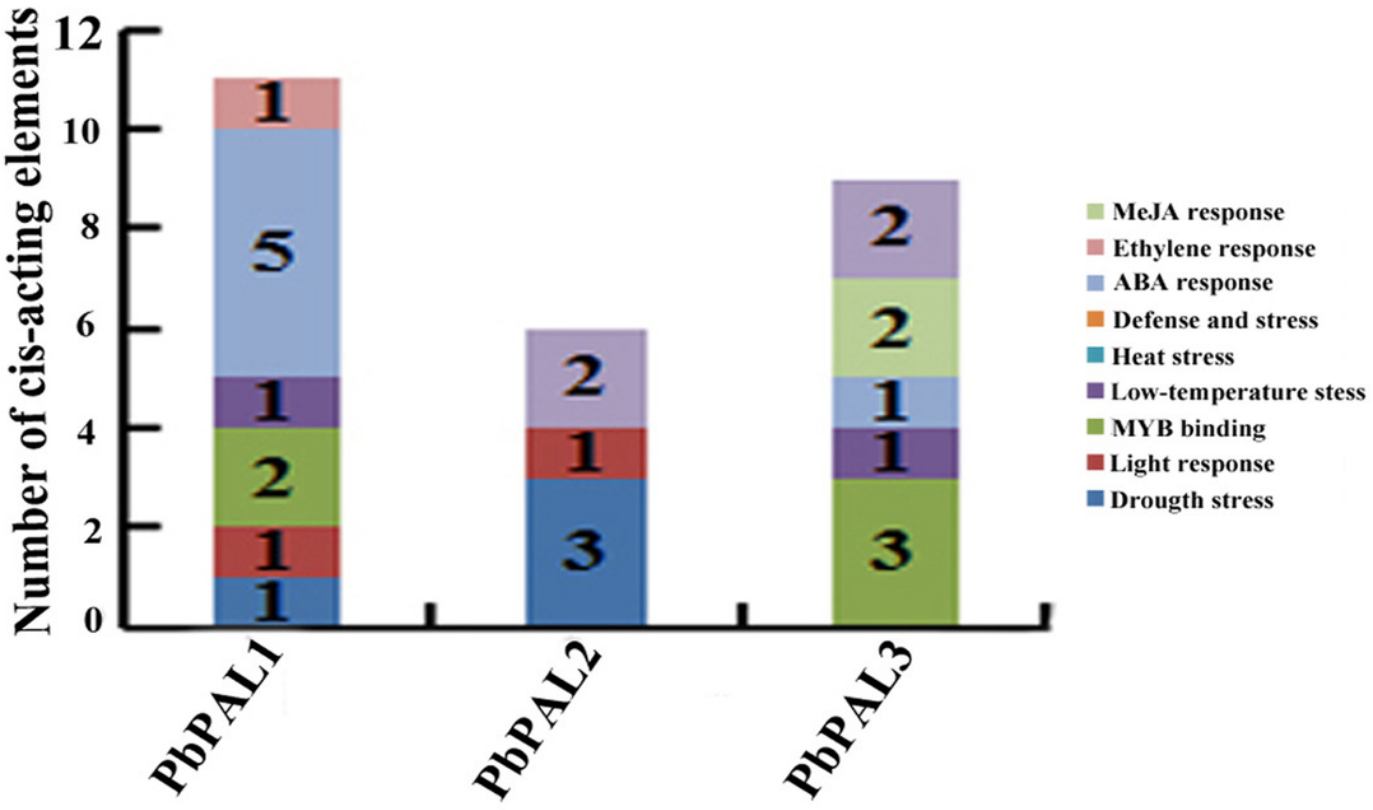

Figure 4

Figure 4 Phylogenetic analysis of *PAL*s constructed by the Neighbor-Joining method

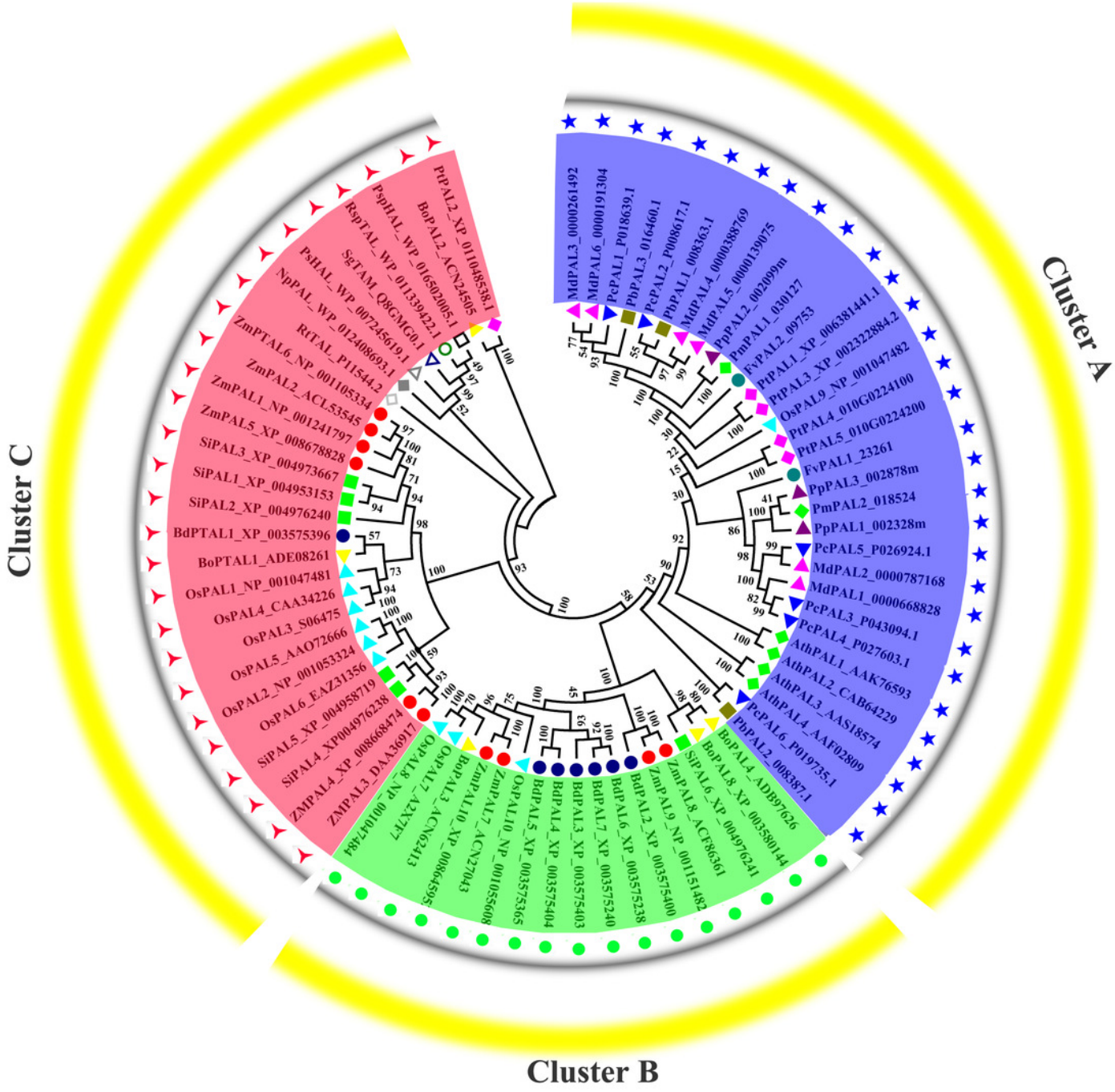

Figure 5

Figure 5 Expression analysis of *PbPAL* genes in various tissues of pear

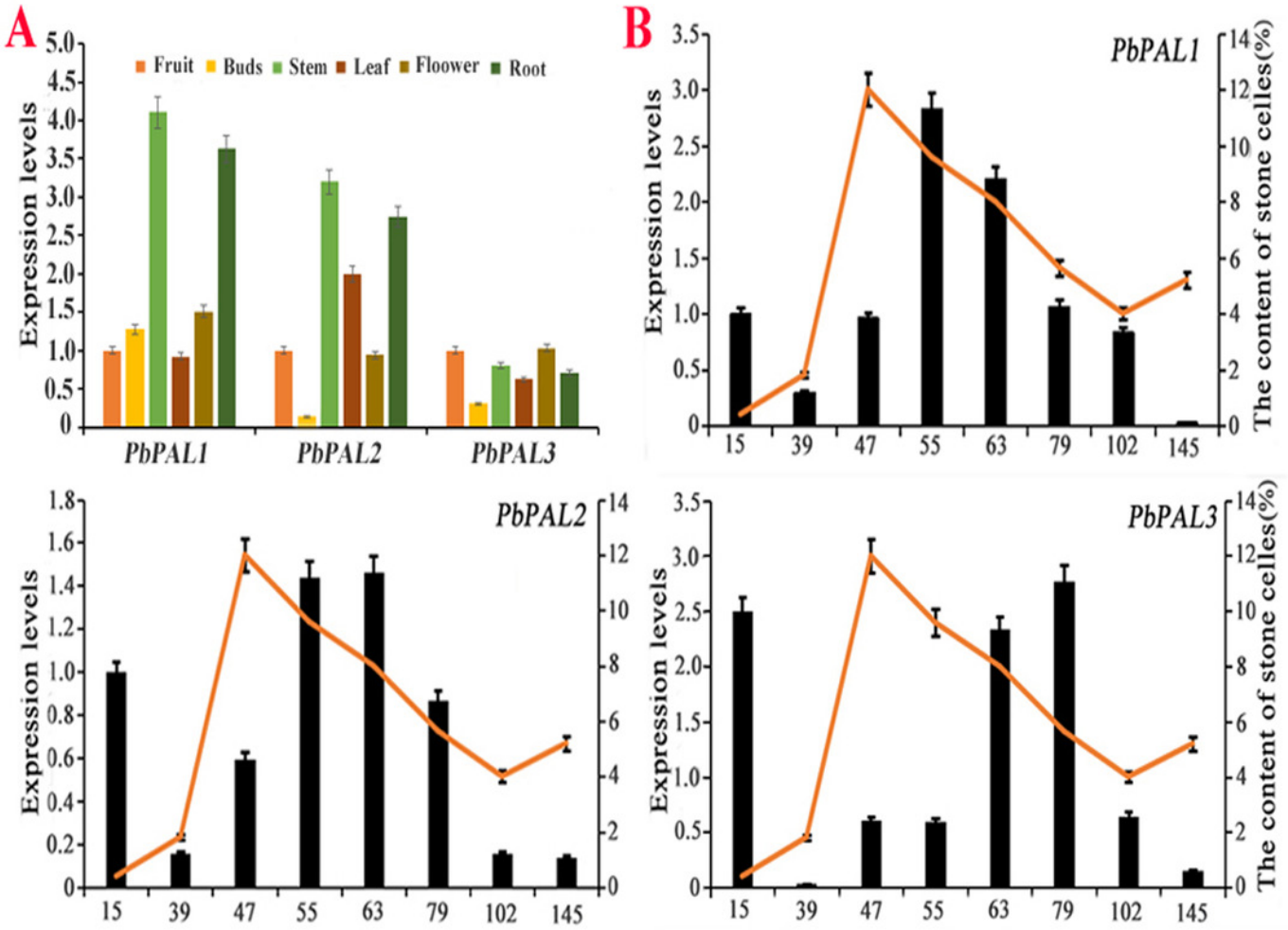

Figure 6

Figure 6 Hormone response pattern analysis of *PbPAL*s. *PbPAL*s expression in pear fruits in response to exogenous hormone (A: ABA; B: MeJA; C: SA;) treatment for 0, 1, 2, and 3 h.

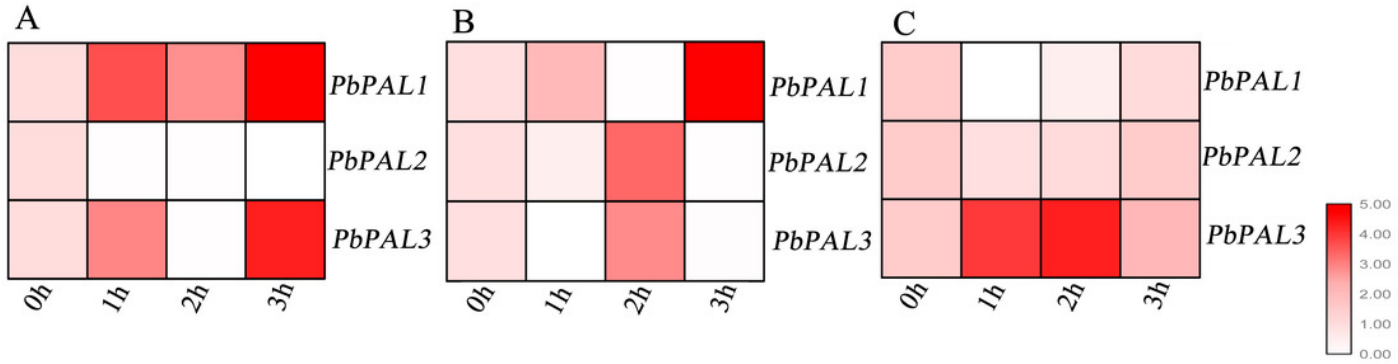

Figure 7

Figure 7 Vector Construction and Overexpression.

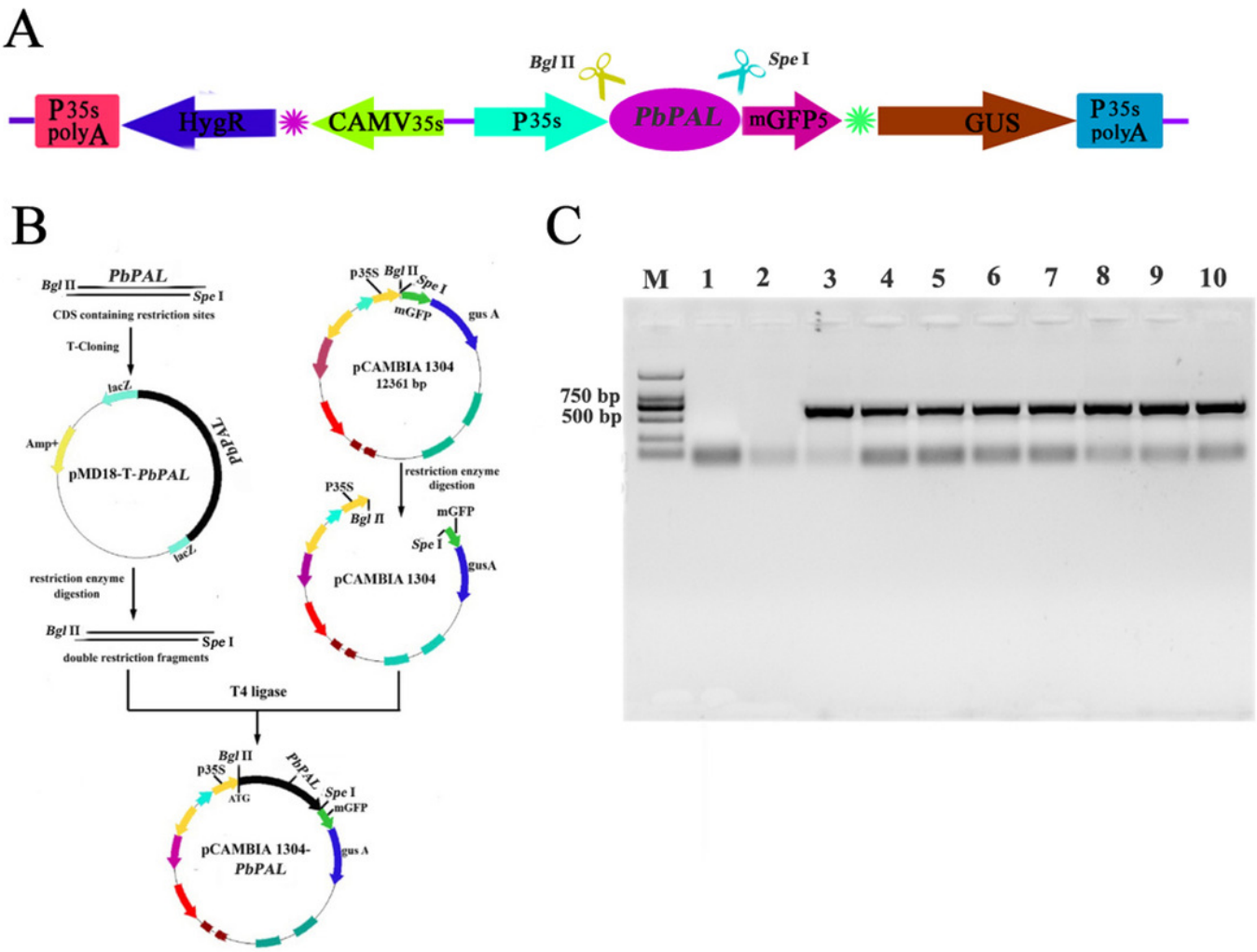

Figure 8

Figure 8 Determination of lignin content in *A. thaliana* stem and leaf .

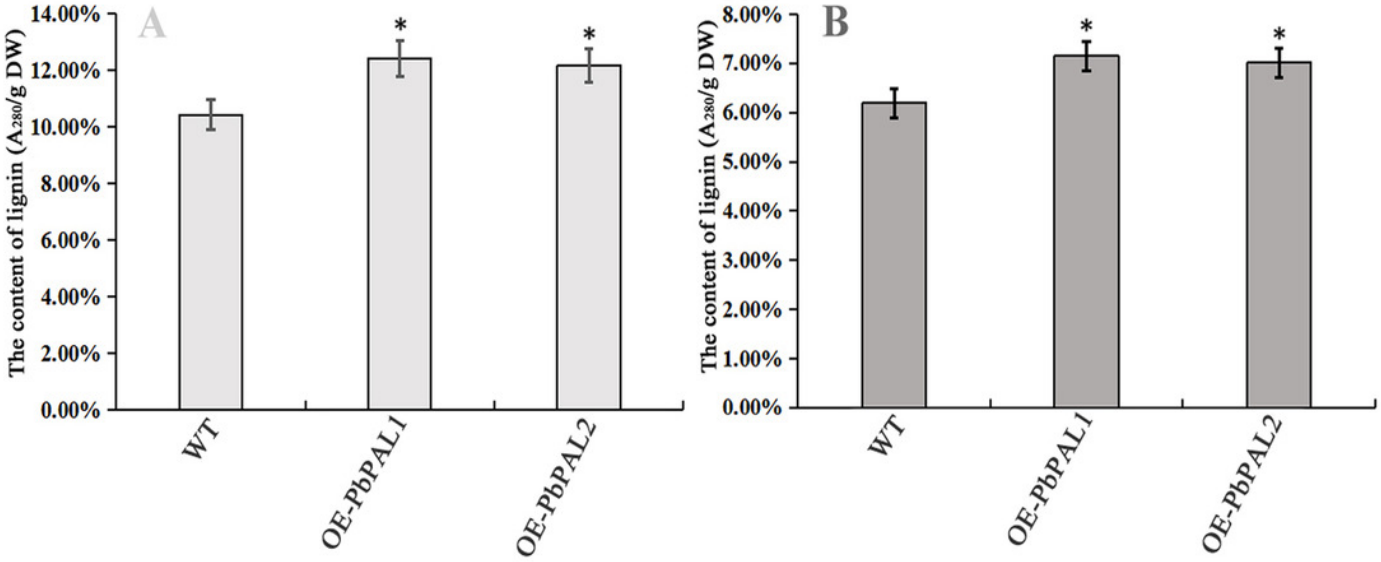

Figure 9

Figure 9 Wiesner cross section staining of *A. thaliana* stem

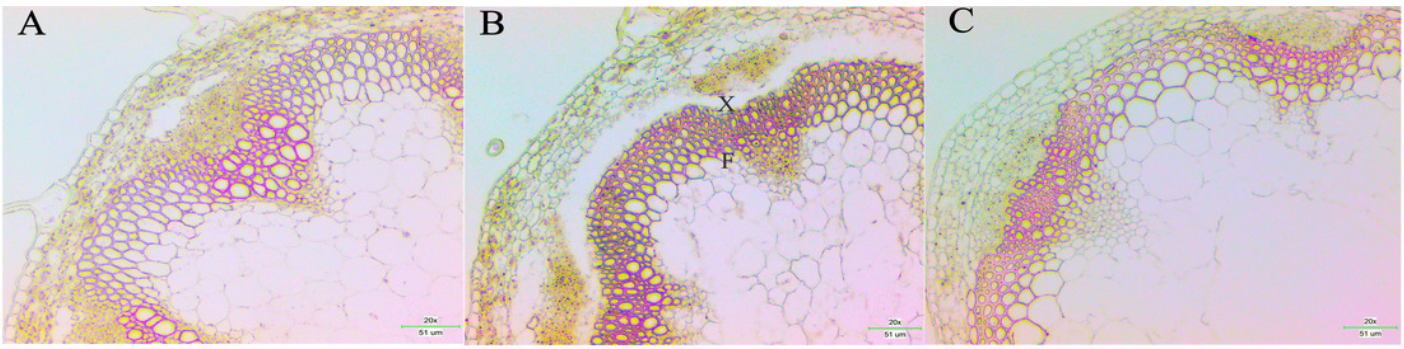

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Figure 10

Figure 10 Toluidine blue ross section staining of the inflorescence stems from WT and transgenic lines