Molecular Cloning and Functional Characterization of Two Divergent 4-Coumarate : Coenzyme A Ligases from Kudzu (Pueraria lobata)

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As part of the efforts to understand isoflavonoid metabolism in Pueraria lobata at the molecular level, the cDNAs encoding two divergent 4-coumarate : coenzyme A ligases (4CLs, designated Pl4CL1 and Pl4CL2, respectively) were isolated from P. lobata roots. Sequence analysis revealed that Pl4CL1 had an N-terminal extension of twenty-one amino acid residues compared to Pl4CL2. Phylogenetic analysis showed that Pl4CL1 and Pl4CL2 fell into angiosperm Class II and Class I, respectively. Through in vitro biochemical assays, both Pl4CLs were found to have the capacity to utilize 4-coumarate and trans-cinnamate as substrates, while neither of them could convert sinapate. Pl4CL2 had a broader substrate specificity than Pl4CL1. The affinity of Pl4CL1 for 4-coumarate was 2.6-fold higher than that of Pl4CL2 (with the K\text{m} values of 3.5\,\mu M and 9.1\,\mu M, respectively). Combining the dataset including gene expression profiles, metabolites measurements, and biochemical properties, our results indicated that Pl4CL1, just as other angiosperm Class II 4CLs, might play a role in isoflavone biosynthesis in P. lobata, while Pl4CL2 belongs to angiosperm Class I, and may function as a housekeeping enzyme concerning lignification.

Key words 4-coumarate : coenzyme A ligase (4CL); isoflavonoid; Pueraria lobata; puerarin

Human have used Pueraria lobata as a herbal drug for many years. Its pharmacological effects are due to the presence of isoflavonoids, which include puerarin, daidzin, and other related metabolites. Puerarin exhibits diverse medicinal properties including hypotensive, hypolipidemic, hypoglycemic, anti-oxidant, anti-ischaemia, vasodilation and estrogen-like effects. As an isoflavonoid, the biosynthesis of puerarin should derive from phenylpropanoid metabolism, in which the enzymes in the early steps such as phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate : coenzyme A ligase (4CL), chalcone synthase (CHS), chalcone reductase (CHR), and chalcone isomerase (CHI) are conserved in many plant species. Although puerarin was proposed to exhibit diverse pharmacological activities, the genes for the enzymes involved in the pathway have limitedly been isolated and characterized from P. lobata. As part of understanding the isoflavonoid metabolism in P. lobata at the molecular level, we have undertaken standard amplifications of cDNAs on PAL, C4H, 4CL, CHS, CHR, CHI and isoflavone synthase (IFS) by degenerate primers from the P. lobata roots that are deemed as the source tissues mostly accumulating puerarin. Interestingly, the partial cDNAs corresponding to two divergent 4CLs, designated as Pl4CL1 and Pl4CL2, were amplified, and showed only 65% amino acid sequence identity between each other. In plants, 4CLs usually have many isoenzymes, e.g. structurally and functionally different 4CLs were discovered in Arabidopsis thaliana, Glycine max, Lithospermum erythrorhizon, Lolium perenne, Oryza sativa, Phyllostachys edulis, Populus tremuloides, Panicum virgatum, and Rubus idaeus. As the last enzyme of the general phenylpropanoid pathway, 4CLs directs carbon fluxes through different pathways into the biosynthesis of monolignols and other phenolic metabolites such as flavones (Fig. 1).

Methyl jasmonate (MeJA) has been suggested to be an important signal for inducing isoflavonoid biosynthesis in P. lobata. Therefore, in our preliminary experiments, the gene expression of PAL, C4H, 4CL, CHS, CHI and IFS were examined in P. lobata suspension cultures in response to MeJA treatment. Interestingly, among the genes, Pl4CL1 was shown to be strongest one to be up-regulated by MeJA treatment (unpublished data). For studying the regulatory mechanism on isoflavonoid biosynthesis in P. lobata, it will be important to experimentally elucidate the role of Pl4CLs. Here we reported on the full-length cDNA isolation and functional characterization of the two distinct 4CLs, Pl4CL1 and Pl4CL2, from P. lobata. Combined with gene expression profiles and in vitro biochemical characterizations, this study demonstrated that Pl4CL1 played a role in isoflavone production in P. lobata while Pl4CL2 most likely was involved in lignin biosynthesis. The connection of the 4CLs primary sequence to their functions was discussed.

MATERIALS AND METHODS

Plant Materials and Chemicals Seeds of P. lobata in this study were collected from Langxi county, Anhui province, China. Seed explants were scarified by grinding with quartz sand in a mortar for 3 min at most, avoiding damage to the embryos. After treatment with 20 mg/L kinetin (KT) for 24 h at room temperature, seeds were surface sterilized by being immersed in 75% (v/v) ethanol for 10–15 s, followed by three washes in sterile distilled water and 10 min immersion in 10% hydrogen peroxide. Afterwards, another three washes were necessary. Axenic seeds were then placed on the agarized (0.8%) Murashige and Skoog (MS) basal medium at 25°C in darkness. The medium was autoclaved at 121°C for 20 min. After germination, the seedlings were incubated under sterile conditions in the growth chamber at 25±2°C with a 14h photoperiod. After 30d, seedlings were divided into two groups. One was for callus induction, the other was harvested under sterile conditions in the growth chamber at 25±2°C with a 14h photoperiod. After 30d, seedlings were divided into two groups. One was for callus induction, the other was harvested

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and partitioned into root, stem, and leaf materials, and stored at \(-80\)°C for further experiments.

Reagents were from Dingguo Company (http://www.dingguo.com/) unless otherwise specified. trans-Cinnamate, 4-coumarate, caffeate, ferulate and sinapate were purchased from Aladdin (http://www.aladdin-reagent.com/). Puerarin standard was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Primer synthesis and DNA sequencing were performed by Tsingke Company (http://www.tsingke.net/). Unstained Protein Molecular Weight Marker was from Fermentas (http://www.fermentas.com).

Establishment and Treatment of \textit{P. lobata} Cell Suspension Cultures For callus initiation, 30-d-old seedlings were cut at the root collar position, the aerial parts were then planted in MS basal medium with 3% sucrose, 0.8% agarose, 1 mg/L \(l\)-naphthylacetic acid (NAA) and 1 mg/L 6-Benzyl-aminopurine (BA) at a pH of 5.8. About one week later, the calli were cut into pieces and subcultured on \(B_4\) medium, supplemented with 0.8% agarose, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L NAA, and 0.5 mg/L KT, at pH 5.5. Calli were subcultured every two weeks and incubated at 25±2°C in darkness.

After about 3 months of subculture, soft, loose and pale green calli were achieved, and transferred to 250 mL Erlenmeyer flasks containing 100 mL liquid medium (\(B_5\) medium with 1 mg/L 2,4-D, 1 mg/L NAA, 0.5 mg/L KT, and 0.2% casein hydrolysate). Flasks were then sealed with silicone rubber plugs and incubated on a rotary shaker (110–130 rpm) in continuous light at 25°C for suspension cultures initialization. Once established, cultures were periodically subcultured into 250 mL Erlenmeyer flasks by transferring 33 mL 18-d-old cells into 67 mL fresh \(B_5\) medium.

For the MeJA treatment, MeJA dissolved in 95% ethanol was added to the cell cultures at a final concentration of 10 \(\mu\)M on the 4th day after subcultures. Equivalent volumes of 95% ethanol were added into the cell culture as the mock treatment. The treated suspension cultures were incubated at 25°C in a continuous light condition. The cells were collected as described at 0 h, 24 h and 48 h, after MeJA or mock treatment and frozen in liquid nitrogen. Then lyophilization was carried out in a LGJ-10 freeze-drier (Songyuan Huaxing, Beijing, China) at 1 Bar. Freeze-dried cells were stored at \(-80\)°C for later experiments.
Puerarin Extraction and HPLC Determination For puerarin quantifications, 20 mg of the freeze-dried suspension cells were directly extracted with methanol. The methanol extracts were centrifuged at 12000 rpm for 5 min and then the supernatant was transferred to new test tubes. The extraction procedure was repeated three times. Methanolic extracts were combined and volatilized to dryness. The residue was redissolved in 1 mL HPLC grade methanol and filtered through 0.22μm nylon syringe filter prior to HPLC analysis.

The reverse-phase high-performance liquid chromatography (HPLC) analysis was carried out using an Agilent 1100 chromatographic system (Wilmington, DE, U.S.A.) equipped with a quaternary pump, an autosampler, a photodiode array detector and an Agilent HC-C18(2) reversed-phase column (4.6 mm×250 mm, 5 μm). The samples were eluted with methanol–water (15:85, v/v) at a flow rate of 0.8 mL/min, and detected at 250 nm. The column temperature was maintained at 30°C. For the quantification of puerarin in samples, a standard calibration curve was made from the puerarin standard in five gradient concentrations (0.1, 1, 10, 100, 1000 μg/mL).

The Isolation of Pl4CL1 and Pl4CL2 cDNAs from P. lobata Roots First-strand cDNA was synthesized from P. lobata total RNAs using SuperScript III Transcriptase (Invitrogen, U.S.A.) with an oligo(dT) primer. Based on high conserved regions, degenerate primers 1 and 2 were designed for amplifying about 950 bp of DNA fragment using EasyTaq DNA polymerase (TransGen, Beijing, China) for P. lobata root cDNAs. The primary PCR was performed using 1 μL of the root cDNA as the template and the thermocycling conditions were as follows: 5 min of initial denaturation at 95°C, followed by 95°C for 1 min, 60°C for 30 s, and 72°C for 1 min in a 35-cycle reaction, and a final elongation step of 72°C for 10 min. One microliter of a 20-fold dilution of the primary PCR product was directly used as the template for the second PCR with the same primers and thermocycling program as the above. The second PCR products were gel-purified and inserted into the pMD18-T vector for sequencing.

Based on the partial sequences obtained, 3‘ and 5‘ rapid amplifications of cDNA ends (3‘RACE and 5‘RACE) were performed to recover the remaining cDNA regions. For 3‘RACE experiments, the root total RNA was reversely transcribed to cDNA using primer No. 3. The first-round PCR was conducted using primers No. 4/8 for Pl4CL1, primers No. 5/8 for Pl4CL2. Then the second-round PCR reactions were performed with 1 μL of a 20-fold dilution of corresponding first-round PCR products using primer set No. 6/9 and No. 7/9 for Pl4CL1 and Pl4CL2, respectively. For 5‘RACE experiments, the first-strand cDNA was synthesized from 2 μg total RNA with oligo(dT) primer, and tailed with Terminal deoxynucleotidyl Transferase (TdT) and dCTP (TaKaRa, Japan) according to the manufacturer’s instructions. Then the first-round PCR was performed using primers No. 10/12 for Pl4CL1 and primers No. 10/13 for Pl4CL2. The secondary PCR was performed using primer set No. 11/14 and No. 11/15 for Pl4CL1 and Pl4CL2, respectively. The open reading frame (ORF) of Pl4CL1 cDNA was then amplified using gene-specific primers No. 16 and 17. The primers No. 18 and 19 were used to amplify the ORF of Pl4CL2 cDNA. The primers used in this study were listed in the supplemental information (Table S1). All the PCR products were separated by electrophoresis on a 1.0% agarose gel, purified, and inserted into the pMD18-T vector (TaKaRa) for sequencing.

Phylogenetic Analysis DNA sequence analysis was performed using Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORFs of the two 4CL genes were predicted using DNA Star software version 7.10. Multiple alignments of amino acid sequences were generated by ClustalW2. An unrooted Neighbor-joining phylogenetic tree of 4CLs from different species was constructed by Molecular Evolutionary Genetics Analysis software (MEGA) version 5.05<sup>33</sup> using the p-distance method with gaps treated by pairwise deletion and a 1000 bootstrap replicate. The GenBank accession numbers of the sequences used for the phylogenetic analysis were shown in supplemental information (Table S2).

The Purifications of Recombinant Pl4CL1 and Pl4CL2 The ORF of Pl4CL1 was PCR-amplified with primers No. 20/21, and subcloned into the p<sub>B</sub>gal-SalI restriction sites of expression vector pQE-30 (QiaGen, Germany) to give the construct pQE30-Pl4CL1. The ORF of Pl4CL2 was PCR-amplified with primers No. 22/23 and subcloned into the NdeI-SacI sites of pET28-a vector (Novagen, Germany) resulting in the construct pET28a-Pl4CL2. For all PCR amplifications, the TransStart FastPfu DNA Polymerase (TransGen) was used, and the amplified DNAs were sequenced to confirm their fidelity. Primers used were shown in the supplemental information (Table S1). For the heterologous expressions, the construct pQE30-Pl4CL1 was transformed into M15 Escherichia coli (E. coli) cells while the construct pET28a-Pl4CL2 was into BL2I(D3E) E. coli cells. Cultures inoculated from a single colony were grown at 37°C until OD<sub>600</sub> reached 0.6 in the presence of corresponding antibiotics (for M15/pQE30-Pl4CL1, both 100 μg/mL ampicillin and 50 μg/mL kanamycin were used; for BL2I(D3E)/pET28a-Pl4CL2, 100 μg/mL kanamycin was used). Protein expressions were induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM for Pl4CL1 and 1 mM for Pl4CL2, followed by incubations on a rotary shaker (220 rpm) at 25°C for 6–8 h.

For purifying the recombinant 4CLs, cells were harvested by a 5 min centrifugation at 6000 × g at 4°C, resuspended in ice-cold PBS (50 mM sodium phosphate, pH 7.5, containing 300 mM NaCl), and lysed at 4°C for 30 min by the addition of lysozyme (0.5 mg/mL). Cells were then sonicated on ice at 40% power for 20 min in 2 s pulses with a 4 s rest period using Scientz-IID 950 W ultrasonic processor (Scientz, China). Lysates were centrifuged at 12000 × g for 30 min at 4°C, and clear soluble fractions were passed through a gravity-flow column packed with HisPur<sup>™</sup> Ni-NTA Resin (Thermo Scientific, U.S.A) according to the manufacturer’s instructions with all the buffers modified. Buffers used in this study were listed as follows: Equilibration buffer (50 mM sodium phosphate, pH 7.5, containing 300 mM NaCl and 10 mM imidazole), wash buffer (50 mM sodium phosphate, pH 7.5, containing 300 mM NaCl and 20 mM imidazole), elution buffer (50 mM sodium phosphate, pH 7.5, containing 300 mM NaCl and 100 mM imidazole). The 4CLs were eluted in 10 mL elution buffer, desalted, and concentrated to about 500 μL using Millipore concentrator with 30-kDa cut-off. The desalted protein was resuspended in 1 mL storage buffer (0.2 M Tris–HCl, pH 7.8, 30% glycerol), and stored at ~80°C prior to use. The purity of recombinant protein was monitored on sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE) following Coomassie Brilliant Blue staining. Recombinant Pi4CL2 was quantified by the Bradford assays.24) For Pi4CL1, there were two major bands visualized on SDS-PAGE, to characterize the protein bands, the two bands were cut off from the gel separately and used for matrix assisted laser desorption/ionization (MALDI) analysis on a MALDI-time-of-flight (TOF)/TOF mass spectrometer (ABI 4700 Proteomics Analyzer, Applied Biosystems). The peptides obtained were identified by searching the NCBI database using the MASCOT search engine (http://www.matrixscience.com).25) For Pi4CL1, the Bradford assays were performed with the integral optical density (IOD) of each of the two bands also being determined using Gel-Pro software version 4.0 (Media Cybernetics, U.S.A.).

**In Vitro Enzyme Assays** Enzyme assays were performed according to the method described by Knobloch and Hahlbrock26,27) with minor modifications. In brief, the reaction mixture of 2 mL contained 200 mM Tris–HCl, pH 7.8, 5 mM ATP, 5 mM MgSO₄, 0.1 mM CoASH, 2–4.5 µg of purified recombinant 4CL proteins, and cinnamate derivatives. Concentrations of cinnamate derivatives were listed as follows: trans-cinnamate (15–2000 µM), 4-coumarate (0.5–150 µM), caffeate (20–1250 µM), ferulate (250–3000 µM) and sinapate (2.5–150 µM). Control mixtures contained no CoASH. The reaction was initialized by adding CoASH and carried out at room temperature. The first measurement was performed at 1.5 min after the addition of CoASH. Spectrophotometric changes resulting from CoA-ester formation were monitored over 5 min at 311 nm for cinnamate, 333 nm for 4-coumarate, 346 nm for caffeate, 345 nm for ferulate, and 352 nm for sinapate.28) Extinction coefficients of these esters were used to calculate enzyme activities according to method established by Lee et al.29) Kₑₒₐ, Vₘₐₓ, kₑₜ and kₑₒₐ/Kₑₒₐ values were determined as described.30) Three replicate assays were conducted for each substrate.

**Gene Expression Analysis of Pi4CL1 and Pi4CL2 by Quantitative Reverse Transcription-Polymerase Chain Reactions (qRT-PCRs)** The cDNAs for qRT-PCRs were generated with an oligo(dT) primer using EASYspin Plant RNA isolation kit (Aidlab, Beijing, China). The constitutively expressed actin mRNA (GenBank accession no. HQ704972) was used as the reference transcript. To investigate the gene expression patterns in different organs, the roots, stems, and leaves derived from one-month-old *P. lobata* seedling were excised from the gel, digested with trypsin and analyzed by MALDI-TOF/TOF mass spectrometry. Thirteen peptide sequences from the upper band and six peptides from the lower one were obtained (see supplemental information Table 1).

**RESULTS**

**The Full-Length cDNAs of Pi4CL1 and Pi4CL2 Were Isolated from *P. lobata* Roots** Based on the conserved regions of plant 4CLs, degenerate primers were used for RT-PCRs using *P. lobata* root cDNAs as the template, which led to the identification of two distinct partial 4CL cDNA sequences. 3′ and 5′-RACE were successfully performed to retrieve the full-lengths of the two 4CL cDNAs designated as Pi4CL1 and Pi4CL2. The cDNA for Pi4CL1 contains an ORF of 1704 bp encoding for 567 amino acids with a predicted molecular mass of 61521. The Pi4CL2 cDNA shows a length of 1638 bp ORF encoding a polypeptide of 545 amino acids with a predicted molecular mass of 59837. According to Fig. 2, Pi4CL1 had an N-terminal extension of twenty-one amino acid residues compared to Pi4CL2. The deduced amino acids of both genes contain the conserved motifs for plant 4CL family, such as the box I “SSGTTGLPKGV” and box II “GEICIRG” motifs (Fig. 2). Between Pi4CL1 and Pi4CL2, there is only 64% amino acid identity to each other in the full-lengths.

A phylogenetic tree was constructed to examine the relationship of Pi4CL1 and Pi4CL2 to other plant 4CL members. The Sc4CL from filamentous bacterium *Streptomyces coelicolor* A3(2)32) was used as outgroup in the tree. As shown in Fig. 3, the selected sequences corresponding to forty-nine 4CL members are partitioned into six groups, which is in accordance with an earlier study.33) The plant 4CL members are divided into the following five groups: angiosperm Class I, angiosperm Class II, angiosperm Class III, Gymnospermae and Pteridophyte/Bryophyta. Pi4CL1 is a member of angiosperm Class II group, which mainly participates in flavonoid biosynthesis pathway.34) By contrast, Pi4CL2 belongs to angiosperm Class I group, which is involved in lignification.10,34) The deduced amino acid sequence of Pi4CL1 shows the highest homology with Gm4CL3 from *G. max* (95% identity) whereas Pi4CL2 shows the highest amino acid sequence identity with Gm4CL2 from *G. max* (89% identity). Gm4CL3 and Gm4CL2 have been reported to participate in flavonoid and lignification biosynthesis, respectively.35)

**Biochemical Characterization of Pi4CL1 and Pi4CL2** For functional characterization, Pi4CL1 and Pi4CL2 were initially constructed in an *E. coli* expression vector pET28a, and heterologously expressed in BL21(DE3) cells. The soluble Pi4CL2 was successfully expressed and purified through a His-trap Ni-NTA column (Fig. 4). However, for Pi4CL1, no soluble form was obtained by the expression system at all the conditions we tried (data not shown). To get the soluble Pi4CL1, Pi4CL1 cDNA was sub-cloned into the vector pQE-30a and expressed in M15 *E. coli* strain. The soluble Pi4CL1 was successfully expressed in M15 cells and purified by a His-trap column. However, for the purified Pi4CL1 elution, there were two major bands observed on the gel with the lower band matching the molecular weight of Pi4CL1 (Fig. 4). To characterize the purified Pi4CL1 solution, the two bands were excised from the gel, digested with trypsin and analyzed by MALDI-TOF/TOF mass spectrometry. Thirteen peptide sequences from the upper band and six peptides from the lower one were obtained (supplemental information Table 1).
Fig. 2. Amino Acid Sequence Alignment of 4CL Isoenzymes

P4CL1 has twenty-one more amino acid residues at the N-terminal than P4CL2. The conserved peptide motifs Box I and Box II are highlighted in yellow. The black arrow indicates the amino acid residues involved in sinapate activation.
S3). When the peptide sequences were analyzed by MASCOT search against a database of NCBInr, the results showed that the upper band corresponded to a HtpG-like chaperone protein while the lower band corresponded to Gm4CL3, which suggested that the lower band was from the recombinant Pl4CL1. The chaperone protein might help the correct folding of Pl4CL1 expressed in M15 cells.

The purified recombinant Pl4CL1 and Pl4CL2 were assayed with various phenylpropanoid substrates including trans-cinnamate, 4-coumarate, caffeate, ferulate, and sinapate. Kinetic parameters of Pl4CL1 and Pl4CL2 for the active substrates were determined and summarized in Table 1. The recombinant Pl4CL2 showed the 4CL activity toward trans-cinnamate, 4-coumarate, caffeate, and ferulate, whereas Pl4CL1 was only able to convert 4-coumarate and trans-cinnamate but not caffeate, ferulate, and sinapate. The $K_m$ value of Pl4CL1 for 4-coumarate was almost 200-fold lower relative to trans-cinnamate, while Pl4CL2 appeared to have broader substrate specificity with 4-coumarate also being the preferred substrate. The catalytic efficiency ($k_{cat}/K_m$) of Pl4CL1 toward 4-coumarate was 3.26 fold to that of Pl4CL2.

The Transcript of Pl4CL1 but Not Pl4CL2 Was Related to the Biosynthesis of Puerarin  As previously reported, the accumulation of puerarin was mostly detected in the roots compared to other organs (Fig. 5a). To compare the 4CL transcripts among the roots, stems and leaves, qRT-PCRs were performed. The transcripts of Pl4CL1 and Pl4CL2 were detectable with different expression intensity in all the organs, the strongest expression of Pl4CL1 was detected in the roots while Pl4CL2 was primarily expressed in the stems (Fig. 5b). Therefore, in spatial matter, the transcript of Pl4CL1 but not Pl4CL2 was related to the accumulation of puerarin in vivo.

To further assess the connection between the 4CLs transcripts and puerarin biosynthesis, the P. lobata suspension culture was established and elicited by MeJA. As shown in Fig. 6, compared to the mock treatment, MeJA treatment triggered a significant increase of puerarin accumulation during the time courses. After 24 h of MeJA treatment, the concentration of puerarin in MeJA-treated cells was increased in about three-folds compared to the mock control. To test the effects of the MeJA treatment on the expressions of 4CLs, quantitative real time PCRs were performed. Pl4CL1 was clearly up-regulated...
in physiological construction (37–41) and environmental resistance (39,42–45) which make phenylpropanoid pathway one of the most important metabolisms for higher plants. As a divergence-point enzyme, 4CL converts 4-coumarate and other cinnamate derivatives to respective CoA esters, connecting general phenylpropanoid metabolism to different subsequent branch pathways. In this study, two 4CL isoenzyme genes, Pl4CL1 and Pl4CL2, were isolated from P. lobata. Phytogenic analysis showed that Pl4CL1 and Pl4CL2 belong to angiosperm Class II and Class I, respectively. A number of the features of Pl4CL1 and Pl4CL2 support this classification. For example, compared to the Class I 4CLs, there is usually an N-terminal extension of amino acid residues in the Class II 4CLs, which also can be shown in the alignment of Pl4CL1 with Pl4CL2 (Fig. 2). It still remains unclear whether this amino acid extension is involved in functional divergence between the two 4CL classes. In addition, the Class I 4CLs are normally expressed in a constitutive manner while most Class II 4CLs could be induced by sorts of environmental factors, such as elicitors, pathogen attacks and UV irradiation.10,11 In P. lobata, Pl4CL1 share the feature with Class II 4CLs and its transcript could be up-regulated by MeJA treatment whereas the expression level of Pl4CL2 was not changed by the treatment (Fig. 6b).

It is well known that the Class II 4CLs are involved in flavonoids biosynthesis while the Class I 4CLs are associated with lignin formations.10,34 Puerarin is the characteristic compound from flavone biosynthetic pathway in P. lobata. It would be reasonable to investigate the connection between puerarin biosynthesis and gene expression levels of Pl4CLs. Our results showed that the transcript level of Pl4CL1 but not Pl4CL2 was in a positive correlation with the accumulation pattern of puerarin (Figs. 5, 6). Therefore, from these data, it would be possible to demonstrate the in vivo role of Pl4CL1 in puerarin biosynthesis.

Biochemical characterizations in vitro are usually used to deduce the in vivo functions of the enzymes in secondary metabolites biosynthesis. The biochemical properties of Pl4CL1 and Pl4CL2 showed distinct differences. 4-coumarate is the preferred substrate for both enzymes among the substrates tested in this study, while compared to Pl4CL1, Pl4CL2 shows a broader substrate specificity (Table 1). Both Pl4CL1 and Pl4CL2 show comparatively lower affinity (higher $K_m$ value) to trans-cinnamate (Table 1), which is consistent with the fact that trans-cinnamate is usually not an acceptable substrate for 4CLs in vivo.18,46 Sinapate is thought to be the precursor for the biosynthesis of the sinapyl alcohol monomers of S lignin.29 While it is a poor substrate for most of the plant 4CL isoenzymes,47 so far, only a few members have been found as natural sinapate-converting 4CL enzymes, including Gm4CL1 from G. max,11 At4CL4 from A. thaliana,34 4CL2/3 from Robinia pseudoacacia48 and 4CL from Erythrina cristagalli.49 Presence of all three amino acid residues (Pro, Val and Leu) in the substrate binding pocket (SBP, marked with black arrow in Fig. 2) was believed to block the capacity of 4CLs to utilize sinapate as substrate.50,51 That might explain why

**DISCUSSION**

Phenylpropanoid-derived end-products play pivotal roles in physiological construction (37–41) and environmental resistance (39,42–45) which make phenylpropanoid pathway one of the most important metabolisms for higher plants. As a divergence-point enzyme, 4CL converts 4-coumarate and other cinnamate derivatives to respective CoA esters, connecting general phenylpropanoid metabolism to different subsequent branch pathways. In this study, two 4CL isoenzyme genes, Pl4CL1 and Pl4CL2, were isolated from P. lobata. Phytogenic analysis showed that Pl4CL1 and Pl4CL2 belong to angiosperm Class II and Class I, respectively. A number of the features of Pl4CL1 and Pl4CL2 support this classification. For example, compared to the Class I 4CLs, there is usually an N-terminal extension of amino acid residues in the Class II 4CLs, which also can be shown in the alignment of Pl4CL1 with Pl4CL2 (Fig. 2). It still remains unclear whether this amino acid extension is involved in functional divergence between the two 4CL classes. In addition, the Class I 4CLs are normally expressed in a constitutive manner while most Class II 4CLs could be induced by sorts of environmental factors, such as elicitors, pathogen attacks and UV irradiation.10,11 In P. lobata, Pl4CL1 share the feature with Class II 4CLs and its transcript could be up-regulated by MeJA treatment whereas the expression level of Pl4CL2 was not changed by the treatment (Fig. 6b).

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**Table 1. Substrate Specificity of E. coli-Expressed Pl4CL Recombinant Proteins**

| Substrate      | $V_{max}$ (µkat/g protein) | $K_m$ (µM) | $k_{cat}/K_m$ (s$^{-1}$M$^{-1}$) |
|----------------|----------------------------|------------|---------------------------------|
| 4-Coumarate    | 21.47±0.23                 | 3.51±0.49  | 1.28±0.01                       |
| trans-Cinnamate| 16.33±0.86                 | 664.10±95.9 | 0.97±0.05                      |
| Caffeate       | ND                         | ND         | ND                              |
| Ferulate       | ND                         | ND         | ND                              |
| Sinapate       | ND                         | ND         | ND                              |

Note: Specific activities were measured with three independent assays. ND means “not detected.”
neither of Pl4CLs can convert sinapate in the kinetic experiments.

In summary, combining primary sequence analysis, gene expression profiles, and biochemical characterizations, we demonstrated that Pl4CL1 isoenzyme might be involved in flavone pathways whereas Pl4CL2 might function as a housekeeping enzyme concerning lignification in *P. lobata*.

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