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The phenylalanine ammonia-lyase gene family in Isatis indigotica Fort.: molecular cloning, characterization, and expression analysis

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[ABSTRACT] Phenolic compounds, metabolites of the phenylpropanoid pathway, play an important role in the growth and environmental adaptation of many plants. Phenylalanine ammonia-lyase (PAL) is the first key enzyme of the phenylpropanoid pathway. The present study was designed to investigate whether there is a multi-gene family in I. Indigotica and, if so, to characterize their properties. We conducted a comprehensive survey on the transcription profiling database by using tBLASTn analysis. Several bioinformatics methods were employed to perform the prediction of composition and physicochemical characters. The expression levels of IiPAL genes in various tissues of I. indigotica with stress treatment were examined by quantitative real-time PCR. Protoplast transient transformation was used to observe the locations of IiP ALs. IiP ALs were functionally characterized by expression with pET-32a vector in Escherichia coli strain BL21 (DE3). Integration of transcripts and metabolite accumulations was used to reveal the relation between IiP ALs and target compounds. An new gene (IiPAL2) was identified and both IiP ALs had the conserved enzymatic active site Ala-Ser-Gly and were classified as members of dicotyledon. IiPAL1 and IiPAL2 were expressed in roots, stems, leaves, and flowers, with the highest expression levels of IiPAL1 and IiPAL2 being observed in stems and roots, respectively. The two genes responded to the exogenous elicitor in different manners. Subcellular localization experiment showed that both IiPALs were localized in the cytosol. The recombinant proteins were shown to catalyze the conversion of L-Phe to trans-cinnamic acid. Correlation analysis indicated that IiPAL1 was more close to the biosynthesis of secondary metabolites than IiPAL2. In conclusion, the present study provides a basis for the elucidation of the role of IiP ALs genes in the biosynthesis of phenolic compounds, which will help further metabolic engineering to improve the accumulation of bioactive components in I. indigotica.

KEY WORDS] Phenylalanine ammonia-lyase; Isatis indigotica Fort.; Phenolic compounds; Lignan; Correlation analysis

Introduction

Isatis indigotica Fort. (I. intintoria) is a biennial herbaceous plant, belonging to the family of Brassicaceae [1]. Its dried roots (Banlangen, Isatis root) and leaves (Daqingye, Isatis leaf) show notable anti-inflammatory, antibacterial, and antiviral activities [2]. Additionally, Banlangen has been demonstrated to have potentials to treat SARS (severe acute respiratory syndromes) [3] and H1N1-influenza [4].

In previous experiments, la riciresinol and larch lignan glycosides isolated from Isatis indigotica have been proven to possess a number of biological activities, such as anti-influenza A1 virus [5], anti-inflammation [6], and anti-fungal effects [7] as well as reducing the risk of cardiovascular
diseases [8]. However, the contents of lariciresinol and larch lignan glycosides in the roots are very low, only 47.14 and 84.67 μg·g⁻¹, respectively [5]. Knowledge of the biosynthetic enzymes and their corresponding genes would enable a much higher production of the valuables in engineered plant or microbial cells [9].

As one of guaiacy lignins, lariciresinol is derived from phenylpropanoid pathway with many enzymes involved [10] (Fig. 1). As the first key enzyme in the phenylpropanoid biosynthesis, PAL links primary and secondary metabolism by catalyzing the conversion of L-phenylalanine to cinnamic acid and is also a rate-limiting step of the phenylpropanoid metabolism [11]. Since the first PAL was discovered from barley by Koukol and Conn in 1961 [12], more and more PAL genes have been cloned in many higher plants, such as Salvia Miltiorrhiza [13], Dendrobium [14], Salix viminalis [14], and Picrorhiza kurrooa [16], and it also have been found in some liverworts [17] and fungi [18].

Fig. 1  Lignin biosynthetic pathway and the involved genes. PAL catalyzes the first step in the conversion of L-phenylalanine (L-Phe) to trans-cinnamic acid. Abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; HCT, p-hydroxycinnamoyl-CoA: quinate shikimate p-hydroxycinnamoyltransferase; C3H, p-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-methyltransferase and CAD, cinnamyl alcohol dehydrogenase. The solid line represents one step reaction; the dotted line represents a multi-step reaction.

In a number of plants, the PAL proteins are encoded by a multi-gene family. The number of PAL genes is three in S. Miltiorrhiza [19] and Coffea canephora [20], four in S. viminalis [15], five in Populus trichocarpa [21], six in Oryza sativa [22], and twelve in Citrullus lanatus [23]. The individual PAL may respond differentially to biotic or abiotic stress, and its different expressions in tissues may be involved in the production of different products under specific conditions. In C. canephora [20], CcPAL2 transcripts appear predominantly in flower, fruit pericarp, roots, and branches, whereas CcPAL1 and CcPAL3 are highly expressed in immature fruits. What’s more, CcPAL1 and CcPAL3 are associated with the accumulation of chlorogenic acids (CGA), whereas CcPAL2 may contribute more significantly to flavonoid accumulation. In S. Miltiorrhiza [19], all three SmPALS are regulated by drought and MeJA treatments, although the time and degree of reactions differ one from another.

In a previous study, we have cloned a new plant PAL gene (designated as IiPAL1) from I. indigotica [24]. The open reading frame (ORF) of IiPAL1 is 2178-bp and it encodes a polypeptide of 725 amino acid residues. IiPAL1 is constitutively expressed in roots, stems, and leaves, with the highest expression being found in stems, and it responds to gibberellin (GA3), abscisic acid (ABA), methyl jasmonate (MeJA), and cold treatments. However, the characteristics of IiPAL1 need to be further investigated and a systematic analysis of different PAL genes in I. indigotica also is needed. Moreover, the relations between IiPALS and synthesis of secondary metabolites remain to be explored.

Under the umbrella of a transcription profiling of I. indigotica [25], one additional gene (IiPAL2) was identified in the present study. This was the first time to report the existence of a small multi-gene family in I. indigotica. The relations between IiPALS and secondary metabolites were also evaluated in the present study. The results from the present study would enable us to further understand the role of IiPALS in the synthesis of phenylpropanoid compounds in I. indigotica at the molecular level, which might be helpful to overcome the low rate of production of secondary metabolites, such as lariciresinol, in the future.

Materials and Methods

Plant materials

The seeds of I. indigotica were collected from the School...
of Pharmacy, Second Military Medical University, Shanghai, China, and authenticated by Professor ZHANG Han-Ming (Department of Pharmaceutical Botany, School of Pharmacy, Second Military Medical University). The seeds were pretreated with 75% alcohol for 5 min, washed thrice with distilled water, treated with 0.1% HgCl₂ for 10 min, and then washed with sterile distilled water four times. The sterilized seeds were incubated between several layers of sterilized wet filter paper and then cultured in MS basal medium for germination. The seedlings were grown at 25 °C under 16-h light/8-h dark photoperiod cycles for 2 months until treatments, for RNA and DNA isolation.

**Hairy root culture and various treatments**

The *I. indigotica* hairy root cultures were derived after the infection of plantlets with *Rhizobium* bacterium (C58C1). Hairy roots developed at cut edges 2–3 weeks after co-cultivation were excised and cultured in solid, hormone-free, half-strength MS medium. After bacteria were eliminated, the hairy roots (0.1 g fresh weight) were cultivated in a 250-mL shake flask containing 200 mL of the liquid basal medium on an orbital shaker at 110 rpm, 3 weeks after co-cultivation were excised and cultured in MS basal medium. The seedlings were grown at 25 °C under 16-h light/8-h dark photoperiod cycles for 2 months until germination. The seedlings were grown at 25 °C under 16-h light/8-h dark photoperiod cycles for 2 months until germination. The seedlings were grown at 25 °C under 16-h light/8-h dark photoperiod cycles for 2 months until germination.

**RNA and DNA isolation**

The roots, stems, leaves, and flowers of *I. indigotica* as well as hairy root samples collected at various times after various treatments were used for RNA isolation. Total RNA was extracted using RNA prep pure plant kit (Tiangen Biotech Co., Beijing, China), according to the manufacturer’s protocol. The genomic DNA was isolated using the modified CTAB method [26]. The quality and concentration of RNA and DNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis on a Helios Gamma ultraviolet spectrophotometer (Thermo electron corporation, Waltham, Massachusetts, USA).

**Discovery of IiP ALs from the transcription profiling database**

In order to obtain PAL genes, we searched the *I. indigotica* transcription profiling database [25] through tBLASTn analysis using protein sequences, nucleotide sequences, and expressed sequence tag (EST) records of target genes of other plants from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). An e-value cut-off of 10⁻² was applied to the homologue recognition.

The Pfam database (http://pfam.janelia.org/) [27] was used to screen the above putative sequences and identify the conserved protein domains using default parameters. As a final quality check, the simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de/) [29] was used to find the PAL domain.

**Molecular cloning of the IiP ALs full-length cDNA**

Total RNA isolated from *I. indigotica* was reversely transcribed using TransScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech Co., Beijing, China). The full length of *IiPAL1* was cloned based on the sequence obtained from Lu [24] with primers for *IiPAL1*-F and *IiPAL1*-R through the PCR reaction under the following conditions: denatured at 94 °C for 2 min, followed by 35 cycles of amplification (94 °C for 35 s, 56 °C for 35 s, and 72 °C for 3 min), and 72 °C for 10 min.

Molecular cloning of *IiPAL2* from *I. indigotica* was based on the sequencing result from transcription profiling. The full length cDNA sequence was obtained by using the first-strand cDNA as the template under the following PCR conditions: 1 min at 95 °C, 35 cycles of amplification (20 s at 95 °C, 20 s at 60 °C, and 75 s at 72 °C), and a final extension of 5 min at 72 °C. The resulting amplified full length ORF was purified and cloned into PMD18-T vector and then sequenced.

**Bioinformatics analysis**

Sequence alignments and molecular mass calculation of the predicted protein were carried out on Vector NTI Advance 11. ORF translation and GenBank Blast were done on NCBI (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis of *IiPAL2* and other known PALS from other plant species retrieved from GenBank were aligned using ClustalX software (version 1.80) and a phylogenetic tree was subsequently constructed using the neighbor joining (NJ) method [29] (1 000 bootstrap replicates) with the MEGA 5.0 software. Protein secondary structures were predicted using NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/) and SOMPA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) [30]. A homology model was generated from the native crystal structure of *Petroselinum crispum* PAL using Discovery studio 2.5 [31].

**Expression profiling of IiP ALs in different tissues under different stresses**

The responses of *IiPAL1* and *IiPAL2* in hairy roots exposed to exogenous MeJA and UV-B and the expression profiles in different tissues were characterized using quantitative real-time PCR (Q-PCR). According to the corresponding sequences of *IiPAL1* and *IiPAL2*, gene specific primers *IiPAL1*-qRT-F, *IiPAL1*-qRT-R, *IiPAL2*-qRT-F and *IiPAL2*-qRT-R were designed. Partial polyubiquitin gene was amplified with primers Actin-F and Actin-R as a control. The Q-PCR assay was carried out in an assay mixture (final volume of 25 µL) containing 12.5 µL of 2 × SYBR Green Real Time PCR Master Mix (TaKaRa, Osaka, Japan), 0.5 µm of each primer, and 2 µL of cDNA. The program for all the Q-PCR reactions was as follows: 10 s pre-denaturation at 95 °C, 1 cycle; 5 s denaturation at 95 °C, 30 s annealing at 60 °C, 40 cycles; and separation reaction (15 s at 95 °C, 30 s
at 60 °C, 15 s at 95 °C). Quantification of the gene expression was done with comparative CT method. All the PCR reactions consisted of three technical replicates.

**Subcellular localization of IiPALS**

The full-length coding regions of IiPAL1 and IiPAL2 were amplified by PCR with sticky BspHI and SpeI ends inserted into the vector pCAMBIA1301-GFP under the control of cauliflower mosaic virus 35S promoter. The expression plasmids were transferred into the rice protoplast cells and were observed under a confocal microscope (Nikon, Tokyo, Japan) with argon laser excitation at 488 nm and a 505–530-nm emission filter set. The red autofluorescence of chlorophylls was imaged at emission wavelength longer than 650 nm [32].

**Expression and characterization of IiPALS in Escherichia coli**

The full-length IiPAL1 and IiPAL2 cDNAs were cloned into plasmid pET32a (+) (Novagen, Copenhagen, Denmark) using EcoRV/EcoRI restriction sites to generate IiPAL1-pET-32a and IiPAL2-pET-32a constructs. The gene-specific primers for IiPAL1-EcoRV-F, IiPAL1-EcoRI-R, IiPAL2-EcoRV-F, and IiPAL2-EcoRI-R were listed in Supplementary Table S1. After sequencing confirmation, the IiPAL1-pET-32a and IiPAL2-pET-32a constructs were transfected into Escherichia coli BL21(DE3) cells using the heat shock method. The E. coli BL21(DE3) cells harboring IiPAL1-pET-32a or IiPAL2-pET-32a in a single colony were inoculated at 37 °C into Luria-Bertani (LB) medium containing ampicillin (100 mg·L⁻¹) and grown with shaking (200 r·min⁻¹) at 37 °C until the optical density (OD600) reached about 0.6. The protein expression was induced for 4 h by an addition of isopropyl-β-D-thiogalactoside (IPTG, Bio-Rad, Berkeley, California, USA) at a final concentration of 1 mmol·L⁻¹. Protein purification was performed on BioLogic DuoFlow using Bio-Scale™ Mini chromatographic column, following the manufacturer’s instructions (Bio-Rad). The purity of the His-tag-fused IiPALS (ht-IiPAL1 and ht-IiPAL2) was assessed by analyzing the total protein on 12% SDS-PAGE, followed by Coomasie Brilliant Blue R250 (Beyotime Biotech Co., Shanghai, China) staining and the protein concentration were determined by the Bradford method [33] using bovine serum albumin as the standard.

**Enzyme activity assay for IiPAL1 and IiPAL2**

The enzyme activities of IiPALS were measured using the method of Yan [34] with minor modifications. The enzyme extract (0.5 mL) was incubated with 50 μL of 0.1 mmol·L⁻¹ L-phenylalanine and 450 μL of 0.01 mmol·L⁻¹ Tris-HCL, pH 7.5, at 37 °C for 60 min. The enzyme reaction was terminated by an addition of 50 μL of 5 mol·L⁻¹ HCL after 1 h. After centrifugation at 25 °C (12 000 r·min⁻¹, 15 min), the compound determination was performed on an Agilent 1260 series liquid chromatography (Agilent, Santa Clara, California, USA) equipped with a quaternary solvent delivery system, an autosampler and a photodiode array detector (DAD). A Diamonsil C₁₈ column (4.6 mm × 250 mm, 5 μm, Dikma Beijing, China) was used for analysis, using a mobile phase consisting of 48% acetonitrile (HPLC grade, Merck KGaA, Darmstadt, Germany) and 52% formic acid (0.1%, HPLC grade, Merck). The flow rate was set at 1.0 mL·min⁻¹ and the injection volume was 10 μL. Elution of the compounds was monitored at 290 nm. Standards of L-phenylalanine and trans-Cinnamic acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

The effects of the reaction time and protein concentration on the enzyme activity were examined. To determine the optimum reaction time, the assays were performed at 37 °C. The assay mixture consisted of 450 μL of 100 mmol·L⁻¹ Tris-HCL (pH 7.5), 500 μL of purified IiPAL1 or IiPAL2 protein and 50 μL of 100 mmol·L⁻¹ L-phenylalanine. 50 μL of 5 mol·L⁻¹ HCL was used to stop reaction at different times (10, 20, 30, 40, and 50 s, and 1, 2, 3, 4, and 5 min). For optimal protein concentration determination, the reaction was carried out with different protein concentrations (10, 20, 40, 60, 80, 90, 100, 200, and 300 mmol·L⁻¹) at the same conditions as above. The IiPAL1 and IiPAL2 activities were determined by measuring absorbance of the reaction solution at 290 nm.

**Transcript abundance of IiPALS in I. indigotica hairy roots treated with MeJA**

The Illumia RNA-Seq data obtained in previous research [25] were utilized to get an insight into the IiPALS’ transcript abundance induced with MeJA in I. Indigotica. The RNA-Seq expression profile data were generated using the Illumina HiSeq™ 2000 platform, after the hairy roots of I. indigotica were treated with MeJA at different times (0, 1, 3, 6, 12, and 24 h). The expression levels at different times were normalized to the level of the control (0 h).

**Metabolite analysis**

MeJA-treated hairy roots of I. indigotica (100 mg) were dried at 45 °C, ground into fine powder, and extracted twice with 10 mL of methanol under sonication for 30 min. After centrifuged at 4 000 r·min⁻¹ for 5 min, the supernatant was diluted with methanol to 10 mL in total volume. The final solution was filtered through a 0.22-μm organic membrane filter prior to analysis.

The LC-MS/MS analysis was conducted on an Agilent 1200 series coupled with an Agilent 6410 triple Quadrupole mass spectrometer and an electrospray ionization source (Agilent). The data were processed with MassHunter Workstation Software. Chromatographic separation was achieved on an Agilent ZORBAX SB-C₁₈ (3.5 μm, 100 mm × 2.1 mm i.d.) at column temperature of 35 °C. The mobile phase consisted of acetonitrile (eluent A) and 5 mmol·L⁻¹ of ammonium acetate solution (eluent B, HPLC grade, Merck KGaA, Darmstadt, Germany), eluted at a rate of 0.3 mL·min⁻¹ with a gradient program as follows: 0–4.00 min, 14% A; 4.00–4.50 min, 50% A, 4.50–8.50 min, 85% A and the run time was 8.5 min. The injection volume was 5 μL. Quantification was achieved in multiple reaction monitoring mode (MRM), and the selected transitions of m/z were
The correlations between two lIPIs and four lignans were calculated using the Pearson correlation coefficient by canonical correlation analysis [33]. Gene-to-metabolite network was visualized to identify probable relation between lIPIs and lignan biosyntheses.

Results
Molecular cloning of the lIPIs full-length cDNA
The decoding of the I. indigotica transcriptome enabled us to identify a novel PAL gene (lIPI2) that had not been reported before. The full-length cDNA sequence of lIPI2 was verified by PCR amplification.

The open reading frame of lIPI2 was slightly shorter than that of lIPI1 (2 115 bp vs. 2 530 bp), but lIPI1 and lIPI2 only shared a 70.06% of sequence identity. The deduced amino acid sequences of lIPI1 and lIPI2 included 725 and 705 amino acids (Fig. 2). The predicted molecular masses of lIPI1 and lIPI2 proteins were 78.65 kDa and 76.89 kDa respectively, and their theoretical pI were 5.96 and 5.63, respectively.

Protein-protein BLAST showed that lIPI1 and lIPI2 had a high degree of similarity (77%−93%) to PALs from other plant species at the amino acid level. A detailed sequence alignment of the lIPIs proteins is shown in Fig. 3.

The results showed that lIPIs contained the conserved Ala-Ser-Gly (216−218) catalytic triad. In addition, sequence alignment showed that lIPIs contained conserved deamination sites (i.e., L-220, V-221, L-269, and A-270) and catalytic active sites (i.e., N-272, G-273, H-350, NDN[396−398 aa], and HNQDV[500−505 aa]), just as Dendrobium candidum [14].

Phylogenetic analysis of the lIPIs
To investigate the evolutionary relationships among the lIPIs and PALs from other plant species, the phylogenetic tree was constructed using the neighbor-joining method. The lIPIs in the present study were most closely related to the dicot PALs, and they were classified as members of this group, with

Fig. 2 Nucleotide sequences and deduced amino acid sequences of lIPIs. (A) lIPI1, (B) lIPI2. The start codon (ATG) is in italics and the stop codon (TGA or TAA) is indicated by an asterisk. The conserved active sites motifs are circled.

The secondary structures are shown in Fig. 4A and the differences between lIPI1 and lIPI2 are listed in Table 1. The three-dimensional structures of lIPIs were predicted using Discovery Studio 2.5 with P. Crispum PAL as a template.
a closest relationship with \( \text{AtPAL1} \) and \( \text{AtPAL2} \), forming a Cruciferae cluster (Fig. 4C). A total of 20 protein sequences used for analysis were from following plant species: \( \text{IiPAL1} \) (DQ468345), \( \text{AtPAL1} \) (L33677), \( \text{AtPAL2} \) (L33678), \( \text{NiPALB} \) (AB008200), \( \text{PiPAL} \) (P52777), \( \text{GmPAL1} \) (X52953), \( \text{CsPAL} \) (D26596), \( \text{MsPAL} \) (X58180), \( \text{CiPAL6} \) (U43338), \( \text{DoPAL1} \) (AY450643), \( \text{PsPAL} \) (D10003), \( \text{RiPAL2} \) (AF237955), \( \text{ShPAL} \) (L36822), \( \text{MePAL1} \) (AY036011), \( \text{BnPAL2} \) (AY795080), \( \text{TdPAL} \) (X99705), \( \text{PaPAL1} \) (AF036948), \( \text{OsPAL} \) (XM473196), \( \text{ZeaPAL} \) (L77912), and \( \text{TpPAL} \) (AB236800).

![Fig. 3](image)

**Fig. 3** Multi-sequence alignment of \( \text{IiPALs} \) with other plant PAL proteins. The sequences shown here are from \( \text{AtPAL1} \) (Arabidopsis thaliana, L33677), \( \text{AtPAL4} \) (A. thaliana, NP187645), \( \text{VvPAL} \) (Vitis vinifera, XP 002268732), \( \text{PtPAL} \) (Populus trichocarpa, ACC63889.1), and \( \text{BnPAL} \) (Brassica napus, AY795080).

**Tissue-specific and induced expression profile of \( \text{IiPALs} \)**

The expression profile of \( \text{IiPAL1} \) and \( \text{IiPAL2} \) in different tissues of \( \text{I. indigotica} \) showed that \( \text{IiPAL1} \) and \( \text{IiPAL2} \) expression could be detected in all tissues with different expression levels. The transcript level of \( \text{IiPAL1} \) was the highest in flowers, whereas that of \( \text{IiPAL2} \) was highest in roots. The expressions of \( \text{IiPAL1} \) and \( \text{IiPAL2} \) in leaves and flowers were almost the same (Fig. 5A).

To understand the role of \( \text{IiPALs} \) in responses to plant defense, the plants were treated with MeJA and UV-B respectively. The results revealed that the transcription levels of \( \text{IiPALs} \) were responsive to different treatments to various degrees. As shown in Fig. 5B, the \( \text{IiPAL1} \) expression under the MeJA induction was rapidly and strongly induced, peaked at 4 h (5-fold of original value), and gradually decreased at 6–8 h. Interestingly, the expression level of \( \text{IiPAL1} \) increased again at 12 h, and then decreased a little after 24 h. For \( \text{IiPAL2} \), the transcript abundance in the hairy root was also increased, peaked at 8 h, and then declined. After UV-B treatment (Fig. 5C), \( \text{IiPAL1} \) and \( \text{IiPAL2} \) displayed the highest expression levels at 10 min with about 4- and 9-fold increases, respectively, and then decreased after 30 min. After UV-B
Bioinformatics analysis of IiPALS. (A) predicts secondary structures of IiPAL1 and IiPAL2. The short-term, green, dotted line and random coil represent alpha helix, beta turn, random coil, respectively; (B) Three dimensional protein model of IiPALs protein. The chains corresponding to the MIO domain (golden), core domain (blue), inserted shielding domain (green) and the MIO group (red) are highlighted; (C) Phylogenetic tree of IiPALS and other plant PAL genes using the Clustal X software and MEGA 5.0 software based on the Neighbour-joining method. IiPAL1 and IiPAL2 are marked by black box.

Table 1 The differences in secondary structures between IiPAL1 and IiPAL2

| Gene | α-helices | β-turns | Extended strands | random coils |
|------|-----------|---------|-----------------|-------------|
| IiPAL1 | 366 (50.48%) | 60 (8.28%) | 81 (11.17%) | 218 (30.07%) |
| IiPAL2 | 388 (55.11%) | 36 (5.11%) | 51 (7.24%) | 299 (32.53%) |

Subcellular localization of IiPALS

As shown in Figs. 5D–5G, the rice protoplast expressing IiPAL1-GFP showed green fluorescent signals, which was localized in the cytoplasm (Fig. 5D). The fluorescence of the IiPAL1-GFP fusion was exclusively distributed in cytoplasm when merged with signals shown in panels D and E (Fig. 5F). The results of IiPAL2-GFP were the same as that of IiPAL1-GFP.

Functional analysis of IiPAL1 and IiPAL2

The in vitro functional activities of IiPAL1 and IiPAL2 were investigated by expressing the genes in E. Coli BL21 (DE3). The fractionation analysis using SDS-PAGE showed molecular masses of about 97 kDa and 95 kDa (including the tags), respectively (Fig. 6). It was in good agreement with that predicted by the bioinformatics method. Enzyme assays were carried out under standard conditions, using purified protein and L-Phe as the substrate. The control reaction with L-Phe using the same buffer without the PAL enzyme gave a peak of L-Phe (Fig. 7A). After L-Phe catalyzed by recombinant proteins ht-IiPAL1 and ht-IiPAL2, respectively, only one peak at about 23 min could be seen, which should be ascribed to the L-Phe conversion to trans-cinnamic acid (Figs. 7C–7D).

Moreover, the time course for expression of the target proteins was also examined. From Fig. 8A, it was evident that, in a short time (6 min), the amount of the protein production increased over time. And the promoting reaction rate of IiPAL2 rose quickly at 30 s, from which IiPAL2 had a stronger catalytic ability than IiPAL1.

Meanwhile, the protein concentration was proportional to enzyme promoting reaction rate (Fig. 8B). The initial activity of IiPAL1 was higher than that of IiPAL2, and the effects of different concentrations of IiPAL2 on the enzymatic reaction were higher than that of IiPAL1. When the protein concentration of IiPAL1 and IiPAL2 reached 100 mmol·L⁻¹, the promoting reaction rate had a rapid increase and reached the peak at 200 mmol·L⁻¹, followed by a gradual decrease.

Integration of transcript and metabolite accumulation analyses

A canonical correlation analysis was performed to ex-
Fig. 5 Characterization of IiPALs. (A) Transcript abundance of IiPALs in different tissues of I. Indigotica; (B) IiPALs expression under the induction of MeJA; (C) Fold changes of IiPALs in hairy roots of I. indigotica treated with UV-B; (D-G) Analysis of IiPAL1 subcellular localization (Bars = 3.0 μm)

Fig. 6 SDS-PAGE analysis of the purified proteins. (M, protein marker; Lane 1, purified ht-IiPAL1; Lane 2, purified ht-IiPAL2)

Palo possibly correlations between the accumulations of four lignans and the expression profiles of IiPAL1 and IiPAL2 (Fig. 9) with the variable correlation coefficient cut-off values being set at 0.6. The variable correlation coefficients between IiPAL1 transcript and four metabolites accumulation (coniferin, lariciresinol, secoisolariciresinol, and pinoresinol) were −0.184, 0.954, 0.573, and 0.873, respectively, whereas, that of IiPAL2 were −0.136, 0.200, 0.503 and 0.072, respectively. These results suggested that IiPAL1 was most likely to be involved in the biosynthesis of lariciresinol and its precursor (pinoresinol). However, IiPAL2 seemed to be unrelated to any of the measured lignans.

Discussion

PAL is one of the branch point enzymes that link primary metabolism to secondary metabolism [35]. It catalyzes the first step in the formation of cinnamic acid, a precursor, to a variety of phenylpropanoid derivatives. In the present study, we isolated a novel I. indigotica PAL gene, IiPAL2. The existence of a small multigene family in I. indigotica was consistent with the results from other plant species such as S. Viminalis, S. Miltiorrhiza and C. canephora [15, 19-20].

IiPAL1 and IiPAL2 encoded 725 and 711 amino-acid proteins, respectively, whose lengths were similar to that of other reported PALS [36]. Sequence analysis and homology modeling revealed that IiPALS shared identical characteristics with many other PALS. IiPALS also possessed a conserved Ala-Ser-Gly (216−218) catalytic triad (Figs. 2 and 3). As reported in other PALS, the Ala-Ser-Gly triad can be converted into the MIO prosthetic group by cyclization and the elimination of water [37]. Meanwhile, the conserved deamination and catalytic sites may participate in substrate selectivity and binding, catalysis and/or the formation of the MIO prosthetic group. This analysis suggested that IiPAL1 and IiPAL2 are members of PAL family and the proteins may have the same catalytic function as other PAL proteins.

The members of the PAL gene family in a plant are usually expressed differently in tissues and appear to be functionally distinct. The RiPAL1 in Rubus idaeus is associated with early fruit ripening events, whereas expression of RiPAL2 is correlated more with later stages of flower and fruit development [38]. In C. lanatus, only six of the 12 CIPALS may have the potential roles of developing fruit color and flavor [23]. Consistently, differential expression patterns were observed from the two IiPALS, although they were expressed in all of the tissues analyzed. IiPAL1 was found to be highly expressed in the stems, while IiPAL2...
predominately showed expressions in the roots. The root of *I. indigotica* is the well-known Chinese medicine “Banlangen” (Radix *Isatis*), and the distribution of *IiPALs* in the tissues suggests that *IiPAL2* may play possible roles in roots through lignin formation and participate in the developmental processes of *I. indigotica*. However, the role of *IiPAL1* cannot be denied, because some genes may mediate active pharmaceutical compounds biosynthesis in aerial organs [39].

**Fig. 7** Representative HPLC profiles of incubation of L-Phe without (A) or with (C, D) purified *ht-IiPALs* enzyme. (B) trans-cinnamic acid standard (Peak a: L-Phe; peak b: trans-cinnamic acid)

**Fig. 8** The effects of the reaction time (A) and protein concentration (B) on the enzyme activity. Trans-cinnamon acid has the maximum absorption at 290 nm

*PAL* is one of the most extensively studied enzymes with respect to plant responses to biotic and abiotic stresses. Expression analyses demonstrated that *PAL* could be widely stimulatated by environmental elicitors such as low temperature, dehydration, and UV irradiation [40]. Plant signaling molecules, including ABA, salicylic acid (SA), and JA [41], have also been demonstrated to elicit *PAL* activity. In general, all the elicitors chosen in the present study could escalate the *IiPALs* expression, but the up-regulations may be caused by different mechanisms.

Consistently, *IiPALs* transcripts in hairy roots were enhanced after MeJA and UV-B treatments (Fig. 5). However, differences were observed for two *IiPALs* in the time and degree of responses after treated with MeJA. Similar results were previously found in three *PALs* from *Salvia Miltiorrhiza*. These differences in response to MeJA might be explained by the complexity and diversity of MeJA. On the other hand, the expressions of *IiPAL1* and *IiPAL2* were similar after irradiation with UV-B. They showed the highest induction at the same time under UV-B induction and the increase didn’t disappear after the removal of UV-B, as seen with *P. Kurrooa*. Many elicitors could modulate the production of many important secondary metabolites of the phenylpropanoid pathway, such as bisbibenzyl in *Plagiochasma appendiculatum* [17], lignins and anthocyanins in *A. thaliana* [42], and phenolics and flavonoids in *Hypericum perforatum* [43]. So it may be used as a new strategy to improve the content of phenylpropanoid compounds.
Subcellular localization analyses indicated that IiPAls were localized in cytoplasm, which was consistent with the results obtained with S. viminalis [15]. At subcellular level, PAL was mainly located in cytoplasm and chloroplast, mitochondria, glyoxysome, peroxisome, and other membrane organelles [44]. Jin Nakashima has proven that PAL activity in the cytosolic is the best [45]. The results of subcellular localization suggested that IiPAls could be localized in cytoplasm, indicating that IiPAls may have a high PAL activity.

To date, many plant PAL genes have been cloned and successfully expressed in vitro, such as PAlS in Artemisia annua [46]. To further confirm its function, IiPAls were expressed in E. coli BL21 (DE3) in the present study. The recombinant protein could catalyze the elimination of ammonia from L-phenylalanine to form cinnamic acid, revealing that IiPAls encoded a functional protein with a high PAL activity. As the gateway enzyme, PAL plays a key role in mediating carbon flux from primary metabolism in the phenylpropanoid pathway. It may be a new target for enhancing the content of pharmaceutical compounds in I. Indigotica, just as Junli Chang reported with Arabidopsis thaliana [47].

In previous studies, the integration of transcriptomics and metabolomics data is used as an important mean to dig crucial genes involved in the synthesis of target compounds [44]. In the present study the correlation analysis indicated that IiPALI transcript was coincident with the accumulation of lariciresinol, suggesting that IiPALI was relevant with the biosynthesis of active pharmaceutical compounds in I. Indigotica. However this result needs to be validated by metabolic engineering in the future.

Taken together, our results showed the existence of another IiPAL gene in I. Indigotica. The cloning and characterization of two distinct I. Indigotica PAL genes provided a basis for further undertaking a detailed molecular and genetic analysis of the regulation of this key gene family in I. Indigotica. The different expression profiles showed that roles of each IiPAL may be different in the biosynthesis of secondary metabolites. As a key step, further studies concerning the roles of IiPAls using Agrobacterium tumefaciens-mediated genetic transformation would help better understand the metabolic network of secondary metabolites in I. Indigotica, which may also provide a new strategy for enhancing the content of pharmaceutical compounds.

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