Combined transcriptome and metabolite profiling reveals that *Ii*PLR1 plays an important role in lariciresinol accumulation in *Isatis indigotica*

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Abstract

A lignan, lariciresinol, is an important efficacious compound for the antiviral effect of *Isatis indigotica*, a widely used herb for the treatment of colds, fever, and influenza. Although some rate-limiting steps of the lariciresinol biosynthetic pathway are well known, the specific roles of gene family members in *I. indigotica* in regulating lariciresinol production are poorly understood. In the present study, a correlation analysis between the RNA sequencing (RNA-Seq) expression profile and lignan content by using *I. indigotica* hairy roots treated with methyl jasmonate (0.5 μM) at different time points as a source implicated that *I. indigotica* pinoresinol/lariciresinol reductase 1 (*Ii*PLR1), but not *Ii*PLR2 or *Ii*PLR3, contributed greatly to lariciresinol accumulation. Gene silencing by RNA interference (RNAi) demonstrated that *Ii*PLR1 indeed influenced lariciresinol biosynthesis, whereas suppression of *Ii*PLR2 or *Ii*PLR3 did not change lariciresinol abundance significantly. *Ii*PLR1 was thus further characterized; *Ii*PLR1 was constitutively expressed in roots, stems, leaves, and flowers of *I. indigotica*, with the highest expression in roots, and it responds to different stress treatments to various degrees. Recombinant *Ii*PLR1 reduces both (±)-pinoresinol and (±)-lariciresinol efficiently, with comparative *K*_cat/*K*_m values. Furthermore, overexpression of *Ii*PLR1 significantly enhanced lariciresinol accumulation in *I. indigotica* hairy roots, and the best line (ovx-2) produced 353.9 μg g⁻¹ lariciresinol, which was ~6.3-fold more than the wild type. This study sheds light on how to increase desired metabolites effectively by more accurate or appropriate genetic engineering strategies, and also provides an effective approach for the large-scale commercial production of pharmaceutically valuable lariciresinol by using hairy root culture systems as bioreactors.

Key words: Correlation analysis, *Isatis indigotica*, lariciresinol, lignan, pinoresinol/lariciresinol reductase, RNA-Seq expression profile.

Abbreviations: AACT, acetoacetyl CoA thiolase; ABA, abscisic acid; CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA N-methyltransferase; CCR, cinnamoyl CoA reductase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, 4-(hydroxy)cinnamoyl CoA ligase; DIR, dirigent protein; dw, dry weight; FPS, farnesyl diphosphate synthase; fw, fresh weight; HMGR, HMG-CoA reductase; ht-*Ii*PLR1, His-tag fused *Ii*PLR1; MeJA, methyl jasmonate; MRM, multiple reaction monitoring; PAL, phenylalanine ammonia-lyase; PLR, pinoresinol/lariciresinol reductase; PrR, pinoresinol reductase; qRT-PCR, real-time quantitative PCR; SARS, severe acute respiratory syndrome; SIRD, secoisolariciresinol dehydrogenase; SMMU, Second Military Medical University; SQE, squalene epoxidase; UGT, UDP-sugar-dependent glycosyltransferase.

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Introduction

Isatis indigotica Fort. (Isatis tinctoria), belonging to the family Cruciferae, is a prevalent Chinese medicinal herb (Zhao, 2007). The root of I. indigotica (Radix Isatidis), named ‘Ban–Lan–Gen’ as a traditional Chinese medicine, has been used for the treatment of influenza, epidemic hepatitis, and epidemic encephalitis B for >1000 years (Wang et al., 2000). During the epidemic period of severe acute respiratory syndromes (SARS) and H1N1 influenza, Ban–Lan–Gen demonstrated its significant antiviral effect (Lin et al., 2005; Sui, 2010; Wang et al., 2011). However, the antiviral compounds of I. indigotica were unclear until Li (2003) determined that lariciresinol isolated from this plant was useful for the treatment of influenza A virus. More recently, Yang et al. (2013) determined that a lariciresinol derivative, clemastin B [7S,8R,8’R-(−)-lariciresinol-4,4’-bis-O-β-d-glucopyranoside], significantly inhibited human and avian influenza A and B viruses.

Lariciresinol is a type of lignan which has been reported to possess a number of biological activities, including antimicrobial, antioxidant, anti-inflammatory, and antioestrogenic properties (Saleem et al., 2005). However, lariciresinol accumulates at a low abundance in plants. For example, its content in I. indigotica is only 68 μg g−1 of dry weight (DW) (Li, 2003), which not only constrains the yield of lariciresinol for the pharmaceutical industry, but also affects the use of the I. indigotica herb itself. Metabolic engineering provides an attractive approach for increasing the production of such health-promoting compounds in plants.

Plant metabolic pathways are complex and often feature multiple levels of regulation, making the consequences of metabolic engineering difficult to predict. The existence of multcopy gene families in the metabolic pathway suggests divergent functional roles for pathway components involved in the biosynthesis of metabolites. For example, in Arabidopsis thaliana, six squalene epoxidases (SQEs) were predicted based on a homology search (SQE1–SQE6) (Benveniste, 2002; Rasbery et al., 2007; Posé et al., 2009); however, only the products of SQE1, SQE2, and SQE3 were able to functionally complement the Saccharomyces cerevisiae squalene epoxidase mutant erg1 (Rasbery et al., 2007). Recently, only SQE3, but not SQE2, was found to functionally complement SQE1 in the promotion of plant squalene epoxidase activity (Laranjeira et al., 2015). At the early stages of Arabidopsis seed development, embryo viability relies on farnesyl diphosphate (FPP) or an FPP-derived isoprenoid/sterol precursor provided by farnesyl diphosphate synthase 1 (FPS1) gene products, but not those of FPS2 (Keim et al., 2012). In contrast, Arabidopsis HMG-CoA reductase 1 (HMG1) and HMG2 are both responsible for the biosynthesis of triterpenes (Ohayama et al., 2007). Arabidopsis acetoacetyl CoA thiolase 1 (AACT1) and AACT2 can both complement the lethal aact deletion allele (DERG10) in yeast despite displaying different spatial and temporal expression patterns (Jin et al., 2012). With respect to lariciresinol biosynthesis, although some enzymatic steps have been proposed to be particularly important for activating the lariciresinol biosynthetic pathway (Fig. 1) (Humphreys and Chapple, 2002; Nakatsubo et al., 2008; Satake et al., 2013), the specific roles of gene family members in regulating this metabolic flux have rarely been investigated. PLR, a gene encoding pinoresinol/lariciresinol reductase, represents one of the most important rate-limiting steps of the lariciresinol biosynthetic pathway (Satake et al., 2013). Hemmati et al. (2010) reported that both LuPLR1 and LuPLR2 are expressed in flax seed tissues, whereas only LuPLR2 is expressed in stem and leaf tissues. Zhao et al. (2014) reported that pinoresinol reductase 1 (PrR1), but not PrR2, impacts lignin distribution during secondary cell wall biosynthesis in Arabidopsis. These studies indicated that PLR family members in plants appeared to play divergent functional roles.

Under the umbrella of a transcription profiling of I. indigotica (Chen et al., 2013), 51 biosynthetic genes involved in the lariciresinol pathway were first identified in I. indigotica in the present study. Correlation analysis based on profiling changes of transcript abundance and accumulation of metabolites under methyl jasone (MeJA) stress indicated that iPLR1 was positively correlated with lariciresinol, whereas iPLR2 or iPLR3 were not. This hypothesis was demonstrated by examining lariciresinol production when iPLR1, iPLR2, or iPLR3 was silenced. iPLR1 was then well characterized, and the effect of iPLR1 overexpression on lariciresinol accumulation was also investigated.

Materials and methods

Plant material

The I. indigotica plant, grown in the gardens of the Second Military Medical University (SMMU), Shanghai, China, was identified by Professor Hanming Zhang, School of Pharmacy, SMMU. The harvested I. indigotica seeds were pre-treated with 75% alcohol for 1 min, washed three times with distilled water, followed by treatment with 0.1% HgCl2 for 5 min and by four rinses with sterile distilled water. The sterilized seeds were then incubated between several layers of sterilized wet filter paper and cultured on Murashige and Skoog (MS) basal medium for germination. The seedlings were grown at 25°C under 12 h light/12 h dark photoperiod cycles.

Transcriptome analysis

The assembly of I. indigotica transcriptome sequences (Chen et al., 2013) was searched for putative genes involved in the lariciresinol biosynthetic pathway. Protein sequences, nucleotide sequences, and expressed sequence tag (EST) records of target genes of other plants were downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). All the sequences were used as queries to search the I. indigotica transcript profiling database through the basic local alignment search tool (BLASTN or BLASTX) to determine candidate gene sequences with an e-value <1e-5. The Pfam database (http://pfam.janelia.org/) (Punta et al., 2012) was used to screen the above putative sequences and identify the conserved protein domains with default parameters. Blastx alignment (e-value <1e-5) between the resulting sequences and protein databases such as the NCBI non-redundant protein database, the Swiss-Prot protein database, the Kyoto Encyclopedia of Genes and Genomes, and the Cluster of Orthologous Groups database was performed, and the best aligning sequences were determined to be the target genes and their functional annotations. Sequences corresponding to the target genes involved in the lariciresinol biosynthetic pathway are listed in Supplementary Table S1 available at JXB online.
To gain insight into the gene expression patterns in response to MeJA treatment, the Illumina RNA sequencing (RNA-Seq) data provided by Chen et al. (2013) were utilized. The RNA-Seq expression profile data were generated using the Illumina HiSeq™ 2000 platform (100 bp, TruSeq SBS kit v3-HS 200 cycles; Illumina), and included the hairy roots of *I. indigotica* treated with MeJA (0.5 μM) at different time points (0, 1, 3, 6, 12, and 24 h). 0 h was used as the control to normalize the expression level of the other time points. A heat map was constructed using the log2-transformed and normalized expression level data in MultiExperiment Viewer (Saeed et al., 2003). Genes with different expression patterns were grouped by hierarchical clustering.

**Metabolite analysis**

MeJA-treated *I. indigotica* hairy roots were freeze-dried and ground into powder. The dried powder sample (100 mg) was extracted twice with methanol (20 ml) under sonication for 30 min, and then centrifuged at 4500 rpm for 5 min. The supernatant was concentrated to 10 ml total volume, and the extract solution was then filtered through a 0.2 μm membrane filter (Woongki Science, Seoul, Korea) before analysis. Lignan contents were determined using an Agilent 6410 triple quadrupole mass spectrometer (Agilent, USA) coupled to an Agilent 1200 high-pressure liquid chromatograph equipped with a pump (Agilent 1200 G1311A) and an autosampler (Agilent G1329A). Chromatographic separation was performed with a Thermo® C18 column (150 mm×2.1 mm, id, 3.5 μm particle size; Agilent). A mobile phase consisting of acetonitrile (solvent A) and 5 mM ammonium acetate (solvent B) was used, with the flow rate set at 0.3 ml min⁻¹. A gradient run of 0–4 min in 15% solvent A, 4–4.5 min in 50% solvent A, and finally 4.5–8.5 min in 85% solvent A was determined as optimal. Multiple reaction monitoring (MRM) mode was used for the quantification, and the selected transitions of m/z were 357→151 for pinoresinol, 359→329 for lariciresinol, 361→164 for secoisolariciresinol, 357→121 for mataireisnol, and 685→523 for secoisolariciresinol diglucoside. All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Correlation analysis of genes and metabolites**

Correlations between the identified biosynthetic genes and five lignans were calculated using the Pearson correlation coefficient using R based on the co-occurrence principle between mRNA and metabolite levels (Saito and Matsuda, 2010). The heat maps of lignans and genes were generated by two-way hierarchical clustering using R. The genes were selected by the criterion that they were correlated with at least one lignan (|r|>0.6). The correlation network was generated using Cytoscape (Cline et al., 2007).

**Generation and analyses of lIPLR1-, lIPLR2-, and lIPLR3-silenced hairy roots**

*lIPLR1*, *lIPLR2*, and *lIPLR3* were each suppressed by RNA interference (RNAi) technology. First, the nucleotide sequences of *lIPLR1*, *lIPLR2*, and *lIPLR3* retrieved from RNA-Seq (Supplementary Table S1 at JXB online) were aligned using CLUSTALX version 2.0.12 (Larkin et al., 2007), and the conserved sites were shaded.
using GeneDoc (Nicholas and Nicholas, 1997). Secondarily, according to the alignment results, an appropriate 400–500bp fragment (Supplementary Table S2) of the coding region of each IiPLR member was selected and amplified by PCR using primers IiPLR1-sNcoI-aSalI and IiPLR1-skpnI-aBamHI for IiPLR1; IiPLR2-sNcoI-aSalI and IiPLR2-skpnI-aBamHI for IiPLR2; and IiPLR3-sNcoI-aSalI and IiPLR3-skpnI-aBamHI for IiPLR3 (Supplementary Table S3) containing two sets of restriction sites at the 5’ end. The PCR product was cloned into pGEM-T vector, sequenced, and then subcloned in opposite orientations on either side of the pDk intron of the pCAMBIA-1300-pHANNIBAL vector (Wesley et al., 2001) to generate plasmids pIiPLR1-RNAi, pIiPLR2-RNAi, and pIiPLR3-RNAi.

After sequencing confirmation, plasmids pIiPLR1-RNAi, pIiPLR2-RNAi, and pIiPLR3-RNAi, together with pCAMBIA-1300-pHANNIBAL as vector control (CK), were introduced separately into I. indigotica leaf explants by using the Agrobacterium tumefaciens C58C1 strain, and the generated hairy root lines were screened using hygromycin (100 mg l^\text{-1}). Some transgenic roots turned brown and aged considerably faster than wild-type root cultures (hairy root lines generated through transformation with the blank C58C1 strain, WT). These lines were discarded, and the remaining hairy root lines were subcultured for 45 d in hormone-free, half-strength MS liquid medium, for DNA extraction, RNA extraction, metabolite analysis, and phloroglucinol-HCl staining.

Genomic DNA was isolated from hairy root samples using the cetyltrimethyl ammonium bromide method (Doyle, 1990). The DNA was then used in PCR analysis for detecting the presence of the inserted IiPLR fragment by primers JDPDK-1F and JDPDKR. The transformed status of hairy roots was also verified by PCR for the genes hpt and rolB (Chilton, 1982). The primer sequences are listed in Supplementary Table S3 at JXB online. The PCR was performed under the following conditions: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 3 min.

The expression profiles of IiPLR1, IiPLR2, and IiPLR3 were investigated by using real-time quantitative PCR (qRT-PCR) analysis. Total RNAs from hairy root samples were extracted using TRizol Reagent (GIBCO BRL) (Jaakola et al., 2001) and then reverse transcribed to generate cDNA as a template. Gene-specific primers (IiPLR1-qRT-F and IiPLR1-qRT-R; IiPLR2-qRT-F and IiPLR2-qRT-R; IiPLR3-qRT-F and IiPLR3-qRT-R) were designed using Primer Premier 5.0 and optimized using oligo 7; the I. indigotica actin gene reported by Li et al. (2014) was used as the internal reference gene (Supplementary Table S3 at JXB online). The qRT-PCR was performed according to the manufacturer’s instructions (TaKaRa, Japan) under the following condition: 30 s pre-denaturation at 95 °C, one cycle; 10 s denaturation at 95 °C, 20 s annealing at 58 °C, 20 s collection fluorescence at 72 °C, 40 cycles. Quantification of gene expression was done with the comparative CT method.

Lariciresinol accumulations were analysed by liquid chromatography–mass spectrometry as described above. The hairy root samples were stained with phloroglucinol-HCl for 20 min for detection of lignans, lignins, or phenolic derivatives according to Hano et al. (2006).

IiPLR1 gene isolation and sequence analysis

Total RNAs of I. indigotica seedlings were extracted using TRizol Reagent (GIBCO BRL) (Jaakola et al., 2001) and then reverse transcribed to generate cDNA. Gene-specific primers for full-length IiPLR1 were designed based on the corresponding sequence retrieved from RNA-Seq (Supplementary Table S1 at JXB online). IiPLR1 was amplified by PCR using Pfu Ultra DNA polymerase (TransGen Biotech), the gene-specific primers IiPLR1-ORF-F and IiPLR1-ORF-R (Supplementary Table S3), and the cDNA as a template. The amplified PCR product was purified and cloned into the PMD18-T vector and then sequenced.

Translation of the open reading frame (ORF) and molecular mass calculation of the predicted protein were carried out on Vector NTI Suite 8. The amino acid sequence alignments of IiPLR1 with other known PLRs (Supplementary Table S4 at JXB online) were performed using CLUSTALX version 2.0.12 (Larkin et al., 2007), and the conserved sites were shaded using GeneDoc (Nicholas and Nicholas, 1997). Phylogenetic relationships were analysed using the Neighbor–Joining method with the pairwise deletion option in MEGA 5.05. Tree reliability was estimated using a bootstrap analysis of 1000 replicates (Tamura et al., 2011).

Expression profile of IiPLR1 in different tissues and under various treatments

Leaves of 2-month-old I. indigotica seedlings were sprayed with 100 μM MeJA or 100 μM abscisic acid (ABA), and then sampled at 0, 2, 4, 6, 8, 12, and 24 h post-treatment. For UV-B treatment, the seedlings were exposed to 1500 J m^{-2} UV-B light for 30 min, and then sampled at 0, 5, 10, and 30 min during treatment, and at 30, 60, and 120 min post-treatment.

The expression profile of IiPLR1 in different tissues (roots, stems, leaves, and flowers) and under various treatments was investigated by using qRT-PCR analysis as described above.

Enzyme assay

Full-length IiPLR1 cDNA was cloned into plasmid pET32a (+) (Novagen, USA) using NcoI/SalI restriction sites to generate the IiPLR1-pET construct. The sequences of primers IiPLR1-pET-F and IiPLR1-pET-R are given in Supplementary Table S3 at JXB online. After sequencing confirmation, the IiPLR1-pET construct was transformed into Escherichia coli BL21(DE3) cells for fusion protein expression. Escherichia coli BL21(DE3) cells harbouring IiPLR1-pET were grown in LB medium at 30 °C. When the A_{600} of the cultures reached 0.6, expression of the gene was induced for 12 h by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Merck, Germany). Protein purification was performed using a His Spin Trap column following the manufacturer’s instructions (GE Healthcare). The purity of the His-tag fused IiPLR1 (ht-IiPLR1) was examined by 12% SDS–PAGE, and the protein concentration was determined by the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as the standard.

IiPLR1 activity was assayed according to Fukuhara et al. (2013) with slight modifications. The assay mixtures (1 ml) consisted of TG buffer (50 mM TRIS-HCl, 10% glycerol; pH 7.0), 150 μM NADPH, 200 μM (±)-pinoresinol, or 3.5 μM (±)-lariciresinol and 5 μg of purified ht-IiPLR1. Protein, buffer, and substrate were pre-incubated for 5 min at 30 °C. The enzyme reaction was initiated by addition of NADPH and terminated by addition of 300 μl of ethyl acetate after 30 min. The assays were extracted with ethyl acetate (3×300 μl in total). The combined ethyl acetate phases were dried under vacuum. The residue was dissolved in 1000 μl of methanol and subjected to LC-MS analysis (see ‘Metabolite analysis’ above). For determination of V_{max} and K_m values, 10 different concentrations of (±)-pinoresinol (between 10 μM and 500 μM) or (±)-lariciresinol (between 0.5 μM and 20 μM) as substrates and 1 μg of purified ht-IiPLR1 were used. Incubations were carried out at 30 °C for 5 min (within the linear kinetic range). The assay mixtures without ht-IiPLR1 were used as controls. The consumption of substrates was calculated for kinetic analysis. V_{max} and K_m values were determined from Lineweaver–Burk plots, and K_{cat} was determined by dividing V_{max} by the enzyme concentration.

Generation and analyses of IiPLR1-overexpressing hairy roots

To construct the IiPLR1 overexpression vector, full-length IiPLR1 cDNA was cloned into plasmid PHB-flag using BglII/XhoI restriction sites to generate the PHB-IiPLR1-flag construct. The sequences of primers IiPLR1-oxv-F and IiPLR1-oxv-R are given in Supplementary Table S3 at JXB online.
After sequencing confirmation, plasmid PHB-IiPLR1-flag, together with PHB-flag as vector control (CK), were separately introduced into *I. indigotica* leaf explants by using the *A. tumefaciens* C58C1 strain and the hairy root lines generated were screened using hygromycin (100 μg ml⁻¹). The vigorous hairy roots (~100 mg) were inoculated into 150 ml conical flasks containing 40 ml of hormone-free, half-strength MS liquid medium, cultured on an orbital shaker (120 rpm) at 25 °C in the dark, and the culture medium was routinely refreshed every 9 d. The fresh weight (fw) of root tissues from flasks of cultures (determined as the difference between the whole flask with and without the harvested root tissues) was recorded at days 9, 18, 27, 36, and 45 after inoculation. At day 45, the hairy roots were harvested for DNA extraction, RNA extraction, protein extraction, metabolite analysis, and phloroglucinol-HCl staining.

Genomic DNA was isolated and then used in PCR analysis for detection of the presence of the inserted *IiPLR1* fragment. Primers *IiPLR1-oxv-1F* and *IiPLR1-oxv-1R* (Supplementary Table S3 at *JXB* online) were designed specifically to cover the gene sequence and the vector sequence for detecting exogenous *IiPLR1* transformations. The transformed status of hairy roots was also verified by PCR for the genes *hpt* and *rolb* (Chilton, 1982).

PCR-positive hairy roots harbouring the *IiPLR1* gene were also analysed for *IiPLR1* expression by western blot assay. Protein was extracted from hairy roots by grinding in ice-cold lysis buffer (50 mM HEPES/pH 7.5, 150 mM NaCl, 1 mM EDTA/pH 8.0, 1% Triton X-100, 1% SDS) supplemented with a cocktail of protease inhibitors (Roche). Aliquots of 30 μg of total soluble proteins per sample, determined by the Bradford method (Bradford, 1976), were fractionated on a 12% SDS–PAGE mini-gel and blotted to a nitrocellulose membrane. The blots were probed with the DYKDDDDK Tag antibody (Mouse) (1:1000, Santa Cruz Biotechnology), washed in PBST three times, reacted with goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000, Amersham), washed, and exposed to X-ray film using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham). For actin detection, anti-actin goat polyclonal IgG (Santa Cruz) and anti-goat IgG, were used as a primary and a secondary antibody at a final dilution of 1:1000 and 1:5000, respectively.

*IiPLR1* transcript and lariciresinol content determination, as well as phloroglucinol-HCl staining, were performed following the methods described above.

### Statistical analysis

Experiments were performed in triplicate, and all results are expressed as the mean ±SEM.

### Results

#### Gene–lignan pathway network analysis

Fifty-one ESTs corresponding to lariciresinol biosynthetic genes (Supplementary Table S1 at *JXB* online) were identified from *I. indigotica* through analysis of transcriptome data. To assess the impact of the expression of these genes on lignan production, variation in gene expression and lignan accumulation was exploited by using MeJA-elicited *I. indigotica* hairy roots harvested at different time points (0, 1, 3, 6, 12, and 24 h after treatment). The RNA-Seq expression profile showed that almost all tested genes except *IiDIR15* responded to MeJA treatment, indicating their important role in MeJA-mediated transcriptional reprogramming. These genes responded to MeJA with different patterns; some (e.g. *IiDIR1* and *Ii4CL12*) were dramatically up-regulated, some (e.g. *Ii4CL11* and *IiCCAOAMT2*) were down-regulated, and others (e.g. *IiDIR6* and *IiDIR7*) showed ‘up and down’ changes but without clear trends (Fig. 2A). Since jasmonates orchestrate a diverse set of physiological and developmental processes in plants (Pauwels et al., 2008), it is suggested that the various response patterns might be caused by several different regulatory mechanisms. The lignan content determination by LC-MS showed, as expected, that the signalling molecule MeJA greatly triggered lignan production but with different patterns. Each lignan showed wide ranges of concentrations in these hairy roots, indicating that the MeJA-elicited *I. indigotica* hairy roots contain significant lignan variation. For example, lariciresinol accumulation first gradually decreased, with a minimum amount of 36.4 μg g⁻¹ dw at 3 h after treatment, and then gradually increased and peaked at 24 h with an amount of 77.0 μg g⁻¹ dw (Fig. 2B).

A correlation matrix of all pairwise comparisons between gene expression and lignan concentrations was created, and the full set of correlation coefficients (Supplementary Table S5 at *JXB* online) is presented as a heat map in Fig. 3A, which makes the genes positively correlated (red), negatively (blue) correlated, or uncorrelated (yellow), with each lignan easily distinguishable. For example, *IiPLR1* is found to be positively correlated with the pharmaceutically important lariciresinol, with a high correlation coefficient value (*r*=0.98), whereas *IiPLR2* and *IiPLR3* seem uncorrelated with any of the measured lignans.

Correlation coefficient cut-off values were applied to construct gene–metabolite correlation networks. *Figure 3B* presents one example with a cut-off |*r|>0.6: a total of 27 unigenes could be correlated with one or more of the five measured lignans, and among them seven (*IiPAL1, Ii4CL7, IiC3H, IiDIR1, IiDIR5, IiDIR9, and IiPLR1*) were positively correlated with lariciresinol (Table 1).

#### RNAi silencing of IiPLR1, IiPLR2, and IiPLR3 in hairy roots

*IiPLR* family members (*IiPLR1, IiPLR2* and *IiPLR3*) were selected to validate the predicted gene–metabolite correlation. A 400–500 bp fragment (Supplementary Table S2 at *JXB* online) selected from the unconserved region of each member (Supplementary Fig. S1) was inserted upstream and downstream of the Pdk intron in both sense and antisense orientations to generate the RNAi vector (*Fig. 4A*). The transformants were identified by PCR analysis: all of the hairy roots contained the *rolb* gene, which was evidence of transformation by pRiA4 (Chilton, 1982). The hygromycin resistance gene *hpt* was detected in both RNAi (*IiPLR1*RNAi, *IiPLR2*RNAi, and *IiPLR3*RNAi) and CK lines. Compared with CK, RNAi lines contained an additional 400–500 bp fragment when amplified by using primers JDPDK-1F and JDPDKR (data not shown).

qRT-PCR analysis showed that the transcript level of the target gene (*IiPLR1, IiPLR2, or IiPLR3*) was significantly suppressed through corresponding RNAi manipulation, with the transcript level of the other two *IiPLR* members displaying a slight fluctuation. There was no significant difference in *IiPLR1, IiPLR2*, and *IiPLR3* transcript abundance between WT and other hairy roots.
CK lines (Fig. 4B). Metabolite analysis showed that the lariciresinol content in the IiPLR1-RNAi lines was significantly reduced compared with that in WT lines, and the reduced lariciresinol accumulation in different IiPLR1-RNAi lines (1-1, 1–3, 1–4, 1–6, and 1–7) was found to correlate well with the corresponding decrease of IiPLR1 mRNA levels. Line 1–4, with the lowest IiPLR1 mRNA level (~5% of that of the WT, Fig. 4B), accumulated the least lariciresinol (2.0 μg g⁻¹ dw), which was ~3.6% of that of the WT control (56.0 μg g⁻¹ dw). However, there was no significant difference in lariciresinol content between the WT and CK, IiPLR2-RNAi, or IiPLR3-RNAi hairy roots (Fig. 4C). These results indicated that IiPLR1 is the only one of the IiPLR members that influences lariciresinol accumulation.

The IiPLR1-RNAi hairy roots presented a weaker browning after staining with phloroglucinol-HCl compared with their CK and WT counterparts (Fig. 4D), indicating a decrease of lignans, lignins, and/or wall-bound or secreted phenolic derivatives (Hano et al., 2006). Since IiPLR1 is involved in the lignin biosynthetic pathway, and lignins share the same precursor monolignols with lignans (Davin and Lewis, 2000), it is speculated that not only lignans (e.g. lariciresinol) but also lignins are strongly impacted by IiPLR1 suppression. However, there was no phenotype difference observed in WT and CK, IiPLR2-RNAi, and IiPLR3-RNAi hairy roots after phloroglucinol-HCl staining (Fig. 4D).

Isolation and expression characteristics of IiPLR1

A full-length cDNA sequence of IiPLR1 (GenBank accession no. JF264893) was isolated from I. indigotica (Supplementary Fig. S2 at JXB online). This gene codes for a predicted polypeptide of 317 amino acids with a calculated molecular mass of 35.6 kDa and an isoelectric point of 5.64. The protein–protein BLAST analysis showed that IiPLR1 has 85% and 82% amino acid identity to A. thaliana pinoresinol reductase AtPrR2 and AtPrR1, respectively. Similarly to other known PLRs and PrRs, IiPLR1 contains an NADPH-binding motif at the N-terminus (Supplementary Fig. S3). Phylogenetic analysis indicated that IiPLR1 has the closest relationship with AtPrR2 from A. thaliana, which also belongs to the family Cruciferae (Supplementary Fig. S4).

The expression profiles of IiPLR1 in different tissues and under different stresses were investigated by qRT-PCR. The results showed the IiPLR1 gene was
Lignan-correlated genes

With the strongest expression in roots (Fig. 5A), IiPLR1 expression increased sharply and peaked at 4 h, with an increase of ~3.8-fold, and then decreased until 24 h (Fig. 5B). When I. indigotica was treated with 100 μM MeJA, the expression level of IiPLR1 increased gradually and reached a maximum at 12 h after treatment, which was almost 14-fold higher than the expression before treatment (Fig. 5C). For UV-B treatment, the response was very fast; IiPLR1 expression increased at 5 min and decreased at 10 min under UV-B, but the highest expression appeared at 30 min after UV-B was turned off (~4.6-fold of that before treatment) (Fig. 5D).

Functional expression of IiPLR1 in E. coli

To carry out the biochemical characterization of IiPLR1, the recombinant enzyme prepared using E. coli was tested in vitro for its activity toward racemic pinoresinol and lariciresinol. The IiPLR1 gene was cloned in pET32a(+) to obtain IiPLR1-pET, and expressed in E. coli BL21(DE3). SDS-PAGE showed the production of a 53 kDa protein. This size is close to the predicted molecular mass of ht-IiPLR1 (52,922.4 Da). ht-IiPLR1 was purified to near homogeneity by Ni affinity chromatography (Fig. 6A).

When purified ht-IiPLR1 (5 μg ml⁻¹ of protein) was incubated with 200 μM (±)-pinoresinol for 30 min, (±)-pinoresinol decreased, whereas (±)-lariciresinol and (±)-secoiariciresinol appeared. No activity was measured in extracts from cells containing the expression vector lacking an insert (CK). It was presumed that IiPLR1 catalysed the sequential steps: first it reduced (±)-pinoresinol to form (±)-lariciresinol, and then it reduced (±)-lariciresinol to give rise to (±)-secoiariciresinol. This assumption was demonstrated by incubating (±)-lariciresinol (3.5 μM) individually with ht-IiPLR1 (5 μg ml⁻¹). After 30 min of incubation, ht-IiPLR1 reduced (±)-lariciresinol efficiently to (±)-secoiariciresinol, whereas no activity was measured in the control extract (Fig. 6B). These results indicate that IiPLR1 is able to catalyse both (±)-pinoresinol and (±)-lariciresinol.

Kinetic analysis of IiPLR1 using both (±)-pinoresinol and (±)-lariciresinol as substrates was conducted. As shown in Table 2, the $K_m$ value of IiPLR1 for (±)-pinoresinol (65.4 ± 2.76 μM) was 26 times higher than that for (±)-lariciresinol (2.50 ± 0.14 μM), but there was no significant difference in activities between (±)-pinoresinol and (±)-lariciresinol with regard to $k_{cat}/K_m$ values [0.91 ± 0.11 μM⁻¹ min⁻¹ for (±)-pinoresinol, 1.59 ± 0.05 μM⁻¹ min⁻¹ for (±)-lariciresinol].

Table 1. Lariciresinol biosynthetic genes positively correlated with the five measured lignans with a cut-off |r|>0.6.

| Lignan                  | Lignan-correlated genes                                                                 |
|-------------------------|----------------------------------------------------------------------------------------|
| Pinoresinol             | IiPAL1, IiI4CL1, IiI4CL2, IiI4CL3, IiI4CL7, IiI4CL10, IiIC3H, IiI4CoAOMT1, IiIDIR1, IiIDIR3, IiIDIR5, IiIDIR9, IiIDIR12, IiPLR1 |
| Lariciresinol           | IiPAL1, IiI4CL7, IiIC3H, IiIDIR1, IiIDIR5, IiIDIR9, IiPLR1                             |
| Secoisolariciresinol    | IiPAL1, IiI4CL1, IiI4CL2, IiI4CL3, IiI4CL7, IiI4CL10, IiIC3H, IiI4CoAOMT1, IiIDIR1, IiIDIR2, IiIDIR3, IiIDIR9, IiIDIR11, IiPLR1 |
| Matairesinol            | IiPAL1, IiI4CL1, IiI4CL2, IiI4CL3, IiI4CL7, IiI4CL10, IiIC3H, IiI4CoAOMT1, IiIDIR2, IiIDIR3, IiIDIR11, IiPLR1 |
| Secoisolariciresinol diglucoside | IiPAL1, IiI4CL1, IiI4CL2, IiI4CL3, IiI4CL7, IiI4CL10, IiIC3H, IiI4CoAOMT1, IiIDIR1, IiIDIR2, IiIDIR3, IiIDIR11, IiIDIR11 |

constitutively expressed in roots, stems, leaves, and flowers of I. indigotica, with the strongest expression in roots and the lowest in flowers (Fig. 5A). IiPLR1 responded to MeJA, ABA, and UV-B treatments, but with different patterns of variation. After treatment with 100 μM MeJA, the expression level of IiPLR1 increased sharply and peaked at 4 h, with an increase of ~3.8-fold, and then decreased until 24 h (Fig. 5B). When I. indigotica was treated with 100 μM ABA, IiPLR1 expression increased gradually and reached a maximum at 12 h after treatment, which was almost 14-fold higher than the expression before treatment (Fig. 5C). For UV-B treatment, the response was very fast; IiPLR1 expression increased at 5 min and decreased at 10 min under UV-B, but the highest expression appeared at 30 min after UV-B was turned off (~4.6-fold of that before treatment) (Fig. 5D).
Overexpression of IiPLR1 in I. indigotica hairy roots

The IiPLR1 overexpression vector PHB-IiPLR1-flag (Fig. 7A) and its vector control PHB-flag were separately introduced into I. indigotica to generate IiPLR1-OVX and CK lines. PCR analyses showed that all these lines contained both rolC and hpt genes, and the exogenous IiPLR1 gene was detected in IiPLR1-OVX lines (data not shown).

qRT-PCR analysis showed that in comparison with levels of the WT control, IiPLR1 transcript levels in all the seven independent IiPLR1-OVX lines (ovx-1, ovx-2, ovx-5, ovx-7, ovx-8, and ovx-9) were significantly enhanced (~5.4-, 10.5-, 2.1-, 3.2-, 3.2-, 8.8-, and 7.3-fold). No significant difference was observed in the IiPLR1 transcript between WT and CK controls (Fig. 7B). Moreover, the accumulation of the flag-tagged IiPLR1 (IiPLR1-flag fusion protein) in IiPLR1-OVX lines was examined by western blot analysis using an antibody against flag. A strong cross-reaction signal corresponding to a polypeptide with an expected mol. wt of 52 kDa for the IiPLR1-flag fusion protein was present in all the seven tested root lines, and the strengths of IiPLR1-flag signal in these independent lines was found to match well with the levels of IiPLR1 transcript. As expected, no signal was observed using total protein extracted from WT and CK controls (Fig. 7C). These results indicated that the exogenous IiPLR1 gene had successfully integrated and was expressed in I. indigotica.

The biomass growth rates of transgenic lines were recorded during the hairy root culture period. All hairy root lines reached the highest growth rate at day 18 and achieved maximum fresh weight at day 45 after inoculation (Supplementary Table S6 at JXB online). Particular attention was paid to lines ovx-2, ovx-8, and ovx-9 because of their relatively higher IiPLR1 expression. As shown in Fig. 7D, lines ovx-2 and
ovx-8 presented growth rates comparable with the WT, while the growth rates of ovx-9, along with CK, were significantly lower than that of the WT.

The phloroglucinol-HCl-stained *Ii* PLR1-OVX hairy roots are shown in Fig. 7E. It was apparent that *Ii* PLR1-OVX hairy roots presented a violet-red colour, whereas CK control presented a strong browning, which was similar to the WT (Fig. 4D). This result indicated that *Ii* PLR1-OVX hairy roots accumulated more lignans, lignins, and/or wall-bound or secreted phenolic derivatives than CK and the WT. In agreement with the speculation derived from phloroglucinol-HCl staining of *Ii* PLR1-RNAi hairy roots, the result also suggests *Ii* PLR1 expression probably also has a marked effect on lignin accumulation. Metabolite analysis showed that the lariciresinol content in the *Ii* PLR1-OVX lines was significantly higher than that in the WT line, and the lariciresinol accumulation in different independent lines was closely correlated with the corresponding *Ii* PLR1 expression levels. Line ovx-2, with the highest *Ii* PLR1 expression (Fig. 7B, C), produced the most abundant lariciresinol (353.9 μg g⁻¹ dw), which was ~6.3-fold
more than in its WT counterpart (56.0 μg g⁻¹ dw); line ovx-8, with a relative higher IiPLR1 expression (Fig. 7B, C), accumulated 310.4 μg g⁻¹ lariciresinol, which was 5.5-fold more that in the WT. There was no significant difference in lariciresinol content between CK (42.9 μg g⁻¹ dw) and the WT (Fig. 7F).

**Discussion**

Plant metabolic pathways are complex and often involve multicopy gene families. It is therefore difficult to target the best intervention points and accurately predict the outcome. Until recently, metabolic engineering in plants relied on the trial and error approach to achieve desirable changes in the metabolic profile. However, technological advances in data mining and modelling are now taking away much of the guesswork by allowing the impact of modifications to be predicted more accurately (Farré et al., 2015). This knowledge-driven approach for engineering requires a well-characterized population as a source of genetic variation. The potency of jasmonates, such as MeJA, to elicit secondary metabolism in cell cultures has made them powerful tools to cause the genetic diversity and help to unravel the complex cellular process (Pauwels et al., 2008). Combined transcript and metabolite profiling approaches to determine the MeJA-mediated regulation of secondary metabolism resulted in the establishment of gene-metabolite networks involved in the biosynthesis of the pharmaceutically valuable terpenoid indole alkaloids in Catharanthus roseus (Rischer et al., 2006), in the characterization of enzymatic steps in the isoflavone biosynthetic pathways in Medicago truncatula (Naoumkina et al., 2007), and in the rosmarinic acid biosynthetic pathways in Salvia miltiorrhiza (Xiao et al., 2009), as well as in the isolation of novel regulators of aliphatic glucosinolate biosynthesis in Arabidopsis (Hirai et al., 2007).

Here, MeJA-elicited I. indigotica hairy roots were employed as a resource for the identification of candidate lignan-correlated genes. These hairy roots obtained from different time points after MeJA treatment harbour genetic diversity, impacting on the chemical composition of lignans, as shown in Fig. 2. Correlation analysis of gene transcripts and metabolite contents resulted in multiple new hypotheses regarding regulatory relationships between the identified biosynthetic genes and lignans (Fig. 3; Table 1). Seven biosynthetic genes were identified to be significantly correlated with lariciresinol, a pharmaceutically valuable compound of I. indigotica.

To validate this network analysis, one of the best-supported Table 2. Kinetic properties of purified recombinant IiPLR1

| Substrate     | $K_m$ (μM) | $V_{max}$ (μM min⁻¹) | $K_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (μM⁻¹ min⁻¹) |
|--------------|------------|----------------------|-------------------|----------------------------|
| (±)-Pinoresinol | 65.4 ± 2.76 | 1.67 ± 0.13          | 59.6 ± 4.52       | 0.91 ± 0.11                |
| (±)-Lariciresinol | 2.50 ± 0.14 | 0.11 ± 0.003         | 3.98 ± 0.09       | 1.59 ± 0.05                |

Fig. 7. Overexpression of IiPLR1 in I. indigotica hairy roots. (A) Schematic representation of the overexpression vector. $P_{35S}$, CaMV 35S promoter; $P_{2×35S}$, CaMV 35S double promoter; $hyg^r$, hygromycin resistance gene; $Bar$, herbicide resistance gene; IiPLR1, the IiPLR1 coding sequence; $polyA$, poly(A) tail; $T_{35S}$, CaMV 35S terminator; $T_{nos}$, nopaline synthase terminator; LB, T-DNA left border; RB, T-DNA right border. The flag-tag and restriction sites are marked. (B) IiPLR1 transcript abundance, (C) IiPLR1 transcript expression, and (F) lariciresinol content in transgenic hairy root lines. (D) Time courses of growth of hairy root lines ovx-2, ovx-8, ovx-9, WT, and CK. (E) Phenotype of CK control (left) and IiPLR1-OVX hairy roots (right) before (upper) and after (lower) phloroglucinol-HCl staining.
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In order to produce a high concentration of lariciresinol, gene constructs containing cDNA clones of *IiPLR1*, driven by a double *Cauliflower mosaic virus* (CaMV) 35S promoter (*P*2×*P*35S), was introduced into *I. indigotica* hairy roots. As expected, the lariciresinol content significantly increased following introduction of the *IiPLR1* gene. Generally, a high content of secondary metabolites in the tissues is associated with poor growth, and the actual total productivity of secondary metabolites therefore remains low (Jouhiokainen et al., 1999). It was found in this study, however, that the growth rate in the high lariciresinol-producing lines ovx-2 (353.9 μg g⁻¹ dw, 6.3-fold that of the WT) and ovx-8 (310.4 μg g⁻¹ dw, 5.5-fold that of the WT) was not reduced as compared with those with low lariciresinol production including WT and CK lines. The current study provides an effective approach for commercial large-scale production of lariciresinol by using the *I. indigotica* hairy root systems as bioreactors.

In conclusion, the current study sheds light on how to engineer the production of target metabolites effectively using more suitable intervention points or by more refined strategies. In the case of PLR, which is undoubtedly a key rate-limiting step of the lariciresinol biosynthetic pathway, three *IiPLRs* were predicted from *I. indigotica* based on homology search (*IiPLR1–IiPLR3*); however, only *IiPLR1* influenced lariciresinol biosynthesis. The comprehensive profiling approach described here provides a good example to locate the most potential member(s) rapidly from a multicopy gene family. Moreover, the present approach of integrated transcriptome and metabolite profiling using MeJA-elicited *I. indigotica* hairy roots as a source of variation of gene expression and metabolites provided insight into multiple previously uncharacterized candidate lignan-correlated genes. One candidate, *IiPLR1*, was demonstrated to indeed be important in lariciresinol biosynthesis, indicating that the analysis is robust and genes identified via this process are worthy of validation.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** Nucleotide sequence alignment of *IiPLR1, IiPLR2*, and *IiPLR3*.

**Figure S2.** Nucleotide sequence and the deduced amino acid sequence of *IiPLR1*.

**Figure S3.** Amino acid sequence alignment of *IiPLR1* with other plant PLRs.

**Figure S4.** Phylogenic analysis of *IiPLR1* with other plant PLRs.

**Table S1.** Sequences corresponding to the lariciresinol biosynthetic genes of *I. indigotica*.

**Table S2.** The inserted fragments for RNAi silencing of *IiPLR1, IiPLR2*, and *IiPLR3*.

**Table S3.** PCR primers used in this study.

**Table S4.** Pinoresinol/lariciresinol reductases used in this study.

**Table S5.** The correlation coefficients between lariciresinol biosynthetic gene expression and lignan concentrations.
Table S6. Biomass accumulation of iPLR1 transgenic lines.

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