

# Expression patterns of an isoflavone reductase-like gene and its possible roles in secondary metabolism in *Ginkgo biloba*

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## Abstract

**Key message** Our results showed that *GbIRL1* belongs to the PCBER protein family. Besides, *IRL1* gene was a novel gene regulating lignin change and also effecting the accumulation of flavonoids in *Ginkgo*.

**Abstract** A cDNA encoding the IFR-like protein was isolated from the leaves of *Ginkgo biloba* L., designated as *GbIRL1* (Accession no. KC244282). The cDNA of *GbIRL1* was 1,203 bp containing a 921 bp open reading frame encoding a polypeptide of 306 amino acids. Comparative and bioinformatic analyses revealed that *GbIRL1* showed extensive homology with IFLs from other gymnosperm species. Phylogenetic tree analysis revealed that *GbIRL1* shared the same ancestor in evolution with other PCBERs

protein and had a further relationship with other gymnosperm species. The recombinant protein was successfully expressed in *E. coli* strain with pET-28a vector. The vitro enzyme activity assay by HPLC indicated that recombinant *GbIRL1* protein could catalyze the formation the TDDC, IDDDC from DDDC, DDC. Tissue expression pattern analysis showed that *GbIRL1* was constitutively expressed in stem and roots, especially in the parts of the pest and fungal infection, with the lower expression being found in 1- or 2-year old stem. The increased expression of *GbIRL1* was detected when the seedlings were treated with Ultraviolet-B, ALA, wounding and ethephon, abscisic acid, salicylic acid. Correlation analysis between *GbIRL1* activity and flavonoid accumulation during *Ginkgo* leaf growth indicated that *GbIRL1* might be the rate-limiting enzyme in the biosynthesis pathway of flavonoids in *Ginkgo* leaves. Results of RT-PCR analysis showed that the transcription level of change in *GbIRL1* power correlated with flavonoid contents, suggesting *IRL1* gene as a novel gene regulating lignin change and also effecting the accumulation of flavonoids in *Ginkgo*.

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## Abbreviations

bp	Base pair
HPLC	High performance liquid chromatography
IPTG	Isopropyl b-D-thiogalactoside, C9H18O5S
IFR	Isoflavone reductase
IRL	Isoflavone reductase-like protein
IRLP	IRLgene promoter
ORF	Open reading frame
PCBER	Phenylcoumaran benzylic ether reductases
PCR	Polymerase chain reaction

RACE	Rapid amplification of cDNA ends
QRT-PCR	Real-time quantitative PCR
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV	Ultraviolet

## Introduction

Plant produces a variety of secondary metabolites to counteract not only environmental stresses but also as part of normal growth and development processes (Gapper and Dolan 2006). As a major component of plant-specialized metabolism, phenylpropanoid biosynthetic pathways provide anthocyanins for pigmentation, flavonoids such as flavones for protection against UV photodamage, various flavonoids and isoflavonoid inducers of *Rhizobium nodulation* genes, polymeric lignin for structural support and assorted antimicrobial phytoalexins (Ferrer et al. 2008). Isoflavonoids, valuable secondary metabolites for important physiological processes, are produced primarily in leguminous plants and are rarely found in gymnosperms (Kim et al. 2010). Although isoflavonoid phytoalexins are confined primarily to legumes, IFR-like (abbreviated hereafter as IRL) proteins from non-leguminous plants are also part of the IFR family (Kim et al. 2010). At present, numerous studies dealing with the biosynthesis pathway to the lignans (Suzuki and Umezawa 2007) and isoflavonoids (Dixon 1999) have resulted in the isolation and characterization of various lignan reductases, namely pinoreosin lariciresinol reductases (PLRs) (Hano et al. 2006), phenylcoumaran benzylic ether reductases (PCBERs) (Turley 2008) and isoflavone reductases (IFRs) (Paiva et al. 1991). Several IRLs have been reported to be involved in responses to biotic or abiotic stress, such as *Nicotiana tabacum* *NtIRL* (Shoji et al. 2002), *Zea mays* *ZmIRL* (Petrucchio et al. 1996), *Oryza saliva* *OsIRL1* (Kim et al. 2003) and *Fagopyrum cymosum* *FcIRL* (Zhu et al. 2009). Phenylcoumaran benzylic ether reductase is a member of the IRL family (Karamloo et al. 2001), which catalyzes the non-enantio-specific NADPH-dependent reduction of the benzylic ether functionality in the 8-5'-linked lignans dehydrodiconiferyl alcohol and dihydro dehydrodiconiferyl alcohol (Gang et al. 1999). Because of the high sequence similarities, both PLR and PCBER are phylogenetically related to isoflavone reductase, a branch point enzyme in the biosynthesis of isoflavonoids (Paiva et al. 1991; Tiemann et al. 1991). Plant PCBER was reported to be up-regulated by abiotic stress (Nam et al. 2003), the circadian clock (Harmer et al. 2000), and reported to be strongly associated with phenylpropanoid biosynthesis in

young xylem, phloem and differentiating xylem ray parenchyma cells (Vander Mijnsbrugge et al. 2000).

In the present time, *Ginkgo biloba* is one of the most popular functional plants, especially as medicinal plants. Extracts of *G. biloba* leaves contain active compounds such as flavonoids and terpene lactones (ginkgolides and bilobalide) and can, therefore, be used to increase peripheral and cerebral blood flow (Smith and Luo 2004; van Beek 2002). Flavonoids metabolism and lignans metabolic belong to phenylalanine metabolism path branch; lignans synthesis might affect the accumulation of *G. biloba* flavonoids. Therefore, to study the medicinal secondary metabolism, a cDNA library derived from high flavonoids producing leaves of *G. biloba* was constructed using SMART-RACE technique to isolate key genes related to biosynthesis of medical components. Here we report the cloning and characterization of a first *GbIRL1* encoding a putative PCBER. We also discuss its potential function in secondary metabolism of *G. biloba*.

## Materials and methods

### Plant materials and treatments

Fourteen-year-old grafted *G. biloba* seedlings were grown in a greenhouse in Huanggang (E, 111°54'–112°19', N, 30°6'–30°39', Hubei province, central of China) were sampled as plant materials. For gene cloning and tissue expression, diverse tissues including young leaves, mature leaves, ovules, stamens, albumen, gynoecia, stems and roots were collected for DNA and RNA extraction as described by Xu et al. (2008b). Tissues were immediately frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  prior to total RNA extraction.

Total RNA was isolated from injured stem tissues of 14-year-old grafted *G. biloba* seedling after they were attacked by *Rhizoctonia solani*. For fungal infections blade, the total RNA was isolated from Ginkgo leaves cells punctured with an infected agent isolate of *Phyllosticta mortonii* Fairman. One-year-old cuttings from the same genotypic strain of *G. biloba* were subjected to treatments with UV-B, wounding (WOU), 5-aminolevulinic acid (ALA), abscisic acid (ABA), salicylic acid (SA) and ethephon (ETH). For UV-B treatment, seedlings were exposed to  $1,500\ \mu\text{J}/\text{m}^2$  UV-B irradiation in a closed chamber, and the control cuttings were placed in a dark closed chamber. The edges of Ginkgo leaves were cut by about 0.6 cm with scissors for wounding treatment; the intact leaves of Ginkgo were as control. The ALA (100  $\mu\text{M}$ ), ABA (10 mM), SA (25 mM), and ETH (20 mM) were dissolved in 0.01 % Tween 20 and sprayed onto young leaves. The control leaves were sprayed with an equivalent volume of 0.01 % (v/v) Tween 20.

## Cloning of the full-length cDNA

Given species conservation of the functional domain of the IRLs protein and the Ginkgo EST database in GenBank, a pair of degenerate primers, IRF and IRR, was designed according to the conserved peptide sequences, LGGAGYR and PGSSILFT, to amplify a conservative fragment of the *GbIRL1* gene. The purified PCR product was cloned into the pMD18-T vector (TaKaRa, Dalian China), and the positive clone was confirmed by a BLASTN query of the NCBI Database to verify the homology of this cloned fragment with *IRL* sequences from other species.

Based on the sequence of cloned *GbIRL1* fragments, the specific primer pairs (IR5R and IR3R) and the nested primer pairs (IR5 N and IR3 N) were designed to amplify the 5' and 3' end of *GbIRL1* using the SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech, USA). The PCR products were purified and cloned into the pMD18-T vector for sequencing. After comparing and aligning the sequence of 5'RACE, 3'RACE, and the internal fragment, the full-length cDNA sequence of *GbIRL1* was obtained.

## Amplification of the 5'-promoter region of GbIRL1

The promoter region was isolated by genomic walk strategy using a Universal Genomewalker<sup>TM</sup> kit (Clontech, USA) according to the manufacturer's instructions. The first turn of PCR was carried out using AP1 and IR5R as primers, and constructed libraries as a template using the following protocol: 94 °C for 4 min; 30 cycles (98 °C for 10 s, 68 °C for 3 min) and 68 °C for 10 min. The products were 20-fold-diluted and used for nested PCR, which was performed using AP2 and IR5 N as primers under the following conditions: 94 °C for 4 min; 32 cycles (94 °C for 30 s, 56 °C for 50 s, 72 °C for 3 min); and 72 °C for 10 min. The PCR products were cloned into pMD18-T for sequencing (Sangon, China).

## Relative quantification by QRT-PCR

The transcription levels of *GbIRL1* were determined in different *G. biloba* tissues, as well as in young seedling leaf samples collected at different time points after stress and hormone treatments. QRT-PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, American) with SYBR Green PCR Master Mix (Applied Biosystems, American), according to the manufacturers protocol. The *G. biloba* glyceraldehydes-3-phosphate dehydrogenase gene (*GbGAPDH*, L26924) (Jansson et al. 1994) was used as the reference gene as described by Xu et al. (2008b).

The gene-specific primers (IRT3, IRT4) and reference primers (GAPU, GAPD) for QRT-PCR were listed in Table 1. The QRT-PCR conditions were: 10 min at 95 °C,

and 40 cycles (95 °C for 15 s, 60 °C for 1 min). Before performing QRT-PCR, primer efficiency was evaluated using both *GbIRL1* and *GbGAPDH* at 100, 150, 200, 250 and 300 nM combinations. A 120 nM concentration was chosen as most suitable combination for both genes. For each plant sample, aliquots of 150 ng total RNA was analyzed for each gene and the two genes (*GbIRL1* and *GbGAPDH*) were always analyzed, simultaneously. Each sample was amplified three times and all reactions were performed on ABI PRISM 7500 Sequence Detection System. With a housekeeping gene *GbGAPDH*, the relative amount of the *GbIRL1* transcript is presented as  $2^{(-\text{ddCt})}$  according to the  $C_T$  method (Schmittgen and Livak 2008). When comparing the expression of *GbIRL1* in different tissues or treatments, the relative expression of *GbIRL1* was achieved by calibrating its transcription level to that of the reference gene, *GbGAPDH*.

## Functional expression of the GbIRL1 in *Escherichia coli*

To clone *GbIRL1* into an expression vector, a pair of primers, IRZF and IRZR, were designed and synthesized to amplify the coding region by RT-PCR with the incorporation of a restriction enzyme site and a protective base to simplify later vector construction. After confirmation by sequencing, the resulting recombinant plasmid was introduced into BL21 (DE3) by the heat shock method. A single colony of *E. coli* BL21 cells harboring the expression plasmid pET28a-GbIRL1 was inoculated at 37 °C in Luria-Bertani medium containing kanamycin (50 mg L<sup>-1</sup>) and were grown with shaking (150 rpm) at 37 °C until the optical density (OD600) reached about 0.6. For induction, IPTG was added at a final concentration of 1 mM and the cells were further cultured at 30 °C for 2 h. The cells were lysed by sonication for 10 s at 4 °C, and centrifuged at 7,000g for 15 min. Supernatants and pellets were analyzed by SDS-PAGE and Coomassie Brilliant Blue R250 staining. The recombinant GbIRL1 protein from induced cells was purified using Nickel-CL agarose affinity chromatography (Bangalore Genei) and used for in vitro enzyme assays.

Western blotting was carried out to verify expression of a GbIRL1 protein having a His-tag in the N-terminus. After electrophoresis, the proteins were electro-transferred onto a PVDF membrane and detected with antimouse RGS-His antibody (Santa Cruz, American), and a secondary antibody (goat anti-mouse IgG), conjugated to alkaline phosphatase (AP). Western Blue Stabilized Color Substrate for AP (Promega, USA) was used for the color reaction.

## Assay of GbIRL1 activity

GbIRL1 activity was analyzed according to Gang et al. (1999) with a slight modification. The reaction mixture

**Table 1** The gene clone and analysis primers of *GbIRL1*

Primer	Sequence (5'–3')	Description
IRU	GATVCTAGAWCTTTGAMCAASACCT	Degenerate primer, forward
IRD	GTRTTCATCYACAGIGGTATHCTTC	Degenerate primer, reverse
IR5R1	ATTACCTGGGAAGGGTGTTCCTG 171	Reverse primer for 5' RACE, outer
IR5R2	TTCTCCCACAGGGCTACAAG 86	Reverse primer for 5' RACE, nested
IR3R1	TTCAATGAGCTTGTAGCCCTGTG 58	Forward primer for 3' RACE, outer
IR3R2	GGAGAAGAAGATTGGGAAGA 81	Forward primer for 3' RACE, nested
IRZ1	<i>ACAGAGCTC</i> ATGGGAAAGAGCAGGATTCTGA	Gene-specific primer, forward
IRZ2	<i>CAACTCGAG</i> TCACACAAATTGGTTGAGGTATTC	Gene-specific primer, reverse
IRQ1	ACTGACATCGATCATGGGCAAAAATG	Gene-specific primer, forward
IRQ2	Poly(T) <sub>20</sub> AATAGTAA	Gene-specific primer, reverse
IRG1	ACCGCACTCGTCTTATCAACATCG	Primer for probe forward
IRG2	CTGCTATCTGTGCTCCTCCAAC	Primer for probe reverse
IRT1	CTTGTCGGAGAACTACAGCCT	Primer for RQPCR, forward
IRT2	TTTCGGGATTAGAGGCTGTAGT	Primer for RQPCR, reverse
GAPU	TAGGAATCCCGAGGAAATACC	Primer for RQPCR, forward
GAPD	TTCACGCCAACAACGAACATG	Primer for RQPCR, reverse

Restriction site sequences are indicated in italic letter and underline

contained 10 mM potassium phosphate, pH 6.5, 2 mM DDC or DDDC, 4 mM NADPH, 5 mM dithiothreitol and the protein (8 µg for bacterial supernatants containing GbIRL1) in a total volume of 15 µl. The reaction was performed at 37 °C for 3 h, and stopped by boiling for 3 min. After centrifugation at 17,000×g for 3 min, an aliquot (12.5 µl) was mixed with 1.66 µl of acetonitrile and analyzed by reversed-phase HPLC under the same conditions as described in Gang et al. (1999), except that a C8-AP column (YMC, Kyoto) was used.

Purified GbIRL1 protein (275 ng) was incubated with 0.2 mM of either NADPH or NADH in 240 µl of 20 mM Tris–HCl pH 7.5 for 1 h at room temperature. The mixtures were concentrated to less than 50 µl on a Centricon-10 (Amicon, Beverly, USA) and then diluted with 350 µl of 20 mM Tris–HCl pH 7.5. After three concentration/dilution cycles, the protein-bound coenzyme was detected by measuring the absorption spectra of the protein solution.

#### Lignin content analysis and histo-chemical lignin staining

Lignin content was quantitatively measured using the Klason method (Kirk and Obst 1988). Briefly, air-dried stem tissues were ground into powder and exhaustively extracted in a Soxhlet apparatus with toluene–ethanol (2:1, v/v), followed by 95 % ethanol and water. Samples were then vacuum-dried and 200 mg was hydrolysed in 3 ml of 72 % H<sub>2</sub>SO<sub>4</sub> at 25 °C for 3 h with occasional stirring. The hydrolysate was diluted with the addition of 190 ml H<sub>2</sub>O and then autoclaved for 1 h. The sample was filtered

through a fritted glass crucible, and then washed with hot water. The crucible was dried at 105 °C and weighed. The filtered solution was diluted to 500 ml and A<sub>205</sub> was determined spectrophotometrically using a 1 cm long cuvette. The Klason lignin was expressed as a percentage of the cell wall residue (CWR). The acid-soluble lignin was calculated by following formula: acid lignin (g l<sup>-1</sup>) = A<sub>205</sub>/110.

For lignin staining observation, hand-cut sections of different sample were treated by 1 % phloroglucinol ethanol and 35 % HCl, and images were captured by Cannon camera (Lu et al. 2010).

#### Extraction and determination of flavonoid content

Flavonoids were extracted, identified and quantified as previously described by Xu et al. (2008b) with some modifications. Briefly, 100 mg of dried leaf powder was dissolved in 40 ml of acidified methanol. The samples solutions were then suitably diluted with methanol and filtered through 0.45 µm filter membranes (Millipore, Nylon) for high performance liquid chromatography analysis. Selected samples were analyzed by HPLC (model LC-10, Shimadzu, Kyoto, Japan) with a SPD-10AVP UV–Vis detector. Flavonoids were analyzed on a Shim-pack VP-ODS column (250 mm 1 × 4.6 mm), using a mobile phase of methanol: 0.4 % (v/v) phosphoric acid (60:40) for elution. The flow rate was 1.0 ml min<sup>-1</sup> and injection volume was 20 µl. The UV detector was set at λ = 370 nm. Three extraction samples were prepared for HPLC analysis and each sample was injected three times. A standard mixture

of quercetin, kaempferol and isorhamnetin was selected, because the many different flavonol glycosides that occur in ginkgo leaves are predominantly derivatives of these three flavonol aglycones. Based on the methods used to determine flavonoids concentrations described by van Beek (2002), flavonoid contents were calculated by multiplication of the total content of quercetin, kaempferol, and isorhamnetin by a factor 2.51 and were expressed as percentages.

#### Bioinformatics analysis and molecular evolution analyses

The obtained sequences were analyzed using bioinformatics tools at websites (<http://www.ncbi.nlm.nih.gov> and <http://www.xpasy.org>). Vector NTI Suite 10 was used for sequence alignment and analysis. The cis-acting element in the promoter region was predicted using PlantCARE (Lescot et al. 2002). Phylogenetic tree analysis of GbIRL1 and known IRLs from other plant species retrieved from GenBank were aligned with Mega 4.0 (Tamura et al. 2007). The phylogenetic tree was constructed by a neighbor-joining (NJ) method and measured by bootstrap analysis with 1,000 replicates. SPSS 17 was used for statistical analysis and graphing.

## Results

### Characterization of the *GbIRL1* gene

A full-length cDNA of the *IRL1* gene (Accession no. KC244282) was obtained from *G. biloba* leaf tissue by degenerate PCR and RACE methods. The cDNA sequence was 1,261 bp with a poly A tail, and suggested it contained a 921 bp ORF. A 115 bp 5' untranslated region was upstream of the start codon, and the coding region was followed by a 225 bp 3' untranslated region downstream of the stop codon. The 5' untranslated region conformed to the predicted translation start site for eukaryotic genes (AX-XATGG) (Kozak 1984; Lütcke et al. 1987), and two stop codons (TAA, positions 89–91 bp and TAG, 84–86 bp) found in the 3' UTR proved that the cDNA contained the entire ORF. One potential polyadenylation signal AATAA was found at 43 bp position downstream from the stop codon. The G+C content of the coding region was 45.1 % similar to SDR family protein genes, such as *VvIFL* (46 %), *PsIFR* (40 %) and *PoPCBER* (46 %). The putative GbIRL1 was 306 aminoacid with a theoretical molecular weight of 33.28 kDa and a *pI* value of 6.18. Subcellular location prediction for GbIRL1 using SignalP 3.0 did not find any secretory pathway signal peptide (SP), and suggested that GbIRL1 was a cytoplasmic protein, as predicted for other members of the SDR family.

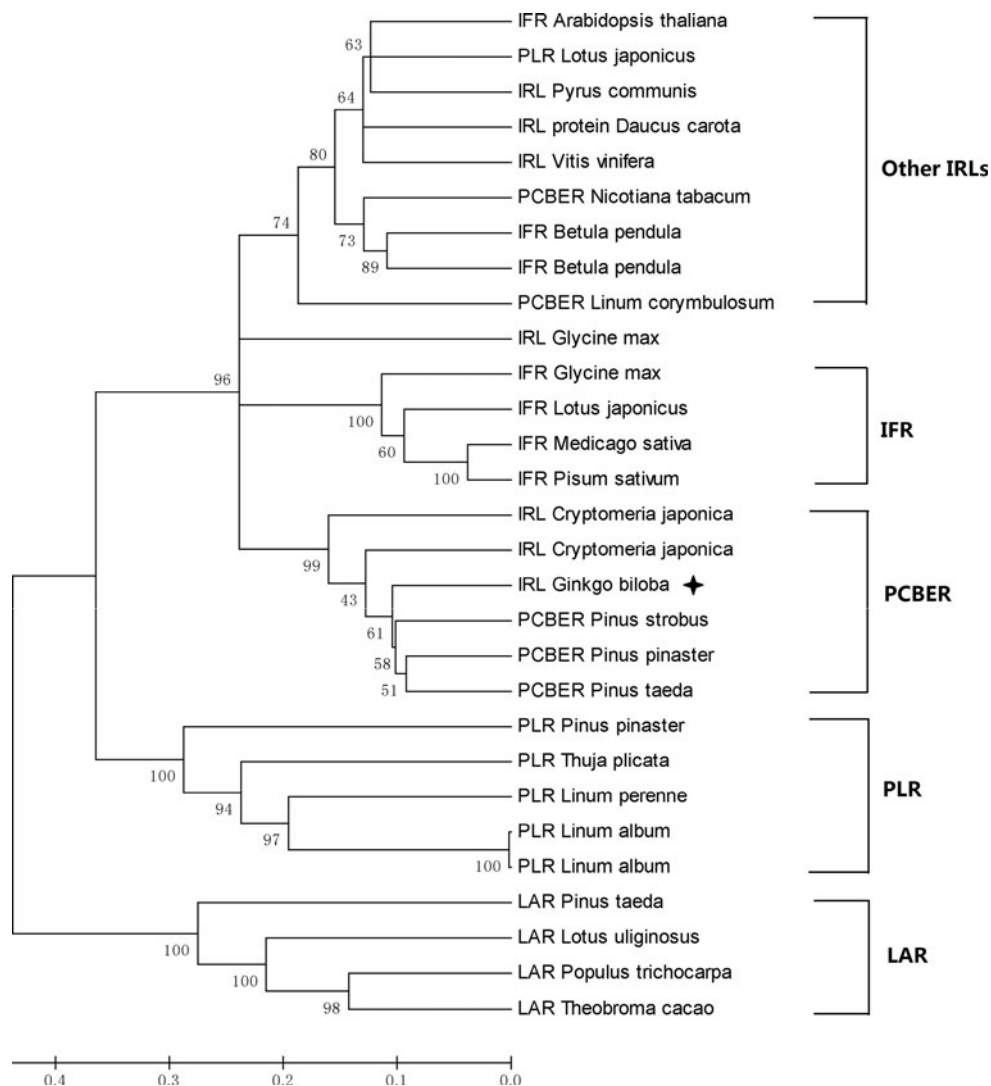
The 1,962 bp regulatory sequence upstream of the IRL gene was obtained by genome walking based on the obtained Ginkgo *GbIRL1* sequences. The IRLP sequences were submitted to the PlantCARE server for analysis. The results indicated that the IRLP transcription site may be located at 249 bp upstream of the translation initiation site (CCGTCCG, Fig. 2). Analysis of the upstream regulatory sequences showed that there was a TATA-box at 117 bp upstream of the transcription initiation site, and a CAAT-box cis element at 292 bp. It was speculated that this area may constitute the core sequences of the *GbIRL1* promoter (Supplementary Figure 1S). The GbIRLP promoter also contained several environmental factors, the cell cycle inductive response elements and cis-acting elements associated with hormone induction.

Multiple sequence alignment with other plant PIPs and their homologs showed that GbIRL1 had higher similarities to PCBERs than to other PIPs (Supplementary Figure 2S). The amino acid sequence of the GbIRL1 shared 82.0 % identity with that of *Tsuga heterophylla*, 80.4 % with *Pinus taeda*, 80.1 % with *Pinus pinaster*, 74.2 % with *Cryptomeria japonica*, 70.9 % with *Vitis vinifera*, 69.5 % with *Betula pendula*, 68.6 % with *Nicotiana tabacum*, 68.3 % with *Pyrus communis*, 67.6 % with *Glycine max*, 66.7 % with *Medicago truncatula*, 65.1 % with *Daucus carota*, 65.0 % with *Ricinus communis* and 63.4 % with *Lotus japonicus*. In all of these protein sequences, the NADPH binding motif was conserved.

A phylogenetic tree generated by the neighbor-joining method based on the putative amino acid sequences of IRLs showed that all members of the IRL family could be sorted into five monophyletic groups. The results indicated that each kind of enzyme with distinct functions formed a separate clade, and the various IRLs fell into four different functional clades (PCBER, IFR, PLR and LAR) (Fig. 1). The PCBERs had a closer relationship with IFRs than with PLRs, and the LAR group was furthest from the PCBERs. The tree suggested that PCBER was among the earliest of these enzymes to arise, and that the IFR and PLR proteins evolved later, which was in agreement with the suggestions by Gang et al. (1999). GbIRL1 was grouped in the PCBER branch, indicating that GbIRL1 belonged to the class of PCBERs.

### The activity of GbIRL1 in vitro

To express *GbIRL1* in *E. coli*, we cloned the coding sequence of *GbIRL1* into pET-28a, an expression vector with the T7 promoter and a His-tag, yielding pET28a-GbIRL1. The expression construct was checked for in-frame fusion by DNA sequencing (Sangon, Shanghai, China). Upon induction by IPTG, GbIRL1 was expressed as a major soluble protein product (Fig. 2). Figure 2, Lane 6 shows the insoluble proteins after IPTG induction for 2.0 h. The



**Fig. 1** Phylogenetic tree based on deduced amino acid sequences of various IRLs. Amino acid sequences were analyzed using the CLUSTALW program. The numbers at each node represented the bootstrap value, with 1,000 replicates. The bars represent evolutionary distance. The polymerization results are divided into five categories, *IRL* isoflavone reductase-like protein; *IFR* isoflavone reductase; *LAR* leucoanthocyanidin reductase; *PCBER* phenylcoumaran benzylic ether reductase; *PLR* pinoreosin lariciresinol reductase. The star indicates the gene studied in this paper. The sequence (accession numbers) are as followed: AAL85023IFR *Arabidopsis thaliana*, BAF34843PLR *Lotus japonicus*, AAC24001IRL *Pyrus communis*, AEY79728IRL *Daucus carota*, CAI56335IRL *Vitis vinifera*, BAG84267PCBER *Nicotiana*

*tabacum*, AAC05116IFR *Betula pendula*, CAA06708PCBER *Populus trichocarpa*, ACA60729 PCBER *Linum corymbulosum*, XP 003537595IRL *Glycine max*, NP 001236037IFR *Glycine max*, BAF34847IFR *Lotus japonicus*, CAA41106IFR *Medicago sativa*, AAB31368IFR *Pisum sativum*, AAK27264IRL *Cryptomeria japonica*, AF242497PCBER *Tsuga heterophylla*, ABF39004PCBER *Pinus strobus*, CCC55425PCBER *Pinus pinaster*, AAC32591PCBER *Pinus taeda*, CCC55424PLR *Pinus pinaster*, AF242506PLR *Thuja plicata*, ABM68630PLR *Linum perenne*, BAI66418PLR *Linum album*, CAH60857PLR *Linum album*, CAI56321LAR *Pinus taeda*, AAU45392LAR *Lotus uliginosus*, EEF06163LAR *Populus trichocarpa*, ADD51358LAR *Theobroma cacao*

molecular weight of the expressed recombinant protein was estimated as a 34 kDa band with a His-tag: this size was in agreement with the value predicted by bioinformatics methods. The recombinant GbIRL1 protein was purified using Nickel-NTA agarose affinity chromatography and the purified protein showed the expected size (Fig. 2, lane 7). Western blotting of the purified recombinant GbIRL1 protein confirmed its specific immune reactivity to anti-His antibodies (Fig. 2, lane Western blot).

The GbIRL1 protein shared considerable amino acid sequence homology to PCBERs from *Pinus taeda* L. and poplar. To test whether GbIRL1 has PCBER activity, recombinant GbIRL1 was expressed in *E. coli* and assayed for PCBER activity. GbIRL1 was further purified to homogeneity through chromatographic steps (Fig. 3a). Two different reductase reactions in the presence of NADPH were measured by reversed-phase HPLC: the formation of tetrahydrodehydrodiconiferyl alcohol (TDDC) from

dihydrodehydrodiconiferyl alcohol (DDDC), and isodihydrodehydrodiconiferyl alcohol (IDDDC) from dehydrodiconiferyl alcohol (DDC). The protein extract from *E. coli* expressing the fusion protein showed clear PCBER activity, with either DDC or DDDC as the substrate. GbIRL1 reduced DDDC to TDDC, and DDC to IDDDC, this agreed well with results reported previously (Gang et al. 1999). Negative controls in which empty vectors were expressed, or in which boiled enzyme preparations were used, produced no reaction products (Fig. 3b). These results indicated that GbIRL1 is a Ginkgo PCBER family protein involved in lignan biosynthesis.

The purified GbIRL1 protein was also incubated with NADPH or NADH and excess unbound cofactors were removed by filtration through a size-exclusion membrane. Absorption spectra of the protein fractions showed that GbIRL1 selectively bound NADPH but not NADH (Fig. 4). These results suggested that GbIRL1 has a typical PCBER activity, and it catalyzes a distinct NADPH-dependent oxidoreductase reaction.

#### Expression profile of the *GbIRL1* gene in different tissues

To determine whether the *GbIRL1* gene was related to lignin or flavonoid synthesis in *Ginkgo*, its expression patterns were analyzed by qRT-PCR. We found that the

transcription level of *GbIRL1* varied according to tissues, growth conditions and tissue developmental stage. The results of qRT-PCR analysis are shown in Fig. 5. *GbIRL1* was expressed most abundantly in fungal-infected blades, followed by damaged or fungus-infected stem segments. The expression levels also varied significantly in different development stages in the stem. The highest level of transcription was found in the third-year stems. The next highest levels of *GbIRL1* were observed in fourth-, and second-year stems, while the lowest level of expression was found in first-year stems.

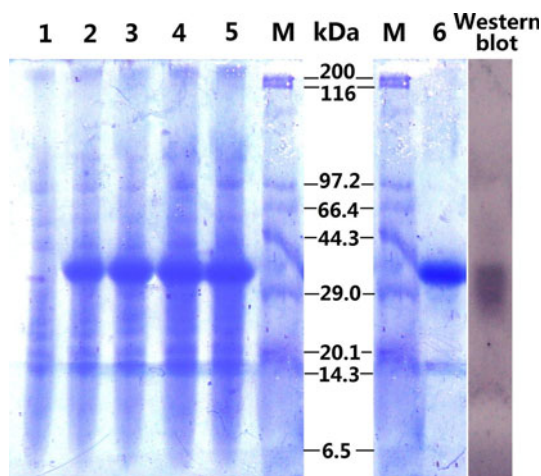
The data obtained were analyzed by linear regression. The results showed that lignin content ( $Y$ ) had a linear correlation with the transcription level of *GbIRL1* ( $X$ ). The correlation coefficient was as follows  $R^2 = 0.517$  (Supplementary Table 1S). Thus, changes in *IRL1* expression could affect the lignin content in different tissues of Ginkgo.

#### UV-B, wounding and hormones regulate the transcription of *GbIRL1*

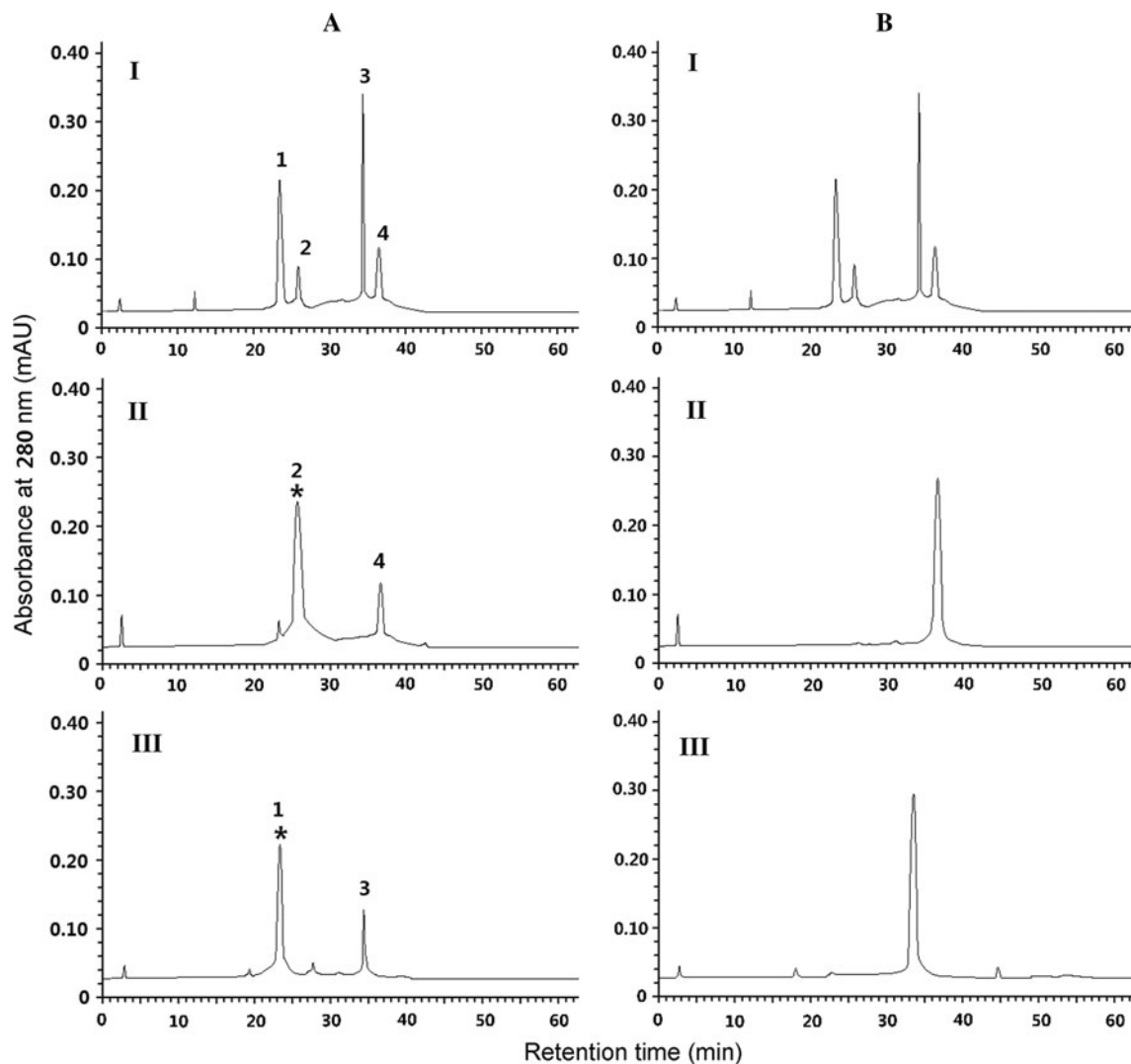
UV-B and plant hormones can induce flavonoid content changes in Ginkgo leaves. To study the expression pattern of the *GbIRL1* gene under UV-B, ultraviolet light of 260 nm, 1,500 Lx light intensity was used to process Ginkgo and sample it in different lighting time for RT-PCR analysis. In the low-dose UV-B treatment, the trend of the transcription level of *GbIRL1* fell after rising and it reached its maximum expression quantity at about 48 h (Fig. 6a). Damage treatment significantly induced *GbIRL1* expression. The transcription level rose slowly in the first 8 h after cut damage treatment, and then increased rapidly and reached its maximum level at 12 h (Fig. 6b).

In the anabolism of Ginkgo leaf flavonoids, endogenous hormones are correlated with flavonoid content changes. The hormones ABA, ALA, SA and ETH were selected for spraying treatments, after which RT-PCR was used to test the transcription of *GbIRL1*.

Salicylic acid is a phenolic hormone that can regulate plant growth and development. It is also involved in plant defense against pathogen as endogenous signal and belongs to the phenylalanine metabolic pathways like flavone and flavodoxin (Ferrer et al. 2008; Hayat et al. 2007). Previous researches had indicated that salicylic acid treatment can significantly increase the expression level of Ginkgo flavonoid-related genes (Xu et al. 2008a, b). Spraying Ginkgo leaf with low concentrations of SA induced the *GbIRL1* gene rapidly, and the transcription level reached 3.8 times that of the control at 4 h after treatment. It reached its maximum level at the 8 h of sampling point and then declined slightly, but was still maintained at about five times the level of the control (Fig. 6c).



**Fig. 2** SDS-PAGE gel and Western blot analysis of GbIFR1 expressed in *E. coli* BL21 (DE3). After IPTG induction, *E. coli* BL21 cells containing pET28a-IFR were grown at 30 °C for 2 h. *lane 1* protein of total cells without IPTG induction, *lane 2* protein of total cells with IPTG induction for 30 min, *3* protein of total cells with IPTG induction for 60 min, *4* induction for 1.5 h, *5* soluble proteins with IPTG induction for 2 h, *6* purified recombinant GbIFR1 protein with Nickel-CL agarose affinity chromatography and used for enzyme activity assay; *M* molecular marker; *Lane Western blot* western blotting of the purified recombinant GbIFR1 protein with an anti-His-tag primary antibody probe



**Fig. 3** **a** GbIRL activities of BL21 pET28a recombinant proteins. **b** HPLC profiles of the products of the control reaction. **a(I)** Reactions of IRL in lignan biosynthesis. DDC and DDDC were enzymatically reduced to IDDDC and TDDC by GbIRL, respectively. Both reactions require NADPH as the hydride donor. **a(II)** reaction products (4. IDDDC) were monitored by absorbance at 280 nm. **a(III)** another

products (1. TDDC) were monitored by absorbance at 280 nm. Chromatographic peaks were identified by comparing their retention times with those of authentic standards. **b(I)** Standard chromatogram (280 nm). **b(II)** The extract of bacteria harboring the empty vector was used for the protein, the reaction with DDC as the substrate. **b(III)** The reaction with DDDC

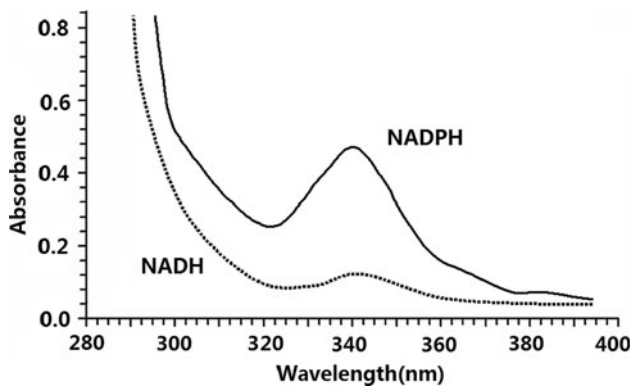
Previous research has indicated that ethephon treatment can effectively increase the accumulation of Ginkgo leaf flavonoids, the basis of which is the increased expression of key flavonoid metabolic genes (Cheng et al. 2004, 2005, 2011; Wang and Cheng 2002; Xu et al. 2008a, b). We found in Ginkgo leaf, which was sprayed with the same concentration of ethephon, that the *GbIRL1* transcription level also increased significantly, but it responded faster than to the other hormones and reached its maximum at 4 h, before declining rapidly to the control level at about 48 h (Fig. 6d).

ALA is not a plant hormone, but it has been reported that spraying ALA appropriately can deepen the color of apples (Wang et al. 2004b). Previous research has indicated

that 100 mg/L ALA can significantly improve the enzymatic activity of Ginkgo PAL, CHS and CHI. Ginkgo leaf was treated with the same concentration of ALA (100 mg/L) in this study. The *GbIRL1* transcription level increased slowly up to 24 h and reached a maximum at 48 h of 2.3 times the control level, then started to decline slowly (Fig. 6e).

ABA is formed by carotenoid degradation and can prevent the promotive growth effects of gibberellic acid and cytokinin. It is also correlated with leaf aging and fruit abscission. Generally, stresses such as drought, cold, hyperthermia, salt and flooding can all make the ABA levels in plants increase rapidly, which enhances plant resistance (Shen et al. 2006). In the *GbIRL1* metabolic





**Fig. 4** Binding of NADPH or NADH to GbIRL1 protein. Absorption spectra of purified GbIRL1 protein incubated with either NADPH (solid line) or NADH (dashed line)

pathway, ABA could induce *GbIRL1* transcription, but it responded slower than the other ABA-responsive processes. After 4 h of treatment, the *GbIRL1* transcription level had increased significantly compared with the control. It continued to rise and reached a maximum at 48 h of about 12 times the level of the control. It then started to decrease, but was still maintained at eight times the control level at 96 h (Fig. 6f).

The relationship between *GbIRL1* expression and flavonoid accumulation during Ginkgo leaf growth

PCBERs belong to the phenylalanine metabolic pathway branch and have a parallel position to flavonoid metabolism. Thus, changes in the IRL expression level may cause changes of flavonoid content in Ginkgo leaves. To study the relationship between flavonoid content in Ginkgo leaf and the *GbIRL1* gene expression level, the annual changes in *GbIRL1* gene expression level and flavonoid content were measured during the growth of Ginkgo leaves (Fig. 7). Grafted Ginkgo leaves were collected at 15 different sampling points (May 3, 18; June 13; July 4, 18, 27; August 1, 13, 30; September 11, 29; October 17; November 1, 14 and 29) for RT-PCR and the flavonoid content measurement.

It can be seen in (Fig. 7) that the annual changes in flavonoid content showed several peak values. Some obvious peak values were found on July 4, September 29, and November 1, and the corresponding contents were 0.8039, 0.9383 and 1.0994, respectively. The highest level was at the sampling point of November 1. The main trend was an increase in flavonoid content. The *GbIRL1* gene expression levels showed two main peak values during the growth of the Ginkgo leaves. The first appeared on July 18, which was close to the time of the first peak in flavonoid content. It descended to a nadir on August 13, and then increased continuously to the highest peak of the year on

October 17, when the relative transcription level was 37.77. Thereafter, the expression level decreased to about 16.33.

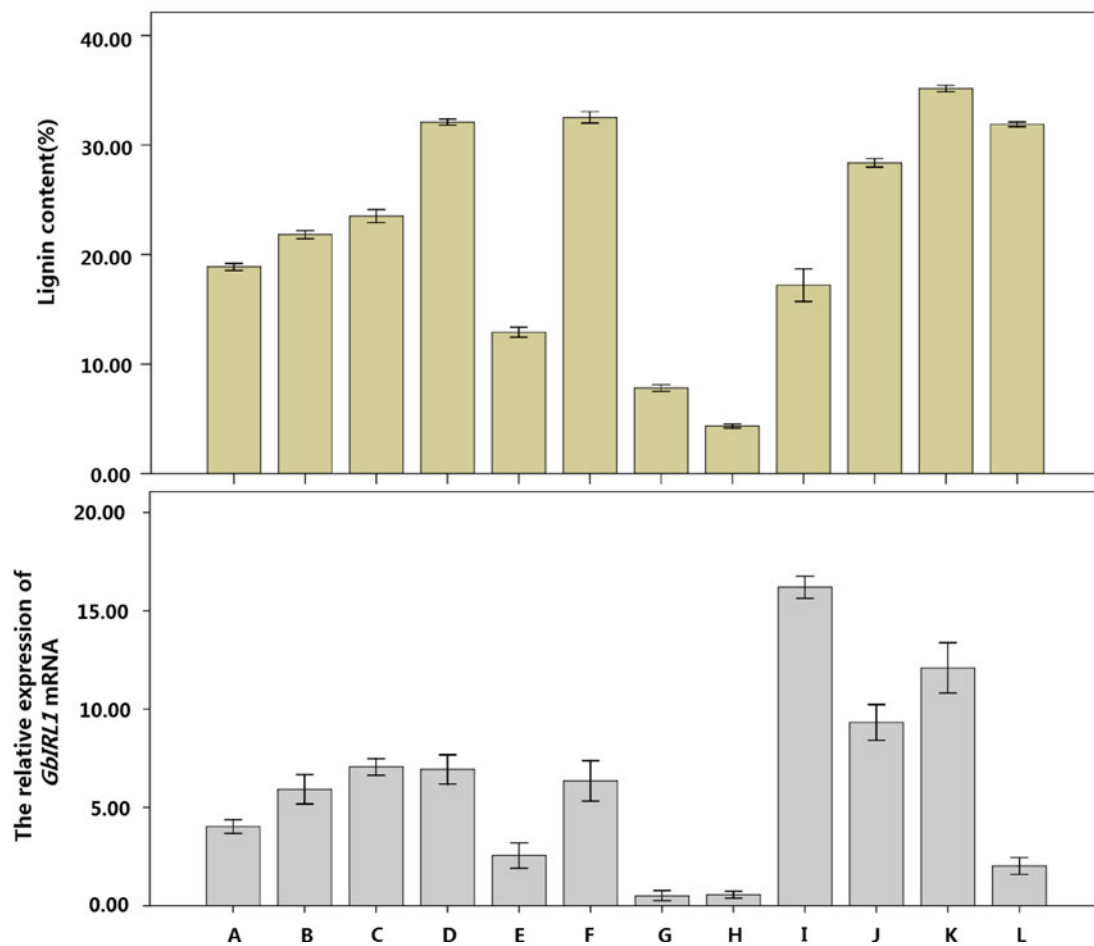
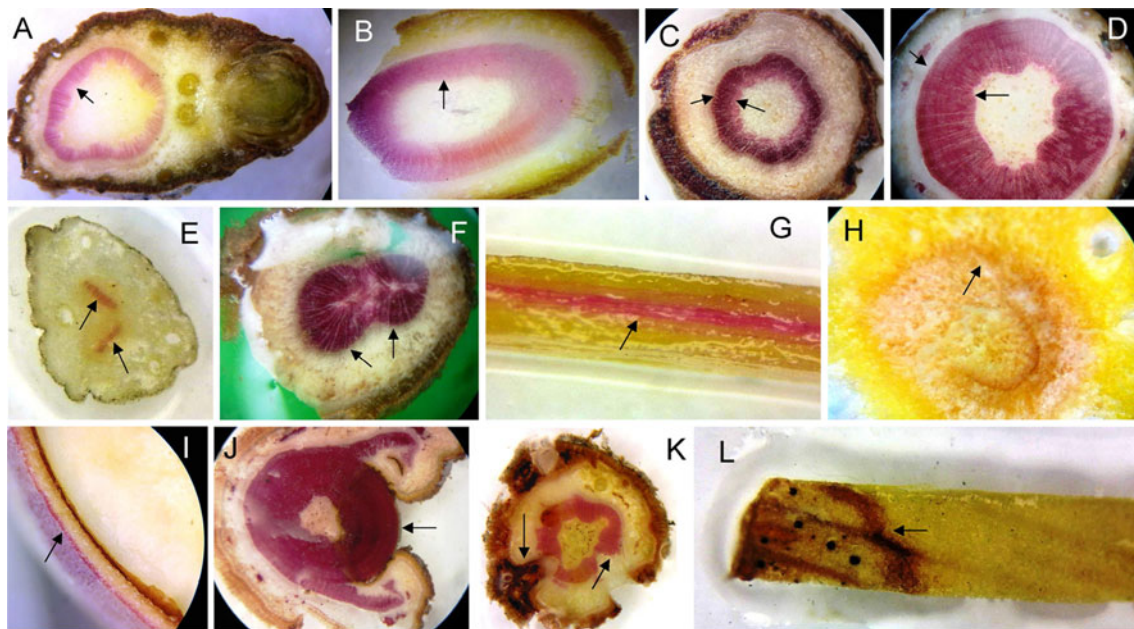
SPSS17 was used to analyze the relationship between the *GbIRL1* transcription level and seasonal variations in flavonoid content (Supplementary Table 2S). Curve fit was conducted according to the test results, among which the goodness of fit of the S-shaped curve, power curve and square curve was high. All three regression models were statistically significant. Taking the goodness of fit and the model's degree of conciseness together, the power curve was most appropriate. According to the results, in the annual changes of flavonoids in Ginkgo leaf, the flavonoid content ( $Y$ ) and the *GbIRL1* expression level ( $X$ ) were power-related, the  $F$  statistic = 208.188, concomitant probability value  $p = 0.000 < 0.001$ , correlation coefficient  $R^2 = 0.610$ . Although the *GbIRL1* expression level and the accumulation of flavonoids were not completely synchronous, the results of curve fitting showed that they were closely related to each other.

## Discussion

The *GbIRL1* (isoflavone reductase-like protein, IRL) gene from Ginkgo was cloned through degenerate PCR and RACE techniques. IRL genes have been identified in several flowering plants and their functions have been analyzed. However, to date, especially for gymnosperms, there are few reports on IRL genes in medicinal plants that have high flavonoid contents.

### Enzyme and recombinant protein analysis

PCBER catalyzes the corresponding reduction reaction in lignans with an 8-5' connection (Min et al. 2003). The analysis of enzyme activity in vitro shows that PCBER depends on NADPH and can catalyze several 7-O-4' reduction reactions of benzyl coumarin lignan related to defense and produce relevant dihydric phenols, such as catalyzing DDC and DDDC to produce corresponding IDDDC and TDDC (Ferrer et al. 2008; Gang et al. 1999). Although the catalysis of the reaction is less efficient, the two isomer forms of the substrate can be recognized by the enzyme and involved in the reaction. PCBER does not catalyze spatial specific reaction of the substrate, but the catalytic substrates of PLRs and IFRs do have spatial specificity. From this it can be predicted that the PCBER protein is evolutionarily older than PLRs and IFRs (Gang et al. 1999). Because lignin-like material is mostly found in ancient plants such as the fern, *Ceratophyllum demersum* L, gymnosperms and early angiosperms, isoflavones exist only in leguminous plants and few are seen in non-leguminous plants. Therefore, it appears that the metabolic



pathway of lignans is older than that of isoflavones (Dinkova-Kostova et al. 1996). Although PLR and IFR catalyze similar reactions, IFR is an evolution of PLR according to evolutionary analysis and substrate specificity inference

(Ferrer et al. 2008). The Ginkgo IRLs evolutionary tree shows that *GbIRL1* and ancient gymnosperm enzymes are of the same species, and the IRL1 sequence alignment shows that *GbIRL1* closely matches the conserved sites of PCBER

**Fig. 5** The expression of *GbIRL1* and lignin contents in different organs. In the *top* panel, hand-cut sections of different sample were stained with phloroglucinol-HCl method. The lignin content of the tissue was determined by modified Kalsom method in the *middle* panel. *Bottom* panel, mRNA levels was measured by QRT-PCR, Expression pattern of *GbIRL1* gene in different tissues with GAPDH gene as control, Data are mean values of triplicate tests  $\pm$  SD **a** annual stem, **b** biennial stem; **c** triennial stem, **d** Four years stem, **e** petiole, **f** root, **g** leaf veins of the longitudinal cutting, **h** endosperm. **i** sclerotesta. **j** injured stems; **k** diseases stems, **l** fungal infections leaves. The *arrow* indicates histo-chemical lignin staining

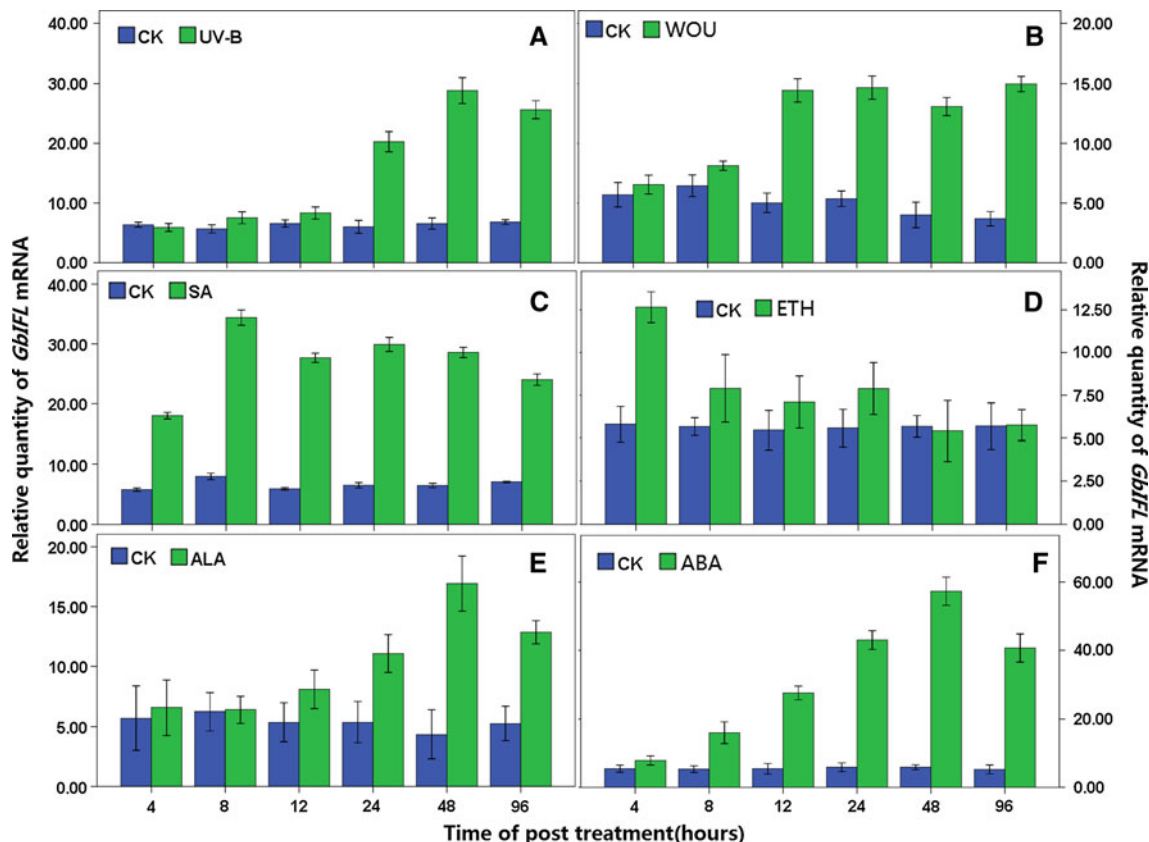
and lacks the insertion sequences. It was confirmed by analyzing the enzyme activity that its recombinant protein possesses the characteristics of PCBER enzymes, which depend on NADPH, and its catalytic function is close to PCBER, Thus, it can be deduced that GbIRL1 belongs to the PCBER protein family.

The expression patterns of the *GbIRL1* gene and accumulation of flavonoids in different tissues and leaf developmental stages

Vander Mijnsbrugge et al. (2000) found that the poplar shoot and root secondary xylem synthesized large amounts of

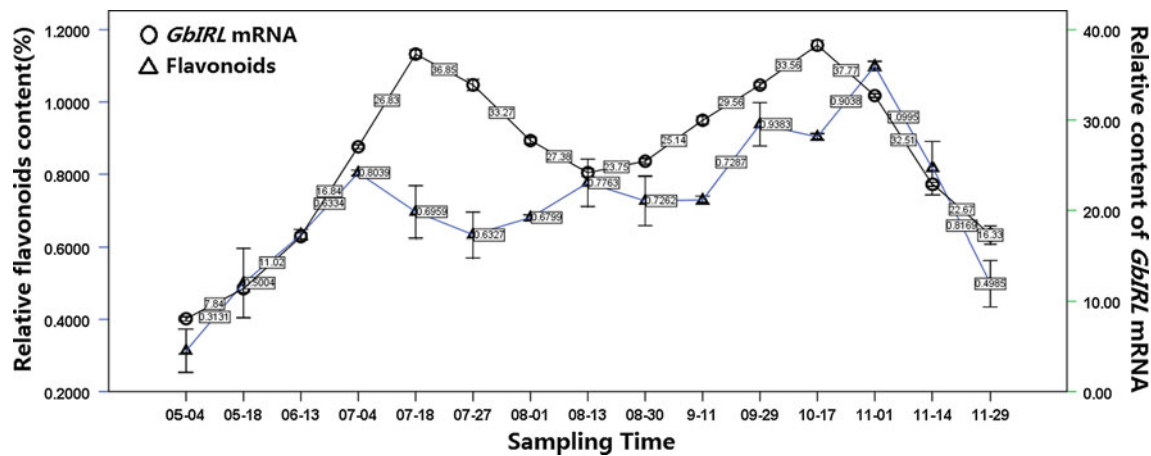
PCBER, suggesting that it was related to protective lignan formation in the xylem, and lignans may be involved in the relevant timber growth metabolism during plant development. The PCBER transcription level in cotton testa is high, and its expression pattern is very important in cotton seed development as it can not only protect the seed structure, but also functions in defending against external infection. Deletion mutations can easily affects the synthesis of xylary fiber lines (Turley 2008). We found from expression analysis of different tissues in Ginkgo that *GbIRL1* is highly transcribed in leaves infected by fungi, as well as in injured and insect-damaged shoot segments, suggesting that *GbIRL1* may be involved in lignification in Ginkgo during its defense against pest and disease damage and to protect non-infected tissues. However, whether it forms a lignin protective barrier or pest-defensive lignans requires further investigation. Its expression in the stem showed that besides involvement in adapting to biotic stress, *GbIRL1* is also related to wooden formation in different tissues as part of its regular expression. Thus, *GbIRL1* is a multi-function gene during Ginkgo growth and development.

Studies have indicated that the reason ultraviolet light can induce flavonoid synthetic gene expression is that



**Fig. 6** Relative quantities of *GbIRL1* mRNA at various time points post-treatment with UV-B (a), wounding (b), SA (c), ETH (d), ALA (e) and ABA (f). Each sample was individually assayed in triplicate.

Values shown represent the mean reading from three treated plants and the error bars indicated the standard errors of the mean



**Fig. 7** Changes in flavonoid content, *GbIRL1* expression level during growth of ginkgo leaves. In the annual change of flavonoid in Ginkgo leaf, the flavonoid content and *GbIRL1* expression level

showed power-related. Data are mean values of triplicate tests and the error bars indicated the standard errors of the mean

flavonoids can reduce UV damage to plants; this response is mediated by ultraviolet responsive elements in the upstream promoters of genes related to flavone metabolism (Bashandy et al. 2009; Cheng et al. 2011). UV-B was shown to induce high level expression of an IRL gene in grape. This grape IRL was simultaneously induced by injury and pest and disease damage (Lers et al. 1998). A Pomelo IRL gene has also been found to be involved in the response to ultraviolet radiation (Min et al. 2003). So, these results suggested that the high level of *GbIRL1* expression after UV-B induction may be related to the accumulation of flavonoids (Xu et al. 2008a).

After *Pyricularia grisea* inoculation of rice, the *OsIRL* gene was induced with an expression pattern similar to MeJA induction (Kim et al. 2003). Studies of the *Coffea arabica* *CaIRL* gene promoter found a damage response element in 0.8 kb upstream of the coding region. However, the damage induction regulation function ceased after deleting the sequence about 0.4 kb upstream of the promoter (in which there was a W-box and an ACCTACC-motif element) (Brandalise et al. 2009). There are several cis-acting elements, including a W-box and a WRKY element, in the upstream regulatory sequence of *GbIRL1* (Supplementary 1S). The W-box is associated with damage induction and ERF responses caused by damage (Nishiuchi et al. 2004). WRKY elements are correlated with pest defense, disease damage induction and abscisic acid signal regulation (Eulgem et al. 1999; Xie et al. 2005). These results offer possible evidence for injury-induced expression of the *GbIRL1* gene in *G. biloba*.

When plants encounter osmotic stress or any other adversity, ABA can regulate relevant genes to adapt to the environmental change (Skriver and Mundy 1990). ABA treatment will improve the transcription level of the Ginkgo leaf flavonoid synthesis genes FLS (Xu et al.

2012), ANS (Xu et al. 2008b), CHI (Cheng et al. 2011) in the flavonoid metabolic pathway. *GbIRL1* is involved in lignan metabolism, which is a branch of phenylalanine metabolism, and has a parallel position to flavonoid metabolism. Thus, the significantly increased transcriptional level induced by ABA could be seen as raising the downstream metabolic level by promoting the phenylalanine metabolic pathway.

ETH can promote fruit maturation and coloring, regulate the relevant gene expression of flavonoids metabolism, and is associated with the accumulation of flavone in Ginkgo (Cheng et al. 2011; Xu et al. 2008a, b, 2012). Ethephon can induce CHS gene expression in Brazilian rubber (Kush et al. 1990) and exogenous ETH can induce both CHS and F3H gene expression in avocado (Ardi et al. 1998). The *GbIRL1* expression level was not obviously increased in Ginkgo sprouts treated with ETH; expression increased slightly in the beginning but then declined rapidly to the control level. To date, there have been few reports about IRL genes that are induced by ETH, but we found ETH induction is not obvious compared with other flavonoids metabolic genes.

5-aminolevulinic acid (ALA) is a key precursor of all porphyrin compound biosynthesis in vivo. The production increase can reach 11–15 % if ALA is used in the wheat growth period or rice panicle and flowering periods (Hotta et al. 1997). A lot of research has proven that a low concentration of exogenous ALA can promote photosynthesis in plants, and photosynthesis can in turn promote lignin synthesis (Nishihara et al. 2001, 2003; Wang et al. 2004a). Moreover, treatment of 100 mg/L ALA can increase the activity of Ginkgo PAL, CHS and CHI (Xu 2008). Therefore, the increased transcription level of Ginkgo IRL in response to ALA we observed may be related to the increased level of substrate in this pathway.

SA is a signaling molecule for plants to react to external environmental stresses and damage from infection by pests and disease (Gaffney et al. 1993). When a plant is damaged by external pests or disease infection, the expression level of flavonoid metabolism genes is improved significantly (Fofana et al. 2002; Meer et al. 1993). Previous studies have indicated that SA treatment can significantly increase the expression level of the *Ginkgo PAL* gene (Xu et al. 2008a). In this study, SA could significantly induce the transcription of the *GbIRL1* gene. Moreover, the increased expression level of *GbIRL1* when leaves were damaged by the outside wounding showed that *GbIRL1* is involved in the SA signaling response pathway induced by pest and disease damage.

Flavonoids such as flavone can protect plants from ultraviolet light and various flavonoids and isoflavone can induce the expression of genes related to root nodules and offer protection for nitrogen-fixing of the plant. The formation of lignin provides structural support for higher plant evolution, and lignans provide protection for the normal growth of plants as antibacterial agents and antitoxins (Ferrer et al. 2008). *Ginkgo* has strong resistance to adverse environments and very high esthetic value, and the flavones in *Ginkgo* leaf have high medicinal value. All of these are closely related to the vigorous phenylalanine metabolic pathway. After the production of coumaroyl-CoA by 4CL catalysis, the flavone synthetic pathway and the lignin and lignan synthetic pathway start to differentiate. In the flavone anabolic pathway, the gene expression levels directly related to *Ginkgo* flavone metabolism have a significant positive correlation with the correlating enzyme activity and flavone accumulation (Cheng et al. 2011). We found that changes in *Ginkgo GbIRL1* expression have some power correlation with the flavone content in the leaf. This result suggests that the carbon flow regulation by the lignan metabolic pathway with *GbIRL1*'s involvement influences the flavone metabolic pathway. Thus, flavone accumulation is not only regulated by key genes in the flavone metabolic pathway itself, but is also related to parallel pathway metabolisms such as the anabolism of lignins and lignans.

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