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Full Length Research Paper

Molecular cloning, characterization and expression of phenylalanine ammonia-lyase gene from *Ginkgo biloba*

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A full-length cDNA and genomic DNA of phenylalanine ammonia-lyase gene, which catalyzes the first step in the flavonoid biosynthetic pathway, were isolated from *Ginkgo biloba* for the first time (designated as *GbPAL*, GenBank Accession No. EU071050). The cDNA and genomic DNA sequences of *GbPAL* were the same, in other words, this gene is intronless. The coding region of the gene was 2172 bp long, and its deduced protein consists of 724 amino acids with a predicted molecular mass of 79.1 kDa and a p*I* of 5.96. The deduced GbPAL protein showed high identities to other plant PALs. Southern hybridization analysis of the genomic DNA indicated that *GbPAL* belonged to a small multi-gene family. Tissue expression analysis by real-time PCR revealed that *GbPAL* constitutively expressed in all the tested tissues, especially highly in leaf and stem. *GbPAL* was also observed to be induced by a variety of stresses including UV-B, wounding, cold and salicylic acid. Temporal expression profiling analyses showed that the transcription levels of *GbPAL* were significantly correlated with flavonoid accumulation, suggesting that GbPAL might play a regulatory role in flavonoid biosynthesis in leaves of *G. biloba* at the transcriptional level.

Key words: *Ginkgo biloba*, phenylalanine ammonia-lyase (PAL), cloning, expression analysis, flavonoids.

INTRODUCTION

Ginkgo biloba is the only living representative of the order *Ginkgoales*, and it is also called a 'living fossil'. *G. biloba* extract possesses interesting pharmacological properties, and it becomes the most widely sold phytomedicine to treat early-stage Alzheimer's disease, vascular dementia and many others (Sierpina et al., 2003). The standardized preparations of *G. biloba*, Egb761, contain 24% ginkgo flavonoids, which have been elucidated to have many pharmaceutical properties for human health (van Beek, 2002; Smith and Luo, 2004). Furthermore, flavonoids can protect plants against UV-B radiation and pathogen attack, attract pollinating insects, and act as signal molecules for initiating plant-microbe symbiotic associations (Parr and Bolwell, 2000; Winkel-Shirley, 2002).

Flavonoids are synthesized through the phenylpropanoid pathway that has been extensively studied (Holton and Cornish, 1995; Dixon and Steele, 1999; Winkel-Shirley, 2001; Koes et al., 2005). The pathway of flavonoid biosynthesis has been well studied and most of the enzymes and the genes involved had been characterized (Li et al., 2006). The first step in the pathway is catalyzed by phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), which is at the gateway from the primary metabolism into the important secondary phenylpropanoid metabolism in plants (Hahlbrock and Scheel, 1989). PAL catalyzes the nonoxidative elimination of ammonia from L-phenylalanine to give *trans*-cinnamic acid, a substrate common to the biosynthesis of different classes of phenylpropanoid products: anthocyanins, flavonoids, ultraviolet (UV) protectants, antimicrobial furanocoumarins, isoflavonoid phytoalexins, ligins and wound phenolic esters (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995; Ritter and Schulz, 2004). Due to the central function of PAL at the branch point of phenylpropanoid derivative metabolism, this enzyme has been considered to be one of the key enzymes in the biosynthesis of flavonoids

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(Hahlbrock and Scheel, 1989; Schijlen et al., 2004). Furthermore, PAL is also a key enzyme in plant stress response. Its biosynthesis is stimulated on pathogenic attack, tissue wounding, UV irradiation, low temperature, or low levels of nitrogen, phosphate, or iron (Dixon and Paiva, 1995). The enzyme is accumulated in the vicinity of the affected tissue (Mauch-Mani and Slusarenko, 1996; Ehness et al., 1997). Along with the gradual elucidation of flavonoid metabolism pathway, new intermediates in the pathway are discovered and each of them is found to possess certain positive roles in different tissues, such as UV protection, resistance against pathogens and insects. The products of the pathway enable plants to better adapt to the environmental stress (Winkel-Shirley, 2002; Pang et al., 2005). Thus, the study on the characterization and expression pattern of the genes involved in the flavonoid biosynthetic pathway, in particular the well-documented genes such as PAL and CHS, is essential to further understanding of the mechanism of stress resistance and the biosynthesis of flavonoids.

Since the first discovery of PAL by Koukol and Conn (1961), the presence of this enzyme has been demonstrated in all higher plants tested, as well as in some yeast species (Hahlbrock and Scheel, 1989; Joos and Hahlbrock, 1992). Because of its crucial role in the biosynthesis of flavonoids, ligins, and phytoalexins and stress responses, PAL and its gene were widely studied (Hahlbrock and Scheel, 1989; Ohl et al., 1990). So far, the genes encoding PAL have been cloned from more than 25 angiosperm species, as well as one gymnosperm (pine) and three fungi (Lu et al., 2006). The *PAL* genes cloned from many plants were found to belong to small multi-gene family. Each member of the family showed a distinctive expression pattern.

Flavonoid accumulation in *G. biloba* leaves requires coordinated expression of genes encoding enzymes in the core phenylpropanoid pathway, such as PAL and 4-coumarate CoA ligase, and enzymes in branch pathways such as chalcone synthase and chalcone isomerase. Our group and Tang's group have cloned and characterized four downstream genes of flavonoid biosynthetic pathway in *G. biloba*, that is *GbCHS1* (Pang et al., 2005), *GbCHS2* (Xu et al., 2007), *GbF3H* (Shen et al., 2006a), *GbANR* (Shen et al., 2006b). However, to the best of our knowledge, there has been no report on cloning and expression profiles of upstream genes in flavonoid biosynthetic pathway including *PAL* gene. In this paper, the full-length cDNA and genomic DNA sequence of *PAL* gene were isolated and characterized from *G. biloba* for the first time. The expression profiles of *GbPAL* in different tissues and under stresses, and relationships between the mRNA transcripts of GbPAL and flavonoid accumulation were also investigated.

MATERIALS AND METHODS

Plant materials

Fresh one-year old seedlings of *G. biloba* grown at 25/18℃ (day/night) in a controlled-growth chamber (16 h light/8 h dark) were subjected to different treatments. The edges of ginkgo leaves were cut by about 1 cm with scissors for wounding treatment, the intact leaves of ginkgo was as control. The cold treatment was performed by placing the seedlings in a 4℃ growth room and the control in a 25℃ growth room. For UV-B treatment, seedlings were exposed with 1500 μJ/m^2 UV-B irradiation in the dark closed chamber, and the control was placing in the dark closed chamber. Salicylic acid (SA) treatment was performed by spraying 500 μM SA, and control plants were sprayed with the equal amount of distilled water. For analyses of relationship between *GbPAL* mRNA transcript and flavonoid content, leaves of three-year-old seedlings of *G. biloba* growing in a controlled-growth chamber were harvested at time points of 20, 40, 60, 80, 100, 120, 140, 160 and 180 days after leaf bud break (DALB). All samples were immediately frozen in liquid nitrogen and stored at -80℃ prior to RNA extraction, PAL activity and flavonoid content analysis. Experiments for investigating the time course expression profile of *GbPAL* were conducted from 2005 to 2006 and all experiments were repeated twice and duplicate samples were taken at each time point.

DNA and RNA extraction

Genomic DNA was extracted from leaves of *G. biloba* according to the protocol as described by Jiang and Cai (2000). Total RNA was extracted separately from all samples by using CTAB method (Liao et al., 2004), followed by incubation with RNase-free Dnase I (TaKaRa, Dalian, China) at 37℃ for 30 min. The quality and concentration of DNA and RNA were measured with electrophoresis and spectrophotometer.

Cloning of the full-length cDNA and genomic DNA of *GbPAL*

To get the internal conservative fragment, primers PAL1 (5'-GCHTCBGGTGATYTRGTY-3') and PAL2 (5'-ACATCTTGGTTRTGYTGCTC-3') were designed and synthesized based on the conserved amino acid and nucleotide sequence of plant *PAL* genes (Shanghai Sangon Biotechnological Company, China). One step Reverse transcription-PCR (RT-PCR) was carried out and a fragment of 862 bp was obtained by using one step RT-PCR kit (TakaRa) under the following PCR program: 50℃ for 30 min and 94℃ for 3 min, followed by 35 cycles of amplification (94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min). The amplified product was purified and cloned into pMD18-T vector (TaKaRa) followed by sequencing. Subsequent BLAST results confirmed that fragment was partial fragment of *PAL*.

Based on the sequence of the internal conservative fragment of *G. biloba* PAL gene, the three specific primers, PAL5 (5'-TGGCAGACATCACTTCAGACAGCACAGC-3') and the nested primer PAL5N (5'-AAGCCGCTCATT TCAGTTCCAT-3'), PAL3 (5'-GCTGTGCTGTCTGAAGTGATGTCTGCCA-3') were designed to amplify the 5' end and 3' end of *GbPAL* gene using the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). The 5'-RACE-PCR and 3'-RACE-PCR was performed according to the manufacture's instructions. The PCR product was purified and cloned into pMD18-T vector for sequencing.

After comparing and aligning the sequence of 5' RACE, 3' RACE and the middle region products, the full-length cDNA sequence of *GbPAL* was obtained through PCR amplification using 3'-Ready cDNA as the template and a pair of primers GbPALC1 (5'-TTCTTCTAATAATCTGCCTCCTCTCCGTCG-3') and UPM under the following condition: 94 for 3 min followed by 35 cycles of amplification (94℃ for 20 s, 68℃ for 30 s and 72℃ for 2 min). After sequenced, the full-length cDNA of *GbPAL* was subsequently analyzed for molecular characterization. Two gene-specific primers, GbPALG1 (5'-TCATCCTCATCGGTATCTTTTCG-3') and GbPALG2

(5'-CCGTTTTGTTCCAGTAGACCGTA-3'), designed based on the cDNA sequence was used to amplify the genomic sequence of *GbPAL*. The obtained sequences were analysed using bioinformatic tools at websites (http://www.ncbi.nlm.nih.gov/ http://www.expasy.org/). The software Vector NTI 10 was used for sequence multi-alignment.

Southern blot analysis

Aliquots of genomic DNA (30 µg per lane) were digested overnight at 37°C with *Hin*d III, *Xba* I and *Bam* HI, respectively. The genomic DNA was fractionated by 1.0% agarose gel electrophoresis and transferred onto a positively charged Hybond-N^+ nylon membrane (Amersham Pharmacia, UK). The membrane was subsequently hybridized with a DIG-labeled *GbPAL* DNA fragment generated with primer GbPALH1 (5'-TGCCAACCCAGTCACTAACCATG-3') and primer GbPALH2 (5'-TCCACCGCCTGAAGCAAATCC-3'). For probe-labeling, an aliquot of 50 ng purified product was used as the template in a total volume of 50 µl. Probe labeling, hybridization and signal detection were performed following DIG High Primer DNA Labeling and Detection Starter Kit II manufacturer's instruction (Roche Applied Science, Mannheim, Germany).

Expression analysis of *GbPAL* by real-time PCR

For quantification of *GbPAL* transcripts in different tissues including root, stem and leave of *G. biloba*, under different elicitor treatments and at different developmental stage of ginkgo leaves, respectively, quantitative real-time PCR was carried out with the forward primer GbPALF (5'-ATGGTTGCAGGAGCAGAAAGGA-3') and the reverse primer GbPALR (5'-TCAGAATGGACCAGGAGTTCCTT-3') specific to the coding sequence of *GbPAL*. The specific primers GAPDHF (5'-ATGAGTTCCACCGGAAAGATT-3') and GAPDHR (5'-TTAGACAGTGGAGGCCATATG-3') were used to amplify the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) of *G. biloba* (Jansson et al., 1994), which served as an internal reference. The reaction was carried out in triplicate for 45 cycles on a Rotor-Gene 2000 Real Time Amplification system (Corbett Research) using a Qiagen Quantitect SYBR Green PCR system. The PCR reaction (total volume 50 µl) consisted of 1×master mix, 0.5 µM each primer, and 0.1 µg cDNA as the template. The cDNAs for quantification were prepared from about 9 plants. The PCR amplification was performed under the following conditions: 95°C for 15 min, followed by 45 cycles of amplification (94°C for 20 s, 61°C for 30 s, 72°C for 40 s). For quantification of standard, the PCR products amplified from cDNA was isolated, and the concentration of the product was measured at 260 nm to calculate the number of cDNA copies in the sample as described by Yin et al. (2001). The range of cDNA concentrations in standardization reactions was 10^4 - 10^8 copies per µl.

Extraction and determination of flavonoid content

Flavonoids were extracted and determined as previously described by Wang et al. (2006) with some modifications. Briefly, the powder of dry leaves of 50 mg was dissolved in 40 ml acidulated methanol. The sample solutions were then suitably diluted with methanol and filtered through 0.45 µm filter membrane (Millipore, Nylon) for high performance liquid chromatography (HPLC) analysis. Extractions of 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 L × 4.6 mm i.d., 5 µm particle size), using a mobile phase consisting of methanol: 0.4% (v/v) phosphoric acid (60:40) for elution. The flow rate was 1.0 ml min^{-1} and injection volume was 20 µl. The UV detector was set at λ = 370 nm. Three extraction samples were prepared for HPLC analysis with each sample injected three times. The quercetin, kaempferol and isorhamnetin were selected as standard sample because that many different flavonol glycosides occur most of them being derivatives of this three flavonol aglycones in ginkgo leaves (van Beek, 2002). Based on the methods of flavonoids concentration described by van Beek (2002), flavonoid contents were calculated by multiplication of total content of quercetin, kaempferol and isorhamnetin by a factor 2.51 and were expressed as percentage.

RESULTS AND DISCUSSION

Characterization of *GbPAL* cDNA, genomic DNA and deduced GbPAL protein

Using RACE method, the full-length cDNA sequence of *GbPAL* (GenBank accession No. EU071050) was obtained by RT-PCR. It was found to be 2886 bp with a poly (A) tailing, and *GbPAL* contained an ORF of 2172 bp encoding 724 amino acids. There was a 5' untranslated region of 303 bp upstream from the start codon, and the coding region was followed by 3' untranslated region that was 411 bp long downstream from the stop codon. A proposed polyadenylation signal (AATAA) was found at 71 bp downstream from the stop codon.

The PCR for genomic sequence of *GbPAL* resulted in a clear band, which was 2617 bp by sequencing and the same as the cDNA sequence; that is, the *GbPAL* gene did not contain introns. Similarly, it was found that the *PAL* gene of *Pinus taeda* (GenBank, PTU39792) did not contain introns. *PAL* gene in gymnosperm might be unique in that it does not contain introns. On the contrary, *PAL* genes from angiosperm plants, such as *Oryza sativa* (Minami et al., 1989), *Lycopersicon esculentum* (Lee et al., 1992), *Pisum sativum* (Yamada et al., 1992), *Arabidopsis thaliana* (Wanner et al., 1995), *Populus kitakamiensis* (Osakabe et al., 1995) and *Beta vulgaris* (Schmidt et al., 2004) have one or two intrones each. Since introns may have some regulatory functions in gene expression, the phylogenetic significance of presence vs. absence of introns in this gene remains to be investigated.

By using the software of Computer pI/Mw Tool at http://www.expasy.org/, the deduced GbPAL protein had a theoretical *p*I at 5.96 and a calculated molecular weight of 79.1 kDa, which was consistent with the size determined for PAL polypeptides from other plants using SDS/PAGE (Schomburg and Salzmann, 1990). A database search with BlastP2.2.3 at http://www.ncbi.nlm.nih.gov/ and the multialignment by Vector NTI 10 showed that the deduced GbPAL had considerable high homology with other plant PALs. Figure 1 showed an alignment of the deduced polypeptide sequence of GbPAL and other PALs from several plant species. It was found that GbPAL presented 84, 83, 75, 70, 67 and 66% identity to *Pinus taeda*, *Pinus pinaster*, *Isoetes lacustris*, *Prunus avium*, *Nicotiana tabacum* and *A. thaliana*, respectively. The high

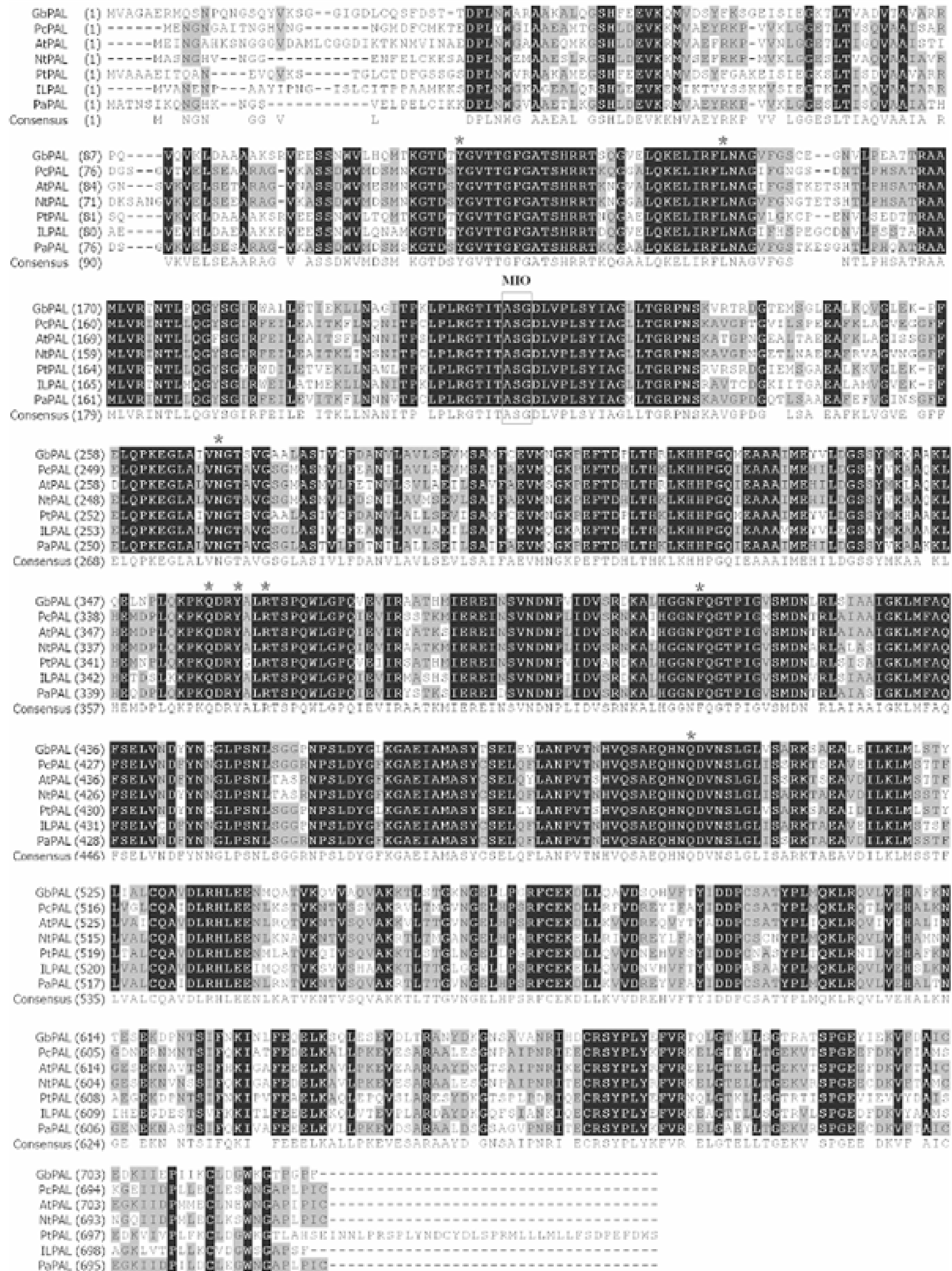

Figure 1. Sequence multi-alignment of the deduced GbPAL protein with other PALs.
The completely identical amino acids are indicated with white foreground and black background. The conserved amino acids are indicated with black foreground and grey background. Non-similar amino acids are indicated with black foreground and white background. The active sites residues are indicated in asterisk (*), and residues Ala-Ser-Gly forming MIO group are boxed in the alignment. PcPAL, PAL from *Petroselinum crispum* (CAA57056); AtPAL, PAL from *A. thaliana* (NP181241); NtPAL, PAL from *N. tabacum* (BAA22948); PtPAL, PAL from *Pinus taeda* (AAA84889); IlPAL, PAL from *Isoetes lacustris* (AAW80637); PaPAL, PAL from *Prunus avium* (AAC78457).

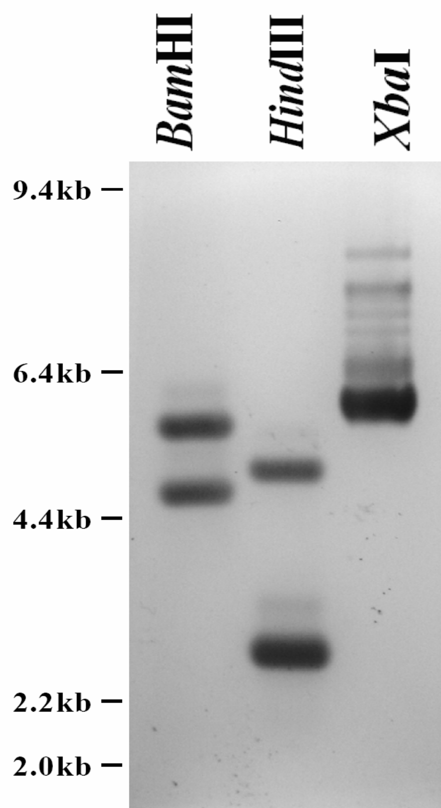

Figure 2. Southern blot analysis. Genomic DNA (30 µg per lane) was digested with *Hind*III, *Xba*I and *Bam*HI, respectively, followed by hybridization with GbPAL fragment probe.

similarity was observed for residues 36 - 723, but the N-terminal region showed some variability in length and composition (Figure 1). It was clear from the multi-alignment that PALs from gymnosperm plants were more similar to each other than to those of angiosperm plants, as confirmed by the phylogenetic analysis. The stringent conservation among evolutionary diverse plant species may indicate the functional significance of these amino acids.

Many sites essential to PAL activities conserved in different plant species were also found in GbPAL. For example, active site amino-acid residues in GbPAL were S213, the predominant precursor of 4-methylidene-imidazole-5-one (MIO) group, as well as Y^{120}, L^{148}, N^{269}, Q^{357}, Y^{360}, R^{363}, F^{409} and Q^{497} (Figure. 1). These active site residues may be important for substrate binding, catalysis or MIO formation. All the active sites mentioned above were the same and at the counterpart positions with those of PAL from *Petroselinum crispum* (Röther et al., 2002), indicating GbPAL was a member of PAL family. Besides, several consensus protein motifs detected in the bean (*Phaseolus vulgaris*) PAL protein (Allwood et al., 1999) were also found in the GbPAL by PROSITE motif search (http://www.expasy.ch/tools/). One example was the MIO group formed autocatalyticallly by cyclization and dehydration of an Ala-Ser-Gly tripeptide, which together with adjacent substrate binding sites and product *E*-cinnamate/ammonia were formed into the active site of PAL model by the σ-complex to give further insight into the role of the amino-acid residues in catalysis (Schulz and Ritter, 2004). Another example was the conserved motif (GTITASGDLVPLSYIAG), which was buried within the core of the enzyme and was predicted to be a typical phenylalanine and histidine ammonia-lyases signature pattern (Schuster and Rétey, 1994).

Southern blot analysis

To examine whether the *GbPAL* gene belongs to a multi-gene family, genomic DNA of *G. biloba* was digested with *Hin*d III, *Xba* I and *Bam* HI, respectively, followed by hybridization with *GbPAL* gene probe. There are no restriction sites of *Hin*d III, *Xba* I and *Bam* HI within the probe region. Southern blot analysis revealed two specific hybridization bands ranging from 2.0 to 9.4 kb were presented in each lane (Figure 2), indicating that *GbPAL* gene belonged to a small multi-gene family. It was well known that in most plants, *PAL* is encoded by a small multi-gene family with two to six members, while its family members are differentially expressed in plant tissues as well as in the response to different stress conditions (Wang et al., 2007).

Expression analysis of *GbPAL* in different tissues and under different stresses

In order to investigate the *GbPAL* expression pattern in different tissues of *G. biloba* seedlings, total RNA was extracted from root, stem and leaf, respectively, and used for quantitative real-time PCR analysis. The result showed that the *GbPAL* was constitutively expressed in all of the tested tissues, with the highest expression in leaf and stem and lowest expression in root (Figure 3). Our data for abundant transcript accumulation in leaf and stem of *G. biloba* were contrary to other studies showing that *PAL* mRNA was typically high in root, low in leaves of other plant, such as *A. thaliana* (Wanner et al., 1995), *Solanum tuberosum* (Joos and Hahlbrock, 1992), *Hordeum vulagare* (Kervinen et al., 1997) and *Isatis indigotica* (Lu et al., 2006). Furthermore, experiments with the gene-specific probes have suggested that most of the isolated *PAL* genes were expressed strongly in roots and notably fewer genes were expressed in leaves (Kervinen et al., 1997; Kumar and Ellis, 2001). The high transcript levels in root compared with other plant tissues may be due to the high rate of lignification which is part of normal root development (Dixon et al., 1994). The contradiction between their results and ours may be explained

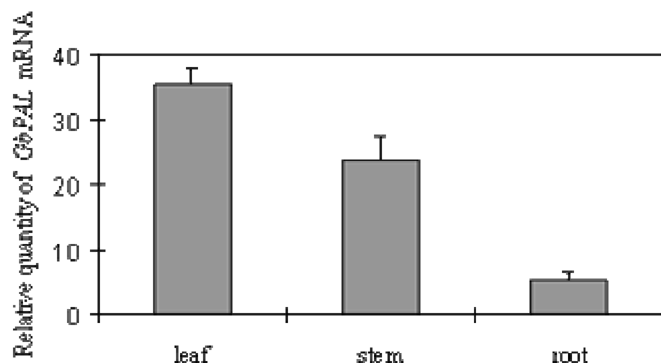

Figure 3. Relative quantities of GbPAL mRNA in different ginkgo tissues. Each tissue sample was individually assayed in triplicate. Values shown represent the mean reading from three plants and the error bars indicate the standard error of the means.

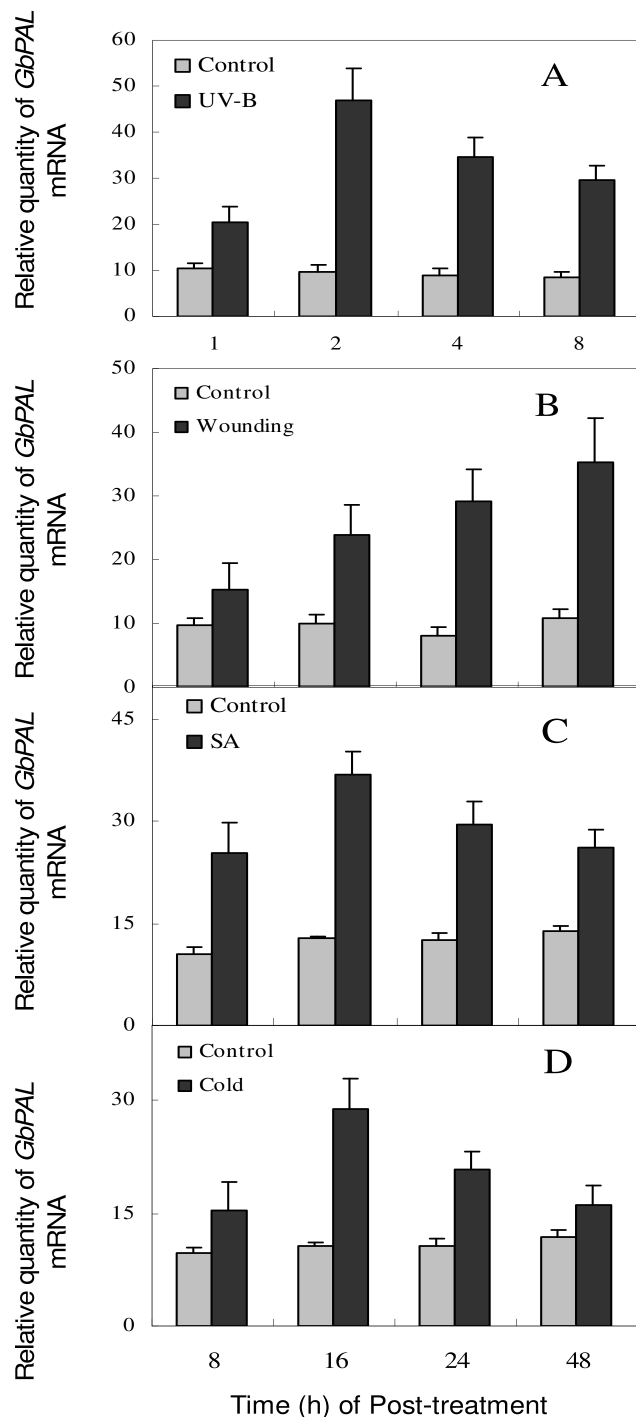

Figure 4. Relative quantities of *GbPAL* mRNA at various time points post-treatment with UV-B (A), wounding (B), SA (C) and Cold (D). Each plant was individually assayed in triplicate. Values shown represent the mean reading from three plants and the error bars indicate the standard error of the means.

by difference in tissue-specific expression of *PAL* gene among different plant species.

Because PAL activity could be induced by different abiotic stresses, we tested whether the expression of *GbPAL* was also affected by these stresses by using quantitative real-time PCR. The seedlings of *G. biloba* were treated with UV-B, wounding, cold and SA respectively for various durations and afterwards leaves were harvested for total RNA extraction. As shown in Figure 4, under UV-B treatment, *GbPAL* transcript accumulation reached the highest level at 2 h and then gradually declined, but still showed higher level than the control. Under wounding treatment, the transcript accumulation increased along with the treatment time and reached the highest level at the end of treatment (48 h). Expression of *GbPAL* upon cold and SA treatments also gradually increased in initial 16 h and then decreased slightly, but also still showed higher level than control. These results were similar to those in leaves of barley under UV-B irradiation (Kervinen et al., 1997), younger leaves of wounded lettuce (Campos-Vargas and Saltveit, 2002), leaves sprayed with SA in parsley and tobacco (Fraissinet-Tachet et al., 1998; Thulke and Conrath, 1998) and leaves of tetraploid *I. indigotica* under cold stress (Lu et al., 2006). The expression of *GbPAL* gene increased during UV-B and wounding treatment, which might be due to an acute need for extra pigments in terms of protection from UV or defensive phenolics in response to wounding. SA is considered as one of the key endogenous signals involved in the activation of numerous plant defense responses (Wen et al., 2005). Application of SA could significantly induce resistance against a variety of biotic and abiotic stresses. PAL is an enzyme at the entry-point of the phenylpropanoid pathway. *Trans*-cinnamic acid, a product of PAL catalysis, is a substrate common to the biosynthesis of different classes of phenylpropanoid products. Because of the nature and defense-related function of these products, expression of *GbPAL* was induced by SA could be considered a part of defense mechanism (Dixon and Paiva, 1995). The results showed that *GbPAL* could be marked-

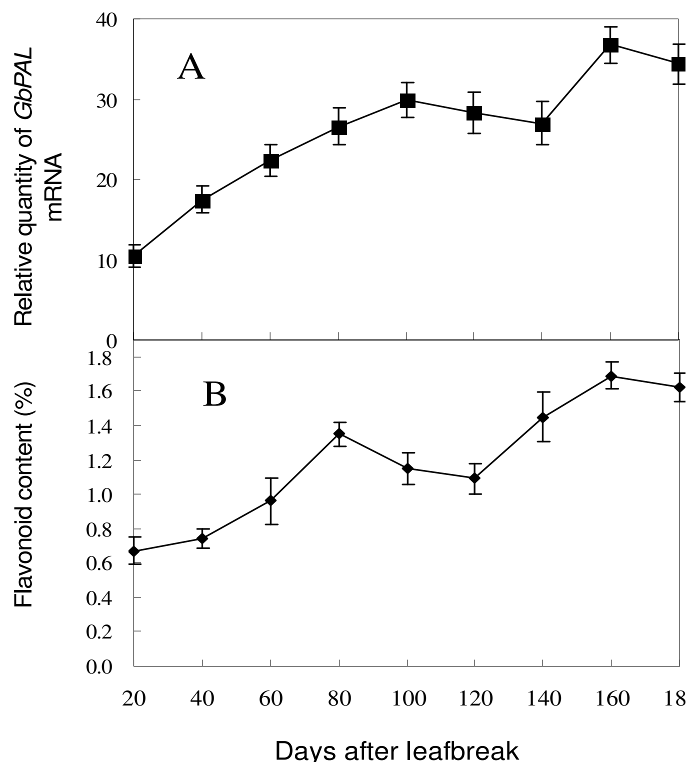

Figure 5. Temporal expression profile of *GbPAL* and flavonoid accumulation during leaf development of *G. biloba*. (A) Temporal expression pattern of *GbPAL* during leaf development. Total RNA samples were isolated from three-year old *G. biloba* seedlings leaves harvested at time points of 20, 40, 60, 80, 100, 120, 140, 160 and 180 days after leaf bud break, respectively. (B) Changes in flavonoid content during leaf development. The flavonoid contents were extracted and determined by HPLC from three-year old *G.* biloba seedlings leaves harvested at time points of about 20, 40, 60, 80, 100, 120, 140, 160 and 180 days after leaf bud break, respectively, and were expressed as percentage (m/m).

ly induced by cold treatment. Keeping in mind that among the cold-responsive genes identified in previous studies, *PAL* and other anthocyanin biosynthesis genes have been considered to be *cor* (cold regulated) genes, and have been proposed as excellent subjects for characterizing plant molecular responses to low temperature (Christie et al., 1994). In addition, the activities of *PAL* genes were reported to be largely regulated at transcription level. For example, *PAL* genes from parsley contained L-box and P-box in their promoter regions, which was identified as UV-B responsive elements (Lois et al., 1989).

Relationship between the transcript level of *GbPAL* and flavonoid content

Because we are interested in the relationship among *PAL* gene expression, PAL activity and accumulation of flavornoids, we examined the time course of expression patterns of *GbPAL* and flavonoid concentrations during leaf development in *G. biloba*. To gain insights into the regulatory role of the GbPAL in the flavonoids biosynthesis, quantitative real-time PCR analyses were carried out to monitor the changes in *GbPAL* expression level in the developmental stages of leaves. The results showed that the expression levels of *GbPAL* varied among different developmental stages of leaves. The expression level of *GbPAL* was lowest at the beginning of leaf growth, increased gradually until 100 DALB and then slightly decreased during 100 to 140 DALB; subsequently, it increased again and reached the maximum at 160 DALB and then remained relatively constant (Figure 5A). The accumulation of flavonoids was almost parallel with the changes in expression level of *GbPAL*, except that it reached the first peak at 80 DALB in comparison to that at 100 DALB in expression level of *GbPAL* (Figure 5B). There was a remarkable correlation between the flavonoid content and the expression level of *GbPAL* using a least-squares linear regression analysis, suggesting that GbPAL might specifically play a regulatory role in flavonoid biosynthesis of *G. biloba* at transcriptional level. We also analyzed the changes in PAL activity during the leaf developmental stages in *G. biloba*, the results showed that no good correlation existed between PAL activity and flavonoid content as well as expression level of *GbPAL* (Data not shown). The PAL activity did not parallel with the *GbPAL* expression level and flavonoids accumulation, which might be explained by (1) complexity of flavonoid biosynthetic process, and (2) the presence of other PAL isoforms in *G. biloba*. Two classes of genes are required for flavonoid biosynthesis, the structural genes encoding the enzymes that directly participate in the formation of flavonoids, and the regulatory genes that control the transcription of structural genes (Holton and Cornish, 1995). The enzyme activities in the various branch pathways are highly regulated. Transcriptional controls play an important role in regulating the overall activity of flavonoid biosynthesis (Jaakola et al., 2002). The flavonoid biosynthetic pathway was also controlled in response to different development and environmental cues (Winkel-Shirley, 2001, 2002). There was also evidence that the enzymes involved in flavonoid metabolism might be acting as membrane-associated multienzyme complexes, which had implications on overall efficiency, specificity, and regulation of pathway (Stafford, 1991; Winkel-Shirley, 2001). The PAL is only one of the enzymes that encoded many structural and regulatory genes involved in flavonoid biosynthetic pathway, incongruous correlation between PAL activity and flavonoid content implied that PAL might not be the predominant factor and cooperate with other enzymes to co-regulate flavonoid metabolism in *G. biloba*. On the other hand, the PAL isoforms encoded by PAL homologous genes which might exhibit different stimulus-specific expression pattern during the development of plant tissues (Liang et al., 1989), and probably contributed to the PAL activity determined, resulting in the difference between change in the expression pattern of *GbPAL* and trend of PAL activity during the leaf develop-

ment in *G. biloba*. One example was the possibility of individual PAL isoforms regulating different branch pathways of phenylpropanoid metabolism was examined by incubating several phenolic compounds with inducible PAL isoforms. The PAL III isoform was thought to be associated with the phenolic acid biosynthetic pathway, whereas PAL II might be the isoform contribu-ting towards flavonoids and anthocyanin biosynthetic pathways (Sarma et al., 1998). Another example was found in barley. Kervinen et al. (1998) reported four PAL genes of barley were all induced by mercuric chloride and fungi, despite considerable variation in their expression level and timing. The four genes account for activation of PAL at different time under elicitor, and *HpPAL4* possibly has a specific role in flavonoid biosynthesis. Our previous work also indicated *GbCHS2* was the specific key-regulating gene involved in flavonoid biosynthesis in *G. biloba* (Xu et al., 2007).

In the present work, our results suggested that *Gb*PAL was another specific key-regulating gene in flavonoid biosynthesis in *G. biloba* and provided useful information for understanding the expression regulation and molecu-lar mechanism of genes encoding related enzymes involved in flavonoid biosynthesis.

In summary, we have successfully isolated and characterized the GbPAL cDNA and genomic DNA from *G. biloba*. Our study indicated that GbPAL was highly expressed in leaf and stem, and was up-regulated by UV-B, wounding, SA and cold. GbPAL might play an important role in the control of flavonoid biosynthesis and is thus an important potential target for the control of flux through this branch of the phenylpropanoid pathway. For further understanding of the GbPAL and its function, a plant expression vector containing the GbPAL has been constructed and a study of the genetic transformation of *G. biloba* is underway in order to test its potential role in improving flavonoid accumulation by genetic engineering.

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