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Isolation and characterization of a phenylalanine ammonia-lyase gene (PAL) promoter from *Ginkgo biloba* and its regulation of gene expression in transgenic tobacco plants

Wei-Wei Zhang[1,†], Jin-Bao Li[2,†], Feng Xu[2,*], Yin Tang[2], Shui-Yuan Cheng[3,*], Fu-Liang Cao[1]

[1]Forest resources and Environment Institute, Nanjing Forestry University, Nanjing 210037, China
[2]College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China
[3]Huanggang Normal University, Hubei Key Laboratory of Economic Forest Germplasm Improvement and Resources Comprehensive Utilization, Huanggang 438000, China

***Corresponding authors: xufeng198@126.com (Feng Xu.); s_y_cheng@sina.com (Shui-Yuan Cheng)**
[†]These authors contributed equally to this work.

Abstract

The *Ginkgo biloba* produces flavonoids and ginkgolides of high interest due to their medical values. Phenylalanine ammonia-lyase (PAL) is a core enzyme in the flavonoid biosynthesis pathway. In this study, we constructed genomic libraries with the DNA from leaves of 18-year-old grafts *G. biloba*. Using nested PCR method, a 1,627 bp 5' flanking region, named *GbPALp* (GenBank: GU968736) of a *PAL* gene (*GbPAL*) was isolated from genomic libraries, and the analysis of the promoter sequence by the PLACE database has revealed the existence of several putative *cis*-elements. To assess the organ-specificity and developmental characteristics of PAL gene expression in *G. biloba*, the GbPALp-driven *GUS* expression in transgenic tobacco was studied. Histological analysis of the transgenic young tobacco plants showed that the cloned *PAL* promoter displayed a tissue-specific GUS staining restricted to root hair region, the cortex of root, the vascular bundle cells, the cortex and the primary xylem of stems, and vascular region of leaves. In transgenic mature plant, *GUS* was expressed in the spire apical meristem of stems but not in leaves and root. The GUS activity in transgenic young tobacco leaves was also observed to be induced by a variety of stresses, including UV-B, abscisic acid, methyl jasmonate and salicylic acid, respectively. The results indicated that *GbPALp* had multiple functions in the expression under the various developmental stages and stress conditions in the transgenic tobacco.

Keywords: *GbPALp*, GUS activity, Tissue-specific expression, Stress induced expression.
Abbreviations: ABA_abscisic acid; GUS_*β* glucuronidase; MeJA_methyl jasmonate; PAL_Phenylalanine ammonia-lyase; PNPG_4-Nitrophenyl-beta-d-glucopyranoside; SA_salicylic acid; X-Gluc_5-bromo-4-chloro-3-indolyl-b-D-glucuronide

Introduction

Ginkgo biloba L., the only surviving representative of the Ginkgophyta division, is the ''living fossil'' that survived geological time from the Jurassic period (Bowe et al., 2000; Chaw et al., 2000). *G. biloba* extract contains valuable compounds such as flavonoids and ginkgolides with pronounced pharmacologic and economic importance (Singh et al., 2008), and it becomes the most widely sold phytomedicine to treat early-stage Alzheimer's disease, vascular dementia, peripheral claudication and tinnitus of vascular origin (Sierpina et al., 2003). Ginkgo leaf extracts also has pharmacologic effects, such as antagonistic action against platelet-activating factor (PAF) receptor (Hosford et al., 1990), and improvement of short-term memory (Polich et al., 2001). Flavonoids own the functions of UV protection, regulation of auxin transport, modulation of flower color in plants (Parr and Bolwell, 2000; Winkel-Shirley, 2002). Flavonoids are synthesized through the phenylpropanoid pathway that has been extensively studied (Holton and Cornish, 1995; 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). Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) has been considered to be one of the key enzymes in the biosynthesis of flavonoids. It catalyzes the first step in the biosynthesis of phenylpropanoid (Hahlbrock and Scheel, 1989; Campos et al., 2004). Furthermore, PAL also serves as a key enzyme in plant stress response. Its activity could be stimulated by tissue wounding, pathogenic attack, low temperature, UV irradiation, or low levels of nitrogen, phosphate, and iron (Dixon and Paiva, 1995). Owing to these essential roles in plant development and defense against various environmental stresses including pathogen ingression, structural and regulatory properties of *PAL* genes have been extensively studied in various higher plants such as *Arabidopsis thaliana* (Cochrane et al., 2004), *parsley* (Lois et al., 1989; Logemann et al., 1995), *poplar* (Subramaniam et al., 1993), *Solanum lycopersicum* (Guo and Wang, 2009), *Pinus taeda* (Whetten and Sederoff, 1992), *sweet potato* (Tanaka et al., 1989), and so on. The expression patterns of the *PAL* gene members are differently regulated, in a spatial and temporal manner, and respond to environmental stimuli. Furthermore, the promoter activities of these *PAL* genes have been found to be cell or tissue-specific in transgenic plants (Kawamata et al., 1997; Osakabe et al., 2009; Wong et al., 2002; Zhang et al., 2014). The transcription of *PAL* genes may be identified in a cell or tissue-specific manner. Studies of several potential *cis*-acting elements and transcription factors show that they might control common mechanisms in the expression of *PAL* genes. These *cis*-elements are known as AC, H-box, L-box, or P-box elements (Murakami et al., 1997; Kaothien et al., 2000; Maeda et al., 2005). Plant R2R3-MYB proteins have been shown to

bind to AC elements to regulate *PAL* gene expression and flavonoid biosynthesis (Broun, 2004; Craven-Bartle et al., 2013). The regulatory effect of *PAL* promoter on gene expression are interrelated at the accumulation site of phenylpropanoid products such as lignin and flavonoids (Kao et al., 2002; Osakabe et al., 2009). Therefore, *PAL* promoter is closely related to the accumulation of lignin and flavonoids. Our group previously had cloned and characterized a gene (*GbPAL*) encoding phenylalanine ammonia lyase from *G. biloba* (Xu et al., 2008a). The study demonstrated that *GbPAL* might play a regulatory role in the flavonoid biosynthesis in leaves of *G. biloba* at the transcriptional level. However, *cis*-acting domains of *GbPAL* promoters have not been fully elucidated. In order to further understand the molecular regulatory mechanisms of *GbPAL* underlying flavonoid biosynthesis in *G. biloba*, here we report isolation of the *GbPAL* promoter (*GbPALp*) and investigation on the pattern of GbPALp-driven *GUS* gene expression in transgenic tobacco plants at two different growth periods. Besides, the reporter activity was also investigated in transgenic tobacco plants in different tissues and various stresses, such as UV-B, abscisic acid, methyl jasmonate and salicylic acid.

Results

Isolation and characterization of GbPALp

With two gene-specific primers based on the coding sequence of *GbPAL* genomic DNA, the 1,627 bp long fragment, 5′-upstream of the putative transcription start site of *GbPAL* gene was isolated through nested PCR amplifications from a constructed genomic DNA library of *Ginkgo biloba*. The nucleotide sequence is shown in Fig 1. The transcriptional start site of *GbPAL* was located 293 bp upstream from the ATG codon. The *GbPALp* was subjected to analysis using PlantCARE software tools, and a series of putative *cis*-acting elements in the promoter was found. Putative TATA box was found at -34bp, which was highly conserved eukaryotic transcriptional activator element. The typical *cis*-acting elements CAAT box in promoter regions, were identified at positions -209bp. A search for the regulatory elements in the *GbPALp* revealed a CGTCA-motif (at -501 bp) and two TGCAG-motif (at -521 bp, -662 bp) were involved in MeJA-responsiveness. Many light responsive elements such as Box 4 (at -559bp), G-box (at-198 bp), GATA-motif (at -302 bp), Sp1 (at -636), GT1-motif (at -331bp) and Box I (at -1492bp), involved in SA responsiveness were noticed. Other putative regulatory elements, SA-responsive element (TCA-element), ethylene-responsive element (ERE), and abscisic acid responsive elements (ABRE) were also detected at -404, -1503, and -102, -519bp, respectively (Fig 1).

Tobacco transformation and screening transgenic

This pBI121-GbPALp vector has been subsequently applied to Agrobacterium-mediated transformation and regeneration of tobacco plants. After Agrobacterium infection and kanamycin selection, a total of putative 50 kanamycin-resistant transformants were obtained and grown in the greenhouse. Putative transgenic tobacco plants were screened by PCR with the corresponding primers. Eight kanamycin-resistant putative transformant seedlings were selected randomly to estimate transgenic efficiency. Five amplification products consistent with the desired size were obtained by PCR (Fig 2, lanes 1-5), and no visible product was detected from wild-type control tobacco plant (Fig 2, lane -).

After digestion of tobacco genomic DNA with *Hind* III, the length of restriction fragments hybridized with the probe was determined. Southern blot results showed that the target fragments had been integrated into some tobacco genome. Different integration sites within the transgenic genome produce different lengths of restriction fragments, the number of which represents the copy number. The transgenic line 1 carried two copies of the transgenes and a single copy in line 2 (Fig 3). There was no hybridization signal was observed in line 3, indicating that line 3 did not carry the transgenic copy. In the wild-type control tobacco plant, we also could not find the hybridization signal.

Expression of GbPALp- GUS in transgenic tobacco

The full-length promoter fragment was fused to the *GUS* reporter gene to characterize its spatial and developmental expression patterns. Histochemical GUS staining was performed on young transgenic tobacco plants (10 days old) and mature plants (40 days old). Non transgenic plants were used as control. In young plants, the *GUS* expression was found in almost all tissue types, and the *GUS* expression was higher in the callus junction to root (Fig 4B). In the mature plants, the blue staining was only visible in the spire apical meristem of the stems (Fig 4F). In other words, the location of *GUS* expression within seedling was varied in different developmental stages. No GUS activity was observed in non-transgenic tobacco plants. Furthermore, according to the tissue slice analysis of young transgenic tobacco, the GUS activity was mainly concentrated in the root hair region (Fig 4H), the cortex of root (Fig 4G), the vascular bundle cells of stems, the cortex and the primary xylem of the stems (Fig 4I, J), and vascular region of leaves (Fig 4K).

Quantitative assay of GUS activity in transgenic tobacco leaves induced by stress treatments

To examine the effect of stress on the *GbPAL* promoter, GUS activity in tobacco leaves transformed with GbPALp-GUS was quantitatively determined after stress treatment. As showed in Fig 5A, SA treatment significantly ($p < 0.05$) increased the activity of GUS in leaves by about 100% of the control up to 8 h post treatment, after which the activity declined to less than the control value until 24 h post treatment. However, the activity of GUS bounced back to 2.0-fold than the control at 48 h. GUS activity in MeJA-treated plants followed similar trends as SA treatment, the treatments caused the GUS activity reached the maximal level at 24 h and then decreased thereafter, but still maintained significantly ($p < 0.05$) higher than control at 48 h (Fig 5C). Unlike SA and MeJA treatments, the activity of GUS in ABA-treated leaves remained significantly ($p < 0.05$) higher than control throughout the 2-day period (Fig 5B). Furthermore, ABA treatment induced a gradual increase of GUS level to 4.1-fold at 48 h after treatment. Fig 5D showed that the expression of GUS was significantly ($p < 0.05$) inhibited in 4 h of the UV treatment, but the activity significantly ($p < 0.05$) increased to 2.6-fold than control at 8 h then gradually decreased to 1.6-fold of control at 24 h. There was no significant ($p > 0.05$) change of GUS activity in the control leaves at different times after treatments.

Discussion

Previous studies have shown that the transcription levels of *GbPAL* were significantly correlated with flavonoid accumulation in *G. biloba* (Xu et al., 2008a). To investigate the regulatory mechanisms of *PAL* gene expression, we isolated the promoter of *PAL* from *G. biloba* and characterized in heterologous

Fig 1. Sequence and bioinformatics of *GbPALp*. The italic indicates the start codon (ATG). The transcription start site is displayed in bold letters. Potentially functional elements predicated by PLACE software are either underlined or boxed.

transgenic tobacco plants. Typical *cis*-acting elements in the promoter regions such as TATA box and CAAT box were identified, and the TATA-box was located in -34 bp upstream the transcription start site. Some putative regulatory elements such as CGTCA-motif, TGCAG-motif, TCA-element, GATA-motif, ABRE that responses to stress were also detected. The cold stress, salicylic acid, UV-B and mechanical damage are known to induce the *GbPAL* transcription (Xu et al., 2008a), which could have correlation with the regulation by *GbPAL* promoter *cis*-elements. In order to understand the spatial and temporal control mechanisms of the gene expression, the *GbPALp* promoter was fused to the *GUS* reporter gene and then transferred to tobacco plants. PCR screening and Southern blot results showed *GbPAL* promoter-GUS expression vector successfully was transferred into the tobacco plant by one or more copies. Hybridization results indicated the sample 3 (Fig 3) had no hybridization signal, which may indicate gene silencing. Studies showed that gene silencing could be mediated by introduction of multiple copies of a transgene (Cervera et al., 2000; Mishiba et al., 2005; Tang et al., 2007). For example, while the maize *A1* gene can normally be expressed in a single-copy transgenic plants, the gene did not function in multi-copy plants because of the promoter methylation (Linn et al., 1990). In transgenic plants carrying *GbPALp-GUS*, histochemical staining showed that *GUS* expression was higher in young tobacco plants than the mature plants. The *GUS* expression was found in almost every type of tissue in young tobacco plants, especially in the callus junction to root. However, in the mature plant, the activity was observed only in spire apical meristem of mature tobacco. As the tobacco plants aged, the *GUS* expression was gradually reduced or even stopped. Similar results were also found in transgenic melon (Dong et al., 1991) and rice (Ishicla et al., 1996) plants. These results might indicate that the PAL activity was higher in tender tissue, and the PAL activity was gradually reduced as the differentiation of the cell. Developmentally regulated *PAL* expression in poplar showed that the highest expression was observed in young stems, apical buds, and young leaves-expression level were lower in mature stems and undetectable in mature leaves (Subramaniam et al., 1993). The observation in poplar is consistent with our histochemical staining results in transgenic tobacco plants. *PAL* promoters from different plants fused to *the GUS* reporter gene were differentially expressed in various tissues of transgenic plants. For example, in bean, the expression of *PAL2* promoter was in very high level in petals with marked accumulation in anthers, stigmas, roots, and shoots, whereas low levels were found in sepals, ovaries, and leaves (Liang et al., 1989). *Pinus taeda PAL* promoter activity was detected in the cortex and the primary xylem in young stems near the shoot buds of transgenic tobacco, while the highest expression level of the *GUS* was observed in the cells surrounding the xylem vessels and ray-parenchyma cells in mature stems (Osakabe et al., 2009). In *Arabidopsis*, the *PAL1* promoter was strongly expressed in the vascular tissues of roots and leaves in adult plants, but was not active in the root tip or the shoot apical meristem. In flowers, expression of *PAL* promoter was observed in sepals, anthers, and carpels, but not in petals (Ohl et al., 1990). Analysis of GbPALp-GUS fusions

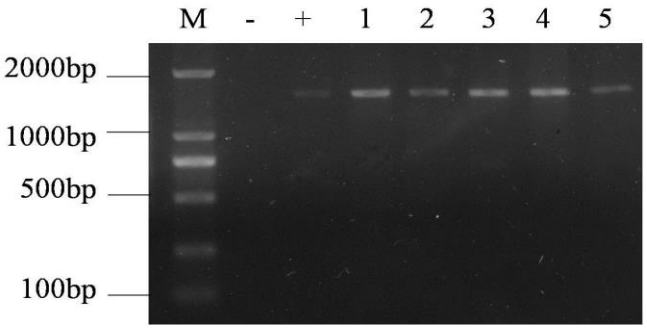

Fig 2. PCR identification of transgenic tobacco plants. M DL2000 marker, + positive control, – wild-type tobacco, 1-5 transgenic tobacco.

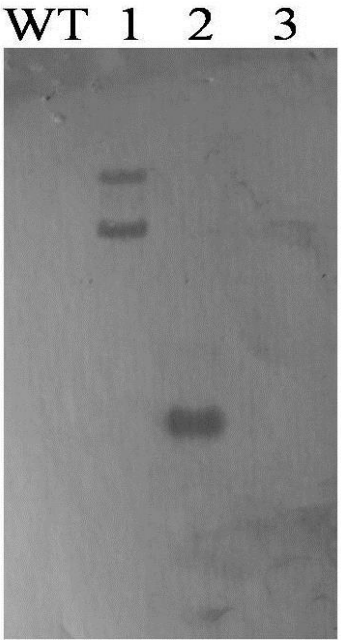

Fig 3. Southern hybridization analysis of transgenic tobacco plants. WT, Non transgenic tobacco; Lanes 1-3, transgenic tobacco plants with pBI121- GbPALp. Lane 1, two hybridization signal detected; Lane 2, single hybridization signal detected; Lane 3, no hybridization signal detected.

clearly demonstrated that the GbPAp -directed GUS expression was organ-specific. In young transgenic tobacco, GUS activity was higher in leaf than in stem, but was lowest in root. GUS activity mainly near the vascular region in leaf, while in stems at the vascular bundle cells, the cortex and the primary xylem. GUS activity was also observed in the hair region and the cortex of the root. The GUS specific expression is correlated with the *GbPAL* expression which was high in leaf and stem, and low in the root (Xu et al., 2008a), indicating that the *GbPAL* gene expression was differentially regulated by *GbPAL* promoter.

In the process of plant growth and development, plant hormones play a very important role. Salicylic acid (SA) acts as an endogenous signal responsible for inducing systemic acquired resistance in plants (Gaffney et al., 1993). SA can specifically bind to a variety of plant proteins affecting their activity (Durner et al., 1997; Wen et al., 2005). It can also activate gene expression/activity by multiple mechanisms and at different steps in plant defense signaling (Feys and Parker, 2000). Previous research has shown that the transcriptional level of *GbPAL* (Xu et al., 2008a), *GbANS* (Xu et al., 2008b), and *GbFLS* (Xu et al., 2012) in *G biloba* were up-regulated by SA treatment. The positive response of these genes under SA-treatment implied the involvement of the genes in the flavonoid biosynthesis. In the present study, GUS was induced up to 2.0-fold by SA at 48 h in the leaf (Fig 5A). Presence of SA-responsive element in *GbPALp* further supports the responsiveness of *GbPAL* to various stress conditions. ABA is known to be a senescence-triggering plant hormone. It acts as an important signal transducer for wound and microbial defense responses in plants. ABA modulates gene expression under osmotic-related stresses such as freezing, drought, and high salt (Skriver and Mundy, 1990). ABA can up-regulate PAL activity during fruit ripening in strawberry (Jiang and Joyce, 2003). The expression of *Salvia miltiorrhiza PAL1* was also found to be increased markedly by ABA treatment (Song et al., 2009). Treatment of *Medicago truncatula* cell cultures with ABA resulted in only a very weak induction of PAL transcripts (Suzuki et al., 2005), while in *A. thaliana*, the expression of *AtPAL1* decreased significantly after ABA treatment (Mohr and Cahill, 2007). In the present study, the exogenous application of ABA triggered a significant induction of GbPALp- GUS (4.1-fold to control at 48 h, Fig 5B). The existence of ABRE response element (Fig 1) in *GbPALp* could explain the responsiveness if *GbPAL* to ABA in *G. biloba*. Methyl jasmonate is a plant hormone that regulates the response of plants against both biotic and abiotic stresses. Up-regulation of a gene under the influence of MeJA has been regarded as evidence of the gene involvement in such responses. Wu and Pan (1997) found the PAL activity in rice seedling leaf was increased after MeJA treatment in parallel with the lignin content. In the present study, MeJA was shown to induce GbPALp-GUS up to 2.6-fold at 24 h in transgenic tobacco leaves (Fig 5C). In addition, *GbPALp* had MeJA responsive *cis*-elements (CGTCA-motif and TGCAG-motif) in the promoter region of *GbPAL* (Fig 1), which supported the responsiveness of *GbPALp* to stress. The result suggests that *GbPAL* is possibly involved in methyl jasmonate-responsive signal transduction. Reports showed that the expressions of *GbPAL* (Xu et al., 2008a), *GbANS* (Xu et al., 2008b), *GbCHI* (Cheng et al., 2011), *GbCHS* (Pang et al., 2005), and *GbFLS* (Xu et al., 2012) was induced due to UV-B exposure. UV-B exposure could induce the expression of flavonoid synthesis-related genes, which might be attributed to flavonoids playing a role in resistance to UV damage (Cheng et al., 2011). In the present experiment, UV irradiation caused the increase of GbPALp-GUS activity in transgenic tobacco leaves to 2.6-fold than control at 8 h (Fig 5D). This phenomenon indicated with presence of light-responsive elements responding to UV-B stress, and indeed several light-responsive elements were identified in the promoter region of *GbPAL*. The similar UV-induced PAL induction was observed in the rice *pal-1* promoter (Peng et al., 2003) and in pea *PAL1* and *PAL2* promoters (Yamada et al, 1994). In sum, we can conclude that the *GbPAL* promoter stimulated GUS activity by UV-B, MeJA, ABA, and SA via response to corresponding *cis*-elements.

Materials and Methods

Plant materials and growth conditions

18-year-old grafts of *G. biloba* were grown in an orchard at Yangtze University. the leaves were collected, immediately frozen in liquid nitrogen, and kept at －80 ° C prior to DNA extraction. Tobacco (*Nicotiana tobacco* cv. NC 89) plants were

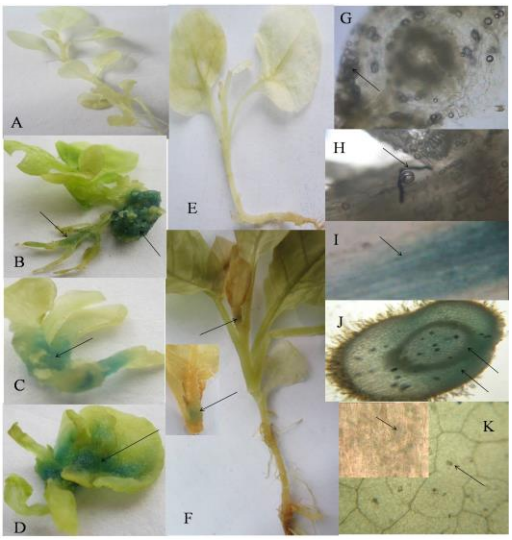

Fig 4. Histochemical analysis of GbPALp-GUS activity in tobacco. Arrows indicated staining site. A, young control tobacco plant. B(Arrows indicated callus junction to root), C, D, young transgenic tobacco with pBI121-GbPALp. E, mature control tobacco plant with no visible blue color. F, mature transgenic tobacco with pBI121-GbPALp, arrows indicated spire apical meristem of the stems. G(Arrows indicated cortex of root), H(Arrows indicated root hair region), root cross-sections. I, stem longitudinal section, arrows indicated cortex and the primary xylem of the stems. J, stem cross-sections, arrows indicated vascular bundle cells of stems. K, tobacco leaf surface, arrows indicated vascular region of leaves.

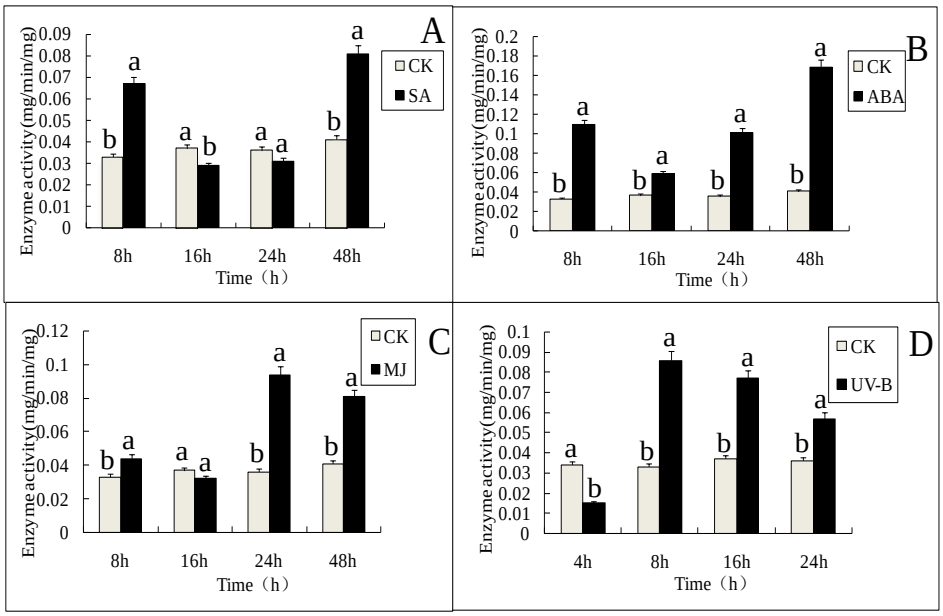

Fig 5. GbPALp-GUS enzyme activities under different stress in leaves of young transgenic tobacco seedlings. CK, Tobacco seedlings without stress. Values are the mean of six samples and bars represent standard errors. Mean with the different letters are significantly difference at $p < 0.05$ by Duncan's multiple range test between the same treatment times.

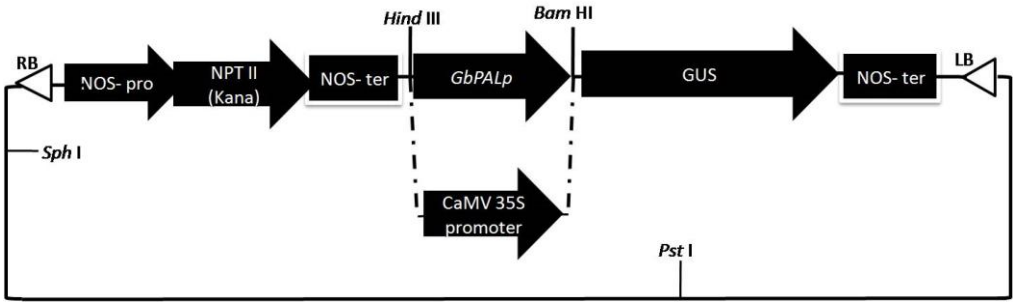

Fig 6. Construction of GbPALp-GUS expression vector. The *CaMV 35S* promoter of the plasmid pBI121 was deleted and replaced by *GbPALp*.

grown on sterile MS medium at 25 ° C under a 16 h light/8 h dark cycle, and leaves were used for leaf-disc transformation.

Construction of genomic library and isolation of GbPALp

Genomic DNA was extracted from the leaves of *G. biloba* seedlings following the CTAB method described by Xu *et al* (2008a). The quality and concentration of the genomic DNA were determined by agarose gel electrophoresis and spectrophotometer analysis. The Genome Walker libraries of *Ginkgo biloba* were constructed using the Universal Genome Walker Kit (Clontech, USA). Total DNA of *Ginkgo biloba* was digested with several restriction enzymes (*Dra* I, *EcoR* V, *Pvu* II, *Ssp* I, respectively) and ligated to the adapters to construct genomic libraries. To clone the 5'-franking regions of *GbPAL*, two rounds of PCR were performed using two gene-specific primers (GSP1:5'-CACAGATCTCCAATTCCGCCACTCTT AACAT-3', GSP2: 5'-TCCGACGGAGAGGAGGCAGATTAT TAGAAG-3') that were constructed according to the sequences of the coding regions of *PAL* gene (EU071050), and adapter primers of the kit (AP1: 5'-GTAATACGACTATAGGGC-3', AP2: 5'-ACTATAGGGCACGCGTGGT-3'). The purified PCR product was cloned into the pMD18-T vector (TaKaRa, Dalian China), and sequenced. The isolated sequence was searched for the putative cis-acting elements previously characterized using the Signal Scan Program PlantCARE (http://bioinformatics.psb. ugent.be/webtools/plantcare /html/) databases (Lescot et al., 2002).

Construction of the GbPALp- GUS expression vector

The *GbPALp* was cloned from pMD18-T vector (included *GbPAL* after sequenced) with Specific Primers (PALproS: 5'-CCCAAGCTTTGTTGTTCCGCATCCATTTC-3', PALproA: 5'-CGGGATCCCTCCTGCTCTGCTTTCATTAGTC-3') containing overhanging *Hind* III and *Bam* HI recognition sites (Underline), respectively. The PCR product was digested with *Hind* III – *Bam* HI and cloned into the binary vector pBI121 replacing the *CaMV 35S* promoter (Fig 6). The resulting constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 and used to transform tobacco leaf discs as described (Horsch et al., 1985). Transgenic tobacco Plants were selected on MS medium containing 80 mg/L kanamycin and transformants were grown in a greenhouse (16 h/8 h light/dark; 25 °C; 70% RH).

Screening transgenic tobacco and Southern blot analysis

In order to Screen transgenic tobacco, the genomic DNA was isolated from leaf tissues of putative transformants according to the method described by Wang, *et al.* (1996) and the PCR analysis were carried out with the primers PALproS and PALproA. To further verify the integration of transgenic tobacco and to determine the transgene copy number, Southern blot analysis was carried out according to the instruction of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany). The fragment isolated by PCR with primers PALproS and PALproA from pBI121- GbPALp vector was used as probe, and synthesized by PCR DIG Probe Synthesis Kit. Both the genomic DNA of transgenic tobacco plants and control tobacco were digested with *Hind III*, separated on 1% agarose gel, and transferred to nylon membranes as described by Khandjian (1987). Then, according to the instruction of DIG High Prime DNA Labeling and Detection Starter Kit I, the hybridization and immunological detection were completed.

Histochemical assay

Histochemical staining of growth chamber-grown transgenic tobacco plants were performed as described by Jefferson et al (1987a). For the histochemical GUS assay, plant samples (Non transgenic plants were used as control) were collected and fixed for 15-20 min in the 90% acetone, washed three times by sterile water before staining with 0.4 mg/ml 5-bromo-4- chloro-3-indolyl-b-D-glucuronide (X-Gluc) for 1 hour at 37 °C, After staining, chlorophyll was removed with decoloring liquid (glacial acetic acid: ethanol (V/V) = 3: 1) for 30 min in 50 °C water bath. Plant samples were kept in the FAA fixed liquid (50% ethanol: 38% formaldehyde: glacial acetic acid (V/V/V) = 18: 1: 1) before observation under a light microscope.

Stress treatments

PCR positive tobacco plants at the four to five leaf stages were used in stress induction treatments. Equally aged sets of tobacco plants were treated with UV-B, abscisic acid (ABA), methyl jasmonate (MeJA) and salicylic acid (SA). The seedlings were irradiated in an UV-B closed chamber (1500 $\mu J/m^2$) and the control seedlings were placed in a dark closed chamber. 4 h, 8 h, 16 h, 24 h after treated, leaves from the tobacco plants were harvested and immediately frozen in liquid nitrogen followed by storage at -80 °C until use. ABA, MeJA and SA were dissolved in 0.01% (v/v) Tween 20 and then sprayed on the tobacco plants leaves at concentrations of 100 μM, 100 μM and 10 mM, respectively. The control leaves were sprayed with an equal amount of 0.01% (v/v) Tween 20. 8 h, 16 h, 24h, 48 h after treated, leaves from the tobacco plants were harvested and immediately frozen in liquid nitrogen followed by storage at -80 °C until use.

Quantitative assay of GUS activity

To quantify GUS activity, the protein was extracted with extraction buffer (0.1% v/v 2-mercaptoethanol),10 mM Na_2EDTA, 0.1% v/v Triton X-100, 50 mM sodium phosphate buffer, pH 7.0) from plant samples, 1.0 g plant samples were grinded in liquid nitrogen with a mortar and pestle. Add powdered tissue to 10 ml centrifuge tubes containing 5 ml protein extraction buffers, fully mixing. The homogenate was centrifuged at 12, 000g for 5 minimums and the supernatant was used for quantification of crude protein (Bradford, 1976), Protein concentrations were determined using Bradford's methods with BSA as a standard (Kruger, 1994). The GUS activity assay was carried out by incubating 300 μl of protein extract with 1ml PNPG (4 mM 4-Nitrophenyl-beta-d-glucopyranoside) as substrate at 37 °C in six tubes. The reaction was stopped after 0 min, 5 min, 15 min, 30 min, 45 min and 60 min by adding 300 ul 2-Amino-2-methyl-1.3-propanediol (1 M). The *p*-nitrophenol as a standard and reaction mix stopped after 0 min as control, the GUS enzyme activity was determined by measuring the fluorescence of *p*-nitrophenol produced by GUS cleavage of PNPG (Jefferson, 1987b). GUS activity was expressed as the concentration of *p*-nitrophenol released per min per mg protein. All the tests were carried out in triplicate, and every experiment was read at 3 times.

Statistical analysis

The data represent the means ± SE. Data were analyzed with one-way ANOVA using SPSS 11.0 for Windows (SPSS Inc., Chicago, Illinois). The means were compared with Duncan's multiple range tests. *P*-value of < 0.05 was considered to be statistically significant.

Conclusions

In conclusion, we isolated and characterized a *PAL* gene promoter from *Ginkgo biloba*. Functional and bioinformatic analyses reveal that the PAL promoter contains a series of *cis*-regulatory elements that could response to stress. Our study indicated that *GbPAL* promoter from *Ginkgo biloba* directs the GUS reporter gene expression in leaf, stem and root in young transgenic tobacco. GUS expression was up-regulated by UV-B, MeJA, SA and ABA. This study provides information required to ascertain the regulated expression characteristic of *GbPAL* gene in flavonoids biosynthesis and would contribute to the progress of molecular breeding in gymnosperm tree species.

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