

# Molecular cloning and function assay of a chalcone isomerase gene (*GbCHI*) from *Ginkgo biloba*

Hua Cheng · Linling Li · Shuiyuan Cheng ·  
Fuliang Cao · Yan Wang · Honghui Yuan

Received: 10 September 2010/Revised: 17 October 2010/Accepted: 19 October 2010/Published online: 3 November 2010  
© Springer-Verlag 2010

**Abstract** Chalcone isomerase (CHI, EC 5.5.1.6) is one of the key enzymes in the flavonoid biosynthesis pathway catalyzing the stereospecific isomerization of chalcones into their corresponding (2S)-flavanones. In this investigation, both the cDNA sequence and the genomic sequence encoding the chalcone isomerase from *Ginkgo biloba* L. (designated as *GbCHI*) were isolated from the leaves. The *GbCHI* gene contained two introns and three exons and encoded a peptide of 244 amino acids with a predicted molecular mass of 26.29 kDa and a pI of 7.76. RQPCR showed that *GbCHI* was expressed in a tissue-specific manner in *G. biloba*. Expression of *GbCHI* was also up-regulated by UV-B irradiation or treatment with 5-amino-levulinic acid or three plant growth regulator—ethylene, abscisic acid, and chlormequat—and these effects were consistent with analysis of the *GbCHI* promoter region. The recombinant protein was successfully expressed in an *E.coli* strain with the pET-28a vector. In vitro enzyme activity, assayed by HPLC, indicated that recombinant

*GbCHI* protein could catalyze the formation of naringenin from 6'-hydroxychalcone. RQPCR analysis showed that CHI activity correlated with changes in transcription level of the *CHI* gene, *GbCHI* activity was also positively correlated with total flavonoid levels in ginkgo leaves, suggesting *CHI* as a key gene regulating flavonoid accumulation in ginkgo leaves.

**Keywords** Chalcone isomerase · Enzyme activity · Gene expression · *Ginkgo biloba* L. · Flavanoids

## Abbreviations

bp	Base pair
HPLC	High performance liquid chromatography
IPTG	Isopropyl $\beta$ -D-thiogalactoside, C <sub>9</sub> H <sub>18</sub> O <sub>5</sub> S
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RQPCR	Real-time quantitative PCR
RT-PCR	Reverse transcriptase polymerase chain reaction
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV	Ultraviolet

Communicated by C. Quiros.

First two authors contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00299-010-0943-4) contains supplementary material, which is available to authorized users.

H. Cheng · L. Li · S. Cheng (✉) · Y. Wang · H. Yuan  
College of Life Science and Engineering,  
Huanggang Normal University, Huanggang  
438000, China  
e-mail: s\_y\_cheng@sina.com

H. Cheng · S. Cheng · F. Cao  
College of Forest Resources and Environment,  
Nanjing Forestry University, Nanjing 210037, China

## Introduction

Flavonoids are ubiquitous plant secondary products that have diverse functions in plant physiology and ecology (Mol et al. 1998; Winkel-Shirley 2002), including protection against UV-B radiation and pathogen attack, attraction of pollinating insects, regulation of male fertility, cell cycle

regulation, and auxin transport. Flavonoids also have many pharmaceutical uses in human health, with pharmacological properties that have been especially valuable in the treatment of early-stage Alzheimer's disease, vascular dementia, and many other diseases (Diamond et al. 2000; Oken et al. 1998; Smith and Luo 2004). Flavonoids are synthesized by the well-studied phenylpropanoid pathway (Koes et al. 2005) and most of the enzymes and genes involved in flavonoid biosynthesis have been characterized (Li et al. 2006).

Chalcone isomerase (also called chalcone flavanone isomerase) is one of the key enzymes in the flavonoid biosynthesis pathway and catalyzes conversion of chalcones to (2S)-naringenin. Although this step can occur spontaneously, CHI catalyzes it  $10^7$ -fold more efficiently (Bednar and Hadcock 1988). CHIs can be classified into two types of isozymes: one is an elicitor-inducible form that acts on both 6'-hydroxychalcone and 6'-deoxychalcone and presumably is involved in the legume-specific 5-deoxyflavonoid pathway. The second form acts on only 6'-hydroxychalcone and is generally found in non-legumes (Kimura et al. 2001; Shimada et al. 2003). At present, CHI genes have been cloned from several plants: *Callistephus chinensis*, *Medicago sativa*, *Petunia hybrida*, *Oryza sativa*, *Hordeum vulgare*, and *Saussurea medusa* (Druka et al. 2003; Kuhn et al. 1978; Li et al. 2006; McKhann and Hirsch 1994; Van Tunen et al. 1988). One previous report has suggested that chalcone isomerase may be a regulatory enzyme in the biosynthesis of flavonoids (Mehdy and Lamb 1987). In *Glycyrrhiza uralensis*, the enhanced accumulation of flavonoids was correlated with the elevated levels of CHI transcripts and CHI activity (Zhang et al. 2009). Overexpression of petunia CHI gene in tomato results in an increase of up to 78-fold in fruit peel flavonols, mainly due to an accumulation of rutin (Muir et al. 2001). Therefore, efforts to study the induction and developmental or tissue-specific regulation of flavonoids biosynthetic activity have been focused mainly on the mechanisms controlling CHI gene expression.

*Ginkgo biloba* is often called "a living fossil" of the plant kingdom, and has been well studied owing to the many active ingredients, such as flavonoids, contained in its leaves. At present, about 38 different flavonoids, especially, multiform glycosides of flavonols, have been isolated from *G. biloba* (van Beek 2002; van Beek and Montoro 2009). However, the concentrations of these economically important compounds are often insufficient to justify commercial exploitation of ginkgo leaves. In recent years, a number of investigations have focused on the biological mechanism of flavonoid formation, the in vitro chemical synthesis of flavonoids, and the use of tissue culture for producing flavonoids, among others aspects (Cheng et al. 2004b; Xu 2008; Xu et al. 2008a, b). Few

studies have yet focused on synergistic effects of genetic modification and the production of flavonoids in *Ginkgo biloba* leaves. In the present study, a gene, designated as *GbCHI*, and shown previously to encode a putative CHI enzyme, was isolated from the leaves of *Ginkgo biloba* as a useful tool for studying the regulation of flavonoid biosynthesis. Based on the catalytic function of CHI at the branch point of (2S)-naringenin metabolism, the expression profile, and the correlation between *GbCHI* expression and flavonoid content in *Ginkgo biloba*, we suggest that *GbCHI* might be a specific key gene in the regulation of flavonoid accumulation in ginkgo leaves, as well as being a crucial in vivo factor in responses to UV stress, hormones, or plant growth regulator.

## Materials and methods

### Plant materials and treatments

Twelve-year old of grafted *G. biloba* seedlings were growing in a greenhouse in Jingzhou (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. (2008a). Tissues were immediately frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  prior to total RNA extraction.

One year old cuttings from the same genotypic strain of *G. biloba* were subjected to treatments with UV-B, chlormequat chloride (CCC), 5-aminolevulinic acid (ALA), abscisic acid (ABA), gibberellin (GA), and ethephon (ETH). For UV-B treatment, seedlings were exposed to  $1500\ \mu\text{J}/\text{m}^2$  UV-B irradiation in a closed chamber, and the control cuttings were placed in a dark closed chamber. The CCC (10 mM), ALA (100  $\mu\text{M}$ ), ABA (10 mM), GA (15 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 and genomic DNA of *GbCHI*

Given species conservation of the functional domain of the CHI protein, a pair of degenerate primers, CHIDF and CHIDR, was designed according to the conserved peptide sequences, LGGAGYR and PGSSILFT, to amplify a conservative fragment of the *GbCHI* 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 *CHI* sequences from other species.

Based on the sequence of cloned *GbCHI* fragments, the specific primer pairs (CHI5R and CHI3R) and the nested primer pairs (CHI5N and CHI3N) were designed to amplify the 5' and 3' end of *GbCHI* 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 *GbCHI* was obtained. Two gene-specific primers, CHIGF and CHIGR, designed based on the cDNA sequence, were used to amplify the genomic sequence of *GbCHI*.

#### Amplification of the 5'-promoter region of *GbCHI*

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 CHI5R as primers, and constructed libraries as a template using the following protocol: 94°C for 4 min; 30cycles (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 CHI5N as primers under the following conditions: 94°C for 4 min; 32 cycles (94°C for 30 s, 56°C for 50 s, and 72°C for 3 min); and 72°C for 10 min. The PCR products were cloned into pMD18-T for sequencing (Sangon, China).

#### 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 *GbCHI* and known *CHIs* 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 1000 replicates. SPSS 16 was used for statistical analysis and graphing.

#### Southern blot analysis

Genomic DNA (20 µg/sample) was digested overnight at 37°C with *Sma* I, *Sac* I, and *Hind* III. The digested DNA was fractionated by 0.9% agarose gel electrophoresis and

transferred onto a PVDF membrane (Roche Applied Science, Germany). At total of 50 ng purified *GbCHI* was used as a template in a total volume of 20 µl for probe labeling. Probe labeling (DIG), hybridization, and signal detection were performed following the manufacturer's instruction for the DIG High Primer DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany).

#### Relative quantification by RQPCR

The transcription levels of *GCHI* 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. RQPCR 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 manufacturer's 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 (2008b).

The gene-specific primers (CHIF, CHIR) and reference primers (GAPF, GAPR) for RQPCR were listed in Table 1. The RQPCR conditions were: 10 min at 95°C, and 40 cycles (95°C for 15 s, 60°C for 1 min). Before performing RQPCR, primer efficiency was evaluated using both *GbCHI* and *GbGAPDH* at 100, 150, 200, 250, and 300 nM combinations. A 150 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 (*GbCHI* 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 *GbCHI* transcript is presented as  $2^{-(dCt)}$  according to the  $C_T$  method ( $dCt = C_{t_{sample}} - C_{t_{control}}$ ) described in the RQPCR Application Guide (Applied Biosystems). When comparing the expression of *GbCHI* in different tissues, the relative expression of *GbCHI* was achieved by calibrating its transcription level to that of the reference gene, *GbGAPDH*.

#### Expression of *CHI* recombinant proteins in *Escherichia coli*

To clone *GbCHI* into an expression vector, a pair of primers, CHIZF and CHIZR, 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

**Table 1** Primers for *GbCHI*

Primer	Sequence (5'–3')	Description
CHIDF	GRIAAVTTYRTIAARTTYAC	Degenerate primer, forward
CHIDR	ACCKTTRGGCRAAMCTGTHAA	Degenerate primer, reverse
CHI5R	CTCGCTACTTTTTTCAGAGTATTGGGT	Reverse primer for 5' RACE, outer
CHI5N	GGACAAGTAACAGCATCCATAA	Reverse primer for 5' RACE, nested
CHI3R	GGCAATCATCCCCATCTCGCTCTCAA	Forward primer for 3' RACE, outer
CHI3N	TGGGAATCTGCAGGTATATATGGT	Forward primer for 3' RACE, nested
CHIZF	<i>ACAGAGTC</i> CATGGGCAAAAATGGCGTTTCTC	Gene-specific primer, forward
CHIZR	<i>CAACTCGAGTT</i> ACATCATAGTGGAAAGCCGCTCA	Gene-specific primer, reverse
CHIGF	ATGGGCAAAAATGGCGTTTCTC	Gene-specific primer, forward
CHIGR	ACATCATAGTGGAAAGCCGCTCA	Gene-specific primer, reverse
CHITF	CTGGAATAGAAGTTGAAGGCGTC	Primer for probe forward
CHITR	AATCACTGCCTCTTCCTTCTCTG	Primer for probe reverse
CHIF	GAAAAAGTAAGCGAGGGGTG	Primer for RQPCR, forward
CHIR	TGGGCGAAACTGTAAAGAGA	Primer for RQPCR, reverse
GAPF	TGTCACGGTTTTTCGGTTGTAG	Primer for RQPCR, forward
GAPR	ACCTTTTTGGCACCTCCCTTA	Primer for RQPCR, reverse

Note: Restriction site sequences are indicated in italic letters and underlined

introduced into BL21 (DE3) by the heat shock method. A single colony of *E. coli* BL21 cells harboring the expression plasmid pET28a-GbCHI 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 (OD<sub>600</sub>) 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 7000×g for 15 min. Supernatants and pellets were analyzed by SDS-PAGE and Coomassie Brilliant Blue R250 staining. The recombinant GbCHI 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 GbCHI protein having a His-tag in the N-terminus. After electrophoresis, the proteins were electrotransferred 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 GbCHI activity

For assay of GbCHI activity, 6'-hydroxychalcone or 6'-deoxychalcone (10 µg each in 10 µl of ethanol) was incubated at 30°C for 5 min with 0.49 ml of 50 mM potassium phosphate (pH 7.5) containing 10 µl of crude enzyme (total 0.5 ml). 6'-hydroxychalcone or 6'-deoxychalcone was purchased from Advanced Technology and Industrial Corporation (Hong Kong, China). The reaction mixture was then

extracted with ethyl acetate and the products were analyzed by HPLC on a Shim-pack CLC-O DS column (6.0 × 150 mm; Shimadzu, Kyoto, Japan). The chromatography conditions were a mobile phase of 40% (v/v) methanol and 4% (v/v) acetic acid in water at a flow rate of 0.8 ml min<sup>-1</sup> at 40°C, and the eluate was monitored at 304 nm. To analyze the stereochemistry of CHI reaction products, 6'-hydroxychalcone and a crude extract from *E. coli* expressing CHI isozymes were incubated at 30°C for 3 h. An extract of *E. coli* transformed by the vector without insert was used for the control under the same reaction conditions. The reaction product was developed on a silica-gel thin-layer chromatography plate (Merck, Darmstadt, Germany) with the solvent toluene:ethyl acetate:methanol:light petroleum (6:3:1:3 [v/v]), and the naringenin spot ( $R_F = 0.44$ ) was recovered. The sample was then analyzed by HPLC on a Chiracel OD-RH column (4.6 × 150 mm; Daicel, Tokyo) with 35% (v/v) aqueous acetonitrile at a flow rate of 0.6 ml min<sup>-1</sup> at 30°C, and the eluate was monitored at 290 nm.

CHI activity in *G. biloba* leaves was measured by the methods of Jez et al. (2000) and Fouché and Dubery (1994), with minor modifications. The standard CHI assay reaction mixture contained the following components in a final volume of 1 ml: 50 mM Tris-HCl buffer (pH 7.6) containing 1% ethanol, 100 µM naringenin chalcone in 2-ethoxy-ethanol (18.5 µM), and an appropriate amount of protein extract. The rate of spontaneous cyclization of the substrate was determined beforehand and was subtracted from the rate in the presence of enzyme. Naringenin chalcone was synthesized from naringenin according to the procedure of Moustafa and Wong (1967) and authentic naringenin was purchased from Herbfine (Nanchang, China).

## Extraction and determination of flavonoid content

Flavonoids were extracted identified and quantified as previously described by Xu et al. (2008a) 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  $\mu\text{m}$  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 ml  $\times$  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  $\text{min}^{-1}$  and injection volume was 20  $\mu\text{l}$ . The UV detector was set at  $\lambda = 370$  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 (van Beek 2002). 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.

## Results

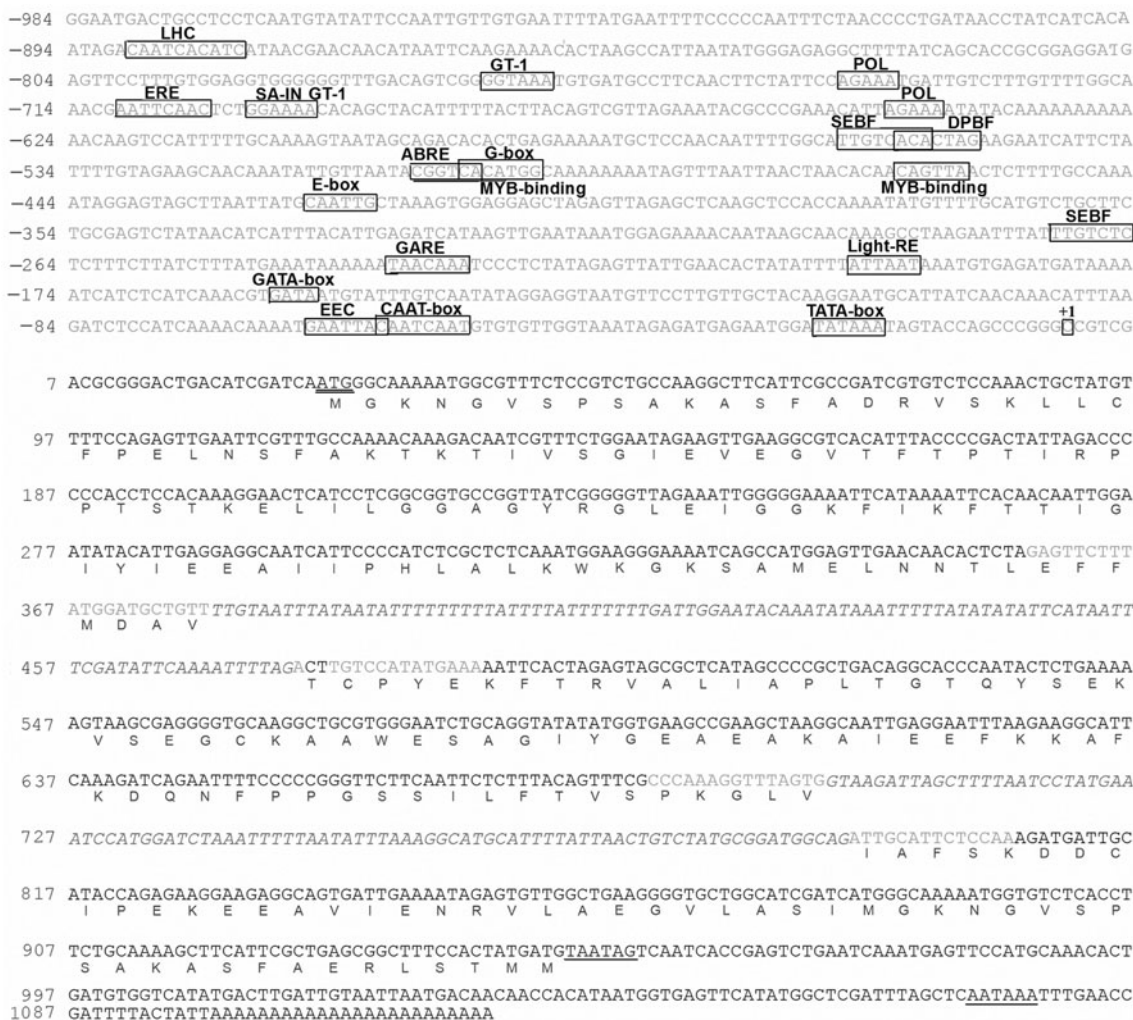
### Characterization of the full-length cDNA, genomic DNA, and promoter region of the *GbCHI* gene

A full-length cDNA of the *CHI* gene was obtained from *G. biloba* leaf tissue by degenerate PCR and RACE methods. The cDNA sequence was 926 bp with a poly A tail, and contained a 732 bp open reading frame (ORF) encoding a 244 amino acid protein. A 20 bp 5' untranslated region was upstream of the start codon, and the coding region was followed by a 160 bp 3' untranslated region downstream from the stop codon (Fig. 1). A phylogenetic tree generated by the neighbor-joining method based on the putative amino acid sequences of CHIs showed that all members of the CHI family could be sorted into three monophyletic group. The *G. biloba* CHI was included in a polyphyletic group with other CHIs from various non-leguminous plants (Fig. 2).

The genomic *GbCHI* gene was isolated from a pair of specific primers derived from the start and stop codon regions of the cDNA. The full-length genomic DNA of *GbCHI* gene was 1290 bp long and had 100% identity with the coding region of the full-length cDNA sequence. Exon 1 (351 bp), exon 2 (225 bp), and exon 3 (156 bp) were

separated by intron 1 (97 bp) and intron 2 (59 bp). The putative splicing site also obeyed the GU/AG rule (Fig. 1). By genome walking, we isolated a 1011 bp fragment upstream of the ATG codon which corresponded to the putative promoter region. The 5' upstream region of *GbCHI* possessed the typically high A + T content (65.6%), commonly found in other plant promoters. Computational analysis revealed six potential TATA box sequences within the upstream region of *GbCHI*, at positions –140, –206, –224, –346, –639, and –963. Several conserved eukaryotic promoter elements, including a CAAT box, were also observed at positions –58, –143, –299, –424, –570, –956, and –968. A number of phenylpropanoid regulation sequences were also noted. Two putative E-boxes were identified: the first one located at positions –950 to –956, and one near the transcription initiation site at position –418 to –424 (Fig. 1). Two MYB-binding sites (EEC) with a consensus sequence GANTTNC were identified on the *GbCHI* 5'-upstream sequence (Solano et al. 1995; Yoshioka et al. 2004). Sequence analysis of the 5'-upstream region of CHI with PLACE revealed two pollen-expression-related sequence motifs (POLRE) (Bate and Twell 1998) at positions –739 and –859. Two putative SEBF-like elements with the central YTGTCWC core (Boyle and Brisson 2001; Yamagata et al. 2002) were present in the promoter region. Four GATA box elements with the core sequence GATA were found at positions –23, –157, –181, and –911 these showed a tissue specific expression-related sequence. Several LHC-related *cis*-elements (CAANNNNATC) (Piechulla et al. 1998) known to play important roles in the tomato circadian rhythms, were also identified in the 5' upstream region of *GbCHI* (Fig. 1).

A core motif (GARE), described as a dehydration and GA-response expression element (Sutoh and Yamauchi 2003), was identified at position –237 to –230. The *GbCHI* contained a conserved 5'UTR sequence AC-GTGCGC similar to the well known ABRE or G-Box elements found in ABA responsive genes such as the wheat *Em* (Guiltinan et al. 1990), rice *rab16* (Mundy et al. 1990), and barley *Hva1* genes (Straub et al. 1994). A DPBF binding core element (ACACNNG) was also found at position –554, which functions as an ABA-responsive *cis*-element in *Arabidopsis* (Lopez-Molina and Chua 2000). Four GT-1 motifs (GRWAAW) were observed at positions –157, –181, –699, and –769 and one SA-IN transcription factor binding site (GGAAAA) was identified at position –699. Both of these are involved in induction of genes by salicylic acid (SA) (Park et al. 2004; Zhou 1999), an important component of the signal transduction cascades that activate plants defense responses against pathogen attack. Four GATA boxes with tissue specific expression-related sequences and an ethylene-responsive element



**Fig. 1** The full-length *GbCHI* sequence, promoter region sequence, intron sequence and deduced amino acid sequence of *GbCHI* gene. The exons are indicated in capital letter and the introns are indicated

in italic letter. The start condon (ATG), the stop (TAG), and putative polyadenylation signals are *underlined*. TBFs and *cis*-elements of the *GbCHI* promoter region are shown in *black box*

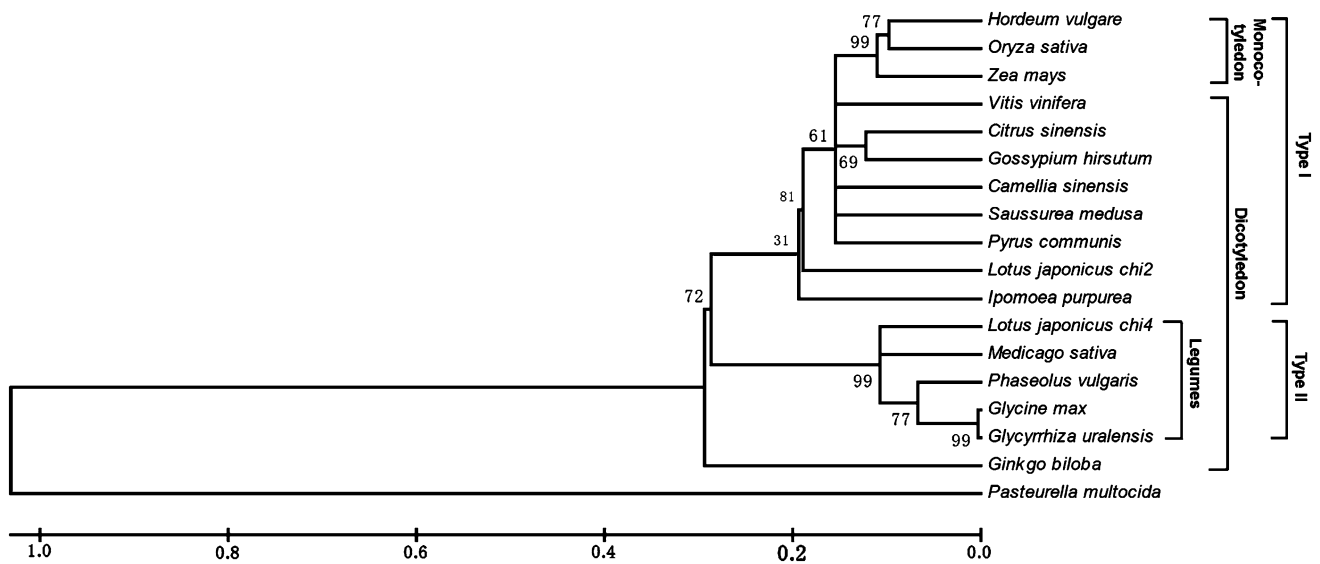
(ERE) motif (Itzhaki et al. 1994; Rawat et al. 2005) were found at positions –23, –157, –181, –911, and –710.

To examine if the *GbCHI* gene belongs to a multigene family, aliquots of 20 µg genomic DNA were digested with *Sma* I, *Hind* III, and *Sac* I, which did not cut within the coding region. These fragments were then hybridized with the coding sequence of *GbCHI* under high stringency conditions. In total, 5–7 specific hybridization bands ranging from 0.5 to 10 kb were recovered in each of the restriction enzyme-digested lanes (Supplementary Fig. 1S), this indicated that *GbCHI* belonged to a multigene family.

The expression of the *GbCHI* gene and accumulation of flavonoids in different tissues

The transcription level of *GbCHI* and accumulation of flavonoids varies according to tissues, growth conditions,

and tissue developmental stage. The expression pattern of the *GbCHI* gene was examined in different tissues of grafted plants by extracting total RNA from young leaves (buds), mature leaves (130 days after budding), ovules, fruit albumen, gynoecia, stamens, stems, and roots. The results of RQPCR analysis are shown in Fig. 3a. The transcription of *GbCHI* was clearly tissue specific and development-dependent. The highest level of *GbCHI* mRNA was observed in mature leaves, with moderate levels in stamens, gynoecia, and stems, but *GbCHI* was only weakly expressed in ovules and young leaves, and expression was lowest in albumen. No *GbCHI* transcripts were detected in the roots. These results agreed with the pattern of flavonoids accumulation in *G. biloba* (Fig. 3b). As seen in Fig. 3b, flavonoids were detected abundantly in mature leaves, and to a lesser extent in the stamens and gynoecia, but was not detected in roots. Thus, expression of



**Fig. 2** A phylogenetic tree based on deduced amino acid sequences of various CHIs. Amino acid sequences were analyzed using the Mega 4.0 program. The numbers at each node represented the bootstrap value, with 1000 replicates. Monocotyledons and most other species are classified into the type I CHI group. Known typeII CHIs form a subgroup, and legumes CHIs belong to this group. GbCHI does not belong to any group. The CHI-like protein of *Pasteurella multocida* is introduced as an external frame of reference. The relative evolutionary time is displayed under the tree. GenBank accession

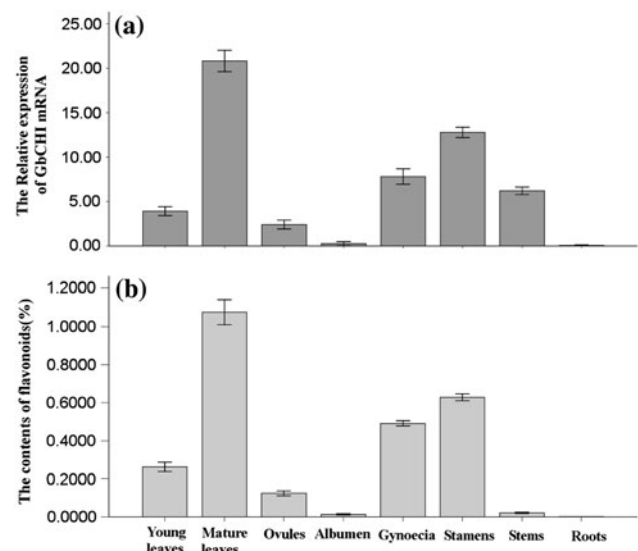
number are as follows: *Hordeum vulgare*, AF474923; *Oryza sativa*, AF474922; *Zea mays*, Q08704; *Vitis vinifera*, P51117; *Citrus sinensis*, BAA36552; *Gossypium hirsutum*, ABM64798; *Camellia sinensis*, AAZ17563; *Saussurea medusa*, AF509335; *Pyrus communis*, ABQ08639; *Lotus japonicus chi2*, CAD69022; *Ipomoea purpurea*, ABW69677; *Lotus japonicus chi4*, BAC53984; *Medicago sativa*, P28012; *Phaseolus vulgaris*, CAA78763; *Glycine max*, ABA86740; *Glycyrrhiza uralensis*, ABM66533; *Pasteurella multocida*, NP245716

the *GbCHI* gene appears to be one of the critical factors determining the flavanone accumulation pattern in *G. biloba*.

Effect of UV-B, GA, CCC, ABA, ETH, and ALA on *GbCHI* expression

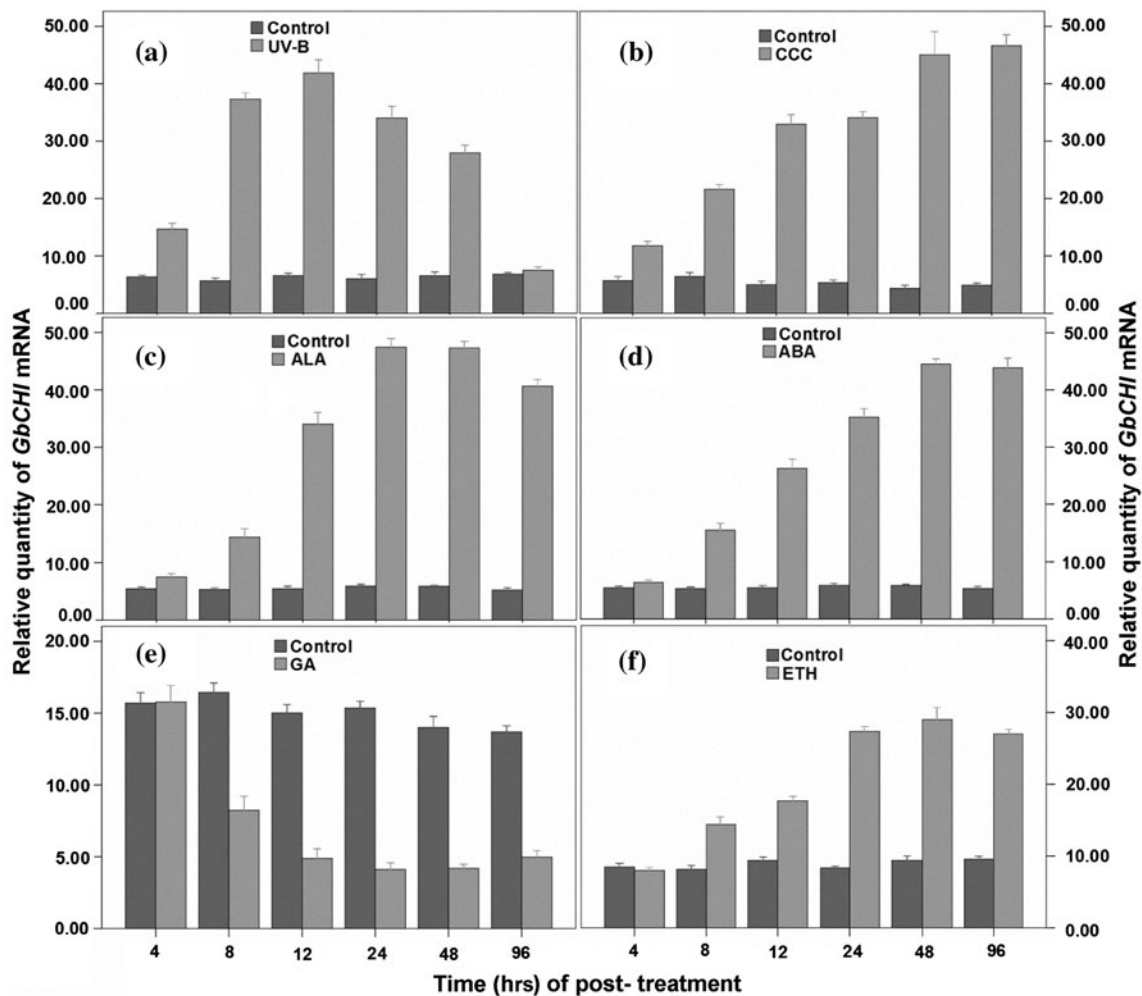
RQPCR of ginkgo seedling following diverse chemical and stress treatments showed that *GbCHI* was inducibly expressed. Following a UV-B treatment, the amount of *GbCHI* mRNA was quickly increased during the 0–12 h of treatment and then declined thereafter, returning to a similar level to that of control at 96 h (Fig. 4a). Exogenous application of CCC caused a rapid and durable induction of the *GbCHI* gene at 12 h, and that level was maintained until 24 h, when the transcript level began to increase slowly, reaching a maximal level at 96 h that was 9.6-fold higher than the control (Fig. 4b).

As shown in Fig. 4c, d, and f, the expression of *GbCHI* was apparently up-regulated by ALA, ABA, and ETH. Transcription levels of *GbCHI* increased along with the treatment time and reached the highest level at the end of 24 or 48 h, at 9.2-fold, 8.2-fold, and 2.8-fold, respectively, compared with the controls. The *GbCHI* transcription was down-regulated by GA and the mRNA transcript showed a quick decrease within the first 24 h, followed by a slow rise



**Fig. 3** Comparison among different tissues in *G. biloba* regarding relative amount of *GbCHI* mRNA and accumulation of flavonoids. **a** Expression pattern of *GbCHI* gene in different tissues with GAPDH gene as control. **b** Accumulation pattern of flavonoids in different tissues. Data are mean values of triplicate tests  $\pm$  SD

(Fig. 4e). Exogenous GA, therefore, appeared to decrease the transcription level of *GbCHI*. Overall, all five tested abiotic stresses appeared to cause significant accumulation of *GbCHI* mRNA.



**Fig. 4** Relative quantities of *GbCHI* mRNA at various time points post-treatment with UV-B (a), CCC (b), ALA (c), ABA (d), GA (e), and ETH (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

#### Characterization of the deduced GbCHI protein

The deduced GbCHI protein sequence of GbCHI consisted of 244 amino acids residues, with an isoelectric point of 7.76. The amino acid sequence of the GbCHI shared 62.7% identity with that of *Camellia sinensis*, 64.4% with *Gossypium hirsutum*, 65.0% with *Pyrus communis*, 52.5% with *Phaseolus vulgaris*, 50.3% with *Medicago sativa*, 52.5% with *Glycyrrhiza uralensis*, and 61.0% with *Lotus japonicus*. Similar to other non-legume plants, the four conserved amino acid residues essential for GbCHI active sites were Thr80, Tyr138, Gly145, and Ser221. This was unlike the case in *Saussurea medusa*, where the corresponding residues are Thr50, Tyr108, Met115, and Ser192, or in *Medicago sativa* (legume), where the corresponding residues are Thr48, Tyr106, Asn113, and Thr190 (Gensheimer and Mushegian 2004; Jez et al. 2000; Li et al. 2006). A GenBank CDD search and function analysis revealed

that GbCHI belongs to the chalcone superfamily (Supplementary Fig. 2S).

#### Expression and purification of GbCHI

In order to express GbCHI in *E.coli*, we cloned the coding sequence of GbCHI into pET-28a, an expression vector with the T7 promoter and a His-tag, yielding pET28a-GbCHI. The expression construct was checked for in-frame fusion by DNA sequencing (Sangon, China). Upon induction by IPTG, GbCHI was expressed as a major soluble protein product (Supplementary Fig. 3S, lanes 2–5). Supplementary Fig. 3S Lane 6 shows the insoluble proteins after IPTG induction for 2.0 h. The molecular weight of the expressed recombinant protein was estimated as a 27 kDa band with a His-tag: this size was in agreement with that predicted by bioinformatics methods. The recombinant GbCHI protein was purified using Nickel-NTA agarose



affinity chromatography and the purified protein showed the expected size (Supplementary Fig. 3S, lane 7). Western blotting of purified recombinant GbCHI protein confirmed its specific immune reactivity to anti-His antibodies (Supplementary Fig. 3S, lane western blot).

#### The activity of GbCHI in vitro

GbCHI activities were assayed using extracts of the recombinant expressing *CHI* cDNA. The reaction product of recombinant GbCHI was detected by HPLC monitored at 304 nm and its identity confirmed as naringenin and naringenin chalcone by comparison to known standards (Supplementary Fig. 4S, a). The elution profiles of ethyl-acetate-extracted reaction products showed that CHI yielded 5-hydroxyflavanone (naringenin) from the incubation with 6'-hydroxychalcone (naringenin chalcone) as substrates (Supplementary Fig. 4S, b). On the other hand, the control added with crude enzyme extract from *E.coli* + pET28a vector had more residual substrate and less spontaneous product (Supplementary Fig. 4S, c). To confirm the enzymatic cyclization by GbCHI proteins, the stereochemistry of naringenin at the C-2 position was estimated by HPLC on a chiral column. As shown in Supplementary Fig. 5S a and b, (2S)-naringenin was the predominant product compared with (2R)-naringenin in the presence of CHI protein, whereas only a racemic mixture of naringenin was found in the control reaction (Supplementary Fig. 5S, c). Compared with a standard sample (Supplementary Fig. 6S, a), the

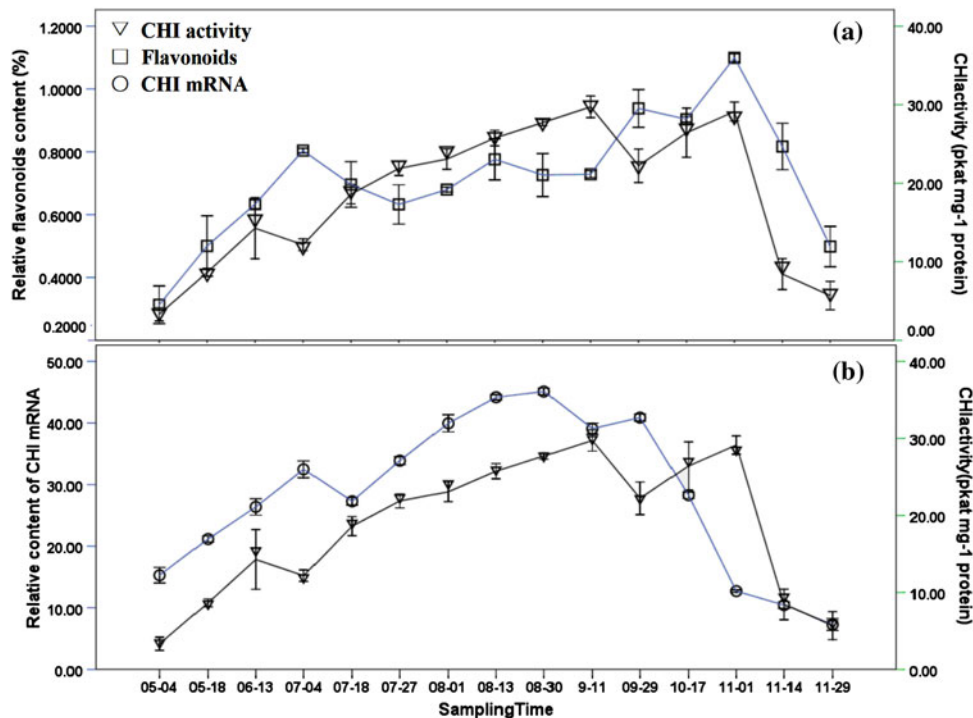
reaction with 6'-deoxychalcone (isoliquiritigenin) as substrate showed no product resulting from GbCHI activity (Supplementary Fig. 6S, b) and control (Supplementary Fig. 6S, c). Thus, our data suggest that GbCHI is a type I CHI, which only accepts 6'-hydroxychalcone as a substrate.

#### The relationship between *GbCHI* expression, GbCHI activity, and flavonoid accumulation during ginkgo leaf growth

To determine the temporal expression pattern of the *CHI* gene in ginkgo leaves, we examined *CHI* gene transcription, flavonoid content, and CHI activities at different leaf developmental stages. RQPCR of leaves at 15 developmental stages (Fig. 5) showed curves for changes in *CHI* expression that paralleled the curves for relative GbCHI activities at different developmental stages. The *CHI* gene mRNA was detected at all stages, with a gradual increase occurring from stage 1 to stage 4 and a peak at stage 4. After a brief decline in July 18, the transcript level continued to rise to an annual peak by August 30; the highest transcript level was 45.1107. A third peak (40.8804) appeared on September 29, followed by a subsequent slow decline.

The GbCHI activity showed a similar pattern during annual leaf growth, again indicating three peaks. CHI activity began to increase upon emergence of the leaf bud. The first peak of activity occurred around June 13

**Fig. 5** Changes in flavonoid content, CHI expression, and activity during growth of ginkgo leaves, the curve for content of CHI expression, calculated from regression equation based on CHI activity which agrees well with the experimental data of flavonoid accumulation



**Table 2** Relationship between flavonoid content and CHI activity

Model summary						
Model	Unstandardized coefficients <sup>a</sup>			Linear regression		
	Regression coefficient	Standard error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	0.459	0.032	0.000	0.614	0.377	0.373
CHI activity	0.014	0.002	0.000			

<sup>a</sup> Dependent variable: Flavonoid

**Table 3** Relationship between *CHI* expression level and CHI activity

Model summary						
Model	Unstandardized coefficients <sup>a</sup>			Linear regression		
	Regression coefficient	Standard Error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	5.352	1.463	0.000	0.649	0.421	0.417
mRNA	0.467	0.047	0.000			

<sup>a</sup> Dependent variable: CHI activity

(14.6167 U/min mg protein) and CHI activity declined thereafter to a minimum by July 4 (12.0816 U/min mg protein). A second peak and the highest yearly activity occurred on September 11 (29.7331 U/min mg protein). A decline followed with minimal activity on September 29. A third peak (28.7272 U/min mg protein) was followed by a rapid decline. The peaks of annual activity showed slight lags behind the peak transcript levels.

Flavonoid content correlated with the seasonal patterns of GbCHI activity. An obvious peak was noted on July 4 (0.8039), on August 13 (0.7763), on September 29 (0.9383), and on November 1 (1.0994), with the annual peak appearing on November 1. The first peak appearing on July 4 also correlated with a peak in *GbCHI* transcript level. The annual peaks of flavonoid content also lagged behind the peaks of GbCHI activity. From a commercial standpoint, the best harvest stage for flavonoids appeared to be from the end of September to early November. Linear regression indicated that flavonoid content ( $Y_1$ ) and CHI activity ( $X_1$ ) were linearly correlated (Table 2), with an  $F$  value of 80.622, a correlation coefficient of 0.373, and a linear equation represented by  $Y_1 = (0.014 \pm 0.002)X_1 + (0.459 \pm 0.032)$ . Linear regression of CHI transcription levels ( $X_2$ ) and CHI activity ( $Y_2$ ) showed a high correlation coefficient  $R^2 = 0.421$ ; the  $F$  value was 96.755, and the linear equation was represented by  $Y_2 = (0.467 \pm 0.047)X_2 + (5.352 \pm 1.463)$  (Table 3). From this, we deduced that the CHI activity and accumulation of flavonoids in *Ginkgo biloba* leaves would be affected by *CHI* gene transcription levels.

## Discussion

### Characterization and function analysis of GbCHI

In this study, a *G. biloba* gene encoding a type I chalcone isomerase was isolated and characterized. Many genes encoding CHI from angiosperm plant species have been isolated and characterized at the genetic, chemical, and enzymological levels. However, cloning of a *CHI* gene from a gymnosperm plant such as *G. biloba* has not yet been reported. This work verifies for the first time the presence of a *CHI* in a gymnosperm. According to the analysis and comparison, GbCHI could be classified as a member of the type I CHI family.

Classification of GbCHI as to type I was not possibly based only on the phylogenetic tree. Studies of the three-dimensional structure of the *Medicago sativa* CHI (Jez et al. 2000) (a type I CHI) indicated the presence of four conserved amino acid residues (Supplementary Fig. 2S) that were essential for its activity. Amino acid analysis and three-dimensional structural configurations of GbCHI indicated that this enzyme was similar to a type I CHI. Previous observations (Dixon et al. 1988; Shimada et al. 2003) have shown that CHI converts both 6'-deoxychalcone and 6'-hydroxychalcone to isoflavonoids and flavonoids (referred to as type II CHI), while GbCHI is capable of converting only 6'-hydroxychalcone to 5-hydroxyflavanone (as a type I CHI). In addition, GbCHI controls the preferred formation of the biologically active (2S)-flavones. Thus, GbCHI was clearly similar to non-leguminous

types, based on amino acid sequence and type I substrate specificity. The non-enzymatic conversion of chalcones yields racemic (2R/S)-flavanones. Since only (2S)-flavanones are intermediates of the subsequent flavonoid pathway, CHI specificity guarantees the efficient formation of biologically active (2S)-flavanones. Only trace amounts of flavonoids were detected in the *Arabidopsis transparent testa 5* mutants lacking CHI activity (Shirley et al. 1995). These results indicate that CHI activity is essential for the in vivo production of flavonoids.

#### Effects of UV-B and plant growth regulator on *GbCHI* expression

The ultraviolet absorbing characteristics of flavonoids have been considered as evidence for a role in UV protection. The induction of *GbCHI* by UV-B should not be surprising, since a light-responsive element and homologous MYB/MYC recognition sites, present in most of the phenylpropanoid biosynthesis gene promoters studied (Solano et al. 1995; Yoshioka et al. 2004; Xu et al. 2008b), were identified in the 5'-upstream region of *GbCHI*. UV exposure could induce the expression of flavonoid synthesis-related genes allowing flavonoids to play a role in resistance to UV damage. The first direct evidence in support of a role for flavonoids in UV protection came from experiments with *Arabidopsis* mutants, which showed that lesions in chalcone synthase or chalcone isomerase resulted in UV-hypersensitive phenotypes (Li et al. 1993). A role for flavonoids in UV protection is further supported by Bieza and Lois' (2001) isolation of an *Arabidopsis* mutant that is tolerant of extremely high UV-B levels. This mutant shows constitutively high levels of a number of phenolics, including flavonoids. Interestingly, the *CHI* mutants were the most sensitive to UV light and showed a corresponding decrease in flavonoids and sinapate esters (Li et al. 1993). Indeed, flavonoids are often present in the epidermal cell layers of leaves and in tissues that are susceptible to UV light, such as pollen and the apical meristem. This may be the reason that the outer leaves of a ginkgo tree have a higher flavonoid content (Cheng 2001). In response to UV irradiation, the leaves of *Ginkgo biloba* showed a rapid increase in the transcription of *GbCHI*, this result indicated that the expression level of *GbCHI* could improve the activity of GbCHI, further increasing the accumulation of flavonoids in the leaves.

Changes in flavonoid levels in response to environmental stimuli and at specific developmental stages were mediated by regulated *CHI* expression. The synthesis of flavonoids compounds in plants is controlled by various factors, with enzymes and internal phytohormones as the major regulators (Cheng et al. 2000; Cheng 2001). However, there have been few studies of exogenous hormone responses with respect to the regulation of *CHI* expression.

Plant hormones play roles in flavonoid formation. For example, ethylene (ETH) has been shown to regulate the expression of several genes in flavonoid biosynthesis. Kush et al. (1990) reported that ETH treatment induced transcript accumulation of CHI, chalcone synthase (CHS), and phenylalanine ammonia lyase (PAL) in the rubber tree (*Hevea brasiliensis*). Ardi et al. (1998) observed that exogenous ETH led to the activation of transcript accumulation of CHS and flavanone-3-hydroxylase (F3H) in *Persea americana*. El-Kereamy et al. (2003) discovered that exogenous ETH could stimulate the transcription of flavonoid biosynthesis gene in grape (*Vitis vinifera*), including CHS, F3H, and anthocyanidin synthase (ANS). The in vitro spraying of ETP (ethephon) promoted ABA and ETH synthesis to various degrees while it hindered GA synthesis, but increased flavonoid content when supplied at various levels (Cheng et al. 2004a). In the present work, transcription of *GbCHI* was induced by ETH, consistent with the observation of ethylene-responsive elements in promoter region of *GbCHI*. These features suggested that *GbCHI* is likely to respond to ethylene-responsive signal transduction.

One common element between the ABA-mediated pathways and the flavonoid pathway is the role of the MYB and MYC classes of transcription activators on target gene expression (Abe et al. 1997). Some ABA-response elements are also present in the 5'-upstream region of the *CHI* genes. Cheng et al. (2004a) reported that treatment with ABA had significantly increased flavonoid content. In our present study, the exogenous application of ABA triggered a significant induction of *GbCHI*, together with the identification of homologous MYB/MYC recognition sites. These results suggested that the increased flavonoid content in ginkgo leaves might be related to the triggering of *GbCHI* transcription by ABA. These correlative data indicate that ABA may play an important role in the accumulation of flavonoids.

5-aminolevulinic acid (ALA) is a key precursor in the biosynthesis of porphyrins such as chlorophyll and heme, and its formation may be the rate limiting step (Von Wettstein et al. 1995). Leaf chlorophyll and flavonoid content are closely related (Awad et al. 2001; Cheng 2001). Treatment with ALA can promote the biosynthesis of flavonoids and anthocyanins (Saure 1990; Xu 2008). The present study showed that the transcription of *GbCHI* could be induced by ALA. Therefore, the expression of *CHI* played an important role in the promotion of flavonoid content.

Gibberellin (GA) speeds growth and reduces CHS activity, lowering the accumulation of flavonoids (Carrier et al. 1990). GA also reduced the content of flavonoids in the root of *Scutellaria baicalensis* (Cai et al. 2008). Reduced GA levels promote flavonoid accumulation (Cheng et al. 2004b). In this experiment, exogenous

gibberellins restrain the gene expression level of *GbCHI*. Meanwhile, a primary study reveals that the exogenous GA process can adjust and control the reduction of accumulated content of ginkgo flavones, which reveals that the reduction of ginkgo flavones by gibberellic has something to do with the adjustment and control of the reduction of *GbCHI* gene transcription level. Therefore, GA treatment was not suitable for the cultivation of ginkgo and other medicinal plants whose leaves are to be used as the medicinal materials.

Cycocel (CCC) is known to inhibit gibberellins responses (Saure 1990) and thus might stimulate formation of flavonoids (Cheng et al. 2004a). Cheng et al. (2004a) reported that CCC greatly increased flavonoid contents of *G. biloba* leaves. In contrast, in *Scutellaria baicalensis*, CCC reduced the content of flavonoids in the root while elevating the content of lignin in the aerial parts (Cai et al. 2008). In our study, CCC greatly induced the transcription of *GbCHI* consistent with accumulation of flavonoids (Cheng et al. 2004a).

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

The pattern of *GbCHI* transcript expression matched the accumulation pattern of flavonoids (Figs. 3, 5), indicating that the *GbCHI* is one of the key enzymes in the flavonoid biosynthetic pathway in *G. biloba*, and is responsible for the formation of flavonoids in different tissues. Two POLRE *cis*-elements were found on the 5' upstream region, which act as *cis* factors to direct pollen-specific expression in dicots (Bate and Twell 1998). Some SEBF-like elements were also found in the upstream region, some of these consensus sequences are present in promoters of fruit-specific expression genes (Boyle and Brisson 2001; Yamagata et al. 2002). Therefore, it can be speculated that high levels of expression of *GbCHI* and enzyme activity serve to allow accumulation of flavonoids in ovules and stamens. Thus, control of the expression level of *CHI* can be viewed as a potential approach to increase flavonoid production in ginkgo.

Little is known regarding the expression of *CHI* genes at different developmental stages in ginkgo leaves. Previous work on temporal expression profiles reported that the transcription of flavonoid synthesis genes was associated with the accumulation of flavonoids (Xu et al. 2007, 2008a). In the present study, we showed that the qualitative differences in competence for *CHI* activity at different developmental stages are primarily the result of the differences in the transcription of *CHI* in developing leaves. Significant correlation was found between expression level of *CHI* genes and *CHI* activity at all of the annual cycle

stages of ginkgo leaves. Transcriptional controls play an important role in regulating the overall activity of flavonoid biosynthesis (Jaakola et al. 2002). The same correlation was seen between *CHI* activity and flavonoid content (Tables 2, 3). In early July, plants have more leaves than they do in November, thus July would be the best time to harvest ginkgo leaves for highest flavonoid recoveries. Our study showed that the mRNA levels of the *CHI* gene and the accumulation of flavonoids both were correlated with *CHI* activity. The *CHI* activity paralleled the *GbCHI* transcription level but not the flavonoid accumulation, which might be explained by (1) The enzyme activities in the various branch pathways are highly regulated, the accumulation of flavonoids is a complex process or (2) Flavonoid concentrations in ginkgo leaves are not only regulated by the involvement of genes, but also by other factors, such as photosynthesis, tree age, season, quantity of fertilizer application, etc. (Cheng et al. 2009).

*Ginkgo biloba* L. is a medicinal plant in China, and its flavonoids can be found in standardized ginkgo leaf extract preparations (24% flavonoids). Thus, the flavonoids are considered to be important for the beneficial pharmacological effects of this plant (Smith and Luo 2004; van Beek 2002). Ecological factors influence flavonoid accumulation primarily during the young stages of ginkgo leaf development. Among all ecological factors, light, seasonal variations, and hormones are the most important (Cheng et al. 2009). This study used *G. biloba* leaves as experimental material to explore the effects of regulating factors on the synthesis of flavonoids compounds and on the modulation of *GbCHI* expression and activity. The overall aim was to provide a theoretical and practical basis for increasing the flavonoid content of *G. biloba* leaves used in commercial production. The best time for application of flavonoid-promoting regulators and the optimum application concentrations still need further study. However, with the development of molecular biology techniques that now allow the successful cloning of genes encoding some key enzymes, the prospect for improved synthesis of flavonoids is optimistic.

**Acknowledgments** This work was supported by the Natural Science Foundation of China (30971974), the Program for New Century Excellent Talents in University (NCET-04-0746), and University–industry Cooperation Fund of Hubei Educational Office (CXY2009B009).

## References

- Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* 9:1859–1868

- Ardi R, Kobiler I, Jacoby B, Keen NT, Prusky D (1998) Involvement of epicatechin biosynthesis in the activation of the mechanism of resistance of avocado fruits to *Colletotrichum gloeosporioides*. *Physiol Mol Plant Pathol* 53:269–285
- Awad MA, Wagenmakers PS, de Jager A (2001) Effects of light on flavonoid and chlorogenic acid levels in the skin of 'Jonagold' apples. *Sci Hortic* 88:289–298
- Bate N, Twell D (1998) Functional architecture of a late pollen promoter: pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant Mol Biol* 37:859–869
- Bednar RA, Hadcock JR (1988) Purification and characterization of chalcone isomerase from soybeans. *J Biol Chem* 263:9582–9588
- Bieza K, Lois R (2001) An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. *Plant Physiol* 126:1105–1115
- Boyle B, Brisson N (2001) Repression of the defense gene PR-10a by the single-stranded DNA binding protein SEBF. *Plant Cell* 13:2525–2537
- Cai G, Guo Y, Yao H, Chen S, Zhou T (2008) Impacts of cycocel and gibberellin on the biomass and flavonoid production in *Scutellaria baicalensis* Georgi. *Chin Agric Sci Bull* 24:213–217
- Carrier D, Cosentino G, Neufeld R, Rho D, Weber M, Archambault J (1990) Nutritional and hormonal requirements of *Ginkgo biloba* embryo-derived callus and suspension cell culture. *Plant Cell Rep* 8:635–638
- Cheng SY (2001) Studies on the main factors influencing flavonoid formation and its regulation in *Ginkgo biloba* leaf. Dissertation, Shandong Agricultural University
- Cheng SY, Gu MR, Shu HR (2000) Advances in research on flavonoids in *Ginkgo biloba* leaf. *Scientia Silvae Sinicae* 36:110–115
- Cheng SY, Wang Y, Fei YJ, Zhu GC (2004a) Studies on the effects of different treatments on flavonoids contents in *Ginkgo biloba* leaves and their regulating mechanism. *J Fruit Sci* 21:116–119
- Cheng SY, Wang Y, Li JK, Fei YJ, Zhu GC (2004b) Study on the relationship between the endogenous hormones and flavonoids. *Scientia Silvae Sinicae* 40:45–49
- Cheng SY, Xu F, Wang Y (2009) Advances in the study of flavonoids in *Ginkgo biloba* leaves. *J Med Plants Res* 3:1248–1252
- Diamond BJ, Shiflett SC, Feiwei N, Matheis RJ, Noskin O, Richards JA, Schoenberger NE (2000) *Ginkgo biloba* extract: mechanisms and clinical indications. *Arch Phys Med Rehabil* 81:668–678
- Dixon RA, Blyden ER, Robbins MP, Van Tunen AJ, Mol JN (1988) Comparative biochemistry of chalcone isomerases. *Phytochemistry* 27:2801–2808
- Druka A, Kudrna D, Rostoks N, Brueggeman R, von Wettstein D, Kleinhofs A (2003) Chalcone isomerase gene from rice (*Oryza sativa*) and barley (*Hordeum vulgare*): physical, genetic and mutation mapping. *Gene* 302:171–178
- El-Kereamy A, Chervin C, Roustan JP, Cheynier V, Souquet JM, Moutounet M, Raynal J, Ford C, Latché A, Pech JC (2003) Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. *Physiol Plant* 119:175–182
- Fouché S, Dubery I (1994) Chalcone isomerase from *Citrus sinensis*: purification and characterization. *Phytochemistry* 37:127–132
- Gensheimer M, Mushegian A (2004) Chalcone isomerase family and fold: no longer unique to plants. *Protein Sci* 13:540–544
- Guiltinan M, Marcotte W Jr, Quatrano R (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* 250:267–271
- Itzhaki H, Maxson JM, Woodson WR (1994) An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-S-transferase (*GST1*) gene. *PNAS* 91:8925–8929
- Jaakola L, Maatta K, Pirttila A, Torronen R, Karenlampi S, Hohtola A (2002) Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant Physiol* 130:729–739
- Jansson S, Meyer-Gauen G, Cerff R, Martin W (1994) Nucleotide distribution in gymnosperm nuclear sequences suggests a model for GC-content change in land-plant nuclear genomes. *J Mol Evol* 39:34–46
- Jez JM, Bowman ME, Dixon RA, Noel JP (2000) Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase. *Nat Struct Biol* 7:786–791
- Kimura Y, Aoki T, Si A (2001) Chalcone isomerase isozymes with different substrate specificities towards 6-hydroxy- and 6-deoxychalcones in cultured cells of *Glycyrrhiza echinata*, a leguminous plant producing 5-deoxyflavonoids. *Plant Cell Physiol* 42:1169–1173
- Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci* 10:236–242
- Kuhn B, Forkmann G, Seyffert W (1978) Genetic control of chalcone-flavanone isomerase activity in *Callistephus chinensis*. *Planta* 138:199–203
- Kush A, Goyvaerts E, Chye ML, Chua NH (1990) Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). *PNAS* 87:1787–1790
- Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Vande Peer Y, Rouzé P, Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucl Acids Res* 30:325–327
- Li J, Ou-Lee TM, Raba R, Amundson RG, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. *Plant Cell* 5:171–179
- Li F, Jin Z, Qu W, Zhao D, Ma F (2006) Cloning of a cDNA encoding the *Saussurea medusa* chalcone isomerase and its expression in transgenic tobacco. *Plant Physiol Biochem* 44:455–461
- Lopez-Molina L, Chua NH (2000) A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*. *Plant Cell Physiol* 41:541–547
- McKhann HI, Hirsch AM (1994) Isolation of chalcone synthase and chalcone isomerase cDNAs from alfalfa (*Medicago sativa* L.): highest transcript levels occur in young roots and root tips. *Plant Mol Biol* 24:767–777
- Mehdy M, Lamb C (1987) Chalcone isomerase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *EMBO J* 6:1527–1533
- Mol J, Grotewold E, Koes R (1998) How genes paint flowers and seeds. *Trends Plant Sci* 3:212–217
- Moustafa E, Wong E (1967) Purification and properties of chalcone-flavanone isomerase from soya bean seed. *Phytochemistry* 6:625–632
- Muir S, Collins G, Robinson S, Hughes S, Bovy A, De Vos C, van Tunen A, Verhoeven M (2001) Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. *Nat Biotechnol* 19:470–474
- Mundy J, Yamaguchi-Shinozaki K, Chua N (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *rab* gene. *PNAS* 87:1406–1410
- Oken BS, Storzbach DM, Kaye JA (1998) The efficacy of *Ginkgo biloba* on cognitive function in Alzheimer disease. *Arch Neurol* 55:1409–1415
- Park H, Kim M, Kang Y, Jeon J, Yoo J, Kim M, Park C, Jeong J, Moon B, Lee J (2004) Pathogen- and NaCl-induced expression of the *SCaM-4* promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol* 135:2150–2161

- Piechulla B, Merforth N, Rudolph B (1998) Identification of tomato *Lhc* promoter regions necessary for circadian expression. *Plant Mol Biol* 38:655–662
- Rawat R, Xu ZF, Yao KM, Chye ML (2005) Identification of cis-elements for ethylene and circadian regulation of the *Solanum melongena* gene encoding cysteine proteinase. *Plant Mol Biol* 57:629–643
- Saure MC (1990) External control of anthocyanin formation in apple. *Sci Hortic* 42:181–218
- Shimada N, Aoki T, Sato S, Nakamura Y, Tabata S, Ayabe S (2003) A cluster of genes encodes the two types of chalcone isomerase involved in the biosynthesis of general flavonoids and legume-specific 5-deoxy (iso) flavonoids in *Lotus japonicus*. *Plant Physiol* 131:941–951
- Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, Goodman HM (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. *Plant J* 8:659–671
- Smith JV, Luo Y (2004) Studies on molecular mechanisms of *Ginkgo biloba* extract. *Appl Microbiol Biotechnol* 64:465–472
- Solano R, Nieto C, Avila J, Canas L, Diaz I, Paz-Ares J (1995) Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB. Ph3) from *Petunia hybrida*. *EMBO J* 14:1773–1784
- Straub P, Shen Q, Ho T (1994) Structure and promoter analysis of an ABA- and stress-regulated barley gene, HVA1. *Plant Mol Biol* 26:617–630
- Sutoh K, Yamauchi D (2003) Two cis-acting elements necessary and sufficient for gibberellin-upregulated proteinase expression in rice seeds. *Plant J* 34:635–645
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- van Beek TA (2002) Chemical analysis of *Ginkgo biloba* leaves and extracts. *J Chromatogr A* 967:21–55
- van Beek T, Montoro P (2009) Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts, and phytopharmaceuticals. *J Chromatogr A* 1216:2002–2032
- Van Tunen AJ, Koes RE, Spelt CE, Van der Krol AR, Stuitje AR, Mol JN (1988) Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: coordinate, light-regulated and differential expression of flavonoid genes. *EMBO J* 7:1257–1263
- Von Wettstein D, Gough S, Kannangara CG (1995) Chlorophyll biosynthesis. *Plant Cell* 7:1039–1057
- Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. *Curr Opin Plant Biol* 5:218–223
- Xu F (2008) Cloning and expression of *GbPAL* and *GbANS* genes and effect of ALA on the content of flavonoids in *Ginkgo biloba*. Dissertation, Shandong Agriculture University
- Xu F, Cheng SY, Cheng SH, Wang Y, Du HW (2007) Time course of expression of chalcone synthase gene in *Ginkgo biloba*. *J Plant Physiol Mol Biol* 33:309–317
- Xu F, Cai R, Cheng SY, Du HW, Wang Y, Cheng SH (2008a) Molecular cloning, characterization and expression of phenylalanine ammonia-lyase gene from *Ginkgo biloba*. *Afr J Biotechnol* 7:721–729
- Xu F, Cheng H, Cai R, Li LL, Chang J, Zhu J, Zhang FX, Chen LJ, Wang Y, Cheng SH (2008b) Molecular cloning and function analysis of an anthocyanidin synthase gene from *Ginkgo biloba*, and its expression in abiotic stress responses. *Mol Cells* 26:536–547
- Yamagata H, Yonesu K, Hirata A, Aizono Y (2002) TGTCACA motif is a novel cis-regulatory enhancer element involved in fruit-specific expression of the cucumis gene. *J Biol Chem* 277:11582–11590
- Yoshioka S, Taniguchi F, Miura K, Inoue T, Yamano T, Fukuzawa H (2004) The novel Myb transcription factor LCR1 regulates the CO<sub>2</sub>-responsive gene *Cah1*, encoding a periplasmic carbonic anhydrase in *Chlamydomonas reinhardtii*. *Plant Cell* 16:1466–1477
- Zhang H, Liu J, Lu H, Gao S (2009) Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the over-expression of chalcone isomerase gene with the elicitation treatment. *Plant Cell Rep* 28:1205–1213
- Zhou D (1999) Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. *Trends Plant Sci* 4:210–214