

An R2R3-MYB transcription factor as a negative regulator of the flavonoid biosynthesis pathway in *Ginkgo biloba*

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Abstract Flavonoids are secondary metabolites that contribute substantially to the quality of *Ginkgo biloba*. Plant flavonoid accumulation is controlled by transcriptional regulation of the genes that encode the biosynthetic enzymes, in which the R2R3-MYB transcription factor is a key factor. In this study, we describe the cloning and functional characterization of a R2R3-MYB transcription factor gene, *GbMYBF2*, isolated from *G. biloba*. *GbMYBF2* encodes a protein belonging to a small subfamily of R2R3-MYB transcription factors. Comparative and bioinformatics analyses showed that *GbMYBF2* is more closely related to the repressor R2R3-MYB subfamily involved in flavonoid biosynthesis. Tissue expression pattern analysis showed that *GbMYBF2* was constitutively expressed in leaves, fruits, stems, and roots, wherein the level of transcription in the roots is significantly higher than that in the stems, leaves, and fruits. During *G. biloba* leaf growth, the transcription of *GbMYBF2* is negatively correlated with the flavonoid content, suggesting that the *GbMYBF2* gene is responsible for the repressed flavonoid biosynthesis. Transgenic *Arabidopsis* plants that overexpress *GbMYBF2* exhibit an inhibition of flavonoid and anthocyanin biosynthesis compared with the untransformed *Arabidopsis* plants. In addition, the overexpression of *GbMYBF2* in *Arabidopsis* clearly downregulates the expression of the structural genes that control the synthesis of flavonoids and anthocyanins. These findings suggest that *GbMYBF2* may have a key role in repressing

transcription in regulating the biosynthesis of flavonoids in *G. biloba*.

Keywords Anthocyanins · Flavonoids · *GbMYBF2* · *Ginkgo biloba* · R2R3-MYB transcription factor · Repressor

Introduction

Ginkgo biloba is the only surviving member of a family of trees, *Ginkgoaceae*, which appeared about 200 million years ago, and is thus called “living fossil.” *G. biloba* extract is among the best-selling herbal medications worldwide. The *G. biloba* extract EGb 761 is obtained from dry leaves extracted with an acetone/water mixture. This extract has been standardized to contain 6 % terpene trilactones and 24 % flavonoids, which have several pharmaceutical properties for human health (van Beek and Montoro 2009). Flavonoids have high antioxidant capacity and contribute to protection against cardiovascular diseases and cancer (Lin and Weng 2006). To increase the flavonoid content in ginkgo leaves, understanding the biosynthesis pathway of these molecules is fundamental.

Flavonoid biosynthesis is derived from the phenylpropanoid pathway, one of the most characterized secondary metabolic routes in plant systems. Although different groups of proteins are responsible for producing, transporting, and storing flavonoids, the two most studied classes correspond to the biosynthetic (structural) enzymes and the transcription factors (TFs) controlling the pathway. Flavonoid biosynthesis regulation is mainly at the transcriptional regulation level of the structural genes by TFs. The structural genes of the flavonoid biosynthetic pathway have been isolated from *G. biloba*, including phenylalanine ammonia lyase (*PAL*; Xu et al. 2008a), chalcone synthase (*CHS*; Pang et al. 2005; Xu et al. 2007), chalcone isomerase (*CHI*; Cheng et al. 2011), flavanone 3-hydroxylase (*F3H*; Shen et al. 2006), flavonol synthase (*FLS*; Xu et al.

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2002), and anthocyanidin synthase (*ANS*; Xu et al. 2008b). The TFs that control flavonoid biosynthesis include MYB TFs, basic helix–loop–helix (bHLH) TFs, and tryptophan–aspartic acid repeat (WDR) protein (Broun 2005; Koes et al. 2005). Plant MYBs have important roles in many secondary metabolic pathways, including flavonoids and anthocyanins (Dubos et al. 2010). MYBs are classified by conserved imperfect repeats in the DNA-binding domain, called R1 or R2R3. *Arabidopsis* has 133 R2R3-MYB genes (Dubos et al. 2010), whereas grape has 108 (Matus et al. 2008). Most R2R3-MYB TFs that regulate flavonoid biosynthesis depend on cofactors including WDR proteins, but most importantly, a small subgroup of bHLH proteins that share a common motif in their N termini that interact with a signature motif in the R3 repeat of the N-terminal R2R3 domain of MYB factors (Grotewold et al. 2000).

To date, MYBs that control flavonoid biosynthesis have been characterized in many plants. The first R2R3-MYBs (MYB11/PFG2, MYB12/PFG1, and MYB111/PFG3) that regulate flavonol synthesis have been described in *Arabidopsis* (Mehrtens et al. 2005; Strack et al. 2007). These MYBs are also cofactor independent and individually regulate flavonol accumulation in different organs of developing seedlings (Strack et al. 2007). In apple, several MYBs that control anthocyanin biosynthesis have been isolated. *MdMYBA* was isolated from a pale-skinned cultivar “Tsugaru” and deep red-skinned “Jonathan” fruit (Ban et al. 2007), whereas *MdMYB1* was isolated from a red-skinned apple “Cripps Pink” (Tacos et al. 2006). The expressions of *MdMYB1* and *MdMYBA* correlate with anthocyanin synthesis in fruit skin. In a red flesh apple variety, Espley et al. (2007) found that *MdMYB10* controlled apple anthocyanin production in the fruits and leaves. In grape, *VvMYBA1* and *VvMYBA2* could activate anthocyanin biosynthesis by regulating the expression of the *UFGT* gene (Kobayashi et al. 2002; Walker et al. 2007), whereas *VvMYBF1* has been identified to regulate flavonol synthesis in developing grape berries (Czemmel et al. 2009). In pears, *PyMYB10* regulates anthocyanin biosynthesis, and the overexpression of *PyMYB10* is sufficient to induce anthocyanin accumulation (Feng et al. 2010). Aside from these activator MYBs, several repressors of the flavonoid pathway, specifically anthocyanins, are also MYB TFs, including a R2R3-MYB repressor from strawberry FaMYB1 (Aharoni et al. 2001), *Arabidopsis* AtMYB4 and AtMYB60 (Jin et al. 2000; Park et al. 2008), *Antirrhinum* AmMYB308 (Tamagnone et al. 1998), and a single-repeat MYB in *Arabidopsis*, AtMYBL2 (Dubos et al. 2008). However, the interaction mechanism of the repressor MYBs with the MBW transcriptional complex still remains to be elucidated (Matus et al. 2008).

In *G. biloba*, although most genes in the flavonoid biosynthesis pathway are known, whether or how the *MYB* genes contribute to flavonoid biosynthesis is largely unknown. In this study, we report on the isolation and molecular characterization

of a putative regulatory gene *GbMYBF2* from *G. biloba*. The expression analysis during leaf development and the transgenic plants suggest that *GbMYBF2* has a key role in downregulating flavonoid biosynthesis.

Materials and methods

Plant materials

Three-year-old seedlings and 14-year-old grafts of *G. biloba* were grown in a greenhouse at Yangtze University, China. To test the expression of *GbMYBF2* and analyze the flavonoid content, the leaves, fruits, stems, and roots of six *G. biloba* grafts were collected in mid-August. In mid-April of 2011, the 3-year-old seedlings were arranged in a randomized, complete block design with three replications. The plots were hand weeded and irrigated regularly. For this study, 90 seedlings were selected as sampling seedlings labeled with tags. The annual leaves from the terminal shoot were collected from the top of the sampling seedling from mid-April to late-November with about 15-day intervals. The labeled seedlings ($n=6$) were sampled, cleaned with a wet cloth, and then mixed. The mixed sample from the labeled seedlings was considered as one sample. All the samples were immediately frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ until further analyses.

Isolation of the full-length cDNA and genomic DNA of *GbMYBF2*

Total RNA was extracted from different tissues from all treatments using the cetyl trimethylammonium bromide (CTAB) method (Cai et al. 2007). Genomic DNA was extracted from the fresh leaves of *G. biloba* seedlings following the CTAB method described by Xu et al. (2008a). The quality and concentration of the RNA and genomic DNA were determined by agarose gel electrophoresis and spectrophotometer analysis.

The primers GbMYBUP and GbMYBDP (Table 1) were designed and synthesized (Shanghai Sangon, China) based on the conserved amino acid and nucleotide sequences of the plant R2R3-MYB genes to obtain the internal conserved fragment. One-step reverse transcription PCR (RT-PCR) was performed, and three approximately 300-bp fragments were obtained using the one-step RT-PCR kit (Dalian TaKaRa, China) under the following conditions: $50\text{ }^{\circ}\text{C}$ for 30 min and $94\text{ }^{\circ}\text{C}$ for 3 min, followed by 35 cycles of amplification at $94\text{ }^{\circ}\text{C}$ for 1 min, $52\text{ }^{\circ}\text{C}$ for 1 min, and $72\text{ }^{\circ}\text{C}$ for 1 min; followed by an extension for 10 min at $72\text{ }^{\circ}\text{C}$. The PCR product was purified, cloned into the pMD18-T vector (Dalian TaKaRa, China), and then sequenced. Subsequent BLAST results confirmed that the amplified products were partial fragments of R2R3-MYB gene.

Table 1 The primers used in the study

Primer	Sequence (5'–3')	Description
MYBUP	TWCCCAARVHTGCAGGHCTGC	Degenerate primer, forward
MYBDP	TCCAGTARTTYTTKATCTCRTT	Degenerate primer, reverse
MYB3	TGTGGAAAGAGTTGTAGATTGCGTTGGA	Forward primer for 3 RACE, outer
MYB3N	AGTTGCCAGGGAGAACGGACAATGAGA	Forward primer for 3 RACE, nested
MYB5	TCTCATTGTCCGTTCTCCCTGGCAACT	Reverse primer for 5 RACE, outer
MYB5N	TGTCCAACGCAATCTACAACCTTTTCCA	Reverse primer for 5 RACE, nested
MYBC1	ATGGGAAGGCAGCCATGC	Gene specific primer, forward
MYBG2	TCACATTCATCAAGTATGGAAGC	Gene specific primer, reverse
18SFP	ATAACAATACTGGGCTCATCG	Gene specific primer, forward
18SRP	TTCGCAGTGGTTCGTCTTTC	Gene specific primer, reverse
MYBFPT	CGGGATCC ATGGGAAGGCAGCCATGC	Gene specific primer, forward
MYBRPT	CGGGATCCTCACATTCATCAAGTATGGAAGC	Gene specific primer, reverse
PALFP	GTCAGGACAGAGTACGAGAACGG	Gene specific primer, forward
PALRP	TCGAATGGTGTGTTTATTCTCG	Gene specific primer, reverse
CHSFP	CGCATCACCAACAGTGAACAC	Gene specific primer, forward
CHSRP	TCCTCCGTCAGATGCATGTG	Gene specific primer, reverse
CHIFP	CCGGTTCATCGATCCTCTTC	Gene specific primer, forward
CHIRP	ATCCCGTTCAGGGATACTATC	Gene specific primer, reverse
F3HFP	CAGATCGTTGAGGCTTGTGAGA	Gene specific primer, forward
F3HRP	GACGAGTCATATCCGCCACTAAGT	Gene specific primer, reverse
FLSFP	CCG-TCGTCGATCTAAGCGAT	Gene specific primer, forward
FLSRP	CGTCGGAATCCCGTGGT	Gene specific primer, reverse
ANSFP	TATGCAAACGATCAAGCCA	Gene specific primer, forward
ANSRP	TGTAATCACTTGGTGTCTTAGG	Gene specific primer, reverse
UBQ10FP	CCCTAACGGGAAAGACGATT	Gene specific primer, forward
UBQ10RP	GAAGATGAGACGCTGCTGGT	Gene specific primer, reverse

Based on the sequence of the internal conservative fragment of the *GbMYBF2* gene, four specific primers, namely, MYB3 and MYB5 and nested primers MYB3N and MYB5N (Table 1), were designed to amplify the 5' and 3' ends of *GbMYBF2* using the SMARTTM rapid amplification of complementary DNA ends (RACE) amplification kit (Clontech, CA, USA). The 5'- and 3'-RACE-PCR were performed according to the instructions of the manufacturer. The PCR products were then purified and cloned into the pMD18-T vector for sequencing.

After comparing and aligning the sequences of the 5' RACE, 3' RACE, and the internal region products, full-length cDNA sequence of *GbMYBF2* was obtained through PCR amplification using 3'-Ready cDNA as template, as well as a pair of primers MYBC1 (Table 1) and UPM (provided in the kit). PCR was performed under the following conditions: 94 °C for 3 min, followed by 35 cycles of amplification (94 °C for 20 s, 53 °C for 30 s, and 72 °C for 2 min). After sequencing, the full-length cDNA of *GbMYBF2* was subsequently analyzed for molecular characterization. Two gene-specific primers, namely, MYBC1 and MYBG2, designed based on the cDNA sequence, were used to amplify the *GbMYBF2* genomic sequence.

Bioinformatics analysis and molecular evolution analyses

The obtained sequences were analyzed using online bioinformatics tools (<http://www.ncbi.nlm.nih.gov> and <http://www.expasy.org>). The software vector NTITM Suite 10 was used for sequence multialignment.

Extraction and analysis of flavonoid content from ginkgo leaves

Flavonoids were extracted and determined as previously described by Xu et al. (2008a). Three extraction samples were prepared for high-performance liquid chromatography (HPLC) analysis, with each sample injected three times. Quercetin, kaempferol, and isorhamnetin were selected as standard samples because they contain different flavonol glycosides, most of which are derivatives of the three flavonol aglycones in ginkgo leaves. Based on the methods of flavonoid concentration described by van Beek (2002), flavonoid contents were calculated by multiplying the total content of quercetin, kaempferol, and isorhamnetin with a factor of 2.51, and were expressed as percentage (m/m).

Construction of *GbMYBF2* expression vector and *Arabidopsis* transformation

The coding region of *GbMYBF2* was amplified using a pair of primers, namely, MYBFPT and MYBRPT, containing *Bam*HI sites at their respective 5' ends. The PCR products were cloned into pMD18-T vector and confirmed by sequencing. Subsequently, the plasmid DNA of the *GbMYBF2* clone was digested with *Bam*HI. DNA fragments containing the full-length cDNA of *GbMYBF2* were recovered and cloned into binary vector pBI121. After PCR and sequencing, the expression vector of *GbMYBF2* was introduced into *Agrobacterium tumefaciens* LBA4404, and *Arabidopsis* (ecotype Columbia) transformation was performed using the floral dip method (Clough and Bent 1998). Transgenic seedlings were selected from half-strength MS media containing 100 mg/L kanamycin. Kanamycin-resistant T₁ seedlings were transferred to soil and grown at 22 °C and 70 % relative humidity, with a 16-h photoperiod. T₂ seedlings were selected from half-strength MS media containing 100 mg/L kanamycin. RT-PCR was used to identify the transgenic plants from T₂ seedlings. RT-PCR analysis was performed using the RNA was extracted from the leaves of 2-week-old wild-type and transformed *Arabidopsis* plants. The wild-type *Arabidopsis* plants had no *GbMYBF2* gene, whereas each of transgenic plants expressed *GbMYBF2* mRNA. Segregation test was used to select homozygote lines from T₃ seedlings.

Extraction and determination of flavonols and anthocyanin in *Arabidopsis* plants

Transgenic and wild-type *Arabidopsis* plants were collected at equivalent ages and underwent freeze drying. The extraction and determination of flavonols and anthocyanin of the *Arabidopsis* seedlings were performed according to the methods of Mehrtens et al. (2005) and Park et al. (2008). The methanolic extract samples were analyzed using HPLC with a DAD SPD-M10avp detector (Shimadzu, Japan). Reverse-phase chromatography was carried out using a Diamonsil™ C18 reverse phase column (250 mm, 9.4 mm, 5 mL) (Agilent, USA). The HPLC parameters were as follows: column temperature, 30 °C; solvent A=0.1 % trifluoroacetic acid in water; solvent B=98 % acetonitrile with 0.1 % trifluoroacetic acid; solvent gradient, 0 min=0 % B, 3 min=6 % B, 12 min=18 % B, 25 min=25 % B, 35 min=100 % B, and 40 min=100 % B. The flow rate was 0.35 mL/min. The flavonol peaks were classified to correspond to kaempferol or quercetin derivatives by UV spectral analysis. The UV detector was set at $\lambda=365$ nm. The areas of the flavonol peaks were normalized to the peak area of the internal standard naringenin, resulting in relative flavonol amounts. For the anthocyanin analysis, the peaks were

classified as cyanidin or delphinidin derivatives by spectral analysis. The UV detector was set at $\lambda=510$ nm.

Relative quantification by real-time PCR

Real-time PCR was carried out to investigate the transcription levels of *GbMYBF2* and the flavonoid structural genes of *Arabidopsis*. RNA was isolated from the ginkgo or *Arabidopsis* samples. First-strand cDNA synthesis was carried out in triplicate for each sample according to the instructions of the manufacturer (PrimeScript™ RT Reagent Kit, Dalian TaKaRa, China). The flavonoid structural gene primers of *Arabidopsis* and *GbMYBF2* are listed in Table 1. Real-time PCR was performed using a Perkin-Elmer 7000 thermal cycler with SYBR Premix Ex Taq™ II Kit (Dalian TaKaRa, China) according to the protocol of the manufacturer. Reactions were performed in triplicate using 2 μ L of Master Mix, 0.5 M of each primer, 2 μ L of diluted cDNA, and nuclease-free water to a final volume of 20 μ L. The PCR reaction conditions were preincubated at 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 3 min. Fluorescence was measured at the end of each annealing step. Raw data were analyzed with Light Cycler software, and expression was normalized to *G. biloba* 18S gene (*Gb18S*, accession no. D16448) or *Arabidopsis* *UBQ10* gene (accession no. NM_178970) to minimize the variation in the cDNA template levels. Real-time PCR data were technically replicated with error bars, representing mean \pm SE ($n=3$).

Results

Isolation and sequence analysis of *GbMYBF2*

To isolate and characterize the ginkgo MYB transcription factor that has a role in flavonoid biosynthesis, two degenerate primers were designed in the R2R3 domain. Three cDNA fragments of putative *R2R3-MYB* gene were isolated and identified: *GbMYBF1* (GenBank accession no. GU985273), *GbMYBF10* (GU985274), and *GbMYBF2*. The amino acid sequence of *GbMYBF2* has strong similarity to the MYB TF repressors of flavonoid biosynthesis, and pilot experiments showed that expression level of *GbMYBF2* had significantly negative correlation with flavonoid content, while those of *GbMYBF1* and *GbMYBF10* had significantly positive correlation with flavonoid content. Therefore, we focused on the molecular characterization of *GbMYBF2* in this study. A full-length cDNA sequence of the *GbMYBF2* gene (JQ068807) was finally obtained from *G. biloba* by RACE method. The cDNA sequence was 1,370 bp, with a poly (A) tail and a 1,041-bp open reading frame (ORF). The ORF encoded a predicted protein sequence of 346 amino acid residues, with a molecular mass of 38.9 kDa and a *pI* of 4.86. BLASTP

analysis in NCBI revealed that the deduced GbMYBF2 amino acid sequence had 96.8 % identity to GbMYB1, another R2R3 MYB TF involved in phenylpropanoid and lignin biosynthesis (Qin 2007). The analysis of the deduced amino acid sequence revealed that GbMYBF2 contains an N-terminal R2R3 repeat that corresponds to the DNA-binding domain of plant MYB-type protein. In addition, the alignment of GbMYBF2 with other MYB TFs at the R2R3 domain indicated a high degree of homology, especially for some MYB TF repressors of flavonoid biosynthesis (Fig. 1). The similarity between the R2R3 domain of GbMYBF2 and that of AmMYB330 and AmMYB308, two transcriptional repressors of phenylpropanoid biosynthesis from *Antirrhinum majus* (Tamagnone et al. 1998), was 83 and 81 % amino acid identity, respectively. Matches against other plant species included flavonoid-repressed R2R3 MYBs with 80 % identity (of the R2R3 domain) to strawberry FaMYB1, 79 % to grape VvMYB4A, 79 % to *Zea mays* ZmMYB42, 78 % to *Gossypium hirsutum* GhMYB6, and 75 and 74 % to *Arabidopsis* AtMYB60 and AtMYB4, respectively. The GbMYBF2 protein contains the motif “[D/E]Lx₂[R/K]x₃Lx₆Lx₃R” in the R3 domain for interaction with R-Like bHLH proteins (Zimmermann et al. 2004), suggesting that specific R-like bHLH cofactors are needed to act as regulatory factors, as well as other plant MYB TFs (Espley et al. 2007). Within the C-terminal, two typical protein motifs, namely, “LIsrGIDPxT/SHRxI/L” and “pdLNLD/ELxiG/S,” which have been proposed to act as transcriptional activators and repressors of the late flavonoid biosynthesis gene, respectively (Aharoni et al. 2001; Matus et al. 2008; Shen et al. 2012), were also found in the GbMYBF2 protein (Fig. 1). Phylogenetic analysis also showed that GbMYBF2 is more closely related to repressor R2R3 MYB TFs, including AtMYB60, FaMYB1, and AmMYB308, than to the well-known activator R2R3 MYB TFs, such as MdMYB10, GhMYB10, PyMYB10, and An2, all of which belong to subgroup-10 MYBs. (Fig. 2) These results suggest that GbMYBF2 may have the same functions as MYBs, which function as a transcriptional repressor of flavonoid biosynthesis.

Genomic *GbMYBF2* (JQ068808) 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 *GbMYBF2* was 1,307 bp long and had 100 % identity with the coding region to the full-length cDNA sequence. Exons 1 (133 bp), 2 (130 bp), and 3 (778 bp) were separated by introns 1 (143 bp) and 2 (123 bp) (Fig. 3). The putative splicing site obeyed the GU/AG rule. The R2 domain spanned exons 1 and 2, and the R3 domain spanned exons 2 and 3. Such configuration is similar to that of other R2R3 MYBs (Jiang et al. 2004).

Expression of *GbMYBF2* in different tissues

The expression profile of *GbMYBF2* was assessed by real-time PCR in leaf, fruit, stem, and root of 12-year-old graft

ginkgo trees (Fig. 4). The highest transcription level of *GbMYBF2* was observed in the roots, which was significantly higher ($p < 0.05$) than that in the stems. Subsequently, the transcript level of *GbMYBF2* in the stems was significantly higher ($p < 0.05$) than those in the leaves and fruits. Although no significant difference between the transcript levels of *GbMYBF2* was observed in the leaves and fruits, the transcript level of *GbMYBF2* was the lowest in the fruits.

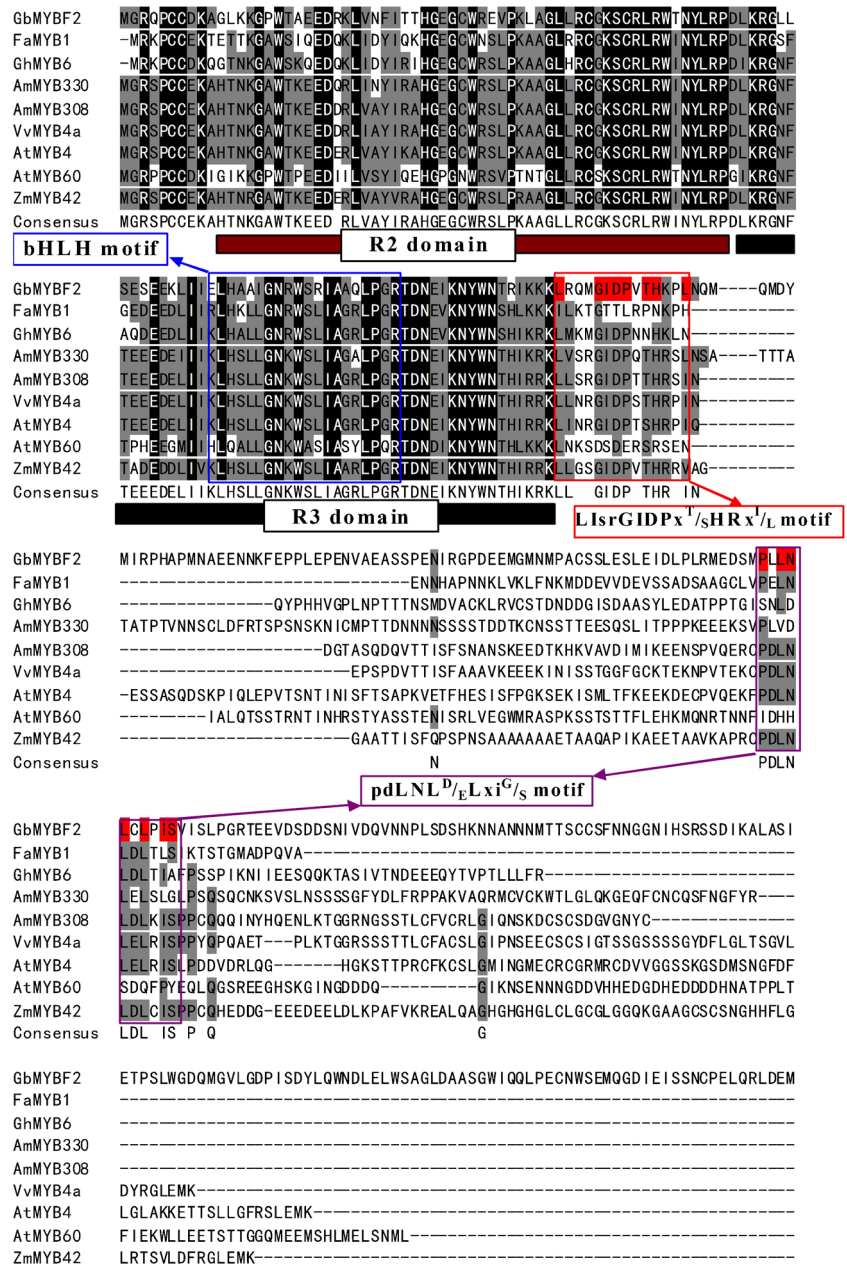
Relationship between *GbMYBF2* expression and flavonoid accumulation during ginkgo leaf age

To determine the temporal expression pattern of *GbMYBF2* gene in ginkgo leaves, the transcript level of *GbMYBF2* and the flavonoid content were examined at 15-leaf developmental stages. The real-time PCR results showed that the transcript level of *GbMYBF2* decreased from April 16 to July 15 and reached the valley value (1.825) on July 15 (Fig. 5). After a brief increase in August 1, the transcript level of *GbMYBF2* remained stable from August 1 to September 19. Subsequently, the transcript level of *GbMYBF2* showed a noticeable decline with the annual minimum value (1.196) on October 6. Afterward, the transcript level of *GbMYBF2* slightly increased from October 20 to November 21. The flavonoid content showed an inverse pattern to the expression profile of *GbMYBF2* with two obvious peaks (Fig. 5). Flavonoid content started to increase with the emergence of the leaf bud and reached the first peak on July 2 (1.286 % DW). Thereafter, the flavonoid content declined to a minimum by August 16 (0.806 % DW). A second peak and the highest yearly content occurred on October 20 (1.429 % DW). From a commercial standpoint, the optimal harvest time of ginkgo leaf flavonoids is thus around mid- and late-October. Linear regression indicated that the relationship between flavonoid content (Y) and *GbMYBF2* expression level (X) was significantly negatively linearly correlated (Table 2), with an F value of 43.57, a correlation coefficient of 0.448, and a linear equation represented by $Y = -(0.487 \pm 0.076)X + (1.985 \pm 0.171)$. These results indicate that the *GbMYBF2* gene is responsible for the repressed flavonoid accumulation.

HPLC analysis of *GbMYBF2* transgenic plants showed that the accumulation of flavonoid compounds was inhibited

To investigate the function of the *GbMYBF2* gene, *GbMYBF2* cDNA was overexpressed in *Arabidopsis*. The heterologous expression of *GbMYBF2* was confirmed by RT-PCR. Several independent lines showed high expression of *GbMYBF2* transcripts (data not shown). Plants of the *GbMYBF2*-overexpression (*GbMYBF2*-OE) and wild-type (WT) lines were analyzed for their flavonoid contents by HPLC. Pilot experiments were carried out based on the design and method of Mehrtens et al. (2005), and the results showed that young *GbMYBF2*-

Fig. 1 Multialignment of the amino acid sequences of GbMYBF2 and other flavonoid-repressed R2R3 MYB proteins. The identical amino acids are indicated with *white foreground* and *black background*. The conserved amino acids are indicated with *black foreground* and *gray background*. Nonsimilar amino acids are indicated with *black foreground* and *white background*. The R2R3 binding domain is *underlined in red and black*, respectively. The bHLH binding motif is *boxed in blue* in the R3 domain. The C-terminal-conserved motifs, “LIsrGIDP_xT/SHR_xI/L” and “pdLNL^D/E_xL_xI^G/S” for flavonoid-repressor MYBs are *boxed in red and purple*, respectively. The GenBank accession numbers of these sequences are below: *Fragaria × ananassa*, FaMYB1, AF401220; *Gossypium hirsutum*, GhMYB6, AAC04720; *Antirrhinum majus*, AmMYB330, P81395; *Antirrhinum majus*, AmMYB308, P81393; *Vitis vinifera*, VvMYB4a, XP_002278222; *Arabidopsis thaliana*, AtMYB4, AF062860; *A. thaliana*, AtMYB60, AF062895; *Zea mays*, ZmMYB42, CAJ42204



OE seedlings contained reduced amounts of flavonoids. For the following analysis, we focused on a series of 2- to 10-day-old developing seedlings grown under continuous white light that were sampled at 2-day intervals. In the WT seedlings, five different quercetins, three different kaempferols, and one naringenin derivative were detected to have originated from different glycosylation patterns of the aglycone (Fig. 6a). These data are consistent with the results of Mehrtens et al. (2005). At all time points, both the quercetin and the kaempferol contents of the GbMYBF2-OE seedlings were

clearly reduced compared with the WT reference (Fig. 6b, c). To check whether the biochemical alterations also extended to anthocyanin, the anthocyanin content of GbMYBF2-OE and WT seedlings was determined. Similar to quercetin and kaempferol, the anthocyanin contents in the GbMYBF2-OE seedlings from 2 to 10 days were significantly lower than those in the WT seedlings (Fig. 6d). Taken together, a clear and negative correlation was found between the *GbMYBF2* expression level and the flavonoid content in developing seedlings.

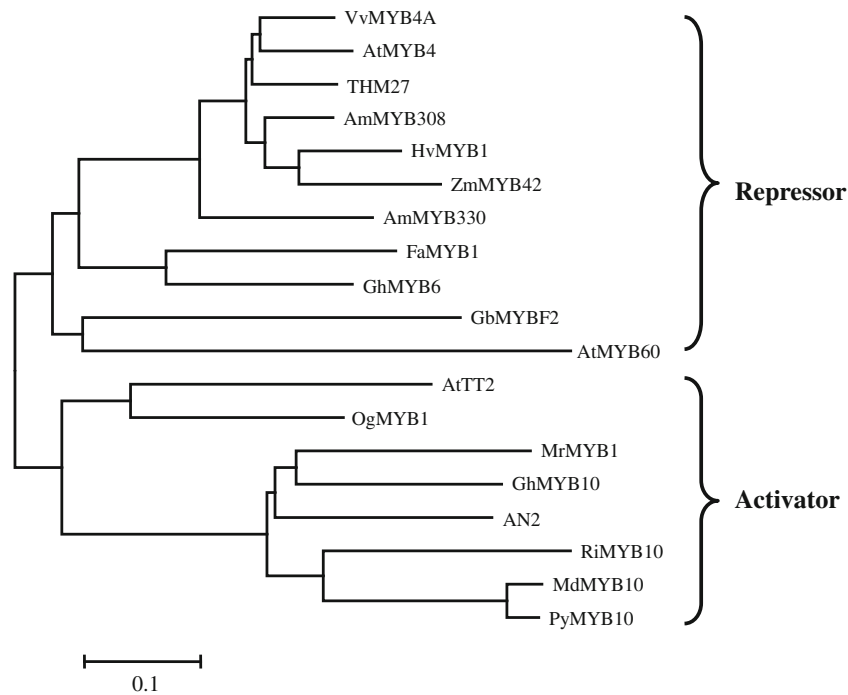


Fig. 2 Phylogenetic relationship of GbMYBF2 and other MYBs. The tree was constructed using MEGA 5.0 and Clustalx 1.83. A minimum evolutionary phylogeny test and 1,000 bootstrap replicates were chosen during analysis. The scale bar represents 0.1 substitutions per site. The GenBank accession numbers of these sequences are below: *Solanum lycopersicum*, THM27, CAA64614; *Hordeum vulgare*, HvMYB1,

P20026; *A. thaliana*, AtTT2, Q9FJA2; *Oncidium gower*, OgMYB1, ABS58501; *Morella rubra*, MrMYB1, ADG21957; *Gerbera hybrid*, GhMYB10, CAD87010; *Petunia × hybrida*, An2, AAF66727; *Rubus idaeus*, RiMYB10, ABX79950; *Malus domestica*, MdMYB10, ABB84753; *Pyrus pyrifolia*, PyMYB10, ADN26574

The expression levels of biosynthetic genes in the flavonoid biosynthetic pathway is affected by GbMYBF2

To verify the effects of the GbMYBF2 protein on the expression of the structural genes involved in flavonoid biosynthesis, quantitative real-time reverse transcription PCR analyses were carried out. The six structural genes, including *PAL*, *CHS*, *FLS*, *CHI*, *F3H*, and *ANS*, which act in the flavonoid biosynthetic pathway, have been well characterized in *G. biloba* in our previous study (Cheng et al. 2011; Shen et al. 2006; Xu et al. 2007, 2008a,b, 2012). The transcript levels of these six flavonoid biosynthetic genes were compared between the GbMYBF2-OE and the WT seedlings sampled from 2- to 10-day-old developing lines. In the GbMYBF2-OE seedlings,

the expression levels of *F3H*, *CHS*, *FLS*, and *ANS* were obviously reduced compared with that of the WT seedlings at all time points (Fig. 7b, d–f), whereas the expression level of *PAL* was significantly higher than that of the WT seedlings (Fig. 7a). However, no significant difference was observed in the *CHI* expression level between GbMYBF2-OE and the WT seedlings at five time points (Fig. 7c).

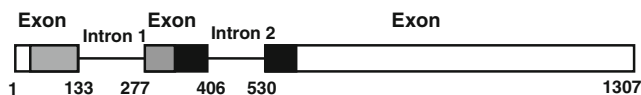


Fig. 3 Characterization of *GbMYBF2* genomic copy. Exons and introns are labeled. Numbers refer to position relative to the first nucleotide of the start codon. Exons are indicated by boxes, and introns are indicated by lines. Location of R2 and R3 domain are highlighted with gray and black backgrounds, respectively

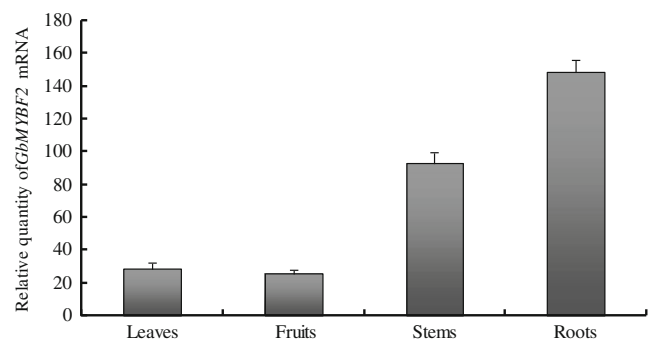
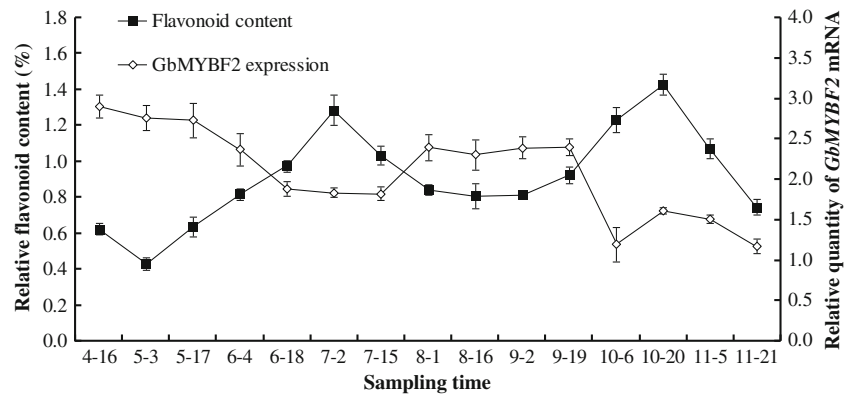


Fig. 4 Expression pattern of *GbMYBF2* gene in different tissues with *18S* gene as control. Total RNA samples were isolated from leaves, fruits, stems, and roots, respectively, and subjected to real-time PCR. Data are mean values of triplicate tests ± SE

Fig. 5 Changes in flavonoid content, *GbMYBF2* expression level during growth of ginkgo leaves. In the annual change of flavonoid in ginkgo leaves, the flavonoid content and *GbMYBF2* expression level showed significantly negatively correlated



Discussion

Our previous study indicated that the accumulation of flavonoids in *G. biloba* leaves was temporally and spatially controlled by the transcription level of flavonoid biosynthetic genes (Cheng et al. 2011; Shen et al. 2006; Xu et al. 2007, 2008a,b, 2012). However, no transcription factors that regulate flavonoid biosynthesis have been identified in *G. biloba* to date. The same holds true in woody plants, such as apple (Takos et al. 2006), pear (Feng et al. 2010), citrus (Cultrone et al. 2010), mango-steen (Palapol et al. 2009), grape (Czemmel et al. 2009), and Chinese bayberry (Niu et al. 2010). However, in *G. biloba*, whether MYB TFs participate in flavonoid biosynthesis remains to be determined. In this study, a 1,370-bp full-length cDNA of the *R2R3-MYB* gene *GbMYBF2* was isolated from *G. biloba*. At the genomic level, *GbMYBF2* was also isolated from *G. biloba*. *GbMYBF2* has three exons, without a signal peptide. The R2 domain spanned exons 1 and 2, whereas the R3 domain spanned exons 2 and 3. The genomic organization and R2R3 domain distribution of *GbMYBF2* were similar to those of the other R2R3 MYBs (Jiang et al. 2004; Feng et al. 2010). The deduced amino acid sequence of *GbMYBF2* showed high identity to other MYB proteins from angiosperm plants, such as AmMYB330, AmMYB308, FaMYB1, VvMYB4A, ZmMYB42, GhMYB6, and AtMY60, which function as inhibitors of flavonoid biosynthesis. The phylogenetic analysis clearly placed the *GbMYBF2* in clades with the

repressor R2R3 MYB TFs, including AtMYB60, FaMYB1 and AmMYB308 (Fig. 2). These results suggested that *GbMYBF2* might be a repressor of the flavonoid biosynthesis pathway. In addition, protein motif analysis showed that *GbMYBF2* has two conserved motifs: “LIsrGIDP \times T/SHR \times L” and “pdLNLD/ELxiG/S.” Motif “LIsrGIDP \times T/SHR \times L” may function as an activation domain, confirming a previous report (Matsui et al. 2008). However, for the motif “pdLNLD/ELxiG/S” sequence, we found the “pdLNLD/ELxiG/S” motif functions as the repression domain, consistent with the results from a similar analysis of AtMYB4 and FaMYB1 (Jin et al. 2000; Aharoni et al. 2001). The coexistence of the activation and repression motifs at the C-terminal of *GbMYBF2* reflects the complexity of the protein structures and potential regulatory network. In addition, one signature motif that specifies the interaction between MYB and bHLH proteins was also found in the *GbMYBF2* protein (Grotewold et al. 2000; Zimmermann et al. 2004). Flavonoid biosynthesis is usually regulated by R2R3-MYB, in combination with bHLH. The ratio and amounts of R2R3-MYB and bHLH transcripts alter the amount of flavonoid production (Nakatsuka et al. 2009). Thus, the spatial and temporal expressions of *R2R3-MYB* and *bHLH* genes in *G. biloba* warrant further study.

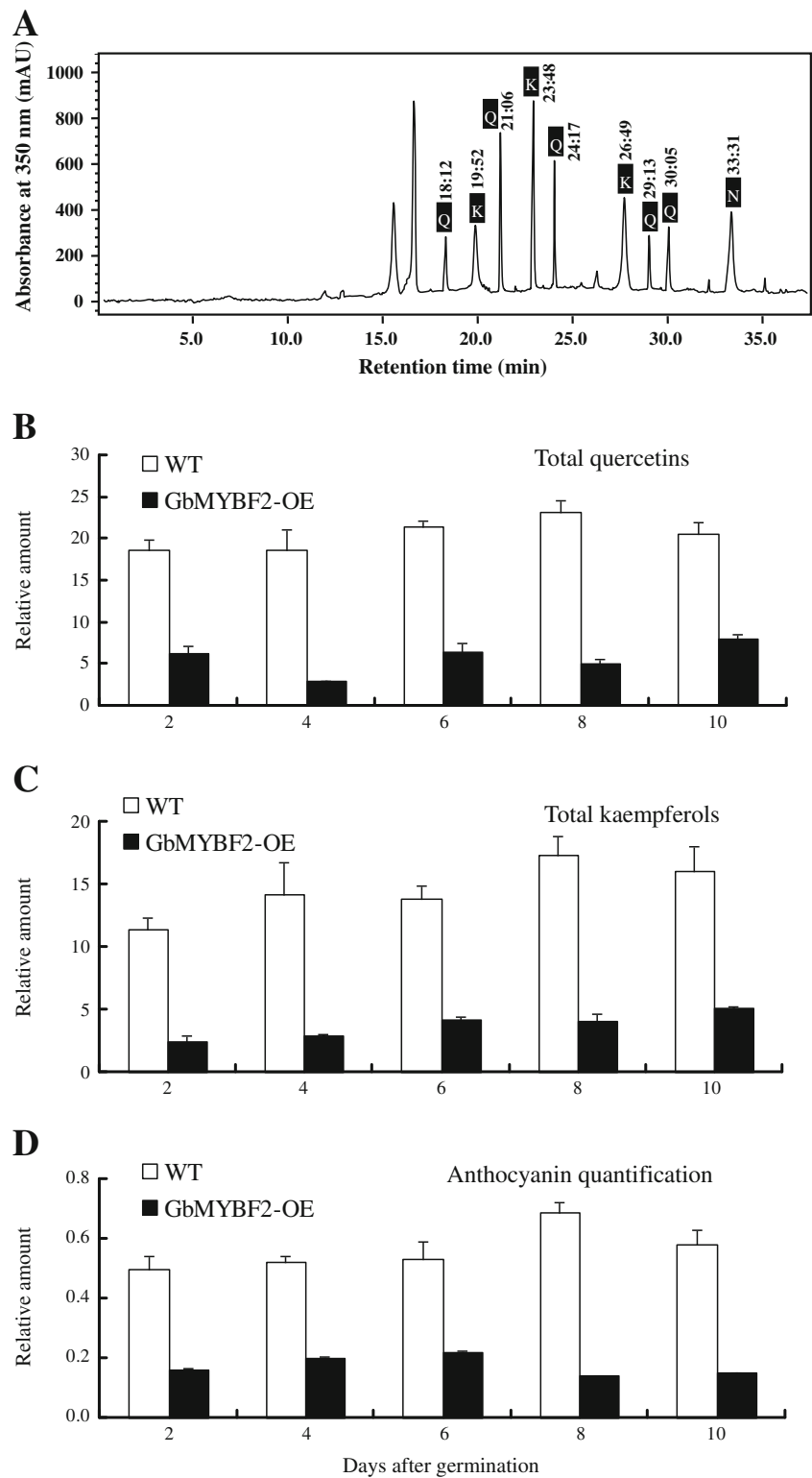
The transcript level of *GbMYBF2* appeared to be different among different *G. biloba* tissues. We previously determined the flavonoid content and the transcript levels of several structural genes in different tissues. The results showed that

Table 2 Relationship between flavonoid content and *GbMYBF2* expression level

Model summary						
Model	Unstandardized coefficients ^a			Linear regression		
	Regression coefficient	Standard error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	1.985	0.171	0.001	0.679	0.461	0.448
<i>GbMYBF2</i> expression level	-0.487	0.076	0.001			

^a Dependent variable: flavonoid content

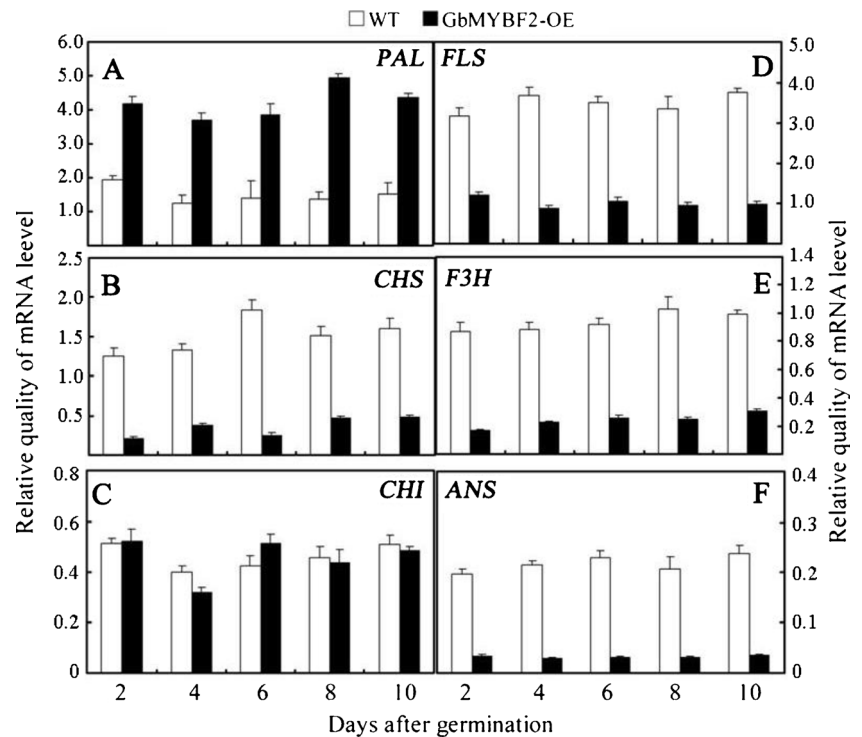
Fig. 6 Flavonoid and anthocyanin contents in wild-type control (WT) and GbMYBF2-overexpression (GbMYBF2-OE) transgenic *Arabidopsis* seedlings. **a** Representation of a selected HPLC result. The example shows the chromatogram obtained from a methanolic extract of 4-day-old WT seedlings. Peaks identified as corresponding to quercetin or kaempferol derivatives and the internal standard naringenin are labeled. Retention times are indicated above the peaks. *K* kaempferol, *N* naringenin, *Q* quercetin. **b** The contents of total quercetins in methanolic extracts of developing *Arabidopsis* seedlings by HPLC analysis. **c** The contents of total kaempferols in methanolic extracts of developing *Arabidopsis* seedlings by HPLC analysis. **d** The contents of total anthocyanins in methanolic extracts of developing *Arabidopsis* seedlings by HPLC analysis. Values shown represent the mean reading from three independent biological replicates and each plant was individually assayed in triplicate



the transcript levels for *GbPAL* (Xu et al. 2008a), *GbFLS* (Xu et al. 2012), *GbANS* (Xu et al. 2008b), and *GbCHI* (Cheng et al. 2011), as well as their flavonoid content, were higher in leaves than in roots. The expression pattern of *GbMYBF2* was in contrast with those of structural genes and flavonoid content

in different tissues. The correlation coefficient analysis of the relationship between the transcript level of *GbMYBF2* with flavonoid content and the transcript level of *GbPAL*, *GbFLS*, *GbANS*, and *GbCHI* indicated a strong negative relationship between the transcript level of *GbMYBF2* with flavonoid

Fig. 7 Expression levels of the genes involved in flavonoid biosynthesis in the WT and GbMYBF2-OE transgenic *Arabidopsis* seedlings. Relative expression was determined in triplicate measurements in three independent biological replicates. *Arabidopsis UBQ10* gene was used as the quantitative control



content, and the transcript level of *GbPAL*, *GbFLS*, *GbANS*, and *GbCHI*, with Pearson correlation coefficients of -0.385 , -0.098 , -0.664 , -0.172 , and -0.191 , respectively. The negative correlation of the *GbMYBF2* expression level compared with flavonoid content and the expression level of the structural genes suggested that *GbMYBF2* may have a negative regulatory role in flavonoid biosynthesis.

To further confirm the function of *GbMYBF2* in flavonoid biosynthesis in *G. biloba*, the overexpression of *GbMYBF2* in the model plant *Arabidopsis* was attempted because no reliable ginkgo transformation system is available at the moment. In addition, *Arabidopsis* has merit as a model plant system to further characterize the functions of the gene products in flavonoid metabolism because most of the biosynthetic enzymes of the flavonoid pathway are encoded by single-copy genes (Winkel-Shirley 2001). Our results showed that flavonoid and anthocyanin accumulations were significantly inhibited in plant ectopic overexpression of *GbMYBF2* compared with that in wild-type plants (Fig. 6). The results suggest that *GbMYBF2* functions as a repressor of flavonoid and anthocyanin biosynthesis. Based on our present findings of the inhibition of flavonoid and anthocyanin biosyntheses, as well as our metabolite analysis of *GbMYBF2*-overexpressed *Arabidopsis* plants compared with the wild type, we speculated that the biosynthesis of flavonoid and anthocyanin was principally regulated by the *GbMYBF2* transcription factor. Thus, this characterization of the function of *GbMYBF2* represents a crucial step in furthering our understanding of the molecular regulation of flavonoid biosynthesis and the transcription of related genes in *G. biloba*.

Notably, MYB-related proteins act as transcriptional activators (Espley et al. 2007; Palapol et al. 2009; Feng et al. 2010; Niu et al. 2010).

The R2R3-MYB proteins activate the transcription of structural proteins that act in different branches of the phenylpropanoid metabolism (Mehrtens et al. 2005; Deluc et al. 2006; Akagi et al. 2009; Palapol et al. 2009; Feng et al. 2010), or those that play roles in the plant response to stress signals (Chen et al. 2005; Cominelli et al. 2005; Liu et al. 2011). Anthocyanin expression in maize is dependent on the presence of both the MYB and MYC types of the regulator factors. Thus, this study showed that the expression of *GbMYBF2* alone under the control of the CaMV 35S promoter was sufficient to repress flavonoid and anthocyanin accumulations in *Arabidopsis* plant.

We also investigated the effects of *GbMYBF2* on the expression of the different structural proteins involved in flavonoid production, which we have identified previously in *G. biloba*. Our results revealed that this MYB protein notably represses the expression of *CHS*, *F3H*, *FLS*, and *ANS*, in contrast to significantly up-regulating the expression of *PAL* in *GbMYBF2*-OE *Arabidopsis* plants. *CHS*, *F3H*, and *FLS* represent important branching points within the flavonoid biosynthesis. *CHS* catalyzes the committing step toward the flavonoids and the formation of 4,2',4',6'-tetrahydroxy-chalcone, whereas *F3H* and *FLS* catalyze the committing steps toward flavonols using (2S)-flavanones and dihydroflavonols as substrate, respectively (Forkmann et al. 1980; Mehrtens et al. 2005). The *ANS* protein represents an

important branching point within the anthocyanin biosynthetic pathway; it catalyzes the committing step in this process, which is the formation of anthocyanidins from leucoanthocyanidins (Cheng et al. 2007). This result indicated that GbMYBF2 specifically inhibited the flux of flavonoid production by repressing the transcription of *CHS*, *F3H*, *FLS*, and *ANS*, with a resulting reduction of flavonoid and anthocyanin contents. PAL catalyzes the first step in the biosynthesis of phenylpropanoids including lignins, flavonoids, coumarins, stilbenoids, and so on. Qin (2007) demonstrated that GbMYB1 may be involved in the control of lignin biosynthesis in *G. biloba*, wherein the deduced GbMYBF2 amino acid sequence had very high identity with GbMYB1 (96.8 %), indicating that GbMYBF2 has a similar role with GbMYB1 related to lignin biosynthesis. Further studies are required to identify the effect of GbMYBF2 on the accumulation of lignin and on the regulation of genes involved in the lignin biosynthetic pathway. Given that the transcription of four structural flavonoid genes was affected by the GbMYBF2 protein, the genetic basis of flavonoid biosynthesis is probably the regulation of GbMYBF2 on the activity of a common regulator of these genes.

The *CHI* gene was the only flavonoid pathway gene unaffected by expression in GbMYBF2-OE seedlings. This observation suggested that CHI is not coregulated with other pathway steps by the regulation of GbMYBF2. However, Cheng et al. (2011) demonstrated that *GbCHI* is one of the key genes related to flavonoid biosynthesis and found that the promoter regions contain several MYB-binding sites, suggesting that *GbCHI* could be controlled by MYB protein. Southern blot analysis has indicated at least three sequences similar to *GbCHI* in the *G. biloba* genome (Cheng et al. 2011). The other *GbCHI* gene isoform that has not been isolated is involved in anthocyanin; thus, analysis of the other putative *GbCHI* genes is needed to clarify whether this step is coregulated.

Various MYB-binding motifs are found in plant MYB TFs, such as MYBCORE (Urao et al. 1993; Solano et al. 1995), MYBPLANT (Grotewold et al. 1994; Tamagnone et al. 1998), MYB2CONSENSUSAT (Abe et al. 2003), and in the PALCE database for plant cis-motifs (Higo et al. 1999; Akagi et al. 2009). The ability of MYB TF to bind to them depends on the sequences of the R2 region (Koes et al. 2005). Therefore, MYB TFs with conserved homologous R2 sequences have the same binding ability. The R2 regions of MYB TFs phylogenetically placed in the same cluster of GbMYBF2, such as AtMYB60, GhMYB6, FaMYB1, VvMYB4A, and AmMYB330, showed high sequence identities up to 65 % (Fig. 1); however, their detailed target motifs have not been identified to date. MYB TFs that regulate the transcription of genes in the flavonoid biosynthetic pathway have been studied previously (Jin and Martin 1999). The MYB-binding sites are present in the promoter regions of the flavonoid biosynthetic genes in *G. biloba*, such as *GbPAL*, *GbANS*, *GbFLS*, and *GbCHS2* (Shen et al. 2006; Xu et al. 2007, 2008a,b, 2012;

Cheng et al. 2011), indicating that the GbMYBF2 TF may also specifically and directly target these genes. Additional target genes related to flavonoid metabolism can be negatively regulated by *GbMYBF2* in *G. biloba*. Taken together, these results strengthen the view of a close correlation between the structural and the functional similarity of R2R3-MYB transcription factors across plant species, with modifications in the target gene set mainly determined by the sequence of the target gene promoter.

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