

Identification of Class 2 1-Deoxy-D-xylulose 5-Phosphate Synthase and 1-Deoxy-D-xylulose 5-Phosphate Reductoisomerase Genes from *Ginkgo biloba* and Their Transcription in Embryo Culture with Respect to Ginkgolide Biosynthesis

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Abstract

Diterpenoid ginkgolides having potent platelet-activating factor antagonist activity are major active ingredients of ginkgo extract. Class 2-type 1-deoxy-D-xylulose 5-phosphate synthase (*GbDXS2*) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*GbDXR*), the first two enzymes in 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, operating in the earlier step of ginkgolide biosynthesis, were cloned from embryonic roots of *Ginkgo biloba* through a homology-based polymerase chain reaction for role assessment of the enzymes. Plasmids harboring each gene rescued the respective knockout *E. coli* mutants. The levopimaradiene synthase gene (*LPS*), responsible for the first committed step in ginkgolide biosynthesis, and *GbDXS2* were transcribed exclusively in embryonic root, suggesting a specific role of *GbDXS2* in ginkgolide biosynthesis. *GbDXR* retained a higher transcription level in roots than in leaves, whereas class 1 *DXS* (*GbDXS1*) showed 30 to 50% higher level in leaves. Ginkgolides and bilobalide were found both in leaves and roots from an earlier stage of the embryo culture. Exclusive transcription of ginkgolide biosynthesis-specific *LPS* and *GbDXS2* in roots and the ap-

pearance of ginkgolides in leaves was consistent with translocation of the compounds from roots to leaves.

Key words

Ginkgo biloba · 1-deoxy-D-xylulose 5-phosphate synthase · 1-deoxy-D-xylulose 5-phosphate reductoisomerase · ginkgolide

Abbreviations

dd: double distilled
DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXS: 1-deoxy-D-xylulose 5-phosphate synthase
LB: Luria-Bertani
LPS: levopimaradiene synthase
ME: 2-C-methyl-D-erythritol
MEP: 2-C-methyl-D-erythritol 4-phosphate
ORF: open reading frame
PCR: polymerase chain reaction
RACE: rapid amplification of cDNA end
RT: real time

Introduction

Terpenoids, found in various plants, have interesting bioactivities suitable for human use, with some finding their ways into the cellular machineries, such as ubiquinone, heme, and phytol,

to execute pivotal roles in life processes. Many examples of terpenoids used in human health care have been reported [1]. Ginkgolides are highly modified diterpene lactones found uniquely in *Ginkgo biloba* (Ginkgoaceae), and, as one of the salient examples, have potent platelet-activating factor antagonist activity [2]. The

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popular use of ginkgo extract, whose estimated world-wide sales in 2004 exceeded US \$ 1 billion, relies, in part, on the inhibition of blood clotting by ginkgolides. Recently, an excellent review on the chemistry and pharmacology of ginkgolides was published [3].

Biosynthesis of the isoprene unit, the building block of terpenoids, had long been believed to originate from the mevalonate pathway. However, a novel 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway has recently been mapped in bacteria and plant plastid; in general, mono-, di-, and tetraterpenoids originate from the MEP pathway, while sesqui- and triterpenoids arise from the mevalonate pathway [4]. Therefore, the biosynthetic precursor of diterpenoid ginkgolides is expected to arise from the MEP pathway (Fig. 1). While cloning and functional identification of the MEP pathway genes were first achieved in a bacter-

ium *E. coli*, studies on the genes of plant origin have been lagging. A number of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) genes from *Arabidopsis thaliana* [5], [6] and tomato [7], [8] have been cloned and functionally identified. While DXR catalyzes the first committed step in MEP pathway, DXS plays a dual role – providing vital precursor to MEP pathway, and thiamine and pyridoxal biosyntheses (Fig. 1). It is noteworthy that Walter et al. [9] recognized two classes of DXS, DXS1 and DXS2, with distinctive amino acid sequences and functions, and, recently, a putative class 1 DXS from *G. biloba* (*GbDXS1*, GenBank Accession Number AY599128) was functionally identified [10].

Levopimaradiene synthase (LPS) performs the first committed step of cyclization of geranylgeranyl diphosphate in ginkgolide

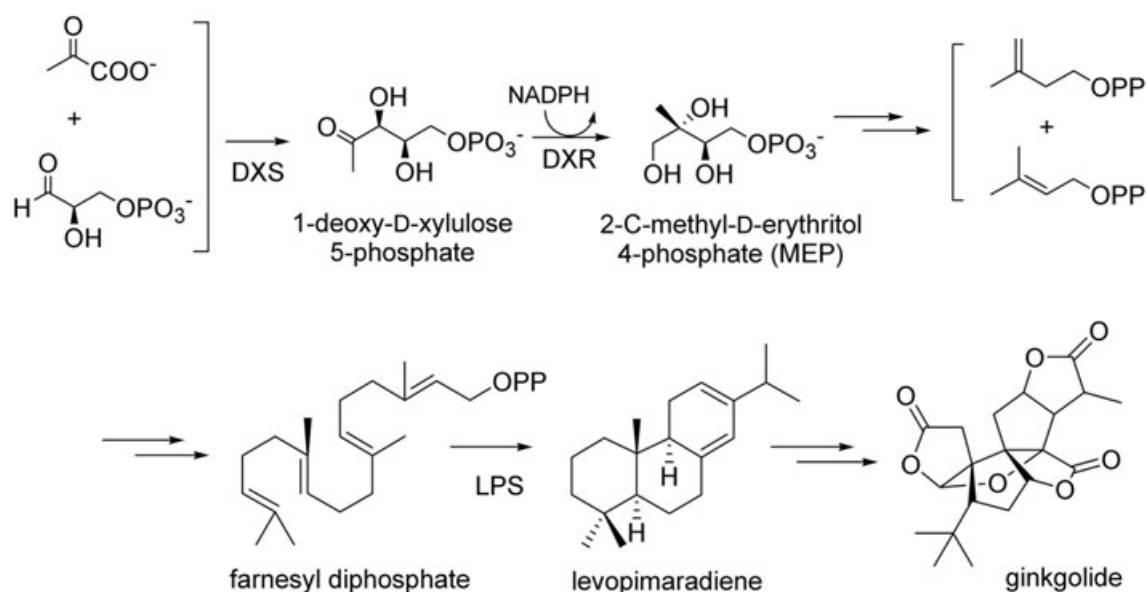


Fig. 1 Biosynthesis of ginkgolides in *G. biloba* through MEP pathway.

Table 1 List of primers used in this study

Usage	Primer name	Sequence (5' → 3')
PCR of the conserved regions	DXS3 DXS4 DXR4 DXR5	TBTTYGARGARCTBGGNHT GYTCNGCDATNCCDACRTC CKCATMTCNGGCCANCC ATHGGNACNCARACNYTNGA
RACE	GeneRacer 5'-nested GeneRacer 3'-nested DXS-F DXS-B DXR-F DXR-B	GGAACTGACATGGACTGAAGGAGTA CGCTACGTAACGGCATGACAGTG GGGCTACCTCCTGCAGAGAAA AGCCGGGTCAAAGTTCACCACA ACCACAAGGTGGTCTGCGGCGTA GAACTGCCGACCTGATCTGCAA
ORF PCR	TDXS-START DXS-STOP TDXR-START DXR-STOP	CGCGGATCCAAAATTGTACACGGCA CGCGGATCCTTAATTACTAAGTTGTAGA CGCGGATCCTGTTCTCTGAATGGC CCGGAATTCTAAACTGCAATGGGC
RT-PCR	RTDXS2-F RTDXS2-B RTDXR-F RTDXR-B RTLevo-F RTLevo-B	GGTTGCAATTTACAAGAAGG CCTAATCAAATCTCCATCCA GAAGTCAAAGTTTCAGACGC TAGTCAGCCCCATAAAGGTA ATTGGACTTCAATATGGTGC ATACTAACCGCCACTGAGAA

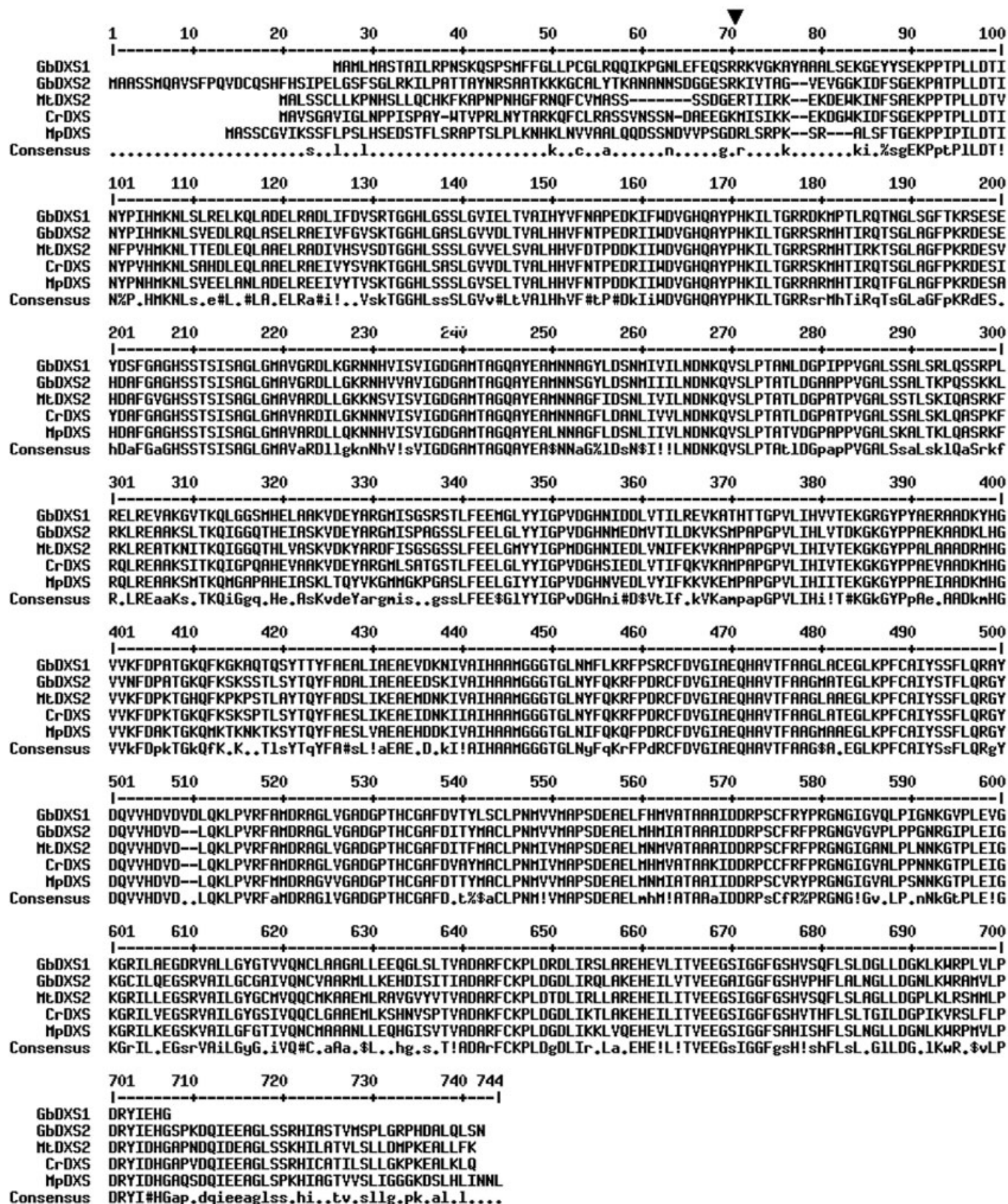


Fig. 2 Alignment of amino acid sequences of plant DXSs. Full-length sequences of *G. biloba* DXSs were compared with those of MtDXS2 (*M. truncatula*, AJ430048), CrDXS (*C. roseus*, AJ011840), and MpDXS (*Mentha piperita*, AF019383). Black arrow indicates the predicted N-terminus of the mature proteins.

biosynthesis [11]. The modification of the initial cyclization product, levopimaradiene, into ginkgolides needs extensive oxidative and skeletal rearrangements (Fig. 1). While the ginkgolides and

their degradation product, bilobalide, are mostly found in leaves, ginkgolides are biosynthesized in roots, which necessitates the translocation of ginkgolides from roots to leaves [12].

This paper describes the cloning and functional identification of class 2 DXS and DXR from ginkgo for the first time, with attempts to correlate the location among organs and timing of the gene transcription with the accumulation of ginkgolides. Functional identification of the genes was achieved through complementation with the knockout *E. coli* mutants. Transcription patterns of DXSs and DXR, as well as LPS, were also assessed with respect to the ginkgolide accumulation.

Material and Methods

Plants

Seeds of ginkgo, purchased from Nammun Market, Suwon, Korea, were carefully dehulled and sterilized in 4% NaOCl solution for 20 min. After extensive washing in distilled water, the seeds were cut to remove the cotyledon, and the embryo was placed on hormone-free MS medium and incubated at 23°C under 16 h/8 h light/dark regimen. Plantlets were harvested every week.

RNA isolation and cDNA synthesis

Total RNA was isolated from a 1-month-old embryo culture through the cetyltrimethylammonium bromide (CTAB) method [13]. Messenger RNA was prepared from total RNA using the PolyAtract mRNA Isolation System (Promega), and cDNA was

prepared using the GeneRacer Kit (Invitrogen). For real-time polymerase chain reaction (RT-PCR), 2 µg total RNA in 12 µL RNase-free water were added with 8 µL reverse transcription reaction solution (2 µL supplied 10× RT buffer, 2 µL of 5 mM each dNTP, 2 µL of 10 µM oligo dT primer, 1 µL of 10 unit/µL RNase inhibitor, 1 µL of 4 unit/µL Qiagen Omniscript Reverse Transcriptase) to make a total 20 µL mixture, which was incubated for 60 min at 37°C. Finally, the temperature was raised to 95°C and held for 5 min to stop the reaction. The mixture was stored at -20°C until use.

Rapid amplification of cDNA end (RACE)

All primers used in the PCR reactions are listed in Table 1. Degenerate primer pairs, DXS-3 and -4, and DXR-4 and -5 for DXS and DXR, respectively, were designed based on the conserved regions of the previously known DXSs and DXRs, and PCR was performed using a cDNA template as follows. The reaction mixture contained 20 µL PCR premix (Bioneer, Daejeon, Korea), 1 µL each primers (100 pmole), 1 µL cDNA (50 ng), and 17 µL dd H₂O. The reaction was run at 94°C for 5 min for the first cycle, 94°C for 1 min, 55°C (DXR) or 50°C (DXS) for 1 min, and 72°C for 1 min for 35 cycles, and for the last cycle 72°C for 5 min. The PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced. New pairs of primers for RACE were designed based on the sequence information. The primer pairs of Generacer 5'-nested

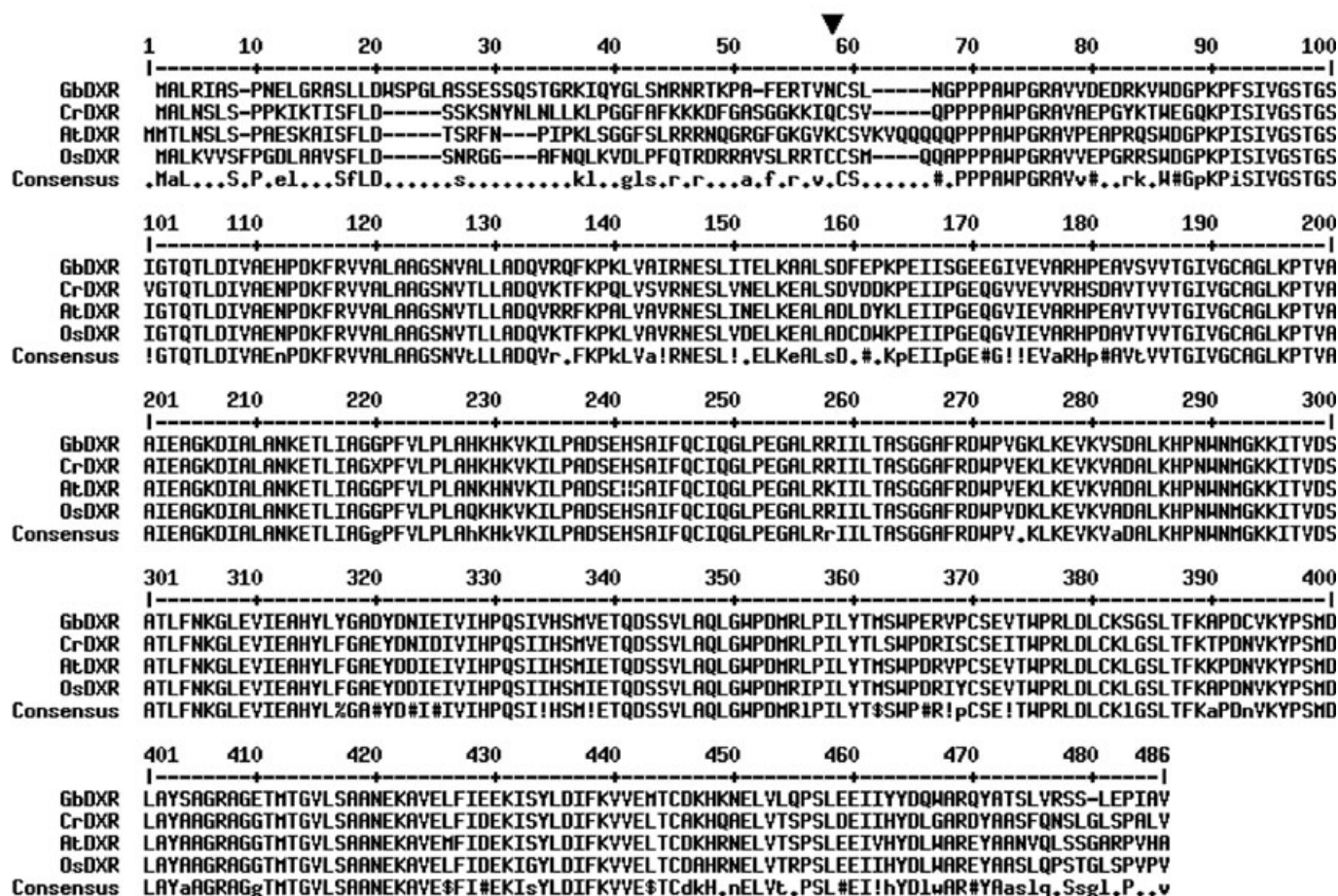


Fig. 3 Alignment of amino acid sequences of plant DXRs. Premature amino acid sequence of *G. biloba* DXR was aligned with those of CrDXR (*C. roseus*, AF250235), AtDXR (*A. thaliana*, AF148852), OsDXR (*Oryza sativa*, AF367205). Black arrow indicates the putative cleavage site of transit peptide sequence.

and B primer of each gene were used for the 5'-RACE, while the primer pairs of Generacer 3'-nested and F primer of each gene were used for the 3'-RACE. The reaction mixture for RACE contained 35 μ L dd H₂O, 5 μ L 10 $\times$ buffer, 3 μ L of 2.5 mM each dNTP, 2 μ L of 10 μ M each primer, 1 μ L cDNA (50 ng), and 0.5 μ L of 5 units/ μ L Takara Ex-Taq. The reaction was run as follows: 5 min at 94°C for the first cycle; 94°C for 1 min; 63°C for DXS 5'-RACE, 65°C for DXS 3'-RACE, 67°C for DXR 5'-RACE or 69°C for DXR 3'-RACE for 1 min, and 72°C for 1 min for 35 cycles; and 72°C for 5 min for the last cycle. The RACE products were cloned and sequenced as above. Open reading frame (ORF) of mature DXS without predicted plastid-target sequence was cloned by PCR using the primer pair of TDXS-START and DXS-STOP. For DXR, the TDXR-START and DXR-STOP primer pair was used. The resulting ORFs were cloned into pMW118 vector (Nippon Gene, Toyama, Japan) and named as pMW-DXS and pMW-DXR.

Complementation assay

E. coli *dxs* disruptant DXM3 [10] and *dxr* disruptant DYM1 [14] were maintained on Luria-Bertani (LB) medium supplemented with 0.01% 2-C-methyl-D-erythritol (ME) and kanamycin at 15 μ g/mL, transformed with pMW-DXS and pMW-DXR, respectively, and selected on LB medium without ME.

RT-PCR

The reaction was run in triplicate for 40 cycles on Rotor-Gene 2000 Real Time Amplification System (Corbett Research) using Qiagen Quantitect SYBR Green PCR system, which consisted of 1 $\times$ master mix, 0.5 μ M each primer, and 100 ng cDNA to a final volume of 50 μ L with RNase-free water. cDNAs for the quantification were prepared from about 10 plants. Temperature program was as follows: 95°C for 15 min for the first cycle, and 94°C for 15 s, 45°C for 30 s, and 72°C for 30 s for the rest of the run. For quantification standard, the PCR products of each gene were obtained from cDNA by PCR, and the concentrations of the products were measured at 260 nm to calculate the number of cDNA copies in the sample as described by Yin et al. [15]. The range of cDNA concentrations in standardization reactions was 10⁴ to 10⁸ copies/ μ L.

Chromatographic analysis of ginkgolides and bilobalide

Ten samples of ginkgo culture were ground, allowed to stand overnight in 10 mL methanol, and sonicated for 10 min. The extracts were filtered through a 0.45- μ m Target Nylon filter (National Scientific, CA, U.S.A.) and concentrated to 1.5 mL under the stream of N₂. The samples were then analyzed in duplicate on a Shimadzu LC-10ADVP HPLC equipped with an Alltech 500 Evaporative Light Scattering Detector operating at a drift temperature set at 110°C and N₂ flow at 3.2 standard liter per minute (SLMP). C18 column (4.6 mm $\times$ 150 mm, 3.5 μ m particle size, ZORBAX SB-C18, Agilent Technologies) was run at 30°C under the following gradient conditions: A:B = 90:10 for initial 20 min, 80:20 for subsequent 20 min, and 75:25 for the last 10 min, where A and B were 10 mM ammonium acetate buffer at pH 5.0 and methanol:isobutyl alcohol = 9:1, respectively. After each analysis, the column was washed with B for 5 min and equilibrated with the initial buffer for 10 min. Flow rate was 0.5 mL/min, and the sample size was 20 μ L. Standard solutions were prepared from ginkgolide A, ginkgolide B, and bilobalide (Sigma) to a final concentration of 30.0 μ g/mL.

Results and Discussion

Initial PCR with the primers designed for the conserved regions gave fragments of the expected sizes: 368 bp-long for DXS and 764 for DXR. Comparison of the amino acid sequences with those of CLA1 and DXR of *A. thaliana* showed high homologies at 84 and 89%, respectively, indicating that the fragments were derived from the putative genes. New pairs of primers for 3'- and 5'-RACE were designed based on the sequences of the conserved regions. By combining the sequence information of 3'- and 5'-RACE fragments, respectively 1345 and 1263 bp-long, ORF sequence of DXS consisting of 2217 bp was obtained and cloned by PCR. In the same manner, the ORF of DXR, 1434 bp-long, was secured from the fragments of 919 bp-long 3'-RACE and 498 bp-long 5'-RACE. The nucleotide sequences, designated as *GbDXS2* and *GbDXR*, were submitted to GenBank with accession numbers AY494185 and AY494186, respectively.

The deduced amino acid sequence of *GbDXS2* consisting of 739 residues (Fig. 2) was compared with those of the known plant DXSs. The phylogenetic clustering revealed that the cloned enzyme belonged to class 2 DXS (Fig. 4). The enzyme formed a separate group from the angiosperm DXSs in the dendrogram. Recently, yet another ginkgo DXS, belonging to class 1, was reported [10]; this gene was also separated from the angiosperm DXS1s (Fig. 4). Interestingly, the putative transit sequence of *GbDXS2*, needed for chloroplast targeting, was the longest among the known plant DXSs, consisting of about 70 amino acid residues. The putative mature protein, cut at R₇₀ to exclude the predicted transit

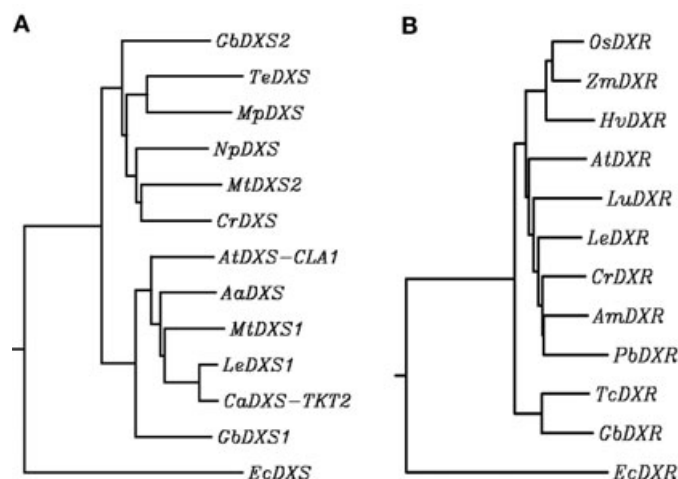


Fig. 4 Phylogenetic trees of DXS and DXR constructed using the program ClustalW with Phylip output format in default parameters. **A** DXS; *AtDXS-CLA1* (*A. thaliana*, U27099), *AaDXS* (*A. annua*, AF182286), *MtDXS1* (*M. truncatula*, AJ430047), *LeDXS1* (*L. esculentum*, AF143812), *CaDXS-TKT2* (*C. annuum*, Y15782), *TeDXS* (*T. erecta*, AF251020), *MpDXS* (*M. piperita*, AF019383), *NpDXS* (*N. pseudonarcissus*, AJ279019), *MtDXS2* (*M. truncatula*, AJ430048), *CrDXS* (*C. roseus*, AJ011840), *GbDXS1* (*G. biloba*, AY505128), *GbDXS2* (*G. biloba*, AY494815), *EcDXS* (*E. coli*, AE005174). **B** DXR; *TcDXR* (*T. cuspidata*, AY575140), *OsDXR* (*O. sativa*, NM_183490), *LeDXR* (*L. esculentum*, AF331705), *ZmDXR* (*Z. mays*, ZMA297566), *AtDXR* (*A. thaliana*, AY091405), *HvDXR* (*H. vulgare* subsp. *vulgare*, AJ583446), *AmDXR* (*A. majus*, AY770406), *CrDXR* (*C. roseus*, AF250235), *PbDXR* (*P. barbatus*, AY515699), *LuDXR* (*L. usitatissimum*, AJ623266), *GbDXR* (*G. biloba*, AY494816), *EcDXR* (*E. coli*, AB013300).

peptide region [16], was expected to have M_r of 71.5 kD and pI of 6.30, and showed about 80% homology with the other class 2 DXSs. The putative GbDXR had 478 amino acid residues (Fig. 3). The mature form predicted by ChloroP 1.1, devoid of a 55 residue-long transit sequence, had M_r of 46 kD and pI at 5.57. GbDXR clustered with *Taxus cuspidata* DXR in the phylogenetic tree and was also distinctively different from the angiosperm DXRs (Fig. 4). The homology of GbDXR to other DXRs ranged from 85 to 92%.

To confirm the functionality of *GbDXS2* in the MEP pathway, the mature protein cut at R_{70} was expressed as His-tagged in *E. coli*. The resulting protein, purified on a Ni-NTA column, failed to show enzyme activity (data not shown). Therefore, complementation of the mature sequence with *E. coli* DXM3, a *dxs* disruptant, was adopted as an alternative strategy to confirm the enzyme function [10]. The result indicated that the gene could rescue the disruptant, an indication that the mature protein was functionally active (Fig. 5). Complementation strategy was again employed for functional identification of *GbDXR*, because the expressed protein was not catalytically competent, and a *dxr* disruptant DYM1 [14] was rescued by the complementation with *GbDXR* (Fig. 5).

Transcription levels of the genes involved in ginkgolide biosynthesis were traced with respect to the age and distribution

among organs of the cultured embryo. Transcriptions of *GbDXS2*, *GbDXR*, and *LPS* in roots were most active 1 week after the initiation of embryo culture and gradually decreased thereafter (Fig. 6). It is noteworthy that *LPS*, specific in the ginkgolide biosynthesis, was exclusively detected in roots. The transcript of *GbDXS2* was also predominantly found in roots. On the other hand, *GbDXR* transcription was found both in the leaves and roots, albeit its level in leaves was 50 to 70% of that in roots. On the contrary, *GbDXS1* was more actively transcribed in leaves than in roots, except during the later stage of culture.

Walter et al., who first recognized the two classes of DXSs, suggested that class 1 DXS performs house-keeping function, while class 2 is involved in the carotenoid biosynthesis in the root of *Medicago truncatula* in response to the microbial invasion [9]. The present data that the transcription pattern of *GbDXS2* faithfully follows the pattern of *LPS* during the time course and organ distribution, strongly support that *GbDXS1* and *GbDXS2* play separate roles. A similar pattern was followed even by *GbDXR*, though in a lesser degree, because active DXR transcription was necessary for the biosynthesis of ginkgolide. The construction of a *GbDXS2*-knockout mutant would provide the proof for the specific involvement of *GbDXS2* in ginkgolide biosynthesis. Whether the detected basal transcription of DXS2 in the leaves represents the biosynthesis of ginkgolide in the leaves, in contrast to the known biosynthesis of ginkgolide in the roots [12],

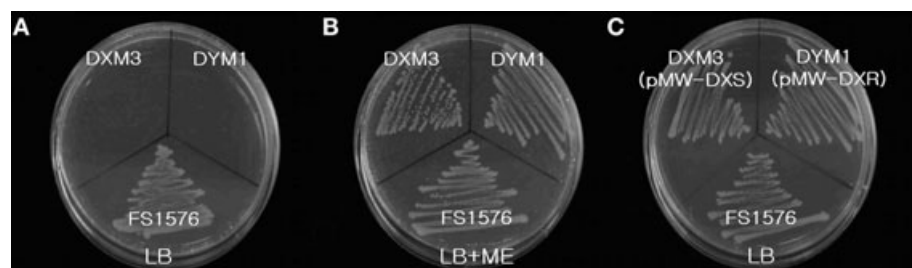


Fig. 5 Complementation assay using *E. coli* *dxs* and *dxr* disruptants DXM3 and DYM1, respectively. **A** Unlike the wild-type FS1576, both disruptants could not grow on the medium without ME. **B** Both disruptants grew on LB medium containing ME. **C** After each disruptant was transformed with the plasmid pMW-DXS or pMW-DXR, transformants could grow without ME.

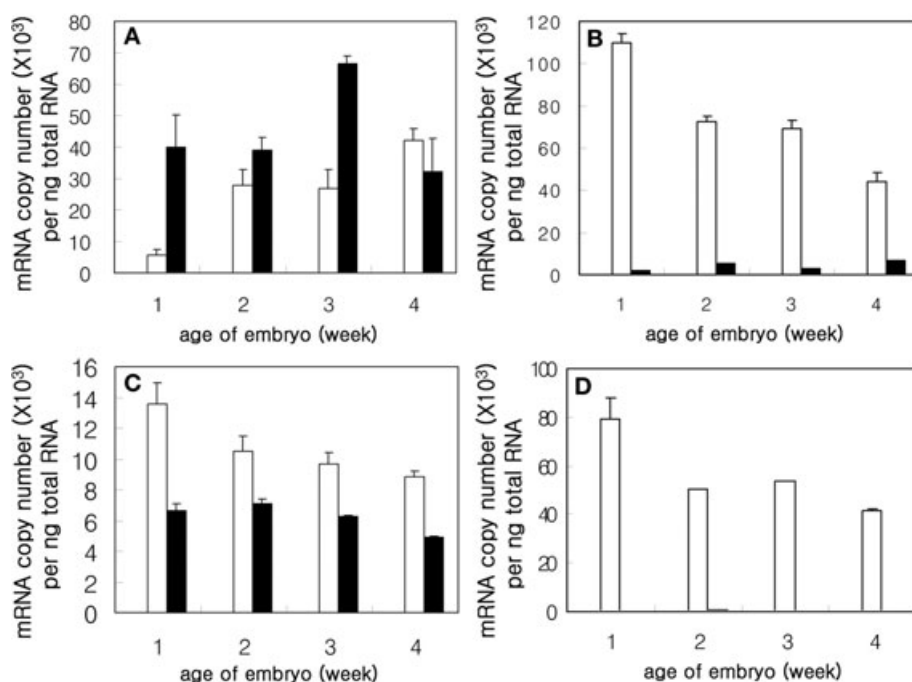


Fig. 6 Gene transcription patterns of DXSs, DXR, and *LPS* in *G. biloba*. White, embryonic roots; black, leaves. **A** *GbDXS1*; **B** *GbDXS2*; **C** *GbDXR*; **D** *LPS*.

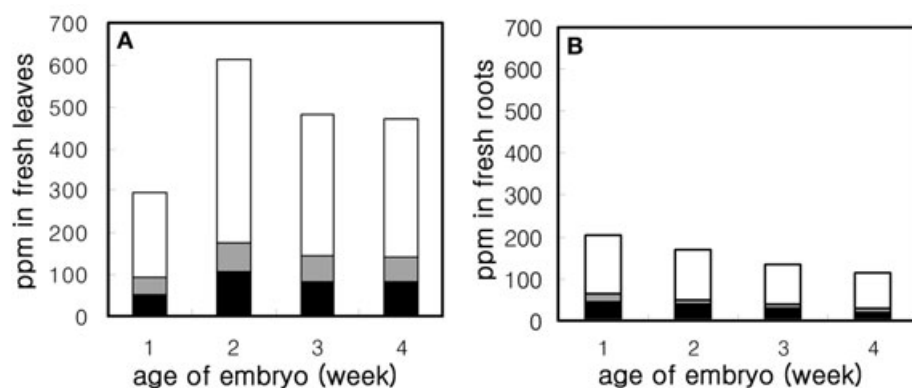


Fig. 7 Contents of ginkgolides and bilobalide in *G. biloba* embryo. White, bilobalide; gray, ginkgolide B; black, ginkgolide A. **A** embryonic leaves; **B** roots.

or cross-reactivity of the PCR primers, although not likely, is yet to be determined.

To further justify the predominant transcriptions of *DXS2* and *LPS* in the roots, contents of ginkgolides and its degradation product bilobalide were measured, and the results showed that ginkgolide content in the root directly reflected the transcription level of the two genes, further supporting the roles of *GbDXS2* and *LPS* (Fig. 7) in the biosynthesis of ginkgolides; however, the root content was 1/3 to 1/4 of that in leaves throughout the culture period, except at week 1, when the contents were comparable. This high content of ginkgolides in leaves, despite the exclusive transcription of ginkgolide biosynthesis genes in roots, could be easily justified by the purported translocation of ginkgolide from root to leaves [12].

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