Plant Mol Biol (2015) 89:451–462
DOI 10.1007/s11103-015-0381-3

Investigating sesquiterpene biosynthesis in *Ginkgo biloba*: molecular cloning and functional characterization of (*E*,*E*)-farnesol and α-bisabolene synthases

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Received: 16 July 2015 / Accepted: 15 September 2015 / Published online: 6 October 2015

Abstract *Ginkgo biloba* is one of the oldest living tree species and has been extensively investigated as a source of bioactive natural compounds, including bioactive flavonoids, diterpene lactones, terpenoids and polysaccharides which accumulate in foliar tissues. Despite this chemical diversity, relatively few enzymes associated with any biosynthetic pathway from ginkgo have been characterized to date. In the present work, predicted transcripts potentially encoding enzymes associated with the biosynthesis of diterpenoid and terpenoid compounds, including putative terpene synthases, were first identified by mining publicly-available *G. biloba* RNA-seq data sets. Recombinant enzyme studies with two of the TPS-like sequences led to the identification of *GbTPS1* and *GbTPS2*, encoding farnesol and bisabolene synthases, respectively. Additionally, the phylogenetic analysis revealed the two terpene synthase genes as primitive genes that might have evolved from an ancestral diterpene synthase.

Keywords Sesquiterpene · Bisabolene synthase · Farnesol synthase · *Ginkgo biloba*

The sequences reported in this study have been deposited in the GenBank database (Accession Numbers: GbTPS1, KM248383 and GbTPS2, KM248384).

Electronic supplementary material The online version of this article (doi:10.1007/s11103-015-0381-3) contains supplementary material, which is available to authorized users.

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Introduction

Terpenoids are a large family of specialized metabolites that are ubiquitous within the plant kingdom, and play essential roles in direct and indirect defenses against herbivores and pathogens, pollinator attraction, and plant–plant interactions. Terpenoid compounds include monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20). Sesquiterpenes represent the largest and most diverse group of terpenoid compounds and carry out some of the most important physiological functions in plants. For example, volatile hydrocarbon sesquiterpenes are responsible for conferring pungent or aromatic flavors to specific plant tissues (Chen et al. 2003; Köllner et al. 2004), and are also released in response to wounding and insect attack (Bohlmann et al. 1998b; Keeling and Bohlmann 2006; Schnee et al. 2006). In gymnosperms, such as *Picea* spp. and *Abies grandis*, the production of abundant and complex mixtures of terpenoids in the form of oleoresin secretions represents a major component of their defensive repertoire (Keeling and Bohlmann 2006; Bohlmann et al. 1998b). In addition, sesquiterpenes are extensively used by humans for various pharmacological purposes and as aromatic and flavouring agents (Kirby and Keasling 2009). Recently, sesquiterpenes have been examined as potential advanced biofuel precursors and farnesene and bisabolene were identified

as biosynthetic alternatives to diesel fuel (Lee et al. 2008; Peralta-Yahya et al. 2011; Renninger and McPhee 2008).

Terpene synthases (TPSs) are the catalytic enzymes responsible for production of an array of terpenes in plants. Structurally, plant mono- and di-terpene synthases typically consist of three different domains—α, β and γ. The terpene synthase Class I active site containing the characteristic DDXXD motif is located within the γ domain, and the functional Class II active site characterized by a conserved DXDD motif is present at the interface of the β and γ domains (Christianson 2006). Within the Class I active site, binding of Mg^{2+} to metal binding motifs initiates the cyclization of substrates such as FPP into cyclic sesquiterpenes via an ionization-dependent mechanism. In contrast, the Class II active site initiates the cyclization of linear isoprenoids via a protonation-dependent mechanism (Keeling et al. 2008, 2011). Although plant mono- and diterpene synthases have all three domains, most plant sesquiterpene synthases have been shown to possess only two of these domains, viz., β and γ (e.g., *Nicotiana tabacum* aristolochene synthase—Starks et al. (1997) and *Gossypium arboreum* cadinene synthase—Gennadios et al. 2009), though the atypical bisabolene synthase from *Abies grandis* is comprised of all three (McAndrew et al. 2011).

Evolutionarily, the majority of plant terpene synthases are classified into three subfamilies: TPSa (sesquiterpene and diterpene synthases from angiosperms), TPSb (monoterpene synthases from angiosperms of the Lamiaceae), and TPSd (gymnosperm monoterpene, sesquiterpene, and diterpene synthases). Three additional minor subfamilies each represented by a single angiosperm terpene synthase type are: TPSc containing copalyl diphosphate synthase, TPSe containing kaurene synthase, and TPSf containing linalool synthase. Terpene synthases from the gymnosperm-specific TPSd subfamily further segregate into the TPSd1, TPSd2, and TPSd3 clades based on the previous phylogenetic analysis of 29 (Martin et al. 2004) and 72 gymnosperm TPSs (Keeling et al. 2011). The TPSd1 subfamily mainly consists of monoterpene synthases, and all diterpene synthases cluster within the TPSd3 clade. The majority of gymnosperm sesquiterpene synthases fall within the TPSd2 clade, although some cluster within TPSd1 or TPSd3 (Martin et al. 2004; Keeling et al. 2011). However, the recently published detailed phylogenetic analysis consisting of previously known gymnosperm TPSs and newly published 83 white spruce TPSs sequenced by Warren et al. (2015), had shown that the gymnosperm TPS family consists of four major clades comprising of hemiterpene synthases, monoterpene synthases, sesquiterpene synthases and diterpene synthases (Warren et al. 2015).

Ginkgo biloba L. (Maidenhair tree), of the order Ginkgoales, originated approximately 150 million years ago and is considered a living fossil (Chamberlain 1957). Ginkgo leaf extracts contain a number of bioactive compounds which include flavonoids, diterpene lactones, terpenoids and polysaccharides, and for this reason have been used for centuries in Traditional Chinese Medicine (Zeng et al. 2013). The two major bioactive terpene lactones of ginkgo leaves, ginkgolides (diterpenes) and bilobalides (sesquiterpenes), have been isolated and studied in detail (Huang et al. 2003; Strømgaard and Nakanishi 2004; Liao et al. 2004). Twelve different enzymes involved in the biosynthesis of terpenes in *G. biloba* have been identified to date, including 11 genes associated with ginkgolide biosynthesis and one encoding a Farnesyl Diphosphate Synthase [FPS] (Zeng et al. 2013; Wang et al. 2004). Despite this progress, relatively little is known concerning the regulation or specific defensive roles played by terpenes within *G. biloba*. Furthermore, characterizations of enzymes associated with the biosynthesis of sesquiterpenes in *G. biloba* have not been reported in the literature. In the present work, publicly-available RNA-seq data was mined to identify expressed members of the ginkgo TPS gene family. We report both the cloning and functional analysis of two previously-uncharacterized sesquiterpene synthases from *G. biloba*.

Materials and methods

Plant materials

Plant materials were collected from authenticated *G. biloba* plants growing in the Medicinal Plant Garden facility at the University of Mississippi. Ginkgo seeds (lot #: S2862-500) were purchased (Richters Herbs, Canada) and were kept at 8 °C for 15 days for cold stratification. The seeds were surface sterilized in 1:8 bleach (6 % sodium hypochloride): water for 10 min and rinsed 3 times in distilled water. Sclerotesta were removed and seeds were covered in vermiculite for germination. The pots were maintained in growth chambers at 25 °C, 16/8 h photoperiod at 150 μmol m^{-2} s^{-1}. The different tissues such as roots, stems, young leaves, (1–3 cm), mature leaves and shoot apices were harvested from 15 seedlings after they reached 7 cm above the soil level. The tissues were snap frozen in liquid nitrogen and stored at −80 °C.

RNA isolation

Ginkgo leaves contain high levels of flavonoid glycosides, terpene lactones, carbohydrates and polyphenolic secondary metabolites which hinder RNA isolation. Therefore, an improved protocol proposed by Wang et al. (2005) for isolation of high quality RNA exclusively from *G. biloba* leaves was used. The addition of polyvinylpolypyrrolidone

(PVPP) instead of polyvinylpyrrolidone (PVP) to cetyltrimethylammonium bromide (CTAB) buffer helped in the separation of nucleic acids from polyphenols which form a complex with PVPP. Furthermore, β-mercaptoethanol added at high concentrations (4 %, v/v) helped in inhibiting RNase activity and prevented sample oxidation. For isolation of high quality RNA from different tissues for RT-qPCR, the protocol from Wang et al. (2005) was used except for shoot apex. Shoot apical RNA was isolated using the MOBIO Power Plant RNA isolation kit as per the manufacturer's instructions. To remove possible contaminant DNA from the RNA samples, the RNAs were treated with RNase-free DNase using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA recovery and purity were determined using the NanoDrop ND-1000 Spectrophotometer and sample integrity was also assessed by agarose gel electrophoresis. Samples where the sharpness of the 26 and 18 S bands were degraded were discarded.

Isolation of cDNA clones for terpene synthases

5′and 3′ cDNAs were synthesized from 1 μg of total RNA using SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA) as per the manufacturer's protocol. The RACE PCR was carried out for two different genes using different primer pairs (Table S1). The single bands were gel-extracted and then cloned into the TOPO-TA cloning vector. The clones were sequenced to confirm the amplified product. The full length ORFs were obtained by secondary amplification from the full length cDNAs. The cDNAs were prepared using ProtoScript M-MULV first strand cDNA synthesis kit (New England Biolabs, Ipswich, MA). GbTPS1 and GbTPS2 DNA sequences were deposited in NCBI GenBank and the accession numbers were obtained. The conceptually translations and alignments with known sesquiterpene synthases from both angiosperms and gymnosperms were performed with ClutalW in GeneDoc (Nicholas and Nicholas 1997).

Production of TPS proteins and enzyme kinetic studies

The ORFs for each of the genes were ligated into the pET28b expression vector (EMD Millipore, Billerica, MA) and transformed to *E. coli* BL21 (DE). Two milliliter cultures initiated from single colonies were grown overnight at 37 °C. The cultures were then diluted 1:50 and grown at 37 °C till the OD_{600} reached ~0.6 (grown for 3 h). Then IPTG was added to a final concentration of 0.5 mM and the cultures were again incubated for 3 h, at 30 °C and 220 rpm. The cells were then collected by centrifuging at 3500*g* for 5 min and resuspended in lysis buffer containing 50 mM Tris–Cl pH-7.5, 5 mM $MgCl_2$ and 1 mM DTT. Lysozyme stock was added to a final 2 mg mL^{-1} concentration and incubated on ice for 30 min. The cells were lysed using a sonicator (Thermo Fisher Scientific Inc., Waltham, MA) at 50 % amplitude, three times for 20 s on ice and the clear supernatant (centrifuged at 13,000 rpm for 10 min at 4 °C) was used for enzymes assays.

Enzyme assays were initiated by mixing 45 μl supernatant with 5 μl of assay buffer (50 mM Tris–Cl, pH 7.5, 5 mM $MgCl_2$) and 0.5 μCi of 1-^{3}H FPP/1-^{3}H GGPP (Perkin Elmer, Waltham, MA) in a total reaction volume of 50 μl. The reactions were incubated at room temperature for 15 min, extracted with 150 μl hexane, un-reacted prenyl diphosphates and prenyl alcohols were removed with silica gel, and incorporation of radioactivity measured by scintillation counting.

For GC–MS profiling of the enzyme reaction products, 45 μl of the cleared supernatant was incubated with 10 μg of FPP in a glass vial and overlaid with equal volume of hexane for 90 min at room temperature (Maille et al. 2004). The reaction mixture was then vortexed vigorously for approximately 2 min and the hexane fraction allowed to separate. Reaction products (in the hexane fraction) were analyzed using an Agilent 7890 GC instrument equipped with an Agilent 5975C mass specific detector and an Agilent 7693 auto-sampler (Agilent Technologies, Santa Clara, CA). A fused silica capillary column (30 m, 0.25 mm i.d.) coated with a 0.25 μm film of cross-linked 5 % phenyl methyl silicone (J&W HP-5MS) was used with helium as the carrier gas at a flow rate of 1 mL/min. In a typical analysis, the oven was held for 2 min at 50 °C, and then programmed at 8 °C/min to a final temperature of 280 °C and held for 10 min. The injector temperature was 260 °C. The split ratio was set to 20:1. Mass spectra with the EI source were recorded at 70 eV from m/z 40 to 650. Compound identification involved comparison of the spectra in the Wiley and NIST databases using a probability-based matching algorithm. Further identification was based on the Relative Retention Indices (RRI) compared with literature and the reference standards isolated in-house or purchased from commercial sources.

The enzyme kinetic studies were carried out with the purified enzymes. The bacterial expressed enzymes were purified using Ni–NTA column (Invitrogen, Grand Island, NY) per the manufacturer's protocol. The purified protein fractions were concentrated using Millipore Ultracentrifugation filter units and stored in 300 mM NaCl, 20 mM Tris–Cl, pH 7.5, 5 mM DTT, 2 mM $MgCl_2$, 50 % glycerol (v/v) at −80 °C. Protein concentrations were estimated by Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. The enzyme assay was carried out with 1.3 μg of purified protein in 50 μl reaction volume. [1-^{3}H] FPP (Perkin Elmer; 23 Ci $mmol^{-1}$) was spiked into 0.1 mM FPP and incubated with the enzyme and assay buffer for 30 min. The products were extracted

in 150 µl hexane and un-reacted prenyl diphosphates and prenyl alcohols removed with silica gel. The incorporation of radioactivity was measured using a liquid scintillation counter. Kinetic properties were determined with substrate concentrations from 10 to 1000 µM for GbTPS1 and 10 to 250 µM for GbTPS2, in triplicate for each concentration. Apparent V_{max} and K_m values were calculated with the Enzyme Kinetics Module in GraphPad Prism 5.

NMR analysis of in vivo products

For expression in yeast, *GbTPS2* was inserted into pYES2 plasmid (Invitrogen) behind the β-galactosidase gene. The pYES2–GbTPS2 construct was then transformed into yeast INVSc1 strain using the lithium-acetate method (Geitz and Woods 2002). Also the pYES2 plasmid without any gene insert was transformed into INVSc1 yeast and served as control. Fifty ml of yeast culture harboring pYES2–GbTPS2 plasmids were grown in CSM medium lacking Uracil overnight, at 30 °C, 210 rpm. The cells were then collected by centrifuging at 3500 rpm for 5 min and resuspended in 1–2 ml induction medium containing 2 % galactose. The cells were then grown in multiple sets of 1 L induction medium at 30 °C, 210 rpm for 6 days. When the sesquiterpene production became saturated, equal volume of acetone was added to the culture broth and incubated for an additional 6 h at 30 °C, shaking at 200 rpm. The culture-acetone mixtures were then extracted with equal volume of hexanes and the hexane extracts were carefully dried using rotavap without heat to avoid losses to volatility of the sesquiterpenes.

The hexane extract (175 mg) was loaded on a SNAP cartridge (KP-Sil 100, Biotage) for separation on a Biotage Isolera Four chromatography system, eluted by hexane/ethyl acetate solvent system with increasing ethyl acetate concentration from 0 to 50 %. The collected fractions were monitored by the UV detector at 210 nm. After verified by thin-layer chromatography (TLC), the fractions containing the same component(s) were combined, and subjected to NMR analysis for structure identification.

TLC was performed on silica gel 60 GF254 plates (EMD Millipore) with hexane/ethyl acetate (8:2) as the solvent system for product separation. The isolates were visualized using UV light (254 nm), followed by spraying with 1 % vanillin-H_2SO_4 reagent.

NMR spectra were recorded at 25 °C on an Agilent DD2-500 NMR spectrometer (Santa Clara, CA) with a OneNMR probe. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.24 ppm, δ_C 77.23 ppm). Specific rotation was measured using a Rudolph Research AutoPol IV polarimeter at room temperature.

Phylogenetic analysis

GbTPS1 and GbTPS2 protein sequences were analyzed phylogenetically by comparing with functionally characterized terpene synthases from other gymnosperm and angiosperm gene sequences retrieved from Genbank. The protein alignments were prepared using ClustalW and the Neighbor joining tree with hundred bootstrap replicates was constructed in MEGA6 software (Tamura et al. 2013). Levopiramadiene synthase, the only terpenoid synthase cloned and characterized from ginkgo (Schepmann et al. 2001), was also included for the analysis.

Gene expression analysis in the tissues of *G. biloba*

RNA samples were treated with DNase I "on column" as per manufacturer's instructions using Qiagen RNeasy® (Qiagen) columns to remove residual DNA contamination. First strand cDNAs were synthesized from 1 µg of total RNA in a 50 µL reaction volume using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. Quantitative real-time PCR reactions were performed in triplicate using the GenAmp® 7300 Sequence Detection System (Applied Biosystems). Independent PCR reactions were performed using the same cDNA for both the gene of interest and 18S rRNA, using the SYBR® Green PCR Master Mix (Applied Biosystems) with the following primer pairs: GbTPS2—forward: 5′-AACCACTCCCAATGTAATTAATATTTGTG-3′, reverse: 5′-TGAAGGAAATCAAACACACACGTATAC-3′; 18S rRNA—forward: 5′-GGCTCGAAGACGATCAGATACC-3′, reverse: 5′-TCGGCATCGTTTATGGTT-3′. Gene-specific primers were designed by first retrieving closely-related *G. biloba* coding sequences via BLASTN analyses using GbTPS2 coding sequences as queries against all publicly-available *G. biloba* transcriptomics data. Sequences thus identified were then aligned with GbTPS2 using ClustalW, and GbTPS2-specific primers were then designed using Primer Express® software (Applied Biosystems).

The PCR conditions consisted of denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the GeneAmp® 7300 sequence detection system. A negative control reaction in the absence of template (no template control) was also routinely performed in triplicate for each primer pair. The change in fluorescence of SYBR® Green I dye in every cycle was monitored by the GenAmp® 7300 system software, and the threshold cycle (C_T) above background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the

gene of interest to obtain a ΔC_T value. The C_T value of an arbitrary calibrator was subtracted from the ΔC_T value to obtain a $\Delta\Delta C_T$ value, and relative gene expression levels are expressed as $2^{-\Delta\Delta CT}$.

Results

Identification and isolation of cDNA clones for terpene synthases

The recent availability of transcriptomic and metabolomic data for *G. biloba*, (Medicinal Plant Genome Resources server (MPGR)—http://www.medicinalplantgenomics.msu.edu; Lin et al. 2011) has significantly expanded the resources available for this species. The metabolic profile of *G. biloba* obtained from MPGR revealed the presence of terpenes in six different tissues with the highest content found in lateral roots, mature leaves and seedlings. Furthermore, when the metabolome was screened for metabolites that yielded ions in the range of m/z 202–206 (m/z 204 is diagnostic for sesquiterpenes), several metabolites with m/z 203–205 were found within roots and exhibited maximal accumulation in mature leaves (Fig. S1).

BLAST searches were then performed against available *G. biloba* transcriptomic data sets using a Levopimaradiene Synthase (LPS) sequence from *G. biloba* as the sequence query (Schepmann et al. 2001). Two predicted transcripts (sequence ID nos. gb58799 and gb19838—http://www.medicinalplantgenomics.msu.edu) were prioritized which showed extensive similarity to known plant terpene synthases, and efforts to obtain the corresponding full-length ORFs using 5′ and 3′ RACE were thus initiated using gene-specific PCR primer pairs (Table S1). High quality total RNAs were first prepared from leaf tissues using an improved protocol devised by Wang et al. (2005) to circumvent problems associated with high levels of flavonoid glycosides, terpene lactones and other secondary metabolites in *G. biloba* leaves. Full-length Open Reading Frames (ORFs) were successfully obtained for both TPS-like sequences, which were confirmed by RT-PCR using 5′ and 3′ flanking primers followed by sequence analysis. Both ORFs encoded proteins which were found to be highly similar to previously-characterized plant TPSs, and in addition contained the signatory TPS DDXXD domains. The second characteristic conserved motif, DXDD is replaced by DLEI in both GbTPS1 and GbTPS2 (Bohlmann et al. 1998a; Fig. 1).

Biochemical characterization of GbTPS1 and GbTPS2

Recombinant enzyme studies were pursued to directly determine the catalytic activities of both putative TPSs. Extracts from *E. coli* cells over-expressing both full-length ORFs were used for enzyme assays. The assays were carried out by incubating the cell free extracts with radiolabeled 30 μM FPP or GGPP, and the conversion of these substrates to hexane extractable products was measured by scintillation counting (Maille et al. 2004; Yeo et al. 2013). The activities of putative terpene synthases were analyzed relative to a negative control, an extract prepared from *E. coli* harboring the same expression plasmid without any putative TPS gene inserts. The activity was negligible when GGPP was used as substrate by extracts prepared from *E. coli* expressing either of the *G. biloba* recombinant genes, but in contrast exhibited high sesquiterpene synthase activity as compared to the empty vector control when incubated with FPP (Fig. 2). The two sequences were subsequently designated GbTPS1 and GbTPS2.

The hexane-extractable products obtained from the in vitro enzyme assays performed with bacterial extracts prepared from cells expressing either of the TPS cDNAs were then analyzed by GC–MS (Fig. 3). The GC–MS profiles of the reaction products generated by the bacterial extracts heterologously expressing either the GbTPS1 or GbTPS2 cDNA showed single dominant peaks for each of the assays with retention times of 24.87 and 22 min, respectively (Fig. 3A, C). The search of the NIST library using the corresponding mass spectra (Fig. 3B, D) identified the GbTPS1 reaction product as farnesol, and bisabolene for GbTPS2. Corroborating evidence for the farnesol assignment was based on comparisons to an authentic standard. The resulting farnesol was identified as (*E,E*)-farnesol (*trans*,*trans*-farnesol). However, while the mass spectrum of the GbTPS2 reaction product matched that for a bisabolene, confirmation for a specific isomer based on retention time was not possible because standards for any of the bisabolene isomers were not available. Hence, we sought sufficient reaction product of the GbTPS2 enzyme for NMR analysis by over-expressing the GbTPS2 gene in yeast.

The GbTPS2 ORF was inserted in a galactose-inducible expression vector and transformed into the yeast INVSc1 strain. Cells from 4 L of recombinant yeast culture grown under induction conditions were subsequently pelleted, lysed with acetone, and the hexane extractable compounds were then examined by TLC. As expected, examination of the yeast extracts from control cells (no TPS2 gene), versus cells expressing GbTPS2, demonstrated a distinct product associated with TPS2-expressing cells on the silica TLC plate, with a retention time close to that of the solvent (Fig. S2). This compound was isolated by conventional silica chromatography from an initial 175 mg extract preparation, yielding 6.9 mg of the purified compound. The purified compound was identified as (−)-α-bisalolene based on 1D and 2D NMR data analysis (Figs. 4 and S3), and its specific optical rotation value [$[\alpha]_D^{20}$ −92.6 (*c* 0.233, $CHCl_3$)] was

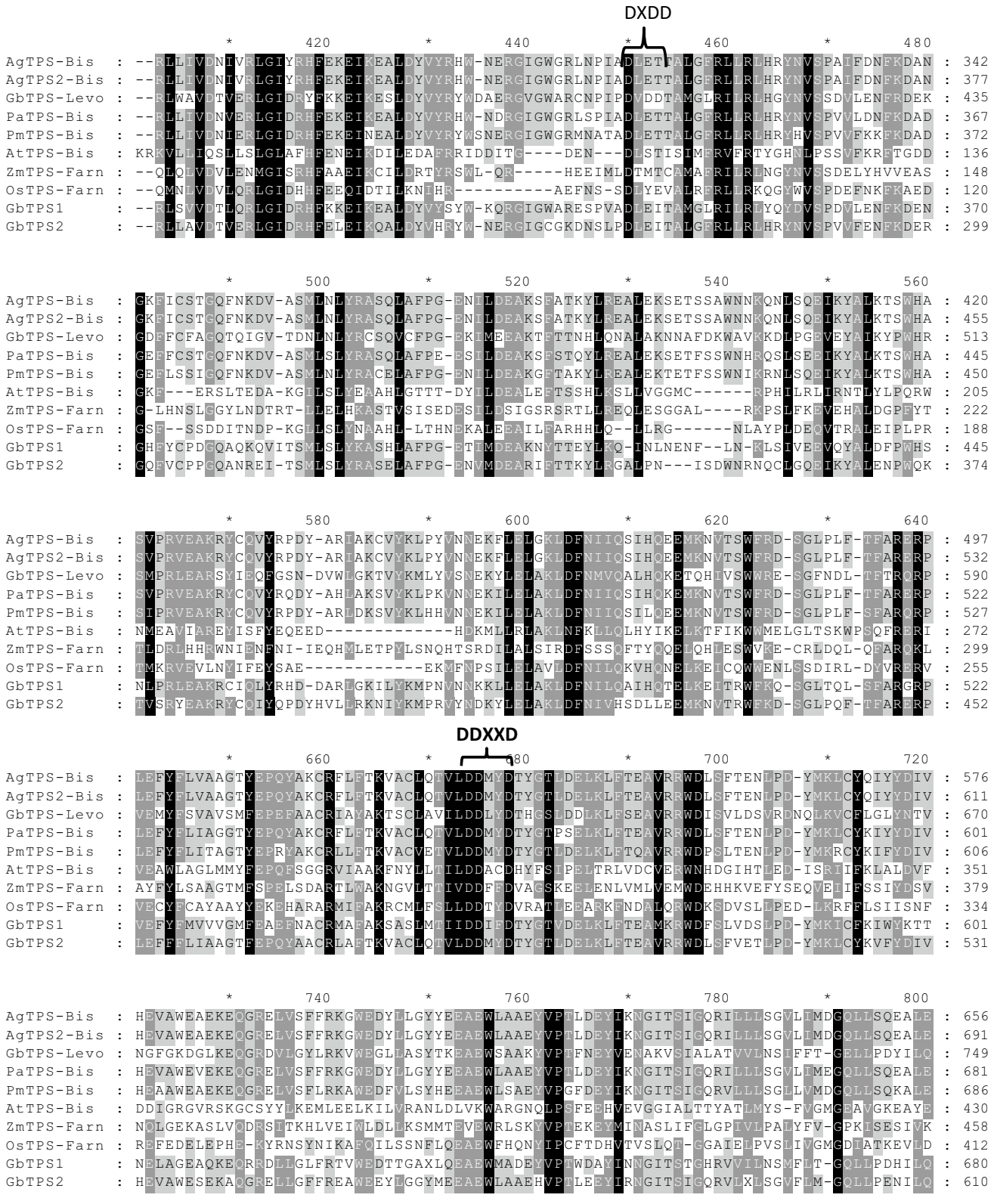

Fig. 1 Amino acid alignments of GbTPS1 and GbTPS2 with sesquiterpene and diterpene synthases from plants generated using ClustalW and Genedoc. Aspartate rich motifs, DXDD and DDXXD, necessary for the binding of cationic cofactors are shown

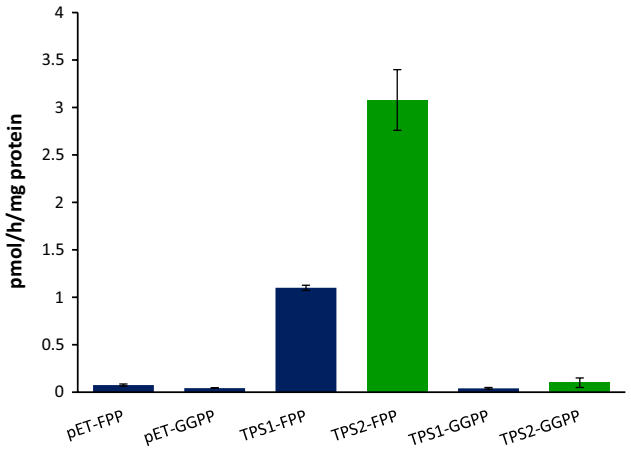

Fig. 2 Substrate specificity of Gb-TPS1 and Gb-TPS2 using ^{3}H-FPP and ^{3}H-GGPP as substrates. Data are expressed as mean ± S.D

also determined, similar to the values reported earlier by Bohlmann et al. (1998a) for the same compound isolated from *Abies grandis*.

Both GbTPS1 and GbTPS2 were subsequently expressed in *E. coli* with carboxy-terminal His-tags to facilitate their isolation, and the expressed proteins were both isolated using a Ni–NTA affinity column. The apparent molecular mass of the purified protein for GbTPS1 was 93kDa (data not shown) and 61kDa for GbTPS2 (Fig. S4), which corresponded to the sizes predicted from the conceptual translations of their respective cDNAs. The purified enzymes were then used to determine their Michaelis–Menten kinetic properties. Both enzymes exhibited a hyperbolic FPP saturation kinetic plot with apparent K_m values of 142.9 μM for GbTPS1 and 77.42 μM for GbTPS2 (Fig. 5). The calculated k_{cat} values were 0.004 s^{-1} for GbTPS1 and 0.01 s^{-1} for GbTPS2.

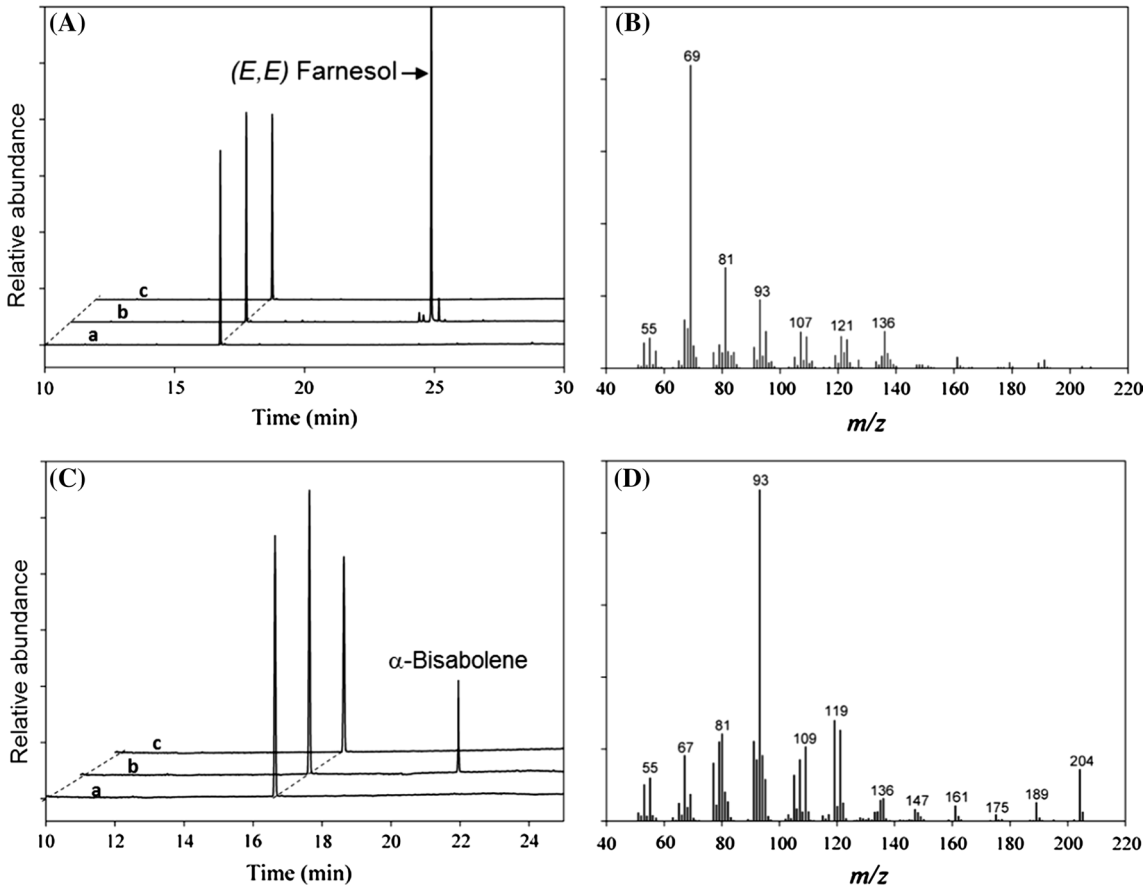

Fig. 3 GC-MS profiles of in vitro reaction products generated by GbTPS1 and GbTPS2. Bacterial lysates prepared with *E. coli* cells harboring 'empty' pET28 vector (*a*) and incubated with FPP, or lysate prepared from bacteria harboring the pET28–GbTPS1 **A** or pET28–GbTPS2 **C** recombinant plasmid and incubated with FPP (*b*) or GGPP (*c*). **B** The corresponding mass spectrum of farnesol (**A**; RT = 24.87 min) produced by recombinant GbTPS1. **D** The corresponding mass spectrum of bisabolene (**C**; RT = 22 min) produced by GbTPS2

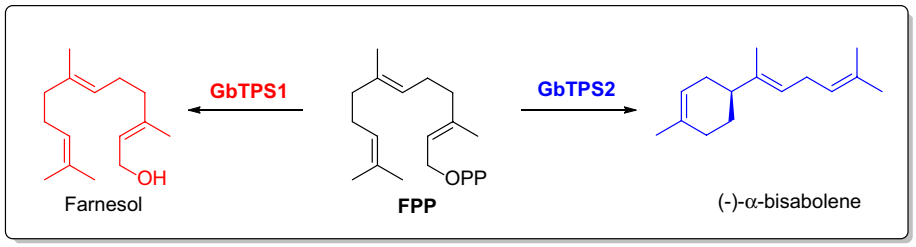

Fig. 4 Products obtained from recombinant GbTPS1 and GbTPS2 in transformed *E.coli* and yeast

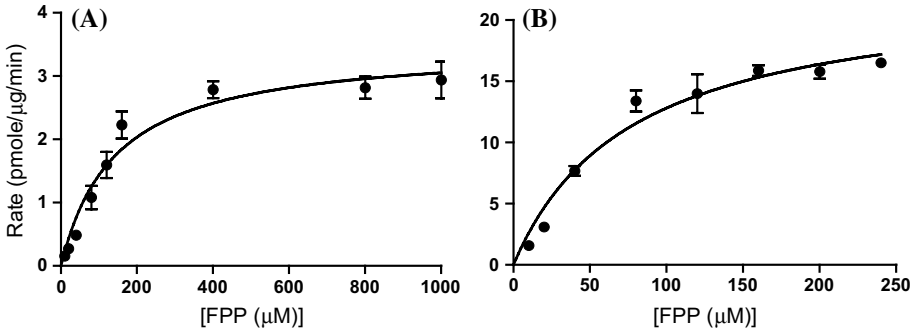

Fig. 5 Plots of **A** GbTPS1 and **B** GbTPS2 reaction rates versus FPP concentration. The FPP saturation curves were determined at pH 7.0 and the Michaelis–Menten model was used for the determination of the K_m and V_{max}. Data are expressed as mean ± S.D

Phylogenetic analysis of GbTPS1 and GbTPS2

GbTPS1 and GbTPS2 were analyzed for their phylogenetic relationship to other terpene synthases broadly as well as bisabolene synthases from gymnosperms. The GbTPS1 and GbTPS2 synthases clustered most closely with the previously characterized gymnosperm sesquiterpene synthases for bisabolene and farnesene belonging to the sesquiterpene synthase clade, as compared to monoterpene and diterpene synthases from the respective clades (Fig. 6). Moreover, the terpene synthases from ginkgo form separate clusters isolated from other major classes of sesqui-TPSs. This was evident from the size of the GbTPS proteins of 804 and 738 amino acids, which appear to harbor amino terminal, ∝-domains of ~100 amino acids of unknown contribution to the catalytic capacity of the enzymes. The phylogenetic alignment also illustrates that other bisabolene synthase and bisabolene synthase-like sequences from *Arabidopsis* and other angiosperms cluster independently into clade TPSa, distant from the *G. biloba* terpene synthases. Of further significance, the phylogeny suggests that the bisabolene synthase class d genes evolved prior to the evolutionary split of conifers from ginkgo (Fig. 6).

GbTPS1 and *GbTPS2* steady-state transcript levels in various *G. biloba* tissues

Relative *GbTPS1* and *GbTPS2* steady-state transcript levels were determined in *G. biloba* shoot apices, immature and mature leaves, seeds, roots, and stem tissues by quantitative real-time RT-PCR (RT-qPCR, Fig. 7). The highest *GbTPS1* transcripts levels were observed in seeds and the lowest were observed in stems followed by roots. *GbTPS1* expression was similar in shoot apices, immature leaves and mature leaves. *GbTPS1* relative transcript levels observed in seeds were approximately 30-fold higher than the levels observed in stem tissues (Fig. 7A). The highest *GbTPS2* transcripts levels were observed in seedling root systems, which were approximately twofold higher than the levels observed in mature leaves. *GbTPS2* transcript levels were substantially higher in these two tissues than all others analyzed, and the lowest levels were observed in mature seeds and stems (Fig. 7B). These analyses also indicated that *GbTPS2* transcript levels are highly regulated during leaf development, as approximately 80-fold higher levels were observed in mature leaves relative to immature leaves.

Discussion

In this study two cDNA clones encoding sesquiterpene synthases (*GbTPS1* and *GbTPS2*) were isolated from *Ginkgo biloba* L. The protein sequences derived from both clones included the DDXXD conserved domain that is characteristic of terpene synthases (Tarshis et al. 1994; Lesburg et al. 1997; Starks et al. 1997; Little and Corteau 2002). The arginine and aspartate residues contained within this motif in terpene synthase Class I active sites are considered to be involved in the binding and cleavage of prenyl diphosphate substrates by chelating a divalent metal ion, typically Mg^{2+} (Little and Corteau 2002). In bisabolene synthase from *A. grandis* (AgBIS), these motifs (DDMYD and DDTKTYKAE) bind Mg^{2+}, which initiates the cyclization of FPP

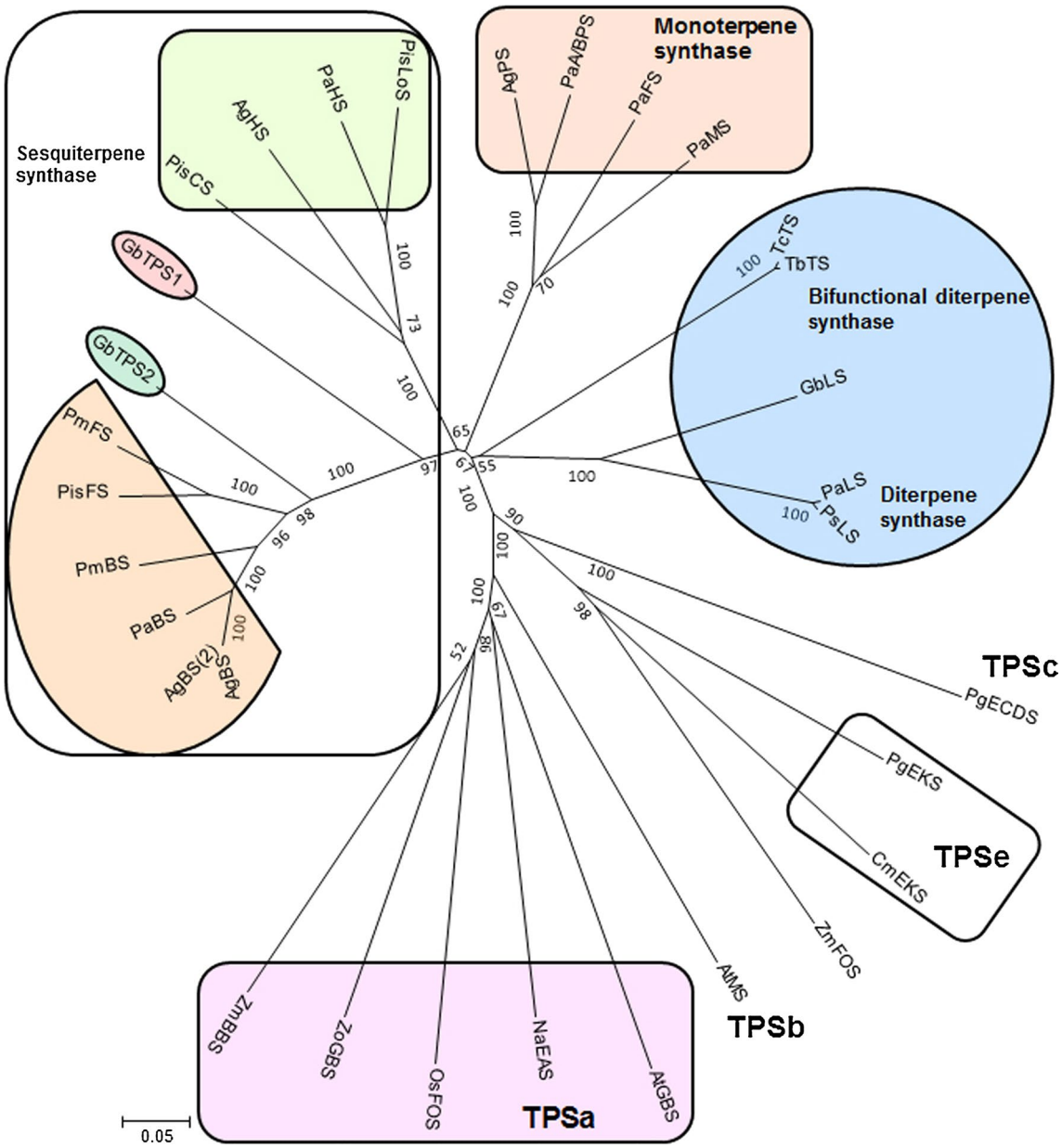

Fig. 6 Phylogenetic relationships of selected functionally characterized terpene synthases from angiosperms and gymnosperms. Protein alignments were prepared using ClustalW and the phylogenetic tree was constructed using the neighbor-joining method with 100 bootstrap iterations in MEGA6. The GbTPS1 and GbTPS2 (*encircled*) form different clades that are distinct from any other previously characterized terpene synthases. (*Abbreviations* AtGBS, *Arabisopsis thaliana* gamma-bisabolene synthase; AtMS, *A. thaliana* myrcene/ocimene synthase; AgBS, *Abies grandis* bisabolene synthase; AgHS, *A. grandis* humulene synthase; AgPS, *A. grandis* pinene synthase; CmEKS, *Cucurbita maxima* ent-kaurene synthase; GbLS, *Ginkgo biloba* levopimaradiene synthase; NaEAS, *Nicotaina arvense* 5-epi aristolochene synthase; OsFOS, *Oryza sativa* farnesol synthase; PaBS, *P. abies* bisabolene synthase; PaFS, *P. abies* Farnesene synthase; PaHS, *P. abies* humulene synthase; PaA/BPS, *Picea abies* (−)-α/β pinene synthase; PaLS, *P. abies* levopimaradiene synthase; PgECDS, *P. glauca* ent-copalyl diphosphate synthase; PgEKS, *P. glauca* ent-kaurene synthase; PisFS, *Pinus sylvestris* farnesene synthase; PisLS, *P. sylvestris* longifolene synthase; PisCS, *Pinus sylvestris* caryophyllene synthase PmBS-*Pseudotsuga menziesii* bisabolene synthase; PmFS, *P. menziesii* farnesene synthase; PsLS, *Picea sitchensis* levopimaradiene synthase; TbTS, Taxus baccata taxadiene synthase; TcTS, *T. chinensis* taxadiene synthase; ZmBBS, *Zea mays* beta-bisabolene synthase; ZmFOS, *Z. mays* farnesol synthase; ZoGBS, Zingiber officinalis gamma bisabolene synthase)

into bisabolene via an ionization-dependent mechanism (McAndrew et al. 2011). The second functional class II active site with characteristic conserved motif DXDD is replaced by DLEI in both GbTPS1 and GbTPS2. Similarly, the AgBIS sequence lacks the DXDD motif and instead contains a DLET sequence, that renders the Class

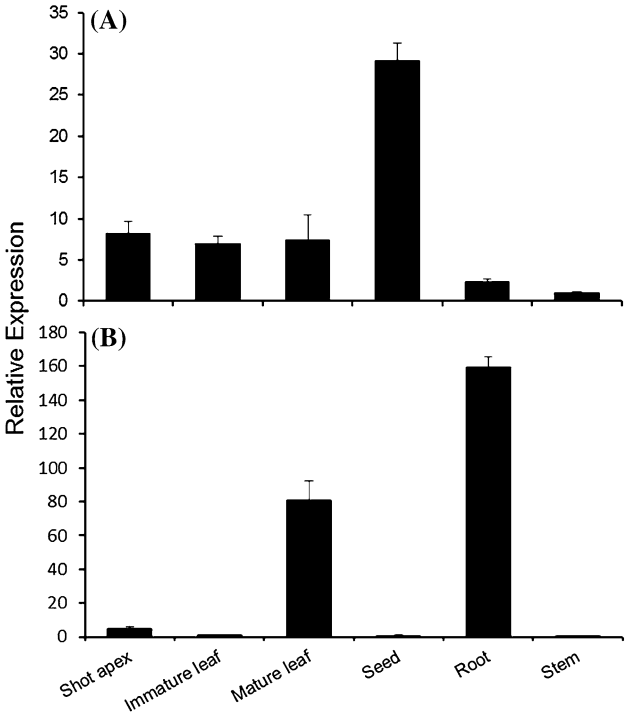

Fig. 7 *Gb-TPS1* (**A**) and *Gb-TPS2* (**B**) transcript levels in various *G. biloba* tissues. Relative steady-state accumulation levels of *GbTPS2* transcripts were determined in different ginkgo tissues by RT-qPCR using gene-specific primers. Data were normalized to an internal control (18S rRNA), and the $\Delta\Delta C_T$ method was used to obtain relative values. Data are expressed as mean ± S.D

II active site nonfunctional (McAndrew et al. 2011). Functional Class II active sites can be found in diterpene synthases, such as *G. biloba* levopimaradiene synthase (LPS; Schepmann et al. 2001) and *A. grandis* abietadiene synthase (AgABI; Vogel et al. 1996). The structural features along with the sequence similarity to previously characterized sesquiterpene synthases from plants suggested that both the *GbTPS1* and *GbTPS2* sequences encode sesquiterpene synthases. Subsequent experiments demonstrated that recombinant GbTPS1 can catalyze the removal of diphosphate from FPP, yielding acyclic farnesol, whereas recombinant GbTPS2 can generate the monocyclic sesquiterpene (−)-α-bisabolene from FPP. The enzyme kinetic analyses revealed high K_m values for FPP for both farnesol synthase and bisabolene synthase, as compared with previously reported K_m values for other plant sesquiterpene synthases (Schnee et al. 2002, Yang et al. 2013). However, the apparent k_{cat} values were found to be similar to previously characterized sesquiterpene synthase enzymes. Although a number of α-bisabolene synthases have been characterized from both angiosperms and gymnosperms to date (Bohlmann et al. 1998a; Fujisawa et al. 2010), there are only two reports of functionally characterized farnesol synthases: one from maize and a second from rice (Schnee et al. 2002; Cheng et al. 2007). The present report represents the first functional characterization of a farnesol synthase from gymnosperms. Moreover, the earlier reports of farnesol synthases in maize (Schnee et al. 2002) and rice (Cheng et al. 2007) do not indicate the potential regulatory nature of this enzyme. Thai et al. (1999) reported the conversion of farnesol to FPP via kinase activity. This suggests that the farnesol synthase described here now closes a loop with the report from Thai et al. (1999) on the kinase activities able to convert FOH back to FPP. FPP and Farnesol are considered regulatory with FPP thought to control flux down the MVA pathway (Vranová et al. 2013). Therefore, the farnesol synthase described here is an unusual synthase with the potential regulatory nature. Plant terpene synthases generally form multiple products, for example the ginger germacrene D synthase produced germacrene D (50.2 %), germacrene B (17.1 %) and a number of minor by-products (Picaud et al. 2006), and in spruce, α-humulene synthase produced α-humulene (43 %) and (*E*)-β-caryophyllene (38 %) along with some minor products (Keeling et al. 2011). Interestingly, the GbTPS1 enzyme produced only the sesquiterpene alcohol farnesol when assayed in the presence of FPP. In contrast, rice farnesol synthase (OsTPS13) converts FPP into (*E,E*)-farnesol (84.2 %), (*E*)-nerolidol (9.7 %) and an unknown compound (Cheng et al. 2007). Similarly, GbTPS2 synthesizes only one major sesquiterpene product (α-bisabolene), analogous to (S)-β-bisabolene synthase from ginger (Fujisawa et al. 2010) and (*E*)-α-bisabolene synthase from Norway spruce (Martin et al. 2004).

The two sesquiterpene synthases described here showed sequence similarity to the gymnosperm TPSd family of terpene synthases (Fig. 6). Gymnosperm TPS enzymes have been previously shown to cluster into three distinct subgroups within the gymnosperm-specific TPS-d family (Martin et al. 2004, Keeling et al. 2011). However, in the recent comprehensive phylogenetic analysis by Warren et al. (2015), the gymnosperm TPSs family is represented by four distinct clades called hemiterpene synthase, monoterpene synthases, sesquiterpene synthases and diterpene synthases. The present phylogenetic investigation of representative gymnosperm TPSs also resulted in the formation of three major clades of mono-, sesqui-, and diterpene synthases with the newly characterized GbTPS1 and GbTPS2 clustering in the sesquiterpene synthase clade. Indeed the GbTPS1 and GbTPS2 sesquiterpene synthases from ginkgo in the present work clustered closely with bisabolene and farnesene synthases from other gymnosperms and this clade is different from the other subgroup consisting of multiproduct spruce sesquiterpene synthases (Fig. 6). Furthermore, gymnosperm (*E*)-α-bisabolene synthase may have evolved from an ancestral diterpene synthase following the loss of an exon encoding a transit peptide (Trapp and Croteau 2001). This was evident from the size of

GbTPS proteins of 804 and 738 amino acids, which appear to harbor amino terminal, ∝-domains of ~100 amino acids of unknown contribution to the catalytic capacity of the enzymes. The presence of ∝-domains similar to diterpene synthases from *Arabidopsis thaliana* and *Taxus brevifolia* confirms the structural similarity of GbTPS proteins to diterpene synthases and perhaps the predecessors of evolutionary advanced mono- and sesqui-terpene synthases. The main point that could be inferred from present analysis is GbTPS1 and 2 form different islands which are isolated from other major classes of TPS genes—this may be because they arose independently a long time ago, but also it could be that they trace back to a common ancestor gene and not clustering is simply a result of the different evolutionary pressure these genes were subjected to.

The RT-qPCR analysis showed preferential accumulation of *GbTPS1* mRNAs in the seeds followed by apical meristems, immature leaves and mature leaves. The latter three vegetative tissues exhibited almost similar expression levels. The GbTPS1 mRNA levels in seed were approximately 20 fold higher than the vegetative tissues, thus indicating a role for GbTPS1 in seed development. GbTPS1 produces (*E,E*)-farnesol, a sesquiterpene that has been previously shown to be produced as a defense compound against herbivory in maize (Schnee et al. 2006). Alternatively, farnesol could be playing some regulatory role to control metabolism or a developmental program. Seeds primarily contain reserve compounds in the form of storage proteins, carbohydrates and lipids for providing nutrition, and also an array defense compounds to protect developing embryos and seedlings from potential predators. The elevated transcript levels of *GbTPS1* in seeds and comparatively low transcript levels in the remaining tissues suggests that farnesol synthase is highly regulated during development in ginkgo, potentially as a tissue-specific chemical defense mechanism. In contrast, *GbTPS2* transcripts preferentially accumulated in root systems, followed by mature leaves. Interestingly, *GbTPS2* steady-state transcript levels in mature leaves were approximately 80-fold higher than those observed in immature leaves, indicating *GbTPS2* is highly regulated during leaf development. The *G. biloba* transcriptome data analysis at MPGR also revealed higher accumulation of gb-19898 (putative transcript for α-bisabolene synthase) in mature leaves as compared with immature leaves. The compound, α-bisabolene produced by GbTPS2, represents one of the sesquiterpene components of the conifer oleoresin complex. The oleoresin complex consists of volatile monoterpenes and sesquiterpenes (turpentine), and non-volatile diterpene resin acids (rosin) which collectively form the major defensive strategy employed by conifers against insect herbivores and fungal pathogens (Langenheim 1969; Bohlmann et al. 1998b). The α-bisabolene synthase from *A. grandis* has been shown to be highly-induced following attack by either insect herbivores or fungal pathogens (Bohlmann et al. 1998b). In maize, (S)-*β*-bisabolene synthesized from TPS10 forms a volatile defense signal against herbivores by attracting their natural enemies (Schnee et al. 2006). Currently, very little is known concerning the specific roles played by bisabolenes and other sesquiterpene compounds produced by gingko, and whether their utilization is similar to that observed in other gymnosperms.

In conclusion, we have cloned and functionally characterized two sesquiterpne synthases from *Ginkgo biloba*. Both the enzymes are capable of synthesizing single respective sesquiterpene product from FPP. The cloning of these terpene synthases has significant implications in understanding the biology of sesquiterpene biosynthesis in primitive extant plant and provides opportunities for further investigation to explore the potentials and applications of these gene products.

Acknowledgments We thank Dr. Franck Dayan for assistance with enzyme kinetic analyses. We also thank Marilyn Ruscoe, Melanie Mask, and Julie Blessitt for excellent technical assistance and Jon Parcher for his revision of the manuscript and suggestions. This research work is supported in part by FDA Grant No. 1U01FD004246-3 and USDA-ARS Grant No. 56-6402-1-612.

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