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Developmental variations in sesquiterpenoid biosynthesis in East Indian sandalwood tree (*Santalum album* L.)

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Abstract The East Indian sandalwood tree, *Santalum album* L. is known for its fragrant heartwood and essential oil. The major bioactive principles of sandalwood oil, i.e., sesquiterpenoids (C_{15} isoprenoids), are known as 'santalols' and are globally used in medicinal, cosmetic, dietary, and aromatherapeutic applications. However, there are no available reports on the biosynthesis and metabolism of isoprenoids in this forest tree. Hence, we provide detailed insights into sesquiterpenoid metabolism across several in vitro and in vivo developmental stages. Since no molecular information was available, several genes encoding enzymes participating in early and critical steps of isoprenoid biosynthetic pathways were isolated using degenerate primers, and their expression patterns across the developmental stages were studied by semi-quantitative reverse transcription PCR. Results indicate that the isoprenoid biosynthetic pathway is differentially regulated with development and in tissue-specific manner. Accumulation of plastidial isoprenoid pigments increased with development, while the amounts of farnesylated intermediates decreased with maturation, thereby possibly indicating conversion into sesquiterpenoids. A differential expression pattern was observed for hydroxy-3-methylglutaryl coenzyme A reductase and 1-deoxyxylulose-5-phosphate synthase at the levels of transcripts and proteins, indicating post-transcriptional regulation. Transcript levels of farnesyl pyrophosphate, sesquiterpene and monoterpene synthases were quantitatively higher in callus, and lower in matured tree leaves. Sesquiterpene synthase activity across different developmental stages indicated a tissue-specific conversion and accumulation. Henceforth, the results would facilitate characterization of routes of sandalwood oil biosynthesis and for future improvement of sesquiterpenoid content in this tree.

Abbreviations

ACT	Actin
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
FPP	Farnesyl pyrophosphate
FPPS	Farnesyl pyrophosphate synthase
GC–MS	Gas chromatography–mass spectrometry
HPTLC	High performance thin layer chromatography
HMG-CoAR	3-Hydroxy-3-methylglutaryl-CoA reductase
MEP	Methyl erythritol phosphate (mevalonate-independent) pathway
MTPS	Monoterpene synthase
MVA	Mevalonate-dependent pathway
sq RT-PCR	Semi-quantitative reverse transcriptase-polymerase chain reaction
STPS	Sesquiterpene synthase

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Introduction

The East Indian sandalwood tree, *Santalum album* L., is sought for its fragrant heartwood and essential oil. This tropical tree species belonging to family *Santalaceae* is known to possess several bioactive properties, such as anti-cancer (Arasada et al. 2008), anti-tumor (Kim et al. 2006), anti-viral (Koch et al. 2008), anti-*Helicobacter pylori* (Ochi et al. 2005), and cytotoxicity (Matsuo and Mimaki 2010), attributed mostly to the heartwood and oil constituents. The essential oil is rich in sesquiterpene alcohols, where α-santalol (40–55 %) and β-santalol (17–27 %) make over 80 % of the oil. These santalene-type sesquiterpenoids (C_{15} isoprenoids) are derived from isoprenoid biosynthetic routes. However, most studies focused on the analyses of commercially available essential oil and its constituents (Howes et al. 2004). Only recently, Jones et al. (2006) studied the sandalwood oil biosynthetic pathway and provided a comparative account of constituents produced from heartwood. Besides, in vitro grown sandalwood callus has been shown to produce sesquiterpenoid constituents (Crovadore et al. 2012). The sesquiterpene synthases implicated in santalol biosynthesis have recently been characterized (Jones et al. 2011). Furthermore, the worldwide natural population of this forest tree has been in decline due to (1) the mycoplasmal seed-born spike disease, (2) illegal poaching, and (3) commercial scale over-exploitation. Hence, biotechnological means of micropropagation for this plant were reported as early as 1963 (Rangaswamy and Rao 1963) with an aim to meet global demands for this expensive natural resource.

Yet, there have been no efforts to study the enzymes, genes, intermediates, and metabolites involved in sandalwood oil biosynthesis either in vivo or in vitro. Thus, we intend to study the sesquiterpenoid metabolism in sandalwood tree using established in vitro developmental stages, i.e., callus, somatic embryo, and seedling to compare with that of in vivo material from oil yielding and non-oil yielding trees.

Materials and methods

Plant materials

All in vitro micropropagated materials of *S. album* were obtained from a highly proliferating cell line (IITKGP/91) maintained in the laboratory in the form of callus. Callus was grown on solid media [WPM (Woody Plant Media) (Lloyd and McCown 1981)] supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D; 1 mg L^{-1}), 3 % sucrose and 0.35 % Phytagel (Sigma, St Louis, MO, USA) and was sub-cultured every 3 weeks. Somatic embryos were maintained on solid WPM supplemented with indole-3-acetic acid (IAA; 0.5 mg L^{-1}), 6-benzylaminopurine (BAP; 0.5 mg L^{-1}), 3 % sucrose, and 0.35 % Phytagel. The somatic embryos were sub-cultured every 4–6 weeks for maintenance. The in vitro raised seedlings obtained from somatic embryos were maintained on solid WPM supplemented with indole-3-butyric acid (IBA; 0.5 mg L^{-1}), benzyl adenine (BA; 0.5 mg L^{-1}), gibberellic acid (GA; 0.5 mg L^{-1}), 2 % sucrose and 0.5 % Phytagel and the plantlets were harvested in 8–12 weeks. Only callus materials were grown in dark, while the somatic embryos and seedlings were exposed to white fluorescent light of 1,000–1,500 lux (16 h light/8 h dark cycle) at 25 ± 2 °C with 50–60 % relative humidity in the tissue culture laboratory. A 7-year-old non-oil yielding and a 15-year-old oil yielding matured tree, growing on the campus of the Department of Biotechnology of the institute, were sampled for fresh twigs. Sandalwood somatic embryo (heart-shape staged) suspension cultures maintained in the laboratory in WPM media supplemented with IAA (0.5 mg L^{-1}), BAP (0.5 mg L^{-1}) and 3 % sucrose in 50 mL volumes were sub-cultured into fresh media in a ratio of 4:1 of fresh media: old culture.

Extraction of enzymes involved in terpenoid biosynthesis

For hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) and 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) enzyme assays, in vitro and in vivo materials were extracted with an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol and 1 % (w/v) polyvinyl pyrrolidone. The tissues were ground in liquid nitrogen, macerated in the extraction buffer (1 g mL^{-1}) for 5 min with mortar and pestle, followed by centrifugation at 14,000*g* and 4 °C for 30 min to obtain a solid-free extract (Ge and Wu 2005). Total protein content was determined by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as a standard.

Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR, EC 1.1.1.34) assay

HMG-CoAR was assayed spectrophotometrically following the method of Toroser and Huber (1998).

1-Deoxy-D-xylulose 5-phosphate synthase (DXS, EC 2.2.1.7) assay

DXS was assayed spectroflourimetrically as described by Querol et al. (2001), which is based on the reaction of 1-deoxy-D-xylulose 5-phosphate (DXP) with 3,3-diaminobenzoic acid (DAB) in an acidic medium to form a highly

fluorescing quinaldine derivative. The fluorescence intensity of the reaction product (excitation, 396 nm; emission, 510 nm) was proportional to DXS activity and was measured with a fluorescence spectrophotometer (LS50B, Perkin Elmer).

Sesquiterpene synthase (STPS, EC 4.2.3.-) assay

Protein extraction

Fresh plant materials were harvested, washed, and ground immediately to a fine powder with a mortar and pestle in liquid nitrogen. Extraction was performed with an extraction buffer containing 50 mM Tris-HCl, pH 6.8, 5 mM ascorbic acid, 5 mM sodium bisulfite, 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (w/v) PVP (Mr 10,000), 4 % (w/v) PVPP, and 0.1 % (v/v) Tween 20 in a ratio of 1:10 (g tissue: mL buffer). The preparations were allowed to shake at 4 °C for 30 min and were then centrifuged at 10,000g for 30 min. The supernatant was then filtered through two layers of cheesecloth, divided into 1 mL aliquots, frozen in liquid nitrogen, and kept at −80 °C until further experiments.

STPS assay

STPS activities were determined by recommended procedures (Bohlmann et al. 1997) with minor modifications. Before assaying enzyme activity, the frozen protein extracts were placed at 37 °C until just thawed. Briefly, the protein extracts were desalted in columns pre-equilibrated with STPS buffer [25 mm HEPES, pH 7.3, 10 mM $MgCl_2$, 10 mM DTT and 10 % (v/v) glycerol]. Enzyme activity was assessed with 1 mL of the desalted extracts with the addition of 30–50 μM farnesyl pyrophosphate (FPP). All enzyme assays were performed in triplicate, overlaid with 1 mL of pentane to collect released volatiles, and incubated at 30 °C for 1.5 h. To stop all enzyme activity, the extracts were immediately frozen. After thawing, the aqueous assay fraction was rapidly extracted with the pentane fraction by vortexing, and separation of the aqueous and organic fractions was achieved by centrifugation at 2,500g for 2 min. The 1 mL pentane overlay (contained 100 ng of β-caryophyllene as an internal standard) was taken and filtered through a Pasteur pipette filled with 0.4 g of silica gel (60 Å, Merck, India) overlaid with 0.6 g of $MgSO_4$ to remove non-specific substrate hydrolysis products and to dry the pentane extract. Each enzyme assay mixture was further extracted with additional two portions each of n-pentane, n-hexane and MTBE, vortexed vigorously and centrifuged as before. These sequential extractions were also passed over the same column and pooled with the initial column eluents. Subsequently, the column was

washed twice (1 mL each) sequentially with n-pentane, n-hexane and MTBE, pooled together, dried in air, redissolved in MeOH and subjected to analyses by gas chromatography. Authenticated sandalwood oil was obtained from Cauvery (Government of Karnataka, Bangalore, India) and was tested by high performance thin layer chromatography (HPTLC) (Camag, Switzerland) for compositional analyses. This oil was used to isolate the major constituent of oil, i.e., α-santalol in a preparative scale using thin layer chromatography to use as a standard for quantification of STPS assay products.

Extraction of terpenoids

About 500 g of all the in vitro and in vivo samples was collected for extraction. The heartwoods of the field grown trees were manually bored at chest height from the ground, up to 10 cm depths to obtain chips and powders. Plant materials were immediately proceeded with solvent extraction with four different solvents in eluotropic series, i.e., n-pentane, n-hexane, diethyl ether, and ethyl acetate (all spectroscopy grade, from E. Merck, Darmstadt, Germany), in four independent sets, in several batches. Solvent extraction was performed in Erlenmeyer flasks of 2L volume, for 2 h each at 25 ± 5 °C, with intermittent shaking in a 10 % (w/v) ratio of tissue to solvent. During extraction, 0.01 % (w/v) BHT (butylated hydroxytoluene) (Sigma-Aldrich, St. Louis, MO, USA) was added as a synthetic antioxidant to protect the phytochemicals from auto oxidation. Following this, extracts were dried over Na_2SO_4 (Hi Media, India), concentrated in vacuuo, in an N–N series rotary evaporator (Eyela, Tokyo) at 40 °C. The extracts were reconstituted in pyridine when required and proceeded with GC–MS analysis.

HPTLC analyses of isoprenoids

For quantification of isoprenoids, an HPTLC-based method was used (Misra and Dey 2012a) prior to further validation by GC analysis. Freshly prepared spray reagent [ethanolic solution of p-anisaldehyde (2.5 %), sulfuric acid (3.5 %), glacial acetic acid (1.6 %)], specific for isoprenoids/sesquiterpenoids was used for derivatization and detection. In addition, α-santalol was bulk-purified from the commercial sandalwood oil sample, validated by GC–MS for identification (data not shown), was eluted from silica using MeOH and used as a standard for quantification methods.

Gas chromatography analyses

The GC system used was a Chemito 100^{TM} workstation. Essentially, three columns were used in the investigation.

Chromatography was performed employing BP 5, BP 21 or BP 20 columns [dimensions: 30 m (*l*) × 0.25 mm (*i. d.*) × 0.25 μm (SGE, Australia)] using an oven program of 40–220 °C at 3 °C min^{-1}. In both cases, the carrier gas was helium (He) at a column flow rate of 1 mL min^{-1} and 1–3 μL injections (with split 1:5, 1:10 and split less modes, respectively for the 3 columns) with injector temperatures of 220–250 °C. Detection was done by flame-ionization detection (FID: 250 °C, 45 mL min^{-1} H$_2$, and 450 mL min^{-1} air). At times, oven temperature programs were as follows; held for 3 min at 40 °C, then 3 °C min^{-1} to 110 °C, 10 °C min^{-1} to 180 °C, then a final ramp of 15 °C min^{-1} to 240 °C, where this temperature was held for 5 min. For BP 5 column, the oven program was held for 1 min at 40 °C, then ramped at 7.5 °C min^{-1} to 250 °C and held for 10 min. The software was Iris^{TM} version 4.1.1. Sandalwood oil and tissue organic extracts were diluted to 1 % (v/v) with diethyl ether (DEE), MeOH, or pyridine (all GC grade solvents) prior to analysis. The relative proportions of the sandalwood essential oil constituents were expressed as percentage obtained by peak area normalization, assuming that total injection was 100 % (10–100 μg of samples). Retention indices (RI) were determined relative to the retention times of a series of *n*-alkanes with linear interpolation, from available standard compounds or the literature.

Gas chromatography–mass spectrometry (GC–MS) analysis

The solvent extracts were analyzed by GC–MS using a Thermo Trace GC Ultra^{TM} gas chromatograph system (Thermo Scientific, USA), equipped with a 30 m × 0.25 mm *i.d.*, 0.25 μm film thickness, non-polar TR-5MS fused silica capillary column, connected to an ion trap quadrupole (ITQ) mass selective detector (unit mass resolution). The split was 1:50, with helium as the carrier gas at a flow rate of 1 mL min^{-1}, while the damping gas flow was 0.3 mL min^{-1}. The initial oven temperature was set to 40 °C for 1 min. The GC oven temperature program was as follows: 40–220 °C, by ramping at 3 °C, and held at 220 °C for 20 min. The injector temperature was 220 °C and the transfer line was held at 220 °C. The detection was performed by a Thermo ITQ 900^{TM} mass spectrometer in the EI mode (ionization energy of 70 eV, ion source temperature of 180 °C, emission current of 220 μA). The acquisition was made in full scanning mode (mass range 50–900 m/z; 3 scan s^{-1}). Maximum ionization time was 25 ms. A solvent delay time of 5 min (setoff) was used to avoid overloading the mass spectrometer with hexane. Data collection, analysis, and integration were performed using the software XCalibur^{TM} (version 2.0.7). Areas were recorded for all detectable peaks, and percent composition was calculated by taking an area of peak divided by total

chromatogram area ×100. For identification of the compounds, a solution of *n*-alkanes (*n*-octane to *n*-hexadecane) was injected in the GC–MS system afterwards and the analysis was performed using the same instrumental conditions. This allowed the calculation of retention indices (RI) for each compound and was compared with the authentic standards (spiking and co-elution experiments) and literature to ensure the correct identification, allowing variation in retention times up to <0.05 min. The sesquiterpenoid constituents were identified according to the National Institute of Standards and Technology (NIST) and Wiley standard mass spectral libraries (spectral fit value of >90) supplied with the instrument. Web resources such as Dr. Duke's Phytochemical and Ethnobotanical Database (http://www.ars-grin.gov/duke/) and Flavornet (http://www.flavornet.org/flavornet.html) were used for confirmation of KI and RI values. The sesquiterpenoid content was calculated from a standard curve made from α-santalol using a similar set up and was expressed as ng g^{-1} FW.

Quantification of terpenoids

The total terpenoid content of the five developmental stages were measured as described (Doneva-Šapceska et al. 2006). Briefly, about 100 μg of extracts was added to 500 μL of 2 % vanilin-H$_2$SO$_4$ in cold and further incubated at 60 °C for 20 min, followed by cooling at 25 °C for 5 min. Absorbance was measured at 608 nm within 20 min for the detection of blue-green colour to express the terpenoid contents in μg L-linalool equivalence per mg of extract (standard curve prepared using 20–100 mg L^{-1}, equation: $y = 0.002x$, $R^2 = 0.988$). A GC-based standard curve was obtained by linear regression analysis, using purified Z-α-santalol to obtain an equation, $y = 2e^{10}x + 30063$, $R^2 = 0.99$, where '*y*' represented the signal/response as area (a.u.) as a function of mV and '*x*' the concentration of α-santalol in ng. Total sesquiterpenoid content was expressed as ng g^{-1} FW material.

Quantification of sesquiterpenoid biosynthesis intermediates

Extraction of farnesylated compounds

For extraction of water-soluble farnesylated isoprenoids from materials, a sequential double-extraction method was followed that involved extraction system, I (0.5 N NH$_4$OH in 75 % EtOH) and II (BuOH/75 mM NH$_4$OH/EtOH in 1:1.25:2.75) for greater efficiency (Tong et al. 2005). The organic solvent was gradually added to suspended cells with constant shaking to avoid cell aggregation. Extracted FPP, farnesene, and farnesol were stable for at least 24 h at 4 °C in the dark.

Preparation of standard farnesylated compounds

Extraction of acid phosphatase (APase) rich fractions from potato

Semi-purified acid phosphatase (APase) was obtained from potato tubers as described (Gellatly et al. 1994).

Acid phosphatase assay

Inorganic phosphate (Pi) released by the APase reaction was measured spectrophotometrically as described (Eibl and Lands 1969).

Enzymatic hydrolysis of prenylpyrophosphates

Prenylpyrophosphates after their hydrolysis to corresponding alcohols were analyzed as described (Fujii et al. 1982).

HPTLC-based analyses for detection and quantification

Tissues extracts and standards were analyzed chromatographically, on silica gel G 60 F_{254} 10 × 10 cm TLC plates and were developed with isopropanol/NH_4OH/H_2O (6:3:1). The FPP, farnesol, and farnesene generated were compared from literature for their log$P$ values, chromatographic mobility (R_f) and absorption spectra data (Thai et al. 1999).

Quantification of chlorophylls, carotenoids, and xanthophylls

Chlorophylls, carotenoids, and xanthophylls were extracted and measured essentially as described (Lichtenthaler 1987) using the following equations:

$$\text{Chlorophyll a} = 0.01261 \times A_{661} - 0.001023 \times A_{534} - 0.00022 \times A_{643}$$

$$\text{Chlorophyll b} = 0.02255 \times A_{643} - 0.00439 \times A_{534} - 0.004488 \times A_{661}$$

$$\text{Carotenoids} = (A_{470} - 17.1 \times (\text{Chl a} + \text{Chl b}) - 9.479 \times \text{anthocyanin})/119.26$$

$$\text{Xanthophyll (weight\%)} = A \times 100 \times F/W \times 2{,}085,$$

where A is the absorbance at 478 nm (optical path length 1 cm), F is the dilution factor, and W is the weight (g) of the sample.

Inhibition studies in suspension cultures

Varying concentrations (5, 10, 25, 50, 100, 250 and 500 μM) of lovastatin (mevinolin) were added to the somatic embryo suspension cultures simultaneously and were incubated at 25 °C for a week in an incubator shaker in darkness with shaking at 80 rpm. The cultures were filtered after 7 days of growth and harvested for further analyses.

Complimentary DNA (cDNA) preparation and reverse transcriptase-polymerase chain reaction (RT-PCR) for gene fragments

First-strand cDNA was synthesized from 5 μg of total RNA using 0.28 mM oligo $(dT)_{16}$ primer (Sigma, St. Louis, USA) in 100 μL volumes, where the mixture of primer and RNA was heated to 70 °C for 10 min, allowed to cool, and the remaining reagents added, i.e., 8 mM DTT, 140 U of human placental RNase inhibitor (Bangalore Genei, India), 0.63 mM dNTPs, 1,000 units of Super-Script^{TM} reverse transcriptase (Qiagen), 50 mM Tris-HCl pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$. The reactions were incubated at 37–45 °C for 30 min and stopped by heating to 94 °C for 10 min. To amplify desirable gene products from sandalwood, RT-PCR was performed using degenerate oligonucleotide primers (Online Resource Table 1) designed against conserved amino acid regions in other plants (Online Resource, Fig. 1–5). One Step Super-Script^{TM} RT-PCR kit (Qiagen) was used to amplify 300–500 ng of first-strand cDNA using the appropriate primers and *Taq* DNA polymerase (Biotaq, Bioline, UK). Reaction steps to amplify the genes were, e.g., reverse transcription at 50 °C for 30 min, initial PCR activation step at 95 °C for 15 min, denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 68 °C for 1 min (35–40 cycles)] and a final extension at 72 °C for 10 min. The products were analyzed on 0.8–1.5 % agarose gels and were subjected to TA-cloning and sequencing using standard protocols (Sambrook et al. 1989).

Cloning and sequencing of fragments of genes

Briefly, the RT-PCR fragments obtained using the degenerate primers were cloned into a commercial TA-cloning vector, i.e., pTZ57 R/T (MBI, Fermentas, India) provided with a one-step InsTAclone^{TM} PCR cloning kit, following the manufacturer's instructions. Following recombinant selection, positive clones were sequenced using vector-specific standard M13/pUC universal primers for sequencing. Automated dye terminator cycle sequencing was done using the ABI PRISM BigDye^{TM} (fluorescence-labeled dideoxynucleotide termination method) Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, USA) in an ABI PRISM 377 DNA sequencer (Applied Biosystems, USA) following manufacturer's instructions.

Sequences were visualized and analyzed using Chromas Lite[TM] version 2.01 software.

Bioinformatics analyses of sequences

Comparative and bioinformatic analyses of the nucleotide sequences obtained from analogous plant genes were carried out online using BLAST search sites (http://www.ncbi.nlm.nih.gov and http://cn.expasy.org). BLASTP, RPS-BLAST, BLASTX and PSI-BLAST (www.ncbi.nlm.nih.gov/BLAST); to identify homologs of the related genes. Nucleotide sequence alignments were performed using Clustal W version 1.82 (Thompson et al. 1994; http://www.ebi.ac.uk/clustalw) and the alignment was edited (Waterhouse et al. 2009).

The partial-length sequences of sandalwood genomic sequences have been submitted under GenBank[TM] accession numbers ACT (HM232850), DXS (HM232852), FPPS (HM232854), MTPS (HM232855), and STPS (HM232858), respectively.

Tissue-level expression profiling by semi-quantitative RT-PCR (sq RT-PCR)

Total RNA was extracted from the tissues representing five developmental stages as described earlier. The template was used in concentration of 1 μg μL^{-1} for reverse transcription. Potential genomic DNA contamination was removed by treatment with RNase-free DNase I and by designing intron spanning primers. First cDNA strands were synthesized using a One Step SuperScript[TM] RT-PCR kit (Qiagen). For semi-quantitative RT-PCR (sqRT-PCR), primers were designed based on the partial clones obtained, using Primer 3 tool (http://frodo.wi.mit.edu/) online. The primers used in this study are described in Table 1. The sqRT-PCR cycling parameters were set as follows: 50 °C for 2 min; [95 °C for 2 min; 95 °C for 15 s, 50 °C for 30 s; 72 °C for 30 s] 28 cycles; and 72 °C for 8 min. The gene expression levels were

quantified as band fluorescence intensity in 1.5 % agarose gels after electrophoresis in a GelDoc[TM] system (Bio-Rad). Plasmids harboring the same GOI were amplified as positive control, while RT-PCRs conducted without the addition of RT-enzyme (-RT) served as the negative control.

Statistical analysis

Statistical analysis was performed to understand the difference in values of parameters obtained from assays using one-way analysis of variance (ANOVA) by SPSS software package (version 17.0) following Duncan's multiple range test (DMRT) (Duncan 1957) as well as Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Results were expressed as mean $\pm$ SD ($n = 6$, interday assays).

Results

## Enzymatic (DXS, HMG-CoAR) routes of core isoprenoid biosynthesis

The DXS activities were determined as a function of the fluorescent quinaldine derivatives. The seedling DXS activity was noted to be 4.7 times higher than the old tree, 2 times higher than the young tree, 1.5 times higher than callus and 1.3 times higher than the somatic embryos (Fig. 1a). The HMGCoAR activity as determined by the oxidation of NADPH was determined spectrophotometrically. No detectable HMGCoAR activity was recorded for seedling and young tree. The callus HMGCoAR activity was higher by 4.2 and 1.6 times than the somatic embryo and old tree, respectively (Fig. 1b).

Terpenoid contents

Total terpenoid contents of the sandalwood developmental stages were determined by GC–MS-based standard curves

Table 1 Primers used in sq-RT-PCR reactions for amplification of five transcripts

Serial no	Gene of interest	Primer name sequences	Amplicon lengths (bp)	Av. melting temp. (T_m)
1	ACT	ACT RT F: 5′-TCAGATCATGTTCGAGACCTTC-3′	460	59 °C
		ACT RT R: 5′-CTGCAGCCTCCATTCCTATC-3′		
2	DXS	DXS RT F: 5′-GACTCGGACATGATTGTCATTCT-3′	277	59 °C
		DXS RT R: 5′-AATAGAGCCCAAGCTTTTCG-3′		
3	FPPS	FPPS RT F: 5′-TGAGAATCTGGACAGCCACA-3′	101	60 °C
		FPPS RT R: 5′- CAGGATGACCAAAGCAGTCC-3′		
4	MTPS	MTPS RT F: 5′-TCGACAATTTGCATGCTACG-3′	187	60 °C
		MTPS RT R: 5′-AAATCCTCACCTTCCCAACC-3′		
5	STPS	STPS RT F: 5′-AGATGACATTTATGATGCTTATGG-3′	144	58 °C
		STPS RT R: 5′-TCATTGTAGAAATCCAAAACTCCTT-3′		
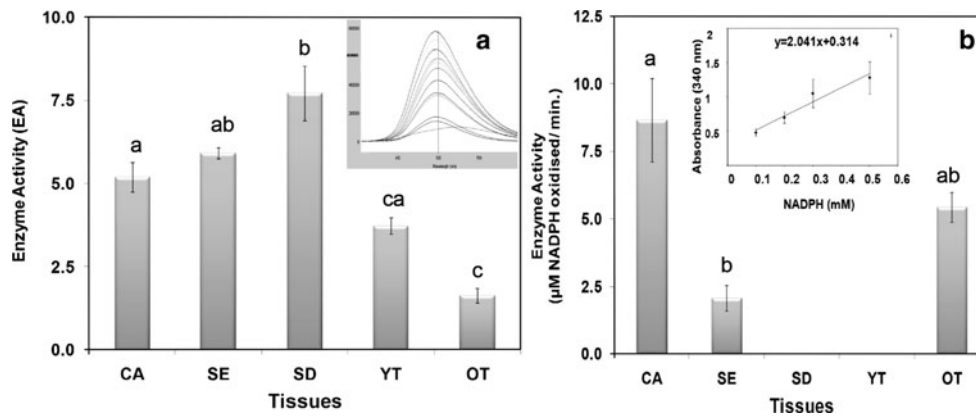
Fig. 1 a DXP synthase activities across sandalwood tree developmental stages. *Inset* shows fluorometric spectra of the quinolidine derivatives. b HMGCoAR activities across sandalwood tree developmental stages. *Inset* shows standard curve of NADPH by plotting concentration against absorbance, where $y = 2.041x + 0.314$, $R^2 = 0.996$. The data,

representative of six interday assays ($n = 6$), are provided as mean ± SD (*error bars*), and the values followed by the same letters are not statistically different ($P < 0.05$). *CA* callus, *SE* somatic embryo, *SD* seedling, *YT* young tree, and *OT* old tree

(i.e., $y = 21303x + 9643$, where y is area in arbitrary units and x is amount of α-santalol in ng; $R^2 = 0.94$) and expressed as ng g^{-1} FW. Evidently, the highest terpenoid content was recorded for the old tree tissues that were 1.4 times higher than young tree, 3.2 times higher than seedling, 1.6 times higher than somatic embryo and 7.2 times higher than callus (Table 2). Initially, sandalwood oil was used as a standard to identify α-santalol, to prepare a standard curve based on the peak area under the GC chromatogram.

Identification and quantification of sesquiterpene synthase assay products

STPS activities measured in cell-free extracts derived from sandalwood tissues for five different developmental stages, revealed that cell-free extracts produced sesquiterpenoids following incubation with FPP. The assay products were identified and quantified using HPTLC, GC-, and GC–MS analyses and compared with that of sandalwood oil constituents for identification. Products with areas below 0.1 % were not considered for measurements and, thus, a

large proportion and numbers of constituents remained unidentified. A marked difference was observed between the products generated in vitro from FPP by the cell-free enzymatic extracts derived from each tissue type. GC–MS analysis of STPS assay products revealed that callus protein extracts, upon incubation with FPP, produced farnesol (49.1 %) and minimal amounts of β-bisabolene and Z-epi-β-santalol. Somatic embryos produced farnesol (13.1 %), followed by Z-lanceol (7 %) and β-santalene. The seedlings yielded Z-lanceol (32.6 %) and Z-nuciferol (21.8 %) followed by little α-bergamotene. The young tree twigs produced α-bergamotene (7.2 %) and α-santalene. The old tree twigs produced Z-lanceol (47 %) and Z-α-santalol. Thus, the prominent identifiable sesquiterpene synthase assay products in sandalwood tree were farnesol, α-bergamotene, Z-nuciferol, and Z-lanceol (Table 3).

HPTLC and GC-based profiling of sesquiterpenoids

Metabolite profiling of sesquiterpenoids across the tissues developmental stages were performed using HPTLC and GC. Results obtained from HPTLC (Table 4) indicate that the dominant constituents in sandalwood oil, callus, somatic embryo, seedlings, young tree, and old tree shoots were Z-α-santalol, Z-epi-β-santalol, Z-α-trans-bergamotol, Z-epi-β-santalol, Z-β-santalol, and α-santalene, respectively. Besides, the sesquiterpenoid alcohol content was found to be highest in sandalwood oil (89.8 %), followed by callus, young and old tree, somatic embryo and seedlings (27.1 %). Similarly, the olefenic sesquiterpenoid content was found to be highest in old tree (17.6 %) and lowest in seedlings (11.2 %). It is also observed that the olefin content was 10.4-fold lower than alcohol content in sandalwood oil, where as only 3.5-fold change was observed for old tree.

Table 2 Total sesquiterpenoid and monoterpenoids content in sandalwood tissues

Serial no	Tissue type	Total sesquiterpenoid content (ng g^{-1} FW)	Total monoterpenoid content (ng g^{-1} FW)
1	Callus	12.25 ± 2.52	2.2 ± 0.83
2	Somatic embryo	55.75 ± 12.36	8.23 ± 1.61
3	Seedling	27.5 ± 6.8	2.3 ± 0.2
4	Young tree	62.5 ± 7.9	15.88 ± 2.67
5	Old tree	88.5 ± 17	40.93 ± 6.8

Table 3 Identification of STPS assay products from various tissues

Sl. no.	Name of tissue	Retention time (min)	Area (%) of total assay products	Identification
1	Callus	18.3	49.1	Farnesol
		14.5	0.4	β-Bisabolene
		22.7	0.2	Z-epi-β-Santalol
		Various	50.3	18 unidentified
2	Somatic embryo	18.3	13.1	Farnesol
		23.8	7.0	Z-Lanceol
		4.8	3.9	β-Santalene
		Various	76	21 unidentified
3	Seedling	23.8	32.6	Z-Lanceol
		21.7	21.8	Z-Nuciferol
		14.8	3.1	α-Bergamotene
		Various	42.5	23 unidentified
4	Young tree	14.8	7.2	α-Bergamotene
		4.0	2.3	α-Santalene
		Various	90.5	28 unidentified
5	Old tree	23.8	47	Z-Lanceol
		19.2	2.9	Z-α-Santalol
		Various	50.1	22 unidentified

Results obtained from GC (Table 5) indicated that the sesquiterpenoid alcohol contents were highest for heartwood (43.4 %), followed by somatic embryo (19.6 %), and seedlings (19.6 %), whereas the olefenic hydrocarbon contents did not vary much among the samples, i.e., 4.5 % in callus to 7.8 % in young tree. Moreover, using GC, 16 sesquiterpenoid constituents could be profiled across the tissues, in comparison to nine constituents' identifiable using HPTLC analysis.

HPTLC-based quantification of farnesylated constituents

Farnesylated isoprenoids were extracted from five developmental stages using solvent extraction method. FPP treated with semi-purified potato acid-phosphatase yielded farnesene and farnesol. The mixtures of FPP, farnesol and α-farnesene were mixed in a volumetric ratio of 1:1:1, and used as HPTLC standards. Their theoretical Log P values (6.6 for farnesene, 5.6 for farnesol and 4.8 for FPP) (Fig. 2a), spectral characteristics (i.e., absorption maxima) (for farnesene (λ_{max}, 220 nm), farnesol and FPP (λ_{max}, 275 nm) (Fig. 2b), and chromatographic migrational patterns [farnesene (R_f, 0.83), farnesol (R_f, 0.5), and FPP (R_f, 0.04)] (Fig. 2c) aided in their identification and further quantification. The FPP content of the callus and somatic embryos were comparable, and was 2.5 times more than the levels in seedling, 4 times more than young tree and 8 times higher than the old tree tissues. The farnesol content of callus, somatic embryo, seedling, and young tree were similar, but 1.5 times lower than the old tree tissues. The farnesene content of the callus, somatic embryo, and young tree tissues was comparable, and 1.3 times higher than the old tree tissue and 2.2 times higher than the seedlings (Fig. 2d).

Photosynthetic pigments

The chlorophylls, carotenoids, and xanthophylls, the downstream products of the isoprenoid biosynthetic pathway were quantified spectrophotometrically. Chlorophyll a, b and total chlorophyll contents were highest for the young tree, followed by old tree and the in vitro stages, except for callus, where no chlorophyll were detected. Total

Table 4 HPTLC-based metabolite profiling of sandalwood sesquiterpenoid constituents

Composition (%)

Peak no	Identification	R_f (max.)	Sandalwood oil	Callus	Somatic embryo	Seedling	Young tree	Old tree
1	α-Santalene	0.08	1.2	–	2.8	–	2.6	0.1
2	Z-α-Santalol	0.2	45.1	–	4.2	–	12.4	5
3	β-Santalene	0.32	1.5	1.6	8.4	–	5.5	14.3
4	E-β-Santalol	0.34	0.4	2.8	7	0.6	0.8	2.1
5	Z-α-trans-Bergamotol	0.5	12	0.2	18.4	1.4	7.7	2.31
6	Z-Nuciferol	0.58	5.2	13.6	–	2.5	8.8	4.3
7	Epi-β-Santalene	0.64	5.9	2.5	–	2.9	–	3.2
8	Z-β-Santalol	0.89	25.2	20.7	17.2	8.6	21.6	34.4
9	Z-epi-β-Santalol	0.97	1.9	30.2	8.7	14	15.2	21.2
Total identified constituents (%)			98.4	54.7	66.7	30.1	74.6	86.9	98.4

Maximum R_f values are provided for identification of constituents from sandalwood oil. Constituents are expressed as percentage (%) of the extract loaded onto the HPTLC plates, 100 μg per tissue extract

Table 5 GC-based profiling of sesquiterpenoids in sandalwood developmental stages

Composition (%)

| Serial no	Name of constituent	Retention time (min.)	Callus	Somatic embryo	Seedling	Young tree	Old tree	Heartwood
Terpene alcohols								
1	Z-α-Santalol	45.9	–	0.3	–	0.5	0.1	22.4
2	Z-β-Santalol	47.9	1.5	0.4	1.5	0.4	1.1	2.4
3	Z-α-trans-Bergamotol	50	0.8	4.4	6.3	0.6	1.2	7.6
4	α-Bisabolol	48.4	0.8	7	0.7	1.3	1.1	0.4
5	Z-Nuciferol	56.8	2.7	–	5.1	2.6	0.9	6.4
6	Z-epi-β-Santalol	58.2	2.1	6.3	2.7	1.3	2.6	1.8
7	E-β-Santalol	48.4	0.8	0.4	1.5	1.3	1.1	2.1
8	Z-Lanceol	49	6.3	9.6	1.8	1.8	1.6	0.8
Total terpene alcohols			15	28.4	19.6	9.8	9.7	43.4
Terpene hydrocarbons								
9	Epi-β-Santalene	38.7	0.3	–	1.1	–	0.1	0.8
10	β-Santalene	23	0.2	0.3	–	3.2	0.6	1.9
11	α-Santalene	22.4	–	0.1	–	0.2	0.2	0.4
12	α-Bergamotene	20.8	0.5	2.8	0.9	0.1	0.4	0.5
13	β-Bisabolene	26.5	–	0.3	–	–	–	0.6
14	γ-Curcumene	35.5	1.3	0.7	0.6	1.7	0.3	2.1
15	β-Curcumene	21	0.5	0.7	0.8	0.1	1.3	0.9
16	α-Curcumene	41.7	1.7	0.5	1.3	2.4	1.3	0.5
Total terpene hydrocarbons			4.5	5.4	4.7	7.8	4.2	7.7

Retention times (min.) are provided for comparison of constituents from sandalwood oil. Constituents are expressed as percentage (%) of the extract
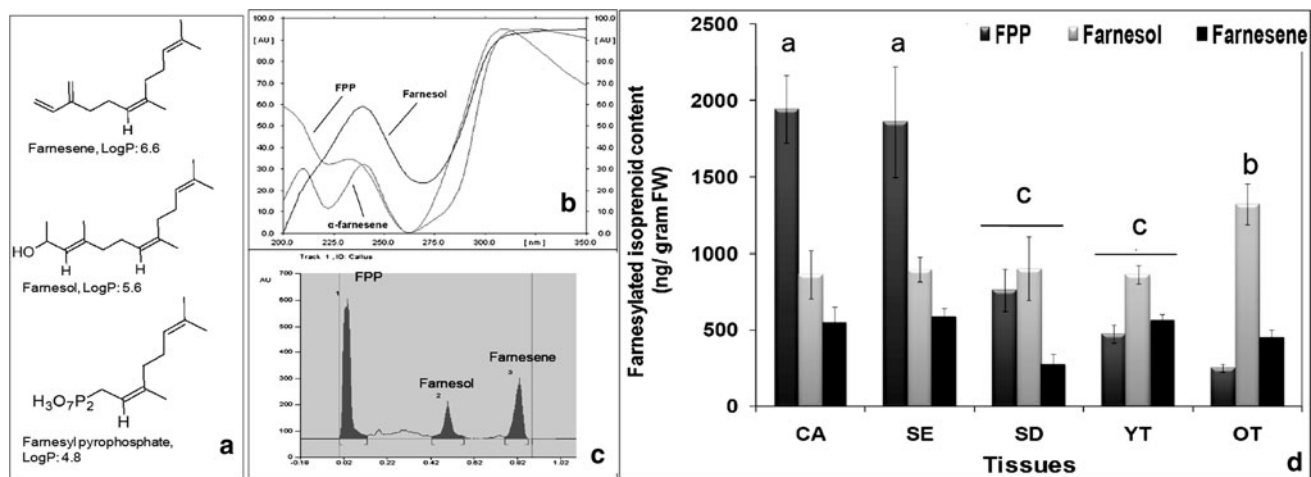
Fig. 2 a Chemical structures and Log P (octanol-water partition coefficient) values of the farnesylated intermediates. **b** The absorption spectra of farnesene, farnesol, and FPP on HPTLC plates scanned using a 208 nm filter. **c** Representative HPTLC chromatogram showing migrational pattern and separation of FPP (R_f, 0), farnesol (R_f, 0.52) and farnesene (R_f, 0.82). **d** Quantification of C_{15} metabolites (farnesene, fanesol and FPP) as detected by HPTLC across various sandalwood tree developmental stages. The data, representative of six interday assays ($n = 6$), are provided as mean ± SD (*error bars*), and the values followed by the *same letters* are not statistically different ($P < 0.05$). *CA* callus, *SE* somatic embryo, *SD* seedling, *YT* young tree, and *OT* old tree
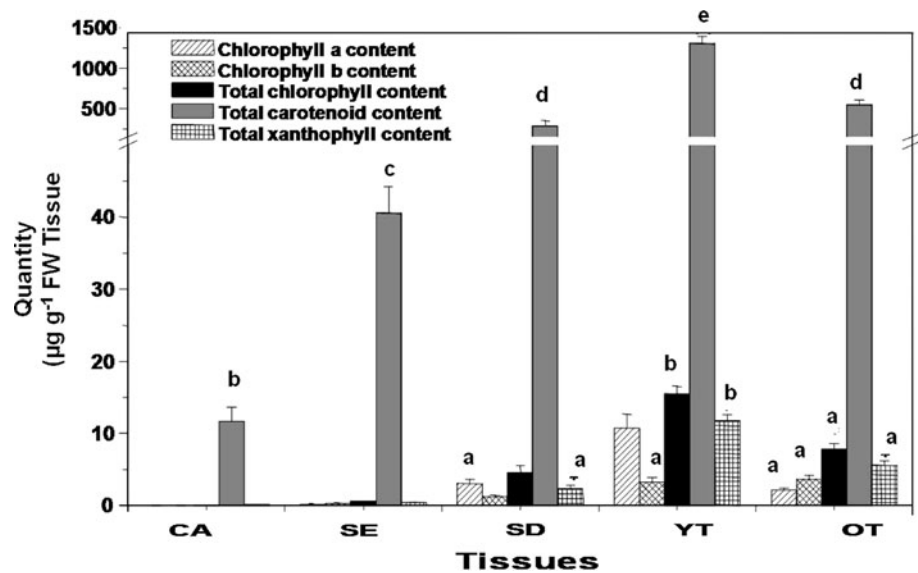
carotenoids and xanthophylls contents increased from callus to young tree stages, with slightly lower values for old tree (Fig. 3).

Inhibition studies

Inhibition of HMG-CoAR in the heart-shaped somatic embryo cell suspension cultures of sandalwood showed a significant effect on the resulting photosynthetic isoprenoid intracellular pigments accumulated viz. chlorophylls a and b, carotenoids and xanthophylls (Fig. 4).

Tissue level expression profiling of the four genes

First, the partial clones of five transcripts (ACT, DXS, FPPS, MTPS, and STPS) were obtained and were submitted to the GenBank[TM] at NCBI. The transcription levels of four genes involved in isoprenoid biosynthesis, i.e.,

DXS, FPPS, MTPS, and STPS were measured by sqRT-PCR. Actin (ACT) transcripts were constitutively expressed in all examined tissues, and hence were used as the positive control for sq RT-PCR studies (Fig. 5). The DXS expression levels were comparable in callus and young tree, while was lowest in seedling. The FPPS expression levels were twice higher in callus than the young tree twig, but could not be detected in any other tissues. The MTPS expression levels in callus were almost three times higher than somatic embryo and twice higher than the young tree. Callus and seedlings only expressed the STPS, at comparable levels and quite high (Table 6).

Discussion

This investigation was intended to understand the terpenoid metabolic events underway across in vitro and in vivo
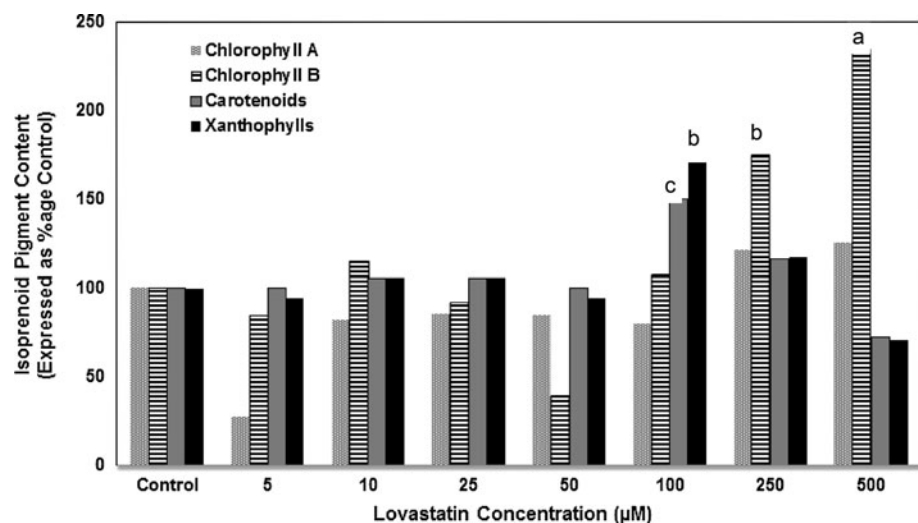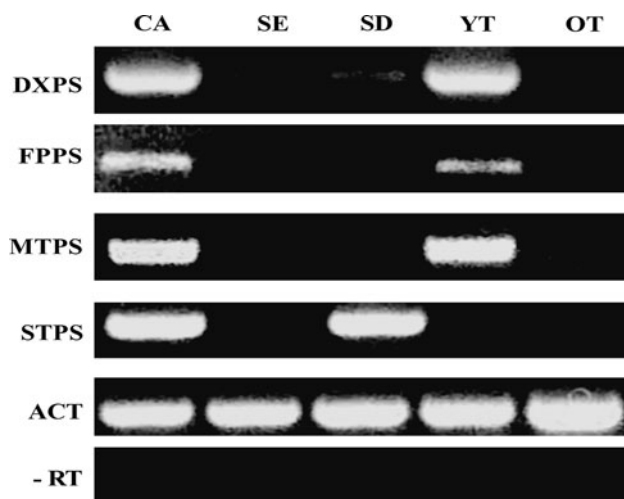
Fig. 5 Semi quantitative RT-PCR measured expression levels of four genes in sandalwood tissues. *CA* callus, *SE* somatic embryo, *SD* seedling, *YT* young tree, *OT* old tree, where DXPS, FPPS, MTPS, STPS are genes of interest, ACT-positive control, RT-negative control

developmental stages in sandalwood tree using enzymes and their inhibitors, genes encoding them and their expression levels and metabolites thereof. Involvement of both mevalonate-dependent (MVA) and independent (MEP) pathways for formation of terpenoids in angiosperms is well known. The DXS reaction is a rate-limiting step of the non-mevalonate pathway. The DXS activity increased with differentiation, which eventually indicated proportionate pigment (chlorophyll, carotenoids, xanthophylls) biosynthesis and accumulation, at least for the first three in vitro developmental stages. Uncorrelated levels of DXS with chlorophyll and carotenoid content could be attributed to the fact that the capacity of various tissues to make and/or degrade carotenoids may differ substantially, in the case of matured tissues (Estevez et al. 2001). The activity of HMG-CoAR, a rate-limiting enzyme of the mevalonate pathway, was found to be highest in callus. Evidently, HMG-CoAR activities are associated with rapidly growing tissues, and much reduced activity is reported in matured tissues (Bach et al. 1980), i.e., reductase activity from mature leaves of pea seedlings was only 7 % of that

observed in the apical buds (Brooker and Russell 1975), thus implying association with rapid division of cells. Similarly, relatively rapid growth of organs and requirement of sterols have been associated with enhanced HMG-CoAR activities (Schaller et al. 1995). This could partly explain the absence of HMG-CoAR activity in seedlings and young tree tissues, where the levels could be below detection limits.

STPSs catalyze the cyclization of FPP into a myriad of cyclic intermediates which are the backbone hydrocarbons for the synthesis 7,000 sesquiterpenoid compounds in nature (Bohlmann et al. 1998), out of which sandalwood boasts 70–80 of such genus-specific sesquiterpenoids with 'santalane' backbones. FPP is the precursor for santalol biosynthesis (Parker et al. 1967) and hence, was used as the substrate in the cell-free assays to measure the STPS activities in sandalwood developmental stages. Reaction products were monitored by HPTLC (dependent on R_f value matches with authentic standards) and GC-FID methods [dependent on Kovat's (KI) (Adams 1985) or linear retention index (LRI) matches]. Proper controls were used in the assays, such as internal controls during extraction of assay supernatant, monitoring assays without FPP feeding, without divalent cations, without proteins from tissues and doing manual subtraction of products from control assay reactions, which did not include FPP, from those reactions inclusive of FPP. In sandalwood developmental stages, tissue-specific variability of assay products, differential quantitative and qualitative product profiles possibly have widespread implications in the tree physiology. Farnesol being the major STPS assay product from callus and somatic embryos, Z-lanceol for seedling and old tree, α-bergamotene for young tree; these implicate the dynamic product conversion ability for the STPS enzymes in general. Negative control assays containing only reaction buffer and FPP produced no farnesol or santalol, indicating that santalol/farnesol production is not a result of the dephosphorylation and incomplete conversion of FPP to sesquiterpenes (Göpfert et al. 2009), rearrangement/transformation of FPP, result of action of endogenous phosphatases, hydroxylation from water, enzymatic solvolysis of FPP and or merely an artefact from higher

Table 6 Relative tissue level expression profiling of sandalwood genes by sqRT-PCR

Gene name	Ampl. length (bp)	Callus	Somatic embryo	Seedling	Young tree	Old tree
ACT	460	0.12	0.12	0.13	0.13	0.12
DXS	277	0.33	0	0.09	0.30	0
FPPS	101	0.67	0	0	0.33	0
MTPS	187	0.49	0.15	0	0.36	0
STPS	144	0.49	0	0.51	0	0

Products from RT-PCR with ACT primers were used as cDNA loading control

column temperatures during GC analyses. Results could also suggest the presence of multiple enzyme isoforms in the cell-free extracts induced by differential tissue-or organ-specificity and possible temporal regulation of gene expression. The number of unidentified products ranged from 18 to 28 for all the five stages of in vitro and in vivo origin. In fact, recently it was shown in case of a chamomile terpene synthase (MrTPS5 from *Matricaria recutita*), apart from the major product germacrene D, the trace amounts of sesquiterpene constituents remained unidentified (Irmisch et al. 2012). Exclusive appearance of lanceols and farnesols in matured tissues, and occurrence of santalenes and santalols in a mutually exclusive manner in some tissues implicate the roles of multiple modifier key enzymes in the crude protein mixtures, such as P450 hydroxylases (Jones et al. 2011), among the most important ones. Lanceols Sesquiterpenoids are known to vary extensively in their abundance among different organs and developmental stages, i.e., presence of approx. 14 tissue-specific olefins (sesquiterpene hydrocarbons) in only pentane extracts (Köllner et al. 2004). However, the differential conversion of cis- or trans-isomers of FPP into completely different chemical entities as products is also known (Takasawa et al. 1997). Recently, the differential effects of solvent extraction on extractability of sesquiterpenoid constituents were observed for immature heartwood samples in sandalwood tree in the author's laboratory (Misra and Dey 2012b). Sandalwood oil differs from the essential oils isolated from mint family plants in having a sesquiterpene base rather than a monoterpene base, as evidences by earlier studies (Howes et al. 2004), while we furthered it by means of HPTLC, GC and GC–MS analyses. For instance, sandalwood oil consists of more than 25 structurally distinct sesquiterpenes, of which 16 were identified and quantified by GC studies that could arise from an equal number of unique STPSs, or a limited number of multi-functional enzymes (Jones et al. 2011). Comparison of the sesquiterpene content of a commercial sandalwood oil to an analysis of sesquiterpenes found in heartwood suggests that several constituents are lost (mostly thermo labile hydrocarbons) or enriched (santalols) during the commercial distillation process. In sandalwood developmental stages, a single product constituting more than 50 % of total products [(i.e., 50 % farnesol in callus, 32 and 47 % Z-lanceol, in seedling and old tree, respectively] and a diversity of products, i.e., in somatic embryo and young tree, were observed. Interestingly, the three reported STPSs, including the putative recombinant sandalwood santalene synthase (SaSSy), did not yield farnesol or Z-lanceol as products under in vitro conditions (Jones et al. 2011). However, in pentane extracts of *Gossypium* cell suspensions fed with radioactivity, nearly 50 % of radioactivity was observed to be incorporated into a single

sesquiterpenoid product (Chappell and Nable 1987). In fact, terpene synthase families are known to utilize a single substrate to generate a variety of products while single-product terpene synthases also exist (Deguerry et al. 2006). The model plant, *Arabidopsis thaliana* is capable of producing a much wider range of natural products than was previously appreciated (D'Auria and Gershenzon 2005). Thus, production of bergamotenes, lanceols, and monoterpenoids in sandalwood is probably owing to the advantage in the plasticity of natural products biosynthesis and conformational mobility within the STPS enzyme. Moreover, many STPS products from various tissues remain unidentified in many cases (Bino et al. 2004). STPSs yield more than one product, i.e., amorpha-4, 11-diene synthase from *Artemisia annua*; δ-selinene synthase and γ-humulene synthase from *Abies grandis*, are known to yield 16, 34, and 52 different sesquiterpenoids, respectively (Picaud et al. 2005). Furthermore, it has been observed that in contrast to the single-product isopimaradiene (diterpene) synthase from Norway spruce, a Sitka spruce diterpene synthase is known to produce minor amounts of sandaracopimaradiene (2 %) in addition to isopimaradiene (98 %) (Keeling et al. 2011). The enzyme (DXS, HMG-CoAR, and STPS) activities obtained across developmental stages are indicative of operative MVA and MEP pathways in sandalwood tree life cycle, where it remains to be seen whether both contribute towards the sesquiterpene-type molecule biosynthesis, through in vitro labeling studies.

Metabolite profiling of the five sandalwood stages was performed by means of both, HPTLC and GC analyses, respectively. HPTLC-based sesquiterpenoid profiling revealed a santalol rich (∼50 %) callus, β-santalol and α-bergamotol dominated somatic embryos, β-santalol rich seedlings, santalol (∼48 %) rich young tree, and β-santalol-rich old tree. Recently, induced sandalwood callus was reported to accumulate santalenes, santalols and α-bergamotol (Crovadore et al. 2012). Interestingly, in case of seedlings, only 30 % of the total constituents could be identified, thus indicating the presence of many obscure and unknown constituents. The developmental stages accumulated different amounts of sesquiterpenoids in various ratios and compositions. The GC analysis of heartwood extractives from the old tree revealed more similar compositions to sandalwood oil than variable constituents. Sesquiterpene alcohol (oxygenated sesquiterpenoids) content in somatic embryo and heartwood were about 44 and 28 %, whereas sesquiterpene olefins (hydrocarbons) constituted only 7–8 %, respectively. Regardless of how these differences in the sesquiterpene profiles come about, either because of selective losses, concentration or secondary transformations occurring during the distillation process, the tissue-specific STPSs described here appear sufficient to account for the majority of terpenes accumulating in the

sandalwood tissues. In fact, a recent study on profiling of sandalwood callus only allowed the 'tentative' identification of several oxygenated sesquiterpenoids (Crovadore et al. 2012).

This constitutes the first report on biosynthetic routes of sesquiterpenoids in in vitro deveopmental stages of sandalwood. When grown on a 12-h light cycle with a minimum temperature of 18 °C, relatively high levels of santalols and several other sesquiterpenes accumulated in the somatic embryo and seedlings (12–56 ng g^{-1} FW). Because of the rather low in vitro activity for STPSs, minor sesquiterpenes (less than 1 % each of them) might have escaped detection. Firmly so, accumulation of terpenoids resulting from de novo synthesis has been demonstrated in tissue cultures of several oil-bearing plants and many culture lines which are extensively reviewed (Banthorpe and Branch 1985) in undifferentiated tissues. Previously, essential oil compositions of parent plants and in vitro cultures in medicinal plants were compared (Chebel et al. 1998). Similarly, in this particular study the sesquiterpenoid profiles were compared among various in vitro and in vivo developmental stages, which though comparable, showed marked differences, in terms of the absence and presence of several constituents, abundance and their chemical nature. Nevertheless, the amount of terpenoids in callus was approx. seven fold lesser than the old tree tissues. The somatic embryos represent a metabolically dynamic stage of development, undergoing rapid differentiation and possess high morphogenetic potential. Thus, the sesquiterpenoid content and profiles of somatic embryos were comparable to sandalwood oil. From a phytochemical point of view, the sesquiterpenoid components obtained from the callus, somatic embryo, seedling, young and old trees vary significantly in diversity and chemical composition in sandalwood.

FPP is the sesquiterpenoid biosynthesis intermediate and a key regulatory molecule for turn-over of sesquiterpenoids. Moreover, the enzyme FPPS regulates the conversion of IPP into both sterols and sesquiterpenoids. Thus, the quantification of FPP, farnesol (hydroxylation product) and farnesene (dephosphorylation product) by HPTLC analysis, based on their log P values, spectral characteristics and chromatographic behaviour, allowed profiling of farnesylated intermediates in the developmental stages. The in vitro tissues indicated accumulation of comparable amounts of farnesylated isoprenoids, while the levels were significantly lower in seedling, young and old tree shoots. Similarly, α-farnesene levels derived from in vitro propagated plantlets were higher than in in vivo ginger plants (Ma and Gang 2006). Furthermore, farnesol formation is known to be catalyzed by terpene synthases (Schnee et al. 2002). Farnesol is the non-sterol regulatory molecule controlling HMG-CoAR activity in a stress-dependent manner in

tobacco cell line, BY-2 (Shearer and Hampton 2005). Thus, farnesol-levels could modulate HMG-CoAR activity, thus regulating terpenoid biosynthesis in sandalwood tissues. Next, it may be concluded that sesquiterpenoids in sandalwood developmental stages are biosynthesized in situ, and this process is developmentally regulated, possibly by the levels of STPS activity. In this study, the activities of the enzyme squalene synthase or the levels of sterols were not measured as possible means for explanation of FPP utilization. However, recently in Artemisia annua L., it was observed that squalenes synthases are significant competitor for FPP in artemisinin-producing tissues in comparison to STPSes (Olofsson et al. 2011). Furthermore, it is noteworthy that the partitioning and metabolic dichotomization in sesquiterpene biosynthesis could be the result of metabolite channelling, for example, through two different FPPS in the cytoplasm, although it is unlikely that MVA precursors are available for the biosynthesis of other terpenoid classes, such as sterols. In addition, the possibility of non-FPP precursor-based biosynthesis of sesquiterpenoids has been proposed (Besser et al. 2009).

Inhibition of the cytosolic MVA pathway enzyme HMG-CoAR, by culturing of suspension cultured cells in medium containing lovastatin (concentration range 1–500 μM), was monitored by an increase in absorption of 80 % acetone extracts at wavelength ranges characteristic of carotenoids (370–520 nm) and chlorophylls (430–460 and 640–670 nm). Furthermore, it is interesting to note that, HMG-CoAR inhibition resulted in enhanced production of photosynthetic pigments beyond doses of 100 μM or more, indicating a possible contribution of plastidial MEP pathway in pigment biosynthesis. These results provided evidence for an increased flux through the plastidial MVA-independent pathway, resulting in an increased production, or alternatively reduced catabolism, of carotenoids and chlorophylls as a response to inhibition of the cytosolic MVA pathway. In fact, an increased accumulation of the carotenoid lycopene was reported for lovastatin-treated tomato fruits (Rodríguez-Concepción and Gruissem 1999), indicating a possible crosstalk between the cytosolic and the plastidial pathways of isoprenoid biosynthesis.

Transcriptional profiling experiments suggest that metabolically active genes are all expressed when the whole plant-body is used for RNA extraction. Unfortunately, in this investigation, owing to numerous constraints, the actual sites of expression could not be distinguished. The higher expression levels of genes in callus may be attributed to their higher proliferation rates and metabolism. In a recent study on olive fruit development, it was observed that the transcripts involved in MVA pathway were predominantly not regulated, while six out of the seven genes coding for MEP pathway were developmentally regulated

(Alagna et al. 2009). Moreover, transcripts of FPPS were up regulated in those tissues, where expression of MTPS and STPS (except for young tree) genes was detected at highest level, as also observed in sunflower glandular trichomes (Göpfert et al. 2009). Disagreement between gene expression and protein or metabolite levels is frequently reported because the later approaches study the individual molecules, whereas conservative sequences used in PCR verification may lead to the quantification of the abundance of several related transcripts or isoforms. An alternative explanation for inconsistent protein, metabolite, and mRNA levels could be different rates in mRNA and protein synthesis, and higher or lower level of protein/metabolite turnover. Moreover, depending upon the plant system under study, there may be developmental changes and expression fluctuations in erratic ways for instance, an increase followed by a decrease and again an increase, as observed in in vitro systems like soybean, maize, *Picea* (Stasolla et al. 2004). The differentiated gene expression patterns are attributed to dynamic changes during development and not always simple increase or decrease in abundance of transcripts. Similarly, in this study, the gene expression levels in young tree showed an upsurge in expression levels. Interestingly, the strong MTPS expression levels in callus and young tree shoots indicate developmental regulation for biosynthesis of monoterpenoids in sandalwood, which are lesser known constituents of the Australian sandalwood oil obtained from *S. spicatum* (Valder and Neugebauer 2003; Braun et al. 2005).

Adopting integrated approaches in sesquiterpenoid profiling, enzymatic assays, quantification of intermediates, gene expression patterns and inhibition studies, a road map towards understanding of sesquiterpenoid metabolism in sandalwood has been put forward. This would allow further inroads in detailed in-depth investigations to understand the regulation of the critical biosynthetic and metabolic events that have significant impact on sandalwood oil deposition, both in terms of quantity and quality. This investigation reveals that degenerate PCR primers corresponding to conserved domains can be successfully used for isolation of genes from sandalwood. The MTPS and STPS represent new genes in the terpene synthase family that are represented in sandalwood. The cloning and full-length characterization of these key sesquiterpenoid biosynthetic pathway genes would offer new perspectives in understanding of sesquiterpenoid biosynthetic pathway machinery in sandalwood tree.

Conclusion

The major aim of our study was to increase our understanding of sesquiterpenoid metabolism in *S. album* life cycle. It is notable that sesquiterpenoid biosynthesis and accumulation patterns in sandalwood tissues are highly polymorphic and their levels might be influenced by climacteric and developmental traits. Traditional experience of having superior quality sandalwood grown only in Indian soil (esp. in southern states) also probably suggests this view. These results support the contention that sandalwood extractives from different sources are in fact likely to possess different properties, both in chemical nature and aroma, but also in potential bioactivity and healthcare benefits.

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