Archives of Biochemistry and Biophysics 477 (2008) 121–130

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Isolation of cDNAs and functional characterisation of two multi-product terpene synthase enzymes from sandalwood, *Santalum album* L.

Christopher G. Jones [a,c,*], Christopher I. Keeling [b], Emilio L. Ghisalberti [c], Elizabeth L. Barbour [d], Julie A. Plummer [a], Jörg Bohlmann [b]

[a] School of Plant Biology (M084), Faculty of Natural and Agricultural Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
[b] Michael Smith Laboratories, University of British Columbia 301-2185 East Mall, Vancouver, BC, Canada V6T 1Z4
[c] School of Biomedical, Biomolecular and Chemical Sciences (M313), Faculty of Life and Physical Sciences, University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia
[d] Forest Products Commission of Western Australia, 117 Great Eastern Highway, Rivervale, WA 6103, Australia

ARTICLE INFO

Article history:
Received 14 March 2008
and in revised form 14 May 2008
Available online 27 May 2008

Keywords:
Santalum album
Terpene synthase
Germacrene D-4-ol
α-Terpineol
Sesquiterpene

ABSTRACT

Sandalwood, *Santalum album* (Santalaceae) is a small hemi-parasitic tropical tree of great economic value. Sandalwood timber contains resins and essential oils, particularly the santalols, santalenes and dozens of other minor sesquiterpenoids. These sesquiterpenoids provide the unique sandalwood fragrance. The research described in this paper set out to identify genes involved in essential oil biosynthesis, particularly terpene synthases (TPS) in *S. album*, with the long-term aim of better understanding heartwood oil production. Degenerate TPS primers amplified two genomic TPS fragments from *S. album*, one of which enabled the isolation of two TPS cDNAs, *Sa*monoTPS1 (1731 bp) and *Sa*sesquiTPS1 (1680 bp). Both translated protein sequences shared highest similarity with known TPS from grapevine (*Vitis vinifera*). Heterologous expression in *Escherichia coli* produced catalytically active proteins. *Sa*monoTPS1 was identified as a monoterpene synthase which produced a mixture of (+)-α-terpineol and (−)-limonene, along with small quantities of linalool, myrcene, (−)-α-pinene, (+)-sabinene and geraniol when assayed with geranyl diphosphate. Sesquiterpene synthase *Sa*sesquiTPS1 produced the monocyclic sesquiterpene alcohol germacrene D-4-ol and helminthogermacrene, when incubated with farnesyl diphosphate. Also present were α-bulnesene, γ-muurolene, α- and β-selinenes, as well as several other minor bicyclic compounds. Although these sesquiterpenes are present in only minute quantities in the distilled sandalwood oil, the genes and their encoded enzymes described here represent the first TPS isolated and characterised from a member of the Santalaceae plant family and they may enable the future discovery of additional TPS genes in sandalwood.

Santalum album L. is a small hemi-parasitic tree found growing in southern India, Sri Lanka, eastern Indonesia and northern Australia. The timber is highly sought after for its fine grain, high density and excellent carving properties. The fragrant wood is usually ground and steam distilled, with the essential oil serving as a fixative for many high-end perfumes. Centuries of over-exploitation has led to the demise of sandalwood in natural stands. Large plantations are being established throughout northern Australia to satisfy demand and conserve remaining reserves. *Santalum album* heartwood contains up to 6% dry wt. sesquiterpene oils [1] predominantly α- and β-santalol, α-*trans*-bergamotol and *epi*-β-santalol, along with the sesquiterpene olefins α- and β-santalene, α-bergamotene and *epi*-β-santalene, β-bisabolene, α-, β- and γ-curcumene [2]. A series of aromatic and phenolic compounds have also been identified in the heartwood of *S. album* [3]. The amount of heartwood oil produced in a tree varies considerably, even under near-identical growing conditions [4]. Causes of this yield variation are not well understood, but it is likely to be the result of both genetic and environmental factors.

Almost nothing is known about the biosynthesis of sesquiterpenoids in *S. album* or how essential oil production is regulated. Biosynthetic schemes were proposed decades ago [5] but these schemes have not been studied biochemically. Recent investigations into co-occurrence patterns of several sesquiterpenes in wood extracts suggest multiple products are formed from common carbocation intermediates [1]. Multiple product formation by single terpene synthase (TPS)[1] enzymes is well documented in the literature [6–10]. Terpenoid biosynthesis is

* Corresponding author. Address: School of Plant Biology (M084), Faculty of Natural and Agricultural Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia. Fax: +61 8 6488 1108.
E-mail address: jonescg@cyllene.uwa.edu.au (C.G. Jones).

[1] *Abbreviations used:* TPS, terpene synthases; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; TEAS, tobacco *epi*-aristolochene synthase; NPP, nerolidyl diphosphate; PVPP, polyvinylpolypyrrolidone; IPTG, Isopropyl-β-D-thiogalactopyranoside.

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.05.008

widely understood to initiate through either the mevalonic acid pathway or the deoxy-D-xylulose pathway to isoprenoid diphosphate precursors like geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), and these are in turn converted by TPS to the terpenoid products typical of many plant essential oils [11]. Plant terpenoids may be involved in defence, and the production of multiple compounds from a single TPS enzyme may provide an evolutionary advantage through increased resistance against potential herbivores or pathogens [12–14]. We proposed that TPS are active in the heartwood and leaves of mature sandalwood trees, and that the diverse mono- and sesquiterpenoid profile of *S. album* likely stems from several multi-product TPS enzymes collectively involved in total oil biosynthesis. Essential oil is located in the heartwood of stems and roots of sandalwood, with biosynthesis probably occurring in parenchyma. Microscopic sections of *S. album* wood show the accumulation of oil and other extractives in ray parenchyma (Fig. 1). The sapwood–heartwood transition zone is therefore a logical place for the isolation of TPS mRNAs and cDNA cloning. We describe here the functional characterization of the first two TPS cDNAs from *S. album*.

Results

Identification of TPS gene fragments amplified from genomic DNA of S. album

Degenerate primers (primers p5F and p8R) were based on conserved regions of several published angiosperm sesqui-TPS genes; valencene synthase from *Vitis vinifera* (grapevine; AAS66358), germacrene D synthase from *Populus trichocarpa x deltoides* (poplar; AAR99061), δ-cadinene synthase from *Gossypium hirsutum* (cotton; AAD51718) and β-caryophyllene synthase from *Artemisia annua* (AAL79181). These primers amplified an intron-free 180 bp fragment from *S. album* genomic DNA. Upon cloning and sequencing of the PCR product, two independent TPS gene sequences were apparent. Based on BLAST searches against the NCBI GenBank nr. database (www.ncbi.nih.gov) the deduced amino acid sequences from these fragments both showed strong similarity to known sesquiterpene synthases from grapevine and cotton. The predicted peptide sequences of the two gDNA fragments cloned from *S. album* differed by 13 amino acids (87% identity, 100% similarity).

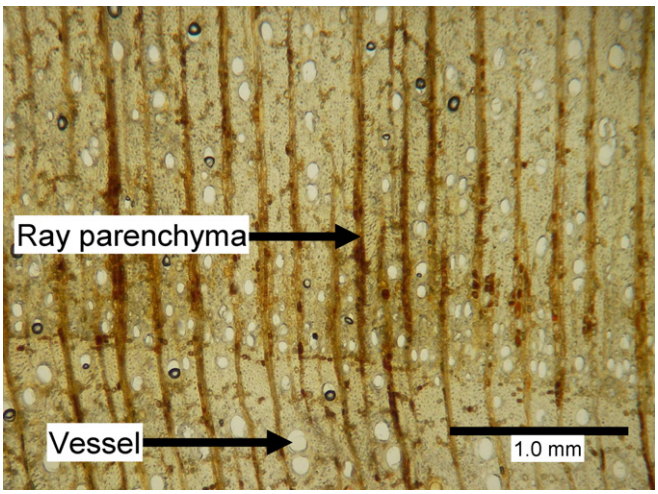

Fig. 1. Transverse section of *S. album* heartwood (lower)–sapwood (upper) transition zone (40× magnification). Essential oil and other extractives are deposited in the ray parenchyma (vertical features) during heartwood formation.

Full-length cDNA cloning of two TPS genes from S. album

The partial genomic TPS sequences enabled the design of PCR primers to be used for iterative rapid amplification of cDNA ends (RACE) which yielded the 3′- and 5′-cDNA ends and eventually the complete cDNA clones for two distinct TPS genes, *Sa*monoTPS1 (GenBank accession no. ACF24767) and *Sa*sesquiTPS1 (GenBank accession no. ACF24768) (Figs. 2 and 3). *Sa*sesquiTPS1 showed highest similarity to other angiosperm sesqui-TPS. *Sa*sesquiTPS1 was isolated from wood cDNA. Although all initial primer design was targeted at sesquiterpene synthases, the other cDNA *Sa*monoTPS1, isolated from leaf cDNA showed highest similarity to previously discovered monoterpene synthases, predominantly grapevine α-terpineol synthase. Alignments of the two *S. album* TPS deduced amino acid sequences with other angiosperm mono-TPS and sesqui-TPS are shown in Figs. 2 and 3, respectively.

*Sa*monoTPS1 features motifs typical of the monoterpene synthase gene TPS-b subfamily [15] including the aspartate rich DDXXD metal ion binding site (positions 323–327) and the RRX_8W motif (positions 33–44) which is implicated in diphosphate group migration [16]. *Sa*sesquiTPS1 also shares the $R(R/P)X_8W$ motif (positions 19–29) and the DDXXD motif. Y538 of *Sa*sesquiTPS1 resides in approximately the same position as Y520 of tobacco *epi*-aristolochene synthase (TEAS) [17,18] (Fig. 3). This residue has been shown to be responsible for protonation of germacrene A in TEAS [19].

Functional characterisation of SamonoTPS1 and SasesquiTPS1

The proteins encoded by cDNAs *Sa*monoTPS1 and *Sa*sesquiTPS1 were expressed as full-length His-tagged fusion proteins (N-terminal His_6) in *Escherichia coli*. Recombinant proteins were purified using Ni^{2+} affinity chromatography and confirmed to be of the expected size of ~66 kDa by SDS–PAGE and Western blot analysis using His_6-specific antibody.

Enzyme assays using *Sa*monoTPS1 recombinant enzyme with GPP as substrate in the presence of Mn^{2+} produced a mixture of mainly (+)-α-terpineol and (−)-limonene as well as a selection of minor monoterpene products as determined by GC–MS (Fig. 4; Table 1). Chemical ionisation assessment of α-terpineol in negative mode revealed an $[M-1]^-$ peak of $m/z = 153$, indicative of the alcohol. When (*E,E*)-FPP was used as substrate, only small amounts of one compound, β-bisabolene, was produced (data not shown). Under the chosen assay conditions, the catalytic activity and product profile of *Sa*monoTPS1 on either substrate was not substantially affected by exchanging the Mn^{2+} metal ion with Mg^{2+} (Table 1).

Enzyme assays of *Sa*sesquiTPS1 with FPP as substrate and Mg^{2+} ion containing buffer produced a complex mixture of mono- and bicyclic sesquiterpenes. The main products as analysed by GC–MS and GC-FID were the cyclic alcohol germacrene D-4-ol, and helminthogermacrene (Fig. 5 and Table 2). On-column injection with chemical ionization in negative mode using methane as the ionization gas confirmed the assignment of alcohol based upon the presence of $[M-1]^-$ of $m/z = 221$ for germacrene D-4-ol.

High GC injector temperatures resulted in a large proportion of β-elemene, and a suite of other bicyclic sesquiterpenes including δ- and γ-cadinene. Cold on-column GC–MS analysis revealed no β-elemene, and considerably less of the cadinenes. Several minor components with mass spectra and retention indices matching α- and β-selinene, α-humulene and nerolidol were detected in proportions below 5% (Fig. 5 and Table 2). In the presence of Mn^{2+}, a similar product profile was detected however the overall yield was almost 10-fold lower. When GPP was used as substrate, *Sa*sesquiTPS1 only produced very small amounts of linalool, regardless of which metal ion was used (data not shown).