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A cDNA clone for 3-carene synthase from *Salvia stenophylla*☆

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Abstract

The essential oil of *Salvia stenophylla* contains (+)-3-carene as the principal monoterpene component. Using an enriched cDNA library prepared from mRNA isolated from *S. stenophylla* peltate glandular trichomes, and a homology-based cloning strategy, a full-length cDNA was isolated that encoded a preprotein of 69.7 kDa which resembled a monoterpene synthase in sequence. Heterologous expression of the gene in *Escherichia coli* provided a soluble recombinant enzyme capable of catalyzing the divalent metal ion-dependent conversion of geranyl diphosphate to (+)-3-carene and to lesser amounts of limonene, myrcene, 4-carene and β-phellandrene. This multiple-product synthase is responsible for the production of all of the essential oil monoterpenes of *S. stenophylla*.

Keywords: *Salvia stenophylla*; Lamiaceae; Glandular trichome isolation; Homology-based cDNA cloning; Recombinant protein expression; Monoterpene synthase; 3-Carene synthase; Monoterpene; Geranyl diphosphate cyclization

1. Introduction

3-Carene **1** is the only commonly occurring member of the carane structural family of monoterpenes characterized by the bicyclo[4.1.0] skeleton (Fig. 1) (Buckingham, 1998), and this unusual monoterpene olefin has been reported as a constituent of the turpentine of several conifer species (Norin, 1972; von Rudloff, 1975). A (+)-(1*S*,6*R*)-3-carene synthase was first isolated from Douglas fir (*Pseudotsuga menziesii*) and lodgepole pine (*Pinus contorta*), and shown to possess physical and kinetic properties typical of other monoterpene cyclases from the Coniferae (Savage and Croteau, 1993). The enzyme converts geranyl diphosphate **2**, the universal precursor of the monoterpenes (Wise and Croteau, 1999), via enzyme bound (3*S*)-linalyl diphosphate **3** to (+)-3-carene **1** by a mechanism involving the initial isomerization and cyclization to the (4*S*)-α-terpinyl cation **4** followed by direct *anti*-1,3-elimination of the 5-*proR* hydrogen of the geranyl precursor to form the cyclopropyl function (Fig. 1).

A cDNA encoding (+)-3-carene synthase was recently isolated from Norway spruce (*Picea abies*) (Fäldt et al., 2003) and shown to resemble most closely in deduced primary structure the terpinolene synthase from grand fir (*Abies grandis*) (Bohlmann et al., 1999), an enzyme which employs a very similar cyclization mechanism (Fig. 1). Although monoterpene synthases responsible for the construction of the *p*-menthane, bornane, pinane, camphane, thujane and fenchane families of monoterpenes have been isolated from various members of the Lamiaceae, Asteraceae and Umbelliferae (Wise and Croteau, 1999), and many of the corresponding genes have been described (Bohlmann et al., 1998), no carane-type terpene synthase of angiosperm origin has been reported.

A survey of the essential oils of a range of *Salvia* species (Kintzios, 2000) revealed that the volatile complex of the South African species *Salvia stenophylla* is very simple in containing principally the monoterpene 3-carene **1** (22%), the sesquiterpenol *epi*-α-bisabolol (43%) and the diterpenol manool (25%). As with most species of *Salvia, S. stenophylla* possesses abundant peltate glandular trichomes in which the terpenoid essential oil is produced. Modification of an established protocol (Gershenzon et al., 1991; Gershenzon et al., 1992) allowed the abrasive isolation of these secretory structures as an

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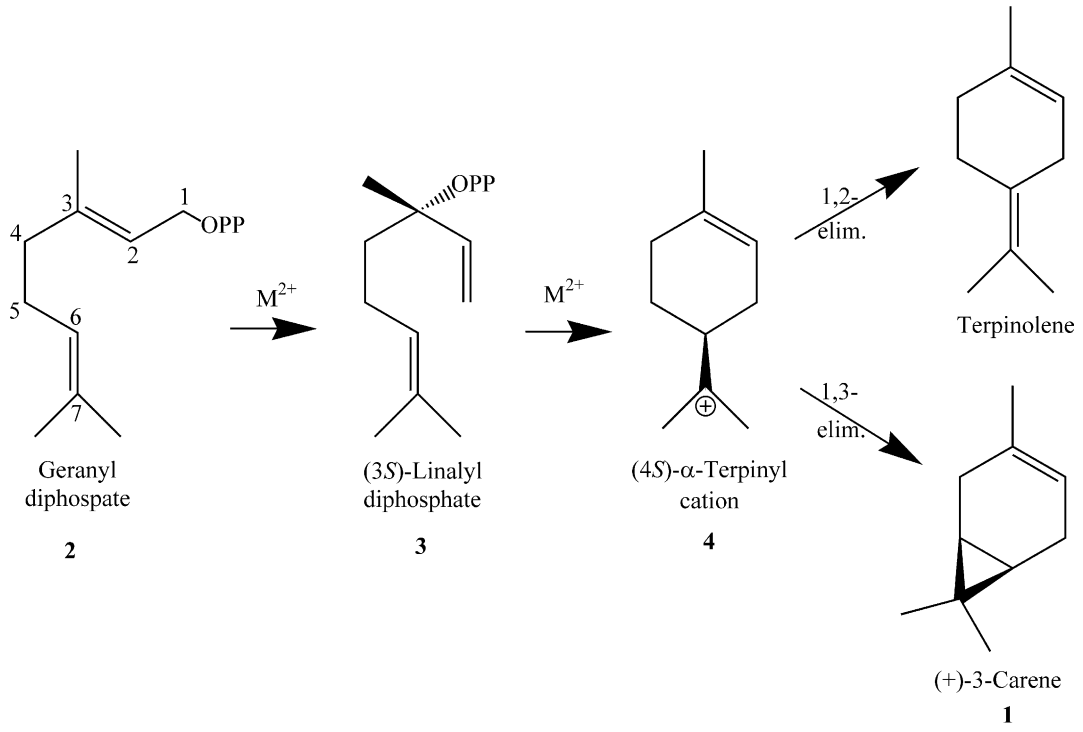

Fig. 1. Cyclization of geranyl diphosphate **2** to (+)-3-carene **1** by metal ion-dependent ionization and isomerization to (3*S*)-linalyl diphosphate **3**, followed by metal ion-dependent ionization and cyclization to the (4*S*)-α-terpinyl cation **4**, and 1,3-elimination of a proton to (+)-3-carene. Terpinolene is similarly formed by 1,2-elimination from the α-terpinyl intermediate.

enriched source of the relevant mRNA species from which a cDNA library was constructed. Using this tool, a homology-based cloning strategy yielded the target cDNA for 3-carene synthase which was characterized and functionally expressed in *Escherichia coli*. The recombinant synthase was shown to catalyze the divalent metal ion-dependent conversion of geranyl diphosphate **2** to (+)-3-carene **1** (73% of the product mix), and to lesser amounts of limonene, myrcene, 4-carene and β-phellandrene, thereby suggesting that this single enzyme is responsible for the production of all of the monoterpenes of *S. stenophylla* essential oil.

2. Results and discussion

2.1. Source material and library construction

Research thus far has been conducted only with 3-carene synthase from the Coniferales (Savage and Croteau, 1993; Fäldt et al., 2002). Since much of our prior work on monoterpene biosynthesis has been focused on members of the mint (Lamiaceae) family, from which cDNAs encoding a range of monoterpene cyclases have been isolated from the genera *Mentha*, *Salvia* and *Perilla* (Colby et al., 1993; Yuba et al., 1996; Wise et al., 1998), a gene for carene synthase was sought from this source. The terpenoid components of the essential oils of several potentially useful *Salvia* species (Kintzios, 2000) were screened by extraction of immature leaves from greenhouse material followed by GC-MS analysis. *Salvia stenophylla* was shown to contain in the extract 22% (+)-3-carene **1**, 6% myrcene, 2.5% limonene, 1% α-phellandrene, <1% 4-carene and a trace of β-phellandrene (Fig. 2), along with the sesquiterpenoid *epi*-α-bisabolol (43%) and the diterpenoid manool (25%). The composition of this terpenoid fraction did not change markedly with leaf maturity, and these analytical results were consistent with those of a previous study (Brunke and Hammerschmidt, 1985). A prior report that α-phellandrene was the principal monoterpene of *S. stenophylla* (Jecquier et al., 1980) could not be verified. *S. stenophylla* was therefore used as a source of mRNA for library construction in a homology-based cloning effort to acquire the 3-carene synthase by first developing a method for the isolation of the glandular trichomes in which the essential oil terpenes are produced.

S. stenophylla produces abundant peltate glandular trichomes on the leaf surfaces which are comprised of a basal cell, a stalk cell and a head of up to 20 radially-distributed secretory cells surmounted by the subcuticular oil storage cavity (G.W. Turner, D.J. Hoelscher and R. Croteau, unpublished). These oil glands are larger than those of peppermint, which normally possess eight secretory cells (Turner et al., 2000), but the similar overall morphology suggested that a previously developed surface abrasion method for isolating *Mentha* oil

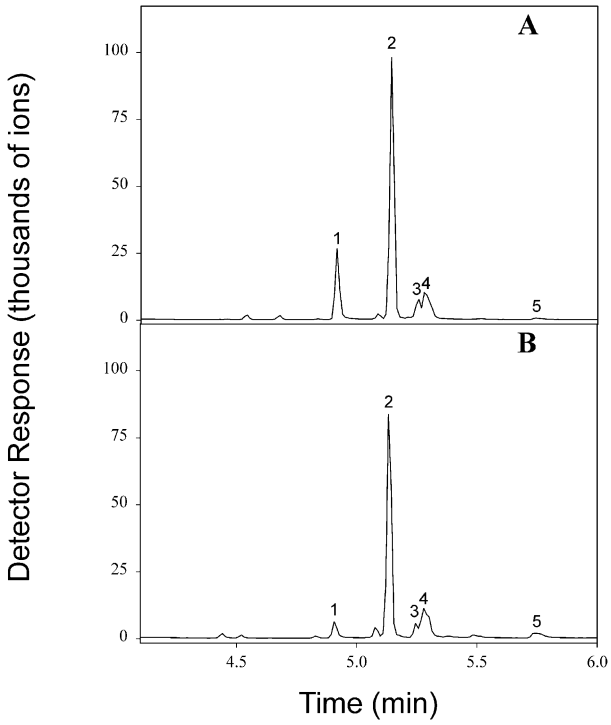

Fig. 2. Total ion chromatograms of the monoterpenes extracted from immature *Salvia stenophylla* leaves (A), and of the monoterpene products derived from geranyl diphosphate by the recombinant 3-carene synthase from the same source (B). The numbered peaks correspond to myrcene (1), 3-carene **1** (2), β-phellandrene (3), limonene (4), and 4-carene (5), and were identified by comparison of retention times and mass spectra to those of authentic standards. The (+)-3-carene **1** and (−)-limonene enantiomers were verified by resolution on a -cyclodextrin chiral phase capillary column.

gland secretory cell clusters (Gershenzon et al., 1991, 1992) could be adapted to *S. stenophylla*. Slight modifications of the viscosity of the isolation buffer and filtration protocol allowed the efficient isolation of peltate gland secretory cell clusters from immature *S. stenophylla* leaves, either with the intact subcuticular storage receptacle (105 μm filtration) or without this structure (50 μm filtration; in roughly half of the isolated cell clusters the storage cavity was ruptured). Starting with approximately 3×10^6 cell clusters as an enriched source of transcripts, mRNA was isolated and purified, and used to construct an oil gland cDNA library of $\sim10^7$ clones with average insert size of 1.6 ± 0.5 kb.

2.2. cDNA isolation and analysis

Based on the assumption that the (+)-3-carene synthase from *S. stenophylla* would resemble limonene synthase from *Mentha* (both of these Lamiaceae monoterpene cyclases catalyze the isomerization and cyclization of geranyl diphosphate **2** to the α-terpinyl cation **4** followed by a terminating deprotonation), a homology-based cloning strategy was employed using the limonene synthase from *M. spicata* (Colby et al., 1993) as a hybridization probe. This approach, involving a moderate stringency screen, yielded abundant positive plaques, and nearly half of the 30 clones purified through two rounds of hybridization appeared to be of full length based on partial sequencing and comparison with other monoterpene synthases. All of these acquisitions (>2.1 kb) possessed identical sequence, with open reading frame of 1791 nt encoding a preprotein of 597 aa with calculated size of 69.7 kDa and pI of 5.3 (~550 aa for the mature protein of 65.0 kDa and pI of 5.9). The sequence (AF527416) falls within the *TpsB* subfamily of plant terpene synthases (Bohlmann et al., 1998). It encodes a typical N-terminal plastidial transit peptide (Williams et al., 1998), the N-terminal pair of arginines ($R_{51}R_{52}$; with another tandem pair at $R_{80}R_{81}$) thought to play an important role in the isomerization step of the monoterpene synthase reaction cycle (Williams et al., 1998), the aspartate-rich motif ($D_{347}D_{348}XXD_{351}$) involved in metal ion-assisted substrate binding and ionization (Davis and Croteau, 2000), and the highly conserved T_{188}, P_{409}, and tandem trypophan pair ($W_{296}W_{297}$). Comparison of the deduced sequence of this putative terpene synthase from *S. stenophylla* (with 50 aa transit peptide deleted) revealed the closest relationship (64–70% I) with (+)- and (−)-limonene synthases (Colby et al., 1993; Yuba et al., 1996; Maruyama et al., 2001, 2002) from other members of the Lamiaceae [*Agastache rugosa* (AY055214), *Schizonepeta tenuifolia* (AF282875), *Perilla frutescens* (AF233894) and *Mentha spicata* (L13459)]. The sequence from *S. stenophylla* does not very closely resemble that of the 3-carene synthase from Norway spruce (Fäldt et al., 2002) (31% I, 41% S); however, it has been shown previously that enzymes from angiosperms and gymnosperms that catalyze ostensibly identical reactions may possess very different primary structures (Bohlmann et al., 1998; Trapp and Croteau, 2001). Thus, it was concluded that a cDNA encoding a monoterpene synthase, presumably 3-carene synthase, had been isolated from the *S. stenophylla* oil gland library.

2.3. cDNA expression and identification

Monoterpene synthases often express more efficiently in soluble form, and with improved kinetics, when the plastidial targeting peptide is deleted to produce a 'pseudomature' form of the enzyme (Williams et al., 1998; Schwab et al., 2001; Crowell et al., 2002). However, in most cases, the preprotein itself can be adequately expressed and assayed directly, with perhaps compromised kinetics but without alteration in product profile (Colby et al., 1993; Wise et al., 1998). Of the many full-length clones acquired, several bore stop codons immediately upstream of the initiating ATG,

thereby permitting direct polycistronic expression from the corresponding pBluescript plasmids as complete preproteins free of the lacZ fusion. Three of these therefore were expressed directly from the cloning vector in the cloning host (pBluescript II in *E. coli* XL1 Blue cells) under conditions previously established for monoterpene synthases from *Salvia* and *Mentha* (Colby et al., 1993; Wise et al., 1998). This procedure yielded a soluble, recombinant enzyme fraction in each case that was assayed by the standard protocol (Schwab et al., 2001) for the divalent metal ion-dependent conversion of [1-^{3}H]geranyl diphosphate to monoterpenoids. These assays produced only a radiolabeled monoterpene olefin fraction, and this material was analyzed by GC–MS (Fig. 2) and shown to contain (+)-3-carene **1** (73%), (−)-limonene (13%), myrcene (6%), 4-carene (4%) and β-phellandrene (1%), thereby confirming that the acquired clone did encode the target (+)-3-carene synthase. Interestingly, the distribution of monoterpenes produced by the recombinant synthase is very similar to the distribution of monoterpene products in the essential oil of *S. stenophylla* (Fig. 2). Multiple-product monoterpene synthases from both gymnosperms and angiosperms are well known (Wise and Croteau, 1999), and, in the present case, it appears that this single enzyme is responsible for producing essentially all of the monoterpenes of *S. stenophylla*.

3. Experimental

3.1. Plant material and terpenoid analysis

Salvia stenophylla (Burch. Ex) Benth (Lamiaceae) seed was obtained from the Botanical Garden, University of California, Berkeley, CA, and plants were grown to flowering under conditions previously described for raising *Mentha* species (Gershenzon et al., 2000). For a rapid survey of essential oil (glandular) terpenoids, intact leaves of various ages were surface extracted with pentane (10 ml/g tissue and 15 min immersion insures complete removal of oil gland contents), and an aliquot of the extract was analyzed directly by GC–MS and by chiral phase capillary GC as previously described (Lewinsohn et al., 1993). Component identification was made by reference to library spectra or by matching of retention times and mass spectra to authentic standards from our own collection.

3.2. Oil gland isolation and cDNA library construction

Secretory cell clusters from *S. stenophylla* peltate oil glands were isolated from young leaves of 2–3 week-old plants (50 g of leaves 10–30 mm in length) using an established surface abrasion technique (Gershenzon et al., 1991, 1992). Following imbibition in ice-cold solution of 2 mM dithiothreitol, 1 mM aurintricarboxylic acid and 5 mM thiourea for several h, the gland heads were removed by agitation with glass beads in buffer consisting of 25 mM HEPES (pH 7.4), 250 mM D-sorbitol, 2 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 1% (w/v) polyvinylpyrrolidone-40, 0.6% (w/v) methyl cellulose, and XAD-4 polystyrene beads (equal in mass to leaf tissue). The suspended material was filtered through 350 μm then 149 μm nylon mesh to remove residual leaves, glass beads and the XAD-4 resin, and the filtrate was passed through 105 μm mesh to collect secretory cell clusters with the oil storage cavity intact, then through 50 μm mesh to collect cell clusters devoid of the storage cavity.

Approximately 3×10^6 secretory cell clusters were frozen in liquid N_2, powdered with a mortar and pestle, and extracted with a commercial reagent (Trizol, Gibco BRL, Carlsbad, CA) using the manufacturer's protocol. The procedure involves phenol extraction in the presence of guanidine thiocyanate and aurintricarboxylic acid with added polyvinylpyrrolidone-40 (0.1%, w/v), phenol/chloroform treatment, isopropanol precipitation at 1.2 M NaCl in 0.9 M Na-citrate to effect separation from polysaccharides, and LiCl precipitation to remove low molecular weight RNA. Yield and quality were evaluated spectrophotometrically and by formaldehyde-agarose gel electrophoresis. Poly(A)$^+$ mRNA was isolated by chromatography on oligo(dT) cellulose (Amersham Pharmacia, Piscataway, NJ), and 5 μg of the resulting material was used to construct a λZAPII cDNA library with Uni-ZAPII vector and packaging using Gigapack Gold III extracts by the Stratagene (La Jolla, CA) protocols. The primary library titer was $\sim10^7$ pfu, and average insert size was determined by PCR of individual plaques to be 1.6 ± 0.5 kb.

3.3. cDNA isolation

Limonene synthase from *M. spicata* (Colby et al., 1993) was employed as hybridization probe prepared by PCR of plasmid template (pLC5.2) as previously reported (Williams et al., 1998). The amplicon was purified as a single 1.4 kb band on a 0.7% agarose gel, extracted with the QIA quick gel (Qiagen, Hilden, Germany), quantified, labeled using a [^{32}P]dCTP Ready-to-Go Oligo labeling bead (Amersham Pharmacia, Piscataway, NJ) and purified by size exclusion chromatography.

The library was plated at 10^4 pfu/90 mm NZY plate and lifts were performed using Hybond N$^+$ nylon membranes (Amersham Pharmacia) by adsorbtion for 2 min, denaturation at 100 °C (2 min; autoclave), and UV crosslinking in a Stratalinker (Stratagene) set to autocrosslink. Membranes were prehybridized twice in 2×SSC/0.1% SDS for 15 min at 65 °C. The denatured probe (3 min at 94 °C) was then added and the membranes were hybridized for 12 h at 65 °C, washed thoroughly in 4×SSC/

0.1% SDS at room temp and with 2×SSC/0.1% SDS at 35 °C, and exposed to Kodak XR-4 film at –80 °C with intensifying screen. Positive plaques were purified through a second round of hybridization, and 30 of these were in vivo excised and plated using helper phage and *E. coli* SOLR cells.

Partial 3′- and 5′-sequencing revealed the presence of a single sequence and indicated that about half of the acquired clones were of full length. Several acquisitions were fully sequenced, and sequence assembly and analysis were conducted using Genetics Computer Group software version 10.

3.4. cDNA expression and enzyme assay

Previously described procedures for heterologous expression of full-length monoterpene synthase clones were employed (Colby et al., 1993; Wise et al., 1998). Recombinant enzyme isolation and assay were by standard protocols for this terpenoid synthase type, involving the divalent metal ion (Mg^{2+} or Mn^{2+})-dependent conversion of [1-^{3}H]geranyl diphosphate to monoterpene products, separation of olefins from oxygenated derivatives by column chromatography, quantification by liquid scintillation counting, and identification by GC–MS (Schwab et al., 2001).

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