PERGAMON

Phytochemistry 60 (2002) 691–702

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

(+)-(10*R*)-Germacrene A synthase from goldenrod, *Solidago canadensis;* cDNA isolation, bacterial expression and functional analysis☆

Ian Prosser[a], Andy L. Phillips[a], Simon Gittings[a], Mervyn J. Lewis[a], Antony M. Hooper[b], John A. Pickett[b], Michael H. Beale[a,*]

[a]*Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol, BS41 9AF, UK*
[b]*Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, UK*

Received 29 November 2001; received in revised form 24 April 2002

Abstract

Profiling of sesquiterpene hydrocarbons in extracts of goldenrod, *Solidago canadensis,* by GC–MS revealed the presence of both enantiomers of germacrene D and lesser amounts of germacrene A, α-humulene, and β-caryophyllene. A similarity-based cloning strategy using degenerate oligonucleotide primers, based on conserved amino acid sequences in known plant sesquiterpene synthases and RT-PCR, resulted in the isolation of a full length sesquiterpene synthase cDNA. Functional expression of the cDNA in *E. coli,* as an N-terminal thioredoxin fusion protein using the pET32b vector yielded an enzyme that was readily purified by nickel-chelate affinity chromatography. Chiral GC–MS analysis of products from of ^{3}H- and ^{2}H-labelled farnesyl diphosphate identified the enzyme as (+)-(10*R*)-germacrene A synthase. Sequence analysis and molecular modelling was used to compare this enzyme with the mechanistically related *epi*-aristolochene synthase from tobacco.

Keywords: Farnesyl diphosphate; Germacrene A; Goldenrod; *Solidago canadensis*; Sesquiterpene synthase

1. Introduction

The sesquiterpenes are a large family of C_{15}-isoprenoid natural products found in higher and lower plants, microbes and some marine organisms. Many have biological activity, including antimicrobial, antitumour, and cytotoxic properties. In plants, they play important ecological roles in interactions with insects and microbes and act as attractants, deterrents, antifeedants and phytoalexins. Furthermore, sesquiterpenes are key components of many essential oils, which are important commercially for the flavour and fragrance industries. The tens of thousands of sesquiterpenes known (Glasby, 1982) are derived metabolically from some 300 distinct C_{15}-hydrocarbon skeleta, which in turn are produced from the single substrate farnesyl diphosphate (FPP) (see Fig. 1 for structures) by the action of sesquiterpene synthases (cyclases). It is known that these enzymic cyclisations proceed by ionisation of the diphosphate group of FPP to form an enzyme-bound allylic cation, which then undergoes isomerisation, before cyclisation to yield new cations. Subsequent rearrangements and final quenching of the carbocations by elimination or by reaction with water yields the various C_{15} hydrocarbon and alcohol products that are typical of a sesquiterpene synthase (Cane, 1985, 1990, 1999). Recently, our ability to study the enzymology of these reactions has increased dramatically as a result of the cloning and functional expression in *E. coli* of a number of cDNAs encoding sesquiterpene synthases of microbial (Hohn and Plattner, 1989; Proctor and Hohn, 1993; Cane et al., 1994) and plant origin (Facchini and Chappell, 1992; Back and Chappell, 1995; Chen et al., 1995, 1996; Crock et al., 1997; Bohlmann et al., 1998; Colby et al., 1998; Steele et al., 1998; Back et al., 1998;

Abbreviations: FPP, farnesyl diphosphate; TEAS, tobacco *epi*-aristolochene synthase; GC–MS, gas chromatography–mass spectrometry; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

☆ The nucleotide sequence reported in this paper has been submitted to the EMBL database with accession number AJ304452.

* Corresponding author. Tel.: +44-1275-549289; fax: +44-1275-394281.

E-mail address: mike.beale@bbsrc.ac.uk (M.H. Beale).

0031-9422/02/$ - see front matter
PII: S0031-9422(02)00165-6

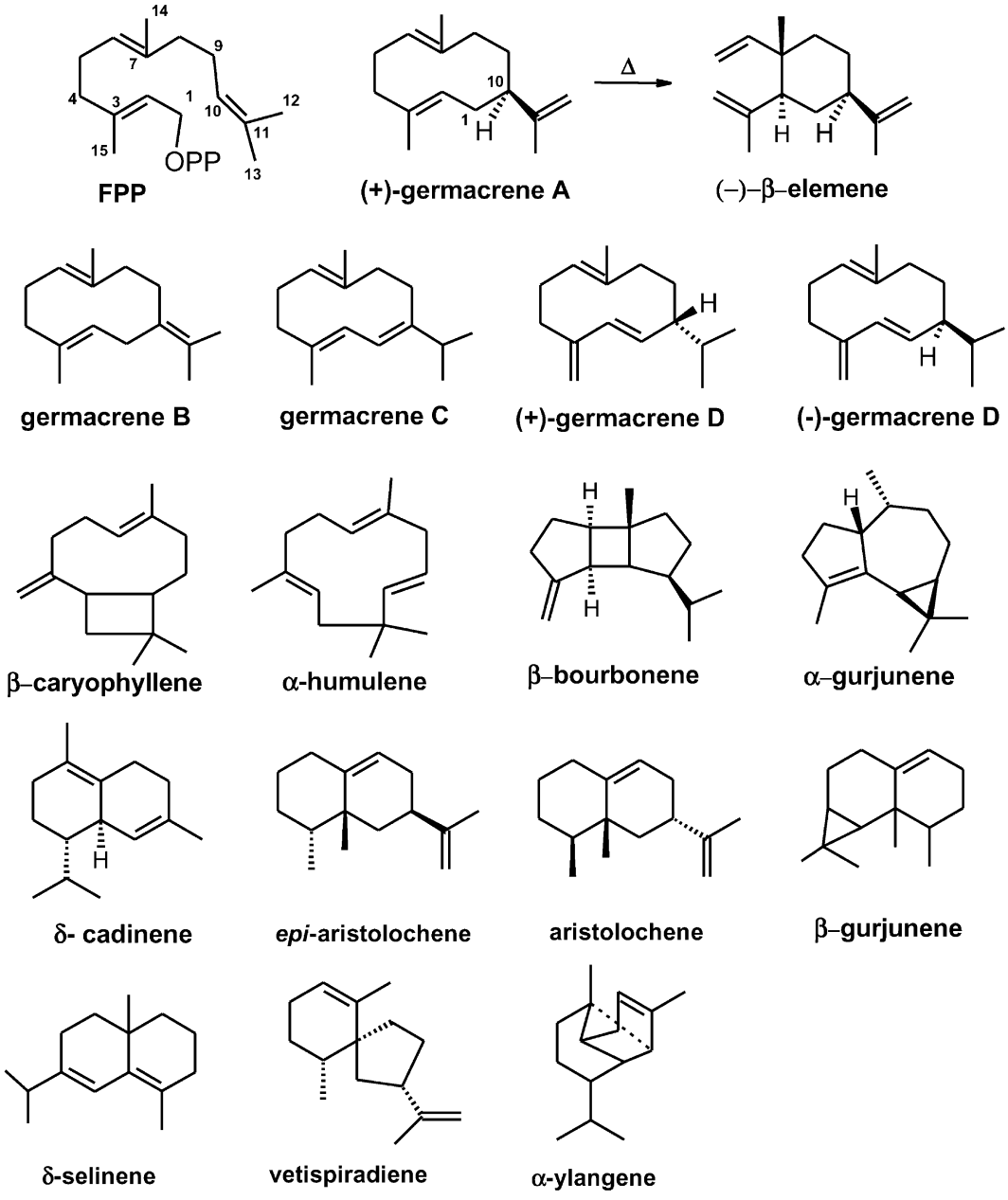

Fig. 1. Structures of sesquiterpene hydrocarbons.

Hua and Matsuda, 1999; Mercke et al., 1999). Furthermore, crystal structures of three recombinant sesquiterpene synthases have given insight into the active site components involved in the binding of FPP and catalysis of its cyclisation (Lesburg et al., 1997; Starks et al., 1997; Caruthers, 2000).

For those sesquiterpene synthases that convert FPP to compounds of the eremophilane and eudesmane classes, including *epi*-aristolochene (Facchini and Chappell, 1992), aristolochene (Proctor and Hohn, 1993), vetispiradiene (Back and Chappell, 1995) and δ−selinene (Steele et al., 1998), it is postulated that the 10-membered-ring germacrenes (isomers A, B, C or D) (Fig. 1) are the initial products of the cyclisation and subsequent elimination and rearrangement reactions. These neutral compounds are apparently not released from the active site of the sesquiterpene synthase but are re-protonated by the enzyme to form new cations, which undergo further cyclisations to yield the final products. Evidence for cryptic germacrene A synthase activity within aristolochene synthase has been provided by Cane and Tsantrizos (1996) who observed the formation of dihydrogermacrene A from the substrate analogue dihydro-FPP. More recently, site-directed mutagenesis of tobacco *epi*-aristolochene synthase (TEAS) has given rise to a mutant protein that produced germacrene A, albeit at lower efficiency compared to the wild-type production of *epi*-aristolochene (Rising et al., 2000).

There are many plant natural products that are derived from oxidative metabolism of germacrene A, indicating that sesquiterpene synthases that produce and release this thermally unstable 10-membered ring hydrocarbon are widespread. Evidence for germacrene A synthase activity in plants has also been presented (de Kraker et al., 1998). Germacrene A synthase is an important biotechnological target involved in the biosynthesis of parthenolide, the active ingredient of feverfew, as well as in the formation of the bitter sesquiterpenoids of lettuce and chicory (Sessa et al., 2000). In this paper we describe the cDNA cloning, functional expression in *E. coli,* and characterisation of this gene — (+)-(10*R*)-germacrene A synthase — from the goldenrod, *Solidago canadensis*.

2. Results

2.1. Solidago sesquiterpene hydrocarbon profile

The presence of both enantiomers of germacrene D has been reported in extracts or hydrodistillates of various species of *Solidago* (*S. altissima, S. canadensis, S. gigantica and S. vigaurea*) (Niwa et al., 1980; Schmidt et al., 1998; Bülow and König, 1997; Kalemba, 1998). Leaves of greenhouse-grown *S. canadensis* var. Gold Baby were extracted with pentane and the sesquiterpene hydrocarbon profile examined by GC–MS. The profile is shown in Fig. 2 and structures are shown in Fig. 1. The compounds were identified by comparison of spectra and Kovat's retention indices with library and published data (Adams, 1995). As expected from previous literature reports (Niwa et al., 1980; Schmidt et al., 1998; Bülow and König, 1997), germacrene D was the major component (71% of total sesquiterpenes). Further analysis by chiral GC-MS using columns and conditions described by Schmidt et al. (1998) showed that both enantiomers were present. The ratio of (+)-germacrene D to (−)-germacrene D was 4:3, based on total ion current. Other sesquiterpenes present in significant amounts were β−caryophyllene (3%), α-humulene (1%), δ-cadinene (7%) and germacrene A (7%). Minor amounts of other compounds such as β-bourbonene, α-ylangene, and β gurjurnene were also detected. There was no significant difference between the profile of previously wounded and that of unwounded tissue.

2.2. Sesquiterpene synthase cDNA isolation and expression

A homology-based PCR cloning strategy was used. Sequence alignments of published sesquiterpene synthase genes were used to design degenerate oligonucleotide primers. PCR using the primer pair corresponding to the amino acid sequences EDIL(D/E)(E/D)A and WWKDLD indicated in Fig. 3 gave a sesquiterpene synthase fragment, designated *si6*, as determined by sequencing and analysis of homology with known terpene synthases. cDNA fragment *si6* was radio-labelled

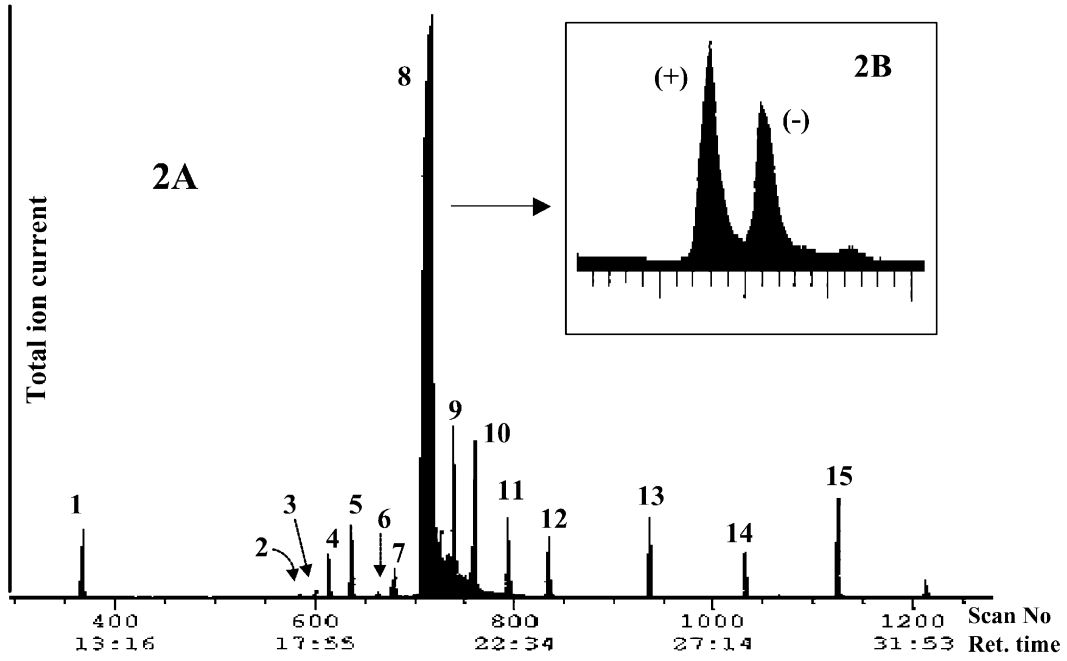

Fig. 2. Sesquiterpene hydrocarbon profile of *Solidago canadensis*. **2A** GC–MS (cold injection on BPX5) of pentane extract. Peaks—**1** C_{12} hydrocarbon standard; **2** α−ylangene; **3** β−bourbonene; **4** C_{14} hydrocarbon standard; **5** β−caryophyllene; **6** β−gurjunene; **7** α−humulene; **8** germacrene D; **9** germacrene A; **10** δ−cadinene; **11** unknown sesquiterpene; **12**–**15** C_{16}-C_{19} hydrocarbon standards. **2B** Separation of germacrene D enantiomers on Cyclosil-B chiral phase.

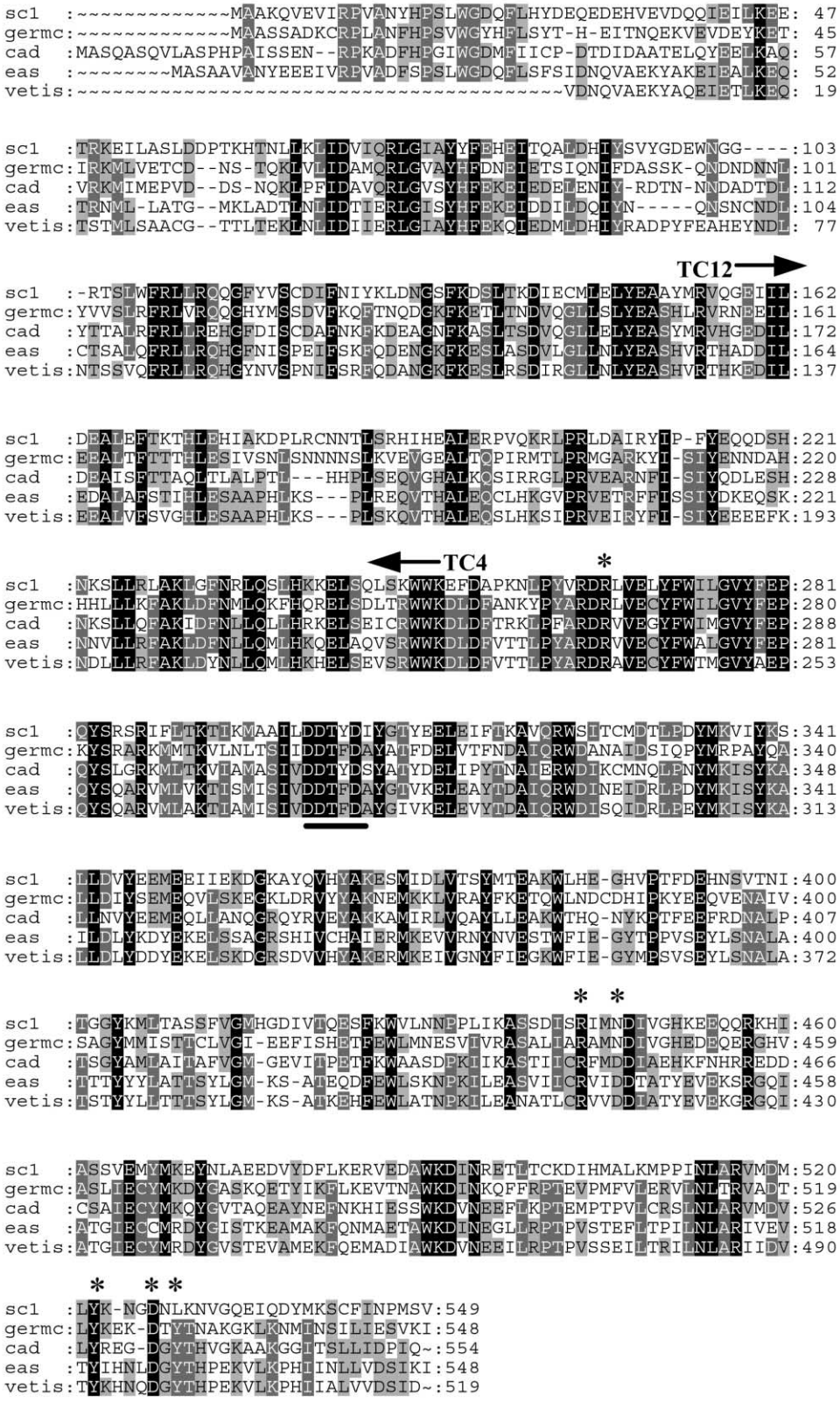

Fig. 3. Amino acid sequence alignment of (+)-germacrene A synthase (*Sc1*) with related plant sesquiterpene synthases; germc = germacrene C synthase (EMBL Accession AF035630) (Colby et al., 1998), cad = cadinene synthase (U27535) (Chen et al., 1996), eas = *epi*-aristolochene synthase (L04680) (Facchini and Chappell, 1992) and vetis = vetispiradiene synthase (U20188) (Back and Chappell, 1995). Sequences corresponding to RT-PCR primers TC12 and TC4 are shown. The characteristic DDXXD motif is underlined. Amino acid residues referred to in discussion of the enzyme mechanism are highlighted with asterisks.

and used to probe a *S. canadensis* cDNA library, giving rise to the full-length clone *Sc1*. The deduced amino acid sequence of *Sc1* is shown in Fig. 3, aligned with selected published sesquiterpene synthase sequences. The homology, length of the sequence and the lack of obvious plastid targeting transit peptide indicated that the cDNA encoded a sesquiterpene synthase rather than a mono- or diterpene synthase.

To determine its function, *Sc1* was expressed in *E. coli*. After some experimentation, the pET32b vector was selected. This vector, which gives rise to proteins containing N-terminal thioredoxin fusions, gave a high level of expression as determined by analysis by SDS–PAGE. As shown in Fig. 4 a soluble protein was induced at a molecular weight of circa 80 KDa. This is consistent with the predicted molecular weight of the sesquiterpene cyclase (~65 kDa) plus thioredoxin and linkers. Incubation of soluble protein from *E. coli* cells expressing *Sc1* with [1-^{3}H]-FPP produced pentane soluble products, as determined by radiocounting. These were identified by GC–MS as germacrene A (98%) and α-humulene (2%) (Fig. 5). Control incubations of [1-^{3}H]-FPP with soluble proteins from cells transformed with vector only did not produce pentane soluble metabolites, as determined by radio-counting. Additionally, no sesquiterpene hydrocarbons were detected by GC–MS in these control experiments. Diethyl ether extracts of the incubations, made subsequently to the pentane extractions, of both control and expressed enzyme-containing solutions contained small amounts of [1-^{3}H]-farnesol and no other sesquiterpene alcohols, as determined by radio-counting and GC–MS. To confirm these results with an isotope traceable by mass spectrometry, the enzyme preparation was incubated with [1-^{2}H]-FPP, containing a small amount of [1-^{3}H]-FPP to enable additional tracking by radio-counting. [$^2H_1$]-germacrene A (98%) and [$^2H_1$]-α-humulene (2%) were the only products detected in the hydrocarbon fraction (Fig. 5). Analysis of the mass spectra indicated that there was total retention of deuterium in both products, thus establishing the cloned sesquiterpene synthase as germacrene A synthase.

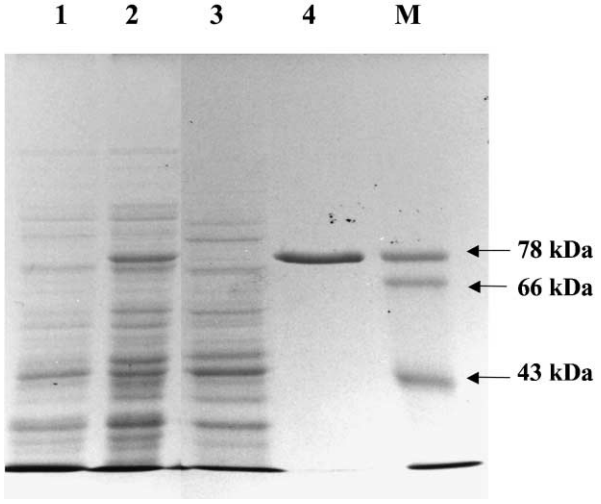

Fig. 4. SDS–PAGE analysis of proteins from *E. coli* expression of (+)-germacrene A synthase. Cultures of *E. coli* strain AD494(DE3)-pLysS containing the pET32b vector with or without the *Sc1* insert were induced with IPTG and protein extracts prepared and purified by affinity chromatography as described in the experimental procedures. Lane 1 Soluble protein from vector only control induction. Lane 2 soluble protein from pET:*Sc1* induction Lane 3 HisBind affinity purification-column wash through Lane 4 purified (+)-germacrene A synthase eluted from HisBind column. Lane M molecular weight markers.

2.3. Chirality of enzyme-produced germacrene A

The chirality of the germacrene A produced by the recombinant enzyme was determined by the chiral GC–MS method of de Kraker et al. (1998). This method relies on the thermal Cope rearrangement of germacrene A to β-elemene in hot GC injectors (structures Fig. 1). The two enantiomers of β-elemene are separable on chiral GC phases based on heptakis (2,3-di-*O*-methyl-6-*O*-*t*-butyl dimethylsilyl)-β-cyclodextrin (König et al., 1994). Standards of (−)-β-elemene in *Piper nigrum* (black pepper) essential oil and (+)-β-elemene from hot injection of a pentane extraction of the liverwort Scapania undulata were used to authenticate the separation (König et al., 1994). As shown in Fig. 6, hot injection of the germacrene A produced by the recombinant sesquiterpene synthase, Sc1, gave a single peak, coincident with (−)-β-elemene standard. Cope rearrangement of germacrene A occurs with retention of configuration and (+)-germacrene A gives rise to (−)-β-elemene (de Kraker et al., 1998). Thus, the cloned *Solidago* sesquiterpene synthase, Sc1, was identified as a (+)-germacrene A synthase.

2.4. Enzyme purification and characterisation

As well as the N-terminal thioredoxin fusion, the recombinant (+)-germacrene A synthase also contains a polyhistidine tag. Single step purification of the enzyme was achieved by affinity purification on a Ni-chelate chromatography column, as shown by SDS–PAGE analysis (Fig. 4). Enzyme purified in this way retained activity after chromatography and after storage frozen at −20 °C in 50% glycerol. Proteolytic cleavage of the fusion protein with thrombin yielded native enzyme that retained germacrene A synthase activity. For convenience, most characterisation was carried out on the affinity purified fusion protein. The enzyme had a K_m for FPP of 2.5 μM at pH 7.0, as determined from a Hanes plot of [FPP]/velocity versus [FPP] (data not shown). This value is typical for higher plant sesquiterpene synthases (Steele et al., 1998). The enzyme consistently produced (+)-germacrene A and α-humulene in the ratio 98:2 from feeds of unlabelled or [1-^{2}H]-FPP. However, when [12,13-$^2H_6$]-FPP was used as a substrate, the proportion

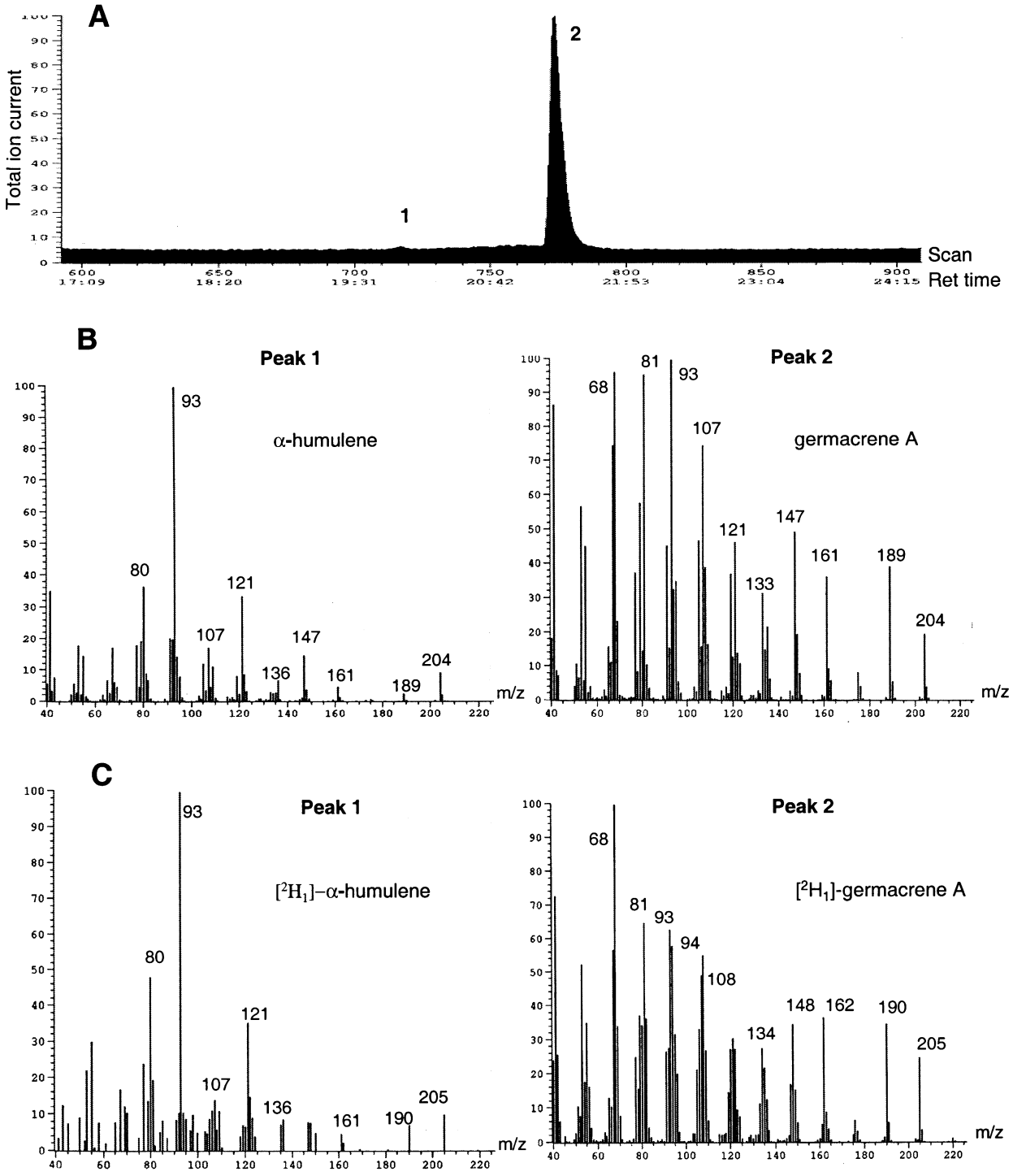

Fig. 5. GC–MS analysis (cold injection) of the products produced by Sc1-thioredoxin fusion from FPP and [$^2H_1$]-FPP substrates. A—total ion current trace from FPP feed. B—mass spectra of peak 1 (α−humulene) and peak 2 (germacrene A) from FPP incubation. C—mass spectra of peaks 1 and 2 from [$^2H_1$]-FPP incubation.

of [$^2H_6$]-α-humulene formed increased to 11%. Additionally [$^2H_6$]-germacrene D (9% of total products) could be detected along with the expected [$^2H_5$]-germacrene A (Fig. 7). Analysis by chiral GC-MS revealed the labelled [$^2H_6$]-germacrene D produced was exclusively the (−) enantiomer.

2.5. Sequence analysis and structural model

The cloned cDNA encoded an open reading frame giving rise to a protein of 549 amino acids, with a predicted molecular weight of 64.7 kDa. The deduced protein sequence is shown in Fig. 3, aligned with other

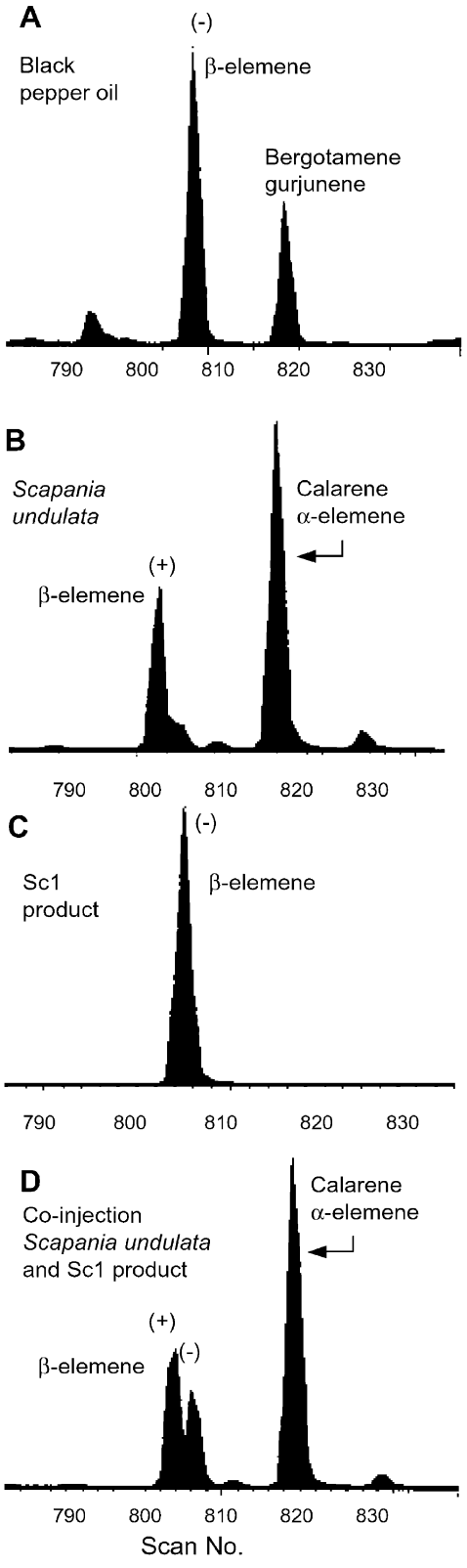

Fig. 6. Determination of the chirality of (+)-germacrene A produced by Sc1-thioredoxin fusion protein by analysis of (−) -β-elemene formed by hot injection GC–MS on Cyclosil-B chiral phase. Relevant region of sesquiterpene profiles of A, black pepper oil; B *Scapania undulata* extract; C germacrene A from FPP feed to Sc1 and D Co-injection of germacrene A from Sc1 and *Scapania undulata* extract.

representative angiosperm sesquiterpene synthases. Two of these, *epi*-aristolochene (TEAS) and vetispiradiene synthases, are postulated to convert FPP to bicyclic hydrocarbon products via germacrene A. Thus, it was useful to compare these sequences with that of the *Solidago* (+)-germacrene A synthase, particularly as the crystal structure of TEAS has been determined. However, (+)-germacrene A synthase has slightly higher similarity and identity with cadinene synthase (58% similarity, 46% identity) and germacrene C synthase (57% similarity, 44% identity) than it has with TEAS (52% similarity, 40.5% identity) and vetispiradiene synthase (54% similarity, 41.5% identity).

The amino-acid sequence of (+)-germacrene A synthase contains the characteristic, DDXXD/E motif, that is highly conserved amongst sesquiterpene synthases (Fig. 3). Structural studies of TEAS (Starks et al., 1997) and aristolochene synthase (Caruthers et al., 2000) have indicated that elements of this motif co-ordinate to a magnesium ion involved in the ionisation of the diphosphate group. Other residues involved in binding and catalysis have been identified by Starks et al. (1997). Some of the amino acid side-chains suggested to be involved in the formation of intermediate germacrene A by TEAS, are present in (+)-germacrene A synthase. For example, Arg^{264} and Arg^{441} which take part in magnesium binding and diphosphate ionisation in TEAS are present in (+)-germacrene A synthase (residues 264 and 443), together with Asp^{525} (position 526 in (+)-germacrene A synthase), a residue that is implicated in proton elimination at C-13 of the germacrenyl cation. However, Tyr^{527}, a residue that was suggested to play a key role in the stabilisation of the germacrenyl cation in TEAS, is a leucine in (+)-germacrene A synthase (position 528). This change may have some bearing on the reaction mechanism. Further indicators were sought by mapping of the (+)-germacrene A synthase sequence onto the TEAS structure using the Swiss-Model service (http://www.expasy.ch/swissmod/SWISS-MODEL.html) to produce an energy minimised model. Examination of overlays of the active site regions of the two enzymes failed to reveal obvious differences in architecture that may indicate why reaction in (+)-germacrene A synthase terminates at germacrene A. From the structure of TEAS, complexed with substrate analogues, further cyclisation of germacrene A appears to be initiated by protonation by the hydroxyl group of Tyr^{520} which itself is polarised by hydrogen-bonding from Asp^{444} and Asp^{525} (Starks et al., 1997). Indications of the role of this tyrosine have also been obtained from mutagenesis studies (Rising et al., 2000). In (+)-germacrene A synthase this tyrosine is conserved (residue 522) along with the second aspartate (at position 526). However the aspartate at position 444 in TEAS has been changed to asparagine (position 446) in (+)-germacrene A synthase. This change is also present in the

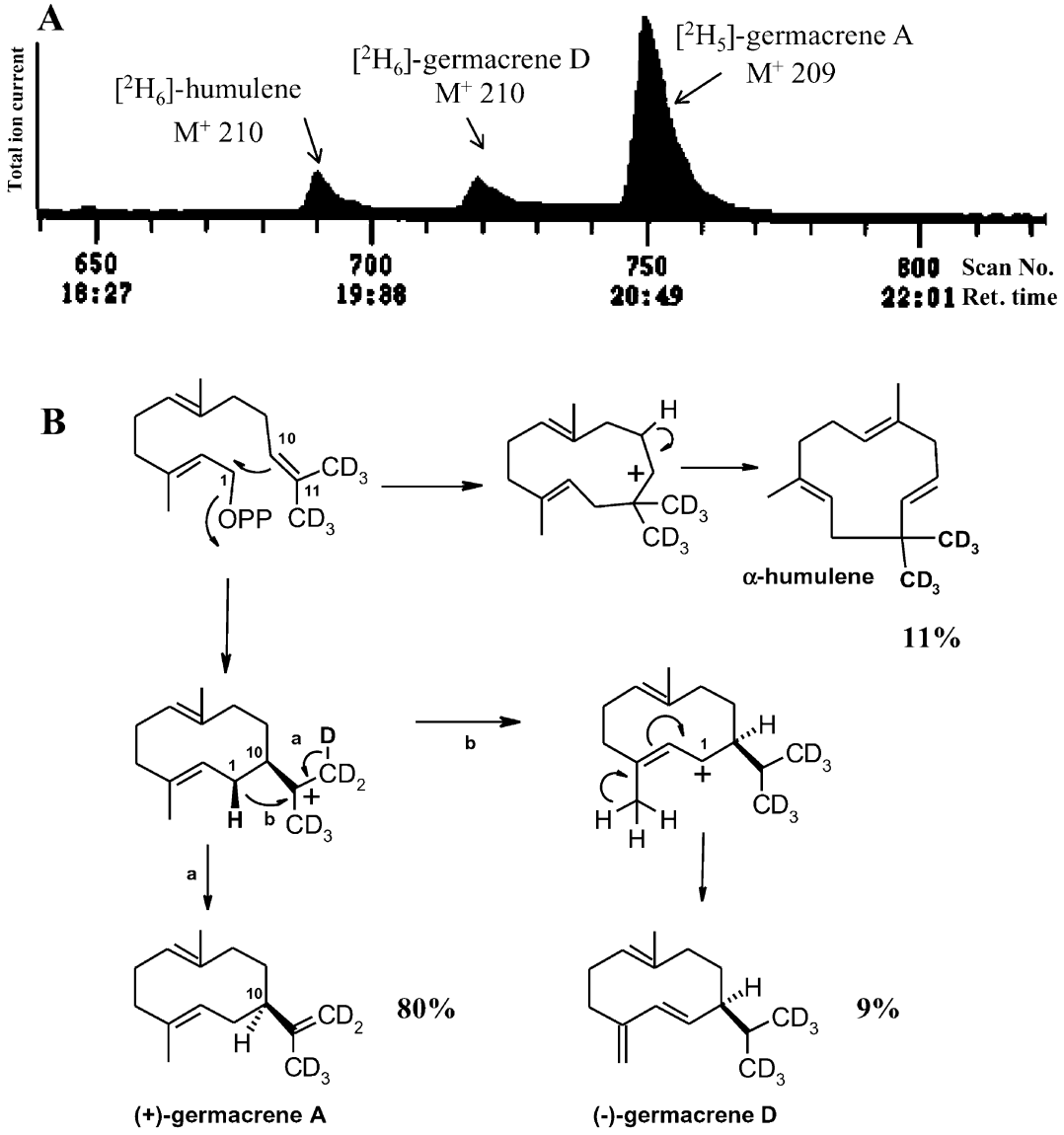

Fig. 7. A GC–MS analysis of products from incubation of [12,13-$^2H_6$]-FPP with Sc1 protein. B Mechanism of formation of the observed products, alternative pathways from the germacrenyl cation are indicated by arrows **a** and **b**.

equivalent position in the sequence of germacrene C synthase, and thus could be correlated with the termination of the reaction at the neutral germacrene. However, aristolochene synthase, which also produces germacrene A as an intermediate also contains this aspartate to asparagine change.

3. Discussion

The sesquiterpene profile observed for the cultivar of *Solidago canadensis* agrees with previous reports on extracts and hydrodistillates of wild collections in that (+)/(−) germacrene D is by far the main constituent. It is interesting that α- and γ-gurjunenes were not present, given that significant gurjunene synthase activity has been reported from this species (Schmidt et al., 1999a). This may be due rapid metabolism of gurjunene to cyclcolerenone or perhaps to varietal differences between the cultivar used and wild populations used by other workers. The second major sesquiterpene of the profile was germacrene A, the product of the enzyme encoded by the cloned *Sc1* cDNA. It was perhaps surprising that the homology cloning strategy yielded a sesquiterpene synthase that produced predominately germacrene A, given the large excess of germacrene D over germacrene A in the plant extract. The profile of sesquiterpene hydrocarbons in the plant may not, however, reflect the relative expression of sesquiterpene synthase genes, as some products of synthase activity could be rapidly metabolised further, while others accumulate.

The sesquiterpene synthase encoded by *Sc1* was expressed to a high level in *E. coli* cells using the pET32 vector. This yielded a soluble thioredoxin fusion protein that was readily purified by affinity chromatography. Although (+)-germacrene A synthase activity was readily detectable in total soluble proteins from the bacterial cultures, most of the characterisation was done on affinity-purified fusion protein. This avoided background phosphatase activity in the crude cell lysates and gave cleaner samples for GC–MS analysis. The recombinant enzyme (fusion protein and native protein generated by thrombin cleavage) converted FPP to almost exclusively (+)-germacrene A. α-Humulene was a by-product produced consistently at the level of 2%. Examples of recombinant sesquiterpene synthases that produce single products have been reported, but often the products are mixtures that are sometimes complex reflecting the many possible reaction paths involving carbocationic rearrangements (Steele et al., 1998). It is not clear at this time whether this enzyme activity is the sole source of the α-humulene present in the profile. Definitive confirmation of the function of *Sc1* protein was obtained using deuterium-labelled substrate and analysis of the products by GC-MS. Deuterium labels from C-1 of FPP were retained in both (+)-germacrene A and α−humulene. However, when [12,13-$^2H_6$]-FPP was used as substrate for *Sc1*, (−)-[$^2H_6$]- germacrene D (9%) was also observed as a product, along with an increased level of [$^2H_6$]- α-humulene (11%), in addition to the expected labelled (+)-[$^2H_5$]- germacrene A. The increased proportion of α−humulene in these experiments, appears to be due to secondary isotope effects on the relative stability of the germacrenyl and humulyl cations (Fig. 7). Hyperconjugative stabilisation of [12,13- $^2H_6$] germacrenyl ion is less than that in the fully protio- species and this may favour increased formation of the humulyl ion in the hexadeuterio analogue. The appearance of (−)-[$^2H_6$]-germacrene D can be attributed to the introduction of a primary isotope effect into the reaction pathway leading to (+)-germacrene A. The outcome is a slowing of the elimination step resulting in the formation of the 11,12 double bond, thereby allowing the alternative pathway leading to (−)-germacrene D to compete more effectively. This type of isotopically sensitive branching is not uncommon in terpene biosynthesis and has been observed before for monoterpene (Croteau et al., 1987; Wagschal et al., 1991, 1994) and diterpene synthases Williams et al. (1970). It is known, from experiments with [10-^2H]-FPP and partially purified germacrene D synthases from *Solidago*, that the formation of (−)-germacrene D from the intermediate germacrenyl cation is accomplished via a 1,3-H shift from C-1 to C-11 (Schmidt et al., 1999b). The chirality of the products obtained from germacrene A synthase is consistent with the intermediacy of the (10*R*)-germacrenyl cation as shown in Fig. 7. This gives rise to (+)-(10*R*)-germacrene A by loss of a C-12 hydrogen (deuterium), or to (−)-germacrene D by the 1,3-H shift and subsequent elimination. The absolute stereochemistry of (+)-(10*R*)-germacrene A follows from that of the (−)-(10*S*)-enantiomer determined by Weinheimer et al. (1970).

Sequence analysis and molecular modelling of (+)-germacrene A synthase was used to try to gain insight into the reaction mechanism, with particular reference to that of TEAS whose structure has been determined (Starks et al., 1997). Comparison of total sequence homologies between known sesquiterpene synthases, particularly across species, is not that informative at present. Homologies amongst plant examples are normally of the order of 50% regardless of similarities in the reaction pathway. Nevertheless, there is conservation of certain key residues that have been implicated as being involved in catalysis. Homologies between plant and microbe sequences are even lower. For example, *epi*-aristolochene and aristolochene synthase show only 16% identity despite much similarity between the reactions catalysed (Caruthers et al., 2000). When considering reaction paths, more informed speculation of the role of certain amino acid side-chains comes from examination of structures predicted from modelling of sequences onto known sesquiterpene synthase structures and consideration of putative active site architecture. The (+)-germacrene A synthase model generated was examined with particular emphasis on identifying differences with respect to the TEAS structure. The overall shape of the active site in germacrene A synthase was very similar to that of TEAS. The analysis failed to reveal obvious differences that could be attributed to termination of the reaction at (+)-germacrene A in the new synthase. The presence of a leucine in place of tyrosine (position 527 in TEAS) could be significant in removing stabilisation of the germacrenyl cation. This may have the effect of accelerating the elimination reaction to (+)-germacrene A, but cannot explain the failure of this molecule to undergo further reaction. The tyrosine at position 520 in TEAS has been implicated in catalysing further reaction of germacrene A by protonation. Mutagenesis of Tyr^{520} gives a mutant TEAS that makes germacrene A (Rising et al., 2000). This, however, does not confirm that wild-type TEAS produces germacrene A as an intermediate. Tyr^{520} is preserved in (+)-germacrene A synthase but only one of two aspartate residues that assist in orientation and polarisation of the hydroxyl group of this tyrosine is present. It is possible that this change may form the molecular basis for the differences in reaction pathway between the two enzymes. However, the aspartate to asparagine change in question is also present in aristolochene synthase, an enzyme that produces (−)-germacrene A as an intermediate. Thus the significance of this change is unclear at present. More definitive data

will come from a crystal structure of (+)-germacrene A synthase. This aspect of the work is currently being pursued, along with mutagenesis of this enzyme and the cloning of further *S. canadensis* sesquiterpene synthases that must be responsible for the biosynthesis of the other sesquiterpenes of the pentane soluble profile.

4. Experimental

4.1. Plant materials, substrates and standards

Seeds of *Solidago canadensis* var. Gold Baby (Asteraceae = Compositae) were purchased from Chiltern Seeds (Cumbria, UK) and germinated in Levington M2 peat-based compost. Plants were grown in 15 cm pots of John Innes No 2 compost with a 16 h photoperiod. [1-^{3}H]-farnesyl diphosphate (FPP) (15–30 Ci/mmol) was purchased from NEN-Dupont and used as supplied. Unlabelled FPP was purchased from Sigma, UK. [1-^{2}H]- and [12,13-$^2H_6$]-FPP were synthesised from the corresponding labelled farnesols by the method of Davisson et al. (1985). [12,13-$^2H_6$]-farnesol was prepared by a published method with minor modifications (DeRopp and Troy, 1984). Sesquiterpene standards were obtained as follows:– (+)/(−)-germacrene D from hexane extraction and silica chromatography of *S. canadensis* aerial tissue; α-humulene was from our reference collection; black pepper oil containing (−)-β-elemene was purchased from Amphora Aromatics, Bristol, UK and (−)-germacrene A was a constituent of the pentane extract of the liverwort *Scapania undulata*, which was kindly collected by David Rycroft, University of Glasgow.

4.2. Extraction of plant tissue

Leaves of *S. canadensis* or whole plants *of Scapania undulata* were frozen in liquid nitrogen and ground to a powder at this temperature. After warming to circa 0°C, the pulverised material was extracted with pentane. The resultant extract was filtered through a short column of silica gel (BDH, 40–63 μm)—magnesium sulfate (10:1), washing through with pentane. The pentane solution was analysed either directly or after concentration of the cooled solution with a stream of nitrogen.

4.3. GC–MS analysis

GC–MS was carried out on Kratos MS80RFA instrument, using cool (~20 °C) on-column injection to a BPX5 (SGE) capillary column (0.32 i.d×25 m). The temperature programme was 45 °C for 1 min, then 5 °C per min to 210 °C which was held for 30 min. A straight-chain alkane standard mix was co-injected with the samples. Spectra and Kovat's retention indices were compared with the Wiley library and published data (Adams, 1995).

Chiral GC–MS was carried out on a 0.25 mm i.d.×30 m fused silica column with a layer (0.25 μm) of Cyclo-Sil-B [30% *heptakis* (2,3-di-*O*-methyl-6-*O*-*t*-butyl dimethylsilyl)-β-cyclodextrin in DB-1701] supplied by J and W Scientific. The column was fitted with a 2 m RGK-1 retention gap (SGE). Injection was either cool on-column as above or at 250 °C. The temperature program was as above.

4.4. mRNA isolation, cDNA synthesis, PCR-amplification, probe generation and library preparation and screening

RNA was extracted from *S. canadensis* leaf tissue by the method of Verwoerd et al. (1989). mRNA was isolated by affinity chromatography on oligo-dT-cellulose (Aviv and Leder, 1972). cDNA was prepared using the Ready To Go kit (Pharmacia) according to the manufacturer's instructions. PCR was performed with the degenerate forward and reverse primers 5′-GAGGATAT(ATC)(TC)T(GATC)GA(GATC)GA(GATC)GC-3′ and 5′-TC(GATC)A(GA)(GA)TC(TC)TTCCACCA-3′ in a 50μl reaction containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin and 2 units *Taq* DNA polymerase (AmpliTaq Gold, Perkin Elmer). Cycling conditions were initial denaturation at 94 °C for 1min; 35 cycles consisting of denaturation 1 min, 94 °C, annealing 1min, 48 °C and extension 3 min at 72 °C; followed by final extension at 72 °C for 30 min before cooling to 4 °C. The resultant PCR product of approx 290 base-pairs was purified by electrophoresis on a 1.5% agarose gel, excised, and electroeluted. The cDNA fragment was ligated into the PCRII vector and cloned using the TA-cloning kit according to the manufacturer's instructions (Invitrogen). Selected colonies containing inserts were grown up in overnight suspension cultures and plasmid DNA was isolated from the cultures using the Wizard Plus SV Miniprep system (Promega). Clones confirmed as containing inserts of the expected size, by restriction analysis, were sequenced. A PCR product designated *Si6* was identified as a sesquiterpene synthase by sequence alignment.

The insert of *Si6* was labelled with ^{32}P-dCTP using Ready-To-Go DNA Labelling Beads (Amersham Pharmacia Biotech) and used to screen a *Solidago canadensis* leaf cDNA library constructed in λ-ZAPII using the ZAP-cDNA Synthesis Kit (Stratagene). Hybridisation and washing conditions were as described by Phillips et al. (1995). Positive plaques hybridising to *Si6* were re-screened through two further cycles until plaque-pure and rescued into phagemids as described by the manufacturer. Representative clones were analysed for insert size by PCR and restriction endonuclease analysis and sequenced.

4.5. E. coli expression

Oligonucleotide primers were used to amplify the coding region of the *Sc1* cDNA and to introduce cloning sites for subsequent subcloning into pET32b (Novagen). The amplified product was digested with EcoRI and XhoI and cloned into pET32b in frame with thioredoxin. The sequence of the insert was verified before expression was attempted. For expression, the recombinant plasmid, designated pET32b:Sc1, was transformed into chemically-competent *E. coli* strain AD494(DE3)pLysS. An overnight starter culture (2 ml) of pET32b:Sc1 transformed cells was used to inoculate 50 ml LB medium containing ampicillin (100 μg/ml). The culture was grown for 3 h at 37 °C ($OD_{600} = 0.5$) before adding IPTG to 1 mM. The incubation was continued for a further 4 h, but at 30 °C. The cells were harvested by centrifugation and resuspended in 2 ml of sesquiterpene synthase assay buffer (SSAB, 100 mM NaPhosphate pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 10% v/v glycerol). The cells were lysed by three cycles of freeze/thaw and the debris was removed by centrifugation at 13,000×*g*. Soluble and insoluble proteins were analysed by SDS–PAGE.

4.6. Enzyme assay

Small-scale assays to test for activity were performed as follows. Up to 100 μl extract was added to 10 μl (10μg) FPP and 1μl [1-^{3}H] FPP (0.5 μCi/μl), made up to 1 ml with SSAB (see above) and incubated for 2 h at 25 °C. The reaction mix was extracted with 1 ml pentane, which was then eluted through a short column of silica gel/$MgSO_4$ (10:1) in a pasteur pipette, washing through with 1 ml pentane and then 2 ml of diethyl ether. Aliquots of the pentane and ether fractions were analysed by liquid scintillation counting (HiSafe scintillant, LKB 1215 Rackbeta). For larger scale assays to enable identification of the products, either crude lysate (500 μl) or purified (+)-germacrene A synthase (30 μl) was added to 50 μg unlabelled or deuterated FPP, 1 μl [1-^{3}H] FPP, made up to 2 ml with SSAB and incubated for 2 h at 25 °C. The reaction mix was extracted three times with 0.5 ml pentane/ether (9:1 v/v) and the organic phases were pooled. The combined pentane/ether extracts were passed through the silica column and washed with 0.5 ml pentane/ether. The pentane/ether fraction was concentrated as described above and was analysed by GC–MS.

4.7. Purification of recombinant (+)-germacrene A synthase

A 50 ml culture of *E. coli* harbouring pET32b:Sc1 was grown and induced as described above. The harvested cells were resuspended in 4 ml binding buffer and then subjected to three cycles of freeze /thaw to lyse the cells. The expressed protein was purified using a His•Bind kit following the supplied protocol (Novagen). Fractions were analysed by SDS–PAGE and those containing the purified protein were pooled and passed through a NAP-10 column (Pharmacia) equilibrated with SSAB. Enzyme was stored at −20 °C in SSAB containing 50% glycerol.

4.8. DNA sequencing

The PCR product S*i6*, the full length cDNA clone *Sc1* and the modified cDNA cloned in pET32b were sequenced on an ABI 377 Prism using a BigDye terminator cycle sequencing system (PE Biosystems). Sequence analysis and alignments used the GCG software.

Acknowledgements

We thank David Rycroft, University of Glasgow, for the collection and identification of *Scapania undulata*, Steve Thomas (LARS) for discussions on cloning techniques and Luke Vincent (LARS) for exploratory expression studies. The Institute of Arable Crops Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

References

Adams, R. P., 1995. Identification of Essential Oil Components by Gas Chromatography–Mass Spectroscopy. Allured Publ. Corp., Carol Stream, IL.

Aviv, H., Leder, P., 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69, 1408–1412.

Back, K., Chappell, J., 1995. Cloning and bacterial expression of a sesquiterpene cyclase from *Hyoscyamus muticus* and its molecular comparison to related terpene cyclases. J. Biol. Chem. 270, 7375–7381.

Back, K.W., He, S.L., Kim, K.U., Shin, D.H., 1998. Cloning and bacterial expression of sesquiterpene cyclase, a key branch point enzyme for the synthesis of sesquiterpenoid phytoalexin capsidiol in UV-challenged leaves of *Capsicum annuum*. Plant Cell Physiol. 39, 899–904.

Bohlmann, J., Crock, J., Jetter, R., Croteau, R., 1998. Terpenoid-based defenses in conifers: cDNA cloning, characterization, and functional expression of wound-inducible (*E*)-α-bisabolene synthase from grand fir (*Abies grandis*). Proc. Natl. Acad. Sci. USA 95, 6756–6761.

Bülow, N., König, W.A., 1997. Germacrene D—a source of rare sesquiterpene hydrocarbons. In: Franz, C.M.A., Buchbauer, G., (Eds). Essential Oils: Basic and Applied Research; Proc. 27th International Symposium on Essential Oils, Vienna. Allured Publ. Corp., Carol Stream, IL, pp. 209–213.

Cane, D.E., 1985. Isoprenoid biosynthesis—stereochemistry of the cyclization of allylic pyrophosphates. Accounts of Chem. Res. 18, 220–226.

Cane, D.E., 1990. Enzymatic formation of sesquiterpenes. Chem. Rev. 90, 1089–1103.

Cane, D.E., Sohng, J.K., Lamberson, C.R., Rudnicki, S.M., Wu, Z., Lloyd, M.D., Oliver, J.S., Hubbard, B.R., 1994. Pentalenene synthase—purification, molecular cloning, sequencing, and high-level expression in *Escherichia coli* of a terpenoid cyclase from *Streptomyces* Uc5319. Biochemistry 33, 5846–5857.

Cane, D.E., Tsantrizos, Y.S., 1996. Aristolochene synthase. Elucidation of the cryptic germacrene A synthase activity using the anomalous substrate dihydrofarnesyl diphosphate. J. Am. Chem. Soc. 118, 10037–10040.

Cane, D.E., 1999. Sesquiterpene biosynthesis: cyclisation mechanisms. In: Cane, D.E. (Ed.), Isoprenoids Including Carotenoids and Steroids, Vol. 2. Elsevier Science, Oxford, pp. 155–200.

Caruthers, J.M., Kang, I., Rynkiewicz, M.J., Cane, D.E., Christianson, D.W., 2000. Crystal structure determination of aristolochene synthase from the blue cheese mold, *Penicillium roqeforti*. J. Biol. Chem. 275, 25533–25539.

Chen, X.Y., Chen, Y., Heinstein, P., Davisson, V.J., 1995. Cloning, expression, and characterization of (+)-δ cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. Arch. Biochem. Biophys. 324, 255–266.

Chen, X.Y., Wang, M.S., Chen, Y., Davisson, V.J., Heinstein, P., 1996. Cloning and heterologous expression of a second (+)-δ-cadinene synthase from *Gossypium arboreum*. J. Nat. Prod. 59, 944–951.

Colby, S.M., Crock, J., DowdleRizzo, B., Lemaux, P.G., Croteau, R., 1998. Germacrene C synthase from *Lycopersicon esculentum* cv. VFNT Cherry tomato: cDNA isolation, characterization, and bacterial expression of the multiple product sesquiterpene cyclase. Proc. Natl. Acad. Sci. USA 95, 2216–2221.

Crock, J., Wildung, M., Croteau, R., 1997. Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha×piperita* L.) that produces the aphid alarm pheromone (*E*)-β-farnesene. Proc. Natl. Acad. Sci. USA 94, 12833–12838.

Croteau, R.B., Wheeler, C.J., Cane, D.E., Ebert, R., Ha, H.J., 1987. Isotopically sensitive branching in the formation of cyclic monoterpenes—proof that (−)-α-pinene and (−)-β-pinene are synthesized by the same monoterpene cyclase via deprotonation of a common intermediate. Biochemistry 26, 5383–5389.

Davisson, V.J., Woodside, A.B., Poulter, C.D., 1985. Synthesis of allylic and homoallylic isoprenoid pyrophosphates. Meth. Enzymol. 110, 130–144.

DeRopp, J.S., Troy, F.A., 1984. Chemical synthesis and ^{2}H NMR investigations of polyisoprenols — dynamics in model membranes. Biochemistry 23, 2691–2695.

Facchini, P.J., Chappell, J., 1992. Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. Proc. Natl. Acad. Sci. USA 89, 11088–11092.

Glasby, J.S., 1982. Encyclopedia of Terpenoids. Wiley, Chichester.

Hua, L., Matsuda, S.P.T., 1999. The molecular cloning of 8-epicedrol synthase from *Artemisia annua*. Arch. Biochem. Biophys. 369, 208–212.

Hohn, T.M., Plattner, R.D., 1989. Expression of the trichodiene synthase gene of *Fusarium sporotrichioides* in *Escherichia coli* results in sesquiterpene production. Arch. Biochem. Biophys. 275, 92–97.

Kalemba, D., 1998. Constituents of the essential oil of *Solidago virgaurea* L. Flavour and Fragrance J. 13, 373–376.

König, W.A., Rieck, A., Hardt, I., Gehrcke, B., Kubeczka, K.-H., Muhle, H., 1994. Enantiomeric composition of the chiral contituents of essential oils. Part 2. Sesquiterpene hydrocarbons. J. High Res. Chrom. 17, 315–320.

deKraker, J.W., Franssen, M.C.R., deGroot, A., König, W.A., Bouwmeester, H.J., 1998. (+)-Germacrene A biosynthesis—the committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. Plant Physiol. 117, 1381–1392.

Lesburg, C.A., Zhai, G.Z., Cane, D.E., Christianson, D.W., 1997. Crystal structure of pentalenene synthase: mechanistic insights on terpenoid cyclization reactions in biology. Science 277, 1820–1824.

Mercke, P., Crock, J., Croteau, R., Brodelius, P.E., 1999. Cloning, expression, and characterization of *epi*-cedrol synthase, a sesquiterpene cyclase from *Artemisia annua* L. Arch. Biochem. Biophys. 369, 213–222.

Niwa, M., Iguchi, M., Yamamura, S., 1980. Co-occurence of (−) and (+) germacrene D in *Solidago altimissa* L. Determination of the optical rotation of optically pure germacrene D. Chem. Pharm. Bull. 28, 997–999.

Phillips, A.L., Ward, D.A., Uknes, S.J., Appleford, N.E.J., Lange, T., Huttly, A.K., Gaskin, P., Graebe, J.E., Hedden, P., 1995. Isolation and expression of three gibberellin 20-oxidase cDNA clones from *Arabidopsis*. Plant Physiol. 108, 1049–1057.

Proctor, R.H., Hohn, T.M., 1993. Aristolochene synthase — isolation, characterization, and bacterial expression of a sesquiterpenoid biosynthetic gene (*Ari1*) from *Penicillium roqueforti*. J. Biol. Chem. 268, 4543–4548.

Rising, K.A., Starks, C.M., Noel, J.P., Chappell, J., 2000. Demonstration of germacrene A as an intermediate in 5-*epi*-aristolochene synthase catalysis. J. Am. Chem. Soc. 122, 1861–1866.

Schmidt, C.O., Bouwmeester, H.J., deKraker, J.W., König, W.A., 1998. Biosynthesis of (+)- and (−)-germacrene D in *Solidago canadensis*: isolation and characterization of two enantioselective germacrene D synthases. Angew. Chem., Int. Ed. 37, 1400–1402.

Schmidt, C.O., Bouwmeester, H.J., Bülow, N., König, W.A., 1999a. Isolation, characterization, and mechanistic studies of (−)-α- gurjunene synthase from *Solidago canadensis*. Arch. Biochem. Biophys 364, 167–177.

Schmidt, C.O., Bouwmeester, H.J., Franke, S., König, W.A., 1999b. Mechanisms of the biosynthesis of sesquiterpene enantiomers (+)- and (−)-germacrene D in *Solidago canadensis*. Chirality 11, 353–362.

Sessa, R.A., Bennett, M.H., Lewis, M.J., Mansfield, J.W., Beale, M.H., 2000. Metabolite Profiling of sesquiterpene lactones from *Lactuca* species. Major latex components are novel oxalate and sulfate conjugates of lactucin and its derivatives. J. Biol. Chem. 275, 26877–26884.

Starks, C.M., Back, K.W., Chappell, J., Noel, J.P., 1997. Structural basis for cyclic terpene biosynthesis by tobacco 5-*epi*-aristolochene synthase. Science 277, 1815–1820.

Steele, C.L., Crock, J., Bohlmann, J., Croteau, R., 1998. Sesquiterpene synthases from grand fir (*Abies grandis*)—comparison of constitutive and wound-induced activities, and cDNA isolation, characterization and bacterial expression of δ-selinene synthase and γ-humulene synthase. J. Biol. Chem. 273, 2078–2089.

Verwoerd, T.C., Dekker, B.M.M., Hoekema, A., 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucl. Acids Res. 17, 2362.

Wagschal, K., Savage, T.J., Croteau, R., 1991. Isotopically sensitive branching as a tool for evaluating multiple product formation by monoterpene cyclases. Tetrahedron 47, 5933–5944.

Wagschal, K.C., Pyun, H.J., Coates, R.M., Croteau, R., 1994. Monoterpene biosynthesis—isotope effects associated with bicyclic olefin formation catalyzed by pinene synthases from sage (*Salvia officinalis*). Arch. Biochem. Biophys. 308, 477–487.

Weinheimer, A.J., Youngblood, W.W., Washecheck, P.H., Karns, T.K.B., Ciereszko, L.S., 1970. Isolation of the elusive (−)-germacrene A from the gorgonian, *Eunicea mammosa*. Chemistry of coelenterates. XVIII. Tetrahedron Lett. 497–500.

Williams, D.C., Wildung, M.R., Jin, A.Q., Dalal, D, Oliver, J.S., Coates, R.M., Croteau, R., 2000. Heterologous expression and characterisation of a "pseudomature" form of taxadiene synthase involved in paclitaxel (taxol) biosynthesis and evaluation of a potential intermediate and inhibitors of the multistep diterpene cyclisation reaction. Arch. Biochem. Biophys. 379, 137–146.