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Cloning and functional characterisation of a *cis*-muuroladiene synthase from black peppermint (*Mentha* × *piperita*) and direct evidence for a chemotype unable to synthesise farnesene

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

Using oligonucleotide primers designed to the known gene sequence of an (*E*)-β-farnesene (*E*βF) synthase, two cDNA sequences (MxpSS1 and MxpSS2) were cloned from a black peppermint (*Mentha* × *piperita*) plant. MxpSS1 encoded a protein with 96% overall amino acid sequence identity with the *E*βF synthase. Recombinant MxpSS1 produced in *Escherichia coli*, after removal of an N-terminal thioredoxin fusion, had a K_m for FPP of 1.91 ± 0.1 μM and k_{cat} of 0.18 s^{-1}, and converted farnesyl diphosphate (FPP) into four products, the major two being *cis*-muurola-3,5-diene (45%) and *cis*-muurola-4(14),5-diene (43%). This is the first *cis*-muuroladiene synthase, to be characterised. MxpSS2 encoded a protein with only two amino acids differing from *E*βF synthase. Recombinant MxpSS2 protein showed no activity towards FPP. One of the two mutations, at position 531 (leucine in MxpSS2 and serine in *E*βF synthase) was shown, by structural modelling to occur in the J–K loop, an element of the structure of sesquiterpene synthases known to be important in the reaction mechanism. Reintroduction of the serine at position 531 into MxpSS2 by site-directed mutagenesis restored *E*βF synthase activity (K_m for FPP 0.98 ± 0.12 μM, k_{cat} 0.1 s^{-1}), demonstrating the crucial role of this residue in the enzyme activity. Analysis, by GC–MS, of the sesquiterpene profile of the plant used for the cloning, revealed that *E*βF was not present, confirming that this particular mint chemotype had lost *E*βF synthase activity due to the observed mutations.

Keywords: *Mentha* × *piperita*; Black peppermint; Farnesyl diphosphate; Sesquiterpene synthase; *cis*-Muuroladiene; (*E*)-β-farnesene

1. Introduction

Sesquiterpenes are a large family of C_{15} isoprenoids, found in bacteria, fungi, insects and plants where they are derived from farnesyl diphosphate (FPP (**1**)) or via nerolidyl diphosphate by the action of sesquiterpene synthases. Synthesis of sesquiterpenes from FPP takes place at the cytosol/endoplasmic reticulum boundary (Belingheri et al., 1988; Gleizes et al., 1980) and one of the products is (*E*)-β-farnesene (*E*βF) which occurs in a number of plants and animals and can be involved in chemical communication. Potentially the most important signalling role of *E*βF is in aphids, where it is secreted from the cornicles when aphids are attacked by

Abbreviations: *E*βF, (*E*)-β-farnesene; FPP, farnesyl diphosphate.

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predators and acts as an alarm pheromone, causing other aphids to disperse(Glinwood and Pickett, 2004; Pickett et al., 1992, 1997).

The biosynthesis of *E*βF in peppermint (*Mentha* × *piperita*, L. cv. 'Black Mitcham') has been studied at the molecular level by Crock et al. (1997)) who identified an *E*βF synthase in a collection of expressed sequence tags (ESTs) from oil-gland cells. The cloned gene encoded a protein most similar to the sesquiterpene synthase, *epi*-aristolochene synthase, from tobacco, and with Mg^{2+} as the co-factor, the recombinant enzyme expressed in *Escherichia coli* from pBluescript was able to convert FPP to *E*βF (85%), (*Z*)-β-farnesene (8%), δ-cadinene (5%) and other minor unidentified sesquiterpene products. With Mn^{2+} as the co-factor, *E*βF (98%) and (*Z*)-β-farnesene (2%) were synthesised.

We have been following up this original work on *Mentha* sesquiterpene synthases with a view to exploring the suggestion (Crock et al., 1997) that *E*βF synthase gene could be used to engineer other plants to produce *E*βF and hence to exploit the pheromonal properties of the natural product for plant defence against aphids. In this paper we report further work on sesquiterpene synthases in *Mentha*, in particular the cloning and functional characterisation of two novel genes.

2. Results

2.1. Isolation of sesquiterpene synthase genes from *Mentha* × *piperita* 'black peppermint'

Primers, designed from the published *E*βF synthase cDNA sequence (Accession No. AF024615, (Crock et al., 1997)), were used successfully in RT-PCR reactions to amplify a fragment from *Mentha* × *piperita* of 1700 bp (data not shown), the same size as would be expected for the *E*βF synthase cDNA. Sequencing of cloned PCR products identified two distinct cDNAs. One sequence, MxpSS1, encoded a predicted protein with 17 amino acids differing from the published *E*βF synthase and also containing an inserted glycine residue at position 95 (MxpSS1, Accession No. AJ786641, 96% overall identity). The other sequence, MxpSS2, encoded a protein almost identical to the reported *E*βF synthase (MxpSS2, Accession No. AJ786642, 99.6% overall identity), except for a leucine to isoleucine substitution at position 471 and a serine to leucine substitution at position 531 (Fig. 1). There were several other differences in the DNA sequences of these clones but these did not affect the predicted protein sequences.

The same primers, used on gDNA from *Mentha* × *piperita* produced an approximately 4500 bp fragment. Cloning and sequencing of this fragment gave only the MxpSS2 sequence and showed that this gene has eight exons of 113, 257, 351, 223, 210, 210, 184 and 305 bp, interspersed by seven introns of approximately 100, 200, 50, 575, 500, 200 and 650 bp (the intron sizes are approximate because in some cases only the borders have been sequenced).

2.2. Activity of MxpSS1 and MxpSS2

The two cDNAs cloned from *Mentha* × *piperita*, with homology to the *E*βF synthase, were cloned into the expression vector pET-32b and the sequences verified. Recombinant thioredoxin fusion proteins were expressed in Codon Plus *E. coli*, purified using Ni-chelate chromatography and then incubated with radiolabelled substrate [1-^{3}H]FPP. The products were extracted and quantified by scintillation counting. MxpSS1 gave significant levels of radiolabelled product (2302 disintegrations min^{-1}), whereas MxpSS2 gave no counts above the background control (28 disintegrations min^{-1}).

The non-polar, volatile products produced from FPP by the activity of MxpSS1 were separated by GC–MS as shown in Fig. 2. This revealed four major sesquiterpene peaks which were initially identified by comparison with published spectra and retention indices (Adams, 2001) as *cis*-muurola-3,5-diene (**9**) (45%) (see Fig. 4), *cis*-muurola-4(14),5-diene (**10**) (43%), γ-cadinene (**6**) (7%) and α-cadinene (**7**) (5%). Since the formation of the muuroladienes was unexpected, their identification was confirmed by GC–MS with authentic muuroladienes from a sample of *Cupressus bakerii* (Kim et al., 1994). Two GC columns were employed and use of the chiral phase confirmed the absolute stereochemistry of the *cis*-muuroladienes as being the same as that described originally (Kim et al., 1994). The same products and product profile were obtained using purified protein that had the N-terminal fusion (thioredoxin + histidine-tag) removed and showed that the fusion did not alter the specificity of the enzyme. Kinetic analysis of MxpSS1 with respect to FPP gave a K_m of 1.91 μM ± 0.1 with the direct linear plot and k_{cat} of 0.18 s^{-1}.

2.3. Mutagenesis of MxpSS2

The clone MxpSS2, which encodes a protein with no activity towards FPP and has two amino acids differing from the reported *E*βF synthase, was subjected to site-directed mutagenesis to give three new sequences MxpSS2a (I471L), MxpSS2b (L531S) and MxpSS2c (I471L and L531S), the latter encoding a predicted protein identical to the published *E*βF synthase.

Recombinant MxpSS2a, MxpSS2b and MxpSS2c were produced in *E. coli* and analysed by SDS–PAGE, confirming that the proteins were expressed and to a similar level (data not shown). The proteins were then

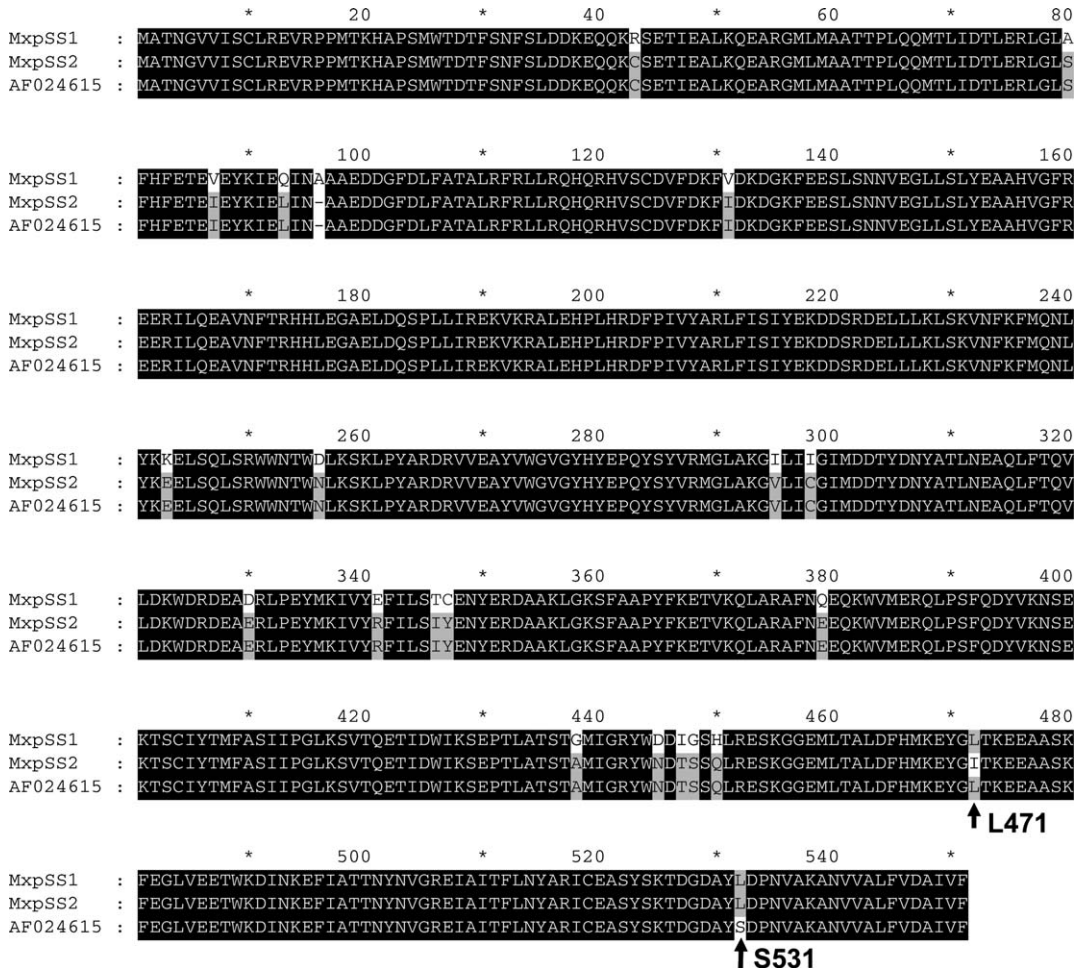

Fig. 1. Predicted amino acid sequences of the (*E*)-β-farnesene synthase (AF024615) reported by Crock et al. (1997), and the proteins encoded by the cDNAs MxpSS1 (Accession No. AJ86642) and MxpSS2 (Accession No. AJ86642). Arrows indicate differences between AF024615 and MxpSS2.

incubated with labelled FPP and the products were extracted and quantified by scintillation counting.

MxpSS2a gave no significant levels of product, whereas MxpSS2b and MxpSS2c produced products which were separated by GC–MS, giving only single peaks corresponding to *E*βF (data for MxpSS2c shown in Fig. 3(a)). Thus, the I471L mutation did not restore activity but L531S and the double mutant I471L/L531S had normal activity.

Kinetic analysis of MxpSS2c with respect to FPP gave a K_m of 0.98 μM ± 0.1 and k_{cat} of 0.1 s^{-1} which is similar to the result for MxpSS1 and demonstrated that normal levels of activity had been restored.

2.4. Structure of EβF synthase

The I471 mutation in MxpSS2a is far removed from the active site and the observation that the single replacement to give I471L did not restore activity, when coupled with the high activity of L531S mutant, which retained the isoleucine at position 47, suggests that the I471 is not involved in catalysis. However, the L531 residue lies in the J–K loop proposed to clamp down over the active site entrance (Starks et al., 1997) and the serine to leucine mutation has a significant impact on the catalytic activity. The crystallography data from the *epi*-aristolochene synthase suggests that this J–K loop exhibits a great deal of flexibility and the mutation in this loop may alter its conformation and affect activity. It should be noted that in the muuroladiene synthase (MxpSS1) there is a leucine at the same position and yet the enzyme is active.

2.5. Sesquiterpenes in extracts of *Mentha* × *piperita* 'black peppermint'

The compounds present in hexane extracts of young and mature leaves of *Mentha* × *piperita* are shown in Fig. 3(b) and (c), respectively. In both cases there is no *E*βF, suggesting that this compound is not synthesised in the plants used in this study. Neither of the muuroladienes were found in the extract. Extraction of plant material with ethyl acetate revealed two puta-

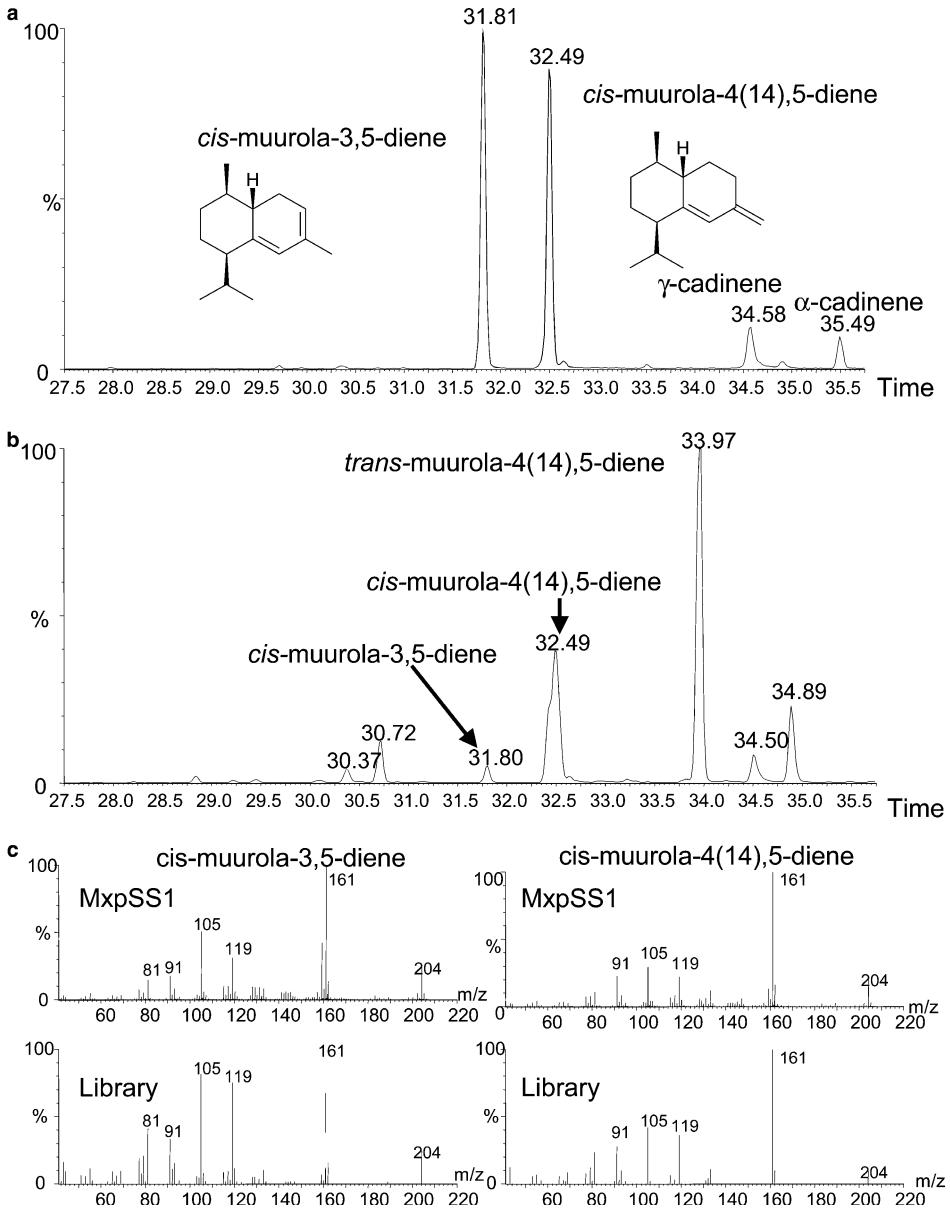

Fig. 2. (a) Total ion current trace of the products synthesised by MxpSS1 from FPP and the structures of the principal products. (b) TIC of hexane extract of *Cupressus bakerii*. (c) Mass spectra derived from the main peaks.

tive oxidised sesquiterpene products. From the mass spectra of these compounds it appears that neither of them are related to muuroldiene.

3. Discussion

One of the cDNAs cloned here (MxpSS1) is a novel member of the sesquiterpene synthase family and encodes an enzyme which can convert FPP to a range of sesquiterpenes. The major products are *cis*-muurola-3,5-diene (45%) and *cis*-muurola-4(14),5-diene (43%) and we therefore name this enzyme a *cis*-muuroladiene synthase. It is interesting to note that we have not been able to identify these compounds in extracts of our plants, indicating that either the *cis*-muurolodiene synthase is expressed at very low levels *in vivo* or that the hydrocarbon products are further metabolised in the plant beyond simple oxygenations. However, this is not without precedent; an *epi*-cedrol synthase has been isolated from *Artemisia annua* but neither cedrol or *epi*-cedrol were found in the sesquiterpene profile of the plant (Mercke et al., 1999). From our *cis*-muuroladiene synthase, the four detected products are biosynthetically related and their biosynthesis via nerolidyl diphosphate can be rationalised following (Kim et al., 1994) as shown

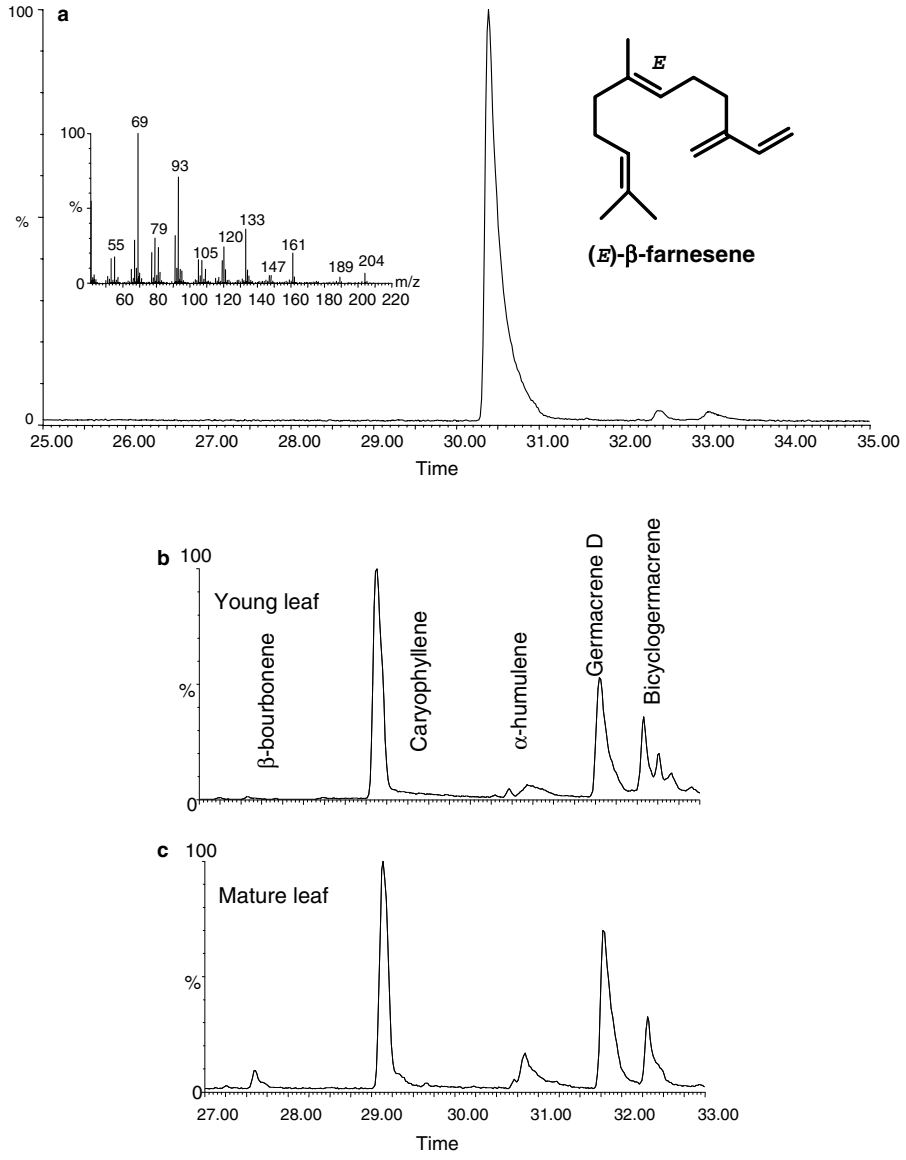

Fig. 3. GC-MS. (a) Total ion current trace of the products from FPP synthesised by MxpSS2c. Inset: mass spectrum of main peak. (b) The compounds present in a hexane extract of young and mature leaves of *Mentha* × *piperita*.

in Fig. 4. FPP (**1**) rearranges (**2**) and ionisation–cyclisation creates a 11-germacryl cation (**3**) which by a 1,3-hydride migration generates the 1-germacryl cation (**4**). This cation, by cyclisation rearranges to the 10-cadinyl cation (**5**), which deprotonates to give the cadinenes (**6** and **7**). However, the majority of the cation (**5**) undergoes two further 1,2-hydride migrations to generate the *cis*-muurolyl cation (**8**), leading to the muuroladiene products (**9** and **10**) by proton elimination. The production of multiple products is in line with other reports that many of the enzymes which act on isoprenoid diphosphates produce a range of products (Steele et al., 1998).

The other cDNA cloned here (MxpSS2) has two amino acids differing to a known *E*βF synthase and shows no activity towards FPP. However, reinstating the serine at position 531 restores activity. This is in line with the work of Deligeorgopoulou and Allemann (2003), showing that a single amino acid substitution could convert an aristolochene synthase into an enzyme which produced 73% EβF. Here it was suggested that differential folding of FPP in the active site was responsible for the change in activity. In our case the change from a serine to a leucine in MxpSS2 may alter the structure of the J–K loop and may therefore abolish catalytic activity by preventing correct closure of the loop after substrate binding. However, this is unlikely since the muuroladiene synthase also has a leucine at this position and is functionally active. The subtle change,is thus either due to a steric effect or the physico-chemical nature of the leucine versus serine residue may impinge on the reaction mechanism.

GC–MS studies showed that both MxpSS2b and MxpSS2c produced a single product from FPP which

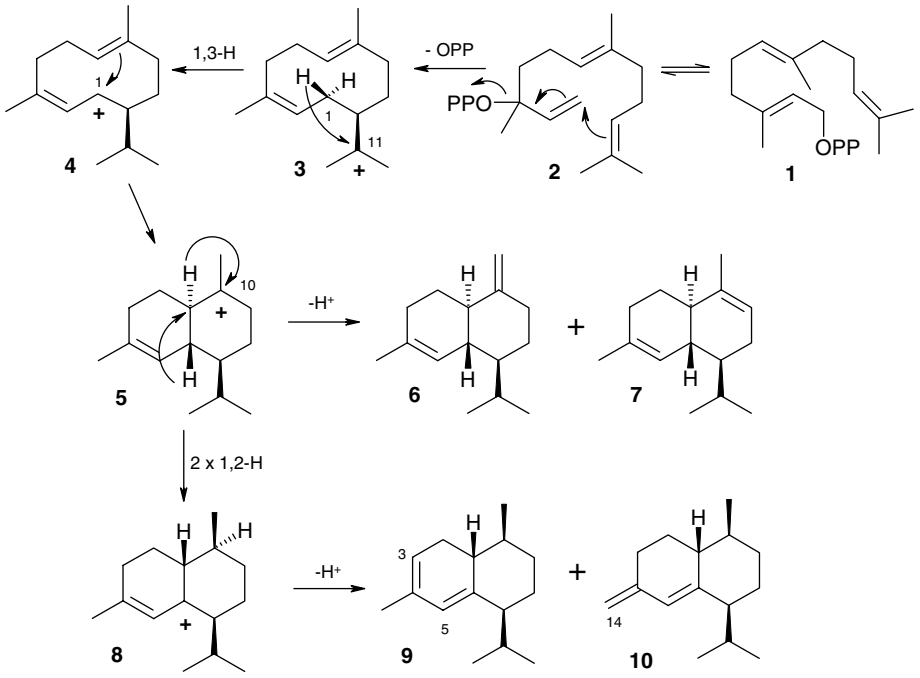

Fig. 4. Putative route for conversion by MxpSS1 of FPP (**1**), via nerolidyl diphosphate (**2**) to γ-cadinene (**6**) α-cadinene (**7**), *cis*-muurola-3,5-diene (**9**) and *cis*-muurola-4(14),5-diene (**10**).

was identified as *E*βF (other small peaks were not sesquiterpenes). Thus, both of these enzymes are *E*βF synthases and interestingly they produce pure product. This is in contrast to the published *E*βF synthase which has the same amino acid sequence and, in the presence of Mg^{2+} (i.e., the conditions used in our study), produced only 85% *E*βF with 8% *Z*βF and 5% cadinene. The reason for the difference is not obvious but our assay conditions may have been slightly different or the different products may be the result of re-arrangement during analysis. Initially, our study used a fusion protein with a large N-terminal region. However, this did not result in any alteration of product specificity or profile when compared to the products from the enzymes with the N-terminal fusions cleaved with thrombin (data not shown).

In a publication by Trapp and Croteau (2001), a new classification of terpene synthase genes was suggested using a comparison of intron/exon patterns; 12–14 introns would denote a class I synthase, nine introns a class II and six introns a class III. Using this guideline, the gene described here with seven introns does not fall in to any of these categories. The closest category is class III, which also contains similar plant sesquiterpene genes such as vetispiradiene synthase and 5-*epi*-aristolochene synthase. Furthermore, (Facchini and Chappell, 1992) isolated two 5-*epi*-aristolochene synthase genes from a genomic source and both of these had five introns.

The fact that we did not clone the original sequence encoding the *E*βF synthase reported by Crock et al. (1997) suggest that we were not using equivalent plant material. The plant used by Crock et al. (1997) was *Mentha* × *piperita* L. cv. "Black Mitcham" and the plant that we purchased was only described as *Mentha* × *piperita*, black peppermint. Extracts of the leaves of our plant did not show any measurable amounts of *E*βF suggesting that it did not have any active *E*βF synthase, in line with the cloning which produced an apparently inactive cDNA. This inactive cDNA was produced by PCR, but the sequence differences between it and the published *E*βF synthase are not PCR-induced errors since the same sequence was found in the gene sequence amplified from the genomic DNA. However, since we cloned MxpSS2 by RT-PCR, the apparently inactive form must be expressed even though it is unlikely to contribute to the sesquiterpene profile of our particular plant. These findings are in line with the known existence of 'chemotypes' of *Mentha* × *piperita* (Piccaglia, 1998) which could arise from mutations in the sesquiterpene synthases leading to different product profiles. If there are any other *E*βF synthase genes in *Mentha* × *piperita* they are either not expressed in the photosynthetic tissues or at such low levels that *E*βF cannot be detected.

4. Experimental

4.1. Plant material

A *Mentha* × *piperita* 'black peppermint' plant was purchased from Harpenden Garden Centre, Harpenden, UK and propagated by root cuttings in compost at

20 ± 5 °C under 400 W HPS mercury vapour lamps. Leaves were excised, frozen in liquid nitrogen and stored at −80 °C. Samples of *Cupressus bakerii* ssp. *mathewsii* (Siskiyou Cypress) were obtained from Castle Howard Arboretum, UK.

4.2. Oligonucleotides

Primers designed to amplify the full-length *E*βF synthase gene were custom synthesised by Gibco-BRL and the concentrations checked by spectrophotometer readings. The sequences were as underlined: Forward primer 5′-CAGAGAGTTTGTTGTAGTGAAAAA**ATG**-3′ and Reverse primer 3′-**ACT**AACGTATTAGTTTCTGGGATATT-5′ where ATG and ACT are the start and stop codons of the published *E*βF synthase cDNA (Crock et al., 1997).

4.3. Extraction of nucleic acids

Plant material stored at −80 °C was used to extract total RNA and gDNA using the RNeasy and DNeasy plant kits (Qiagen) according to the manufacturer's instructions and including the QIA shredder method for tissue disruption.

4.4. PCR amplifications

For RT-PCR reactions, total RNA (200 ng) was used to synthesise first strand cDNA from an oligo(dT) primer. The RNA and primer were heated at 65 °C for 10 min, chilled on ice for 2 min and then added to reaction mixes each containing dNTPs (1 mM each), DTT (10 mM), Superscript™ II Rnase H^- reverse transcriptase (200 U, Gibco-BRL), buffer supplied with the enzyme and RNA guard (10 U, Amersham Pharmacia Biotech) in a total volume of 20 μl. The reaction proceeded for 1 h at 37 °C. PCR amplifications were done in 25 μl reactions containing either cDNA (200 ng) or gDNA (100 ng), $MgCl_2$ (1.5 mM), dNTPs (0.2 mM), primers (31.25 mg each), *Pfu* DNA polymerase (0.75 U, MBI Fermentas) and buffer supplied with the enzyme. Reactions continued for 35 cycles of 94 °C; 30 s, 45–55 °C; 60 s and 72 °C for 60 s kb^{-1} template. This was followed by heating at 72 °C for 10 min. Products were run on agarose gel electrophoresis (alongside suitable markers to check the size and the approximate amounts) and then purified from the gel using the CONCERT™ gel purification kit (Gibco-BRL).

4.5. Cloning and sequencing

PCR fragments were cloned into pGEM-T-Easy (Promega) according to the manufacturer's instructions and transformed into competent *E. coli* XLI Blue cells prepared by the method of Alexander et al. (1984). Plasmid DNAs were isolated using the Qiaprep® Spin Miniprep Kit (Qiagen) and inserts sequenced using a BigDye™ Termination Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). The reactions were run on an ABI 373A automated sequencer.

4.6. Expression of recombinant protein

cDNAs were subcloned into the pET32b vector (Novagen) and transferred into competent *E. coli* DH5 alpha cells, from which recombinant plasmids were isolated and checked. The plasmids were then transferred into *E. coli* Codon Plus (Stratagene). Expression and purification of the recombinant protein followed the procedure described in Prosser et al. (2002).

4.7. Cleavage of N-terminal fusion protein

HisBind-purified MxpSS1 and MxpSS2 were digested with thrombin (1 unit mg^{-1} protein) in 10 mM Tris pH 7.5, 150 mM NaCl at 23 °C for 16 h. The resulting products were analysed by SDS–PAGE to ensure complete digestion and a sample of the cleaved protein was assayed and the products analysed by GC–MS.

4.8. Site-directed mutagenesis

Site-directed mutagenesis was done using the Quickchange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The forward primers used were: I 471 L: TTTCCACATGAAAGAATATGGACTAACAAAGGAAGAGGCG and L531 S: AAACTGACGGAGACGCTTATTCAGATCCTAATGTTGCCAA, where the mutation sites are underlined. Reverse primers were exact complements of the above. The amplification consisted of 12 cycles with an annealing temperature of 55 °C and extension time of 14 min.

4.9. Extraction and identification of sesquiterpenes from plant material

Fresh young or mature leaves of *Mentha* × *piperita* were extracted sequentially with redistilled hexane and then ethyl acetate by grinding with a pestle and mortar aided with a pinch of sand. The hexane extract was applied a short column of silica gel (BDH, 40–63 μm):magnesium sulphate (10:1), eluted with hexane, and then sequentially eluted with ether and ethyl acetate and the eluates collected. The ethyl acetate extract was similarly applied to the column and eluted with ethyl acetate. The column eluates were analysed by GC–MS on a Micromass GCT instrument using a BPX5 (SGE) capillary column. The temperature programme was 50 °C hold for 2 min then an additional 3 °C/min to 246 °C which was held for 4.67 min. Identification of the individual products

was done by comparing spectra and Kovat's retention indices with published data (Adams, 2001), which defined geometric isomers but not the absolute stereochemistry, and by comparison of mass spectra and retention indices with sesquiterpenes from *C. bakerii* (Kim et al., 1994). Additionally, the samples were analysed using a Cyclosil-B (J and W Scientific) chiral column. The temperature programme was 50 °C held for 2 min then an additional 3 °C per min to 230 °C which was held for 4.67 min. Terpenes were similarly extracted from 2 g *C. bakerii* leaf material and analysed by GC–MS.

4.10. Sesquiterpene synthase assays

For product identification, recombinant enzymes were added to reactions containing sodium phosphate pH 7.2 (20 mM), $MgCl_2$ (5 mM), glycerol (10%), [1-^{3}H]FPP 850 GBq mmol^{-1} (0.2 μM) and FPP (36 μM). This was overlaid with hexane and incubated at 27 °C for 3 h. Then the hexane was removed, vortexed, applied to a silica gel column, eluted with hexane and the amount of radioactivity representing the sesquiterpene products was counted in a Rackbeta Scintillation counter. The sesquiterpenes produced were identified by GC–MS as above. A final elution of the column with ethyl acetate gave no measurable radioactivity.

Kinetic analysis used the same core buffer but 5 μl of the purified enzyme (2.4 pmoles MxpSS1 or MxpSS2) was added to different substrate concentrations and incubated for 10 min before the reaction was stopped by the addition of 50 μl of 0.5 M EDTA. The reaction was overlaid with hexane and processed as described above. These quantities of enzyme were in the linear part of a V_{max} vs. enzyme concentration plot (not shown).

4.11. Modelling of protein structures

Three-dimensional structures of the predicted proteins encoded by the cloned genes were modelled using the ExPASy SWISS-MODEL program (Schwede et al., 2003) with 5-*epi*-aristolochene synthase as the template and refining with DeepView. RMS values of 0.25 were computed over 515 residues (of 550 total and 532 from modelling) which indicates reasonable structural alignment.

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