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Unusual features of a recombinant apple α-farnesene synthase

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Abstract

A recombinant α-farnesene synthase from apple (*Malus* × *domestica*), expressed in *Escherichia coli*, showed features not previously reported. Activity was enhanced 5-fold by K^+ and all four isomers of α-farnesene, as well as β-farnesene, were produced from an isomeric mixture of farnesyl diphosphate (FDP). Monoterpenes, linalool, (*Z*)- and (*E*)-β-ocimene and β-myrcene, were synthesised from geranyl diphosphate (GDP), but at 18% of the optimised rate for α-farnesene synthesis from FDP. Addition of K^+ reduced monoterpene synthase activity. The enzyme also produced α-farnesene by a reaction involving coupling of GDP and isoprenyl diphosphate but at <1% of the rate with FDP. Mutagenesis of active site aspartate residues removed sesquiterpene, monoterpene and prenyltransferase activities suggesting catalysis through the same active site. Phylogenetic analysis clusters this enzyme with isoprene synthases rather than with other sesquiterpene synthases, suggesting that it has evolved differently from other plant sesquiterpene synthases. This is the first demonstration of a sesquiterpene synthase possessing prenyltransferase activity.

Keywords: α-Farnesene; Prenyltransferase; Sesquiterpene; Terpene synthase; Farnesyl diphosphate; *Malus domestica*; Apple

1. Introduction

(*E*,*E*)-α-Farnesene (3,7,11-trimethyldodeca-1,3*E*,6*E*,10-tetraene) is a sesquiterpene hydrocarbon produced by many plant species in a range of tissues, in response to pathogens (Huang et al., 2003), or on wounding by herbivores (Boevé et al., 1996; Mercke et al., 2004; Pare and Tumlinson, 1999; van den Boom et al., 2004; Vuorinen et al., 2004). Production of α-farnesene is thought to play a role in plant defence by attracting predators and parasitoids (Pare and Tumlinson, 1998); however, α-farnesene is also an attractant to codling moth (Yana et al., 2003) and a sex pheromone in mice (Morgan et al., 2004). In plants (*E*,*E*)-α-farnesene is often produced along with other sesquiterpenes (Köllner et al., 2004) or as a minor product resulting from genetic engineering (Deligeorgopoulou and Allemann, 2003).

Rupasinghe et al. (2000) partially purified α-farnesene synthase from apple skin as a 108 kDa oligomer with an optimum pH for activity of 5.6 and an absolute requirement for either Mg^{2+} or Mn^{2+}. Kinetic studies on this impure native protein showed optimal activity at 20 °C while half-maximal activity was retained at 0 °C. A cDNA encoding a α-farnesene synthase, essentially identical to the enyme we are now reporting (sharing 574/576 amino acids) was isolated from ripe apple skin by Pechous and Whitaker (2004). The predicted molecular mass was 66 kDa. Protein expressed in vitro from lysed cells, but without further purification, showed α-farnesene synthase activity using (*E*,*E*)-farnesyl diphosphate (FDP) as a precursor, with minor quantities of β-ocimene produced when incubated with

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the monoterpene precursor geranyl diphosphate (GDP). The α-farnesene produced was 99.5% the (*E*,*E*)-isomer. From attempts to solubilise in vitro-produced protein, the authors suggested that improperly folded protein was also capable of synthesising (*E*)-nerolidol. Trace amounts of (*E*)-nerolidol and β-farnesene were also produced in assays with impure soluble enzyme. In contrast to the native partially purified enzyme, maximal activity was observed at pH 7.0–8.0.

Additional α-farnesene synthase genes have been isolated from angiosperms: pear (GeneBank Accession No. AY566286) and cucumber (Mercke et al., 2004), and the gymnosperms: loblolly pine (Phillips et al., 2003), and spruce (Martin et al., 2004). However, with the exception of the pear gene, the sequences have little similarity to those from apple. In each case, (*E*,*E*)-α-farnesene is the main product from FDP. The cucumber enzyme also uses GDP to make the monoterpene, β-ocimene. A mammalian or insect equivalent does not yet appear to have been isolated. With the importance of α-farnesene production to apple (Pare and Tumlinson, 1999; Pechous et al., 2005; Rowan et al., 2001; Rupasinghe et al., 2000; Yana et al., 2003), we carried out extensive kinetic studies on a purified recombinant enzyme from 'Royal Gala' apple. These studies revealed major differences in enzymic properties from those reported for the native apple α-farnesene and other sesquiterpene synthases and revealed a hitherto unreported prenyltransferase activity associated with this sesquiterpene synthase.

2. Results and discussion

2.1. Characterisation of recombinant α-farnesene synthase

The recombinant enzyme, which functioned as a monomer (Fig. 1), demonstrated a broad pH optimum (pH 7–8.5) and K_m values for FDP (~3 μM), Mg^{2+} (~700 μM) and Mn^{2+} (~15 μM), similar to other sesquiterpene synthases (Crock et al., 1997; Steele et al., 1998). Activity with FDP was enhanced 5-fold on addition of K ions (Table 1). Optimal K^+ concentrations were determined to be 30–50 mM ($K_m \sim 3$ mM). The addition of Na^+ did

Table 1
Effect of metal ions on the relative activity of semi-purified recombinant α-farnesene synthase in the presence of saturating (25 μM FDP)

Metal ion	V_{rel} (%)
Mg/K	100
Mn/K	41
Mg/Mn/K	69
Mg	16
Mn	13
Mg/Mn	18
Mg/Na	23
Mn/Na	15
Mg/Mn/Na	24

Concentrations used were 7 mM $MgCl_2$, 150 μM $MnCl_2$, 50 mM KCl, 50 mM NaCl. Data were the mean of three independent measurements and the experiment was carried out twice. SEM was <5% of the mean.

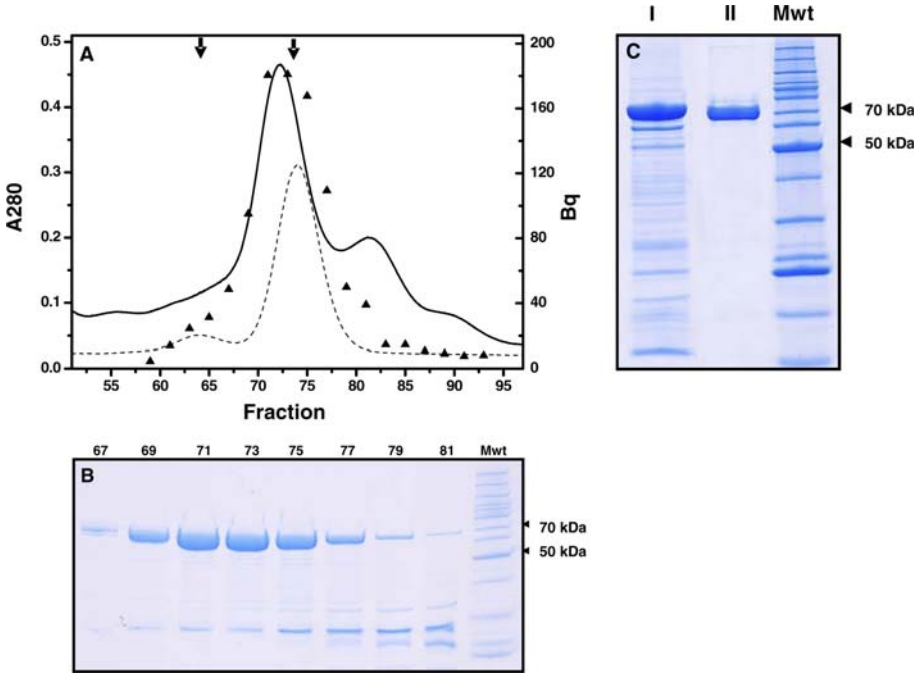

Fig. 1. Purification of recombinant α-farnesene synthase. (A) Gel filtration profile of purified recombinant apple $(His)_6$-α-farnesene synthase (——), BSA standard (------) and corresponding activity profile (▲) on G200 Superdex. BSA monomer and dimer peaks are indicated by down arrows. (B) SDS–PAGE gels of a subset of corresponding fractions from the gel filtration purification of recombinant apple $(His)_6$-α-farnesene synthase with molecular mass markers indicated. (C) Semi-purified (I) and purified (II) recombinant α-farnesene synthase used in kinetic analysis. Specific activity determinations and experiments with deuterated precursors were carried out using (II). Molecular mass markers are indicated.

not significantly increase activity indicating that rate enhancement by K^+ was not a general salt effect. The recombinant enzyme's specific activity was calculated to be 1.6 μmol h^{-1} mg^{-1} under optimal conditions for farnesene production. Inhibition of the recombinant apple enzyme's pyrophosphatase activity was also investigated (data not shown) as reported for the partially purified native enzyme (Rupasinghe et al., 2000). This indicated comparable inhibition (96%) in the presence of 10 mM sodium molybdate. Interestingly, the lack of soluble recombinant protein reported using a C-terminal myc tag (Pechous and Whitaker, 2004) was not seen when the protein was expressed with a N-terminal His tag.

Both (*E*,*E*) and (*Z*,*E*)-α-farnesene are found in apple fruit skin in a ratio varying typically between 100–300:1 (Bengtsson et al., 2001; Hern and Dorn, 2003; Matich et al., 1996). When FDP, synthesised from 96% (*E*,*E*)-farnesol, was incubated with recombinant protein, (*E*,*E*) and (*Z*,*E*)-α-farnesene were produced in a ratio of 96:4, respectively. Incubation of the recombinant enzyme under optimal conditions, including K^+, with FDP prepared from farnesol with an isomeric composition of 41 (*E*,*E*): 29

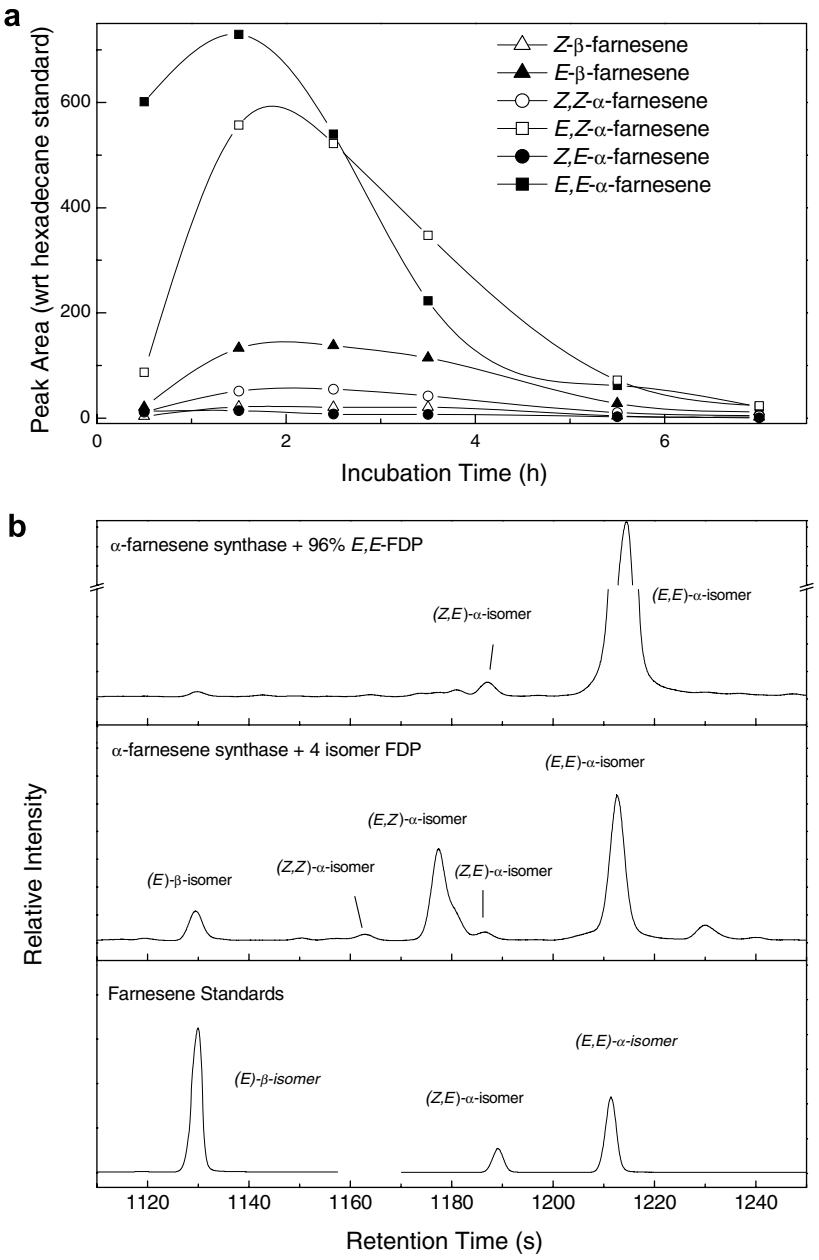

Fig. 2. Time course incubation of α-farnesene synthase with a mixture of FDP isomers ((*E*,*E*) 41%, (*E*,*Z*) 29%, (*Z*,*E*) 25%, (*Z*,*Z*) 5%). Replicate tubes were left open to the air after addition of precursor and just prior to SPME headspace sampling (3 min) hexadecane internal standard was added. (a) Peak areas of farnesene isomers relative to hexadecane at each sampling time. (b) Chromatogram showing the products of α-farnesene synthase with 96% (*E*,*E*)-FDP and a 4 isomer FDP mixture compared with *E*-β-farnesene, and (*Z*,*E*)- and (*E*,*E*)-α-farnesene standards. (*E*,*Z*)- and (*Z*,*Z*)-α-farnesene were identified based on MS and relative retention times.

(*E*,*Z*): 25 (*Z*,*E*): 5 (*Z*,*Z*), showed the enzyme preferentially synthesised (*E*,*E*)-α-farnesene (Fig. 2). (*E*,*Z*)-FDP was the second most favoured substrate. However, over the 7 h incubation, all six isomers of α- and β-farnesene were produced. These results indicate that, although (*E*,*E*)-FDP was the preferred substrate, the enzyme will accept all four isomeric forms of the FDP precursor. Interestingly both isomers of β-farnesene were also synthesised by the enzyme presumably from a specific FDP isomer.

2.2. Recombinant α-farnesene synthase uses GDP to synthesise monoterpenes

Recombinant α-farnesene synthase produced (*E*)-β-ocimene (90% of total), linalool, β-myrcene and smaller quantities of other monoterpenes when incubated with GDP. Incubation with deuterated GDP (d_2-GDP) gave deuterated monoterpenes (Fig. 3). Synthesis of monoterpenes from GDP (100 μM) was at 18% of the optimised rate for α-farnesene synthesis from FDP (25 μM). Potassium ions reduced monoterpene synthase activity (Table 2) and trace quantities of α-farnesene were also found when GDP was added to purified recombinant enzyme. Neither isopentenyl diphosphate (IDP), nor dimethyl allyl diphosphate (DMAPP), singly or together, were substrates.

2.3. Recombinant α-farnesene synthase can use GDP and IDP together to form α-farnesene

When both GDP and IDP were added to purified recombinant enzyme, up to 14 times more α-farnesene was produced than with GDP alone (data not shown), suggesting

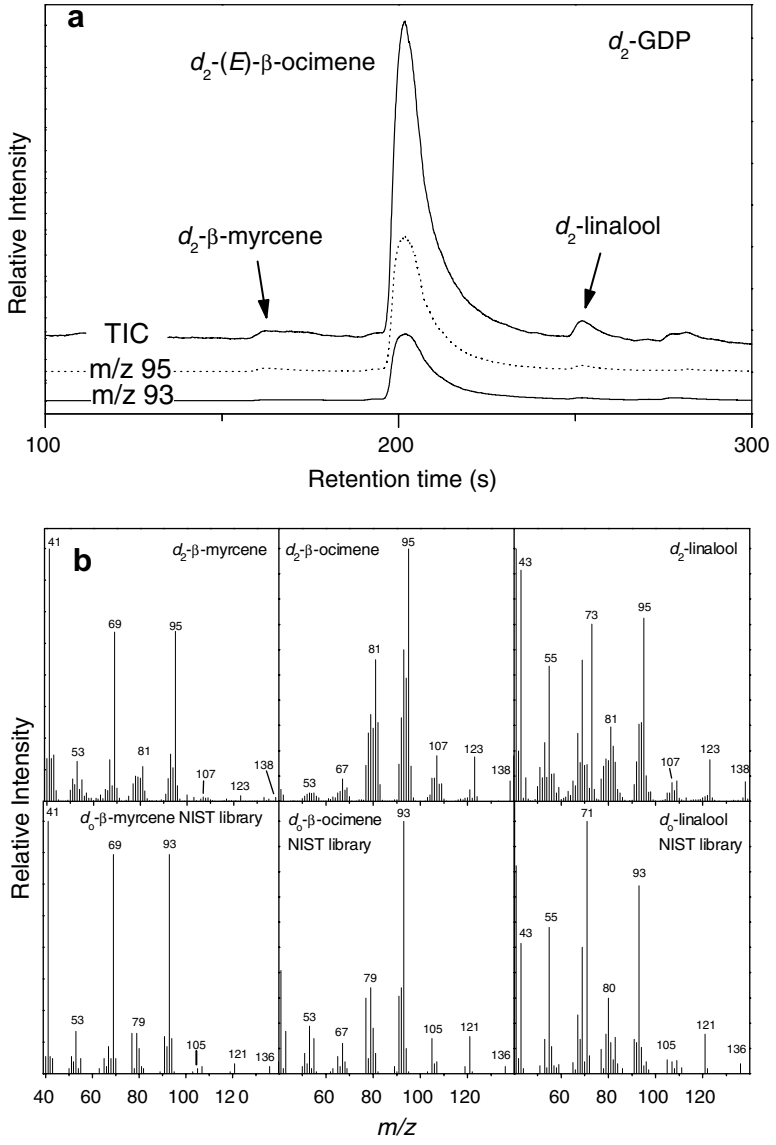

Fig. 3. Monoterpene biosynthesis by recombinant α-farnesene synthase. (a) Headspace volatiles produced by purified α-farnesene synthase incubated with d_2-GDP showing synthesis of deuterated (*E*)-β-ocimene, β-myrcene and linalool. (b) Mass spectra of deuterated monoterpenes and comparison with mass spectra from NIST MS library.

Table 2
Effect of K^+ on the formation of (*E*)-β-ocimene and α-farnesene from GDP or GDP and IDP

Precursor	Product	With K^+	Without K^+
GDP	(*E*)-β-ocimene	16.1[a]	23.2
	(*E*,*E*)-α-farnesene[b]	1.8	3.3
GDP + IDP	(*E*)-β-ocimene	13.8	30.4
	(*E*,*E*)-α-farnesene	2.8	4.8

Amounts are average GCMS peak areas as percent of the hexadecane internal standard.
[a] Duplicates differed by less than 10%.
[b] Contribution from (*E*,*E*)-α-farnesene, or precursor, bound to enzyme.

either that the enzyme could use these two precursors to form α-farnesene or that bound FDP precursor or α-farnesene product was being displaced from the enzyme. Headspace trapping before and after acid hydrolysis of the GDP and IDP substrates showed no sesquiterpene contaminants. Similar testing of assay solutions showed no contaminating compounds. Hence, if α-farnesene was being synthesised from GDP and IDP, the product would be deuterated if deuterated precursors were used. We therefore synthesised the deuterated precursors, d_2-IDP, d_2-GDP and d_8-GDP. Headspace analysis after acid hydrolysis showed that these precursors contained no detectable sesquiterpene impurities by GCMS.

When GDP and d_2-IDP were used together as substrates for α-farnesene synthase, the α-farnesene produced was deuterated (Fig. 4a) but the monoterpenes were not (data not shown). The diagnostic GCMS ions, *m*/*z* 93 (d_0-α-farnesene) and *m*/*z* 95 (d_2-α-farnesene), showed sufficient differences in retention times to indicate clearly the presence of both d_0- and d_2-α-farnesenes. When d_2-GDP and d_2-IDP, both deuterated at C-1 next to the pyrophosphate group, were used together as substrates, both deuterated monoterpenes (data not shown) and d_4-α-farnesene were produced (Fig. 4b). When d_8-GDP and d_2-IDP were used together as substrates, d_{10}-α-farnesene was produced (Fig. 4c), providing additional evidence that the enzyme could indeed synthesise α-farnesene from precursors other than FDP, although at a rate between 0.5% and 1% of that optimised for FDP. Diagnostic ions indicate that a single d_2-IDP becomes joined to the head of the d_0-, d_2- or d_8-GDP moiety. The d_8-GDP used in these experiments was a 89:11 ratio of (*E*):(*Z*) isomers, but only (*E*,*E*)-d_{10}-α-farnesene was found.

To check for the presence of a co-purifying prenyltransferase derived from *Escherichia coli*, the protein bands visible on an SDS–PAGE gel of both purified (Fig. 1C, column II) and semi-purified recombinant α-farnesene synthase (Fig. 1C, column I) were sequenced by nano-electrospray LC MS/MS. Data analysis using TurboSEQUEST

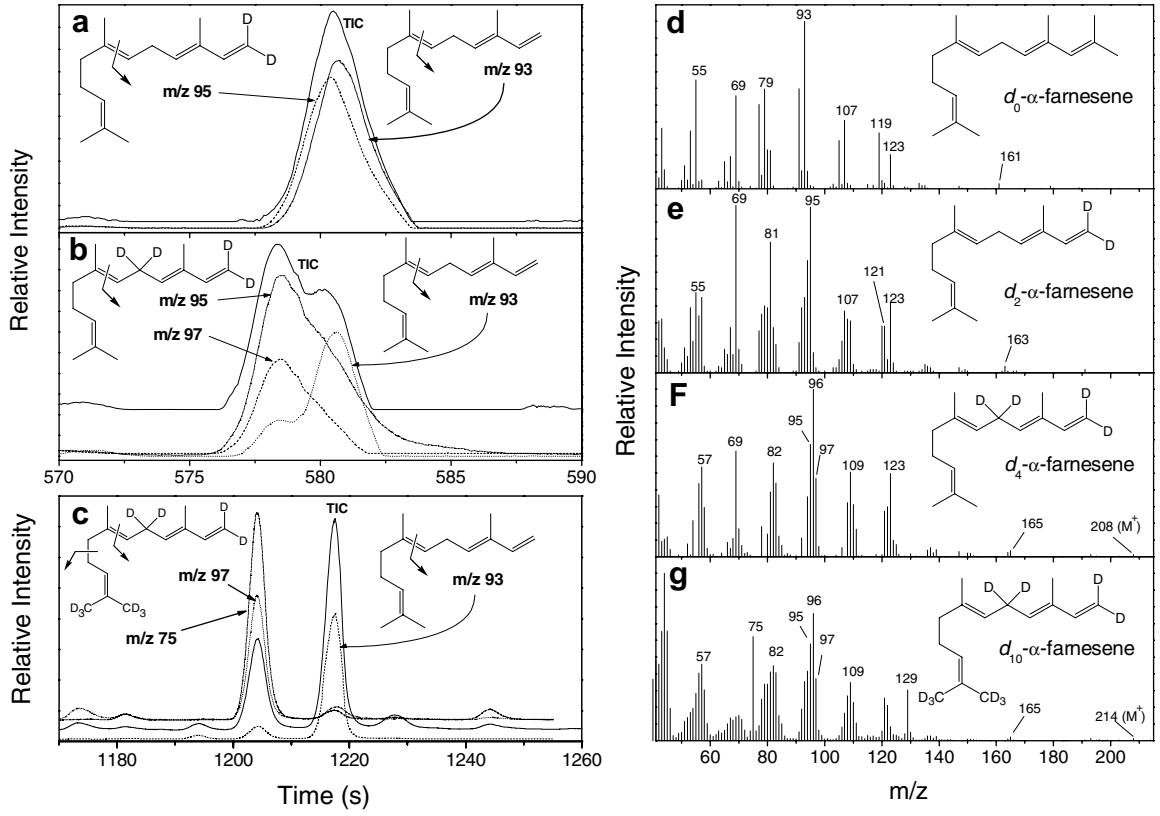

Fig. 4. GCMS profiles of deuterated and non-deuterated α-farnesene produced from purified α-farnesene synthase on incubation with (a) d_0-GDP and d_2-IDP, (b) d_2-GDP and d_2-IDP and (c) d_8-GDP and d_2-IDP, and mass spectra of (d) d_0-α-farnesene, (e) d_2-α-farnesene, (f) d_4-α-farnesene, and (g) d_{10}-α-farnesene. Characteristic ions for d_0-α-farnesene, *m*/*z* 93 $[C_7H_9]^+$; d_2-α-farnesene, *m*/*z* 95 $[C_7H_7D_2]^+$, d_4-α-farnesene, *m*/*z* 97 $[C_7H_5D_4]^+$; and d_{10}-α-farnesene, *m*/*z* 75 $[C_5H_3D_6]^+$. A slower GC oven ramp was used in (c).

software against the latest version of the NCBI non-redundant protein database (NRPD) did not identify any bacterial prenyltransferases.

2.4. Recombinant α-farnesene synthase carries bound FDP or α-farnesene

A complication in the above experiments was the presence of trace amounts of d_0-α-farnesene when using deuterated diphosphate precursors. As all sources, other than the purified recombinant protein, were found to be free of both FDP and α-farnesene, the presence of d_0-α-farnesene must be due to either FDP or α-farnesene that is bound to the enzyme (or to a co-purifying *E. coli* derived prenyltransferase). The passive binding of either FDP, or α-farnesene, to the enzyme, was confirmed using enzyme assays (Fig. 5) in the absence of added substrate. Addition of d_8-GDP, both at the beginning and again at the end of the time course experiment showed that there was no loss of enzyme activity over time. Release of α-farnesene from the enzyme, in the absence of substrates, peaked within the first 4 h then declined over 19 h (Fig. 5).

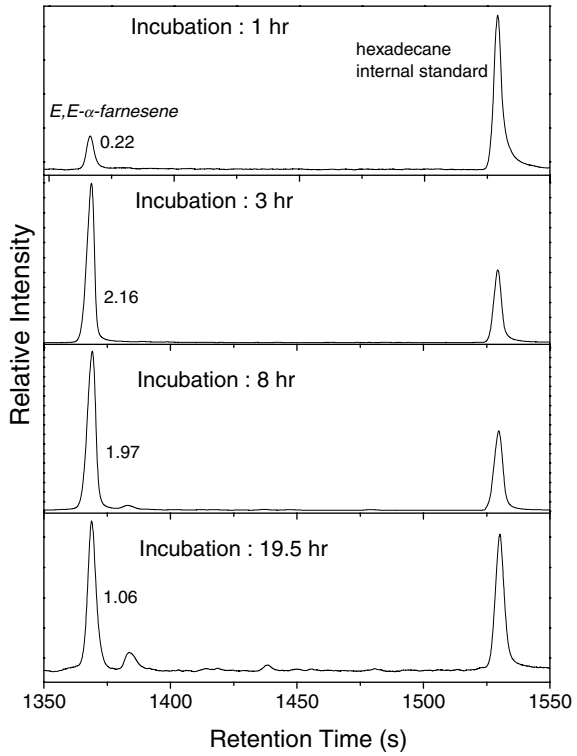

Fig. 5. Release with time of α-farnesene from purified α-farnesene synthase under optimised assay conditions in the absence of precursor. Enzyme was present at 80 μg ml^{-1}. Hexadecane standard was added to each sample prior to SPME headspace sampling at each time point. Panes show GCMS peaks for α-farnesene (*m*/*z* 93) and hexadecane standard (*m*/*z* 226) and ratios of peak areas at four time points during a 19.5 h experiment. Addition of GDP to samples at the beginning and end of the experiment demonstrated that the enzyme retained activity throughout the experiment.

2.5. Mutagenesis of the active site abolishes terpene synthase and prenyl transferase activities

To confirm that the one enzyme contained both terpene synthase and prenyltransferase activities, directed mutagenesis of the enzyme's active site was undertaken. Activity in terpene synthases is associated with a conserved DDXXD motif required for metal ion binding (Köllner et al., 2006; Whittington et al., 2002). The first aspartate residue of this motif has also been implicated in FDP synthase activity (Marrero et al., 1992). Mutations were targeted to convert either the first aspartate, or both the first and the last aspartate residues, of the DDVYD motif to alanines culminating in the D326A and D326A/D330A mutants. As predicted, α-farnesene synthase, monoterpene synthase and prenyltransferase activities were all lost in the mutants. This was demonstrated both by the failure of the mutant enzymes to convert tritiated FDP and GDP to pentane soluble products (Fig. 6), and by SPME headspace GCMS analysis following incubation with FDP or GDP (data not shown), and GDP and IDP (Fig. 7), respectively. The absence of farnesol following acid hydrolysis indicated no free FDP had been formed.

Interestingly, although recent evidence indicates the role of first aspartate residue of the DDXXD motif in Mg^{2+} binding may not always be essential for terpene synthase function (Prosser et al., 2004), our results show that, for the apple enzyme at least, this first aspartate residue is crucial for terpene production.

To confirm that prenyltransferase activity was intrinsic to the α-farnesene synthase and not due to a contaminating bacterial prenyltransferase, α-farnesene synthase mutants were analysed by ESI-LCMS/MS for the accumulation of FDP after incubation with GDP and IDP. No FDP was detected (Fig. 8). Assuming a specific prenyltransferase activity of 8 nmol of FDP formed mg^{-1} protein hour^{-1} (0.5% of the specific activity for α-farnesene synthase) some 0.8 nmol of FDP should have been produced in the assay. Analysis of an assay mixture spiked with 0.08 nmol FDP

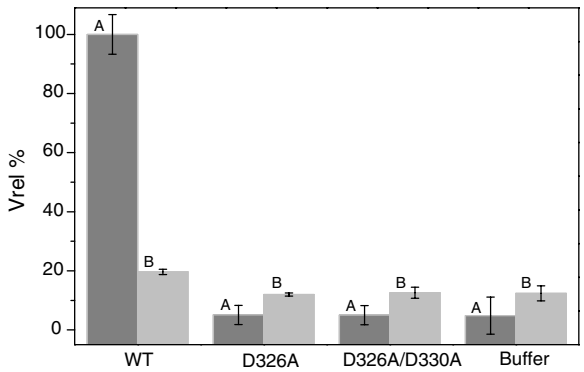

Fig. 6. Relative activities of non-mutated (WT) and mutant α-farnesene synthases in synthesis of sesqui (a) and mono terpenes (b) measured using ^{3}H-FDP and ^{3}H-GDP, respectively, in a solvent extraction assay. Differences in buffer activities are due to difference in specific activities of the tritiated FDP and GDP precursors. α-Farnesene synthase (WT) V_{rel} 100% = 1.6 μmol h^{-1} mg^{-1}.

gave a clearly detectable signal (Fig. 8d) and from this we concluded that no significant concentration of free FDP was produced by the mutant enzymes. Together these results confirm that the DDXXD motif is essential for the monoterpene synthase, sesquiterpene synthase and prenyltransferase activities of the apple α-farnesene synthase and that both terpene synthase and prenyltransferase activities are associated with the same active site.

2.6. Apple α-farnesene synthase gene is phylogenetically most similar to angiosperm isoprene synthases

Comparison of various α-farnesene and isoprene synthases, and mono- and sesquiterpene synthases, from gymnosperms and angiosperms (based on the phylogenetic analysis by Martin et al. (2004)), showed that they tend to be only 30–39% identical (48–54% similar) to apple α-farnesene synthase. Phylogenetic analysis (Fig. 9) indicated that both the apple and pear α-farnesene genes cluster in a well-supported manner with poplar and Kudzu isoprene synthases whereas the cucumber α-farnesene synthase clusters with other sesquiterpene synthases and the two pine α-farnesene synthases cluster with pine monoterpene synthases. The clustering arrangement of the apple enzyme remained the same when both the N-terminus and the C-terminus of the protein (C-terminus starting at residue D242) were compared as fragments, indicating that the active site region did not bias the relationships (data not shown).

3. Conclusions

Apple α-farnesene synthase shows a number of previously unreported features for a sesquiterpene synthase: the enhancement of sesquiterpene synthase activity by K^+, the conversion of isomeric forms of FDP to both α- and β-farnesenes, and the ability to use GDP plus IDP, in addition to FDP, to produce α-farnesene.

Monoterpene synthases from gymnosperms require K^+ for activity whereas those from angiosperms have shown either no effect or some enhancement of activity (Savage et al., 1994). The finding that K^+ causes a 5-fold enhancement of a sesquiterpene synthase activity is, to our knowledge, new. In the apple enzyme, rate enhancement was

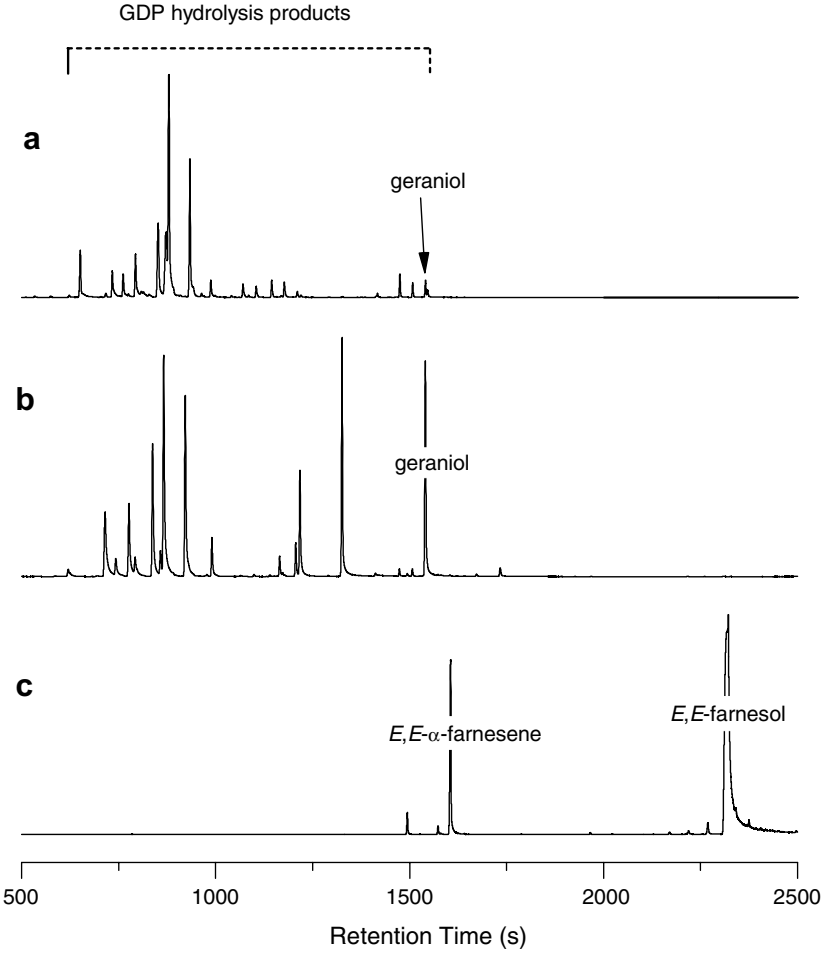

Fig. 7. Selected ion (*m*/*z* 93) SPME headspace GCMS profiles showing loss of prenyltransferase activity in mutant α-farnesene synthases following acid hydrolysis after GDP plus IDP feeding. Profiles are for: (a) double D326D/D330A mutant and (b) single D326A mutant α-farnesene synthases, and C α-farnesene and farnesol standards.

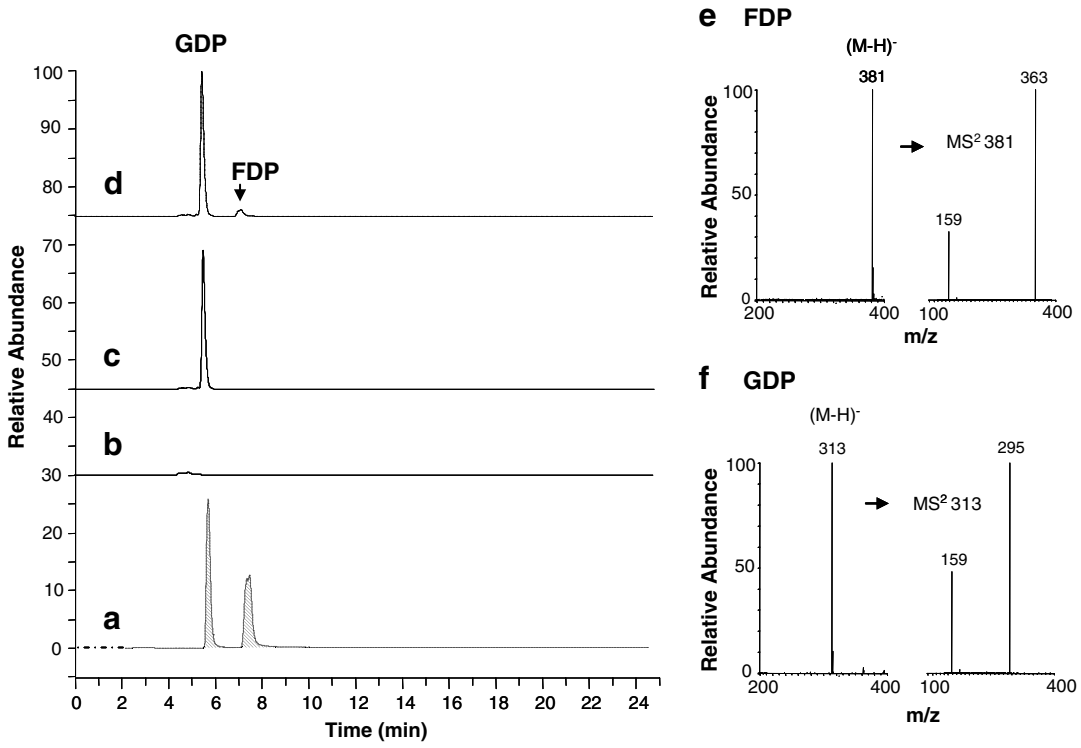

Fig. 8. LC-ESI-MS/MS analysis for farnesyl diphosphate (FDP) produced during the conversion of GDP and IDP to α-farnesene by apple α-farnesene synthases using selective reaction monitoring (SRM) in negative ion mode. SRM plots for GDP and FDP were generated by monitoring the distinctive daughter ions formed by fragmenting the precursor ions $(M-H)^-$ for both target compounds (GDP: SRM m/z 313 > m/z 295, 159 and FDP: SRM m/z 381 > m/z 363, 159). (a) Standard of GDP (0.6 nmol) and FDP (0.5 nmol), (b) D326A only, (c) D326A + GDP/IDP, (d) D326A + GDP/IDP spiked with 0.08 nmol of FDP, (e) fullscan and MS2 data for FDP and (f) fullscan and MS2 data for GDP.

associated only with sesquiterpene synthase activity and not monoterpene or prenyltransferase activities. As assays for sesquiterpene synthase enzymes in the literature generally do not generally include K^+ (Greenhagen et al., 2006; Mercke et al., 1999; Prosser et al., 2002) it will be of interest to see if this effect is unique for the apple enzyme or more widespread.

Unlike maize sesquiterpene synthases (Köllner et al., 2004) the apple α-farnesene synthase does not confer isomeric specificity. Instead various geometric isomers of both α- and β-farnesene are synthesised dependent on the isomeric form of the FDP substrate. Both (*E*,*E*)- and (*Z*,*E*)-FDP are substrates for the maize sesquiterpene synthase TPS4 and, in conjunction with site directed mutagenesis, have been used to probe the mechanism of this enzyme (Köllner et al., 2006). It seems likely that previous reports of isomer production for α-farnesene synthases (Mercke et al., 2004; Pechous and Whitaker, 2004) reflect the isomeric composition of the FDP precursors. Similarly in vivo production of α-farnesene isomers may also reflect the in vivo availability of the various FDP isomers.

Particularly intriguing is the ability of the apple α-farnesene synthase to act as a prenyltransferase producing α-farnesene directly from GDP and IDP. We are unaware of any other similar report of such an activity in a sesquiterpene synthase and these results suggest that crystallisation and further mutagenesis studies should provide valuable insight into the active site topology involved. The isoprene synthase (Miller et al., 2001) that most closely resembles the apple enzyme has been shown to produce not only isoprene from DMAPP, but also the monoterpene limonene from GDP, again demonstrating some flexibility both in the binding and processing of allylic diphosphate substrates. Unfortunately many studies with recombinant genes have not included detailed kinetic analysis, so it is difficult to find any further common features between the different enzymes. From the phylogenetic associations shown here and by others (Martin and Bohlmann, 2004), and from characteristics of the recombinant enzyme, it is probable that the apple α-farnesene synthase gene has evolved differently from other angiosperm sesquiterpene synthase genes, including the cucumber α-farnesene synthase. The observed bifurcation from "sesquiterpene synthase-like" enzymes was further demonstrated from in silico analysis using SWISS_PROT modeller; this resulted in the selection of the monoterpene bornyl-diphosphate synthase (Whittington et al., 2002) as the preferred structure rather than the sesquiterpene *epi*-aristolochene synthase (Starks et al., 1997). The functional commonalities between the apple α-farnesene synthase the isoprene synthases, and prenyltransferases taken together with the phylogenetic associations offer intriguing clues to a possible common ancestral origin. More categorical assertions

regarding this are not possible until significant new terpene synthase structural data are available.

Over 20 years ago, evidence was presented, using a liver farnesyl diphosphate synthase (Davisson et al., 1985; Saito and Rilling, 1981), that a prenyltransferase could, with suitable substrates, carry out a cyclising reaction producing monoterpene like products. Our work now shows that the converse can also be true – the apple α-farnesene synthase is capable of making α-farnesene with either FDP alone or using GDP and IDP together in a prenyltransferase reaction. How widespread this activity is remains to be discovered.

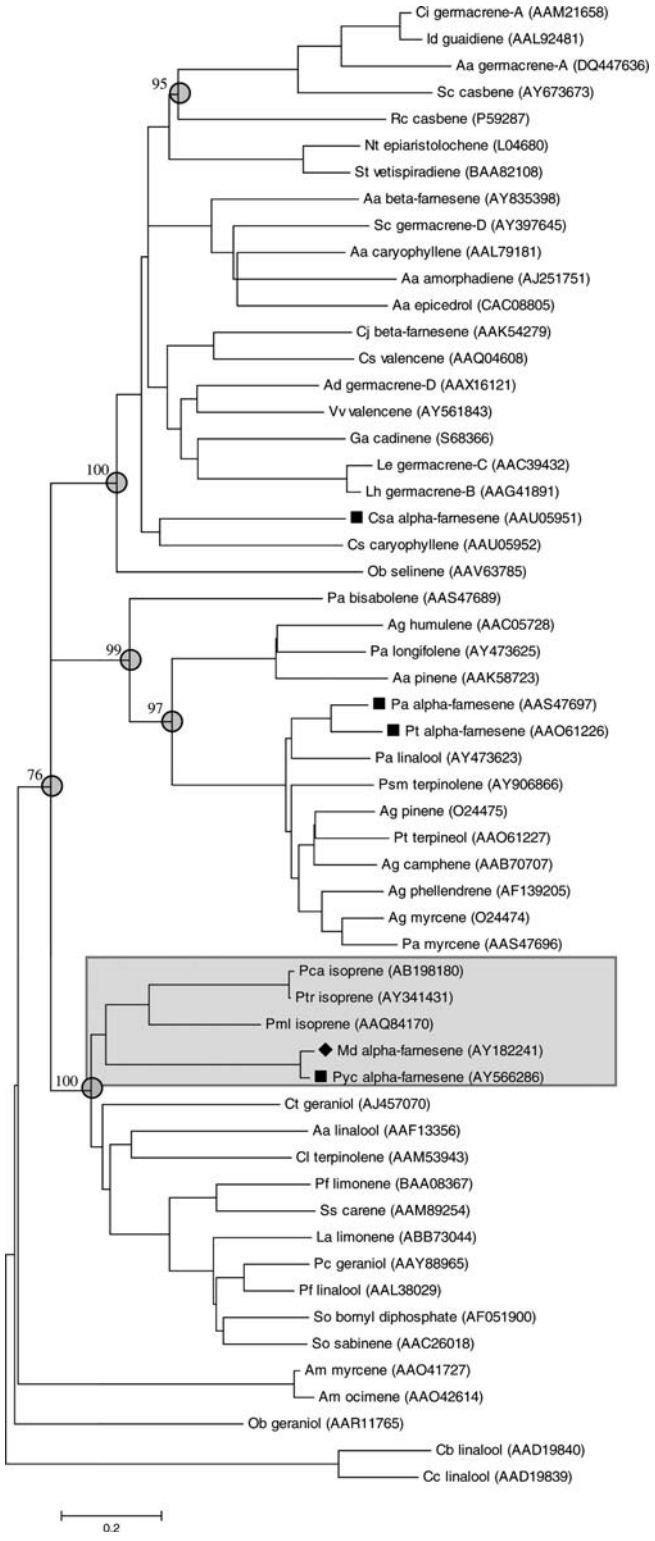

Fig. 9. Unrooted phylogenetic tree of full-length terpene synthase protein sequences. Selected bootstrap values are shown as a percentage of 1000 replicates. Apple α-farnesene synthase is indicated by a black diamond (◆) and additional α-farnesene synthases are indicated by black squares (■). Highlighted region shows clustered isoprene synthases and apple and pear α-farnesene synthases. Species abbreviations used are: Aa, *Artemisia annua*; Ad, *Actinidia deliciosa*; Ag, *Abies grandis*; Am, *Antirrhinum majus*; Cb, *Clarkia breweri*; Cc, *Clarkia concinna*; Ci, *Cichorium intybus*; Cj, *Citrus junos*; Cs, *Citrus sinensis*; Csa, *Cucumis sativus*; Ct, *Cinnamomum tenuipilum*; Ga; *Gossypium arboretum*; Id, *Ixeris dentate*; La, *Lavandula angustifolia*; Le, *Lycopersicon esculentum*; Lh, *Lycopersicon hirsutum*; Md, *Malus domestica*; Nt, *Nicotiana tabacum*; Ob, *Ocimum basilicum*; Pa, *Picea abies*; Pc, *Perilla citriodora*; Pca, *Populus* × *canescens*; Pf, *Perilla frutescens*; Pml, *Pueraria montana* var. *lobata*; Psm, *Pseudotsuga menziesii*; Pt, *Pinus taeda*; Ptr, *Populus tremuloides*; Pyc, *Pyrus communis*; Rc, *Ricinus communis*; Sc, *Solidago Canadensis*; So, *Salvia officinalis*; Ss, Salvia *stenophylla*; St, *Solanum tuberosum*; Vv, *Vitis vinifera*.

4. Materials and methods

4.1. Cloning of apple α-farnesene synthase

EST libraries (Newcomb et al., 2006), constructed from a range of tissues of apple (*Malus* × *domestica* Borkh.), were screened for putative terpene synthases. A candidate α-farnesene synthase from the 'Royal Gala' cDNA library where fruit skin was sampled 150 days after full bloom (DAFB) was selected and fully double strand sequenced (Genbank Accession No. AY787633).

4.2. Expression and purification of recombinant terpene synthase proteins

Recombinant N-terminal His-tagged proteins were expressed from pET-30a plasmids in *E. coli* BL21-CodonPlus™-RIL cells. Cultures were grown in a ZYM-5052 autoinducible media (Studier, 2005) at 37 °C for 4 h at 300 rpm. The temperature was then lowered to 16 °C and incubation continued for a further 60 h. Cells were pelleted by centrifugation, resuspended in chilled binding buffer (5 mM imidazole, 500 mM NaCl, 5 mM dithiothreitol (DTT), 20 mM Tris–HCl pH 7.9) with added EDTA-free inhibitor cocktail tablets (Roche) and disrupted by two passes through an EmulsiFlex®-C15 high-pressure homogeniser (Avestin) (pressure setting 15,000–20,000 psi). Following centrifugation, the supernatant was filtered through a 0.45 μm filter (Amicon) and applied to a 5 ml His-Trap chelating HP column (Amersham-Pharmacia Biotech) charged with Ni^{2+} and equilibrated with binding buffer. Bound proteins were washed following the manufacturer's specifications and eluted using a

continuous 0–250 mM imidazole gradient at 2 ml min^{-1}. Chromatographic peaks corresponding to recombinant proteins were collected (7–10 ml total volume) and concentrated to 1 ml in 20 ml of 30,000 MWCO concentrators (Vivaspin). The presence of recombinant protein was confirmed by SDS–PAGE and/or activity analysis. The concentrate was then applied to a 1.6 times 40 cm G200 Superdex (prep. grade) gel filtration column pre-equilibrated with 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 5 mM DTT at a flow rate of 1 ml min^{-1}. Fractions possessing recombinant proteins were confirmed by SDS–PAGE (Fig. 1) and western analysis. Highest purity fractions were pooled, concentrated to 2 ml using 30,000 MWCO concentrators (to remove remaining lower Mr proteins), adjusted to 15% glycerol and stored at −80 °C. A pET-30a vector-only control was expressed and purified as above.

4.3. Electrophoresis and western analysis

Protein extracts were analysed by SDS–PAGE, using 10% polyacrylamide gels. Protein bands were either visualised using a modified Neuhoff stain (Neuhoff et al., 1988) or transferred to an Immobilon-P PVDF membrane (Millipore) for western analysis with Anti-His_6 monoclonal antibodies (Roche).

4.4. Kinetic evaluation of recombinant α-farnesene synthase

Dimethylallyl diphosphate (DMAPP), isoprenyl diphosphate (IDP), geranyl diphosphate (GDP) and farnesyl diphosphate (FDP) were synthesised by phosphorylation of the corresponding alcohols (Keller and Thompson, 1993). Diphosphates, tritium-labelled on C-1, were synthesised by MnO_2 or Dess-Martin periodinane (Comeskey et al., 2004) oxidation of the appropriate alcohol to the aldehyde, then reduction (Croteau et al., 1994) with NaB^3H_4 (Amersham) prior to phosphorylation. Deuterium labelling on C-1 of the alcohol involved Jones oxidation to the corresponding acid, esterification to the methyl ester, followed by reduction with $LiAlD_4$ to the d_2-alcohol which was phosphorylated. Deuteration was >98% by ^1H NMR. Headspace trapping after acid hydrolysis of the diphosphates detected no contaminants of differing carbon number. d_8-Geranyl diphosphate (96% d_8 with the remaining 4% a mixture of d_7 and d_6-GDP) was synthesised from d_2-geraniol (Comeskey et al., 2006).

α-Farnesene synthase was added to an assay buffer containing 50 mM BisTrisPropane (BTP) (pH 7.5), 10% (v/v) glycerol, 5 mM DTT. Tritiated FDP precursor and metal ions were added, dependent on the experiment. Assays (500 μl) containing 250–500 ng of the recombinant protein were overlaid with 0.5 ml pentane and incubated for 1 h at 30 °C with gentle shaking. Following addition of 20 mM EDTA (final concentration) to stop enzymatic activity an aliquot of the pentane was removed for scintillation analysis.

Assays for determination of K_m values, pH and temperature optima, and sodium molybdate inhibition, using tritiated FDP, were carried out as follows: ^3H-FDP (10.1 MBq ml^{-1} or 2.04 MBq ml^{-1}) was added (concentration range 1–100 μM with saturating Mg^{2+}) to determine the Michaelis constant (K_m). Enzyme activity in the presence of Mg^{2+} (25 μM to 25 mM $MgCl_2$) and Mn^{2+} (1 μM to 1 mM $MnCl_2$) at a saturating FDP (25 μM) allowed determination of kinetic constants for each metal ion. The effect on enzyme activity of metal co-factors with and without salts was also tested. Mg^{2+} and Mn^{2+} were added in the presence and absence of 50 mM KCl and 50 mM NaCl in all possible combinations. The response to (concentration range 0.1–250 mM KCl) was determined in assays with saturating FDP and Mg^{2+}. Controls included incubation without enzyme, with enzyme but without metal ion cofactors and with enzyme and cofactors in the presence of 20 mM EDTA. The pET-30a control extract was assayed to determine potential utilisation of precursor by co-purifying bacterial proteins. Kinetic constants were calculated from DPM data by non-linear regression of the Michaelis–Menton equation using the Origin 5.0 (Microcal Software Inc. Northampton, MA) graphics package. For pH optima, activity was assayed in a tri-buffer system (Ellis and Morrison, 1982) at pH values between 5.85 and 9.15 with 10 mM $MgCl_2$, 25 μM FDP, 10% (v/v) glycerol, 5 mM DTT. Optimal temperatures for enzyme activity in the range 18–50 °C was established under optimal buffer conditions for farnesene production as was determination of specific activity which was carried out based on the methods of Greenhagen et al. (2006).

4.5. SPME analysis of volatile production by recombinant α-farnesene synthase

Purified recombinant enzyme (50 ng to 300 μg protein) was added to 5 ml of an optimised assay buffer (50 mM BTP pH 7.5, 7 mM $MgCl_2$, 50 mM KCl, 10% glycerol (v/v), 5 mM DTT). FDP, GDP, DMAPP or IDP (unlabelled or deuterated) was added at a final concentration of 25 μM FDP, 100 μM GDP or 50 μM IDP and DMAPP. Headspace volatiles were collected after addition of precursor with incubation times from 0 to 20 h at 30 °C and continuous agitation (110 rpm). Headspace sampling times using solid phase microextraction (SPME) were from 3 to 60 min. To quantify products during time course experiments, matching flasks were prepared with assay solution minus internal control and precursor. These were kept uncapped until sampling. At each time point, precursor and hexadecane (internal standard) were added to the enzyme assay just prior to trapping and the headspace sampled. The SPME fibres (65 μm PDMS/DVB, Supelco, Australia) were preconditioned for 45 min at 270 °C. Fibres were tested for volatile contaminants prior to use using GCMS. Before analysis by GCMS, the fibres were stored at ambient temperature in septum-sealed glass vials.

To verify the SPME results, headspace associated with the purified recombinant protein was also sampled by purge and trap methodology (Matich et al., 2003) or by layering pentane over the enzyme solution at the end of the assay as in Colby et al. (1998). In all cases results were as for SPME analysis.

4.5.1. GCMS analysis of headspace volatiles

Separation was effected using a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-5 MS capillary GC column (J&W Scientific, Folsom, USA) in an Agilent HP6890 GC coupled to a Pegasus III TOF mass spectrometer (Leco, St. Joseph, USA). GC temperature programmes were either 30 °C for 3 min, then 5 °C min^{-1} to 220 °C (hold 10 min), or 60 °C for 3 min, then 8 °C min^{-1} to 150 °C and then 3 °C min^{-1} to 205 °C. Mass spectra (*m/z* 33–320) were collected at an acquisition rate of 20 spectra s^{-1}. The transfer line was maintained at 240 °C. Volatiles were identified by comparison with NIST, Wiley and our own mass spectra libraries and confirmed by comparison of retention indices with those of authentic standards and the literature values (Davies, 1990). Sample peak area was measured relative to an internal hexadecane standard.

4.6. Site directed mutagenesis

The QuickChange II site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. The PCR-based mutagenesis protocol was performed using the pBluescriptSK (−) vector harbouring the α-farnesene synthase as template cDNA. For each mutation, the following forward and the reverse mutagenenic primers were used:

D326A:CAACTTAGTACTGATCATA**GCC**GACGTCTATG (fwd)
CATAGACGTC**GGC**TATGATCAGTACTAAGTTG (rev)
D326A/D330A:TACTGATCATA**GCC**GACGTCTAT**GCT**ATTTATGGCTCT (fwd)
AGAGCCATAAAT**AGC**ATAGACGTC**GGC**TATGATCAGTA (rev)

Mutagenized α-farnesene gene inserts were fully sequenced before expression. Mutated sequences were cloned into the pET-30a vector, expressed in *E. coli* and the mutated proteins purified as for the original α-farnesene synthase.

4.6.1. Analysis of mutant α-farnesene synthases for prenyltransferase activity

GCMS method: Aliquots of purified protein (100 μg) were incubated in optimised assay buffer with 50 μM GDP and 50 μM IDP in a total volume of 5 ml in 50 ml glass tubes at 30 °C. After 1 h, 6 M HCl (520 μl) was added and incubations were continued for a further 30 min to hydrolyse any FDP present to extractable farnesol. The headspace above the assays were sampled by SPME and analysed by GCMS for the presence of farnesol (Brodelius et al., 2002).

LCMS method: For direct measurement of FDP, incubations were set up as above. LCMS employed an LTQ linear ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA, USA) coupled to an Ettan™ MDLC (GE Healthcare Bio-Sciences). Separation of GDP and FDP was achieved using a Zorbax Extend-C18 column (Agilent, Palo Alto, CA, USA), 150 × 2.1 mm maintained at 40 °C. Solvents were (A) 25 mM ammonium bicarbonate and (B) methanol. The flow rate was 200 μl min^{-1}. The initial mobile phase, 20% B was ramped linearly to 100% D at 10 min, then held for 5 min. Retention times for GDP and FDP were 5.6 min and 7.2 min, respectively. MS data were acquired in the negative mode using a selective reaction monitoring (SRM) method with two segments: (1) 1–6.4 min, GDP: SRM *m/z* 313 > *m/z* 295, 159 and (2) 6.4–20 min, FDP: SRM *m/z* 381 > *m/z* 363, 159. This method monitors the distinctive daughter ions formed by fragmenting the precursor ions $(M-H)^-$ for GDP and FDP and maximises sensitivity by screening out any chemical noise from other compounds present. The ESI voltage, capillary temperature, sheath gas pressure, sweep gas and auxiliary gas were set at −9 V, 275 °C, 35 psi, 5 psi, and 0 psi, respectively.

4.7. Sequence analysis and tree construction

Multiple alignment analysis was done on selected full-length terpene synthase protein sequences obtained from GeneBank. Alignments were performed using ClustalX (Thompson et al., 1997) with a gap opening penalty of 15 and gap extension penalty of 0.3. Unrooted phylogenetic trees were generated from manually realigned sequences using MEGA3.1 software (Kumar et al., 2004) and constructed using the neighbour-joining method (Saitou and Nei, 1987). Validity testing was based on 1000 bootstrap replicates.

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