RESEARCH PAPER

Identification, functional characterization, and regulation of the enzyme responsible for floral (E)-nerolidol biosynthesis in kiwifruit (Actinidia chinensis)

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Abstract

Flowers of the kiwifruit species Actinidia chinensis produce a mixture of sesquiterpenes derived from farnesyl diphosphate (FDP) and monoterpenes derived from geranyl diphosphate (GDP). The tertiary sesquiterpene alcohol (E)-nerolidol was the major emitted volatile detected by headspace analysis. Contrastingly, in solvent extracts of the flowers, unusually high amounts of (E,E)-farnesol were observed, as well as lesser amounts of (E)-nerolidol, various farnesol and farnesal isomers, and linalool. Using a genomics-based approach, a single gene (AcNES1) was identified in an A. chinensis expressed sequence tag library that had significant homology to known floral terpene synthase enzymes. In vitro characterization of recombinant AcNES1 revealed it was an enzyme that could catalyse the conversion of FDP and GDP to the respective (E)-nerolidol and linalool terpene alcohols. Enantiomeric analysis of both AcNES1 products in vitro and floral terpenes in planta showed that (S)-(E)-nerolidol was the predominant enantiomer. Real-time PCR analysis indicated peak expression of AcNES1 correlated with peak (E)-nerolidol, but not linalool accumulation in flowers. This result, together with subcellular protein localization to the cytoplasm, indicated that AcNES1 was acting as a (S)-(E)-nerolidol synthase in A. chinensis flowers. The synthesis of high (E,E)-farnesol levels appears to compete for the available pool of FDP utilized by AcNES1 for sesquiterpene biosynthesis and hence strongly influences the accumulation and emission of (E)-nerolidol in A. chinensis flowers.

Key words: Actinidia, farnesol, floral volatiles, kiwifruit, nerolidol, terpene, terpene synthase.

Introduction

Terpenes (also referred to as terpenoids or isoprenoids) represent the most abundant group of plant volatiles and are common components of floral scent and herbivore-induced volatile mixtures (Pichersky and Gershenzon, 2002; Shearer and Hampton, 2005; Dudareva and Pichersky, 2006). The volatility of plant terpenes enables them to act as chemical messengers that are received by organisms at different trophic levels. Terpenes not only underlie the complex communication system that exists between plants, pollinators, herbivores, and their parasites and predators (Unsicker et al., 2009; Dicke and Baldwin, 2010), but ultimately serve to increase plant fitness (Harborne, 1991).

The diversity of terpene compounds in nature derives from the C5 compound isopentenyl diphosphate (IDP) and its allylic isomer dimethylallyl diphosphate (DMADP). Successive condensations of IDP with DMADP that proceed via the action of prenyltransferase enzymes, result in the formation of linear elongated prenyldiphosphates, including the C10 monoterpane precursor geranyl diphosphate (GDP), the C15 sesquiterpene precursor farnesyl diphosphate and the C20 diterpene precursor geranylgeranyl diphosphate (GGDP). In higher plants, the synthesis of IDP is compartmentally segregated (Lichtenhaler et al., 1997) with the cytosolic mevalonate (MVA) pathway...
produced by male and female Actinidia deliciosa volatiles (E) include those responsible for the predominant sesquiterpene (Nieuwenhuizen et al., 2009) and an (S)-linalool synthase, required for the production of floral lilac compounds in Actinidia arguta (Chen et al., 2010).

In this study, we describe the identification and functional characterization of the enzyme responsible for the production of (E)-nerolidol that constitutes the major floral volatile in the commercially important kiwifruit species Actinidia chinensis. We also provide evidence that the formation of the dephosphorylated FDP derivative (E,E)-farnesol influences the capacity of A. chinensis to synthesize floral sesquiterpenes.

Materials and methods

Chemicals and chemical synthesis

2-Methyl-but-3-en-2-ol, 3-methyl-but-2-enol, (E)-hex-2-en-1, rac-linalool, rac-(E,Z)-nerolidol, and (E,E)-farnesol were obtained from the Aldrich Chemical Company. (S)-Linalool and geranilylalnalo were obtained from Fluka. Geranial was from Acrs Organics and geranylgeraniol was obtained from the Sigma Chemical Company. (S)-(E)-nerolidol was obtained from neroli essential oil (Neroli, Citrus aurantium, Lotus Essential Oils, Auckland, New Zealand). The essential oil (0.25 ml) was partially purified by flash chromatography on silica gel (pentane:DCM:MeOH, 100:99:1) and (E)-nerolidol identified by GC-MS comparison with (E,Z)-nerolidol (Aldrich). Neroli contains exclusively (E)-nerolidol and predominantly (S)-(E)-nerolidol (Mondello et al., 2002). Enantioselective GC-MS of the partially purified neroli essential oil determined it to be 14:86 (R):(S)-(E)-nerolidol.

8-Hydroxylinalool was synthesized according to a previously described method (Kreck et al., 2002). Prenyldiphosphate substrates were synthesized by phosphorylation of the appropriate alcohols (Keller and Thompson, 1993), viz. DMADP from 3-methyl-but-2-enol, linalyl diphosphate (LDP) from rac-linalool, GDP from geraniol, (E,E)-FDP (FDP) from (E,E)-farnesol, and GGDP from geranylgeraniol. The isotopically-labelled substrates, [Cl,1-H]FDP and [Cl,2-H]GDP were made by phosphorylation of tritium-labelled farnesol and geraniol, respectively (Green et al., 2007). The labelled alcohols were synthesized by MnO2 or Dess-Martin periodinane (Comeskey et al., 2004) oxidation of the appropriate alcohol to the aldehyde, then reduction (Croteau et al., 1994) with NaBH4 (Amersham).

Headspace volatile trapping and solvent extractions

A. chinensis Planch. var. chinensis (‘Hort16A’) flowers were harvested from the Plant & Food Research Actinidia orchard in Te Puke, New Zealand. Headspace volatiles were collected on site from whole flowers according to Match et al. (2003) with minor modifications. Five fully opened flowers were harvested in triplicate every 4 h from noon (12:00 h on 18 October 2007) until 08:00 the following day. The harvested flowers were placed in 50 ml Quickfit tubes and the flower volatiles trapped for 3 h in direct thermal desorption tubes (ATAS GL International, Eindhoven, The Netherlands) packed with 80 mg Chromosorb absorbent (Shimadzu Co, Kyoto, Japan), using purified air at a flow rate of 25 ml min-1.

Solvent extractions were carried out in triplicate on flowers samples (5 g) harvested at the equivalent time points to the headspace sampling. Flowers were transferred to 50 ml Quickfit tubes and extracted twice with 10 ml pentane:Et3O (1:1 v/v) for 30 min with gentle shaking. The two extractions were combined and stored overnight at -20 °C. The following day the upper solvent layer was carefully removed from the lower frozen water layer using a glass pipette and reduced to 2 ml under a gentle stream of N2. The concentrated extract was then passed through a column of anhydrous MgSO4 to remove any remaining water.
Qualitative and semi-quantitative analysis of floral compounds

Headspace volatiles were desorbed directly from the thermal desorption tubes with a temperature ramp of 45–175 °C at 16 °C s⁻¹ and cryofocused on the front of the capillary column by a liquid nitrogen-cooled cryogenic trap at −110 °C. After cryofocusing, the trap temperature was ramped to 175 °C at 50 °C min⁻¹ (Optic 3 thermal desorption system, ATAS GL). A 15:1 split was employed while the volatiles were transferred into the capillary column at a column flow of 1 ml min⁻¹. The GC oven ramp was 35 °C for 2 min, 3 °C min⁻¹ to 60 °C, 5 °C min⁻¹ to 100 °C, 8 °C min⁻¹ to 170 °C, 10 °C min⁻¹ to 200 °C, and held for 13 min. GC separations were on a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-Wax (J & W Scientific, Folsom, CA, USA) capillary column in a HP6890 GC (Agilent Technologies) with helium as the carrier gas. The GC was coupled to a TOF-MS (Leco Pegasus III, St. Joseph, MI, USA). The ion source temperature was kept at 200 °C and ionization energy of 70 eV was used for electron impact ionization. The detector voltage was 1700 V, and ion spectra from 33 to 320 atomic mass units were collected with a data acquisition rate of 20 Hz. The total ion chromatograms were processed using the LECO ChromaTOF software.

Analysis of solvent extracted volatiles was carried out using the GC-MS system described above with the following modifications. Solvent extracts (1 μl) were injected into the GC with a 15:1 split and an initial port temperature of 200 °C. The GC oven ramp was 35 °C for 2 min, 3 °C min⁻¹ to 60 °C, 5 °C min⁻¹ to 100 °C, 8 °C min⁻¹ to 190 °C, 10 °C min⁻¹ to 230 °C, and held for 15 min. The transfer line temperature was 220 °C.

Terpenes were identified using the following reference compounds: α-pinene, linalool, 1,8-cineole, and β-myrcene (Aldrich), limonene (BDH), β-pinene (K & K Laboratories), and sabinen (Phompenex). Other compounds for which we did not have authentic standards (Tables 1 and 2) were identified by the ChromTOF software (version 2.3, Pegasus, Leco Australia) using the National Institute of Standards and Technology (NIST, version 2.0d, 2005) mass-spectral database, in combination with comparing the retention indices with those of a series of straight-chain hydrocarbon standards (C₈–C₂₃, 0.005 μl⁻¹ for each hydrocarbon). Peaks were selected and integrated manually using the molecular ion and/or specific diagnostic ions of each compound. The terpenes were quantified by measuring the m/z 93 peak areas against an average response factor for the m/z 93 ions of each compound. The terpenes were quantified by measuring their peak areas of 1,8-cineole (0.0366 μl⁻¹), linalool (0.0263 μl⁻¹), and caryophyllene (0.03 μl⁻¹), contained in an external standard. Calibration curves determined that the GC-MS system gave a linear response of the mass spectrometer over the concentrations of analytes in the different samples. An enantioselective GC-MS analysis of the linalool and (E)-nerolidol aglycones (data not shown) was performed on the Waters-Agilent GC-MS system and the β-Dec 325 GC column described above, using the oven temperature ramp employed for linalool.

Sequence analysis

Multiple amino acid sequence alignments of TPS genes were performed with Clustal X (Thompson et al., 1997), using default parameters, and were manually adjusted in GeneDoc (www.nrbsc.org/gfx/genedoc/). Prediction of the AcNES1 protein open reading frame (ORF) was carried out using the EMBOSS (http://www.ch.embnet.org/EMBOSS/index.html) translation tool Transq (Rice et al., 2000). The bioinformatics tools ChloroP (Emanuelsson et al., 1999) and TargetP (Emanuelsson et al., 2000) available at http://www.cbs.dtu.dk/services/ were used to predict the intracellular targeting of AcNES1. Evolutionary relationships for TPS enzymes were inferred using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965). Phylogenetic analyses were conducted in MEGA5.1 (Tamura et al., 2007). The TPS accession numbers are given in Supplementary Table S1, available at JXB online.

Production of AcNES1 protein

An AcNES1 fragment possessing 5' EcoRI and 3' XhoI endonuclease sites was amplified by PCR from 1978-bp AcNES1 cDNA (GenBank accession JN242243) and cloned directly into the pET-30a expression vector (Novagen) as an EcoRI-XhoI fragment. Details of the primers are given in Supplementary Table S2. Recombinant proteins were expressed from the pET-30a plasmid following transformation into BL21-CodonPlus-RIL cells (Stratagene). Cultures (500 ml) were grown in ZYM-5052 autoinduction media (Staudt, 2005) at 16 °C for 48–72 h at 300 rpm. Recombinant AcNES1 protein was extracted and purified according to previous methods (Green et al., 2007). Eluted recombinant proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and terpene synthease activity measured. Protein concentrations were determined by Experion (BioRad) automated electrophoresis analysis, according to the manufacturer’s instructions.

AcNES1 product analysis

AcNES1 solvent extraction assays for terpene product identification were carried out in 5 ml activity buffer (50 mM Bis-Tris propane (pH 7.5), 10 mM MgCl₂, and 5 mM DTT), in 50 ml glass tubes typically containing 50–100 μg purified recombinant AcNES1 protein and 20 μM FDP or GDP precursor. Assays were overlaid with 5 ml pentane:Et₂O (1:1 v/v) and incubated in a 30 °C water bath for 30 min. An additional 15 ml of solvent was added to each test tube and the tubes vortexed vigorously for 30 s to...
addition of [C1–3H1]-FDP (4.0 GBq mmol⁻¹) and 150 nmoles AcNES1 protein were initiated by the GC-MS analysis. The oven temperature ramp was 1 min at 35 °C, 5 °C min⁻¹ to 240 °C, and hold for 15 min. The retention time and mass spectrum agreed with those of the authentic compounds obtained commercially and synthesized in-house. Enantioselective GC-MS separations were performed on the above GC-MS system on a 30 m × 0.25 mm i.d. × 0.25 µm film thickness β-Dex 325 (Supelco) capillary column with a helium flow rate of 1 ml min⁻¹. For analysis of AcNES1 products in vitro (after 1 µl, 1 ml splitless injections), the oven temperature ramps were: 2-methyl-but-3-en-2-ol: 5 min at 28 °C and 5 °C min⁻¹ to 230 °C; linalool: 1 min at 35 °C, 5 °C min⁻¹ to 230 °C, and hold for 5 min; and (E)-nerolidol: 1 min at 50 °C, 3 °C min⁻¹ to 130 °C, 7 °C min⁻¹ to 230 °C, and hold for 10 min. For flower solvent extracts the oven temperature ramp for (E)-nerolidol and linalool was 1 min at 35 °C, 5 °C min⁻¹ to 230 °C and hold for 5 min.

Enzyme kinetic analysis

Quadruplicate assays (50 µl) containing 50 mM BIS-TRIS propane (pH 7.5) and 150 nmoles AcNES1 protein were initiated by the addition of [C1–3H1]-FDP (4.0 GBq mmol⁻¹) or [C1–3H1]-GDP (6.2 GBq mmol⁻¹) precursors and incubated for 20 s at room temperature. Reactions were stopped with the addition of three volumes of 0.1M KOH/0.2 M EDTA solution and labelled products extracted with 0.5 ml pentane:Et2O (1:1, v/v) and an aliquot taken for scintillation analysis. Enzymatic activity was determined by the amount in 20 mM MgCl₂ or 20 mM MnCl₂ in separate assays. Cofactor determinations for Mg²⁺ and Mn²⁺ were carried out in the presence of 10 µM [C1–3H1]-FDP. Control assays using boiled enzyme were used to determine background radioactive counts. Kinetic constants were calculated from Bq data by non-linear regression of the Michaelis–Menten equation using the Origin 7.5 (Microcal Software. Northampton, MA, USA) graphics package. Data were calculated from three independent experiments.

Alternative substrate analysis

Relative velocity data (V_rel) for AcNES1 was also determined using prenyl phosphates (50 µM) that were not tritium labelled, using semi-quantitative GC-MS. Assays were set up in the same manner as the 5 ml solvent extraction assay described above with the following exceptions: the duration of incubation was increased to 1 h and the enzyme concentration was decreased to 55 nmoles. For semi-quantitative GC-MS, sample peak areas were measured relative to authentic compounds (external standards) with tetradecane used as an internal standard in all samples to correct for day-to-day variation in the sensitivity of the GC-MS system. Enzyme activities were linear with time and enzyme concentration in all cases and were measured at least twice.

Real-time gene expression analysis

RNA was extracted from flowers, flower parts, fruit, and leaves according to Nieuwenhuizen et al. (2007) and treated with 10 U of DNaseI (Roche Applied Science, Mannheim, Germany) before cDNA synthesis. First-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s instructions and diluted 50-fold before use. Relative quantitation real-time gene expression analysis of targets and the housekeeping gene elongation factor 1α were performed (four technical replicates) on a LightCycler 480 platform using the LightCycler 480 SYBR Green master mix and results were analysed using the LightCycler 480 software (Roche). The analysis was carried out using the –ΔΔCt method according to Montefiori et al., (2011), enabling a comparison of the level of expression of multiple target genes normalized to a common reference gene considered stable and unchanging in the different samples. The thermal programme was 5 min at 95 °C and 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C, followed by melting curve analysis at 95 °C 5 s and 65 °C 60 s, and then ramping at 0.18 °C s⁻¹ to 95 °C. Primers for real-time gene expression analysis are listed in Supplementary Table S2.

Transient expression and subcellular targeting

In planta transient expression was carried out according to the methods of Nieuwenhuizen et al. (2009) utilizing the Agrobacterium tumefaciens strain GV3101 harbouring the pHEX2 binary vector (Hellens et al., 2005). For transient expression vector construction, AcNES1 was amplified by PCR using universal primers (Hellens et al., 2005) and cloned into the pHEX2 binary vector.

For the AcNES1 subcellular targeting analysis, an in-frame N-terminal green fluorescent protein (GFP) fusion was constructed according to the methods of Nieuwenhuizen et al. (2009). Primers for the AcNES1 PCR amplification step are listed in Supplementary Table S2. The amplified DNA was digested with BamHI and XbaI and cloned into the p326-SGFP vector (Lee et al., 2001), culminating in the p326-AcNES1/S GFP vector.

Results

Volatile emission and accumulation in A. chinensis flowers

The emission of floral terpenes by A. chinensis was investigated using a combination of dynamic headspace sampling and solvent extraction followed by GC-MS analysis. Volatile sampling, which was carried out over the course of a 24 h day/night cycle, revealed a mixture of monoterpenes, sesquiterpenes, and various terpene derivatives to be present in the headspace of the flowers (Table 1). (E)-farnesene emission was the next prevalent terpene compound, with an emission rate of 6.2 ng gFW⁻¹ h⁻¹, followed by melting curve analysis at 95 °C 5 s and 65 °C 60 s, and then ramping at 0.18 °C s⁻¹ to 95 °C. Primers for real-time gene expression analysis are listed in Supplementary Table S2.

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midnight onwards. Generally, the concentrations of the different monoterpenes (Supplementary Fig. S1) were low (< 3 ng gFW^{-1} h^{-1}) and showed little variation over the course of the day/night cycle, although cumulative amounts of monoterpenes and sesquiterpenes (predominantly (E)-nerolidol) were similar (Table 1).

Solvent extractions of flowers taken at the equivalent time points to the above headspace analysis were analysed for the presence of accumulated volatiles. In contrast to the emitted volatiles, the profile of accumulated volatiles was significantly less complex and primarily consisted of sesquiterpenes (Table 2). On a whole-flower fresh-weight basis, the dephosphorylated FDP derivative (E,E)-farnesal was by far the most prevalent compound occurring at ~18,700 ng gFW^{-1}, or ~72%, of the total extracted terpene volatiles. The accumulated terpenes, presumed to be produced by TPS enzymes, were dominated by linalool and (E)-nerolidol, with ~1500 and ~890 ng gFW^{-1} respectively. Time point analysis of these terpenes (Fig. 2) showed that (E,E)-farnesol and (E)-nerolidol were largely accumulated during the day, with peak concentrations occurring at noon. Notably, the highest concentrations of accumulated (E)-nerolidol corresponded to the lowest rate of emitted (E)-nerolidol and vice versa, while linalool accumulation remained relatively constant over the course of the day/night cycle.

Table 1. Actinidia chinensis flower semi-quantitative headspace terpene analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sesquiterpenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-Nerolidol</td>
<td>7.50</td>
<td>29.90</td>
</tr>
<tr>
<td>(E,E)-Farnesal</td>
<td>1.30</td>
<td>5.18</td>
</tr>
<tr>
<td>(E,E)-α-Farnesene</td>
<td>1.15</td>
<td>4.58</td>
</tr>
<tr>
<td>(Z,E)-Farnesal</td>
<td>0.93</td>
<td>3.71</td>
</tr>
<tr>
<td>(E,E)-Farnesol</td>
<td>0.26</td>
<td>1.04</td>
</tr>
<tr>
<td>(Z,E)-α-Farnesene</td>
<td>0.14</td>
<td>0.56</td>
</tr>
<tr>
<td>(Z,E)-Farnesol</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>11.31</td>
<td>45.1</td>
</tr>
<tr>
<td><strong>Monoterpenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-β-Ocimene</td>
<td>1.55</td>
<td>6.18</td>
</tr>
<tr>
<td>Linalool</td>
<td>1.46</td>
<td>5.82</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>1.40</td>
<td>5.58</td>
</tr>
<tr>
<td>Sabinene</td>
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</tr>
<tr>
<td>β-Pinene</td>
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</tr>
<tr>
<td>1,8-Cineole</td>
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<tr>
<td>(Z)-β-Ocimene</td>
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<tr>
<td>Limonene</td>
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</tr>
<tr>
<td>β-Myrcene</td>
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</tr>
<tr>
<td>α-Thujene</td>
<td>0.22</td>
<td>0.88</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>8.21</td>
<td>32.73</td>
</tr>
<tr>
<td><strong>Terpene metabolites</strong></td>
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</tr>
<tr>
<td>6-Methyl-5-hepten-2-one^{a}</td>
<td>4.18</td>
<td>16.62</td>
</tr>
<tr>
<td>(E)-Geranyl acetone^{a}</td>
<td>0.73</td>
<td>2.90</td>
</tr>
<tr>
<td>4,8-Dimethyl-1,3,7-nonatriene^{b}</td>
<td>0.63</td>
<td>2.51</td>
</tr>
<tr>
<td>(Z)-Geranyl acetone^{b}</td>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>5.56</td>
<td>22.17</td>
</tr>
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</table>

^{a} Oxidative breakdown product (E,E)-α-farnesene (Anet, 1972).

^{b} Oxidative breakdown products of nerolidol (Donath and Boland, 1996; Boland et al., 1996).

Table 2. Actinidia chinensis flower semi-quantitative solvent-extracted terpene analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sesquiterpenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E,E)-Farnesol</td>
<td>18700</td>
<td>72.4</td>
</tr>
<tr>
<td>(E,E)-Farnesal</td>
<td>1600</td>
<td>6.2</td>
</tr>
<tr>
<td>(Z,E)-Farnesol</td>
<td>1070</td>
<td>4.1</td>
</tr>
<tr>
<td>(E)-Nerolidol</td>
<td>890</td>
<td>3.4</td>
</tr>
<tr>
<td>(Z,E)-Farnesol</td>
<td>820</td>
<td>3.2</td>
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<tr>
<td>(E,Z)-Farnesol</td>
<td>580</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Monoterpenes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linalool</td>
<td>1500</td>
<td>5.8</td>
</tr>
<tr>
<td>Geraniol</td>
<td>400</td>
<td>1.5</td>
</tr>
<tr>
<td>Geranyl acetate</td>
<td>280</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Volatile glycosylation in A. chinensis flowers

Differences in the accumulation and emission patterns for (E)-nerolidol and (E,E)-farnesol in A. chinensis flowers suggested that these compounds might be sequestered in non-volatile glycosylated form for later release. Floral glycosides were extracted from A. chinensis and analysed by GC-MS after enzymatic hydrolysis (Fig. 3). This analysis showed that glycosylated forms of (E)-nerolidol, (E,E)-farnesol, and linalool were present in the flowers, as well as high levels of glycosylated 8-hydroxylinalool. Glycosylated (E)-nerolidol was detected at midnight, at ~290 ng gFW^{-1} (or ~44% of the total (E)-nerolidol pool), and increased to 340 ng gFW^{-1} at noon (although this level now only equated to ~12% of the total (E)-nerolidol pool). There was no glycosylated (E)-nerolidol detected following the period of peak (E)-nerolidol accumulation at noon (Fig. 2), suggesting that between noon and 16:00 h the sequestered (E)-nerolidol had been remobilized and released from the flower. The pattern of (E,E)-farnesol glycosylation (Fig. 3B) was similar to that of (E)-nerolidol, although it only comprised a minor component (~0.1–6%) of the total (E,E)-farnesol pool. Glycosylated linalool accounted for between ~2.5% and 6.5% of the total linalool pool with peak accumulation occurring between midnight and noon. Unexpectedly, 8-hydroxylinalool (Fig. 3C), was by far the most predominant A. chinensis glycosylated floral terpene, with levels declining from ~87% (~11,300 ng gFW^{-1}) of the total linalool pool at midnight to 62% (~2600 ng gFW^{-1}) at 20:00 h.
Identification of terpene synthase-like sequences from A. chinensis flowers

A homology-based gene mining approach was taken to identify putative TPS enzymes that could account for the production of the terpene compounds identified in A. chinensis flowers. A search of an Actinidia expressed sequence tag database (Crowhurst et al., 2008) identified a single contig from floral tissue with sequence homology to known floral TPS sequences (Supplementary Fig. S2). This transcript represented a full-length cDNA (termed AcNES1) of 1978 nucleotides encoding a predicted protein of 573 amino acids and ~65.4 kDa. AcNES1 was not predicted to contain a plastid-targeting peptide according to the ChloroP and TargetP prediction programmes. Phylogenetic analysis showed that AcNES1 clustered in a well-supported manner with the TPS enzymes in the TPS-g subgroup (Bohlmann et al., 1998; Dudareva et al., 2003) (Fig. 4). The TPS-g enzymes are

Fig. 1. Rates of sesquiterpene and homoterpene release from A. chinensis flowers during a day/night cycle. Terpene volatiles were trapped from triplicate flower samples by dynamic headspace sampling at 4-h intervals and analysed by GC-MS. Data are presented as mean ± SEM (n = 3). Means labelled without a common letter are significantly different (P < 0.05) based on LSD of one-way ANOVA.

Fig. 2. Rates of sesquiterpene and monoterpene accumulation in A. chinensis flowers during a day/night cycle. Terpene volatiles were solvent extracted at 4-h intervals over the course of 24 h. Data are presented as mean ± SEM (n = 3). Means labelled without a common letter are significantly different (P < 0.05) based on LSD of one-way ANOVA.
characterized by the absence of an N-terminal RRX₈W motif, which is reported to be essential for cyclic monoterpene production in the angiosperm TPS-b and gymnosperm TPS-d enzyme subgroups (Savage et al., 1994). AcNES1 was most similar to the Actinidia polygama and A. arguta linalool synthases (Chen et al., 2010), with ~78% amino acid identity.

Enzymatic properties of AcNES1

Purified recombinant AcNES1 protein was analysed for activity with GDP and FDP precursors in separate solvent extraction assays (Fig. 5). GC-MS analysis of the pentane/Et₂O extracted volatiles showed that AcNES1 could catalyse the conversion of both FDP and GDP precursors to the respective terpene alcohols (E)-nerolidol and linalool. The smaller amount of (Z)-nerolidol produced by AcNES1 was assumed to derive from (Z,E)-FDP contamination of the (E,E)-FDP used. Kinetic evaluation of recombinant AcNES1 enzyme (Table 3) showed that it had a higher binding affinity for FDP compared with GDP (Kₘ values of 0.80 and 1.89 μM respectively) and an approximate four-fold increase in catalytic efficiency (kₗ/kₘ) in the presence of FDP (300 s⁻¹ mM⁻¹) compared with GDP (69 s⁻¹ mM⁻¹). AcNES1 also showed an approximate 8-fold increase in its affinity for Mn²⁺ (Kₘ ≈ 14.2 μM compared with Mg²⁺ (Kₘ ≈ 117 μM) as the divalent metal cofactor. However, in the presence of Mn²⁺ AcNES1, activity was only ~22% of that observed with FDP and Mg²⁺ together and ~54% of that observed with GDP and Mg²⁺ together. The kinetics for substrate and divalent metal ion preference are similar to those observed for other TPS-g enzymes (Nagegowda et al., 2008; Chen et al., 2010).

Enantiomeric analysis of terpene compounds from AcNES1 and A. chinensis flowers

Enantioselective GC-MS analysis of (E)-nerolidol and linalool produced by AcNES1 determined that (S)-(E)-nerolidol and (S)-linalool (Fig. 6) were the predominant enantiomers. AcNES1 was also observed to produce (S)-linalool (Fig. 6F) from racemic LDP, while the small amount of (R)-linalool observed was assumed to have derived from LDP breakdown. This assumption was based on the fact that other racemic monoterpene products were seen in this analysis (data not shown). Equivalent analysis of an A. chinensis whole-flower extract (Fig. 6D) also showed that (S)-(E)-nerolidol was the predominant enantiomer and hence supports the assumption that AcNES1 is likely to be responsible for floral (S)-(E)-nerolidol biosynthesis in A. chinensis. In contrast to the (S)-linalool produced by AcNES1, the predominant linalool enantiomer in A. chinensis flowers, as previously shown by Matich et al. (2010) was (R)-linalool.

Alternative substrate usage by AcNES1

The sequence homology of AcNES1 to TPS-g nerolidol/linalool and linalool/nerolidol/geranyllinalool synthases from grape (Martin et al., 2010) provided a rational basis for testing additional prenyldiphosphate substrates ranging from C₅ to C₂₀. This analysis (summarized in Table 4)
showed that AcNES1 was able to catalyse the conversion of GGDP (C20) to the corresponding terpene alcohol geranyl-linalool at ~10% of the optimized rate for (E)-nerolidol from FDP but was unable to convert the C5 hemiterpene (isoprene) precursor DMADP to its corresponding alcohol. This in vitro plasticity for GDP, FDP and GGDP prenyldiphosphate usage in AcNES1 is mirrored in the activity of the three grape TPS-g sesqui-TPS enzymes (Martin et al., 2010) and again demonstrates an inherent capacity for TPS enzymes to evolve different product and substrate specificities.

**Gene expression analysis**

Relative gene expression analysis was carried out to investigate the spatial and temporal regulation patterns of AcNES1 expression in A. chinensis (Fig. 7). The highest AcNES1 expression was observed in the petals of fully open flowers, with lower expression in the stamens and pistils (Fig. 7A). AcNES1 expression was not observed in unopened flowers. Over the course of a day/night cycle, AcNES1 expression followed a weak diurnal rhythm with peak expression...
expression levels occurring between 20:00–0:00 h and 8:00–12:00 h (Fig. 7B).

Gene expression analysis was also carried out to determine how genes in the MVA pathway might modulate FDP flux and consequently sesquiterpene emission and/or accumulation in A. chinensis flowers (Fig. 8). The profiles of three family members encoding the key regulatory enzyme HMG-CoA reductase (HMGR) (Maurey et al., 1986; Gondet et al., 1992; Hemmerlin et al., 2003; Hey et al., 2006; Muñoz-Bertomeu et al., 2007) and the mevalonate-5-diphosphate decarboxylase gene (MDC) peaked in expression at 08:00 h. This peak in expression preceded maximal (E,E)-farnesol and (E)-nerolidol accumulation at noon (Fig. 2), and suggests a link between FDP substrate flux and sesquiterpene accumulation in A. chinensis flowers. Interestingly, the reduction of floral HMGR gene expression between 08:00 h and noon also coincided with increasing (E,E)-farnesol concentrations (Figs. 8 and 2, respectively). Expression analysis for the other MVA genes (Fig. 8) showed that peak expression for the 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), phosphomevalonate kinase (PMK), and isopentenyldiphosphate isomerase (IDPI) genes all occurred at 20:00 h.

Subcellular localization and in planta activity of AcNES1

The subcellular localization of AcNES1 was investigated using an AcNES1-GFP fusion protein in Arabidopsis protoplasts. Confocal laser scanning microscopy (Fig. 9) revealed a diffuse GFP fluorescence pattern for AcNES1-GFP protein distribution exclusively present in the cytosol. Similar fluorescence patterns were observed for a control GFP expression product, while no fluorescence was observed in untransfected protoplasts. These results are in agreement with the lack of a predicted AcNES1 plastid-targeting signal (ChloroP) and confirm that this enzyme is located in the cytosol.

A. tumefaciens-mediated transient expression (Hellens et al., 2005) in Nicotiana benthamiana leaves was used to confirm that AcNES1 acted as a (E)-nerolidol synthase in planta. N. benthamiana leaves infiltrated with a CaMV 35S-driven binary AcNES1 vector construct produced significant

Table 3. Kinetic profiles of recombinant AcNES1 protein

<table>
<thead>
<tr>
<th> </th>
<th>Km (μM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (s⁻¹ mM⁻¹)</th>
<th>Vrel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDP (Mg²⁺)</td>
<td>0.80 ± 0.26</td>
<td>0.24 ± 0.013</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺ (FDP)</td>
<td>117 ± 37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn²⁺ (FDP)</td>
<td>14.2 ± 2.70</td>
<td>0.053 ± 0.005</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>GDP (Mg²⁺)</td>
<td>1.89 ± 0.20</td>
<td>0.13 ± 0.002</td>
<td>69</td>
<td>54</td>
</tr>
<tr>
<td>GDP (Mn²⁺)</td>
<td>NA</td>
<td>0.03 ± 0.003</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. AcNES1 GC-MS analysis. (A) Pentane/Et₂O samples extracted from assays containing purified recombinant AcNES1 in the presence of FDP. (B) Authentic (Z)- and (E)-nerolidol standards. (C) Linalool produced by AcNES1 from GDP. (D) Authentic linalool standard. The butylhydroxytoluene contaminant seen in (A) is derived from the diethyl ether used in the terpene volatile extraction.
amounts of nerolidol (Fig. 10). (E)-nerolidol was not produced in control N. benthamiana leaves that had been infiltrated with buffer or a GUS-containing binary vector. Small amounts of linalool were also identified in both the control and AcNES1 infiltrated leaves and were therefore assumed to be derived from an endogenous linalool synthase.

Discussion

Terpene emission by A. chinensis flowers is dominated by the tertiary sesquiterpene alcohol (E)-nerolidol. (E)-nerolidol is a common volatile constituent of plants and nerolidol synthases have been characterized in a number of plant species including strawberry (Aharoni et al., 2004), grape (Martin et al., 2010), snapdragon (Nagegowda et al., 2008), and maize (Schnee et al., 2002). (E)-nerolidol is likely to play a role in attracting pollinators or seed dispersal agents and is also produced by plants in response to insect herbivory (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000; Danner et al., 2011). Our data shows that, in A. chinensis, (E)-nerolidol mostly accumulates during the day, while its release occurs mostly during the night (Figs. 1 and 2, respectively). This not only contrasts to the emission of (E)-nerolidol in snapdragon flowers (Dudareva et al., 2005) and (E,E)-farnesene and germacrene-D in A. deliciosa (Nieuwenhuizen et al., 2009), which occurs during the day, but also suggests a possible role in attracting night-time insect pollinators.

One feature that differentiates A. chinensis floral terpene metabolism from that of other kiwifruit species is the low rates of floral terpene emission. Peak (E)-nerolidol emission in A. chinensis is more than 3-fold lower than the equivalent (E,E)-α-farnesene emission in A. deliciosa (i.e. ~24 v. 76 ng gFW$^{-1}$ h$^{-1}$, respectively) (Nieuwenhuizen et al., 2009), while total monoterpene emissions in A. chinensis (~8.2 ng gFW$^{-1}$ h$^{-1}$) equate to only ~9% of the linalool emission in A. arguta (Chen et al., 2010). Although the level of

Table 4. Semi-quantitative GC-MS analysis of AcNES1 alternate substrate usage

<table>
<thead>
<tr>
<th>Product (%)</th>
<th>LDP</th>
<th>GDP</th>
<th>FDP</th>
<th>GGDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-Linalool</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)-Linalool</td>
<td>2.2</td>
<td>58.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E)-(E)-Nerolidol</td>
<td>100$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geranyl linalool</td>
<td>10.3</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

$^a$ 100% = $k_{cat}$ $\approx$ 0.127 s$^{-1}$.
Moreover, during the peak accumulation of (E)-nerolidol at noon and then during its steady decline from noon to 20:00 h, glycosylated (E)-nerolidol was not detected.

Interestingly, glycosylation is likely to be impacting on floral linalool emission in *A. chinensis*. Significant amounts of linalool are produced (Fig. 3C and D); however, the majority is either glycosylated directly or enzymatically converted to 8-hydroxylinalool and then glycosylated. The presence of 8-hydroxylinalool has also been observed as an unexpected consequence of over-expressing a *Clarkia* (S)-linalool synthase in tomato (Lewinsohn et al., 2001) and was put down to the presence of a hydroxylase able to act on (S)-linalool.

**The function of AcNES1 in *A. chinensis* flowers**

Subcellular protein localization can control terpene biosynthesis *in planta* by modulating terpene precursor accessibility. Although it is generally accepted that GDP and FDP biosynthesis are segregated to plastidial and cytosolic compartments, respectively, low concentrations of plastidial FDP and cytosolic GDP have been identified in *Arabidopsis*, tobacco, and potato (Aharoni et al., 2003, 2006; Wu et al., 2006). It is therefore possible that an enzyme that shows bifunctional behaviour *in vitro* can also exhibit the same behaviour *in planta*. This appears to be true for the bifunctional, cytosolically targeted FaNES1 enzyme in strawberry, which is reported to synthesize both (E)-nerolidol and linalool in cultivated strawberry fruit (Aharoni et al., 2004). However in *Antirrhinum majus* flowers, the bifunctional cytosolically-targeted AmNES/LIS1 enzyme has been shown to produce (E)-nerolidol exclusively from cytoplasmic FDP pools, whilst the chloroplast-targeted AmNES/LIS2 enzyme exclusively produces linalool from plastid (specifically leuoplasts) GDP pools (Nagegowda et al., 2008). The cytosolically targeted AcNES1 enzyme from *A. chinensis* flowers is also likely to act exclusively as a (E)-nerolidol synthase, as *N. benthamiana* leaves transiently over-expressing AcNES1 (Fig. 10) produced only (E)-nerolidol. Two additional results support the contention that AcNES1 acts as an (S)-(E)-nerolidol synthase and not a linalool synthase in *A. chinensis* flowers. The (S)-(E)-nerolidol enantiomer produced by AcNES1 matches the predominant floral (E)-nerolidol enantiomer (Fig. 6A and D, respectively) and AcNES1 produces (S)-linalool from GDP, not (R)-linalool as found in flowers.

**Regulation of terpene biosynthesis in *A. chinensis* flowers**

An unusual feature of *A. chinensis* flowers is the high amounts of (E,E)-farnesol that they accumulate (Table 2) compared with flowers from other species, including other kiwifruit (Matich et al., 2003; Crowhurst et al., 2008). It is also notable that the delicate floral tissue is not adversely affected by (E,E)-farnesol concentrations that can reach 42,600 ng gFW⁻¹ (or ~190 µM), considering that 25 µM is reported to be toxic to tobacco BY-2 cells (Hemmerlin and
Bach, 2000). The accumulation of \((E,E)\)-farnesol in *A. chinensis* flowers suggests that farnesyl pyrophosphatase activity (Nah et al., 2001) is elevated and/or that the ability to regenerate FDP through the successive phosphorylation of farnesol to farnesyl phosphate and farnesyl phosphate to FDP (Thai et al., 1999; Hemmerlin and Bach, 2000; Hemmerlin et al., 2006; Fitzpatrick et al., 2011) is decreased. However, the later explanation seems less

Fig. 8. Relative expression analysis of mevalonate (MVA) pathway genes in *A. chinensis* flowers. All genes were identified from an in-house *Actinidia* expressed sequence tag database collection (Crowhurst et al., 2008). The indicated enzymes are acetyl-CoA/acetyl-CoA C-acetyltransferase (AACT), 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), mevalonate-5-diphosphate decarboxylase (MDC), and isopentenyl diphosphate isomerase (IDPI). Expression levels of the MVA pathway genes were determined using the primers listed in Table S2. Primer efficiencies were between 1.94 and 2.0. MVA expression levels were compared to the reference gene *EF1a*, as indicated in Fig. 7. Data are presented as mean ± SEM \((n = 4)\). Means labelled without a common letter are significantly different \((P < 0.05)\).
plausible given that there is significant turnover of the accumulated farnesol occurring (Fig. 2). The uptake of farnesol by plant cells and incorporation into sterols, proteins, and ubiquinone is likely to account for most of this turnover (Thai et al., 1999; Hemmerlin and Bach, 2000; Hartmann and Bach, 2001), although 7–10% can be accounted for by the conversion of farnesol to farnesal (Supplementary Fig. S3) and up to ~6% is glycosylated (Fig. 3D). Although reasons for the high levels of (E,E)-farnesol accumulation remain to be determined, it is likely that the consequence of this accumulation will be a depleted pool of FDP available to AcNES1 and hence a diminished capacity for floral sesquiterpene production in A. chinensis.

Our work also shows that the regulation of FDP flux in A. chinensis flowers and hence sesquiterpene production is likely to be modulated by a number of different MVA pathway genes (Fig. 8). Specifically, the peak levels of HMGR RNA expression (Fig. 8) were found to correlate with the day-time accumulation profile of (E)-nerolidol and (E,E)-farnesol (Fig. 2). Moreover, the inverse correlation that exists between the day-time increases in (E,E)-farnesol accumulation and decreases in HMGR expression levels (Figs. 2 and 8 respectively) indicates that high (E,E)-farnesol concentrations may down-regulate floral HMGR expression and/or HMGR enzyme activity. While farnesol treatment has been shown to increase both HMGR mRNA and apparent enzyme activity levels in tobacco cells (Hemmerlin and Bach, 2000) it has also been shown to down-regulate HMGR enzyme activity in yeast as a consequence of induced structural changes following farnesol binding (Shearer and Hampton, 2005).

In this study we have identified a sesqui-TPS gene that accounts for (E)-nerolidol production in A. chinensis flowers. Moreover, we have provided evidence to suggest that elevated (E,E)-farnesol production in A. chinensis flowers reduces their capacity for sesquiterpene biosynthesis. In the future it will be interesting to establish the reasons why such high amounts of the MVA pathway carbon flux ends up as (E,E)-farnesol and how the enzymes involved in the dephosphorylation of FDP, its salvage from (E,E)-farnesol, and
subsequent reincorporation into primary metabolism are influencing the availability of a precursor crucial for secondary plant metabolic processes.

Supplementary material

Supplementary data are available at JXB online.  
Supplementary Fig. S1. Rates of monoterpene release from *A. chinensis* flowers during a day/night cycle.  
Supplementary Fig. S2. Sequence alignment of AcNES1 with TPS enzymes from fruit and flowers.  
Supplementary Fig. S3. *(E,E)*-farnesol and *(E,E)*-farnesal accumulation in *A. chinensis* flowers.  
Supplementary Table S1. Terpene synthase accession numbers.  
Supplementary Table S2. Primers.

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