Two terpene synthases are responsible for the major sesquiterpenes emitted from the flowers of kiwifruit (Actinidia deliciosa)

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Received 3 March 2009; Revised 15 April 2009; Accepted 20 April 2009

Abstract
Kiwifruit vines rely on bees for pollen transfer between spatially separated male and female individuals and require synchronized flowering to ensure pollination. Volatile terpene compounds, which are important cues for insect pollinator attraction, were studied by dynamic headspace sampling in the major green-fleshed kiwifruit (Actinidia deliciosa) cultivar ‘Hayward’ and its male pollinator ‘Chieftain’. Terpene volatile levels showed a profile dominated by the sesquiterpenes α-farnesene and germacrene D. These two compounds were emitted by all floral tissues and could be observed throughout the day, with lower levels at night. The monoterpene (E)-β-ocimene was also detected in flowers but was emitted predominantly during the day and only from petal tissue. Using a functional genomics approach, two terpene synthase (TPS) genes were isolated from a ‘Hayward’ petal EST library. Bacterial expression and transient in planta data combined with analysis by enantioselective gas chromatography revealed that one TPS produced primarily (E,E)-α-farnesene and small amounts of (E)-β-ocimene, whereas the second TPS produced primarily (+)-germacrene D. Subcellular localization using GFP fusions showed that both enzymes were localized in the cytoplasm, the site for sesquiterpene production. Real-time PCR analysis revealed that both TPS genes were expressed in the same tissues and at the same times as the corresponding floral volatiles. The results indicate that two genes can account for the major floral sesquiterpene volatiles observed in both male and female A. deliciosa flowers.

Key words: Actinidia, α-farnesene, floral volatiles, germacrene D, kiwifruit, ocimene, terpene, terpene synthases.

Introduction
Many important crop plants including alfalfa, canola, apple, cherry, and strawberry rely on insect pollination to ensure good seed or fruit yield (Klein et al., 2007). Insect pollination is particularly important for kiwifruit (Actinidia species) vines, as all Actinidia species are functionally dioecious, bearing either male flowers that contain stamens with inviable pollen or male flowers that lack a central pistil (Goodwin and Steven, 1993). Efficient pollination between spatially separated male and female individuals can be achieved by placing a high density of beehives in kiwifruit orchards during flowering and by planting male pollinator vines that flower simultaneously with female vines.

Volatile compounds are important cues for attracting pollinators such as insects, bats, and birds (Dobson, 2006), and have many other diverse roles in plant biology and ecology (Degenhardt, 2008; Pichersky and Gershenzon, 2002). A large portion of flower volatiles consists of mono- and sesquiterpene compounds, also known as the isoprenoids (Knudsen and Gershenzon, 2006). Apart from

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pollinator attraction, terpenoid compounds can also act as feeding deterrents to insects (Aharoni et al., 2003), molluscs (Frank et al., 2002), and mammals (Vourc’h et al., 2002) or exhibit toxicity to insects (Raffa et al., 1985), bacteria (Chorianopoulos et al., 2004), and fungi (Terzi et al., 2007). They also act through tritrophic interactions by promoting host location behaviour by parasitic insects when plant volatile release is triggered upon feeding by herbivores (Pare and Tumlinson, 1999; Pichersky and Gershenzon, 2002). Another role for terpene compounds is allelopathy against neighbouring plant species through various phytotoxic mechanisms (Nishida et al., 2005) or through indirect mechanisms such as modification of microbial composition of the rhizosphere (Weir et al., 2004).

Geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) represent the two principal substrates for mono- and sesquiterpenes, respectively. They are produced in plants by two compartmentally separated pathways (Lichtenthaler et al., 1997; Lange et al., 2000a) from isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) by the action of short chain prenyltransferases. In the plastid, the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion and Boronat, 2002) leads to the production of GPP, while in the cytoplasm, the mevalonate (MVA) pathway provides precursors for FPP formation. Alternatively, sesquiterpene precursors can be derived from the MEP pathway alone (Dudareva et al., 2005), or have a mixed origin (Adam et al., 1999) because of the existence of metabolic ‘crosstalk’ between the two IPP biosynthetic pathways (Laule et al., 2003; Schruer et al., 2003). GPP and FPP are then converted into terpenes by the action of terpene synthases (Tholl, 2006), producing the wide range of terpene skeletons found in nature. The primary terpene skeletons can subsequently be modified further by enzymes catalysing oxidation, hydroxylation, double bond reduction, acylation, glycosylation, or methylation, thus increasing terpenoid diversity as well as altering their volatility and olfactory properties (Lange et al., 2000b; Dudareva et al., 2004).

Despite the importance of insect pollination for fruit formation in kiwifruit, studies on floral volatiles produced by the Actinidia genus are limited. Analysis of volatiles emitted by flowers of four Actinidia species revealed that terpenoid fractions vary in their composition and constitute from 13% to 46% of total scent output (Crowhurst et al., 2008). Flowers of A. delicosa ‘Hayward’ (the most widely grown and economically important kiwifruit cultivar) produce as much as 30% of the sesquiterpene α-farnesene (Tatsuka et al., 1990). By contrast, a minute amount of α-farnesene was detected in the floral volatiles emitted from flowers of several A. arguta accessions, which emit a scent dominated by β-linalool and its derivatives, including lilac alcohols and lilac aldehydes (Matich et al., 2003).

To date, terpene synthases have been identified and characterized in many flowering species including Arabidopsis, Clarkia, snapdragon, and rose (Dudareva et al., 1996, 2003; Cseke et al., 1998; Guterman et al., 2002; Chen et al., 2003; Tholl et al., 2005; Nagegowda et al., 2008); however, little is known about the enzymes responsible for the production of the major terpene compounds in dioecious flowers. A detailed comparative analysis of the terpene volatiles produced in male and female kiwifruit flowers (A. delicosa ‘Hayward’) is described here, and the isolation and functional characterization of two terpene synthases responsible for the formation of major terpenoid compounds produced in these flowers is reported. This work links together ecology and plant physiology with terpene chemistry, biochemistry, and molecular biology for the first time in a dioecious plant species.

Materials and methods

Plant material and headspace volatile trapping

Actinidia delicosa Lindl. var. delicosa (A. Chev.) C.F. Liang et A.R. Ferguson female ‘Hayward’ and male ‘Chieftain’ vines were grown at the Plant and Food Research orchard in Te Puke, New Zealand. Flowers were harvested every 4 h from noon (22 November 2007) until 08.00 h the following day (23 November 2007). Headspace volatiles were collected on site from the whole flowers according to Matich et al. (2003) with minor modifications. Four fully-opened flowers were harvested for each time point, in triplicate and placed in 50 ml Quickfit™ tubes. Flower volatiles were trapped for 3 h, in direct thermal desorption (DTD) tubes (ATAS GL International, Eindhoven, The Netherlands) packed with 80 mg of 60–80 mesh Chromosorb™ 105 absorbent (Shimadzu Co. Ltd, Kyoto, Japan), using purified air at a flow rate of 25 ml min⁻¹. For volatile analysis of flower parts, whole flowers were harvested on the morning of 23 November 2007, stored on wet tissue paper, dissected at noon, and trapped for 20 h as per above, in duplicate. Identical samples of flowers and flower parts were also collected for RNA extraction, snap frozen in liquid nitrogen immediately after collection, and stored at −80 °C.

Headspace volatile analysis

Headspace volatiles were desorbed directly from the DTD tubes, using an Optic 3 thermal desorption system (ATAS GL), onto a 30 m×0.25 mm×0.25 μm film thickness DB-Wax (J&W Scientific, Folsom, CA, USA) capillary column in a HP6890 GC (Agilent Technologies, Santa Clara, CA, USA). Peaks were identified by time-of-flight mass spectrometry (TOF-MS, Leco Pegasus III, St Joseph, MI, USA). Thermal desorption from the DTD tubes was at 60 °C for 2 s, followed by 16 °C min⁻¹ to 175 °C. During desorption the volatiles were cryofocused on the GC column for 100 s, at −110 °C using cold nitrogen gas. The focused volatiles were then flushed down the column by heating the cryofocuser at 50 °C min⁻¹ to 175 °C, with 1 ml min⁻¹ He carrier gas. The GC oven temperature programme 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 hold for 13 min. The MS interface was at 210 °C and the ion source was at 200 °C. The detector voltage was 1700 V, the electron impact ionization potential was –70 eV, the acquisition rate was 20 spectra s$^{-1}$, and the mass range was 32 to 320 (m/z). The amount of each chemical was calculated as ng h$^{-1}$ g$^{-1}$ fresh weight with the use of an external standard containing the major floral terpenes. Only terpenes representing over 1% of the total terpene content are shown.

**Germacrene D cold on-column injections**

Cold on-column injections: GC–MS separations were carried out on an Agilent 6890N GC coupled to a Waters GCT TOF mass spectrometer. Chromatographic separations of 1 μl samples were on a 30 m×0.25 mm i.d. ×0.25 μm film thickness DB-5 (J&W Scientific) capillary column with a He flow of 1 ml min$^{-1}$. The GC was installed with a Gerstel CIS-4 PTV injection port equipped with a cold on-column injection port liner. A 15 cm length of 0.53 mm i.d. DB-5 capillary column was inserted into the on-column liner and attached to the analytical DB-5 column via a capillary column ‘MiniUnion’ (Phenomenex, Torrance, CA, USA). Thus, injections were directly into the on-column liner and attached to the analytical DB-5 column via a capillary column ‘Mini Union’ (Phenomenex, Torrance, CA, USA). Therefore, injections were directly into the on-column injection port liner, and without the 0.53 mm i.d. column liner and attached to the analytical DB-5 column. The injection port temperature was 320 °C, hold for 10 min, 3 °C min$^{-1}$ to 200 °C; 4 °C min$^{-1}$ to 155 °C, 10 °C min$^{-1}$ to 240 °C, and hold for 15 min. The oven temperature program was 1 min at 35 °C, 4 °C min$^{-1}$ to 155 °C, 10 °C min$^{-1}$ to 240 °C, and hold for 15 min.

α-Farnesene and (E)-β-ocimene injections: These analyses were performed upon the above GC–MS system, but with a standard splitless injection port liner instead of the cold on-column injection port liner, and without the 0.53 mm i.d. pre-column. The injection port was maintained at 240 °C and 1 min splitless injections were employed.

**Germacrene D enantioselective GC-MS analysis**

Enantioselective GC separations were carried out on a HP5890 GC (Agilent Technologies) coupled to a VG-70SE magnetic sector MS (VG-Micromass, Manchester, UK) with an electron impact ionization potential of 70 eV. A 1 min splitless injection of a 3 μl sample was made onto a 30 m×0.25 mm i.d. ×0.25 μm film thickness β-Dex™ 325 (Supelco Inc, Bellefonte, PA, USA) capillary column. The phase on this column was 25% 2,3-di-O-methyl-6-O-tert-butylidemethylsilyl-β-cyclodextrin in SPB™-20 (poly-(20% dimethylsiloxane/80% dimethylsiloxane)). The He head pressure was 52 kPa (7.5 psig), the injection port was at 220 °C, and the transfer line from the GC to the mass spectrometer was at 310 °C. The oven temperature programme was 1 min at 40 °C, increasing by 3 °C min$^{-1}$ to 100 °C, held for 10 min, 3 °C min$^{-1}$ to 180 °C, and held for 10 min. (+/-)-Germacrene D was obtained from goldenrod (Solidago canadensis) plants. One entire plant was cut off at ground level and placed in a 2.0 l jar. Air was drawn through at a rate of 80 ml min$^{-1}$ for 18 h and trapped on 200 mg of Tenax TA (Alltech, Grace, Deerfield, IL, USA) packed into 0.5 mm i.d. glass tubing. The trapped volatiles were eluted from the Tenax with 4 ml of Et$_2$O and the volume reduced to 1 ml under a gentle stream of N$_2$. Enantiomers were assigned according to Schmidt et al. (1999).

**Sequence identification and phylogenetic analysis**

An A. deliciosa ‘Hayward’ flower petal library containing 9950 ESTs (Crowhurst et al., 2008) was BLAST-searched (Altschul et al., 1990) for sequences with homology to known terpene synthases (expect value <exp$^{-9}$). A full-length cDNA of AdAFS1 was obtained by multiple rounds of overlapping 5′-RACE using ‘Hayward’ petal cDNA as a template according to the manufacturer’s instructions (5′-RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Invitrogen, Carlsbad, CA, USA). The final sequence obtained by 5′-RACE was confirmed by reamplification and cloning of a full-length AdAFS1 cDNA from petal cDNA. Full-length cDNA clones for AdAFS1 and AdGDS1 were fully double-strand sequenced.

Sequence alignments were constructed using ClustalX (Thompson et al., 1997) and visualized using GeneDoc v2.6 (Nicholas and Nicholas, 1997). Chloroplast signal peptide predictions were made using ChloroP (Emanuelsson et al., 1999).

**Real-time gene expression analysis**

For each sample, RNA from a mixture of four or more flowers, leaves, and flower parts was extracted according to Nieuwenhuizen et al. (2007) and treated with 10 U of DNaseI (Roche Applied Science, Mannheim, Germany) prior to 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 prior to use. Real-time gene expression analysis of AdGDS1, AdAFS1, and the housekeeping gene EF1α 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). Program: 5 min at 95 °C; 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C; followed by melting curve analysis: 95 °C 5 s, 65 °C 60 s then ramping at 0.18 °C s$^{-1}$ to 95 °C. The following primer pairs were used for real-time PCR analysis [product size in base pairs (bp) in parenthesis]: AdGDS1 (99 bp): 5′-AAGTTGCAATTAGTAAACCGC-GGTATA-3′ (forward) and 5′-TGACACCAATCA-AAGGCCCTTTT-3′ (reverse); AdAFS1 (90 bp): 5′-GAAGTCATGTTGGGAAACGTCAA-3′ (forward) and 5′-CATATCGGTTGGTCCATGTT-3′ (reverse) and EF1α (118 bp): 5′-GCACTGTCAATGGTATCCTCT-3′ (forward) and 5′-CCAGCTTAAAAACCACAGT-3′ (reverse).
Expression and purification of recombinant terpene synthase proteins

The complete open reading frame (ORF), including the stop codon for AdAFS1 and AdGDS1, was amplified by PCR using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen). Primers used for PCR amplification were for AdAFS1: 5′-GGATCCATGGGACCCCTATGTTTTT-3′ (forward) and for AdGDS1: 5′-GAATTCCAGGTACCCCTTGTTTCC-3′ (reverse), with the restriction sites underlined. The resulting PCR product was subcloned into the pGEM®-T Easy vector (Promega Madison, WI, USA) and sequence verified. In each case the coding region was cut out of the vector with the restriction enzymes introduced by the primers (BamHI+NotI or EcoRI+XhoI, respectively) and cloned into the final expression vector pET30a (Novagen, EMD Biosciences, Beeston, UK). The encoded N-terminal poly-His fusion proteins were expressed by autoinduction in E. coli BL21 (DE3) Codon Plus RIL cells (Stratagene, La Jolla, CA, USA) according to Studier (2005). Expressed culture pellets were disrupted by two passes through an EmulsiFlex®-C15 high-pressure homogenizer (Avestin Inc, Ottawa, Canada), cleared and applied to a 5 ml Ni²⁺ charged His-Trap chelating HP column (Amersham, GE Healthcare, Buckinghamshire, England) and eluted with a continuous 0–500 mM imidazole gradient according to Green et al. (2007). Relevant fractions were combined and further purified by gel filtration using a preparative grade G200 Superdex column (GE Healthcare).

Enzyme kinetics and product identification

GPP and FPP were synthesized by phosphorylation of the corresponding alcohols (Keller and Thompson, 1993). [Cl]⁻⁻Cl]-FPP (4.0 GBq mmol⁻¹) and [Cl]⁻⁻Cl]-GPP (6.2 GBq mmol⁻¹) were synthesized by MnO₂ or Dess-Martin periodinane (Comeskey et al., 2004) oxidation of the appropriate alcohol to its aldehyde, then reduction to the tritiated alcohol (Croteau et al., 1994) with NaBH₄ (Amersham) prior to phosphorylation (Green et al., 2007).

All kinetic determinations using [Cl⁻⁻Cl]-FPP were performed at room temperature in triplicate in 100 μl buffer containing 50 mM bis-tris propane (pH 7.5) and 25 mM (AdAFS1) or 100 mM (AdGDS1) enzyme. For kinetic determinations using [Cl⁻⁻Cl]-GPP (AdAFS1), the same buffer was used with 500 mM enzyme. Reactions were stopped after 1 min by adding 3 vols of stop buffer (0.1 M KOH and 0.2 M EDTA) and labelled products were extracted with 0.7 ml hexane and scintillation counted according to Green et al. (2009). For GPP and FPP kinetics, 25 mM MgCl₂ was included. Cofactor determinations for Mg²⁺ and Mn²⁺ were performed in the presence of 25 μM [Cl⁻⁻Cl]-FPP. Kinetic constants were calculated from Bq data by non-linear regression of the Michaelis–Menten equation using the Origin 7.5 (Microcal Software Inc. Northampton, MA, USA) graphics package. Data were calculated from three independent determinations.

For product identification 100 μg of recombinant enzyme was incubated at 30 °C with 50 μM FPP in a 5 ml reaction containing 20 mM MgCl₂ and 50 mM bis-tris propane pH 7.5 and incubated at 30 °C for 1 h with a 5 ml pentane:ether (1:1 v/v) overlay. Products were extracted after 1 h with 20 ml pentane:ether (1:1 v/v) and blown down under nitrogen gas prior to GC-MS analysis.

Transient expression of terpene synthases in N. benthamiana

Four to six-week-old greenhouse-grown seedlings were infiltrated in triplicate with Agrobacterium tumefaciens strain GV3101 harbouring the pHEX2 binary vector of choice according to the method of Hellens et al. (2005). Freshly-grown cultures were mixed 1:1 with A. tumefaciens GV3101 carrying the viral suppressor p19 (pBIN61 P19) (Voinnet et al., 2003). Agrobacterium cells were suspended in 10 ml infiltration media (10 mM MgCl₂, 10 μM acetyl-syringone) to an OD₆₀₀ of 5–8, incubated for 2 h at room temperature and injected into the three youngest N. benthamiana leaves >1 cm using a 1 ml syringe. After 10–14 d the leaves were detached and analysed for volatiles for 20 h as described above for headspace volatile trapping and analysis. Three plants were analysed for each construct.

Vector construction: AdAFS1 was amplified by PCR with primers AFF1 5′-CACCATAAGGTTTGTGGCA-TGGAGCCCTTATTGCTTTTT-3′ (forward) and AFR1 5′-GG-TGTCAAAAGGAGAACATAGACGCT-3′ and cloned into the p326-SGFP vector (Invitrogen) into the binary destination pENTR-gus (Invitrogen) entry vector. The resulting clone was recombined into the pHEX2 vector of choice according to the method of Hellens et al. (2005). AdGDS1 cDNA was cloned from the original EST clone by PCR using universal primers designed to the multiple cloning site region of pBluescript SK- according to Hellens et al. (2005) to create pHEX2-AdGDS1. pHEX2-GUS was created by recombining the pENTR-gus (Invitrogen) entry vector into the pHEX2 destination vector as described above.

Subcellular localization

N-terminal in-frame GFP fusions were made of AdAFS1 and AdGDS1 in the p326-SGF vector (Lee et al., 2001) which contains the CaMV 35S promoter and octopine synthase terminator (Hellens et al., 2005). AdGDS1 cDNA was cloned into the binary destination as described above. AdAFS1 was amplified by PCR with primers GDS2F 5′-CACAATAGGGTTTGTGGCA-TGGAGCCCTTATTGCTTTTTT-3′, bordering the CaMV 35S promoter and octopine synthase terminator. AdAFS1 was amplified by PCR with primers AFF1 5′-CACCATAAGGTTTGTGGCA-TGGAGCCCTTATTGCTTTTT-3′ (forward) and AFR1 5′-GG-TGTCAAAAGGAGAACATAGACGCT-3′ and cloned into the p326-SGFP vector (Invitrogen) entry vector. The resulting clone was recombined into the pHEX2 destination vector as described above.
in a separate PCR reaction with AFF3 5’-AATGGAGGCCCCCTATTGTATTTCATATT-3’ and AFR2. Both PCR reactions were purified, digested with BamHI, mixed together in equimolar amounts, heat denatured, and then re-annealed to generate the XbaI overhang. The resulting insert was cloned into the XbaI and BamHI sites of the p326-SGFP vector to create p326-AdAFS1/SGFP. Plasmids were sequence verified prior to transformation. DNA from each construct (15 μg) was used for PEG-mediated transformation of 100 μl of ice-cold protoplasts as described by Sheen (2002). Transient expression of GFP fusion proteins was observed 16–20 h after transformation as described in Nagegowda et al. (2008) using a MRC-1024 UV/VIS system (Bio-Rad Laboratories, Hercules, CA, USA) on a Diaphot 300 inverted microscope (Nikon, Tokyo, Japan) with a 60 x 1.4 numerical aperture lens. Fluorescence was collected with a 522/35 bandpass filter. Chlorophyll fluorescence was excited using the 568 nm line of the krypton–argon laser, and emission was collected with a 680DF32 filter.

Results

Volatile analysis of A. delicosa flowers

Volatile terpenes emitted by female A. delicosa ‘Hayward’ and male A. delicosa ‘Chieftain’ flowers and individual floral organs were sampled by dynamic headspace trapping and analysed by gas chromatography–mass spectrometry (GC–MS). Emission of terpenoids from female A. delicosa ‘Hayward’ flowers was about 1.3-fold higher than that from male A. delicosa ‘Chieftain’ flowers when calculated based on g−1 fresh weight (gFW) h−1 and taking into account the relative contribution of the individual flower organs (Fig. 1). When the flower mass was also considered (‘Hayward’ flowers are significantly larger and heavier than ‘Chieftain’; 2.96 gFW versus 1.2 gFW, respectively), then female flowers released approximately 3.3 times more terpenoids per hour. Both profiles were dominated by two sesquiterpenes, (E,E)-a-farnesene and germacrene D, whose levels varied in different flower organs from 18 to 76 ng gFW−1 h−1 and from 1.2 to 4 ng gFW−1 h−1, respectively. These two

Fig. 1. Rates of terpenoid release by different flower parts of Actinidia delicosa female ‘Hayward’ and male ‘Chieftain’ flowers. Parts from ‘Hayward’ (top) and ‘Chieftain’ (bottom) flowers were dissected and volatiles analysed by dynamic headspace trapping onto Chromosorb™ 105 absorbent. Measurements were made in duplicate ±SE and were converted to ng g FW−1 h−1. Average flower weights: ‘Hayward’: 2.96 g FW ±0.36 (SE): sepal 0.40 g, petal 1.60 g, stamen 0.26 g and pistil 0.70 g, ‘Chieftain’: 1.20 g FW ±0.05 (SE): sepal 0.32 g, petal 0.68 g, stamen 0.21 g. Photographs: scale bar 1 cm.
sesquiterpenes were found in all floral organs examined, with lower levels released from ‘Hayward’ pistils and ‘Chieftain’ stamens and sepals. Small amounts of the a-farnesene oxidation product 6-methyl-5-hepten-2-one (Anet, 1972) were also detected in all tissues as well as some (Z,E)-a-farnesene. In addition to sesquiterpenes, petals of both male and female flowers emitted the monoterpene (E)-β-ocimene, at levels which were much higher in female A. deliciosa ‘Hayward’ than in male A. deliciosa ‘Chieftain’ petals (Fig. 1).

The rate of terpene release from whole flowers was analysed under natural orchard conditions during a 24 h day–night cycle (Fig. 2). In flowers of both cultivars, emission of all terpenoid compounds occurred predominantly during the daytime. In ‘Hayward’ flowers, (E,E)-a-farnesene levels dropped from a peak of ~55 ng gFW⁻¹ h⁻¹ at mid-day to less than half that amount at 20:00 h. By contrast, the male ‘Chieftain’ flowers showed a sharp drop at the mid-day sampling followed by a delayed peak at 16:00 h. (Z,E)-a-farnesene accounted for about 1–4% of total a-farnesene emissions and showed similar fluctuation in emission levels to (E,E)-a-farnesene. Germacrene D levels were highest between 08:00 h and noon in both sexes, with ‘Chieftain’ producing higher peak levels (2.4 ng gFW⁻¹ h⁻¹) than ‘Hayward’ (0.5 ng gFW⁻¹ h⁻¹). (E)-β-ocimene was mainly produced during the daytime, with a maximum around noon, when the rates of production for both cultivars were at around 2.5 ng gFW⁻¹ h⁻¹. Interestingly, (E)-β-ocimene levels released from ‘Hayward’ petals (Fig. 1) were much higher (>10 fold) than from ‘Chieftain’ petals; however, these differences were not observed in intact flowers (Fig. 2). Germacrene D levels from ‘Hayward’ flower parts were also higher than those measured from intact flowers, suggesting that these

Fig. 2. Rates of terpenoid release during a day–night cycle by Actinidia deliciosa female ‘Hayward’ and male ‘Chieftain’ flowers. Major terpene volatiles were analysed by dynamic headspace trapping onto Chromosorb™ 105 absorbent at four-hourly time points. Data are presented as mean ± SE, n=3.
differences may be wounding-induced in the detached ‘Hayward’ petals, although this was not observed in ‘Chieftain’ petals.

Identification and cloning of AdGDS1 and AdAFS1 genes

An A. deliciosa expressed sequence tag (EST) library made from ‘Hayward’ flower petal cDNA (Crowhurst et al., 2008) was searched for sequences with homology to known terpene synthases. Two distinct classes of ESTs were identified. The first class contained 10 overlapping petal ESTs as part of a contig (data not shown). A single representative full-length cDNA clone was selected for full-length sequencing and designated as AdGDS1 for A. deliciosa germacrene D synthase 1 (see below; GenBank accession number AY789791). AdGDS1 encodes a protein of 565 amino acids (aa) (Fig. 3) with a calculated molecular mass of 65.2 kDa and a predicted pI of 5.52. The second class of kiwifruit terpene synthase identified from the petal-specific EST library consisted of a single partial cDNA clone. A full-length cDNA was obtained by multiple rounds of 5’-RACE and designated as AdAFS1 for A. deliciosa alpha farnesene synthase 1 (GenBank accession number FJ265785). AdAFS1 encodes a larger protein of 768 aa (Fig. 3) with a calculated molecular mass of 88.1 kDa and a pI of 5.93.

Although the level of amino acid sequence similarity between AdGDS1 and AdAFS1 proteins was relatively low (42%), both proteins contained characteristic sequence motifs of the TPS family, including the highly conserved DDXXD motif crucial for divalent cation (typically Mg\(^{2+}\) or Mn\(^{2+}\))-assisted substrate binding (Davis and Croteau, 2000). AdGDS1 also contains the conserved RXR motif, while AdAFS1 has an RXK variation positioned 35 amino acids upstream of the DDXXD (Fig. 3). This motif is thought to assist in directing the diphosphate anion away from the reactive carbocation after ionization (Davis and Croteau, 2000). AdGDS1 also contains the RRX\(_8\)W motif that is commonly found in cyclizing monoterpene synthases. Both AdGDS1 and AdAFS1 protein sequences lacked predicted N-terminal transit peptide-like sequences for chloroplast targeting.

Phylogenetic comparison (Fig. 4) showed that AdGDS1 belongs to the Tps-a subfamily of angiosperm sesquiterpene synthases (Bohlmann et al., 1998b). By contrast, AdAFS1 belongs to the Tps-f subfamily, previously consisting of exclusively linalool (monoterpene) synthases of the genus Clarkia (Cseke et al., 1998; Dudareva et al., 1996). All Tps-f subfamily proteins including AdAFS1 isolated in this study contain the conifer diterpene internal sequence (CDIS) domain (Bohlmann et al., 1998b) of 221 aa (Fig. 5), which is very rare among mono- and sesquiterpene synthases but is commonly found in diterpene synthases.

Expression analysis of AdAFS1 and AdGDS1 genes

The tissue-specific gene expression of AdAFS1 and AdGDS1 was determined by real-time PCR in ‘Hayward’ and ‘Chieftain’ leaves, flowers, flower buds, and floral tissues of...
open flowers (Fig. 6A, C). Expression of both *AdAFS1* and *AdGDS1* was significantly higher in flowers than in leaf tissue. Interestingly, while *AdAFS1* expression was similar in leaves of both cultivars and decreased with leaf expansion (Fig. 6A), the level of *AdGDS1* transcripts was about 30-fold higher in ‘Chieftain’ leaves, both young and expanded, than in ‘Hayward’ (Fig. 6C). Within floral tissues, expression of both genes was highest in petals and stamens. *AdAFS1* and *AdGDS1* expression was much lower in sepals in both sexes and also in ‘Hayward’ pistils. *AdAFS1* expression in ‘Hayward’ was co-ordinated with anthesis, with low levels of expression in unopened flower buds and high levels detected in open flowers (data not shown). However, no similar trend was found for the *AdGDS1* gene, which was already highly expressed pre-anthesis.

**Enzymatic properties of AdGDS1 and AdAFS1**

To determine the terpene products produced by *AdGDS1* and *AdAFS1*, recombinant proteins were heterologously expressed in *Escherichia coli* and purified by Ni²⁺ affinity chromatography followed by gel filtration. Analysis of the products formed by recombinant *AdGDS1* enzyme from FPP using cold on-column injection techniques showed the formation of only a single product, germacrene D (Fig. 7A). Initially, multiple products were observed using hot injection, most of which were the result of on-column thermal rearrangement and oxidation of the germacrene D (Match et al., 2008). The absolute configuration of the germacrene D product was determined by enantioselective GC-MS analysis. *AdGDS1* exclusively synthesized the (+)-configuration of germacrene D from FPP (Fig. 7B), as was shown by comparison with standards (see Fig. 7C, D) obtained from goldenrod (*Solidago canadensis*) which produces both (+) and (−)-germacrene D enantiomers (Schmidt et al., 1999). Only the (+)-germacrene D enantiomer is produced by ‘Hayward’ flowers (Fig. 7E). *AdGDS1* recombinant enzyme showed no activity when GPP was used as a substrate (data not shown). Overall, these data indicate that *AdGDS1* is a sesquiterpene synthase producing exclusively (+)-germacrene D in the presence of FPP.
AdAFS1 exclusively produced the sesquiterpene α-farnesene from FPP (Fig. 8A), as confirmed by comparison with a reference compound (Anet, 1970) obtained from ‘Granny Smith’ apples (Fig. 8B). The product consists primarily of (E,E)-α-farnesene (>95%) with trace amounts of (Z,E)-α-farnesene. When incubated with GPP, AdAFS1 also exhibited significant monoterpene synthase activity, producing exclusively (E)-β-ocimene (Fig. 8C), as confirmed by comparison with a reference compound produced by photolysis of α-pinene (Fig. 8D) (Kropp, 1969). These data indicate that AdAFS1 can function as both a sesqui- and a monoterpene synthase producing (E,E)-α-farnesene from FPP and (E)-β-ocimene from GPP.

Biochemical characterization of purified recombinant AdGDS1 and AdAFS1 proteins revealed that they require a divalent cation co-factor, Mg\(^{2+}\) or Mn\(^{2+}\) for their activities. Both enzymes showed a preference for Mg\(^{2+}\) in the presence of FPP with \(K_m\) of 0.25 and 0.3 mM, respectively (Table 1). They could also use Mn\(^{2+}\) for their activities. Although \(K_m\) values for Mn\(^{2+}\) were significantly lower than that with Mg\(^{2+}\) (Table 1), the maximal velocities were 43–50% of that with Mg\(^{2+}\). Higher levels of Mn\(^{2+}\) (>30 μM) inhibited sesquiterpene synthase activities of both enzymes (data not shown), an observation previously noted for other sesquiterpene synthases (Bohlmann et al., 1998a; Picaud et al., 2006; Tholl et al., 2005). In contrast to an apple α-farnesene synthase (Green et al., 2007), K\(^+\) had no effect on the activities of both enzymes.

Kinetic characterization of AdGDS1 and AdAFS1 revealed that both recombinant proteins have a high affinity towards FPP, with the corresponding apparent \(K_m\) values of 2.5 μM and 9.5 μM (Table 1), which are within the range of \(K_m\) values previously reported for sesquiterpene synthases (0.1–10 μM) (Cane, 1999). The sesquiterpene activities of both enzymes were inhibited by FPP concentrations >100 μM. The catalytic efficiency (\(k_{cat}/K_m\) ratio) of AdGDS1 with FPP was 7.6-fold lower than that of AdAFS1, despite its lower \(K_m\) values for FPP. The apparent \(K_m\) value of AdAFS1 for GPP was three times lower than that for FPP (2.8 μM versus 9.5 μM, respectively; Table 1); however, its catalytic efficiency was almost five times higher with FPP than GPP (Table 1).

**Transient expression of AdGDS1 and AdAFS1 in planta**

Transient *Agrobacterium tumefaciens*-mediated plant expression (Hellens et al., 2005) was used to investigate the terpene products produced by AdGDS1 and AdAFS1 in planta. Leaves were detached from *Nicotiana benthamiana* plants 10–14 d after *Agrobacterium* infiltration with binary vector constructs containing AdGDS1 and AdAFS1 under the control of the 35S promoter and emitted volatiles were
analysed by dynamic headspace sampling. Leaves infiltrated with the \textit{AdAFS1} construct produced a large amount of (\textit{E,E})-\textit{a}-farnesene, with smaller amounts of (\textit{Z,E})-\textit{a}-farnesene and (\textit{E})-\textit{b}-ocimene (Fig. 9A). No \textit{\alpha}-farnesene was observed in control leaves infiltrated with buffer or binary vector containing GUS. By contrast, a range of minor terpene products were found in leaves infiltrated with the \textit{AdGDS1} construct. However, the majority of these compounds were observed in leaves infiltrated with binary vector control or buffer-only. Germacrene D was the only product produced exclusively by leaves infiltrated with the \textit{AdGDS1} construct (Fig. 9B). A preceding peak was tentatively identified as \textit{\gamma}-muurolene, but this was also found in leaves infiltrated with control constructs. These results show that both AdGDS1 and AdAFS1 produced the same terpene products in planta as were found for the purified recombinant enzymes in vitro.

Subcellular localization of terpene synthases

Sesquiterpene synthesis is believed to take place in the cytosol, whilst monoterpene synthesis is believed to occur primarily in the plastids. The biochemical characterization of AdAFS1 and AdGDS1 revealed that AdAFS1 can function as a bifunctional enzyme possessing both sesquiterpene and monoterpene synthase activities, whereas AdGDS1 acts as a sesquiterpene synthase. To determine experimentally the exact subcellular localization of these proteins, transient expression of GFP fusion proteins was performed in \textit{Arabidopsis} protoplasts. The full-length coding regions were each fused to the N-terminus of the GFP reporter gene and the fusion constructs were then transferred into \textit{Arabidopsis} protoplasts, where corresponding transient GFP expression was analysed by confocal laser scanning microscopy (Fig. 10). GFP fluorescence for both AdAFS1 and AdGDS1 GFP fusions was observed as a diffused signal exclusively in the cytosol (Fig. 10A, B). Expression of a control GFP construct was also localized in the cytoplasm (Fig. 10C) and no GFP fluorescence was detected in untransfected protoplasts (Fig. 10D). These results confirm the predicted cytosolic localization of both enzymes, and are in agreement with a lack of plastid-targeting signal peptides predicted for these terpene synthase proteins.

Fig. 7. GC-MS analysis of the terpene products produced by AdGDS1 and ‘Hayward’ flowers. Purified AdGDS1 recombinant enzymes were obtained by Ni\textsuperscript{2+} affinity and gel filtration chromatography. The sesquiterpene products produced by AdGDS1 using FPP as substrate were analysed by GC-MS by cold on-column injection (A). The enantiomeric composition of germacrene D produced by recombinant AdGDS1 and found in ‘Hayward’ flowers was determined by enantioselective GC separation and analysis by mass spectrometry. (B) Germacrene D produced by AdGDS1 from FPP. (C) (+/-)-Germacrene D enantiomers isolated from goldenrod (\textit{Solidago canadensis}). (D) Co-injection of the enantiomers isolated from goldenrod with germacrene D produced by AdGDS1. (E) Germacrene D extracted from ‘Hayward’ flowers. (B–E) total ion current (TIC). (F) The fragmentation pattern of the germacrene D peak at 50:53 min isolated from ‘Hayward’ flowers shows the characteristic base peak at m/z 161 (M-C\textsubscript{3}H\textsubscript{7}), the molecular ion at m/z 204, m/z 133 (M- C\textsubscript{3}H\textsubscript{7}-C\textsubscript{2}H\textsubscript{4}), and the consecutive losses of CH\textsubscript{2} to produce m/z 119, 105, and 91.
In a kiwifruit orchard, efficient pollination is required to obtain good quality fruit and when bee pollination is prevented, fruit size is much reduced (Palmer-Jones and Clinch, 1974). Kiwifruit flowers are considered a poor source of nectar, so to obtain good bee pollination, high pollinator densities are required (Palmer-Jones and Clinch, 1974). Little is known about the cues that insects use for visiting kiwifruit flowers. In the orchard, bees appear to return to flowers of the same plant and same sex. Although the sterile pollen on the female plants offers much less in terms of nutritional value to the hive, bees show a preference for pistillate flowers (Goodwin and Steven, 1993), but this is balanced by the larger numbers of staminate flowers on a male vine (Goodwin and Steven, 1993). In China, where kiwifruit originated, *A. deliciosa* kiwifruit are pollinated by a large range of insects, mainly bees, including honey bees, carpenter bees, smaller native bees, and bumble bees, but other insects such as hoverflies have also been implicated (Steven, 1988).

In this paper it is shown that the flowers of both male and female *A. deliciosa* genotypes display a similar terpene profile (Fig. 1) dominated by the sesquiterpene *α*-farnesene, along with smaller amounts of germacrene D, (E)-β-ocimene, (*Z,E*)-α-farnesene and 6-methyl-5-hepten-2-one, an auto-oxidation product of *α*-farnesene (Anet, 1972). Female flowers were more scented and emitted 1.3 times more terpenoids than male flowers on a gFW/C0 basis. Previously, it has been shown that *α*-farnesene and germacrene D are involved in the attraction and conditioning of honeybees (Le Metayer et al., 1997) and other insects (Sutherland et al., 1977; Mozuraitis et al., 2002). Indeed, emission of these terpenoids, as well as (E)-β-ocimene over a light/dark cycle occurs during the daytime when potential pollinators are active (Fig. 2). Using a functional genomics approach, we have shown that the terpenes produced by *A. deliciosa* flowers are synthesized by two distinct enzyme activities, AdAFS1 and AdGDS1, that are highly specific for the production of these terpenes (Fig. 3).

**Table 1.** Enzyme kinetic properties of AdAFS1 and AdGDS1

|        | K<sub>m</sub> (μM) | V<sub>max</sub> (pkat mg<sup>-1</sup>) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (s<sup>-1</sup> mM<sup>-1</sup>) |
|--------|-------------------|-------------------------------|-----------------|--------------------------|
| **AdAFS1** |                   |                               |                 |                          |
| GPP (Mg<sup>2+</sup>) | 2.8±0.1 | 316±63 | 0.030±0.005 | 11±0.7 |
| FPP (Mg<sup>2+</sup>) | 9.5±2.3 | 4715±502 | 0.440±0.046 | 51±9.6 |
| Mg<sup>2+</sup> (FPP) | 248±11 | 100% | | |
| Mn<sup>2+</sup> (FPP) | ~20 | 50% | | |
| **AdGDS1** |                   |                               |                 |                          |
| FPP (Mg<sup>2+</sup>) | 2.5±0.3 | 236±8 | 0.017±0.001 | 6.7±0.5 |
| Mg<sup>2+</sup> (FPP) | 317±38 | 100% | | |
| Mn<sup>2+</sup> (FPP) | 17±0.9 | 43% | | |

**Discussion**

**Terpene formation and pollinator attraction in kiwifruit**

In a kiwifruit orchard, efficient pollination is required to obtain good quality fruit and when bee pollination is prevented, fruit size is much reduced (Palmer-Jones and Clinch, 1974). Kiwifruit flowers are considered a poor source of nectar, so to obtain good bee pollination, high pollinator densities are required (Palmer-Jones and Clinch, 1974). Little is known about the cues that insects use for visiting kiwifruit flowers. In the orchard, bees appear to return to flowers of the same plant and same sex. Although the sterile pollen on the female plants offers much less in terms of nutritional value to the hive, bees show a preference for pistillate flowers (Goodwin and Steven, 1993), but this is balanced by the larger numbers of staminate flowers on a male vine (Goodwin and Steven, 1993). In China, where kiwifruit originated, *A. deliciosa* kiwifruit are pollinated by a large range of insects, mainly bees, including honey bees, carpenter bees, smaller native bees, and bumble bees, but other insects such as hoverflies have also been implicated (Steven, 1988).

In this paper it is shown that the flowers of both male and female *A. deliciosa* genotypes display a similar terpene profile (Fig. 1) dominated by the sesquiterpene *α*-farnesene, along with smaller amounts of germacrene D, (E)-β-ocimene, (*Z,E*)-α-farnesene and 6-methyl-5-hepten-2-one, an auto-oxidation product of *α*-farnesene (Anet, 1972). Female flowers were more scented and emitted 1.3 times more terpenoids than male flowers on a gFW<sup>-1</sup> basis. Previously, it has been shown that *α*-farnesene and germacrene D are involved in the attraction and conditioning of honeybees (Le Metayer et al., 1997) and other insects (Sutherland et al., 1977; Mozuraitis et al., 2002). Indeed, emission of these terpenoids, as well as (E)-β-ocimene over a light/dark cycle occurs during the daytime when potential pollinators are active (Fig. 2). Using a functional genomics approach, we have shown that the terpenes produced by *A. deliciosa* flowers are synthesized by two distinct enzyme activities, AdAFS1 and AdGDS1, that are highly specific for the production of these terpenes (Fig. 3).
approach, two terpene synthases, AdAFS1 and AdGDS1, have been identified which can account for the production of the major sesquiterpene volatiles in both male and female *A. deliciosa* flowers. AdGDS1 uses only FPP as a substrate with the formation of germacrene D as the sole product (Fig. 7), while AdAFS1 is a bifunctional terpene synthase and catalyses the biosynthesis of α-farnesene (Fig. 8A, B) and (E)-β-ocimene (Fig. 8C, D), depending on the type of precursor used, FPP and GPP, respectively. mRNA transcripts for both genes were found predominantly in flower organs of ‘Hayward’ and ‘Chieftain’ kiwifruit cultivars (Fig. 6A, C) and their accumulation was positively correlated with the level of the corresponding terpenoids produced by the floral organ. Transient expression of AdAFS1 and AdGDS1 genes in *N. benthamiana* leaves resulted in the formation of α-farnesene and germacrene D, respectively, with minor amounts of the monoterpane (E)-β-ocimene in the case of AdAFS1, providing direct evidence that AdAFS1 and AdGDS1 are capable of producing these terpenes in planta.

(E,E)-α-farnesene, (E)-β-ocimene and (-)-germacrene D have been shown to be important floral scent components in other flowers including mistletoe, carob and oilseed rape (Le Metayer et al., 1997; Bungert et al., 2002; Custodio et al., 2006) but can also be produced in other tissues. All three volatile compounds are released locally and systemically from hybrid poplar leaves after caterpillar herbivory (Arimura et al., 2004), suggestive of a role in indirect defence. Both (E)-β-ocimene and (E,E)-α-farnesene are released in cucumber upon herbivory by spider mites as part of the volatile blend that attracts predators (Mercke et al., 2004). (E,E)-α-farnesene also contributes to the aroma properties of some fruits including apples (Pechous and Whitaker, 2004; Green et al., 2007) and melons (Portnoy et al., 2008). Several farnesene synthases have been isolated from different plant species and characterized. Kiwifruit AdAFS1 characterized in this study is similar to cucumber and apple α-farnesene synthases (Mercke et al., 2004; Pechous and Whitaker, 2004) in its ability to form (E,E)-α-farnesene from FPP and GPP, respectively. Although the apparent AdAFS1 *K_m* value for GPP was three times lower than that for FPP (∼2.8 versus ∼9.5 μM for GPP and FPP, respectively), its catalytic efficiency with FPP was five times higher than that with GPP (Table 1). In this respect, AdAFS1 is similar to many other sesquiterpene synthases, which are able to accept both GPP and FPP and that show higher sesquiterpene synthase activity relative to monoterpane synthase activity.

**Sequence characteristics of kiwifruit floral sesquiterpene synthases**

AdAFS1 was shown to encode an α-farnesene synthase and to be a new member of the Tps-f subgroup of terpene synthases which previously consisted only of *Clarkia* monoterpene synthases (Fig. 4). α-Farnesene synthases have previously been identified in three other TPS subgroups: Tps-a (e.g. *Cucumis melo*, Portnoy et al., 2008); Tps-b (e.g. apple, Pechous and Whitaker, 2004), Tps-d (e.g. *Pinus taeda*, Phillips et al., 2003), suggesting that this enzyme functionality has arisen multiple independent times in evolution. AdAFS1 and other members of the Tps-f subgroup are unique in that they contain a conserved CDIS domain near the N-terminus (Fig. 5) that is rare among mono- and sesquiterpene synthases but conserved among diterpene synthases. It is postulated that this structural element existed in a common diterpene synthase ancestor, but was lost during the evolution of most mono- and sesquiterpene synthases (Bohlmann et al., 1998b). Attempts to delete this domain from AdAFS1 using a series of

![Fig. 9. Transient expression of AdAFS1 and AdGDS1 in planta.](image-url)
progressive N-terminal deletions resulted in the production of insoluble inclusion bodies in *E. coli*, from which no active protein could be recovered (results not shown). This result, taken together with the N-terminal location of the CDIS domain distant from the terpene synthase catalytic domain, suggests its role is in enzymatic stability or folding rather than catalysis. It would be interesting to compare kinetic properties of α-farnesene synthases belonging to the different TPS subfamilies; however, to date this has not been possible, because of the absence of detailed biochemical characterization of α-farnesene synthases from different plant species.

*AdGDS1* was shown to encode a germacrene D synthase which clustered to the *Tps-a* subfamily (Bohlmann et al., 1998b). This subfamily consists of angiosperm sesquiterpene synthases and includes several other germacrene D synthases (GDS), some of which are involved in floral volatile production (e.g. in *Vitis vinifera*: Lucker et al., 2004; and

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**Fig. 10.** Subcellular localization of AdAFS1 and AdGDS1 in Arabidopsis leaf protoplasts. The full-length coding regions of AdAFS1 and AdGDS1 were fused to the GFP reporter gene in the vector p326S-GFP to produce the constructs p326-AdGDS1/SGFP and p326-AdAFS1/SGFP. The fusion constructs were then transferred into *Arabidopsis* protoplasts and analysed by confocal laser scanning microscopy for GFP expression. Chlorophyll autofluorescence detected in the red channel is shown in the ‘Red’ column; the fluorescence of GFP detected in the green channel is shown in the ‘Green’ column; the ‘Merged’ column shows combined red and green channels; the ‘Transmission’ column shows light-microscopy images of the intact protoplasts. (A) p326-AdGDS1/SGFP, in duplicate from two independent experiments; (B) p326-AdAFS1/SGFP, in duplicate; (C) control p326-SGFP construct (expressed in the cytosol), and (D) no DNA control. Diffuse GFP fluorescence localized outside the plastids is the same for the AdAFS1, AdGDS1 and p326-SGFP constructs, indicating cytosolic localization of the proteins. Scale bars: 25 μm.
Rosa hybrida: Guterman et al. (2002). Both kiwifruit and rose germacrene D synthases catalyse the formation of germacrene D as the sole product from FPP, in contrast to a grapevine multi-product germacrene D synthase, which produces germacrene D (92%) along with a second product δ-cadinene (8%) (Lucker et al., 2004). Analysis of the stereochemistry of germacrene D products revealed that the characterized germacrene D synthases exhibit enantiomer-specific synthesis [(+] or [−]). To date, only AdGDS1 and goldenrod (+)-germacrene D synthase (Prosser et al., 2004) are capable of exclusively (+)-germacrene D formation. In goldenrod, two different germacrene D synthase enzymes produce the germacrene D enantiomers (Schmidt et al., 1999) and several key amino acid residues are thought to be important for determining the enantiomer specificity (Prosser et al., 2004). Analysis of AdGDS1 showed a mix of (−)-enantiomeric (Y420) as well as (+)-enantiomeric (G457) determinants (equivalent to Goldenrod residues 406 and 444), indicating that further structural characterization may be required to locate the actual enantiomeric determinants.

Substrate availability and in planta function

The formation of terpene compounds is dependent on the availability of substrates for the terpene synthase enzymes. Monoterpene and diterpene synthases are primarily located in the plastids to access GPP and GGPP (Lichtenthaler, 1999), whilst sesquiterpene synthases are located in the cytosol (Fig. 10) and therefore are likely to be important for determining the enantiomer specificity. The formation of terpene compounds is dependent on the availability of substrates for the terpene synthase enzymes. Both AdGDS1 and AdAFS1 are localized in the cytosol (Fig. 10) and therefore are likely to be mainly acting as sesquiterpene synthase enzymes in planta. In the cytoplasm, both enzymes compete for the same substrate, FPP, and the proportion of the corresponding sesquiterpene produced depends on the enzyme concentration inside the cell, $K_m$ values of enzyme for FPP, and enzyme turnover. AdGDS1 has a lower $K_m$ for FPP than AdAFS1 (2.5 versus $\sim$9.5 μM, respectively); however, its catalytic efficiency ($k_{cat}/K_m$ ratio) with FPP is 7.6-fold lower than that of AdAFS1 (Table 1), suggesting that when these two proteins are competing for the same FPP pool, higher flux is directed towards (E,E)-α-farnesene formation. Indeed, both male and female flowers emit significantly (>25-fold) more α-farnesene than germacrene D (Figs 1, 2).

Biochemical characterization of the AdAFS1 enzyme revealed that, in addition to sesquiterpene synthase activity, it exhibits monoterpene synthase activity and is capable of producing (E)-β-ocimene. Taking into account the cytosolic localization of AdAFS1, its ability to form a monoterpene (E)-β-ocimene would depend on a GPP pool present in the cytosol. Recent metabolic engineering provided experimental evidence for the existence of a small pool of GPP in the cytosol of tobacco leaves (Wu et al., 2006). Small amounts of (E)-β-ocimene produced as a result of AdAFS1 transient expression (Fig. 9A) further proved that a small cytosolic pool of GPP exists in N. benthamiana leaves as well.

In Antirrhinum majus flowers, formation of the monoterpene linalool and the sesquiterpene nerolidol was shown to occur by two nearly identical linalool/nerolidol synthases that were targeted to the plastid and cytosol respectively (Nagegowda et al., 2008). Differential subcellular localization was the determining factor for the selective product formation. It is possible that a similar situation exists within A. delicosa and a spliced variant or parologue of AdAFS1 may be directed to the plastid and produce (E)-β-ocimene, found mainly in petal tissue. Alternatively, an as-yet unidentified ocimene synthase may be expressed in A. delicosa petal tissue and be responsible for most of the ocimene formation. Several other kiwifruit species produce floral (E)-β-ocimene without associated (E,E)-α-farnesene release (Crowhurst et al., 2008) which supports the idea of an additional unidentified petal specific (E)-β-ocimene synthase in kiwifruit. This synthase may be strongly upregulated by wounding in ‘Hayward’ petals, as shown by the high levels of (E)-β-ocimene released from the petal samples. (E)-β-ocimene is one of the most common herbivory-induced plant volatiles (Pare and Tumlinson, 1999; Pichersky and Gershenzon, 2002). In Phaseolus lunatus (lima bean), diurnal β-(E)-ocimene release increased greatly during the daytime by feeding larvae of Spodoptera littoralis or by mechanical wounding and was shown to be controlled by a jasmonate induced β-(E)-ocimene synthase (PLOS) gene. Substrate biosynthesis was almost exclusively fueled by daytime photosynthetic fixation along the plastidal MEP pathway (Arimura et al., 2008).

The ability to produce floral scent or to change the scent profile appears to evolve relatively rapidly and contributes to speciation. In Clarkia, moth pollination evolved through elevated linalool release by changes in the expression domains of a floral linalool synthase (LIS) gene (Dudareva et al., 1996). In rose, a single amino acid polymorphism in the substrate binding site of an OOMT resulted in the evolution of novel scent in Chinese roses (Scalliet et al., 2008). Dioecy has evolved to promote outbreeding, and to generate heterozygosity and genetic variation, but may also be influenced by other ecologic factors such as allocation of resources for male and female functions, sexual selection, seed dispersal, pollination, and predation (Bawa, 1980). The results presented here provide the molecular basis for the production of major sesquiterpenes in dioecious kiwifruit. This work reinforces the importance of volatile terpene cues for insect pollinator attraction and conditioning, a requirement for efficient pollen transfer between the staminate and pistillate plants, and ultimately the survival of any dioecious species.

Acknowledgements

The p326-SGFP vector was kindly provided by Inhwan Hwang, POSTEC, Korea. We would like to thank Sakuntala Karunairetnam for construction of pHEx2-AdGDS1, Ramon Lopez-Perez for construction of pHEx2-AdAFS1, Michael Sullivan for construction of the p326-AdGDS1/S GFP and p326-AdAFS1/SGFP, Julie Nicholls and Wade Wadasinghe for maintaining the N. benthamiana plants, and Daryl Rowan and Andrew Kralicek for critically reviewing.
the manuscript. We thank Jennifer Sturgis for her assistance with the confocal microscope. Confocal microscopy data were acquired from Purdue Cancer Center Analytical Cytometry Laboratories. Work at Plant and Food Research was funded by the New Zealand Foundation for Research, Science, and Technology (C06X0403). Work in ND’s laboratory was supported by grants from the National Science Foundation (Grant no. MCB-0615700) and the Fred Goeckner Foundation, Inc. Purdue Cancer Center Analytical Cytometry Laboratories were supported by the Cancer Center NCI core grant no. NIH NCI-2P30CA23168.

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