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RESEARCH PAPER

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

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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 female 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.*, 2000*a*) 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; Schuhr *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.*, 2000*b*; 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. deliciosa* '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. deliciosa* '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 eco- 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 deliciosa Lindl. var. *deliciosa* (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 'Mini Union' (Phenomenex, Torrance, CA, USA). Thus, injections were directly into the 0.53 mm i.d. GC column. The injection port temperature program was 30 °C for 0.5 min, 10 °C min^{-1} to 35 °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*-butyldimethylsilyl-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, hold for 10 min, 3 °C min^{-1} to 180 °C, and hold 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_2O 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. Relative quantitation 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′-AAGTTGCATTAGTAACCGCGGGTTA-3′ (forward) and 5′-TGACACCCAATCAAAGGCCTCTTT-3′ (reverse); *AdAFS1* (90 bp): 5′-GAAGTCATGTTGCGGAAAACGTCAA-3′ (forward) and 5′-CATATCCGGTGTGGCTCCATGTT-3′ (reverse) and *EF1α* (118 bp): 5′-GCACTGTCATTGATGCTCCT-3′ (forward) and 5′-CCAGCTTCAAAACCACCAGT-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′-GGATCCATGGAGCCCTTATTGTTTTC-3′ (forward) 5′-GCGGCCGCTCAAAGAGGAACATAGAACGCT-3′ (reverse) and for *AdGDS1*: 5′-GAATTCCAACTACCTTGTGCTCAAGC-3′ (forward) and 5′-CTCGAGCCTCCACTTCAGTGTCTTG-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 (*Bam*HI+*Not*I or *Eco*RI+*Xho*I, 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 Ni^{2+} 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). [C1-$^3H_1$]-FPP (4.0 GBq mmol^{-1}) and [C1-$^3H_1$]-GPP (6.2 GBq mmol^{-1}) were synthesized by MnO_2 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 NaB^3H_4 (Amersham) prior to phosphorylation (Green *et al.*, 2007).

All kinetic determinations using [C1-3H_1]-FPP were performed at room temperature in triplicate in 100 µl buffer containing 50 mM *bis-tris* propane (pH 7.5) and 25 nM (AdAFS1) or 100 nM (AdGDS1) enzyme. For kinetic determinations using [C1-$^3H_1$]-GPP (AdAFS1), the same buffer was used with 500 nM 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 $MgCl_2$ was included. Cofactor determinations for Mg^{2+} and Mn^{2+} were performed in the presence of 25 µM [C1-$^3H_1$]-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 $MgCl_2$ 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 $MgCl_2$, 10 µM acetosyringone) to an OD_{600} nm 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′-CACCAATAGGGTTTTGTTGGCATGGAGCCCTTATTGTTTT CA-3′ and AFR1 5′-GGTGTCAAAGAGGAACATAGAACGC-3′ and cloned into pENTR/D-TOPO (Invitrogen). The resulting clone was transferred by Gateway LR reactions as recommended by the manufacturer (Invitrogen) into the binary destination vector pHEX2, creating pHEX2-AdAFS1. pHEX2 contains the CaMV 35S promoter and octopine synthase terminator (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-SGFP vector (Lee *et al.*, 2001) which contains the CaMV 35S promoter, S65T GFP (Niwa *et al.*, 1999) and a nopaline synthase terminator. *AdGDS1* was amplified by PCR with primers GDS2F 5′-AATTTCTAGAATGCAACTACCTTGTGCTCAAGCT-3′ and GDS2R 5′-TAATGGATCCATTGATTGGCATAGAATCGATAA-3′, digested with *Xba*I and *Bam*HI and cloned into *Xba*I and *Bam*HI cut p326-SGFP creating p326-AdGDS1/SGFP. *AdAFS1* was amplified by PCR with primers AFF2 5′-pCTAGAATGGAGCCCTTATTGTTTTCAATT-3′ and AFR2 5′-AATAGGATCCAAGAGGAACATAGAACGCT-3′ and

in a separate PCR reaction with AFF3 5′-AATGGAGCCCTTATTGTTTTCAATT-3′ and AFR2. Both PCR reactions were purified, digested with *Bam*HI, mixed together in equimolar amounts, heat denatured, and then re-annealed to generate the *Xba*I overhang. The resulting insert was cloned into the *Xba*I and *Bam*HI 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. deliciosa *flowers*

Volatile terpenes emitted by female *A. deliciosa* 'Hayward' and male *A. deliciosa* '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. deliciosa* 'Hayward' flowers was about 1.3-fold higher than that from male *A. deliciosa* '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*)-α-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

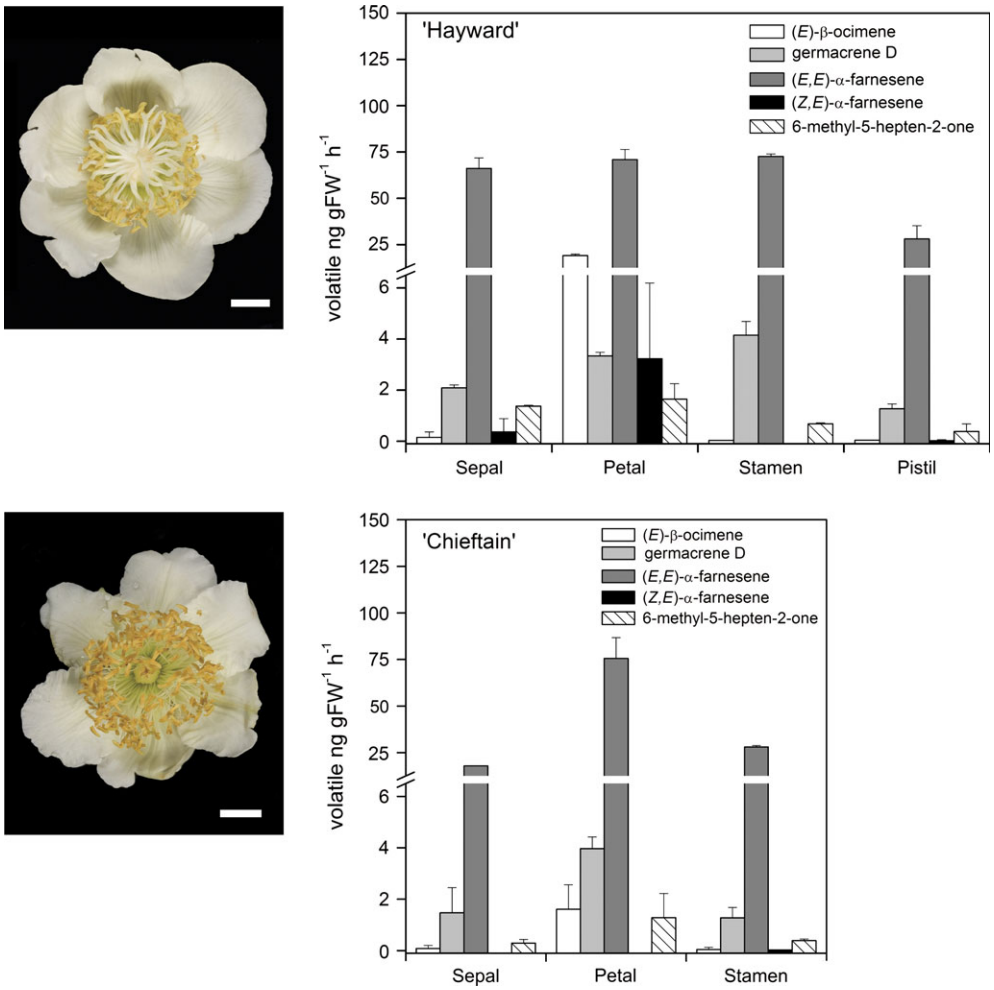

Fig. 1. Rates of terpenoid release by different flower parts of *Actinidia deliciosa* female 'Hayward' and male 'Chieftain' flowers. Parts from 'Hayward' (top) and 'Chieftain' (bottom) flowers were dissected and volatiles analysed by dynamic headspace trapping onto Chromosorb™ 105 absorbent. Measurements 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 α-farnesene oxidation product 6-methyl-5-hepten-2-one (Anet, 1972) were also detected in all tissues as well as some (*Z*,*E*)-α-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*)-α-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*)-α-farnesene accounted for about 1–4% of total α-farnesene emissions and showed similar fluctuation in emission levels to (*E*,*E*)-α-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

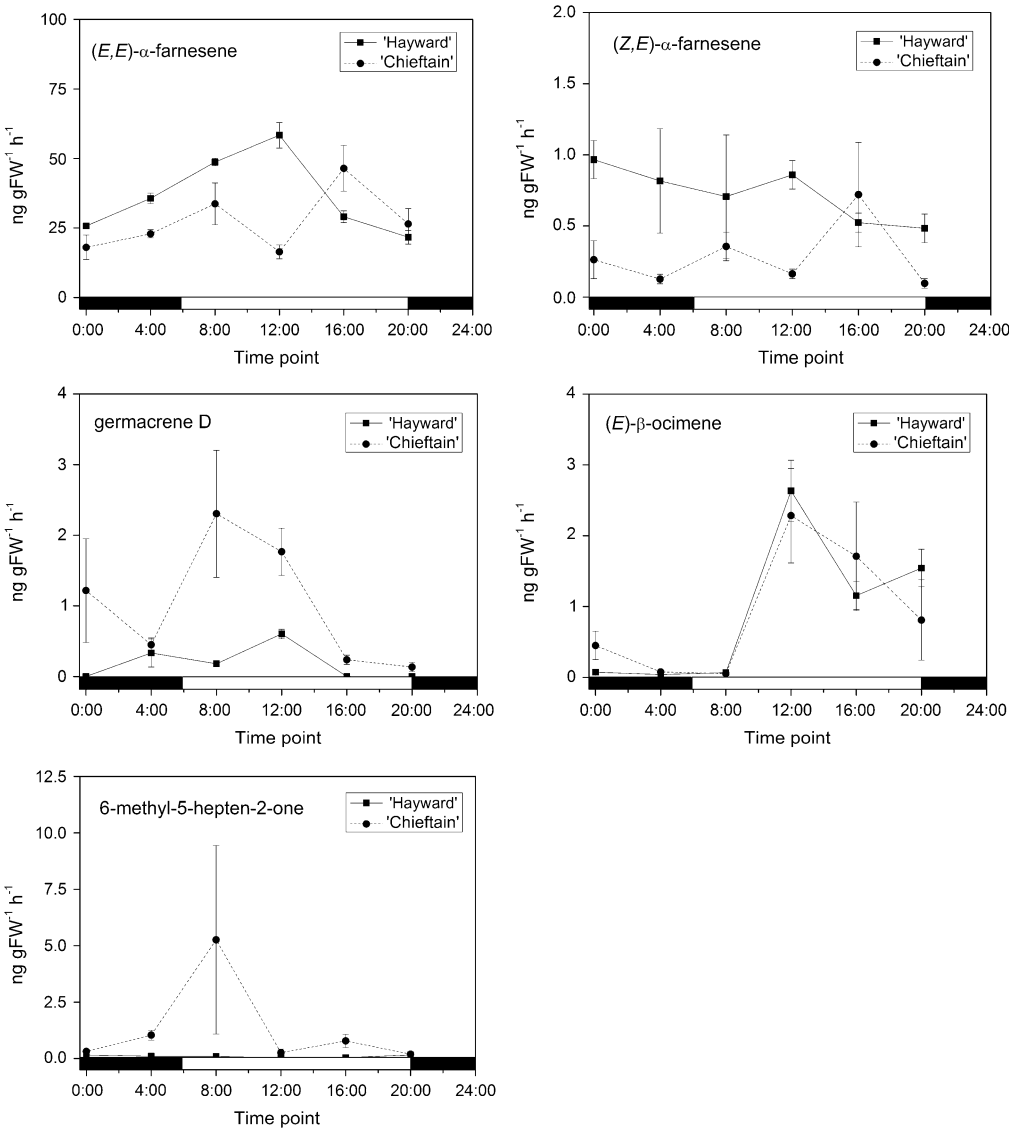

Fig. 2. Rates of terpenoid release during a day–night cycle by *Actinidia deliciosa* female 'Hayward' and male 'Chieftain' flowers. Major terpene volatiles were analysed by dynamic headspace trapping onto Chromosorb™ 105 absorbent at four-hourly time points. Data are presented as mean ±SE, n=3.

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 p*I* 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 p*I* 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_8W 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.*, 1998*b*). 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.*, 1998*b*) 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

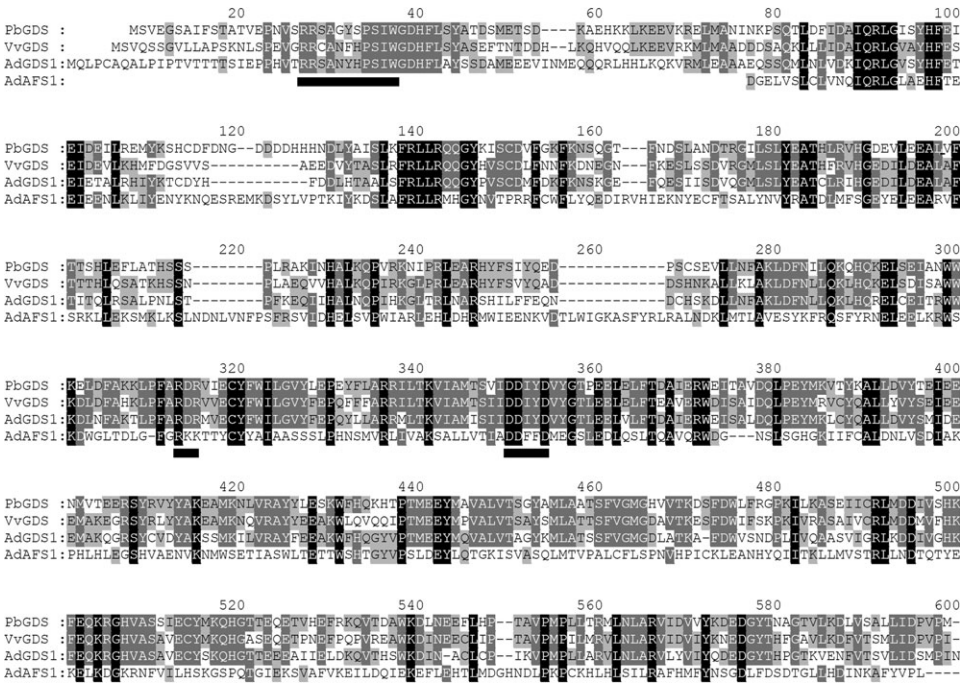

Fig. 3. Amino acid alignment of kiwifruit and other sesquiterpene synthases. Full-length sequences for PbGDS (*Populus balsamifera*, accession number AAR99061); VvGDS (*Vitis vinifera*, AAS66357); AdGDS1 (*Actinidia deliciosa*, AAX16121), and AdAFS1 (*Actinidia deliciosa*, FJ265785, amino acids 252–768, excluding N-terminus). Black boxes contain residues with 100%, dark grey 75%, and light grey 50% identity, respectively. Dashes indicate gaps inserted for optimal alignment. Horizontal lines mark the highly conserved RRX_8W, RXR, and DDXXD motifs, which are conserved in angiosperm monoterpene synthases but show variation in sesquiterpene synthases.

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.

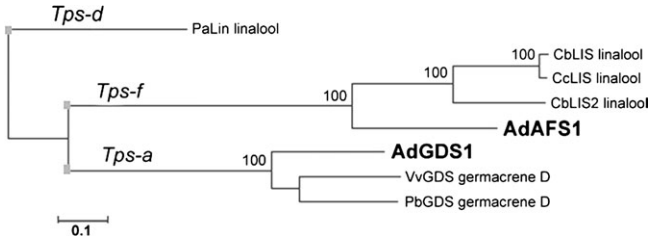

Fig. 4. Phylogenetic analysis of kiwifruit and other sesquiterpene synthases. A rooted Neighbor–Joining tree of selected angiosperm *Tps-a* subfamily terpene synthases and all subfamily *Tps-f* terpene synthases were constructed in Mega 4 and rooted with the *Tps-d* subfamily linalool synthase PaLIN (*Picea abies*, accession number AAS47693). Bootstrap values are shown as a percentage based on 1000 replicates. Gene designations and GenBank accession numbers are the same as in Figs 3 and 5.

The temporal accumulation patterns of *AdAFS1* and *AdGDS1* mRNA were also analysed through a full day–night cycle in whole flower tissue (Fig. 6B, D). *AdAFS1* showed constitutive expression, while *AdGDS1* expression increased at 08.00 h and then dropped to about a half by 20.00 h. For both genes there was essentially no difference in expression between male and female flowers.

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 Ni^{2+} 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 (Matich *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.

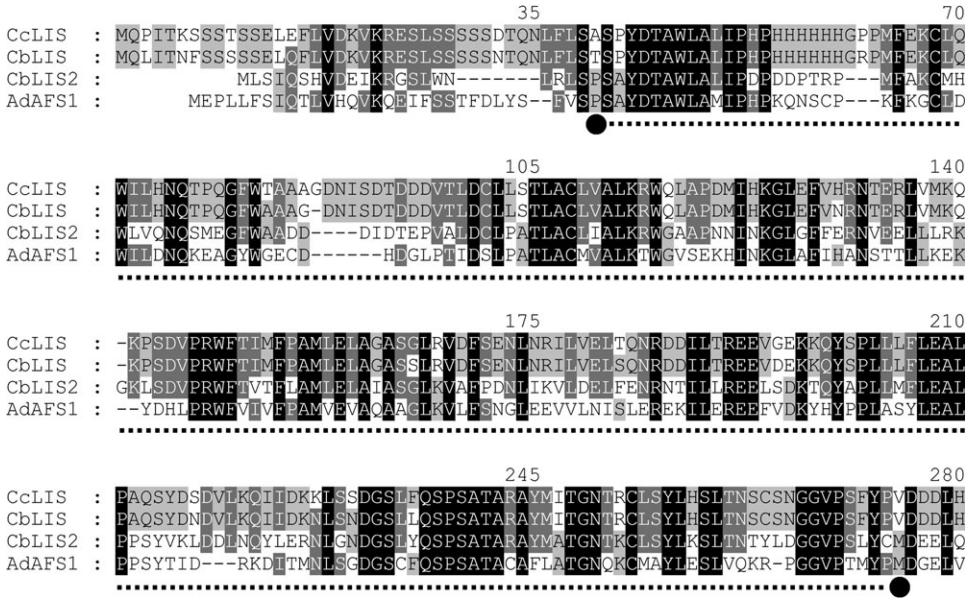

Fig. 5. N-terminal protein sequence alignment of the *Tps-f* subfamily terpene synthases. CcLIS (*Clarkia concinna*, accession number AAD19839); CbLIS (*C. breweri*, AAC49395); CbLIS2 (*C. breweri*, AAD19840), and AdAFS1 (*Actinidia deliciosa*, FJ265785). The conifer diterpene internal sequence (CDIS) is indicated by a dotted line. Black boxes contain residues with 100%, dark grey 75%, and light grey 50% identity, respectively. Dashes indicate gaps inserted for optimal alignment.

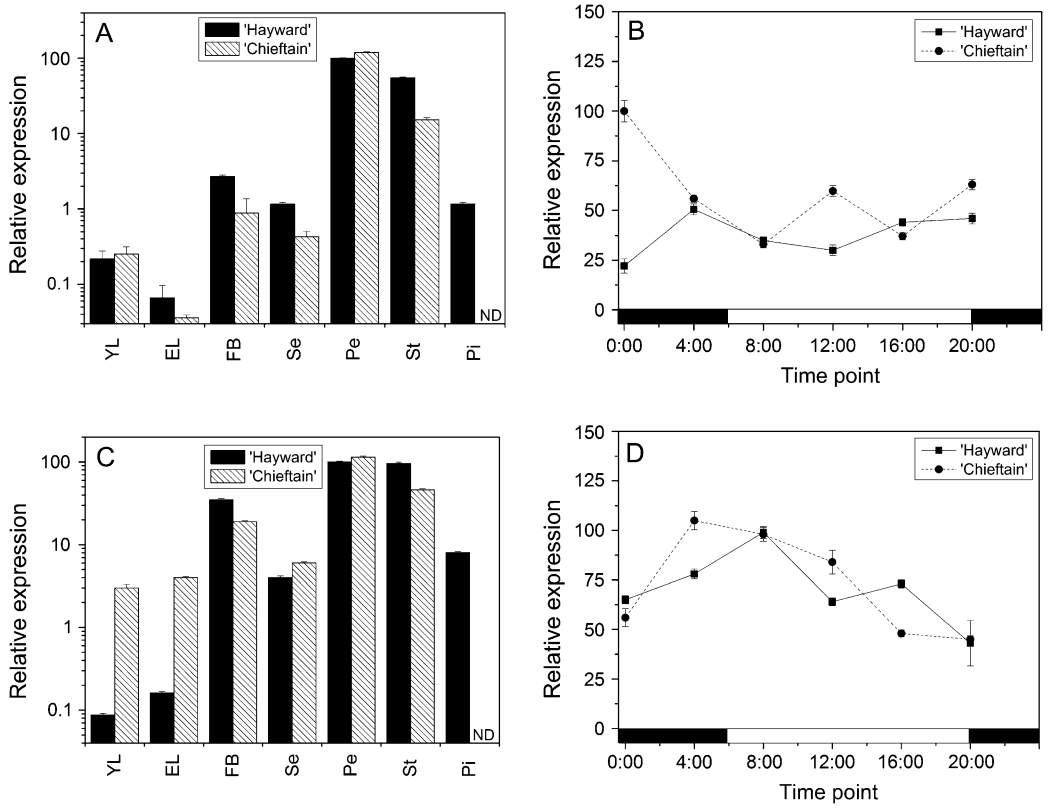

Fig. 6. Real-time PCR gene expression analysis of *AdAFS1* and *AdGDS1* in kiwifruit flowers and leaves. Gene expression levels were determined in 'Hayward' and 'Chieftain' vegetative and floral parts for *AdAFS1* (A) and *AdGDS1* (C); and in whole flowers during a 24 h day/night cycle for *AdAFS1* (B) and *AdGDS1* (D). For both genes, the highest transcript level in the petals of 'Hayward' flowers was set as 100. Error bars are based on four technical replicates. YL, young leaf; EL, expanded leaf; FB, floral bud; Se, sepal; Pe, petal; St, stamen; and Pi, pistil.

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.*, 1998*a*; 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

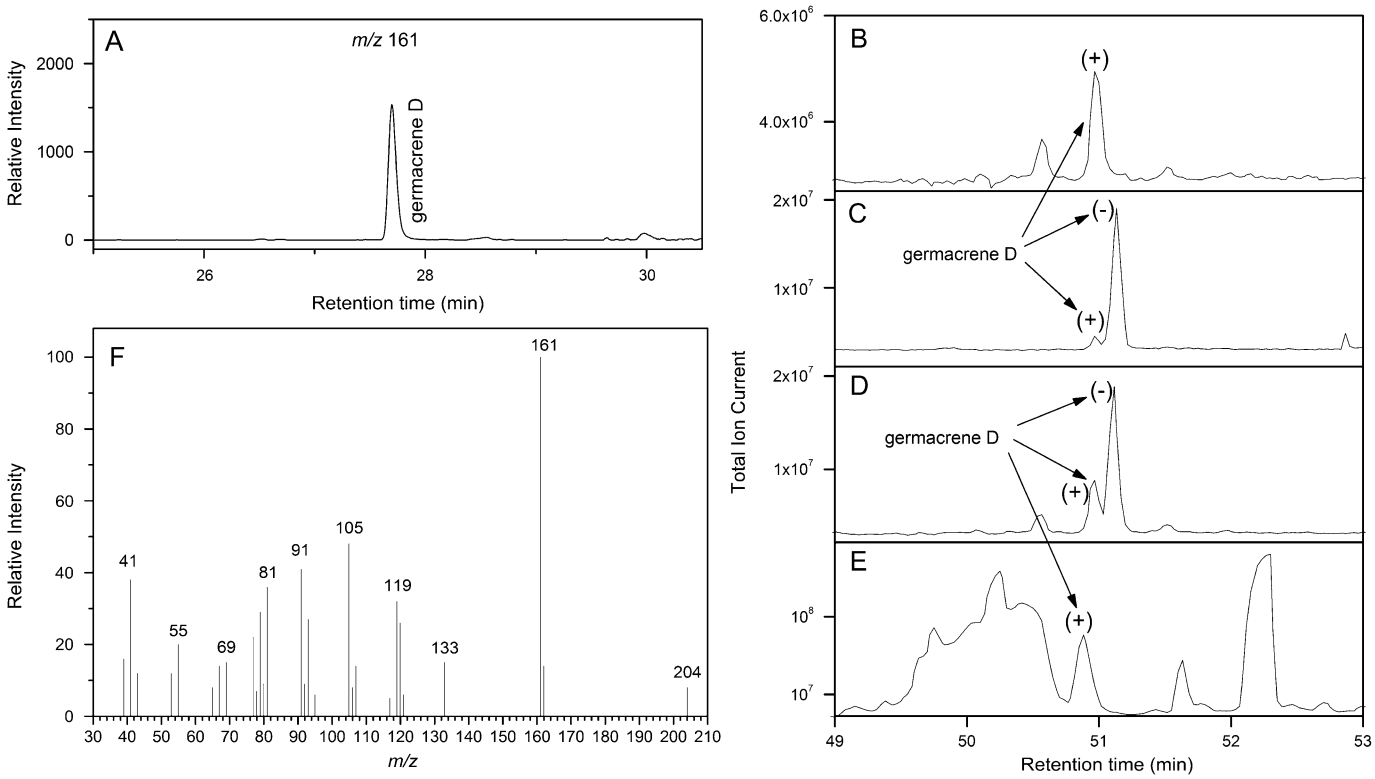

Fig. 7. GC-MS analysis of the terpene products produced by AdGDS1 and 'Hayward' flowers. Purified AdGDS1 recombinant enzymes were obtained by Ni^{2+} affinity and gel filtration chromatography. The sesquiterpene products produced by AdGDS1 using FPP as substrate were analysed by GC-MS by cold on-column 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 (*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_3H_7), the molecular ion at *m/z* 204, *m/z* 133 (M- C_3H_7-C_2H_4), and the consecutive losses of CH_2 to produce *m/z* 119, 105, and 91.

analysed by dynamic headspace sampling. Leaves infiltrated with the *AdAFS1* construct produced a large amount of (*E*,*E*)-α-farnesene, with smaller amounts of (*Z*,*E*)-α-farnesene and (*E*)-β-ocimene (Fig. 9A). No α-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 *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 *AdGDS1* construct (Fig. 9B). A preceding peak was tentatively identified as γ-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 *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 *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.

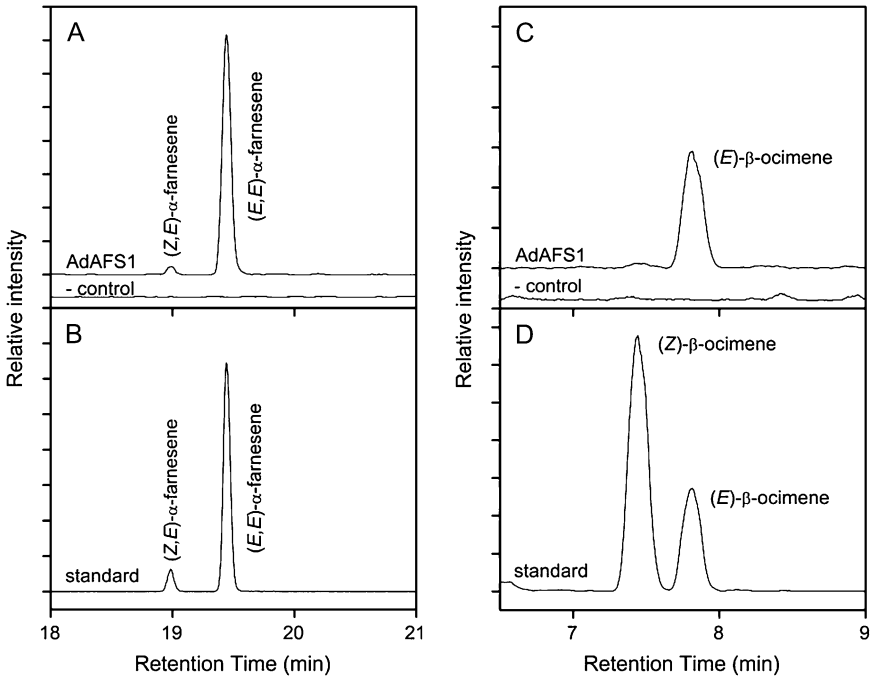

Fig. 8. GC–MS analysis of the terpene products produced by AdAFS1. Purified AdAFS1 recombinant enzymes was obtained by Ni^{2+} affinity and gel filtration chromatography. The products produced using FPP or GPP were analysed by GC–MS versus reference compounds. (A) The terpenes produced by AdAFS1 using FPP as the substrate (upper trace) compared with a 'control' no enzyme reaction (lower trace). (B) (*Z,E*)- and (*E,E*)-α-farnesene reference compounds isolated from 'Granny Smith' apples. (C) The terpenes produced by AdAFS1 using GPP as the substrate (upper trace) compared to a 'control' no enzyme reaction (lower trace). (D) (*Z*)- and (*E*)-β-ocimene reference compounds obtained by photolysis of α-pinene (Kropp, 1969).

Table 1. Enzyme kinetic properties of AdAFS1 and AdGDS1

Purified recombinant AdAFS1 and AdGDS1 enzymes were obtained by Ni^{2+} affinity and gel filtration chromatography. Kinetic parameters with FPP (1–100 μM) and GPP (1–100 μM) both in the presence of 25 mM $MgCl_2$, Mg^{2+} (0.2 to 25 mM) and Mn^{2+} (0 to 0.6 mM) in the presence of 25 μM FPP and all containing 50 mM *bis-tris*-Propane buffer pH 7.5. All values represent mean ±SE, n=3. K_m, Michaelis constant; V_{max}, maximum velocity; k_{cat}, turnover.

	K_m (μM)	V_{max} (pkat mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
AdAFS1				
GPP (Mg^{2+})	2.8±0.1	316±63	0.030±0.005	11±0.7
FPP (Mg^{2+})	9.5±2.3	4715±502	0.440±0.046	51±9.6
Mg^{2+} (FPP)	248±11	100%		
Mn^{2+} (FPP)	~20	50%		
AdGDS1				
FPP (Mg^{2+})	2.5±0.3	236±8	0.017±0.001	6.7±0.5
Mg^{2+} (FPP)	317±38	100%		
Mn^{2+} (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^{-1} 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

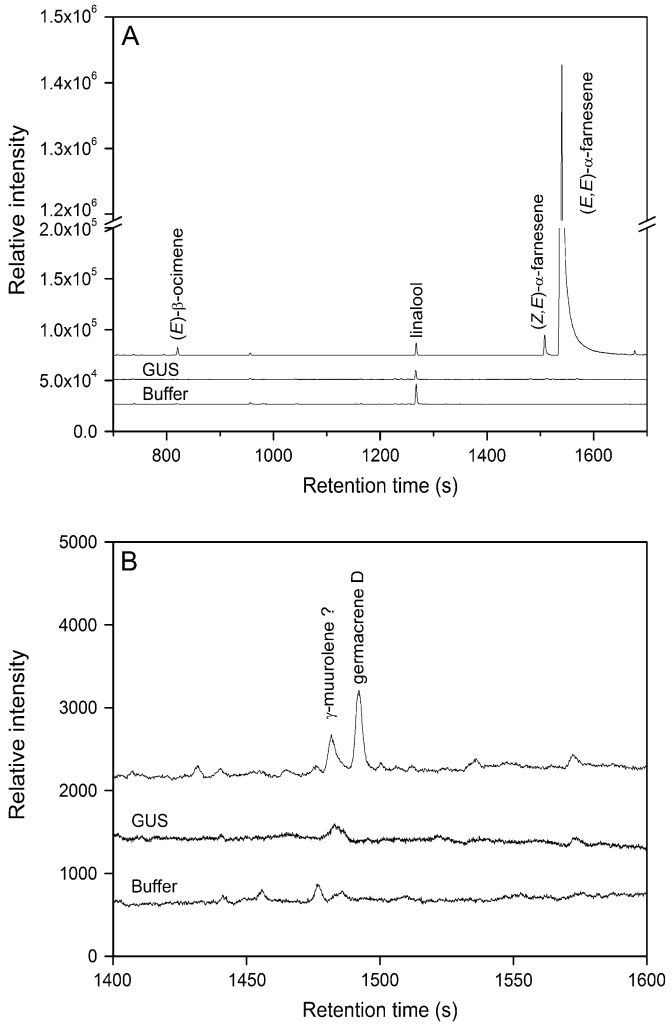

Fig. 9. Transient expression of *AdAFS1* and *AdGDS1 in planta.* The complete open reading frames of *AdAFS1* and *AdGDS1* were cloned into the binary vector pHEX2 and transformed into *Agrobacterium tumefaciens*. Bacterial cell suspensions were infiltrated into the adaxial side of three young *Nicotiana benthamiana* leaves and volatiles were analyzed two weeks after injection by dynamic headspace sampling of detached leaves. Leaves were infiltrated with buffer-only and with a vector expressing the GUS reporter gene (pHEX2-GUS) as controls. Volatiles transiently expressed *in planta* after infiltration with (A) *AdAFS1*, upper trace; pHEX2-GUS, middle trace; buffer-only, lower trace. (B) *AdGDS1*, upper trace; pHEX2-GUS, middle trace; buffer-only, lower trace.

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 monoterpene (*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 monoterpene 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.*, 1998*b*). Attempts to delete this domain from AdAFS1 using a series of

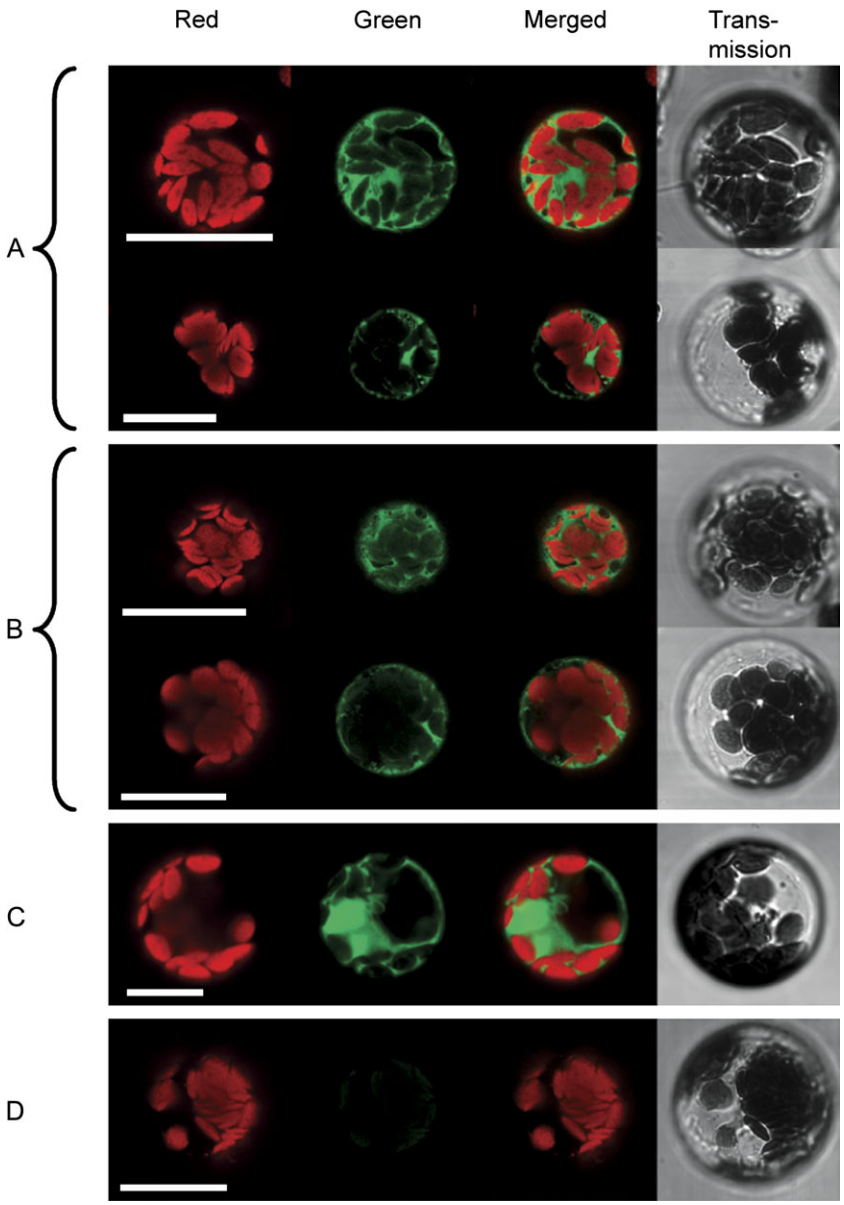

Fig. 10. Subcellular localization of AdAFS1 and AdGDS1 in *Arabidopsis* leaf protoplasts. The full-length coding regions of AdAFS1 and AdGDS1 were fused to the GFP reporter gene in the vector p326S-GFP to produce the constructs p326-AdGDS1/SGFP and p326-AdAFS1/SGFP. The fusion constructs were then transferred 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.

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.*, 1998*b*). 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

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 enantiomeric 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 where FPP is synthesized. 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 ~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. deliciosa* and a splice variant or paralogue 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. deliciosa* 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 leves 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.

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