The Plant Journal (2005) **42**, 757–771 doi: 10.1111/j.1365-313X.2005.02417.x

Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from Arabidopsis flowers

Dorothea Tholl[1,2,*,†], Feng Chen[1,‡], Jana Petri[2], Jonathan Gershenzon[2] and Eran Pichersky[1]
[1]*Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA, and*
[2]*Max Planck Institute for Chemical Ecology, Hans Knöll Strasse 8, D-07745, Jena, Germany*

Received 15 December 2004; revised 8 March 2005; accepted 18 March 2005.
*For correspondence (fax +49 540-231-7126; e-mail tholl@vt.edu).
†Present address: Department of Biology, Virginia Polytechnic Institute and State University, 2006 Fralin Biotechnology Center, West Campus Drive, Blacksburg, VA 24061-0346, USA.
‡Present address: Department of Plant Sciences, University of Tennessee, 2431 Joe Johnson Drive, Knoxville, TN 37996-4561, USA.

Summary

Despite the fact that Arabidopsis is largely self-pollinating, its flowers emit a complex mixture of terpene volatiles consisting predominantly of a large group of over 20 sesquiterpenes. Here we report that only two terpene synthases, encoded by the florally expressed genes At5g23960 and At5g44630, are responsible for the formation of virtually all sesquiterpenes found in the Arabidopsis floral volatile blend. Two independent mutant lines with T-DNA insertions in the previously identified At5g23960 gene lacked the emission of three sesquiterpenes, including the main sesquiterpene volatile (*E*)-β-caryophyllene, confirming the previous *in vitro* functional assignment for this gene. Flowers of a mutant line carrying a T-DNA insertion in gene At5g44630 emitted these three sesquiterpenes, but did not emit any of the remaining sesquiterpene volatiles. An At5g44630 cDNA was expressed in *Escherichia coli* and the produced protein catalyzed the conversion of farnesyl diphosphate into over 15 sesquiterpenes in similar proportions to those found in the floral volatile blend. At5g23960 and At5g44630 promoter-β-glucuronidase (GUS) fusion experiments demonstrated that both genes are expressed in several parts of the Arabidopsis flower, with strong At5g23960 promoter-GUS activity in the stigma and strong expression of At5g44630 in intrafloral nectaries. Given the previously reported antimicrobial activity of terpenes, their production in stigmas and nectaries may serve to inhibit microbial infection at these vulnerable sites. A survey of 37 *Arabidopsis thaliana* ecotypes revealed quantitative, but almost no qualitative, variations of floral monoterpene and sesquiterpene emissions suggesting that floral terpene volatiles must play some significant role in the life of the Arabidopsis plant.

Keywords: floral volatiles, terpenes, terpene synthase, nectary, Arabidopsis, ecotype.

Introduction

Numerous plants emit volatile compounds from their flowers. These volatiles belong primarily to three major groups of compounds: phenylpropanoids, fatty acid derivatives and terpenes (Dudareva and Pichersky, 2000; Knudsen *et al.*, 1993). Floral scents play important roles in the attraction of a variety of insect and other animal pollinators and hence have important implications for plant reproduction (Dobson, 1994; Pichersky and Gershenzon, 2002). Although *Arabidopsis thaliana* is considered to be mainly self-pollinating, the flowers of this species release a complex mixture of volatile monoterpenes (C10) and sesquiterpenes (C15), with (*E*)-β-caryophyllene as the dominant component (Chen *et al.*, 2003). The rate of volatile emission from *A. thaliana* is low compared to those of primarily insect-pollinated plants (Chen *et al.*, 2003), but visits by pollinators including solitary bees, flies and beetles have been observed in naturalized Arabidopsis populations (Hoffmann *et al.*, 2003). By monitoring the daily visitation patterns of these insects, a cross-pollination rate of approximately 1% was estimated, which was in the range of outcrossing rates determined in previous studies (Abbott and Gomes, 1989; Hoffmann *et al.*, 2003; Snape and Lawrence, 1971).

Based on these findings, it has been hypothesized that Arabidopsis floral volatiles may play a role in the attraction of floral visitors, leading to low levels of cross-pollination and increasing reproductive fitness in natural populations

© 2005 Blackwell Publishing Ltd

(Chen *et al*., 2003). Insect-mediated cross-pollination in this species is also supported by the protogynous mode of Arabidopsis floral development (Jones, 1971), and by the presence of sugar-secreting intrafloral nectaries (Davis *et al*., 1998). However, given the low levels of volatile emission, it is quite possible that the terpenes in Arabidopsis flowers have a function besides, or in addition to, pollinator attraction. For example, recent studies have given evidence that volatile terpenes, including the hemiterpene (C5) isoprene, monoterpenes and sesquiterpenes, released from vegetative tissues of different plant species under high light and high temperature could serve as mediators of thermotolerance or in protecting the cell against oxidative stress (Loreto and Velikova, 2001; Sharkey and Yeh, 2001). In addition, terpenes have often been described to exhibit antimicrobial activities *in vitro* (Cowan, 1999; Deans and Waterman, 1993), and thus may protect reproductive organs against bacterial or fungal attack *in vivo*.

Arabidopsis, with its extensive genetic and genomic resources, provides an ideal model system to study the fundamental roles of terpene floral volatiles *in planta*. These resources allow for the relatively easy localization of the sites of expression of the biosynthetic genes, identification of mutant lines lacking volatile emission, and the comparison of floral volatile profiles in different Arabidopsis accessions. Moreover, the information available for Arabidopsis facilitates elucidating the molecular processes responsible for regulation and variability of floral volatile biosynthesis and emission. Previous studies of floral volatile biosynthesis in *Clarkia breweri* and closely related species revealed that floral scent is a variable and rapidly evolving trait (Dudareva *et al*., 1996; Raguso and Pichersky, 1995).

The Arabidopsis genome contains a large family of 32 predicted terpene synthase (*AtTPS*) genes (Aubourg *et al*., 2002; Chen *et al*., 2003), of which several are exclusively or almost exclusively expressed in the flowers (Chen *et al*., 2003). Terpene synthases (TPSs) catalyze the formation of C5, C10, C15 and C20 terpene skeletons from allylic prenyl diphosphate intermediates of the terpene biosynthesis pathway, including dimethylallyl diphosphate (DMAPP, C5), geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) (Davis and Croteau, 2000; Gershenzon and Kreis, 1999). Analysis of the expression of three florally expressed *AtTPS* genes, At3g25810, At1g61680, and At5g23960, combined with biochemical analysis of the proteins they encode (Chen *et al*., 2003), showed that the first gene specifies a TPS that can form several monoterpenes *in vitro* with (−)-α-pinene, myrcene and (*E*)-β-ocimene as the main products. Nearly all of the monoterpene products identified *in vitro* were found as Arabidopsis floral volatiles. The second gene is responsible for the synthesis of the monoterpene alcohol (+)-linalool that was also identified in the Arabidopsis floral volatile blend. The third gene was shown to encode a sesquiterpene synthase that catalyzes *in vitro* the formation of (−)-(*E*)-β-caryophyllene, the major sesquiterpene emitted from Arabidopsis flowers, as well as the sesquiterpenes α-humulene, (−)-α-copaene, and β-elemene, also found in the floral volatile blend.

Besides these sesquiterpene compounds, Arabidopsis flowers emit over 15 additional sesquiterpenes. Here we report the characterization of a second *A. thaliana* sesquiterpene synthase gene At5g44630 that appears to be expressed exclusively in the flowers, and we show that it encodes the enzyme that catalyzes the formation of essentially all of the remaining sesquiterpenes emitted from Arabidopsis floral tissues. Analysis of mutant lines carrying T-DNA insertions in At5g44630 and the other sesquiterpene synthase gene, At5g23960, as well as natural variation found among 37 Arabidopsis accessions examined, demonstrate that these two genes control the synthesis of nearly all sesquiterpenes emitted from Arabidopsis flowers. In addition, examination of the specific location within the flower where these sesquiterpene synthase genes are expressed showed that transcription of the two genes occurred in distinct parts of the flower, providing valuable hints on the roles of sesquiterpenes in this species.

Results

At5g44630 and At5g23960 are responsible for the biosynthesis of virtually all detectable sesquiterpenes emitted from Arabidopsis flowers

Previous genomic analysis indicated that the Arabidopsis genome contains 32 genes with homology to TPSs. Of these genes only four, At5g23960, At5g44630, At4g13280 and At4g13300, are likely to encode sesquiterpene synthases rather than monoterpene or diterpene synthases, as their predicted proteins lack N-terminal transit peptide-like sequences (Aubourg *et al*., 2002; Chen *et al*., 2003) and thus would presumably be present in the cytosol. The cytosol is thought to be the site of formation of the sesquiterpene precursor FPP, whereas the monoterpene and diterpene precursors, GPP and GGPP, respectively, are thought to be made in the plastids (Lichtenthaler, 1999). Transcript analysis indicated that At5g23960 and At5g44630 are expressed exclusively in the flowers, while At4g13280 and At4g13300 showed no expression in floral tissues (Chen *et al*., 2003).

We also previously demonstrated (Chen *et al*., 2003) that the recombinant protein encoded by the cDNA of At5g23960 is capable of converting FPP into the sesquiterpene products (−)-α-copaene, α-humulene, and (−)-(*E*)-β-caryophyllene (referred to here as group A sesquiterpenes) as well as β-elemene, all of which are constituents of the Arabidopsis floral volatile blend (Figure 1). While this result suggested that At5g23960 is responsible for the synthesis of these sesquiterpenes in the flower *in vivo*, it

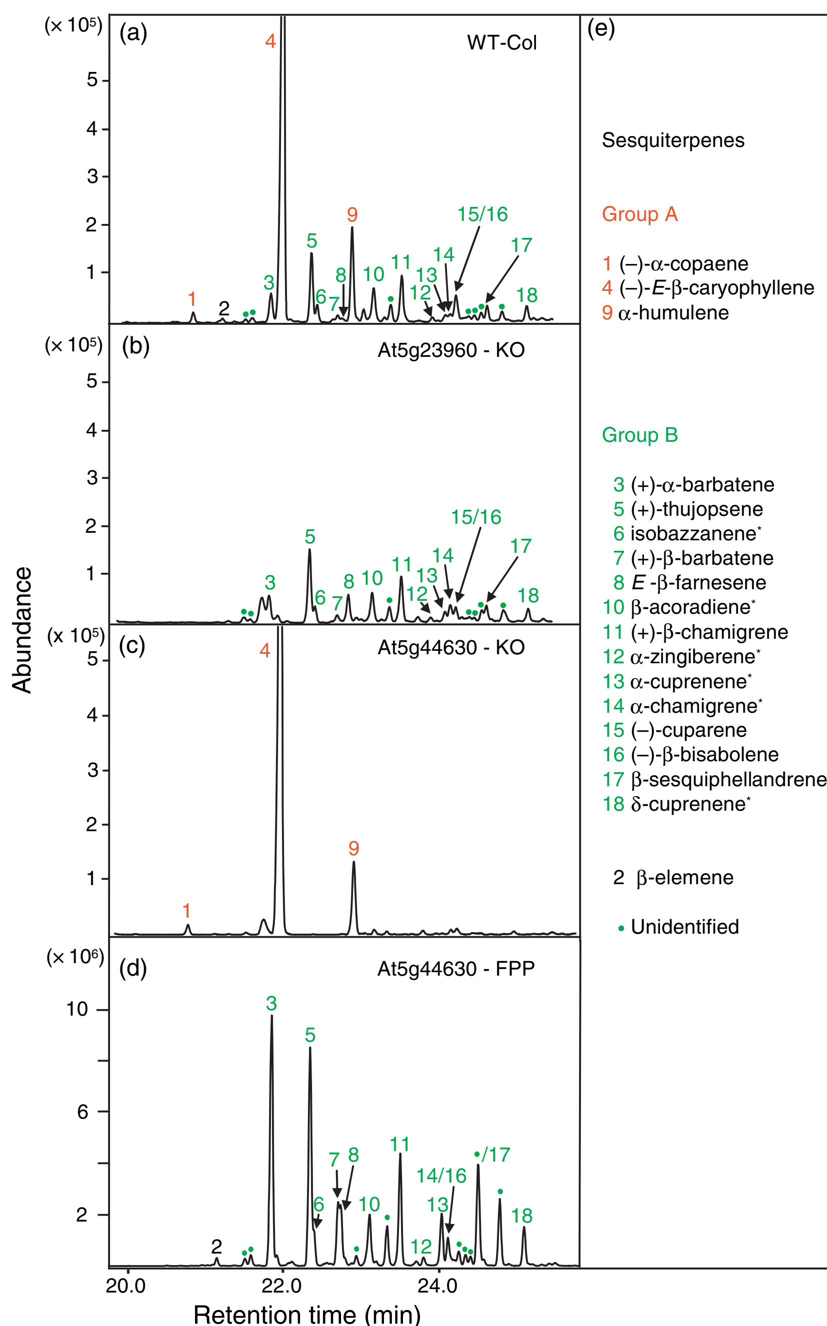

Figure 1. Sesquiterpene volatiles emitted from inflorescences of Arabidopsis Col wild type and At5g23960 and At5g44630 T-DNA insertion lines, and sesquiterpene volatiles produced by the At5g44630 TPS enzyme.
Gas chromatography-mass spectrometry analysis of sesquiterpene hydrocarbons collected from 70 detached inflorescences of Col wild-type plants (a) and T-DNA insertion mutant lines of the genes At5g23960 (b) and At5g44630 (c) during 8 h of closed-loop stripping (Donath and Boland, 1995). (d) GC-MS chromatogram of sesquiterpenes produced by the *Escherichia coli*-expressed protein encoded by At5g44630 after incubation with FPP. Only a portion of the chromatogram of each sample is shown. (e) Identified compounds of the sesquiterpene volatile fraction. The number of each compound corresponds to the numbered peaks in (a), (b), (c), and (d). Flowers of the At5g23960 knock out (KO) line do not emit sesquiterpenes of group A, labeled with red numbers, indicating that these compounds are formed *in vivo* only by the At5g23960 TPS enzyme. Flowers of the At5g44630 knock out line lack the emission of compounds categorized as group B sesquiterpenes and labeled with green numbers, suggesting that they are exclusive products of the At5g44630-encoded enzyme. Although β-elemene was found as an *in vitro* product of the enzymes encoded by both genes, this compound was only detected as a volatile from flowers of WT-Col but not of either mutant. It is likely that β-elemene is the product of a complete rearrangement of germacrene A which could occur at injection port temperatures above 200°C (de Kraker *et al*., 1998). (–)-cuparene (15) was not detected as enzymatic product of At5g44630 TPS but emitted from WT-Col flowers and is presumably a conversion product of α-cuprenene (13). Compounds were identified by mass spectrometry and comparison with authentic standards. Absolute configurations of some sesquiterpenes were determined by gas chromatography on a chiral column as described in Experimental procedures. For compounds labeled with asterisks no enantiomeric composition was determined because of the lack of available standards. Coelution of compounds is indicated by separation of numbers with slashes. Coeluted compounds were separable by selective changes of chromatography conditions. Peaks labeled with dots in (a), (b), and (d) represent sesquiterpenes that could not be unambiguously identified.

could not rule out the contribution of other enzymes to the biosynthesis of some or all of these compounds. In addition, it was unknown whether all of the remaining floral sesquiterpenes (defined here as group B sesquiterpenes, Figure 1) were produced by one or more than one enzyme, although At5g44630 was a likely candidate for the formation of these volatiles.

To evaluate the role of At5g23960 and At5g44630 in the formation of floral sesquiterpenes, we first obtained T-DNA insertional mutant lines for these genes. Headspace collection and GC-MS analysis of volatiles emitted from cut inflorescences of two independent At5g23960 mutant lines clearly showed the complete lack of all group A sesquiterpene compounds (Figure 1b), indicating that At5g23960 alone is responsible for the synthesis of these particular floral volatiles. The floral volatile profile of the At5g23960 mutant line had no significant qualitative and quantitative changes in group B sesquiterpene volatiles (Figure 1a,b) or in any of the floral monoterpenes (data not shown) in comparison with Col wild type.

In contrast, the volatile blend from flowers of an At5g44630 T-DNA insertion line contained only group A

sesquiterpenes, including (−)-(*E*)-β-caryophyllene, in similar ratios as the wild type, but none of the group B sesquiterpene compounds (Figure 1c) and no change was seen in monoterpene production (data not shown). In addition, a full-length cDNA of At5g44630 expressed in *Escherichia coli* resulted in a protein that accepted FPP as a substrate and converted it into over 15 different sesquiterpenes that could be detected and identified by comparison of their gas chromatographic retention times and mass spectra to those of authentic standards (Figure 1d and Figure S1). Nearly all of the enzymatically formed compounds were identical to the floral group B sesquiterpenes and present in similar ratios as those found in the headspace of *A. thaliana* flowers (Figure 1a,d), further supporting the notion that At5g44630 TPS alone is responsible for the synthesis of the entire sesquiterpene group B in the Arabidopsis floral volatile blend.

The enzymatic properties of the proteins encoded by At5g44630 and At5g23960

To investigate the enzymatic formation of Arabidopsis floral sesquiterpene volatiles, we undertook a detailed characterization of the proteins encoded by the two florally expressed *AtTPS* genes At5g44630 and At5g23960. The full-length 1674 bp cDNA of At5g44630 whose heterologous expression was described above was obtained from floral tissue of Ler by RT-PCR. The open reading frame of the At5g44630 cDNA encodes a protein of 557 amino acids with a calculated molecular mass of 64 908 Da and a pI of 5.2, and is identical to the sequence from Col-0 except for changes in two amino acids (Figure 2a). The protein encoded by the open reading frame of the previously isolated 1644 bp At5g23960 cDNA contains 547 amino acids giving a calculated molecular mass of 63 379 Da and a pI of 5.2 (Figure 2a). The protein sequences of both enzymes are only 33% identical to each other. The At5g44630 TPS is more closely related to a larger group of proteins encoded by the other Arabidopsis putative sesquiterpene synthase genes At4g13280 and At4g13300 (52 and 46% identity, respectively), and the putative Arabidopsis diterpene synthase genes (Figure 2b, group 4), while At5g23960 TPS differs significantly from Arabidopsis group 4 TPS and shows some similarities to sesquiterpene and diterpene synthases characterized from other angiosperm species (Figure 2b, group 5). The similarities of both enzymes to monoterpene synthases (from Arabidopsis and other plant species) are comparatively low (Figure 2a,b, group 1). Both proteins contain characteristic sequence motifs of the TPS family including the highly conserved DDXXD motif, involved in binding the diphosphate moiety of the substrate by chelation of bridging Mg^{2+} ions, and the conserved RXR motif 35 amino acids upstream thought to help direct the diphosphate anion away from the reactive carbocation after ionization (Figure 2a).

The enzymatic properties of the *E. coli*-expressed and partially purified proteins encoded by At5g44630 and At5g23960 were further investigated *in vitro*. Both At5g44630 and At5g23960 TPS enzymes showed similar low apparent K_m values of 1.2 and 2.1 μM, respectively, for the substrate FPP. While At5g23960 TPS, as previously reported, was not able to use GPP as substrate (Chen *et al.*, 2003), the At5g44630 enzyme accepted GPP with an apparent K_m value of 0.21 μM. GPP was converted, with equal velocity as FPP (V_{max} ratio GPP/FPP approximately 1), into seven different monoterpene products, including myrcene, limonene, (*Z*)-β-ocimene, (*E*)-β-ocimene, terpinolene, and α-terpineol, as determined by GC-MS analysis. However, the formation of these compounds by the enzyme *in vivo* is rather unlikely, as the At5g44630 TPS protein lacks a transit peptide and is therefore not expected to be present in plastids, where GPP is thought to be produced.

Catalysis of both At5g44630 TPS and At5g23960 TPS was dependent on divalent metal ions. Maximum activity of At5g23960 TPS was reached at approximately 20 mM Mg^{2+}, while with Mn^{2+} only half maximum activity levels were obtained at a concentration of 10 μM with gradual inhibition of activity at increasing Mn^{2+} concentrations. Compared with the At5g23960 enzyme, a desalted At5g44630 TPS fraction was active without external addition of Mg^{2+} or Mn^{2+}. However, the enzyme requires divalent metal ion cofactors as the addition of 1 mM EDTA led to a complete loss of enzyme activity.

Figure 2. Sequence comparison of At5g44630 TPS and At5g23960 TPS with other Arabidopsis and plant terpene synthases.
(a) Protein sequence alignment of At5g44630 TPS (Ler ecotype) and At5g23960 TPS with exemplary other terpene synthases, including the Arabidopsis monoterpene synthase At3g25810, producing mainly (−)-α-pinene, myrcene and (*E*)-β-ocimene, a casbene synthase from *Ricinus communis* (L32134), and a caryophyllene synthase from *Artemisia annua* (AF472361). Amino acids shaded in black indicate conserved identical residues in at least three of five sequences shown. Dashes indicate gaps inserted for optimal alignment. Horizontal lines mark the highly conserved DDXXD and RXR motifs and the RRX_8W motif that is conserved in angiosperm monoterpene synthases and shows variation in sesquiterpene and diterpene synthases. L32134 and At3g25810 have putative N-terminal plastid targeting sites preceding this arginine-containing motif.
(b) A neighbor-joining tree based on degree of sequence similarity between Arabidopsis TPSs and selected TPSs from other plants. Functionally characterized TPSs from Arabidopsis are circled. Group 1 contains angiosperm monoterpene synthases of the TPS-b and g families (class III) (Aubourg *et al.*, 2002; Dudareva *et al.*, 2003; Trapp and Croteau, 2001). Group 2 represents enzymes of the TPS-d family (class I/II) of gymnosperms (Bohlmann *et al.*, 1998). Group 3 contains primarily diterpene synthases including copalyl diphosphate synthases (TPS-c, class I), ent-kaurene synthases (TPS-e, class I), and monocot diterpene synthases. Group 4 comprises a clade of mostly uncharacterized putative sesquiterpene and diterpene synthases from Arabidopsis. Group 5 represents sesquiterpene and diterpene synthases from dicots, and group 6 contains sesquiterpene synthases from maize. Enzymes of groups 4, 5, and 6 belong to TPS-a or class III terpene synthases. Small circles indicate nodes supported by bootstrap values higher than 800 out of 1000 replicates.

Localization of At5g44630 and At5g23960 promoter activities in floral organs

Using RT-PCR At5g44630 and At5g23960 were shown to be expressed exclusively in Arabidopsis flowers, although the spatial distribution of the transcripts within the flowers was not previously determined (Chen *et al*., 2003). To investigate the tissue-specific sites of At5g44630 and At5g23960 gene expression in greater detail, we created At5g44630 and At5g23960 promoter-β-glucuronidase (GUS) fusions and determined GUS activity in Arabidopsis Col plants transformed with these constructs. GUS staining was exclusively

© Blackwell Publishing Ltd, *The Plant Journal*, (2005), **42**, 757–771

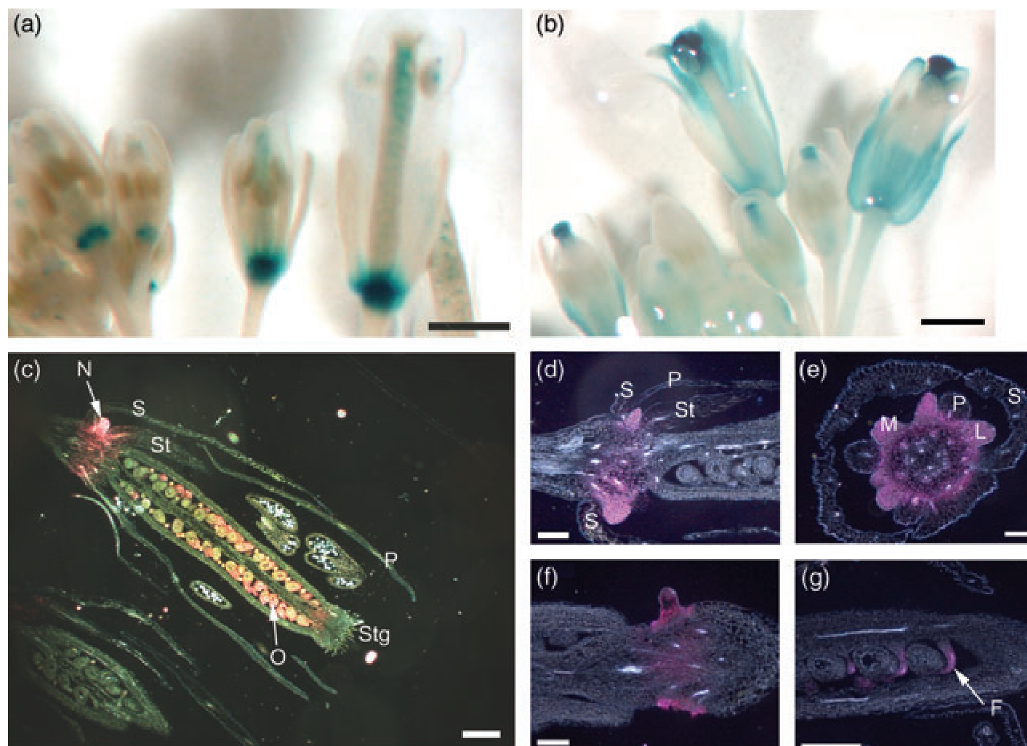

Figure 3. Expression patterns of the At5g44630 and At5g23960 promoter-GUS fusion genes in Arabidopsis Col flowers.
(a) At5g44630 promoter-GUS activity was observed at the base of flower buds and open flowers and in the ovary, particularly in proximity to the stigma.
(b) At5g23960 promoter-GUS activity was detected in the stigma of unopened flowers and newly opened flowers but was strongest in the stigma of older flowers. GUS staining was also observed in sepals of older flowers.
(c–g) Dark field microscopy of longitudinal and transversal sections of flowers of At5g44630 promoter-GUS lines. The GUS assay product is visible by the red or purple color. (c) In longitudinal sections, GUS activity was evident in the intrafloral nectaries at the base of the stamens and in ovules of the upper part of the ovary. (d, e) GUS staining in nectaries of longitudinal and transversal sections, respectively. GUS activity is visible in lateral (L) and median (M) nectaries. (f) GUS staining at the abscission zone of developing siliques. A nectary is visible in one part of the abscission zone. (g) GUS activity observed in funiculi of ovules. F, funiculus; N, nectary; O, ovule; P, petal; S, sepal; St, stamen; Stg, stigma.
Bars = 1 mm (a, b), 200 μm (c, g), and 100 μm (d–f).

observed in flowers, in agreement with previous results. At5g44630 promoter-GUS activity occurred primarily at the base of flower buds and open flowers, with higher intensity in older flowers (Figure 3a). Lateral and transverse sections of flowers revealed that staining at the flower base was restricted primarily to intrafloral lateral and median nectaries, sugar-producing organs located at the base of the stamens (Figure 3c–e). At the stage of silique development, GUS activity was also present in nectaries and in the abscission zone (Figure 3f). Furthermore, GUS staining was found in the ovules of older flowers and developing siliques (Figure 3a,c). Within the ovules, activity seemed to be restricted primarily to the funiculus (Figure 3g), with stronger staining often observed in ovules in closer proximity to the stigma (Figure 3a,c).

In plants transformed with the At5g23960 promoter-GUS fusion construct, strong GUS activity was visible at the stigma of open flowers with lower activity in mature flower buds (Figure 3b). Staining was also evident in sepals and anther filaments of some lines, but was absent in other floral organs.

Variation of floral volatile emission among Arabidopsis ecotypes

To survey natural variation in Arabidopsis floral terpene emission, we analyzed volatile profiles of detached inflorescences from 37 ecotypes from a range of different geographic regions (Table 1). There were no significant differences between the emission profiles of detached inflorescences and intact flowering plants of the same accessions (data not shown), although this was not checked for every ecotype. In total, eight different monoterpenes including myrcene, (*E*)-β-ocimene and linalool, small amounts of α-pinene and limonene and traces of α-thujene, sabinene and δ-3-carene were identified, while almost all the sesquiterpenes belonging to groups A and B, as described for the Columbia ecotype, were also detected. More than

Table 1 Emission of selected monoterpenes and sesquiterpenes from flowers of different *Arabidopsis thaliana* ecotypes

	Compound[a]					
Ecotype	myrcene	(*E*)-β-ocimene	linalool	(*E*)-β-caryophyllene	thujopsene	β-chamigrene
Oy-1	3.7 ± 1.7	3.7 ± 1.4	1.7 ± 0.7	n.d.	3.4 ± 1.2	1.9 ± 0.6
CVI	2.2 ± 0.5	tr	1.5 ± 0.4	tr	n.d.	n.d.
Can-0	0.3 ± 0.0	tr	2.0 ± 0.4	1.1 ± 0.4	1.1 ± 0.2	0.6 ± 0.1
Kas-1	0.8 ± 0.1	2.0 ± 0.2	1.9 ± 0.1	2.8 ± 0.8	3.3 ± 0.6	1.6 ± 0.3
Kil-0	2.3 ± 0.6	7.3 ± 1.6	6.6 ± 2.0	4.0 ± 1.0	2.6 ± 0.6	1.4 ± 0.3
Hodja	0.4 ± 0.1	1.9 ± 0.3	2.0 ± 0.3	5.0 ± 0.3	3.4 ± 0.2	1.7 ± 0.1
Ang-0	0.7 ± 0.3	7.3 ± 1.8	n.d.	5.6 ± 1.6	2.9 ± 0.6	1.5 ± 0.3
Pog-0	3.2 ± 1.0	2.8 ± 0.1	2.3 ± 0.6	5.8 ± 1.8	5.7 ± 1.0	2.9 ± 0.5
Sah-0	0.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	7.7 ± 1.3	0.9 ± 0.2	0.5 ± 0.1
Ws	3.1 ± 1.0	12.1 ± 3.0	4.8 ± 1.8	9.1 ± 2.8	5.4 ± 2.0	2.8 ± 1.0
Mr-0	0.5 ± 0.0	6.6 ± 0.5	3.4 ± 0.4	9.7 ± 1.2	2.2 ± 0.2	0.9 ± 0.1
Shakdara	1.1 ± 0.1	1.0 ± 0.2	0.4 ± 0.0	9.9 ± 0.7	3.2 ± 0.2	1.5 ± 0.1
Lip-0	2.0 ± 0.3	tr	4.7 ± 0.1	10.8 ± 1.3	2.2 ± 0.3	1.1 ± 0.2
Condara	1.6 ± 1.0	3.3 ± 0.4	4.1 ± 2.2	11.3 ± 5.7	3.3 ± 1.4	1.7 ± 0.7
Ag-0	3.3 ± 0.3	5.7 ± 1.0	3.2 ± 0.3	11.6 ± 1.4	2.3 ± 0.2	1.2 ± 0.1
Tsu-1	1.7 ± 0.1	0.2 ± 0.0	1.3 ± 0.2	13.8 ± 0.8	2.4 ± 0.3	1.3 ± 0.2
Jl-3	1.4 ± 0.2	3.7 ± 1.1	3.2 ± 0.6	14.1 ± 2.1	3.5 ± 0.9	1.8 ± 0.5
Di-g	0.9 ± 0.2	1.1 ± 0.4	0.7 ± 0.2	14.2 ± 1.7	3.2 ± 0.2	1.8 ± 0.1
Tu-1	1.6 ± 0.4	2.3 ± 0.5	1.8 ± 0.5	14.2 ± 2.5	3.4 ± 1.0	1.8 ± 0.5
Rsch-0	2.5 ± 0.7	2.1 ± 0.3	1.5 ± 0.4	17.7 ± 4.0	4.5 ± 0.7	2.5 ± 0.4
An-1	1.5 ± 0.3	4.5 ± 1.2	1.3 ± 0.3	18.4 ± 3.4	4.3 ± 1.3	2.1 ± 0.6
Pi-0	1.2 ± 0.2	tr	5.7 ± 0.2	18.6 ± 4.3	2.4 ± 0.3	1.1 ± 0.2
Col-0	1.3 ± 0.1	0.2 ± 0.1	1.1 ± 0.1	18.6 ± 2.7	2.0 ± 0.2	1.1 ± 0.1
Co-1	2.7 ± 1.0	3.4 ± 0.8	3.2 ± 1.4	18.6 ± 6.0	n.d.	n.d.
Bla-1	3.7 ± 0.3	11.7 ± 3.0	3.1 ± 0.7	20.1 ± 2.0	2.9 ± 0.4	1.4 ± 0.2
Stw-0	0.9 ± 0.2	0.5 ± 0.1	2.4 ± 0.4	20.5 ± 3.2	5.4 ± 1.3	2.7 ± 0.6
Ty-0	2.9 ± 0.3	1.9 ± 0.3	2.0 ± 0.0	21.4 ± 1.6	6.6 ± 0.6	3.3 ± 0.3
C-24	3.5 ± 0.2	3.4 ± 0.6	3.7 ± 0.7	22.3 ± 1.7	n.d.	n.d.
Aa-0	5.2 ± 1.5	0.4 ± 0.1	2.6 ± 09	27.4 ± 4.5	2.0 ± 0.3	1.1 ± 0.2
Tul-0	1.9 ± 0.3	1.2 ± 0.2	1.2 ± 0.2	27.9 ± 6.2	1.7 ± 0.4	0.9 ± 0.2
Sei-0	2.8 ± 1.3	0.9 ± 0.4	3.4 ± 1.2	28.0 ± 7.0	9.0 ± 1.4	4.5 ± 0.7
Mt-0	1.8 ± 0.1	2.8 ± 0.7	1.9 ± 0.4	34.6 ± 4.2	2.5 ± 0.4	1.3 ± 0.2
Bl-1	1.6 ± 0.3	n.d.	0.8 ± 0.1	34.8 ± 7.0	2.3 ± 0.3	1.1 ± 0.2
Ri-0	2.7 ± 0.5	0.9 ± 0.2	2.0 ± 0.3	38.1 ± 8.2	3.1 ± 0.3	1.7 ± 0.2
Wei-0	1.4 ± 0.2	3.7 ± 0.7	1.3 ± 0.3	47.2 ± 9.9	2.3 ± 0.6	1.2 ± 0.3
Est-0	1.7 ± 0.1	13.6 ± 1.3	7.5 ± 1.4	69.3 ± 8.5	8.6 ± 1.9	4.0 ± 0.9
Lu-1	4.3 ± 0.6	2.2 ± 0.3	1.7 ± 0.2	71.2 ± 12.3	n.d.	n.d.

[a]Volatiles were collected for 8 h from 70 detached inflorescences by the closed-loop stripping procedure. Emission was determined in ng h^{-1} per 70 inflorescences. Values are averages and standard errors of three to six independent collections. Only emissions of the most abundant monoterpenes and selected group A and B sesquiterpenes are shown. The order of ecotypes corresponds to increasing β-caryophyllene emission rates. tr, traces (emission equal or below 0.2 ng h^{-1}). n.d., not detected.

80% of the investigated ecotypes showed a similar composition, emitting most of the monoterpenes and the group A and B sesquiterpenes from their flowers (Figure 4 and Table 1). However, quantitative analyses revealed extensive differences in the emission rates of (*E*)-β-caryophyllene with an approximately 70-fold difference between Can-0 (1.1 ng $\times$ h^{-1} per 70 inflorescences) and Lu-1 (71 ng $\times$ h^{-1} per 70 inflorescences). Quantitative variation was less pronounced among the sesquiterpenes of group B. Among the monoterpenes, emission rates varied especially for linalool and (*E*)-β-ocimene, with both compounds having highest emission rates of approximately 7–14 ng $\times$ h^{-1} per 70 inflorescences. Other floral terpenes found in small amounts were α-farnesene, the C16-homoterpene 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and caryophyllene oxide.

Three ecotypes (Lu-1, Co-1, and C-24) were found to have a complete lack of emission of group B sesquiterpenes (Figures 4 and 5a), while the flowers of the ecotype Oy-1 did not emit any (*E*)-β-caryophyllene and the rest of the group A sesquiterpenes (Figures 4 and 5b). Additionally, no sesquiterpene volatiles were detected from flowers of the CVI ecotype besides traces of (*E*)-β-caryophyllene (Figure 4 and Table 1).

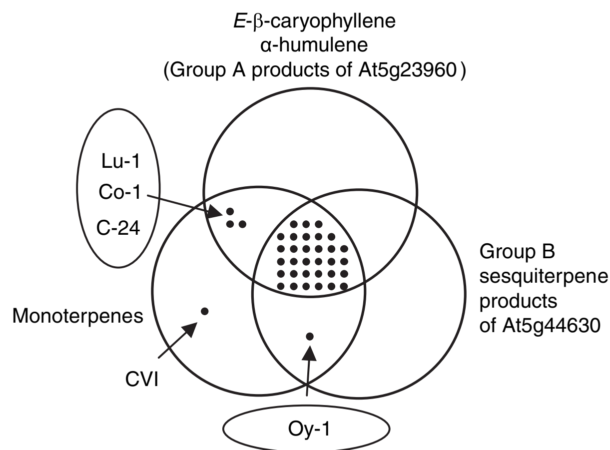

Figure 4. Division of Arabidopsis ecotypes according to floral monoterpene and sesquiterpene emissions.
The diagram groups 37 investigated ecotypes by their ability to emit monoterpenes and group A and group B sesquiterpenes from their flowers. Each dot represents one ecotype. Eighty-six percent of the ecotypes show floral emission of all three groups of terpenes.

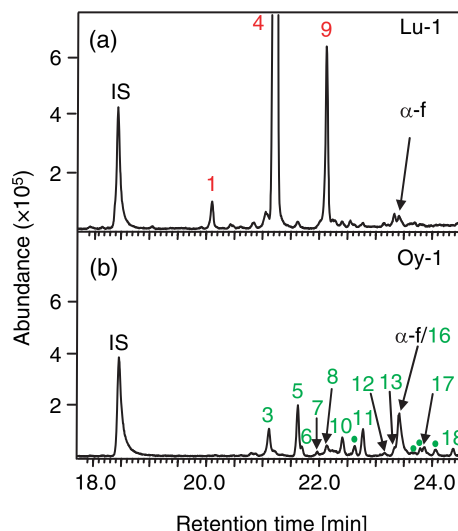

Figure 5. Sesquiterpene volatiles emitted from flowers of Lu-1 and Oy-1 ecotypes.
GC-MS chromatograms of sesquiterpene olefins collected from inflorescences of the ecotypes Lu-1 (a) and Oy-1 (b) as described in Experimental procedures. Only a portion of each chromatogram is shown. Numbers of individual peaks refer to numbers of compounds in Figure 1(e). Compounds labeled with red numbers in (a) are group A sesquiterpenes formed by the At5g23960 enzyme. Green numbers in (b) indicate group B products of At5g44630 TPS. Both ecotypes emit α-farnesene (α-f) from their flowers. Some minor group B sesquiterpenes could not be identified, most likely due to coelution with α-farnesene. Coelution of compounds is indicated by slashes. Peaks labeled with dots represent unidentified sesquiterpenes. IS, internal standard.

To examine whether the absence of group A or B sesquiterpenes in flowers of these unusual ecotypes was caused by a lack of expression of At5g23960 and At5g44630, we analyzed transcript levels of those genes in floral tissue extracts of these ecotypes by RT-PCR. In flowers of ecotypes Oy-1 and CVI, which lack emission of group A sesquiterpenes, expression of At5g23960 was detected with one pair of primers but not with a second primer pair combination (Figure 6a). To examine the reasons for the failure of the second set of primers in more detail, a full-length cDNA of At5g23960 was isolated by RT-PCR from flowers of the Oy-1 ecotype with the appropriate primers, and the protein sequence encoded by the open reading frame of this cDNA compared with the equivalent sequence of the Col ecotype. This comparison revealed a highly divergent region between F520 and V538 that included a 33-nucleotide deletion and sequence differences for five amino acids in the Oy-1 sequence relative to that of the Col ecotype (Figure 6c). These differences cause the loss of one of the PCR primer-binding sites (Figure 6c). Expression of the cDNA of the Oy-1 At5g23960 homolog in *E. coli* and enzyme assays of the purified recombinant protein with FPP as substrate did not show any TPS activity (data not shown) indicating that the lack of group A sesquiterpene emission from Oy-1 flowers is most likely due to a non-functional At5g23960 TPS enzyme.

Expression of the gene At5g44630 responsible for group B sesquiterpene formation was found by RT-PCR in flowers of all accessions (Lu-1, Co-1, C-24, and CVI) that lacked the floral emission of these terpenes (Figure 6b). A full-length cDNA of At5g44630 isolated from floral tissue of the Lu-1 ecotype encoded a protein that was almost identical to the equivalent proteins of Ler and Col with only two and four single amino acid differences, respectively. Expression of this cDNA in *E. coli* resulted in the formation of a protein that was capable of converting FPP to sesquiterpene products of group B as shown for the At5g44630 enzyme (data not shown). Hence, the lack of emission of group B terpenes from flowers of the Lu-1 ecotype is apparently not caused by the presence of a non-functional At5g44630 gene, but is instead the result of some specific transcriptional, post-transcriptional, or post-translational regulation in this particular ecotype.

Discussion

Two TPS enzymes produce all the sesquiterpene volatiles emitted by Arabidopsis flowers

Terpene volatile mixtures emitted from both floral and vegetative tissues of many plant species have been shown to serve as important olfactory cues in plant–animal interactions (Dicke and Van Loon, 2000; Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002; Turlings *et al.*, 1995). The mixture of terpenes released by flowers of most of the Arabidopsis ecotypes is dominated by a complex group of sesquiterpene hydrocarbons comprising over 20

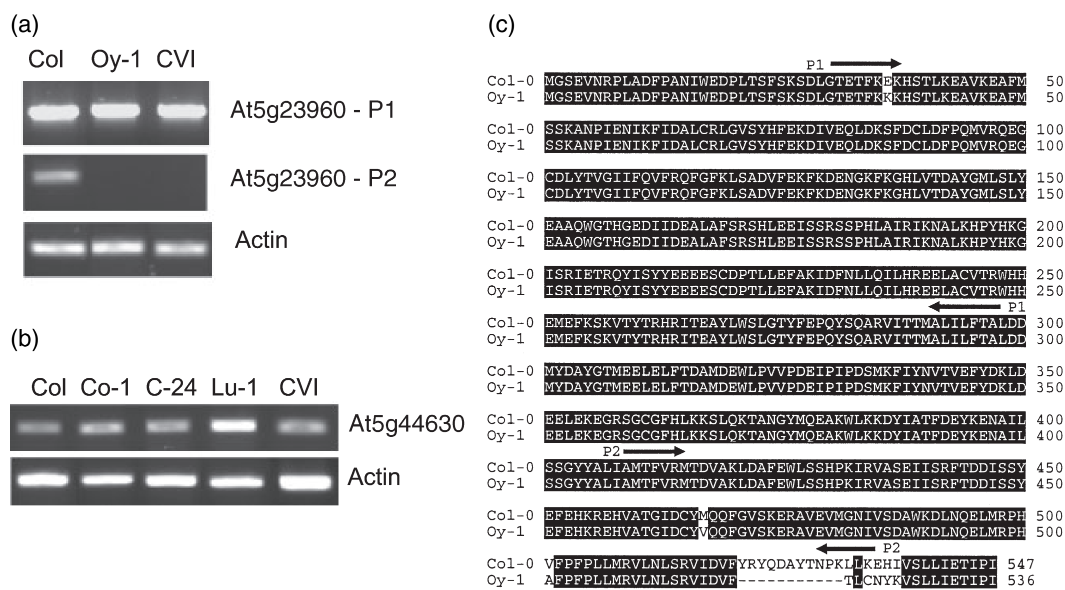

Figure 6. Expression of At5g23960 and At5g44630 alleles in flowers of Arabidopsis ecotypes lacking either group A or group B sesquiterpene emissions.
(a) RT-PCR analysis of transcript levels of At5g23960 in flowers of the Columbia ecotype and ecotypes Oy-1 and CVI which show no emission of group A sesquiterpenes. PCR reactions were performed with two different primer pairs P1 and P2 derived from the At5g23960 sequence (shown in c). Gene-specific PCR products of similar length were obtained for all ecotypes only with primer pair P1.
(b) Expression of At5g44630 in flowers of Col and ecotypes Co-1, C-24, Lu-1, and CVI lacking group B sesquiterpene emissions. RT-PCR reactions with primers derived from the Col At5g44630 sequence resulted in gene-specific products of equal length in all ecotypes. RT-PCR reactions with primers for *actin-8* were performed to judge equality of the cDNA template concentration.
(c) Alignment of amino acid sequences encoded by the At5g23960 gene of Col and its equivalent gene in Oy-1. Underlined is a sequence region between F520 and V538 of the Col protein that shows a deletion and changes of five amino acids in the Oy-1 sequence. Arrows indicate the positions of primer pairs P1 and P2 (shown above the corresponding gene sequence) applied in RT-PCR reactions for analysis of At5g23960 gene expression in flowers of Col and Oy-1.

different compounds and also contains a minor fraction of monoterpenes, including myrcene and (+)-linalool (Figure 1a, Aharoni *et al.*, 2003; Chen *et al.*, 2003). The number of enzymes involved in the formation of such terpene mixtures is often difficult to determine for several reasons. First, many TPSs have been shown to catalyze the formation of multiple products from a single prenyl diphosphate substrate (GPP for monoterpene synthases, FPP for sesquiterpene synthases) (Köllner *et al.*, 2004; Steele *et al.*, 1998). In addition, many plants, including Arabidopsis, contain large families of *TPS* genes, and different TPS enzymes active within the same tissue may synthesize partially overlapping mixtures of compounds. Thus, the precise identification of individual TPS enzymes responsible for the production of particular terpene volatiles requires a combination of methods, including terpene profiling, genetic analysis, and *in vitro* biochemical characterization of the enzymes encoded by candidate *TPS* genes.

The *in vitro* assays and product analyses of recombinant enzymes encoded by the two florally expressed genes At5g23960 and At5g44630 indicated that virtually all Arabidopsis floral sesquiterpene volatiles could be produced by these two TPS enzymes, with the first enzyme responsible for the synthesis of group A sesquiterpenes and the second enzyme responsible for the synthesis of group B sesquiterpenes. Consistent with the biochemical results, floral volatile analyses of plant lines with a T-DNA insertion in gene At5g23960 showed a lack of floral emissions of the group A sesquiterpene products, including the major volatile (*E*)-β-caryophyllene, while flowers of a At5g44630 T-DNA mutant line lacked the sesquiterpene products of group B (Figure 1b,c).

Properties and mechanisms of the recombinant At5g23960 and At5g44630 enzymes

The enzymatic properties of the At5g23960 and At5g44630 TPSs are comparable to those determined for other plant sesquiterpene synthases (Cane, 1999). Both enzymes have a low K_m value for the substrate FPP, in the 1–2 μM range, and a requirement for a divalent metal cofactor for catalysis. The At5g446300 TPS, but not the At5g23960 TPS, also accepts GPP as substrate *in vitro* with similar binding affinities and conversion rates. TPS enzymes reported from other plants are capable of acting on both GPP and FPP *in vitro* (Köllner *et al.*, 2004). Whether these enzymes produce monoterpenes or sesquiterpenes *in planta* depends on their subcellular location and the substrate availability in the particular cell compartment. Hence, the formation of monoterpenes from GPP by the At5g44630 TPS enzyme *in vivo* is probably not physiologically relevant as the protein is most likely located in the cytosol, a compartment

that is not believed to contain significant levels of GPP. This conclusion is also supported by floral volatile analysis of the At5g44630 T-DNA insertion line, which showed no difference in monoterpene emission profiles in comparison with the wild type.

At5g23960 TPS produces only two main products, *E*-β-caryophyllene and α-humulene, and the formation of these compounds via a humulyl cation has been previously described (Figure 7; Cane, 1999). In comparison, At5g446300 TPS catalyzes the formation of over 15 different sesquiterpene hydrocarbons with different molecular structures including acyclic, mono-, bi-, and tricyclic compounds (Figure 7). While several of these products like (*E*)-β-farnesene and β-chamigrene are well-known compounds emitted from flowers or leaves of many other angiosperm species, other products such as the terpenes isobazzanene, (+)-α-barbatene and (+)-β-barbatene are common in liverworts (Warmers and König, 1999) but have only been rarely reported from vascular plants (König *et al.*, 1996).

The formation of all products of the At5g44630 TPS can be accounted for by a carbocationic mechanism which follows closely those proposed for other sesquiterpene synthases (Cane, 1999). Ionization of the substrate FPP and subsequent ring closure between C1 and C6 (numbered as in FPP) lead to a bisabolyl cation, the first cyclic intermediate and precursor to a large range of sesquiterpene skeletons (Figure 7, Cane, 1999). The major fate of the bisabolyl cation is conversion to the cuprenyl cation via 11,7-cyclization followed by an unusual 1,4-hydride shift, well-documented by feeding of tritium-labeled precursors to organisms making similar compounds (Cane, 1981). All of the major products of the enzyme are formed via the cuprenyl cation.

While At5g44630 makes approximately 15 different products, this pales in comparison with the abilities of two *Abies grandis* multi-product sesquiterpene synthases that make 34 and 52 products, respectively (Steele *et al.*, 1998). However, nearly all products of At5g44630 (except (*E*)-β-farnesene) are formed via initial C1–C6 cyclization while the *A. grandis* enzymes make products via C1–C6, C1–C10 and C1–C11 cyclization. The ability of TPSs to make multiple products is thought to be a consequence of the carbocationic mechanism that involves a large number of intermediates, many of which can have multiple metabolic fates. However, the formation of multiple products is not an unavoidable outcome when many carbocationic intermediates are involved. For example, the fungal TPS trichodiene synthase has a mechanism that involves many of the same steps as At5g44630 employs leading to isobazzanene, but only a single product is formed (Cane, 1999). Curiously, site-specific mutagenesis of trichodiene synthase led to the formation of several additional sesquiterpenes which are also part of the product spectrum of At5g44630 (Cane and Xue, 1996).

At5g446300 is the first characterized gene of a large clade of Arabidopsis putative sesquiterpene and diterpene synthases (Figure 2b, group 4). This gene cluster is clearly distinct from sesquiterpene and diterpene synthases of other angiosperms (group 5) and most likely evolved independently in a lineage leading to Arabidopsis by multiple gene duplications and diversifications. In contrast, At5g23960 has evolved separately from group 4 TPSs, and the protein encoded by this gene shows some similarities but no close clustering with TPSs of group 5 (Figure 2b), including (*E*)-β-caryophyllene synthases of *Artemisia annua* (Cai *et al.*, 2002) and *Cucumis sativus* (Mercke *et al.*, 2004) (39% sequence identity with both enzymes) and a casbene synthase from *Ricinus communis* (40% sequence identity).

Arabidopsis floral sesquiterpene synthase genes are expressed in different floral organs and may have various ecological roles

Floral volatiles play important roles in pollinator attraction (Dudareva and Pichersky, 2000). Compared with typical insect-pollinated plants such as *C. breweri*, whose flowers are strongly scented, single flowering plants of the mainly self-pollinating *A. thaliana* emit approximately 200-fold less volatiles per hour (Chen *et al.*, 2003). Nevertheless, in nature, Arabidopsis flowers are often visited by insects, including flies and solitary bees (Hoffmann *et al.*, 2003). These visits most likely contribute to the infrequent outcrossing events that have been observed in natural populations (Abbott and Gomes, 1989; Snape and Lawrence, 1971). Such outcrossing might stabilize these populations by reducing inbreeding depression. Whether the sesquiterpene volatiles released by Arabidopsis flowers play a role in attracting insect pollinators, and the exact role of such attraction is yet to be determined.

In most plants, petals are the main source of floral volatile emission. For example, in flowers of *C. breweri*, the monoterpene alcohol (+)-linalool is synthesized and emitted mostly by petals (Pichersky *et al.*, 1994). In contrast, GUS reporter fusion experiments conducted with the promoter of the At5g23960 gene encoding caryophyllene synthase demonstrated strongest promoter activity in the stigma of the open flower (Figure 3b). No gene expression was observed in flower petals, but some activity was evident in sepals (Figure 3b). Similar patterns of expression had been

Figure 7. Proposed reaction mechanism for the formation of sesquiterpene products by At5g44630 and At5g23960 TPSs.
The substrate FPP and reaction products of At5g44630 TPS, identified by GC-MS, are enclosed in solid boxes. Reaction products of At5g23960 TPS are enclosed in dashed boxes. α-copaene, a minor product of the At5g23960 enzyme, is not shown. WMR indicates a Wagner–Meerwein rearrangement. OPP indicates diphosphate moiety.

α-humulene

$H^{\oplus}$

2,10 closure

$H^{\oplus}$

(*E*)-β-caryophyllene

11,1 closure

14 7 8 6 5 4 9 3 10 2 15 1 13 11 12 OPP

farnesyl diphosphate

$^{\ominus}$OPP

OPP

rotation

nerolidyl diphosphate (transoid)

OPP $^{\ominus}$OPP

nerolidyl diphosphate (cisoid)

$H^{\oplus}$

(*E*)-β-farnesene

6,1 closure

10,6 closure

1,2 $H^{\ominus}$ shift

rotation

$H^{\oplus}$

bisabolyl cation

β-bisabolene

$H^{\oplus}$

β-acoradiene

1,3 $H^{\ominus}$ shift

$H^{\oplus}$

α-zingiberene

1,3 $H^{\ominus}$ shift

$H^{\oplus}$

β-sesquiphellandrene

11,7 closure

α-cuprenene

$H^{\oplus}$

1,4 $H^{\ominus}$ shift

1,2 $H^{\ominus}$ shift

1,2 methyl shift

$H^{\oplus}$

$H^{\oplus}$

cuprenyl cation

isobazzanene

δ-cuprenene

rotation

1,3 methyl shift

WMR (8,6)

WMR (11,6)

≡

WMR (1,7)

$H^{\oplus}$ $H^{\oplus}$

α-chamigrene

β-chamigrene

2,11 closure

1,2 methyl shift

$H^{\oplus}$

2,7 closure

$H^{\oplus}$

α-barbatene

β-barbatene

thujopsene

observed with promoter-GUS fusion lines of the florally expressed monoterpene synthase gene At3g25810 (Chen *et al.*, 2003). Plant lines carrying GUS fusions with the promoter of the second floral sesquiterpene synthase gene, At5g44630, also did not show any GUS activity in petals. Instead, activity appeared to be strongest at the base of unopened and opened flowers (Figure 3a), where GUS staining was observed in lateral and median intrafloral nectaries (Figure 3c–f). Arabidopsis nectaries secrete small amounts of glucose and fructose containing nectar (Davis *et al.*, 1998) that may serve as a reward for small insect visitors. Sesquiterpenes emitted from floral nectaries of the Arabidopsis flower might provide insects with a short-range guide to the site of nectar secretion.

Sugar-containing solutions secreted on the nectary surface also represent a preferred medium for bacterial or fungal growth, particularly after dilution by rain water (Buban *et al.*, 2003). For example, flowers of several species in the Rosaceae family, including apple and pear, can be infected in the vicinity of nectaries by the bacterial pathogen *Erwinia amylovora* entering the tissue through stomata-like openings called nectarthodes (Buban *et al.*, 2003). It is therefore possible that plants have developed defense mechanisms to prevent microbial entrance at the site of floral nectaries. EST and DNA microarray analyses of nectaries from ornamental tobacco revealed an increased expression of defense-related genes in these floral organs (Thornburg *et al.*, 2003). As many terpenes have antimicrobial properties (Deans and Waterman, 1993), terpenes present at the site of nectar secretion might serve as disinfectants.

The sesquiterpenes, including (*E*)-β-caryophyllene, as well as the monoterpenes synthesized in the Arabidopsis stigma may serve similar defensive functions. The papillae at the surface of stigmas are thought to help create a humid microenvironment to optimize pollen hydration and germination (Heslop-Harrison, 2000). As these conditions could also promote the growth of microorganisms, the presence of antimicrobial defenses on the stigma surface may have been a product of natural selection. The presence of terpenes in the stigma is not restricted to Arabidopsis. Linalool and linalool oxide are produced in pistils of *Clarkia* species (both in the strongly scented, hawkmoth-pollinated *C. breweri*, and in the non-scented, bee-pollinated *C. concinna*, which does not emit monoterpenes from its petals), suggesting that these have a function unrelated to pollinator attraction, such as antimicrobial defense (Dudareva *et al.,* 1996; Pichersky *et al.*, 1994).

Emission of monoterpenes and sesquiterpenes shows some variation among A. thaliana *ecotypes*

Natural intraspecific variation in vegetative and floral terpene profiles of many domesticated and non-domesticated species has been noted (Croteau and Gershenzon, 1994), but the molecular basis for such variation has been previously investigated only in cultivars of basil (Iijima *et al.*, 2004), maize (Köllner *et al.*, 2004), and tomato (van der Hoeven *et al.*, 2000), where human selection has occurred. In the present study, the majority of the 37 investigated ecotypes of A. thaliana contained virtually the same set of monoterpenes and sesquiterpenes (Table 1). However, some ecotypes lacked either group A or group B sesquiterpene volatile emission. Expression analyses of the *TPS* genes At5g23960 or At5g44630 in the flowers of these ecotypes and determination of enzyme activities of the corresponding recombinant proteins from two ecotypes suggested that the lack of biosynthesis of group A or group B sesquiterpenes appeared to be the result of gene mutation or post-transcriptional/translational mechanisms and not simply down-regulation of these genes (Figure 6). The near-ubiquitous presence of the same set of monoterpenes and sesquiterpenes in the flowers of different *A. thaliana* ecotypes suggests that the role of such terpenes is widespread in this species. It remains to be determined whether the quantitative and rare qualitative variations of terpene volatile emissions reflect selective adaptations to particular pollinator populations or to other biotic or abiotic factors present in different habitats.

Experimental procedures

Plant material

Seeds of all *A. thaliana* ecotypes except of Col-0, Ws-2 and CVI were obtained as bulk lines from the NASC (http://arabidopsis.info/) and ABRC (http://www.arabidopsis.org/abrc/) stock centers. Seeds of the At5g44630 T-DNA insertion line Salk_126868 (N626868) and the At5g23960 T-DNA insertion lines Salk_136913 (N636913) and Salk_138212 (N638212) (Alonso *et al.*, 2003) were obtained from the NASC stock center. If not stated otherwise, all plants were grown, to the flowering state on soil in a controlled climate-chamber [22°C, 55% relative humidity, and 100 μmol m^{-2} sec^{-1} photosynthetically active radiation (PAR)] under long-day conditions (16 h light/8 h dark photoperiod) for up to 6 weeks.

Reagents

[1-^{3}H]-FPP (37 GBq mol^{-1}) and [1-^{3}H]-GPP (37 GBq mol^{-1}) were products of American Radiolabeled Chemicals (St Louis, MO, USA). Unlabeled GPP and FPP were from Echelon Research Laboratories Inc. (Salt Lake City, UT, USA).

Volatile collection and analysis from Arabidopsis flowers

Volatile collections from flowers were performed under controlled temperature and light conditions (22°C, 160 μmol m^{-2} sec^{-1} PAR) in 1 l bell jars with 70 detached inflorescences placed in a small glass beaker filled with tap water. Inflorescences of most ecotypes had an average of four to five open flowers. Emitted volatiles were collected for 8 h on 25 mg Super Q (Supelco; Sigma-Aldrich, St Louis, MO, USA) traps in a closed loop stripping procedure according to

Donath and Boland (1995). Volatiles were eluted from the traps with 100 μl CH_2Cl_2, and 120 ng of nonyl acetate were added as an internal standard. Sample analysis and quantification of terpenes was performed by gas chromatography–mass spectrometry (GC-MS), under conditions described previously (Chen *et al.*, 2003). Compounds were identified by comparison of retention times and mass spectra with those of authentic standards and with reference spectra in the NIST and Wiley libraries (Agilent Technologies, Palo Alto, CA, USA). A standard of racemic α-barbatene was kindly provided by Wilfried A. König (University of Hamburg, Germany). Isobazzanene, β-barbatene, β-acoradiene, α-cuprenene, and δ-cuprenene were identified by comparison with sesquiterpene constituents of the essential oil from the liverwort *Bazzania trilobata*, characterized by Warmers and König (1999). α-Zingiberene was identified by comparison with the essential oil of *Zingiber officinale* obtained by Tobias Köllner (Max Planck Institute for Chemical Ecology). All other terpene standards were obtained commercially from Fluka (Seelze, Germany), Roth (Karlsruhe, Germany) and Sigma. The absolute configurations of β-chamigrene, cuparene, β-bisabolene, α-barbatene and thujopsene were determined by separation on chiral columns and under temperature conditions as described in detail by Chen *et al.* (2003). Chirality of β-barbatene was determined under comparable conditions as those applied for α-barbatene, but at a constant column temperature of 90°C and the use of only the (+)-enantiomeric standard.

Isolation and cloning of At5g44630 and At5g23960 cDNAs and protein expression in E. coli

Total RNA was isolated from Arabidopsis flowers and transcribed into cDNA as described previously (Chen *et al.*, 2003). Alternatively, RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions, and 2 μg of this RNA was reverse transcribed into cDNA in a 20 μl reaction with 0.5 μg poly(dT) primer, 0.2 mM of each desoxynucleoside triphosphate (dNTP), and 200 U Superscript II Reverse Transcriptase (Invitrogen). Two primers, 5′-AATGGAAGCATTAGGAAACTTTG-3′, and 5′-CTAAAGAAGTATAGGATCTACGAG-3′, which correspond to the start and the end of the coding region of At5g44630 were used for PCR amplification of At5g44630 cDNAs from Ler and Lu-1 ecotypes. The coding region of At5g23960 was amplified from Col and Oy-1 ecotypes by PCR using the forward primer 5′-CCATGGG-GAGTGAAGTCAACC-3′ which introduced an *Nco*I site at the initiating ATG codon in combination with the reverse primer 5′-GGATCCCGAATGGGTATAGTTTCAATGAG-3′ containing a *Bam*HI site which allowed a translational fusion to a carboxyl-terminal His_6-tag. Amplified At5g44630 cDNA products were inserted into the pCRT7/CT-TOPO expression vector (Invitrogen), while amplified cDNA products of At5g23960 were inserted into *Nco*I and *Bam*HI sites of the expression vector pET28A (Novagen, EMD Biosciences, Madison, WI, USA). Sequencing revealed that no errors had been introduced during PCR amplifications. For ecotypes other than Col, sequences of products from three independent PCR reactions were analyzed to verify the ecotype-specific gene sequence. The resulting plasmids were transformed into *E. coli* BL21 Codon Plus cells. Bacterial growth conditions and induction of gene expression were as previously described (Chen *et al.*, 2003).

Purification of At5g44630 and At5g23960 recombinant proteins

The At5g44630 TPS protein was expressed in *E. coli* without fusion to a histidine-tag as addition of a His_6-tag led to a complete loss of enzyme activity. Extraction and partial purification of the recombinant Ler At5g44630 protein essentially followed the procedures described by Chen *et al.* (2003, 2004). At5g23960 TPS proteins, carrying a C-terminal His-tag, were purified from crude bacterial extract on 1 ml Ni-NTA-agarose columns (Qiagen, Hilden, Germany) as described by Tholl *et al.* (2004) and according to the manufacturer's protocols. Following desalting into assay buffer (10 mM Mopso, pH 7.0, 10% glycerol, 5 mM DTT), active fractions were used for determination of enzyme properties.

Terpene synthase enzyme assays and enzyme characterization

Terpene synthase enzyme assays and identification of TPS products by solid phase microextraction and GC-MS analysis were performed under standard conditions as previously described (Chen *et al.*, 2003). For enzyme characterizations, assays were carried out for 20 min in 50 μl reaction volumes with 0.5–1 μg partially purified At5g44630 TPS or At5g23960 TPS enzyme and 4 μM [1-^{3}H]-FPP (17 MBq μmol^{-1}). Reaction products were extracted with 250 μl hexane and their total radioactivity was determined by scintillation counting. Absence of phosphatase activity was verified by GC-MS analysis of extracted TPS products from assays with unlabeled substrates. To determine the divalent metal ion requirement, assays were incubated under standard conditions and increasing $MgCl_2$ (without Mn^{2+}) and $MnCl_2$ (in the absence of Mg^{2+}) concentrations, and for evaluation of the K_m values for FPP and GPP, six to eight different concentrations of [1-^{3}H]-FPP (17 MBq μmol^{-1}) and [1-^{3}H]-GPP (4.2 MBq μmol^{-1}), respectively, were applied. Assays were conducted in three replicates, and apparent K_m values were determined by Hanes-Plot analysis with the Hyper 1.01 program (J.S. Easterby, University of Liverpool).

Construction of At5g44630 and At5g23960 promoter-GUS reporter gene fusions and histochemical localization of GUS activity

The 2.3 kb promoter of At5g44630 was isolated from genomic DNA of Col via PCR using the forward primer 5′-TCTAGAGGGCCAA-CAATGGAG-3′, containing an *Xba*I site, and the reverse primer 5′-GGATCCATAGATAGAGAGAGATGTGG-3′, containing the At5g44630 start codon and a *Bam*HI site. PCR reactions were performed and the resulting PCR product inserted into the *Xba*I and *Bam*HI cloning sites of the GUS gene containing binary vector pDW137 (Blázquez *et al.*, 1997) according to Chen *et al.* (2004). A 1.96-kb At5g23960 promoter-GUS fusion construct was obtained accordingly. For PCR amplification of the At5g23960 promoter the forward primer 5′-CTGCAGTATTGTTGGAGATGTTCTTACCG-3′, containing a *Pst*I site, and the reverse primer 5′-GGATCCATGC-TAAAAATTTCTGGTAGAAG-3′ were used. Transformations of both promoter-fusion constructs into Arabidopsis Col plants, selection of transformed lines and GUS enzyme assays were conducted as described previously (Chen *et al.*, 2003, 2004). For sectioning of GUS-stained flowers, single flowers were embedded in Paraplast according to Blázquez *et al.* (1997) with modifications for an automatic Leica ASP300 embedding device (Wetzlar, Germany). Ten micrometer thick sections were prepared on a Leica microtome and transferred to slides. Paraffin was removed with Histo-Clear solution (Fisher Scientific, Schwerte, Germany) and sections were mounted with Permount medium (Fisher Scientific). Microscopy of

sections was performed under dark-field illumination with a Zeiss Axioskop (Jena, Germany) microscope.

Determination of At5g44630 and At5g23960 gene expression by RT-PCR

For expression analysis of the At5g44630 gene in floral tissue of different ecotypes, RT-PCR reactions were performed with At5g44630-specific primers 5′-CGTGGAGGTCATGTTCCTAG-3′ and 5′-GTGCATAATTGACGGATTGC-3′. For analysis of expression of the gene At5g23960, two primer pairs, P1 with forward primer 5′-GAACTGAGACGTTCAAAGAG-3′ and reverse primer 5′-GCGCTGTGAATAAGATTAGTGC-3′ and P2 with forward primer 5′-GCGATGACATTCGTGAGAATG-3′ and reverse primer 5′-CCTTCAAGAGCTTGGGGTTG-3′, respectively, were used. RT reactions were conducted as described for cDNA cloning. PCR reactions were carried out with 0.5 μM of each primer, 0.2 mM of each desoxynucleoside triphosphate, and 0.5 U of Platinum Taq Polymerase (Invitrogen). To insure the application of equal amounts of cDNA in comparative PCR reactions among different ecotypes, control PCR reactions were performed with primers 5-ATGAAGATTAAGGTCGTGGCAC-3′ and 5′-GTTTTTATCCGAGTTTGAAGAGGC-3′ for *actin-8* and different amounts of cDNA template of each ecotype, and each reaction was carried out in 24–30 cycles. Amplified products were separated on 1% agarose gels and quantified using the Gene Tools Analysis Software (SynGene, Cambridge, UK). The optimal amounts of cDNA templates for PCR reactions with *TPS*-specific primers were chosen according to equal amplification of the *actin-8* PCR products for all compared ecotypes. Amplifications of At5g44630 products were performed with 28 cycles, while PCR reactions for amplification of At5g23960 products were carried out with 24 cycles for primer pair P1 and 27 cycles for primer pair P2. *TPS* gene-specific PCR products were confirmed by sequencing. The relative positions of the At5g44630 and At5g23960 alleles in the genome of other ecotypes were confirmed by PCR reactions with Col-specific genomic primers.

Acknowledgements

We thank Katrin Heisse and Bettina Raguschke for excellent technical assistance, and Tom Mitchell-Olds for the seeds of all the ecotypes except Col-0, Ws-2, and Cvi. We are grateful to W. König for providing several sesquiterpene standards and to Markus Schmid and Detlef Weigel for sharing results before publication and help in embedding and sectioning of Arabidopsis flowers and microscopic documentation. We thank Manfred Barth (Carl Zeiss Jena GmbH) for the opportunity to conduct microscopy under dark field illumination. This work was supported by National Science Foundation Grant IBN-0211697 to E.P. and by funds of the Max Planck Society to J.G.

Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2417/TPJ2417sm.htm

Figure S1. Identification of enzyme products of At5g44630 TPS catalysis.

Mass spectra of (+)-α-barbatene (a), (+)-thujopsene (c), β-acoradiene (e), and (+)-β-chamigrene (g) produced by the incubation of FPP with a cell-free extract of *E. coli* expressing the At5g44630 protein and separated by gas chromatography. Mass spectra of corresponding authentic standards obtained under the same conditions as in (a), (c), (e), and (g), respectively, are shown in (b), (d), (f), and (h), respectively. *m/z*, mass-to-charge ratio.

References

Abbott, R.J. and Gomes, M.F. (1989) Population genetic structure and outcrossing rate of *Arabidopsis thaliana* (L) Heynh. *Heredity*, **62**, 411–418.

Aharoni, A., Giri, A.P., Deuerlein, S., Griepink, F., de Kogel, W.J., Verstappen, F.W.A., Verhoeven, H.A., Jongsmaa, M.A., Schwab, W. and Bouwmeester, H.J. (2003) Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. *Plant Cell*, **15**, 2866–2884.

Alonso, J.M., Stepanova, A.N., Leisse, T.J. ***et al.*** (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Aubourg, S., Lecharny, A. and Bohlmann, J. (2002) Genomic analysis of the terpenoid synthase (*AtTPS*) gene family of *Arabidopsis thaliana*. *Mol. Genet. Genomics*, **267**, 730–745.

Blázquez, M.A., Soowal, L.N., Lee, I. and Weigel, D. (1997) *LEAFY* expression and flower initiation in *Arabidopsis*. *Development*, **124**, 3835–3844.

Bohlmann, J., Meyer-Gauen, G. and Croteau, R. (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proc. Natl Acad. Sci. USA*, **95**, 4126–4133.

Buban, T., Orosz-Kovacs, Z. and Farkas, A. (2003) The nectary is the primary site of infection by *Erwinia amylovora* (Burr.) Winslow et al.: a mini review. *Plant Syst. Evol.* **238**, 183–194.

Cai, Y., Jia, J.-W., Crock, J., Lin, Z.-X., Chen, X.-Y. and Croteau, R. (2002) A cDNA clone for β-caryophyllene synthase from *Artemisia annua*. *Phytochemistry*, **61**, 523–529.

Cane, D.E. (1981) Biosynthesis of sesquiterpenes. In *Biosynthesis of Isoprenoid Compounds*, Vol. 1 (Porter, J.W. and Spurgeon, S.L., eds). New York: John Wiley & Sons, pp. 283–374.

Cane, D.E. (1999) Sesquiterpene biosynthesis: cyclization mechanisms. In *Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and Steroids*, Vol. 2 (Barton, S.B., Nakanishi, K. and Meth-Cohn, O., eds). Oxford: Pergamon, pp. 155–200.

Cane, D.E. and Xue, Q. (1996) Trichodiene synthase. Enzymatic formation of multiple sesquiterpenes by alteration of the cyclase active site. *J. Am. Chem. Soc.* **118**, 1563–1564.

Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E. and Gershenzon, J. (2003) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. *Plant Cell*, **15**, 481–494.

Chen, F., Ro, D.K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E. and Tholl, D. (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. *Plant Physiol.* **135**, 1956–1966.

Cowan, M.M. (1999) Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **12**, 564–582.

Croteau, R. and Gershenzon, J. (1994) Genetic control of monoterpene biosynthesis in mints. In *Genetic Engineering of Plant Secondary Metabolism, Recent Advances in Phytochemistry*, Vol. 28 (Ellis, B.E., Kuroki, G.W. and Stafford, H.A., eds). New York: Plenum Press, pp. 193–229.

Davis, E.M. and Croteau, R. (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. In *Topics in Current Chemistry: Biosynthesis – Aromatic Polyketides, Isoprenoids, Alkaloids* (Leeper, F.J. and Vederas, J.C., eds). Heidelberg: Springer-Verlag, pp. 53–95.

Davis, A.R., Pylatuik, J.D., Paradis, J.C. and Low, N.H. (1998) Nectar-carbohydrate production and composition vary in relation to

nectary anatomy and location within individual flowers of several species of Brassicaceae. *Planta*, **205**, 305–318.

Deans, S.G. and Waterman, P.G. (1993) Biological activity of volatile oils. In *Volatile Oil Crops: Their Biology, Biochemistry and Production* (Hay, R.K.M. and Waterman, P.G., eds). Essex, England: Longman Scientific and Technical, pp. 97–111.

Dicke, M. and Van Loon, J.J.A. (2000) Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomol. Exp. Appl.* **97**, 237–249.

Dobson, H.E.M. (1994) Floral volatiles in insect biology. In *Insect–Plant Interactions* (Bernays, E.A., ed.). Boca Raton: CRC Press, pp. 47–81.

Donath, J. and Boland, W. (1995) Biosynthesis of acyclic homoterpenes – enzyme selectivity and absolute configuration of the nerolidol precursor. *Phytochemistry*, **39**, 785–790.

Dudareva, N. and Pichersky, E. (2000) Biochemical and molecular genetic aspects of floral scents. *Plant Physiol.* **122**, 627–633.

Dudareva, N., Cseke, L., Blanc, V.M. and Pichersky, E. (1996) Evolution of floral scent in *Clarkia* – novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell*, **8**, 1137–1148.

Dudareva, N., Martin, D., Kish, C.M., Kolosova, N., Gorenstein, N., Fäldt, J., Miller, B. and Bohlmann, J. (2003) (*E*)-β-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. *Plant Cell*, **15**, 1227–1241.

Gershenzon, J. and Kreis, W. (1999) Biochemistry of terpenoids: monoterpenes, sesquiterpenes, diterpenes, sterols, cardiac glycosides and steroid saponins. In *Biochemistry of Plant Secondary Metabolism, Annual Plant Reviews* (Wink, M., ed.). Sheffield: Academic Press, pp. 222–280.

Heslop-Harrison, Y. (2000) Control gates and micro-ecology: the pollen–stigma interaction in perspective. *Ann. Bot.* **85**(Suppl. A), 5–13.

van der Hoeven, R.S., Monforte, A.J., Breeden, D., Tanksley, S.D. and Steffens, J.C. (2000) Genetic control and evolution of sesquiterpene biosynthesis in *Lycopersicon esculentum* and *L. hirsutum*. *Plant Cell*, **12**, 2283–2294.

Hoffmann, M.H., Bremer, M., Schneider, K., Burger, F., Stolle, E. and Moritz, G. (2003) Flower visitors in a natural population of *Arabidopsis thaliana*. *Plant Biol.* **5**, 491–494.

Iijima, Y., Davidovich-Rikanati, R., Fridman, E., Gang, D.R., Bar, E., Lewinsohn, E. and Pichersky, E. (2004) The biochemical and molecular basis for the divergent patterns in the biosynthesis of terpenes and phenylpropenes in the peltate glands of three cultivars of basil. *Plant Physiol.* **136**, 3724–3736.

Jones, M.E. (1971) Population genetics of *Arabidopsis thaliana*. 1. Breeding system. *Heredity*, **27**, 39–50.

Kessler, A. and Baldwin, I.T. (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, **291**, 2141–2144.

Knudsen, J.T., Tollsten, L. and Bergstrom, L.G. (1993) Floral scents – a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry*, **33**, 253–280.

Köllner, T.G., Schnee, C., Gershenzon, J. and Degenhardt, J. (2004) The variability of sesquiterpenes emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. *Plant Cell*, **16**, 1115–1131.

König, W.A., Rieck, A., Saritas, Y., Hardt, I.H. and Kubeczka, K.-H. (1996) Sesquiterpene hydrocarbons in the essential oil of *Meum athamanticum*. *Phytochemistry*, **42**, 461–464.

de Kraker, J.-W., Franssen, M.C.R., de Groot, A., König, W.A. and Bouwmeester, H.J. (1998) (+)-Germacrene A biosynthesis. The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. *Plant Physiol.* **117**, 1381–1392.

Lichtenthaler, H.K. (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 47–65.

Loreto, F. and Velikova, V. (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiol.* **127**, 1781–1787.

Mercke, P., Kappers, I.F., Verstappen, F.W.A., Vorst, O., Dicke, M. and Bouwmeester, H.J. (2004) Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. *Plant Physiol.* **135**, 2012–2024.

Pichersky, E. and Gershenzon, J. (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* **5**, 237–243.

Pichersky, E., Raguso, R.A., Lewinsohn, E. and Croteau, R. (1994) Floral scent production in *Clarkia* (Onagraceae). 1. Localization and developmental modulation of monoterpene emission and linalool synthase activity. *Plant Physiol.* **106**, 1533–1540.

Raguso, R.A. and Pichersky, E. (1995) Floral volatiles from *Clarkia breweri* and *C. concinna* (Onagraceae): recent evolution of floral scent and moth pollination. *Plant Syst. Evol.* **194**, 55–67.

Sharkey, T.D. and Yeh, S.S. (2001) Isoprene emission from plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 407–436.

Snape, J.W. and Lawrence, M.J. (1971) Breeding system of *Arabidopsis thaliana*. *Heredity*, **27**, 299–301.

Steele, C.L., Crock, J., Bohlmann, J. and Croteau, R. (1998) Sesquiterpene synthases from grand fir (*Abies grandis*). Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization, and bacterial expression of δ-selinene synthase and γ-humulene synthase. *J. Biol. Chem.* **273**, 2078–2089.

Tholl, D., Kish, C.M., Orlova, I., Sherman, D., Gershenzon, J., Pichersky, E. and Dudareva, N. (2004) Formation of monoterpenes in *Antirrhinum majus* and *Clarkia breweri* flowers involves heterodimeric geranyl diphosphate synthases. *Plant Cell*, **16**, 977–992.

Thornburg, R.W., Carter, C., Powell, A., Mittler, R., Rizhsky, L. and Horner, H.T. (2003) A major function of the tobacco floral nectary is defense against microbial attack. *Plant Syst. Evol.* **238**, 211–218.

Trapp, S.C. and Croteau, R.B. (2001) Genomic organization of plant terpene synthases and molecular evolutionary implications. *Genetics*, **158**, 811–832.

Turlings, T.C.J., Loughrin, J.H., McCall, P.J., Rose, U.S.R., Lewis, W.J. and Tumlinson, J.H. (1995) How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl Acad. Sci. USA*, **92**, 4169–4174.

Warmers, U. and König, W.A. (1999) Sesquiterpene constituents of the liverwort *Bazzania trilobata*. *Phytochemistry*, **52**, 99–104.