The lack of floral synthesis and emission of isoeugenol in *Petunia axillaris* subsp. *parodii* is due to a mutation in the *isoeugenol synthase* gene

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SUMMARY

Floral scent has been extensively investigated in plants of the South American genus *Petunia*. Flowers of *Petunia integrifolia* emit mostly benzaldehyde, while flowers of *Petunia axillaris* subsp. *axillaris* emit a mixture of volatile benzenoid and phenylpropanoid compounds that include isoeugenol and eugenol. Flowers of the artificial hybrid *Petunia hybrida*, a cross between *P. integrifolia* and *P. axillaris*, emit a similar spectrum of volatiles as *P. axillaris* subsp. *axillaris*. However, the flowers of *P. axillaris* subsp. *parodii* emit neither isoeugenol nor eugenol but contain high levels of dihydroconiferyl acetate in the petals, the main scent-synthesizing and scent-emitting organs. We recently showed that both isoeugenol and eugenol in *P. hybrida* are biosynthesized from coniferyl acetate in reactions catalyzed by isoeugenol synthase (PhIGS1) and eugenol synthase (PhEGS1), respectively, via a quinone methide-like intermediate. Here we show that *P. axillaris* subsp. *parodii* has a functional *EGS* gene that is expressed in flowers, but its *IGS* gene contains a frame-shift mutation that renders it inactive. Despite the presence of active EGS enzyme in *P. axillaris* subsp. *parodii*, in the absence of IGS activity the coniferyl acetate substrate is converted by an as yet unknown enzyme to dihydroconiferyl acetate. By contrast, suppressing the expression of *PhIGS1* in *P. hybrida* by RNA interference also leads to a decrease in isoeugenol biosynthesis, but instead of the accumulation of dihydroconiferyl acetate, the flowers synthesize higher levels of eugenol.

Keywords: pollination, plant volatile, scent, evolution, petunia.

INTRODUCTION

Many plant species produce and emit from their flowers a mixture of scent compounds to attract pollinators. Floral bouquets are usually a blend of aromatic compounds. Plants have evolved their floral scent to maximize their reproductive potential under specific ecological conditions and with specific pollinators. It appears likely that individual chemical components of these scents allow pollinators, most often insects, to distinguish between even closely related plant species (Ando *et al.*, 2001). Analysis of the variation in floral scents among genetically closely related plant species may lead to a better understanding of the mechanisms underlying the generation of scent

diversity. For example, the flowers of the California annual *Clarkia breweri* emit a mixture of volatiles that includes linalool, while a closely related species, *Clarkia concinna*, does not emit this compound. Notably, the genomes of both species contain a gene for linalool synthase but the gene is highly expressed in flowers of *C. breweri* and not in flowers of *C. concinna*. The emission of linalool from *C. breweri* flowers (and additional scent compounds, which contain eugenol and isoeugenol, missing from the *C. concinna* flowers) correlated with the attraction of a hawkmoth pollinator that does not regularly visit *C. concinna* flowers.

The coevolution of scent and pollinators has also been studied in the South American genus *Petunia*. *Petunia integrifolia* flowers are pigmented, emit mostly benzaldehyde, and are pollinated by bees. Flowers of the related species *Petunia axillaris* are white, emit a mixture of several benzenoids and phenylpropenes, and are pollinated by various species of hawkmoth (Hoballah *et al.*, 2005, 2007). Moreover, some variation in scent composition among different accessions of *P. axillaris* was noted, and in particular it was observed that the flowers of the subspecies *P. axillaris parodii* did not emit eugenol and isoeugenol, while *P. axillaris axillaris* flowers did (Hoballah *et al.*, 2005).

Flowers of several lines of the artificial hybrid species *Petunia hybrida*, a cross between *P. integrifolia* and *P. axillaris*, have been examined for volatile emission. It has been found that they emit a similar spectrum of volatiles as *Petunia axillaris* subsp. *axillaris* (Verdonk *et al.*, 2005; Sagae *et al.*, 2008). In particular, most *P. hybrida* cultivars emit varying amounts of eugenol and isoeugenol. The synthesis of isoeugenol and eugenol was recently elucidated in *P. hybrida* cv. Mitchell. It was shown that isoeugenol was formed by the action of isoeugenol synthase 1 (PhIGS1) using coniferyl acetate as the substrate, which itself is biosynthesized from coniferyl alcohol and acetyl-CoA in a reaction catalyzed by coniferyl alcohol acetyltransferase (PhCFAT) (Koeduka *et al.*, 2006; Dexter *et al.*, 2007). The enzyme responsible for the synthesis of eugenol in *P. hybrida* flowers was also identified (Koeduka *et al.*, 2008). This protein, designated eugenol synthase 1 (PhEGS1), is homologous to PhIGS1 and also uses coniferyl acetate as the substrate but biosynthesizes eugenol (Koeduka *et al.*, 2008).

Kondo *et al.* (2007) also examined scent biosynthesis and emission in *P. axillaris*, confirming that flowers of several *P. axillaris parodii* accessions as well as some *P. axillaris axillaris* accessions emitted little or no isoeugenol and eugenol. They also demonstrated that the petals of these flowers, unlike the high isoeugenol-emitting *P. axillaris axillaris* accessions, contained high levels of dihydroconiferyl acetate (Kondo *et al.*, 2007). Given the obvious similarity in chemical structure between dihydroconiferyl acetate and coniferyl acetate, the precursor of eugenol and isoeugenol, Kondo *et al.* (2007) hypothesized that the lack of eugenol and isoeugenol synthesis and the synthesis of dihydroconiferyl acetate were linked, but no molecular mechanism has been identified to date.

Here we present data indicating that the *IGS* gene in the genome of *P. axillaris parodii* contains a mutation that renders the encoded IGS enzyme non-functional. This natural mutation in *PapIGS* leads to the production of a non-functional IGS enzyme, and despite the presence of EGS activity in the flowers the coniferyl acetate substrate is mostly converted to dihydroconiferyl acetate by the action of an unidentified reductase. By contrast, experimentally reducing *PhIGS1* expression in *P. hybrida* by using RNA interference (RNAi) suppression leads to a reduction in isoeugenol synthesis and the concomitant increase in eugenol biosynthesis.

RESULTS

Analysis of floral scents in *Petunia axillaris parodii*

We obtained the same *P. axillaris parodii* line as analyzed by Hoballah *et al.* (2005) and confirmed that it does not emit isoeugenol or eugenol (Figure 1a). The flowers also do not emit dihydroconiferyl acetate (Figure 1a). On the other hand, extraction of the petal tissue with hexane revealed that the petals contained dihydroconiferyl acetate at a concentration of 183.5 ± 4.6 ng mg^{-1} fresh weight (FW) (Figure 1b).

Sequence analysis of *P. axillaris parodii* isoeugenol synthase

To determine the cause of the lack of isoeugenol biosynthesis in *P. axillaris parodii*, we first isolated cDNAs encoding IGS (*PapIGS*) from these plants by RT-PCR, using oligonucleotides specific for the 5′ and 3′ ends of the *P. hybrida IGS1* sequence. Complementary DNAs were obtained from multiple independent RT-PCR reactions, and their sequences all showed differences at eight nucleotide positions relative to the previously reported *PhIGS1* cDNA (Figure 2). Most significantly, sequence alignment demonstrated that two nucleotides, T and C at position 250 and 251 in *PhIGS1*, were substituted with a single A, creating a reading frame shift in the *PapIGS* gene which if expressed and translated would lead to a significantly shortened protein. Determination of the sequence of the *PapIGS* gene (accession FJ609836), which was obtained by PCR of genomic DNA, showed that this mutation occurs in the middle of the second exon.

The *PapIGS* cDNA was spliced into a bacterial expression vector and transferred to *Escherichia coli*, and upon induction of gene expression followed by lysis of the bacterial cells and *in vitro* biochemical assays, no IGS activity was detected in the lysate. The RT-PCR isolation of *IGS* cDNAs from several *P. axillaris axillaris* accessions yielded cDNAs that encoded proteins whose sequences were either identical to that of PhIGS1 or different at no more that five positions. We expressed all these cDNAs in the heterologous *E. coli* system and in all cases the *P. axillaris axillaris* proteins were enzymatically active (Figure S1).

Suppression of *PhIGS1* in *P. hybrida* leads to emission of eugenol

Petunia hybrida is an artificial hybrid obtained by crossing *P. integrifolia* and *P. axillaris*. Its flowers emit high levels of isoeugenol (12.0 nmol g^{-1} FW h^{-1}) and significantly lower levels of eugenol (0.8 nmol g^{-1} FW h^{-1}). Moreover, some

Figure 1. Gas chromatography-mass spectrometry (GC–MS) analysis of volatile compounds from *Petunia axillaris parodii* flowers.

(a) Separation by GC of volatile compounds emitted from *P. axillaris parodii* petals and collected by a headspace sampling system. Compounds were identified based on their mass spectra and retention time: 1, benzaldehyde; 2, benzyl alcohol; 3, methyl benzoate; 4, benzyl acetate; 5, methyl salicylate; 6, benzyl benzoate; 7, benzyl salicylate (but not coniferyl derivatives). Relative abundance is shown in arbitrary units, based on total ion current (TIC) measurement. The GC program for this analysis was according to Koeduka *et al.* (2008).

(b) The GC–MS analysis of hexane-soluble compounds extracted from *P. axillaris parodii* petals. The arrow shows the peak of dihydroconiferyl acetate (DCA), with its mass spectra shown in the inset; 6, benzyl benzoate; 7, benzyl salicylate.

(c) The GC–MS of authentic dihydroconiferyl acetate (DCA) standard. Its structure is also shown.

(d) The GC–MS and structures of authentic isoeugenol (IEG) and eugenol (EG) standards.

(e) The GC–MS and structures of authentic coniferyl acetate (CA) standard.
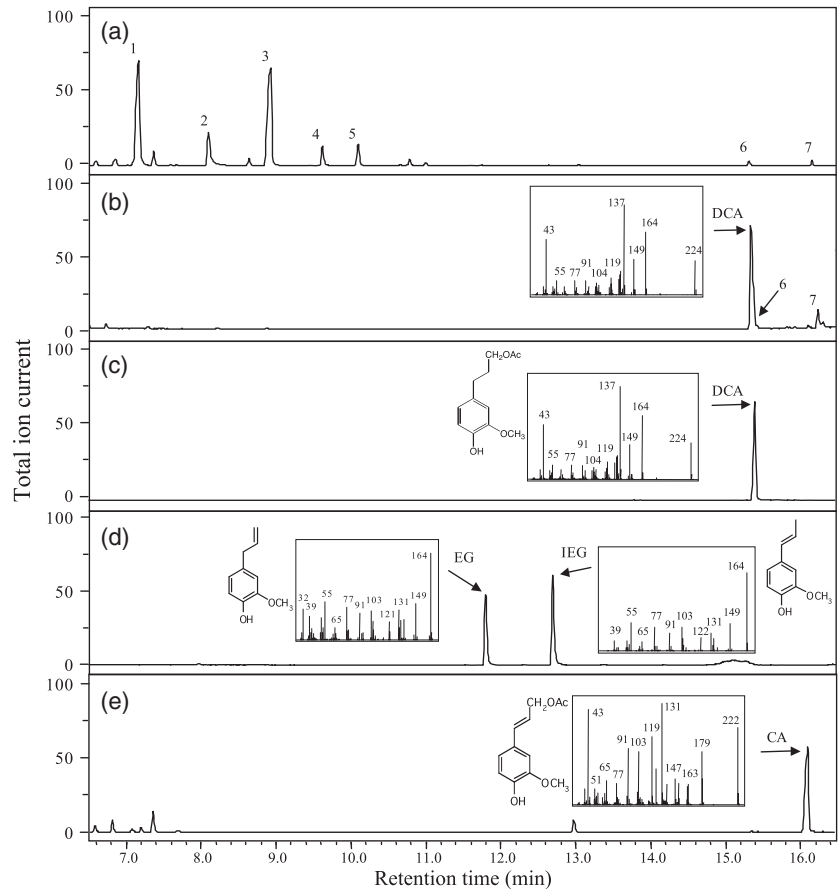
P. hybrida cultivars are amenable to genetic engineering manipulations (Jorgensen *et al.*, 1996). To test if suppression of the *PhIGS1* gene might result in the accumulation of dihydroconiferyl acetate in *P. hybrida* flower tissues, we employed two RNAi constructs containing gene-specific fragments from either the 5′ or 3′ ends of the coding region under the control of the *C. breweri* linalool synthase promoter, which is highly active in flower tissues but also displays low levels of activity in roots and stems (Orlova *et al.*, 2006). Several independent transgenic *P. hybrida* cv. Mitchell lines were generated for each construct. Four independent lines were chosen for further analysis. Two lines expressing the *PhIGS1* RNAi fragment from the 5′ end of the coding region (lines I-4 and I-14) and two independent transformed lines expressing the *PhIGS1* RNAi fragment from the 3′ end of the coding region (lines II-4 and II-5) showed substantially reduced *PhIGS1* transcript levels (Figure 3a) but no significant differences in the transcript levels of *PhEGS1* (Figure 3b). In line II-5, which showed the most severe decrease in *IGS1* mRNA levels, to 32% of the wild-type levels, the measured IGS activity levels were 35% of the wild-type levels (not shown), a corresponding reduction. Analysis of the floral volatiles emitted by these four lines revealed moderate to strong (45–90%) reduction in isoeugenol emission compared to wild-type flowers (Figures 3c and

4), and with corresponding increases in eugenol emission (Figures 3d and 4), so that the combined emission output of isoeugenol and eugenol per flower remained very similar to the combined isoeugenol and eugenol emission of a wild-type flower. The progeny of these four transgenic plants showed near identical decreases in isoeugenol emission and increases in eugenol emission with the corresponding parents, indicating that this change was heritable (Figure S2). Hexane extraction of the petal tissue of these transgenic plants showed no accumulation of dihydroconiferyl acetate, and none was found in the headspace.

Isolation and characterization of *eugenol synthase* and *coniferyl alcohol acetyltransferase* genes from *P. axillaris parodii* and the proteins they encode

Since transgenic *P. hybrida* plants in which the *PhIGS1* gene was suppressed did not produce dihydroconiferyl acetate but instead showed increased production of eugenol, it appears that reduction in IGS activity allowed the PhEGS1 enzyme molecules in the petal cells, whose concentration has not changed, better access to the coniferyl acetate substrate. To understand why an elevated production of eugenol was not observed in *P. axillaris parodii* flowers, which also lack a functional IGS protein, we examined

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         M  T  T  G  K  G  K  I  L  I  L  G  A  T  G  Y  L  G  K  Y
PhIGS1   1 ATGACTACTGGGAAGGGAAAAATATTGATTCTTGGAGCAACTGGTTATCTTGGAAAATAT
PapIGS   1 ..........................................................

         M  V  K  A  S  I  S  L  G  H  P  T  Y  A  Y  V  M  P  L  K
PhIGS1  61 ATGGTGAAAGCCAGTATTTCTTTGGGACATCCAACGTATGCCTATGTCATGCCATTGAAG
PapIGS  61 ..........................................................

         K  N  S  D  D  S  K  L  Q  L  L  K  E  F  E  S  L  G  V  T
PhIGS1 121 AAGAACTCTGATGATTCTAAGCTTCAGCTTCTCAAGGAATTTGAGTCCTTGGGAGTAACT
P1pIGS 121 ..........................................................

         I  F  Y  G  E  L  S  E  H  D  K  L  V  A  V  F  K  E  V  D
PhIGS1 181 ATATTTTATGGAGAGCTTAGTGAACATGATAAACTTGTTGCAGTGTTTAAAGAGGTTGAT
PapIGS 181 ..........................................................
                         I  L  *
         I  V  I  S  T  L  A  V  P  Q  Y  L  E  Q  L  K  V  I  E  A
PhIGS1 241 ATTGTGATATCTACTTTAGCAGTGCCTCAATATCTTGAACAACTCAAGGTGATTGAGGCC
PapIGS 241 .........A▣.................................A...

         I  K  E  A  G  N  I  K  R  F  V  P  S  E  F  G  N  E  V  D
PhIGS1 301 TCAAAGAAGCTGGTAACATTAAGAGGTTTGTTCCTTCTGAATTTGGGAATGAAGTGGATA
PapIGS 300 ..........................................................

         R  V  R  A  L  P  R  F  Q  A  V  L  D  N  K  K  K  I  R  R
PhIGS1 361 GGGTAAGAGCGTTACCACGTTTCCAAGCTGTTCTTGACAACAAGAAGAAGATTAGGAGAG
PapIGS 360 ..........................................................

         A  T  E  A  A  G  I  P  F  T  F  V  S  A  N  S  L  T  A  Y
PhIGS1 421 CAACTGAAGCAGCAGGAATACCATTCACATTTGTATCTGCAAATTCATTAACAGCTTATT
PapIGS 420 ..........................................................

         F  V  D  Y  L  L  H  P  R  Q  K  S  E  Q  V  T  I  Y  G  S
PhIGS1 481 TTGTTGATTATTTGCTTCATCCACGTCAAAAGAGTGAGCAAGTTACCATTTATGGAAGTG
PapIGS 480 ..........................................................

         G  D  K  A  V  L  N  Y  E  E  D  V  A  A  Y  T  I  K  A  A
PhIGS1 541 GGTGATGCAAAGGCTGTGTTGAACTATGAGGAAGATGTCGCAGCCTACACAATTAAAGCA
PapIGS 540 .C........................................................

         A  D  D  P  R  A  A  N  R  V  L  I  I  K  P  P  K  N  I  V
PhIGS1 601 GCAGATGATCCAAGGGCAGCAAACCGTGTCCTAATTATAAAACCCCCTAAAAACATTGTG
PapIGS 600 ..................T.......................................

         S  Q  L  D  L  V  S  S  W  E  K  T  T  G  S  T  L  K  M  T
PhIGS1 661 TCACAATTAGATTTGGTATCTTCTTGGGAGAAAACCACTGGTAGCACTTTGAAAATGACT
PapIGS 660 .................................G......C............T...

         H  I  S  E  Q  E  I  I  K  L  S  E  S  I  N  F  P  E  N  I
PhIGS1 721 CATATCTCTGAACAAGAAATCATCAAACTCTCCGAGAGCATAAATTTCCCAGAGAACATA
PapIGS 720 ......C...................................................

         H  A  S  I  L  H  N  I  F  I  A  G  A  Q  L  S  F  E  L  T
PhIGS1 781 CATGCATCAATCCTACACAATATATTCATAGCAGGAGCCCAACTAAGCTTTGAACTTACA
PapIGS 780 ........................................A.................

         Q  D  H  D  L  E  A  S  E  L  Y  P  N  Y  N  Y  T  S  V  D
PhIGS1 841 CAGGATCATGACTTGGAAGCATCAGAGCTCTATCCTAATTACAACTACACCTCTGTTGAT
PapIGS 840 ...................................C......................

         E  Y  L  K  I  C  L  V  N  P  P  K  P  K  L  A  T  Y  A  Q
PhIGS1 901 GAATATCTCAAAATTTGTCTGGTTAACCCTCCGAAGCCAAAATTGGCAACTTATGCCCAA
P1pIGS 900 ..C.......................................................

         P  S  T  *
PhIGS1 961 CCATCCACTTAA
PapIGS 960 ............
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Figure 2. Sequence of *Petunia axillaris parodii* isoeugenol synthase (IGS). The sequence of the PhIGS1 protein and its gene is shown in full. A dot represents a nucleotide in the *PapIGS* sequence that is identical to that found in *PhIGS1*. The position of the single nucleotide deletion in *PapIGS*, which leads to the frame shift, is indicated with a highlighted, framed dash. The sequence of the abbreviated protein encoded by the *PapIGS* gene is shown in italics above the sequence of PhIGS1 only when it differs from it.

whether *P. axillaris parodii* flowers possessed similar levels of *CFAT* and *EGS* transcripts and CFAT and EGS enzymatic activities to those found in *P. hybrida*. We therefore isolated cDNAs of *PapCFAT* and *PapEGS* from *P. axillaris parodii* petal RNA by RT-PCR and expressed these cDNAs in *E. coli* cells. The sequences of the proteins encoded by *PapEGS* and *PapCFAT* cDNAs differ in two and nine positions, respectively, relative to PhEGS1 and PhCFAT1 proteins (Figure S3). However, PapEGS displayed apparent K_m and k_{cat} values with coniferyl acetate of 155.6 μM and 0.85 sec^{-1}, respectively, that are very similar to those of PhEGS1 (Koeduka *et al.*, 2008). In addition, a coupled *in vitro* assay using PapEGS and PapCFAT led to the synthesis of eugenol (Figure 5).

Figure 3. The effect of decreasing the levels of *PhIGS1* mRNA in *Petunia hybrida* petals on eugenol and isoeugenol emission.

(a, b) The relative *PhIGS1* (a) and *PhEGS1* (b) transcript levels in control and transgenic *PhIGS1* RNAi petunia flowers. The relative mRNA transcript levels were analyzed in corollas of 48-h-old control and *PhIGS1* RNAi petunia flowers by qRT-PCR and the expression levels of *PhIGS1* and *PhEGS1* in control plants were each set as 1. Each graph represents the average of three replications for each of two biological samples. Bars indicate SE.

(c, d) Isoeugenol (c) and eugenol (d) emission in control and transgenic *PhIGS1* RNAi petunia flowers. Floral volatiles were collected from 48-h-old petunia flowers using the closed-loop stripping method. For each plant, an average of five to eight independent measurements were obtained. Bars indicate SE.
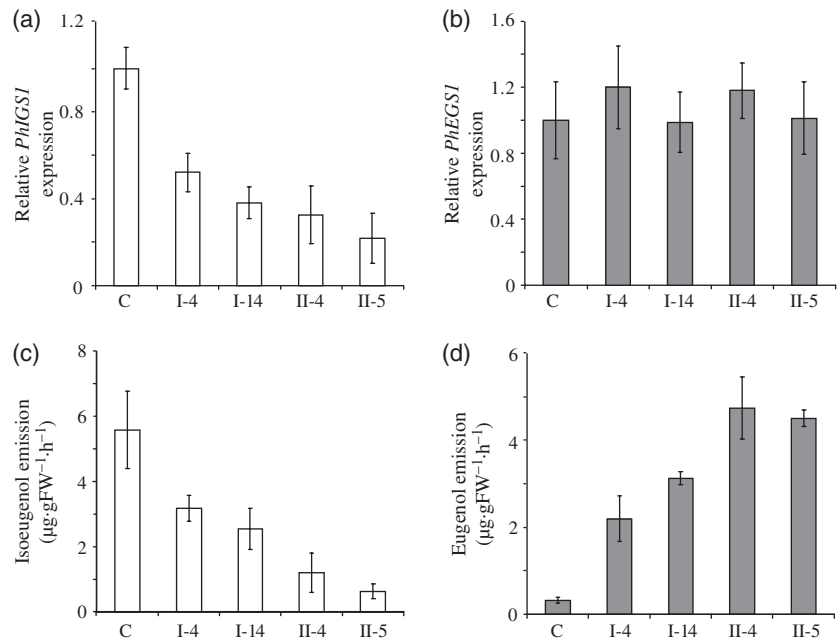
Transcript levels of *CFAT*, *IGS* and *EGS* were determined by quantitative (q)RT-PCR in both *P. axillaris parodii* and *P. hybrida* plants (Figure 6). *EGS* expression levels were similar in both species. Levels of *PhIGS1* transcripts were approximately 70% of those of *PhEGS1*, whereas levels of *PapIGS* transcripts were extremely low. The significant decrease in accumulation of the mutated *PapIGS* transcripts is similar to what has been observed in other systems where transcripts encoding non-functional proteins are quickly degraded (Nam *et al.*, 1999). In contrast, *PapCFAT* transcript levels were approximately twofold higher than levels of *PhCFAT* transcripts. CFAT, IGS and EGS activities in crude extracts from petals of both species were also measured. The CFAT and EGS activities were found to be 4-fold and 1.5-fold higher in *P. axillaris parodii* than in *P. hybrida* (Figure 7). As expected, petals of *P. axillaris parodii* had no IGS activity (Figure 7).

DISCUSSIONS

Pollination syndromes have been described in which a set of floral traits such as shape, pigmentation and scent (or lack thereof) render the flower most suitable to a specific type of pollinator, such as bees, hawkmoths or birds (Knudsen and Tollsten, 1993; Raguso *et al.*, 2003; Stuurman *et al.*, 2004). There has been considerable discussion about how many genetic changes are required to effect a change in pollinator. Several studies on floral scent in *C. breweri* (Raguso and Pichersky, 1995; Dudareva *et al.*, 1996, 1998; Wang *et al.*, 1997; Ross *et al.*, 1999) have shown that the cause of the high levels of synthesis and emission of the scent compounds that render the *C. breweri* flowers attractive to hawkmoth

pollinators, is the high levels of expression of the genes that encode the scent-biosynthetic enzymes. At least in some cases, these genes also exist in the essentially non-scented, bee-pollinated, close relative *C. concinna*, but they are not appreciably expressed in the flowers of the latter species.

Studies on the evolution of floral scent in the *Petunia* genus have also had the aim of understanding the molecular mechanisms that control the biosynthesis of compounds important for the attraction of pollinators, and how changes in these pathways occur over time. Hoballah *et al.* (2007) showed that whereas in flowers of *P. integrifolia* – which typically emit only benzaldehyde and are pollinated by bees – the gene *AN2* controls the production of floral anthocyanin pigments, *P. axillaris* accessions contain a mutated, inactive copy of this gene and as a consequence the flowers are white. On the other hand, flowers of *P. axillaris axillaris* emit many more benzenoid and phenyl-propene volatiles than flowers of *P. integrifolia*, including eugenol and isoeugenol. It has also been shown that flowers of *P. hybrida*, the artificial hybrid between *P. axillaris* and *P. integrifolia*, emit all the volatiles typically emitted by flowers of *P. axillaris axillaris* and may be pigmented similarly to *P. integrifolia*. In *P. hybrida* the shikimate pathway is responsible for the synthesis of the precursors of the benzenoid scent compounds and is regulated by a myb transcription factor encoded by the gene *ODORANT1* (Verdonk *et al.*, 2005). However, the activity of the ortholog of *ODORANT1* in *P. axillaris* has not been investigated to date.

Our data show that when IGS activity is reduced in *P. hybrida*, the coniferyl acetate substrate that would have been used by PhIGS1 is instead used by PhEGS1, leading to increased eugenol formation relative to control flowers. It is
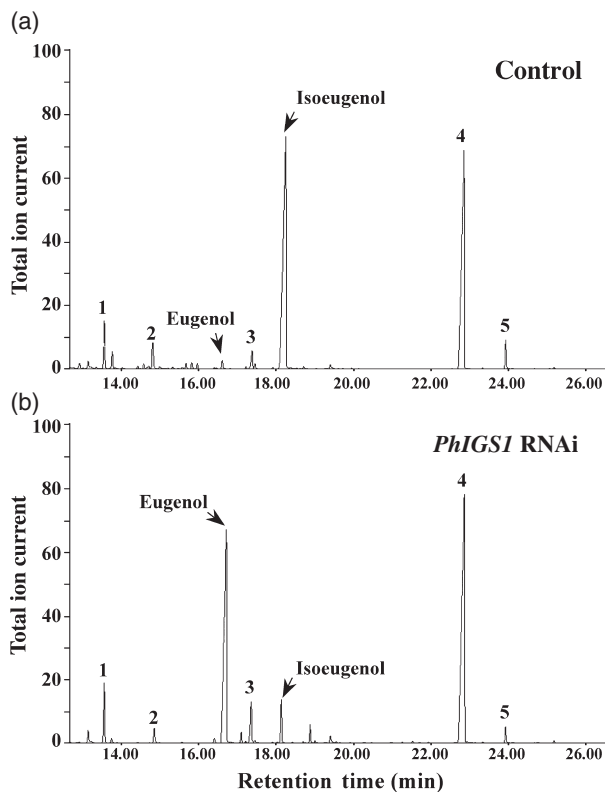
Figure 4. Metabolic profiling of volatile compounds emitted from *Petunia hybrida* flowers of control and *PhIGS1* RNAi II-5 transgenic plants.
(a) Headspace analysis of control flowers.
(b) Headspace analysis of *PhIGS1* RNAi II-5 transgenic flowers. Collected headspace volatiles were analyzed by gas chromatography-mass spectrometry (GC–MS). Compounds were identified based on their mass spectra and retention time: 1, internal standard (naphthalene); 2, phenylethylacetate; 3, vanillin; 4, benzylbenzoate; 5, phenylethylbenzoate. Relative abundance is shown in arbitrary units, based on total ion current (TIC) measurement. The GC program for this analysis was according to Orlova et al. (2006), resulting in longer retention times for the compounds than observed in Figure 1.

interesting that 'wild-type' control (non-transgenic) flowers produce 15–30 fold more isoeugenol than eugenol, despite similar *PhIGS1* and *PhEGS1* transcript levels and kinetic parameters of the corresponding enzymes, with the k_{cat} value of PhEGS1 being only twofold lower than that of

PhIGS1 (Koeduka et al., 2008). It is possible that the levels of isoeugenol and eugenol synthesized reflect the levels of enzymatic activity of PhIGS1 and PhEGS1 in non-transgenic flowers, indicating that synthesis of PhIGS1 and PhEGS1 is regulated post-transcriptionally. On the other hand, a close association between CFAT and IGS (i.e. channeling) may explain the preponderance of isoeugenol produced in these flowers.

Since *P. axillaris parodii* lines from several natural habitats have been found to accumulate dihydroconiferyl acetate (Kondo et al., 2007), it is likely that the mutation in *PapIGS* is widespread in this subspecies and must therefore have occurred some time ago, before significant radiation of the species. While the identification of this natural mutation in the *PapIGS* gene provides a clear explanation for the lack of isoeugenol biosynthesis in this subspecies, we have not yet been able to determine why *P. axillaris parodii* plants do not make eugenol, nor identify the enzyme involved in the reduction of the coniferyl moiety to dihydroconiferyl. Our data indicate that CFAT as well as EGS are still active in flowers of *P. axillaris parodii*. Indeed, *CFAT* transcript levels as well as CFAT enzymatic activity in *P. axillaris parodii* petals are two and fourfold higher than those in *P. hybrida*, respectively, while EGS activity (but not transcript levels) is also approximately twofold higher. Yet, in contrast to the outcome observed in *P. hybrida* flowers in response to artificial suppression of *PhIGS1*, the flowers of *P. axillaris parodii* do not make eugenol but instead they reduce coniferyl acetate (or coniferyl alcohol – dihydroconiferyl alcohol can be acetylated by PapCFAT, see Figure S4) to dihydroconiferyl acetate. An NADPH-dependent double-bond reductase that uses similar substrates has been identified in several plant species (Youn et al., 2006), and it will be of interest to determine if a homologous enzyme is responsible for the synthesis of dihydroconiferyl acetate in *P. axillaris parodii* petals. However, while we were able to isolate several homologs of this gene from *P. axillaris parodii* by RT-PCR of petal cDNAs, none of the enzymes encoded by these genes were able to reduce coniferyl alcohol, coniferyl aldehyde or coniferyl acetate (data not shown).
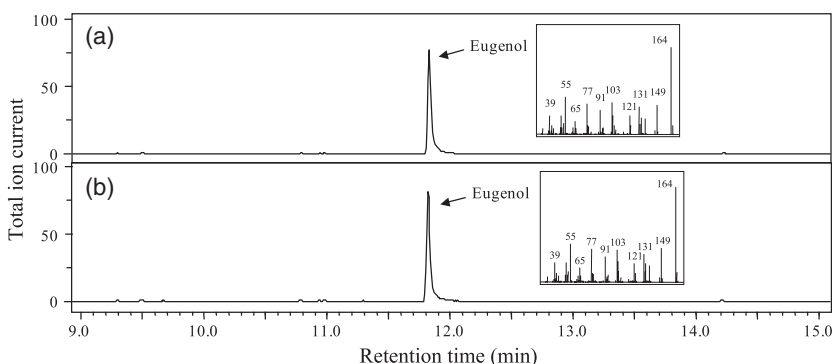

Figure 5. Gas chromatography–mass spectrometry (GC–MS) analysis of the reaction products in reactions catalyzed by *Petunia axillaris parodii* eugenol synthase (EGS) and coniferyl alcohol acetyltransferase (CFAT).
(a) The reaction mixture contained coniferyl acetate, NADPH, and purified PapEGS.
(b) The reaction mixture contained coniferyl alcohol, NADPH, acetyl-CoA, and purified PapEGS and PapCFAT. The eugenol peak was identified by MS (inset), and matched to the mass fragmentation pattern and retention time of authentic eugenol standard.
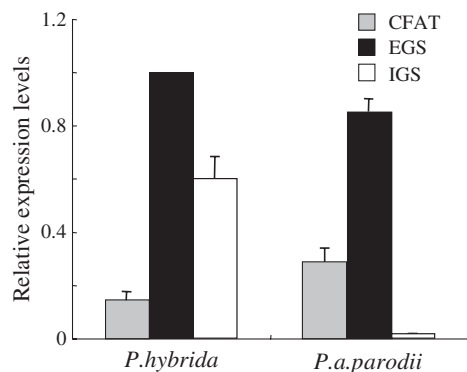
Figure 6. Detection of *EGS*, *IGS*, and *CFAT* transcripts in *Petunia hybrida* and *Petunia axillaris parodii* floral tissues. The relative mRNA transcript levels were analyzed in corollas of 48-h-old flowers by qRT-PCR and the expression of *PhEGS1* in *P. hybrida* plants was set as 1. Each bar represents the average of three replications.
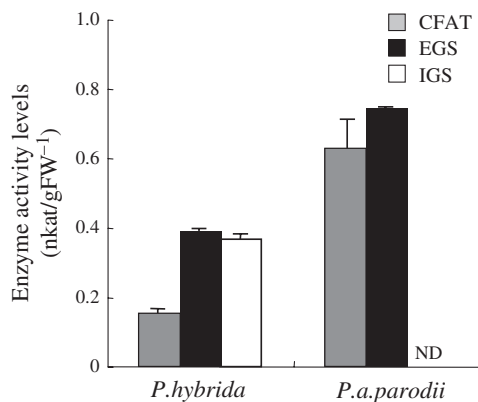

Figure 7. Eugenol synthase (EGS), isoeugenol synthase (IGS) and coniferyl alcohol acetyltransferase (CFAT) activities in *Petunia hybrida* and *Petunia axillaris parodii* floral tissues. Total activity levels were analyzed in corollas of 48-h-old flowers. Each bar represents the average of three replications. ND indicates no detectable activity.

Gas chromatography–mass spectrometry (GC–MS) analysis of petal extracts indicate that *P. axillaris. parodii* petals do not accumulate coniferyl alcohol or coniferyl acetate (Figure 1), suggesting that the reduction of coniferyl acetate (or coniferyl alcohol) to the dihydro derivative is quick and that the enzyme responsible for it competes well with EGS for the substrate. Since the mutation in *PapIGS* occurred many generations ago, it is possible that selection for an increased expression of a double-bond reductase has occurred over time to favor the synthesis of dihydroconiferyl acetate over an increase in the levels of eugenol, perhaps due to interactions with specific pollinators. Given the apparent linkage between the floral bouquet and pollinators in the *Petunia* genus, it will be of interest to determine if *P. axillaris parodii* flowers are visited by different pollinators from those visiting *P. axillaris axillaris*.

EXPERIMENTAL PROCEDURES

Plant materials and growth condition

Petunia axillaris parodii, *P. hybrida* cv. Mitchell (Ball Seed, http://www.ballhort.com/), and the transgenic petunia plants were grown in the greenhouse or growth chamber with a 16-h light period (supplemented to 100 µmol m^{-2} sec^{-1}) and a temperature of 21°C, and an 8-h dark period at 16°C.

Preparation of dihydroconiferyl acetate

To synthesize 3-(4-hydroxy-3-methoxyphenyl)propyl acetate (dihydroconiferyl acetate), dihydroconiferyl alcohol (182.2 mg, 1.0 mM), *Candida antarctica* lipase B (25 mg), vinyl acetate (401 µl, 5.0 mmol), and dry diethyl ether (50 ml, 0.2 M) were added to a 125-ml Erlenmeyer flask at room temperature. The reaction was stirred at room temperature for 2 h. The solution was filtered through glass wool and concentrated *in vacuo*. The reaction yielded a colorless oil (222 mg, quantitative yield): ^{1}H NMR (500 MHz, CDCl$_3$) δ 6.83 (d, J = 8.6 Hz, 1H), 6.68 (m, 2H), 5.52 (br s, 1H), 4.08 (t, J = 6.6, 2H), 3.87 (s, 3H), 2.61 (t, J = 7.3 Hz, 2H), 2.06 (s, 3H), 1.92 (dt, J = 6.6, 7.3 Hz, 2H); ^{13}C NMR (125 MHz, CDCl3) δ 171.2, 146.4, 143.8, 133.1, 120.9, 114.3, 110.9, 63.8, 55.8, 31.8, 30.4, 21.0. LCMS [M + Na]$^+$ calculated for C$_{12}$H$_{16}$NaO$_4$: 247.09, found: 247.17.

Enzyme assays

The reaction to detect CFAT activity contained 50 mM MES/KOH, pH 6.5, 1 mM acetyl-CoA, 0.5 mM coniferyl alcohol and 75 µl crude protein (= 10.0 mg FW, for crude enzyme assays) or 5.9 µg PapCFAT (reaction A). The reaction for EGS activity (total volume 150 µl) contained 50 mM MES/KOH, pH 6.5, 1 mM NADPH, 0.5 mM coniferyl acetate and 3.5 µg PapEGS (for the product identification) or 75 µl crude protein (= 10.0 mg FW, for crude enzyme assays). For the coupled reaction with PapCFAT and PapEGS, 3.5 µg PapEGS and 1 mM NADPH were added into reaction A. The crude enzyme solution was prepared from 48-h-old flowers by grinding under the protein extraction buffer (50 mM Bis-2-amino-2-(hydroxymethyl)1,3-propanediol (TRIS)–HCl, pH 7.0, 5 mM Na$_2$S$_2$O$_5$, 10 mM β-mercaptoethanol, 1% polyvinylpyrrolidone, 10% glycerol, and 100 mM phenylmethanesulfonylfluoride), followed by centrifugation at 12,000**g** for 10 min. Each enzyme reaction was carried out at room temperature for 30 min, and the reaction solution was extracted with 1 ml of hexane and concentrated with a mild stream of N$_2$ gas. A concentrated fraction (2 µl) was injected into a GC–MS for analysis as described by Koeduka *et al.* (2008).

Quantitative RT-PCR

To determine transcript levels, total RNA was isolated from petal tissues 2 h before the dark period. For the relative expression analysis of petunia genes, 48-h-old petunia corolla tissues of control and *PhIGS1* RNAi transgenic plants were used. In all cases, total RNA was extracted from collected tissues as previously described (Dudareva *et al.*, 1996). The RNA was subjected to DNase treatment using the DNA-free kit (Ambion, http://www.ambion.com/), and first-strand cDNA was synthesized by Superscript II reverse transcriptase (Invitrogen, http://www.invitrogen.com/) with poly-T primers in parallel with a negative control reaction in which no Superscript II reverse transcriptase was added. The qPCR reactions utilizing SYBR-Green I dye (Molecular Probes,

Table 1 Primer used in this study

Construct	Direction	Primer sequence (5′→3′)
qRT-PCR		
For CFAT		
CFAT/F1	Forward	GATGTCCTCACCAGTCGTGTCTACT
CFAT/R1	Reverse	GAATCGTCAATACCTCCCTCTGCT
For EGS		
EGS/F1	Forward	GGATGGAAACGCTAAGGCTGTA
EGS/R1	Reverse	CTCCCAAATAGCCACAAGCTCA
For IGS		
IGS/F1	Forward	GAGCAACTGAAGCAGCAGGAATAC
IGS/R1	Reverse	GTAGGCTGCGACATCTTCCTCATA
For UBQ		
UBQ/F1	Forward	CAGACCAGCAGAGGCTGATTTT
UBQ/R1	Reverse	GACGAAGCACCAAGTGAAGAGTAG
cDNA amplification (for C-terminal His-tag expression)		
For CFAT		
CFAT/F2	Forward	ATGGGAAACACAGACTTTCATGTGA
CFAT/R2	Reverse	ATAAGTAGCAGTAAGGTCCAAAT
For EGS		
EGS/F2	Forward	ATGGCTGAGAAAAGCAAAATTC
EGS/R2	Reverse	GGCAAAGTGACTAAGGTACTCC
For IGS		
IGS/F2	Forward	ATGACTACTGGGAAGGGAAAAATA
IGS/R2	Reverse	AGTGGATGGTTGGGCATAAGTT GCCAATTTT
RNAi constructs		
For LIS promoter		
LIS/F1	Forward	GAGCTCGCGGCCGCAAGCTTATCTAAT AATGTATCAAAA
LIS/R1	Reverse	CTCGAGCCCGGGATGGTTGTCTTGT
For 5′IGS		
IGS/F2	Forward	ATGACTACTGGGAAGGGAAAAATA
5′IGS/R1	Reverse	ATCATGTTCACTAAGCTCTCC
For 3′IGS		
3′IGS/F1	Forward	TCTGAACAAGAAATCATCAAAC
3′IGS/R1	Reverse	TTAAGTGGATGGTTGGGCATAAG

http://www.invitrogen.com/), Taq polymerase (New England Biol-abs, http://www.neb.com/), fluorescein (Bio-Rad, http://www.bio-rad.com/), the specific primers (Table 1), and a dilution series of each cDNA standards, were performed as previously described (Varbanova *et al.*, 2007).

Generation of RNAi silencing constructs

PhIGS1 RNAi constructs driven by the *C. breweri LIS* (linalool synthase) promoter constructed essentially as described in Orlova *et al.* (2006). The promoter was amplified using the forward and reverse primers indicated in Table 1, and the 1038-bp fragment was transferred to pART27 (Gleave, 1992), modified for Gateway cloning (Invitrogen). Two *PhIGS1* RNAi constructs were made, one with a fragment from the 5′ end of the gene and another with a fragment from the 3′ end of the gene. These RNAi constructs were made by first amplifying fragments of the *PhIGS1* cDNA corresponding to bases 1–210 and bases 725–972 by PCR with the specific primers listed in Table 1 and separately splicing them into the Gateway entry vector pENTR/D-TOPO (Invitrogen). Each fragment was then separately spliced into the destination vector (pART27 with the *LIS* promoter) in forward and reverse positions flanking the intron. Transgenic petunia plants were obtained via *Agrobacterium*

tumefaciens strain EHA105 carrying a pLISG-*PhIGS1* RNAi construct using the standard leaf disk transformation method (Horsch *et al.*, 1985). Rooted plants were screened for the presence of the *LIS* promoter by PCR with the specific primers indicated in Table 1. The PCR-positive T_0 plants were transferred to the greenhouse and analyzed for floral headspace volatiles. The level of silencing in each transgenic plant was measured by qRT-PCR as described above. The T_0 transformants were also self-pollinated manually and the T_1 seeds were analyzed for segregation by germination on MS medium supplemented with kanamycin (150 mg L^{-1}).

Analysis of volatiles from petunia flowers

Floral volatiles were collected from control and *PhIGS1* RNAi flowers, using a closed-loop stripping method under growth-chamber conditions (21°C, 50% relative humidity, 150 μmol m^{-2} sec^{-1} light intensity and 12-h photoperiod) as described previously (Orlova *et al.*, 2006). We collected floral headspace from detached *P. axillaris parodii* flowers (48-h-old) using a push–pull headspace collection system (Hoballah *et al.*, 2005). Five petunia flowers were placed in a 9.0 L glass vessel and the emitted volatiles were collected for 24 h by using a trap containing 125 mg Porapak Q (0.65 × 1.5 cm, 80/100 mesh; Alltech, http://www.alltech.com/) using a vacuum pump at a flow rate of 1.0 L min^{-1}. After headspace collection, the volatiles were eluted from the Porapak Q filter with 3 ml of hexane, concentrated by a mild nitrogen stream, and analyzed by GC–MS (Koeduka *et al.*, 2008). Dihydroconiferyl acetate was extracted from petals using hexane, as previously described (Pichersky *et al.*, 1994). The GC–MS analysis was performed as described in Koeduka *et al.* (2006) and Orlova *et al.* (2006).

SUPPORTING INFORMATION

The following supporting information is available for this article online:

Figure S1. Sequence and enzymatic properties of isoeugenol synthase (IGS) from several *Petunia axillaris* accession lines.

Figure S2. Emission levels of isoeugenol and eugenol in progeny of the first generation *PhIGS1* RNAi transgenic plants.

Figure S3. Comparison of protein sequences from *Petunia axillaris parodii* and *Petunia hybrida* eugenol synthase (EGS) and coniferyl alcohol acetyltransferase (CFAT) genes.

Figure S4. Comparison of coniferyl alcohol acetyltransferase (CFAT) activities from *Petunia axillaris parodii* using coniferyl alcohol (CA) and dihydroconiferyl alcohol (DCA).

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