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Characterization of a petunia acetyltransferase involved in the biosynthesis of the floral volatile isoeugenol

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Summary

Petunia flower petals emit large amounts of isoeugenol, which has been shown to be synthesized by isoeugenol synthase (PhIGS1) from an ester of coniferyl alcohol, hypothesized to be coniferyl acetate. This paper describes the identification and characterization of a novel petunia gene encoding an enzyme belonging to the BAHD acyltransferase family whose expression correlates with isoeugenol biosynthesis. RNAi suppression of this gene results in inhibition of isoeugenol biosynthesis. Biochemical characterization of the protein encoded by this gene showed that it has acetyltransferase activity and is most efficient with coniferyl alcohol among the alcohol substrates tested. Overall, these data support the conclusion that coniferyl acetate is the substrate of isoeugenol synthase.

Keywords: isoeugenol, floral volatiles, BAHD acyltransferase, coniferyl acetate, *Petunia* × *hybrida*.

Introduction

The scent of petunia flowers consists almost exclusively of volatile benzenoid/phenylpropanoid compounds, whose emission levels change rhythmically through a daily light/dark cycle, with a maximum at midnight (Kolosova *et al.*, 2001; Verdonk *et al.*, 2003). Phenylpropanoid compounds, including phenylethyl alcohol, phenylacetaldehyde and phenylethyl acetate, have recently been shown to be derived from phenylalanine (Kaminaga *et al.*, 2006; Tieman *et al.*, 2006). Benzenoid compounds in petunia scent, such as methyl benzoate, benzyl alcohol, benzylaldehyde and benzyl benzoate, are also most likely derived from phenylalanine (Boatright *et al.*, 2004).

Recently, it has been shown that isoeugenol, a prominent floral scent component in petunia, is synthesized from an ester of coniferyl alcohol (Koeduka *et al.*, 2006). Isoeugenol synthase 1 (PhIGS1), the enzyme catalyzing the formation of isoeugenol in petunia, has been demonstrated to efficiently use coniferyl acetate as a substrate *in vitro* (Koeduka *et al.*, 2006). To date, direct proof of the formation of coniferyl acetate or similar coniferyl esters in petunia has not yet been presented, nor has the enzyme responsible for the formation of such an ester been identified. It is possible that the synthesis of coniferyl esters is catalyzed by a member of the BAHD acyltransferase family. This class of plant enzymes was found to be involved in the acylation of numerous plant secondary compounds (D'Auria, 2006). The BAHD enzymes transfer an acyl moiety of an acyl CoA compound to an alcohol, thus forming an ester.

We have previously reported the construction of petal-specific EST databases for petunia (Boatright *et al.*, 2004; Negre *et al.*, 2003; Underwood *et al.*, 2005), and have used bioinformatics analysis, an RNAi-based loss-of-function approach, and biochemical characterization to define the involvement of specific proteins encoded by these ESTs in floral scent biosynthesis. Thus, we were able to identify one type of EST that encodes an enzyme belonging to the BAHD family. Subsequent RNAi gene inactivation and biochemical characterization showed that this protein, named PhBPBT (benzyl alcohol/phenylethanol benzoyl transferase), catalyzes the formation of benzyl benzoate and phenylethyl benzoate

(Boatright *et al.*, 2004). Transcripts encoding PhBPBT as well as other petunia scent-synthesizing enzymes accumulate predominantly in the petal limbs, and change rhythmically during a daily light/dark cycle.

Here we report the isolation and characterization of a petunia cDNA encoding another member of the BAHD family, coniferyl alcohol acyltransferase (PhCFAT). *PhCFAT* transcript analysis revealed an expression pattern typical of a gene involved in the biosynthesis of floral scent. Inactivation of the *PhCFAT* gene by RNAi-induced gene silencing resulted in petunia flowers that neither synthesized nor emitted isoeugenol. The biochemical characterization of the protein encoded by this gene revealed that it can catalyze the formation of coniferyl acetate from coniferyl alcohol and acetyl CoA.

Results

Identification of a flower-specific putative BAHD acyltransferase

To identify genes involved in floral scent biosynthesis in petunia, we searched our petunia petal EST database (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=petunia) for cDNAs encoding proteins with sequence similarity to known scent biosynthetic enzymes. In this search we identified a cDNA whose complete open reading frame encoded a protein of 454 amino acids with homology to several biochemically characterized BAHD acyltransferases involved in scent production, including PhBPBT from petunia (26% identity), benzyl alcohol acetyl transferase (BEAT) from *Clarkia breweri* (23% identity), and an alcohol acyltransferase AAT1 from *Rosa hybrida* (22% identity). Additionally, this petunia protein, which, based on the biochemical characterization described below, was designated acetyl CoA: coniferyl alcohol acetyltransferase (PhCFAT), was 28% identical with anthranilate *N*-hydroxycinnamoyl/benzoyltransferase from *Dianthus caryophyllus*, an enzyme involved in phytoalexin biosynthesis (Yang *et al.*, 1997). However, it had the highest sequence identity, 55%, with two uncharacterized members of the BAHD family from *Arabidopsis thaliana* (At5g16410 and At1g78990) (Figure 1).

To determine whether this BAHD acyltransferase is involved in scent biosynthesis in petunia flowers, we first examined the expression pattern of the *PhCFAT* gene. Real-time RT-PCR measurements (Figure 2a) indicated that *PhCFAT* transcripts are primarily present in the limbs of the petals, and that the expression was much higher during the night. This pattern of expression is similar to that of five previously characterized floral scent genes in petunia: two benzoic acid/salicylic acid methyltransferases involved in the synthesis of methylbenzoate (*PhBSMT1* and *PhBSMT2;* Negre *et al.*, 2003; Underwood *et al.*, 2005), phenylacetaldehyde synthase (*PhPAAS*) responsible for phenylacetaldehyde formation (Kaminaga *et al.*, 2006), *PhIGS1* (Koeduka *et al.*, 2006) and *PhBPBT* (Boatright *et al.*, 2004).

To further characterize *PhCFAT* transcript accumulation, RNA was analyzed from whole flowers at various developmental stages collected at 20:00 h (Figure 2b). At early stages of floral development (small bud, medium bud and tube expansion), low levels of *PhCFAT* transcript were detected; however, at later stages of development (anthesis and 2 days post-anthesis), a substantial increase in *PhCFAT* transcript was observed. This pattern of *PhCFAT* transcript abundance coincides with peak floral volatile emission (Verdonk *et al.*, 2003), and is indicative of a gene involved in floral volatile biosynthesis.

Suppression of PhCFAT *expression leads to a decrease in the synthesis and emission of isoeugenol and several other volatiles*

To determine whether *PhCFAT* is involved in floral scent biosynthesis, RNAi-induced gene silencing was employed to decrease *PhCFAT* transcript levels and subsequent protein activity. Three independent transformed lines with reduced *PhCFAT* levels (*PhCFAT* RNAi lines 6, 15 and 16) were obtained, and the T_2 generation was analyzed for reduced transcript levels and altered volatile profiles (Figure 3). *PhCFAT* transcript analysis of whole-flower tissue collected at 20:00 h (1 h after onset of darkness) revealed a substantial decrease in *PhCFAT* transcript in all transgenic lines compared with controls (Figure 3, lower right). Analysis of *PhBPBT*, *PhPAAS*, *PhBSMT,* and *PhIGS1* transcript levels in the *PhCFAT* RNAi lines indicated that the RNAi-induced suppression of the *PhCFAT* transcript did not affect the transcript levels of known petunia genes involved in floral volatile biosynthesis (Figure S1). This decrease in *PhCFAT* transcript levels coincided with a substantial decrease in isoeugenol emission as measured over a 1 h period starting at 20:00 h in all transgenic lines analyzed (Figure 3). In addition to a decrease in isoeugenol emission, the emission of five other volatiles also decreased in the RNAi lines compared with control (MD; Figure 3). These volatiles included phenylacetaldehyde, phenylethyl alcohol, phenylethyl acetate, phenylethyl benzoate and benzyl acetate. Analysis of the internal pools of volatiles in the flowers further supported the emission results, showing reduced levels of isoeugenol, phenylethyl alcohol, phenylethyl benzoate and benzyl acetate compared to MD. No consistent differences in emission and internal levels were observed for benzaldehyde, benzyl alcohol, benzylbenzoate, methyl benzoate or methyl salicylate, while inconsistent results were observed for internal pools of phenylacetaldehyde (line 15 contained wild-type levels) and phenylethyl acetate (not detected) (Figure 3). In addition, two compounds found in

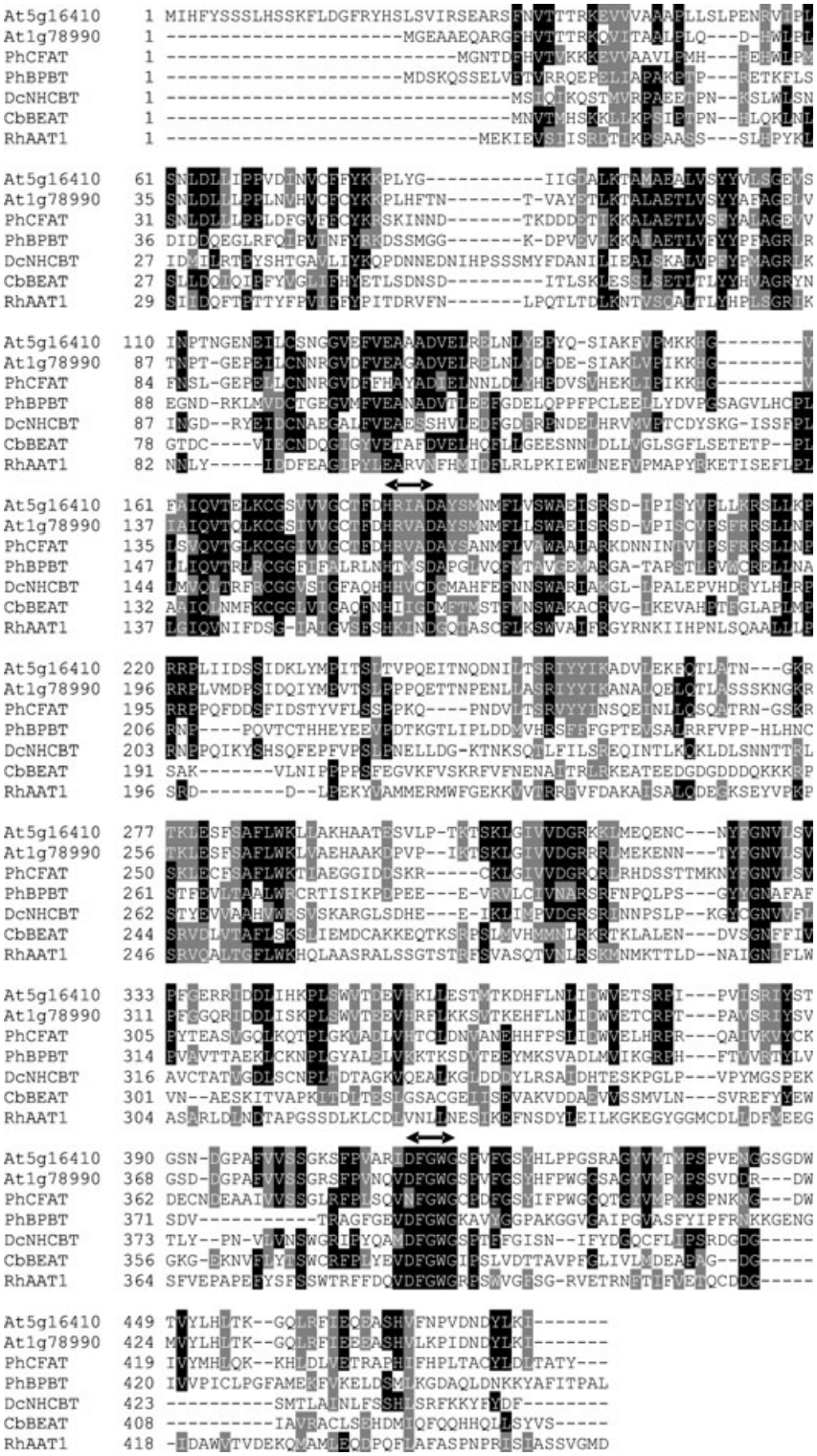


Figure 1. Peptide sequence alignment of PhCFAT and related proteins.
Sequences represented are PhCFAT (GenBank accession no. DQ767969) and proteins encoded by genes At5g16410, At1g78990, DcNHCBT (accession no. CAB06429), CbBEAT (accession no. AAC18062), PhBPBT (accession no. AAU06226) and RhAAT1 (accession no. AY850287). Sequences were aligned using CLUSTALX and shaded using BOXSHADE 3.21. Residues highlighted in black represent identical matches in at least four of the sequences, while residues highlighted in gray represent similar matches. Dashes indicate gaps in the sequence inserted to attain optimal sequence alignment. The HxxxD and DFGWG conserved motifs are identified by horizontal arrows.

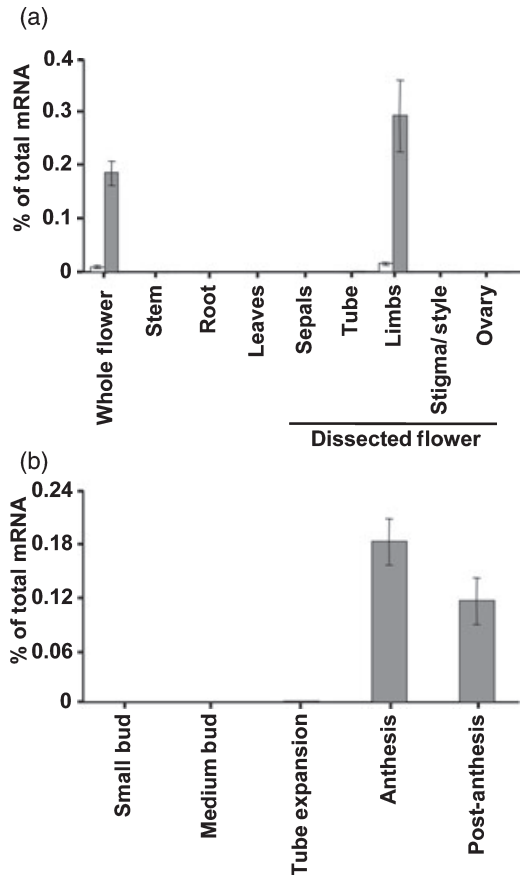


Figure 2. Characterization of spatial and developmental *PhCFAT* transcript accumulation in petunia.
(a) *PhCFAT* transcript levels (mean ± SE, $n = 3$) in root, stem, leaf and flower (whole and dissected) tissue collected 1 h after onset of subjective light (08:00 h; white) and 1 h after onset of subjective darkness (20:00 h; gray), and analyzed by real-time RT-PCR.
(b) *PhCFAT* transcript levels (mean ± SE, $n = 3$) from whole-flower tissue collected at five developmental stages (small bud, medium bud, tube expansion, anthesis, and 2 days post-anthesis) at 20:00 h (1 h after the onset of darkness), and analyzed by real-time RT-PCR.

the petal tissues, homovanillic acid and coniferyl aldehyde, were present at levels that were >1000% and >500% higher, respectively, in the RNAi lines compared with the control (341 μg g^{-1} FW of coniferyl aldehyde in *PhCFAT* RNAi lines versus 51 μg g^{-1} FW in MD, and 445 μg g^{-1} FW of homovanillic acid in *PhCFAT* RNAi lines versus 27 μg g^{-1} FW in MD).

PhCFAT acetylates coniferyl alcohol and several other substrates in a pH-dependent manner

To determine the enzymatic activity of the putative petunia PhCFAT protein, the complete coding region of a *PhCFAT* cDNA was subcloned into the expression vector pET-28a and expressed in *Escherichia coli*. The recombinant protein was purified to 99% homogeneity using nickel-based affinity chromatography. The purified recombinant protein was evaluated for its ability to acetylate coniferyl alcohol as well as a variety of other alcohols using acetyl CoA as a source for the acetyl moiety. Analysis of recombinant PhCFAT activity across a pH range from 3.5 to 8.5 showed the highest activity with coniferyl alcohol at pH 6.0, 200% higher than at pH 7.5 (33.2 and 17.0 nkat mg^{-1} protein, respectively). It should be noted that, at a more basic pH, coniferyl alcohol is possibly deprotonated, which may be noted by a change in the color of the reaction mixture before addition of the enzyme. At pH 6.0, PhCFAT displayed a narrow substrate preference, efficiently accepting only coniferyl alcohol and sinapyl alcohol (71% of the activity with coniferyl alcohol). Activities with all other tested substrates did not exceed 12% of the activity with coniferyl alcohol at this pH (Figure 4). To evaluate PhCFAT activity at a more physiological pH, assays with various alcohol substrates were also performed at pH 7.5. At this pH, PhCFAT displayed broader substrate preference. Although activity with coniferyl alcohol was still the highest, the enzyme could also use 1-octanol, cinnamyl alcohol, geraniol and sinapyl alcohol (activities ranging from 26% to 54% relative to coniferyl alcohol, respectively; Figure 4). The activities with other substrates, such as eugenol, benzyl alcohol, 2-phenylethyl alcohol, 2-hexanol, *p*-coumaryl alcohol, 3-hydroxybenzyl alcohol and isoeugenol, were not higher than 3% of the activity utilizing coniferyl alcohol.

Kinetic characterization of the purified recombinant PhCFAT protein revealed that the apparent K_m value for acetyl CoA with coniferyl alcohol is slightly lower at pH 6.0 than at pH 7.5 (30.6 ± 0.2 and 44.1 ± 1.0 μM, respectively; mean ± SD, $n = 3$), while the apparent K_m value for coniferyl alcohol at pH 6.0 is twice that at pH 7.5 (56.5 ± 1.0 and 27.5 ± 0.6 μM, respectively, mean ± SD, $n = 3$; Table 1). Despite the higher apparent K_m value for coniferyl alcohol at pH 6.0, the apparent catalytic efficiency of PhCFAT (K_{cat}/K_m ratio) is virtually the same at both pH values due to the higher turnover of the enzyme at pH 6.0 (Table 1).

Coniferyl alcohol is converted to isoeugenol by PhCFAT and PhIGS1 in an in vitro *coupled reaction*

Petunia isoeugenol synthase 1 (PhIGS1) converts coniferyl acetate to isoeugenol and cannot use coniferyl alcohol as the substrate (Koeduka *et al.*, 2006). As biochemical characterization of PhCFAT showed that it was able to catalyze the formation of coniferyl acetate, we performed an *in vitro* coupled assay that included both purified PhCFAT and PhIGS1 with coniferyl alcohol, acetyl CoA and NADPH. In this reaction, isoeugenol was produced (Figure 5a). When PhCFAT was replaced by PhBPBT (another member of the BAHD

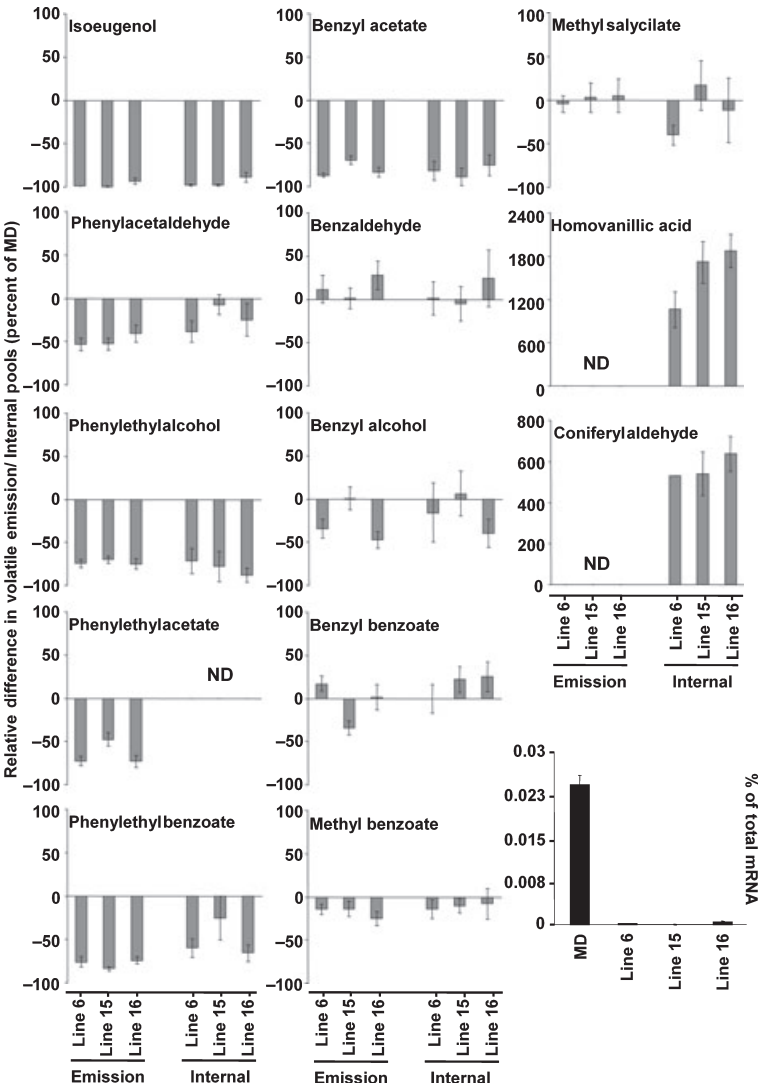


Figure 3. Effect of RNAi suppression of *PhCFAT* on emitted and internal volatiles.
Each graph shows the amount of specified volatile in a given *PhCFAT* RNAi line (6, 15 or 16) relative to that found in MD. For each volatile, emission values (mean ± SE, $n = 10$) and internal pool values (mean ± SE, $n = 12$, except for homovanillic acid and coniferyl aldehyde, where $n = 4$) were determined 1 h after the onset of darkness (20:00 h). Volatiles were collected from whole flowers for 1 h (emission) or extracted from corolla tissue (internal) and analyzed by flame-ionization gas chromatography, or, in the case of homovanillic acid and coniferyl aldehyde, by GC-MS. ND, not detected. *PhCFAT* transcript values (mean ± SE, $n = 3$) are shown at the lower right. Total RNA was extracted from whole flowers collected at 20:00 h, 1 h after the onset of darkness, and analyzed by real-time RT-PCR.

acyltransferase family in petunia) or omitted from the reaction, no isoeugenol was obtained (Figure 5b,c).

PhCFAT expression is responsive to ethylene, and shows a diurnal rhythm

In addition to spatial regulation, the results of further analysis of *PhCFAT* expression are indicative of a gene involved in the biosynthesis of floral volatiles in petunia. Following exogenous ethylene treatment, volatile emission in petunia has been shown to decrease in MD tissue compared to line 44568 (*ert1-1*), a transgenic petunia line with reduced ethylene sensitivity (Underwood *et al.*, 2005; Wilkinson *et al.*, 1997). Similarly, following exogenous ethylene treatment, *PhCFAT* transcript levels in MD petal limb tissue decreased 1000% 2 h after treatment, compared with levels in line 44568 (Figure 6). A similar correlation between ethylene perception and *PhCFAT* transcript levels was also observed following a successful pollination. At 24 h after pollination, a period of rapid ethylene biosynthesis was observed first in the stigma/style and ovary, and later in the corolla. This coincides with decreased volatile emission and ultimately petal senescence (Tang and Woodson, 1996; Wilkinson *et al.*, 1997; Jones *et al.*, 2003; Underwood *et al.*, 2005). Prior to fertilization (<24 h after pollination), *PhCFAT* transcript levels in all tissues were similar; however, by 36 h after pollination, perception of endogenous ethylene resulted in decreased transcript levels in MD pollinated petal limb tissue when compared with both non-pollinated controls and line 44568 (Figure 6). By 60 h after pollination, MD *PhCFAT* transcript levels were 500% lower than observed for all other treatments.

Floral volatile emission has previously been shown to rhythmically oscillate throughout an entire day (Kolosova *et al.*, 2001; Verdonk *et al.*, 2003). Diurnal *PhCFAT* transcript

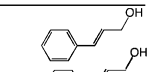
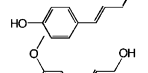
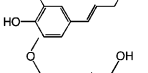
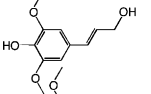
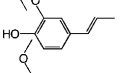
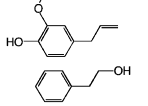
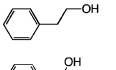
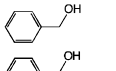
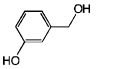
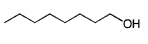
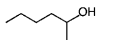
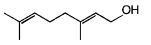
Substrate[a]	Relative Activity pH 6.0 (%)	Relative Activity pH 7.5 (%)	Structure[b]
Cinnamyl alcohol	7.0	41.6	
p-Hydroxycinnamyl alcohol	1.8	1.3	
Coniferyl alcohol	100	100	
Sinapyl alcohol	70.6	25.9	
Isoeugenol	0.4	0.7	
Eugenol	2.2	2.9	
2-Phenylethyl alcohol	0.1	1.6	
Benzyl alcohol	0.3	2.7	
3-Hydroxybenzyl alcohol	0.1	1.1	
1-Octanol	6.2	54.2	
2-Hexanol	0	1.4	
Geraniol	11.9	40.4	

Figure 4. Relative activity of PhCFAT with selected alcohol substrates. Activity was measured at pH 6.0 and 7.5. In both cases, activity with coniferyl alcohol was set at 100% and was 33.2 and 17.0 nkat mg^{-1} protein at pH 6.0 and 7.5, respectively. [a]Alcohols were used at 2 mM; [b]The molecular structure of the alcohols added.

analysis revealed 1000–1500% differences in transcript accumulation between the highest transcript levels measured after the onset of darkness (20:30–02:30 h) and the lowest levels measured after sunrise (08:30–14:30 h; Figure 7a). Emission of isoeugenol, a downstream product dependent on PhCFAT activity, was also rhythmic, with highest levels during darkness (20:30–08:30 h) and lowest levels in the afternoon (14:30 h; Figure 7b), coinciding with transcript accumulation. When the plants were subsequently moved to complete darkness, both transcript accumulation and isoeugenol emission continued to be rhythmic. However, peak accumulation and emission had shifted by 6 h and began to lose rhythmicity by 3 days in complete darkness, indicating a role for both light-dependent and circadian factors in the regulation of the *PhCFAT* transcript (Figure 7).

Discussion

PhCFAT is a BAHD acyltransferase critical to the production of isoeugenol in Petunia × hybrida

The BAHD acyltransferase family represents a diverse group of enzymes that utilize CoA thioesters to produce a variety of secondary metabolites critical to many physiological processes in plants including floral volatile emission (St Pierre and De Luca, 2000; D'Auria, 2006). Members of the BAHD family contain two conserved motifs (HxxxD and DFGWG) as defining characteristics (St Pierre and De Luca, 2000). The HxxxD motif (specifically His160) was shown to be directly involved in catalysis at the active site of the enzyme, while the DFGWG motif was shown to play a more structural role in stabilizing the enzyme (Ma *et al.*, 2005). The PhCFAT sequence also contains the conserved HxxxD motif and four out of five of the conserved residues in the DFGWG motif, as well showing significant sequence identity with BAHD family members in other parts of the protein, indicating that it also belongs to the BAHD acyltransferase family.

RNAi-based loss-of-function analysis in conjunction with *in vitro* biochemical characterization provides a powerful tool for identifying the biochemical function of an unknown enzyme *in planta*. Here we have used these two approaches to effectively characterize the biochemical function of PhCFAT in petunia. Initial analysis of *PhCFAT* RNAi transgenic petunia revealed reduced biosynthesis and emission of the floral volatile isoeugenol (Figure 3). The recent discovery of the novel petunia NADPH-dependent reductase, PhIGS1, capable of reducing coniferyl acetate to isoeugenol (Koeduka *et al.*, 2006), highlighted the importance

Table 1 Kinetic parameters of PhCFAT

	Substrate	K_m(μM)	V_{max} (nkat mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
pH 6.0	Coniferyl alcohol (with acetyl CoA)[a]	56.5 ± 9.5	40.1 ± 2.7	2.05 ± 0.14	36.8 ± 4.5
	Acetyl CoA (with coniferyl alcohol)[b]	30.6 ± 0.2	45.3 ± 1.8	2.32 ± 0.09	75.7 ± 2.6
pH7.5	Coniferyl alcohol (with acetyl CoA)[a]	27.5 ± 5.5	15.9 ± 1.6	0.81 ± 0.08	30.3 ± 6.5
	Acetyl CoA (with coniferyl alcohol)[b]	44.1 ± 10.1	29.5 ± 3.5	1.51 ± 0.18	34.7 ± 3.4
	Benzyl alcohol (with acetyl CoA)[c]	4,500 ± 300	16.0 ± 1.3	0.82 ± 0.07	0.2 ± 0.0
	Acetyl CoA (with benzyl alcohol)[d]	22.6 ± 2.7	22.1 ± 3.2	1.13 ± 0.16	48.6 ± 1.4
	2-phenyl ethanol (with acetyl CoA)[e]	3,600 ± 200	17.8 ± 1.7	0.91 ± 0.1	0.25 ± 0.0
	Acetyl CoA (with 2-phenyl ethanol)[f]	45.1 ± 3.4	18.3 ± 2.3	0.93 ± 0.12	20.7 ± 1.0

[a] at 143 μM; [b] at 2 mM; [c] at 90 μM; [d] at 16 mM; [e] at 300 μM; [f] at 16 mM

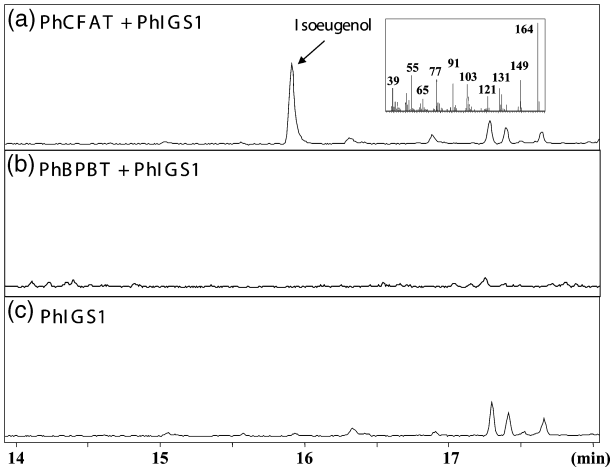


Figure 5. The coupled *in vitro* reaction of PhCFAT and PhIGS1 leads to the production of isoeugenol from coniferyl alcohol.
Gas chromatograms of the hexane-soluble compounds present in the reaction mixtures after incubation for 20 min at room temperature.
(a) The reaction mixture contained coniferyl alcohol, acetyl CoA, NADPH, and purified PhCFAT and PhIGS1. The isoeugenol peak was identified by MS (inset), and matched to the mass spectrogram and retention time of authentic isoeugenol.
(b) The reaction mixture contained coniferyl alcohol, acetyl CoA, NADPH, PhIGS1 and PhBPBT.
(c) The reaction mixture contained coniferyl alcohol, acetyl CoA, NADPH and PhIGS1, but omitted PhCFAT.

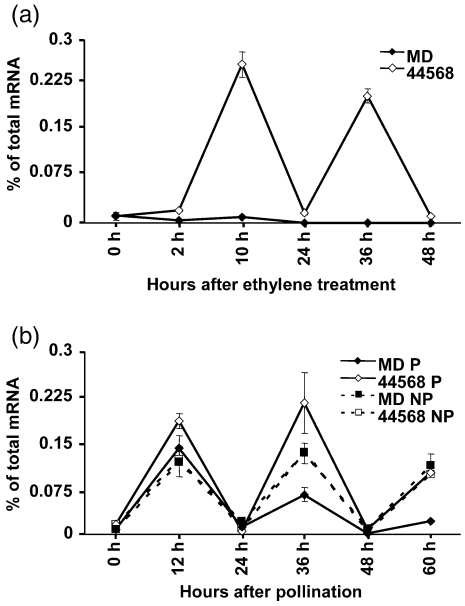


Figure 6. Ethylene-dependent regulation of *PhCFAT* transcript levels.
(a) *PhCFAT* transcript levels (mean ± SE, $n = 3$) in MD (black) and line 44568 (*etr1-1;* white) petal limb tissue treated with exogenous ethylene, collected at 0, 2, 10, 24, 36 and 48 h after treatment, and quantified by real-time RT-PCR.
(b) *PhCFAT* transcript levels (mean ± SE, $n = 3$) from pollinated and non-pollinated MD (black) and line 44568 (*etr1-1*; white) petal limb tissue collected at 12 h intervals after a successful pollination, and analyzed by real-time RT-PCR.

of an acyltransferase that can form the hypothesized precursor of isoeugenol synthesis. To determine whether PhCFAT could catalyze this reaction, *in vitro* PhCFAT activity assays were used to identify potential substrates (Figure 4). PhCFAT was shown to have the highest activity with coniferyl alcohol and acetyl CoA to form coniferyl acetate (Figure 5). These observations, in conjunction with an over 90% reduction in isoeugenol synthesis and emission in the *PhCFAT* RNAi lines, support a role for PhCFAT in the acylation of coniferyl alcohol to coniferyl acetate. Furthermore, a coupled *in vitro* reaction using coniferyl alcohol, acetyl CoA and NADPH with purified PhCFAT and ISG1 yielded isoeugenol, further indicating the role of PhCFAT in isoeugenol biosynthesis.

Our measurements of internal pools of volatiles and some of their precursors in wild-type plants did not identify coniferyl alcohol or coniferyl acetate, suggesting that these compounds are quickly turned over and do not accumulate. However, in the petals of *PhCFAT* RNAi lines, the internal concentration of coniferyl aldehyde was on average >500% higher than in wild-type, and a second compound, homovanillic acid, also accumulated at levels >1000% higher than in wild-type plants. While a block in the conversion of coniferyl alcohol to coniferyl acetate would be expected to cause the accumulation of coniferyl alcohol, it appears that this compound cannot stably accumulate in the cell and instead is oxidized back to coniferyl aldehyde. Homovanillic acid is probably derived from further oxidation and decarboxylation of coniferyl aldehyde.

Silencing of PhCFAT *in petunia affects the emission of other floral volatiles in addition to isoeugenol*

The production of several other volatiles was also affected in *PhCFAT* RNAi transgenic petunia lines (Figure 3). Levels of benzyl acetate and phenylethyl acetate (derived from the acetylation of benzyl alcohol and phenylethyl alcohol, respectively) were substantially decreased compared to levels in MD. Additionally, levels of phenylacetaldehyde, and compounds derived from it (phenylethyl alcohol and its esters), were also decreased in *PhCFAT* RNAi lines. The biochemical characterization of PhCFAT (Figure 4; Table 1) shows that it can acetylate benzyl alcohol and phenylethyl alcohol to benzyl acetate and phenylethyl acetate, respectively. However, K_m values for these two alcohols are relatively high (4.5 + 0.3 and 3.6 + 0.2 mM for benzyl alcohol and phenylethyl alcohol, respectively) and most likely exceed their availability in the cell. In conjunction with a decrease in the level of non-acetylated compounds, including phenylacetaldehyde, phenylethyl alcohol and phenylethyl

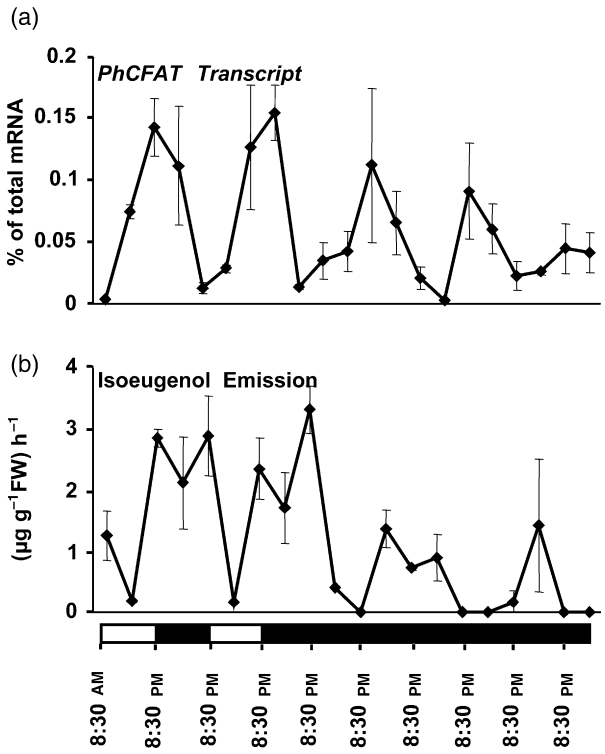


Figure 7. Daily light/dark fluctuations in *PhCFAT* transcript and isoeugenol emission.
(a) *PhCFAT* transcript levels (mean ± SE, $n = 3$) from whole flowers collected at 6 h increments for 5 days, and analyzed by real-time RT-PCR. Plants were grown under standard greenhouse conditions for 2 days and then transferred to complete darkness.
(b) Isoeugenol emission (mean ± SE, $n = 3$) from whole flowers at 6 h increments for 5 days as described above.

benzoate, in *PhCFAT* RNAi lines, this suggests that other factors (including co-suppression of other floral volatile-related genes or enzyme inhibition) could instead explain reduced levels of these petunia volatiles.

In petunia, only one other BAHD acyltransferase (PhBPBT) has been identified. RNAi-based loss-of-function analysis of this gene (*PhBPBT*) resulted in plants with reduced levels of benzyl benzoate and phenylethyl benzoate, but did not affect isoeugenol synthesis (data not shown). Additionally, transcript levels of *PhBPBT* and other floral volatile-related genes were not affected in *PhCFAT* RNAi lines (Figure S1). This suggests that co-suppression of other floral volatile-related genes does not contribute to the observed decrease in petunia volatiles, although it is not possible to test for the co-suppression of any unknown genes that could contribute to volatile production in petunia.

In *PhCFAT* RNAi lines, reduction of PhCFAT function resulted in a substantial increase in coniferyl aldehyde and homovanillic acid in the corolla (Figure 3). It is possible that high levels of these compounds could also inhibit the function of enzymes involved in floral volatile biosynthesis resulting in the observed phenotype. To determine whether elevated levels of coniferyl aldehyde and homovanillic acid are inhibitory to PhBPBT and PhPAAS, corresponding enzyme assays were performed in the presence of these compounds. Coniferyl aldehyde (2 mM) inhibited PhBPBT by 37% and PhPAAS by 65%, while homovanillic acid (2 mM) inhibited the PhBPBT and PhPAAS enzymes by 15 and 63%, respectively. Thus, the decrease in benzyl acetate and phenylethyl acetate as well as phenylacetaldehyde and compounds derived from it could be the result of PhBPBT and PhPAAS inhibition by coniferyl aldehyde and homovanillic acid, although some small, direct contribution of PhCFAT to benzyl acetate and phenylethyl acetate formation could not be excluded.

PhCFAT *transcription patterns are indicative of a petunia floral scent gene*

The biosynthesis and emission of floral volatiles is a process that depends on many factors including tissue type, time of day, floral development and ethylene perception. In the case of petunia, these volatiles are synthesized and emitted primarily from the petal limbs throughout the evening during and shortly after anthesis, and are downregulated in response to both exogenous and post-pollination-derived ethylene (Boatright *et al.*, 2004; Kolosova *et al.*, 2001; Negre *et al.*, 2003; Underwood *et al.*, 2005; Verdonk *et al.*, 2003). Recently we reported the pattern of transcript accumulation of five floral volatile biosynthesis genes, *PhBSMT1*, *PhBSMT2*, *PhPAAS*, *PhIGS1* and *PhBPBT*, in *Petunia* × *hybrida* cv. MD (Boatright *et al.*, 2004; Kaminaga *et al.*, 2006; Koeduka *et al.*, 2006; Negre *et al.*, 2003; Underwood *et al.*, 2005). In all cases, transcripts were primarily localized to the petal limb, and rhythmically expressed with the highest levels in the afternoon and the lowest levels early in the morning. Their expression was also developmentally regulated. Additionally, transcription of *PhBSMT1* and *2* was shown to decrease in response to both exogenous and post-pollination-derived ethylene (Negre *et al.*, 2003; Underwood *et al.*, 2005).

Here we show that *PhCFAT* expression is similarly restricted to the petal limb, occurs in a rhythmic manner (although with a slightly different temporal optimum than that observed for *PhBPBT* and *PhBSMT*), increases during later stages in floral development, and decreases in response to ethylene perception (Figures 2, 6 and 7). These observations provide additional support for a shared general mechanism that regulates all floral volatile biosynthesis genes in *Petunia* × *hybrida*. Identifying the components of this mechanism, in addition to the *ODORANT1* gene recently described (Verdonk *et al.*, 2005), and its mode of action, will provide critical tools for the future discovery of genes, enzymes and pathways directly involved in biosynthesis of floral scent.

Experimental procedures

Plant materials

Petunia × *hybrida* 'Mitchell Diploid' (MD) was utilized as the control for all experiments and served as the genetic background for the production of *CaMV 35S*:*etr1-1* line 44568 (Wilkinson *et al.*, 1997) and *PhCFAT* RNAi transgenic petunias. Plants were grown in a glass greenhouse without artificial lighting and an average temperature of 20 ± 5°C, dependent on the time of day. Plants were grown in 1.2-l pots with Fafard 2B potting material (Fafard Inc., Apopka, FL, USA), and fertilized four times a week with 150 mg l^{-1} nitrogen in Scott's Excel 15-5-15 (Scotts Co., Marysville, OH, USA).

cDNA isolation

A 0.9 kb partial cDNA (C2H4-7RR-B05) was selected, and a full-length cDNA was obtained by 5′ RACE using a BD SMART™ RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA, USA). The resulting 1.8 kb full-length cDNA encoded a 454 amino acid unknown transferase [subsequently named acetyl CoA: coniferyl alcohol acetyl transferase (PhCFAT); GenBank accession no. DQ767969], with 55% identity to an unknown *Arabidopsis thaliana* transferase (NP178020).

Generation of PhCFAT RNAi transgenic petunia

To determine the *in vivo* function of PhCFAT, RNAi-induced gene-silencing technology was utilized. Two fragments of the *PhCFAT* cDNA (corresponding to bases 1010-1344 and 1010-1671) were amplified using PCR and ligated end to end in a sense/antisense orientation. The resulting construct was ligated downstream of the *pFMV* (Richins *et al.*, 1987) constitutive promoter and upstream of the *nopaline synthase* (*nos*) 3′ terminator sequence. The subsequent RNAi construct was subcloned into a binary transformation vector containing the kanamycin resistance gene, *neomycin phosphotransferase* (*NPTII*), and mated with *Agrobacterium tumefaciens* strain ABI. Six-week-old MD leaves were transformed as previously described (Jorgensen *et al.*, 1996), and 16 primary transformants were recovered, transferred to soil, grown to maturity, and screened for an altered volatile profile using flame-ionization gas chromatography and reduced *PhCFAT* transcript abundance using quantitative RT-PCR (see below). Plants showing reduced isoeugenol emission and *PhCFAT* transcript levels when compared to MD were self-pollinated and T_1 progeny grown. T_1 lines were screened for segregation of the transgene using PCR (verifying the presence of the *NPTII* gene), reduced *PhCFAT* transcript and reduced isoeugenol emission. Flowers from lines exhibiting 3:1 segregation were again self-pollinated and T_2 seeds produced. Screening was repeated as above, and two 3:1 segregating lines (lines 6 and 16) and one homozygous line (line 15) were identified and used for subsequent research reported here.

PhCFAT expression analysis by real-time RT-PCR

For spatial transcript analysis, total RNA was isolated from stem, root, leaf, whole flower (at anthesis), petal limb, petal tube, stigma/style, ovary and sepal tissues collected at 08:00 h (1 h after the onset of light) and at 20:00 h (1 h after the onset of darkness). To determine transcript levels during floral development, total RNA was isolated from whole flowers collected at 20:00 h (1 h after the onset of darkness) at various developmental stages including small bud (1 cm), medium bud (3–4 cm), tube expansion (just prior to anthesis), anthesis (complete limb expansion just prior to pollen release) and post-anthesis (2 days after pollen release). For rhythmic and ethylene-regulated expression, tissue was collected as previously described (Underwood *et al.*, 2005). For post-pollination transcript analysis, MD and line 44568 flowers were pollinated 1 day prior to anthesis or set aside as a non-pollinated control. Beginning at 10:00 h, both pollinated and non-pollinated MD and 44568 flowers were collected, and the petal limb removed as spatial analysis revealed the petal limb as the target tissue primary containing *PhCFAT* transcript. This process was repeated at 12 h intervals up to 60 h after pollination. For *PhCFAT* RNAi transcript analysis, whole flowers (at anthesis) from both MD and *PhCFAT* RNAi lines were collected at 20:00 h (1 h after the onset of darkness).

In all cases, total RNA was extracted from collected tissues as previously described (Underwood *et al.*, 2005). Quantitative RT-PCR utilizing Taqman One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA), the following primers and probe (*PhCFAT* forward primer, 5′-GCAAGTGTTGGACAGCTCAAGCAA-3′; *PhCFAT* reverse primer, TCTTGTTAGGGCTAGGCATTGGCA; *PhCFAT* probe, FAM – TGATGAAGCAGCCATCGTTGTCTCCT-3BHQ1), a series of *in vitro*-transcribed *PhCFAT* standards, and 1 μl of 100 ng μl^{-1} RNA were then used to quantify mean *PhCFAT* levels as previously described (Underwood *et al.*, 2005).

To determine the transcript levels of other floral volatile-related genes (*PhBPBT*, *PhBSMT1*, *PhPAAS,* and *PhIGS1*) in *PhCFAT* RNAi lines and MD whole flowers, total RNA (see above) was analyzed utilizing real-time RT-PCR (Applied Biosystems, Foster City, CA, USA) or One-Step RT-PCR (Qiagen Inc., Valencia, CA, USA). For *PhBPBT* transcript analysis, real-time RT-PCR analysis utilizing the following primers and probe (*PhBPBT* reverse primer, 5′-GAAATAAGAAAGGTGAGAATGGGATT-3′; *PhBPBT* forward primer, 5′-AGCTCCTTGACGAATTTTTCCA-3′; *PhBPBT* probe, 5′-/56FAM/TGGTCCCTATATGTTTGCCTGGCTTTGC/3BHQ_1/ -3′), and a dilution series of *in vitro*-transcribed *PhBPBT* standards, was used to determine mean *PhBPBT* transcript levels in *PhCFAT* RNAi and MD petunia. For *PhBSMT1* transcript analysis, real-time RT-PCR (as described by Underwood *et al.*, 2005) was utilized to determine mean *PhBSMT1* transcript levels in *PhCFAT* RNAi and MD petunia.

To determine *PhPAAS* and *PhIGS1* transcript levels, *PhCFAT* RNAi and MD total RNA was analyzed using One-Step RT-PCR. Utilizing the following primers (*PhPAAS* forward primer, 5′-TCCTTGTAGTTCTAGTACTGCTGGAA-3′; *PhPAAS* reverse primer, 5′-TCAACAGCAGTTGTTGAAGTAGTTC-3′; *PhIGS1* forward primer, 5′-GCCTATGTCATGCCATTGAA-3′; *PhIGS1* reverse primer, 5′-TCTTTAATTGTGTAGGCTGC-3′; ubiquitin forward primer, 5′-AACATACAGAAGGAGTCAACAC-3′; ubiquitin reverse primer, 5′-AGAAGTCACCACCACGAAG-3′), 100 ng of whole-flower total RNA from two separate MD and *PhCFAT* RNAi plants of lines 6, 15 and 16 was analyzed. The RT-PCR reaction was then amplified utilizing the following program: 50°C for 30 min; 94°C for 15 min; 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min; final incubation at 72°C for 10 min. Aliquots (15 μl) of each product were then run (20 min at 100 V) on a 1% agarose gel, and photographed using a Polaroid Fotodyne camera (Polaroid Corporation, Pasadena, CA, USA). Transcript levels of a *poly-ubiquitin* petunia homolog were analyzed to provide an RNA loading control.

Volatile emission

For the rhythmic emission experiment, volatiles were collected and analyzed as previously described (Underwood *et al.*, 2005). For comparative analysis of volatiles emitted from *PhCFAT* RNAi and MD, seven flowers each were collected from five plants of each line

(*PhCFAT* RNAi lines 6, 15 and 16, and MD) at 20:00 h (1 h after the onset of darkness) for two consecutive nights that followed sunny days. In all cases, volatiles were then collected for 1 h and analyzed as previously described (Schmelz *et al.*, 2001). The eluted volatiles were quantified using flame-ionization gas chromatography (model 5890, series II; Hewlett-Packard, Palo Alto, CA, USA), and the resulting data were converted to ($\mu g\ g^{-1}$ fresh weight) h^{-1}. To determine a percentage difference for each compound, measured values of volatile emitted by flowers from *PhCFAT* RNAi lines were then divided by the average value of the corresponding volatile emitted by MD flowers over the same sample period. The percentage difference from MD (mean ± SE) was then calculated for each transgenic line.

Internal volatile extraction

To determine *PhCFAT* RNAi internal volatile levels, three flowers each were collected from five plants of each line (*PhCFAT* RNAi lines 6, 15 and 16, and MD) at 20:00 h (1 h after the onset of darkness) following each of two sunny days. In each case, the corollas (petal limb and tube) were removed, immediately frozen, and ground using mortar and pestle. Internal volatiles were then extracted as previously described (Schmelz *et al.*, 2004) with two deviations (no HCl added to sample, and 170°C instead of 200°C during the vapor-phase extraction). Samples were supplemented with nonyl acetate as an internal standard and analyzed by flame-ionization gas chromatography with the resulting data converted to $\mu g\ g^{-1}$ fresh weight. For coniferyl aldehyde and homovanillic acid, tissue was ground and extracted with dichloromethane overnight. The extract was analyzed by GC–MS and compared with authentic standards. *PhCFAT* RNAi sample replicates were then divided by average MD values for corresponding compounds obtained from the same day to determine a percentage difference for each sample. Mean percentage differences from MD (±SE) were then calculated for each transgenic line.

Expression of PhCFAT *in* Escherichia coli *and purification of recombinant protein*

The coding region of petunia PhCFAT was amplified by PCR using forward and reverse primers (5′-CACATATGGGAAACACAGACTTTCATG-3′ and 5′-CATGGATCCTCAATAAGTAGCAGTAAGGTCC-3′, respectively), and subcloned into the *Nde*I–*BamH*I site of the expression vector pET-28a containing an N-terminal hexa-histidine tag (Novagen, Madison, WI, USA). Sequencing revealed that no errors were introduced during PCR amplifications. Expression was performed in *E. coli* Rosetta cells grown in LB medium with 50 $\mu g\ ml^{-1}$ kanamycin and 37 $\mu g\ ml^{-1}$ chloramphenicol at 18°C. Induction, harvesting, and protein purification by affinity chromatography on nickel–nitriloacetic acid agarose (Qiagen Inc.) were performed as described previously (Negre *et al.*, 2002). Eluted fractions with the highest PhCFAT activity were desalted on Econo-Pac® 10DG columns (Bio-Rad Laboratories, Hercules, CA, USA) into 50 mM Tris–HCl buffer (pH 7.5) with 2% glycerol, and examined by SDS–PAGE gel electrophoresis followed by staining of the gel with Coomassie brilliant blue. The purity of the isolated protein (99%) was taken into account for K_{cat} determination. Total protein concentration was determined by the Bradford method (Bradford, 1976) using Bio-Rad protein reagent and BSA as a standard.

Enzyme assays

Enzyme activity was measured by determining how much of the ^{14}C-labelled acetyl group of acetyl CoA was transferred to the side chain of coniferyl alcohol. The standard reaction mixture (50 μl) contained purified PhCFAT protein (18 μg) and 140 μM acetyl CoA (containing 0.08 μCi; Amersham Biosciences UK Ltd, Buckinghamshire, UK) in assay buffer (50 mM citric acid, pH 6.0, 1 mM DTT or 50 mM Tris-HCl, pH 7.5, 1 mM DTT). After incubation for 15 min at room temperature, the product was extracted with 100 μl hexane, and 50 μl of the organic phase was counted in a liquid scintillation counter (model LS6800; Beckman, Fullerton, CA, USA). The raw data (cpm, counts per minute) were converted to nanokatals (nkat, nanomoles of product produced per second) based on the specific activity of the substrate and the efficiency of counting. Controls included assays using boiled protein with and without the alcohol substrate. For pH optimum determination, assays were performed in 50 mM Tris/Na phosphate/Na citrate buffer at pH values ranging from 3.5 to 8.5. Once the pH optimum had been determined, other buffers were used to maximize coniferyl alcohol acyltransferase (CFAT) activity with coniferyl alcohol as a substrate. At pH 6.0, CFAT activity was significantly higher in 50 mM citrate buffer than in 50 mM Tris/Na phosphate/Na citrate buffer at pH 6.0, while at pH 7.5, CFAT activity was significantly higher in 50 mM Tris–HCl than in 50 mM Tris/Na phosphate/Na citrate buffer at pH 7.5. Assays performed at overlapping pH values between two buffers (50 mM citrate and 50 mM Tris–HCl) gave very similar activities, and ratios between activities at pH 6.0 and 7.5 were consistent with those in 50 mM Tris/Na phosphate/Na citrate buffer at corresponding pH values. Thus, 50 mM citrate, pH 6.0, and 50 mM Tris–HCl, pH 7.5, buffers were used in the biochemical analysis of CFAT protein.

Product verification was performed using TLC. For TLC analysis, the standard reaction was scaled to 1 ml using non-radiolabeled acetyl CoA (Sigma, St Louis, MO, USA). The reaction product was extracted in 1 ml hexane, concentrated to approximately 10 μl, spotted onto a pre-coated silica gel TLC plate (PE SIL G/UV; Whatman, Maidstone, UK), and co-chromatographed with authentic standards using ethyl acetate:hexane (7:3 v/v) as a solvent.

For kinetic analysis, an appropriate enzyme concentration was chosen so that the reaction velocity was proportional to the enzyme concentration and linear with respect to incubation time for at least 15 min. Kinetic data were evaluated by hyperbolic regression analysis. PhBPBT and PhPAAS activities in the presence of 2 mM coniferyl aldehyde and homovanillic acid were determined as described previously (Boatright *et al.*, 2004; Kaminaga *et al.*, 2006).

Coupled in vitro *reaction*

The coupled reaction with PhCFAT and PhIGS1 was carried out in a 100 mM MES-KOH buffer (pH 6.5) containing 0.5 mM coniferyl alcohol, 0.3 mM acetyl CoA, 0.5 mM NADPH and 2 μg of each purified enzyme in a total volume of 150 μl. After incubation at room temperature for 20 min, the reaction solution was extracted with 1 ml of hexane, and the hexane solution was concentrated with liquid N_2. A fraction (2 μl) was injected into the GC-MS apparatus for analysis. Control reactions (substituting PhBPBT for PhCFAT, or omitting PhCFAT from the PhIGS1 coupled reaction) were carried out and analyzed in an identical fashion.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Transcript levels of related genes in PhCFAT RNA: transgenic lines.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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