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A petunia chorismate mutase specialized for the production of floral volatiles

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SUMMARY

In Petunia *i* hybrida cv. 'Mitchell Diploid' floral fragrance is comprised of 13 volatile benzenoids/phenylpropanoids derived from the aromatic amino acid phenylalanine. Several genes involved in the direct synthesis of individual floral volatile benzenoid/phenylpropanoid (FVBP) compounds, i.e. at the end of the pathway, have been isolated and characterized in petunia through reverse genetic and biochemical approaches. In an effort to understand the regulation of 'upstream' components in the FVBP system, we have cloned and characterized two CHORISMATE MUTASE (PhCM1 and PhCM2) cDNAs from petunia. PhCM1 has a transcript accumulation profile consistent with known FVBP genes, while PhCM2 showed a constitutive transcript accumulation profile. The plastid-localized PhCM1 is allosterically regulated by tryptophan but not phenylalanine or tyrosine. The total FVBP emission in PhCM1 RNAi knockdown petunias is reduced by approximately 60–70%, and total chorismate mutase activity in corolla tissue is reduced by 80–85% compared to control plants. These results show that PhCM1 is the principal CHORISMATE MUTASE responsible for the coupling of metabolites from the shikimate pathway to the synthesis of FVBPs in the corolla of Petunia *i* hybrida cv. 'Mitchell Diploid'.

Keywords: Chorismate mutase, petunia, benzenoid/phenylpropanoid, volatiles, flower, chloroplast.

INTRODUCTION

Flowering plant species have developed several mechanisms for attracting pollinating organisms. Flower shape, color and fragrance all factor into an increased specialization of the floral phenotype aimed at attracting a pollinator (Fenster et al., 2004). Floral fragrance comprises an assortment of volatile organic molecules, commonly referred to as a scent bouquet. These volatile organic compounds are not only involved in plant reproductive processes, but also in plant–plant interactions, defense and abiotic stress responses (Dudareva et al., 2006). The majority of volatile compounds are lipophilic liquids with high vapor pressures that freely cross biological membranes in the epidermal cells of the petal (Pichersky et al., 2006). Floral volatiles are generally differentiated into three main groups: benzenoids/phenylpropanoids, terpenoids and fatty acid derivatives.

Petunia i hybrida cv. 'Mitchell Diploid' (MD) synthesizes and emits 13 known floral volatile benzenoid/phenylpropanoid (FVBP) compounds (Figure 1) (Kolosova et al., 2001; Verdonk et al., 2003, 2005; Boatright et al., 2004; Koeduka et al., 2006). The majority of FVBP compounds are derived from the aromatic amino acid phenylalanine (Boatright et al., 2004; Schuurink et al., 2006). Eight genes that are known to participate in FVBP synthesis have been isolated from petunia: S-ADENOSYL-L-METHIONINE:BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1 and 2 (PhBSMT1 and PhBSMT2), BENZOYL-COA:BENZYL ALCOHOL/PHENYLETHANOL BENZOYLTRANSFERASE (PhBPBT), PHENYLACETALDEHYDE SYNTHASE (PhPAAS), CONIFERYL ALCOHOL ACYLTRANSFERASE (PhCFAT), ISOEUGENOL SYNTHASE 1 (PhIGS1), and EUGENOL SYNTHASE1 (PhEGS1) (Figure 1) (Negre et al., 2003; Boatright et al., 2004; Underwood et al., 2005; Verdonk et al., 2005; Kaminaga et al., 2006; Koeduka et al., 2006, 2008; Orlova et al., 2006; Dexter et al., 2007, 2008). All of these gene products are involved in the direct formation of a FVBP compound, except PhODO1 (Verdonk et al., 2005), which is a transcriptional regulator, and PhCFAT (Dexter et al., 2007), which produces substrate for PhIGS1 and PhEGS1.

Regulation of the petunia FVBP system is complex and very specific. Emission of MD FVBPs is mostly confined to

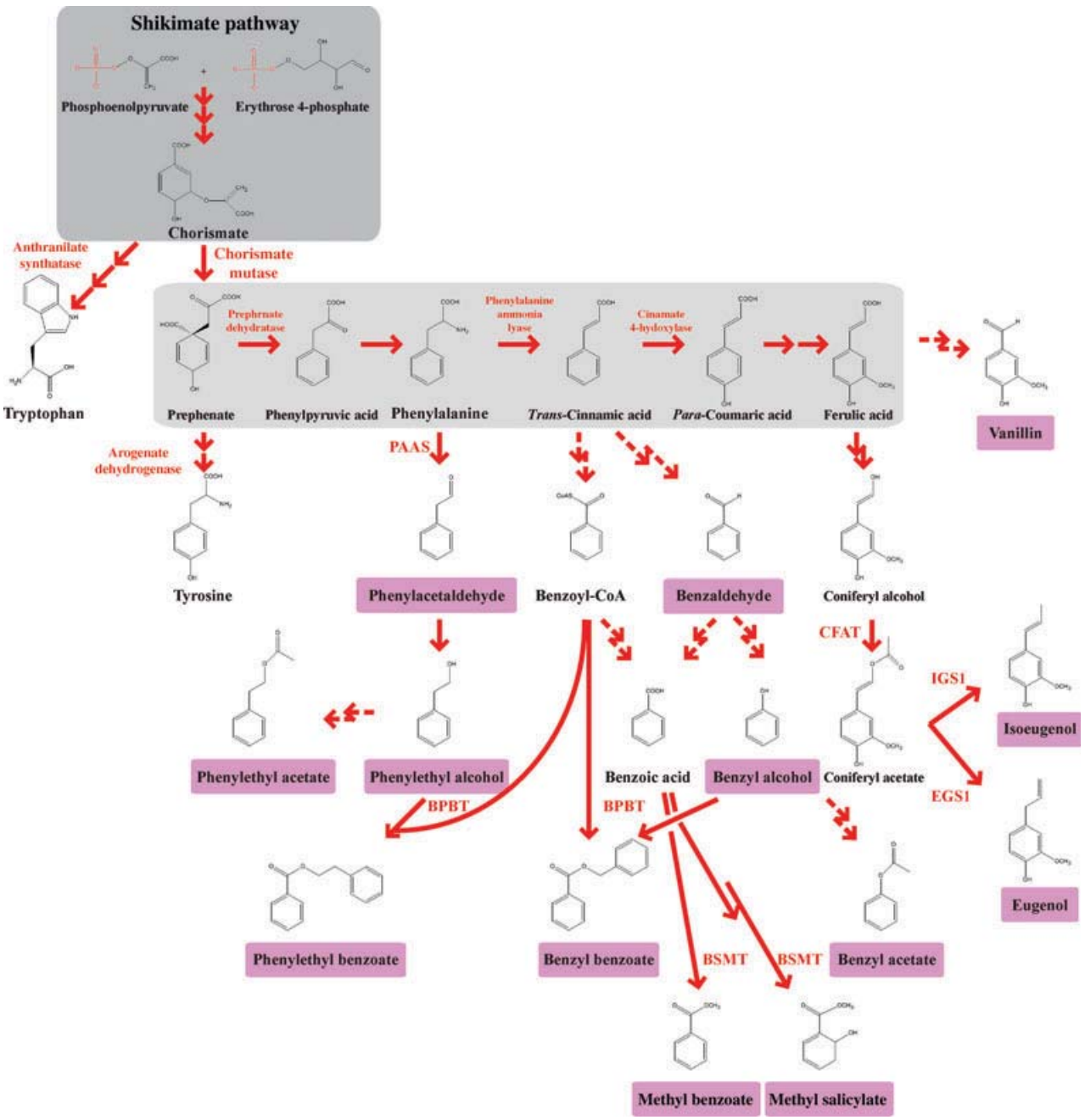

Figure 1. The floral volatile benzenoid/phenylpropanoid pathway in petunia.
The shikimate pathway (dark gray) concludes with the formation of chorismate. Chorismate mutase catalyzes the rearrangement of chorismate to prephenate, directing the flux of metabolites to the production of phenylalanine and tyrosine. Starting with the phenylpropanoid backbone (light gray), FVBP production consists of three main branches: phenylalanine, trans-cinnamic acid and ferulic acid. Floral volatile compounds derived from each branch point are highlighted in pink, and known FVBP genes are abbreviated at the appropriate enzymatic positions. Enzymes are shown in red. Solid red arrows indicate established biochemical reactions. Multiple arrows indicate multiple biochemical steps. Dashed arrows indicate possible biochemical reactions.

the corolla limb tissue during open flower stages of development, which coincide with presentation of the reproductive organs (Verdonk et al., 2003). Accumulation and emission of the MD FVBP internal substrate pool are diurnal, with the highest level detected during the dark period (Kolosova et al., 2001; Verdonk et al., 2003, 2005; Underwood et al., 2005; Orlova et al., 2006). FVBP synthesis and emission, FVBP gene transcript accumulation and PhBSMT activity are greatly reduced following a successful pollination/fertilization event or exogenous treatment with ethylene (Hoekstra and Weges, 1986; Negre et al., 2003; Underwood et al., 2005). Subsequent to a successful fertilization event,

the corolla tissue senesces as the petunia flower shifts from pollinator attraction to supporting seed set.

The shikimate pathway couples metabolism of carbohydrates to the formation of aromatic amino acids (Figure 1) (Herrmann and Weaver, 1999). CHORISMATE MUTASE (CM) catalyzes an intramolecular, [3,3]-sigmatropic rearrangement of chorismic acid to prephenic acid, the initial committed step in phenylalanine and tyrosine biosynthesis (Haslam, 1993). In Arabidopsis thaliana, three CM genes have been identified and characterized: AtCM1, AtCM2 and AtCM3 (Eberhard et al., 1996b; Mobley et al., 1999). AtCM1 and AtCM3 encode putatively plastid-localized CM isoforms, which are allosterically down-regulated by phenylalanine and tyrosine, but up-regulated by tryptophan. AtCM2 encodes a CM isoform that is not regulated by aromatic amino acids and appears to be located in the cytosol.

The majority of floral volatile studies in petunia have focused on identification of gene products involved in the formation of individual, emitted FVBP compounds (i.e. genes at the end of the FVBP pathway). As FVBPs are derived from phenylalanine and CM is the first committed step in phenylalanine biosynthesis, we identified and characterized two petunia CM cDNAs (PhCM1 and PhCM2). Additionally, we identified the principal CM responsible for the production of FVBP compounds in petunia.

RESULTS

Identification of two distinct CM cDNAs

To identify putative CM genes, we searched a publicly available petunia EST database (http://solgenomics.net/index.pl) and a petunia root EST collection (courtesy of Dr Didier Reinhardt of the University of Fribourg) for sequences with homology to any of the three CM genes from Arabidopsis thaliana. The in silico analysis identified two partial ESTs, whose full-length sequences were recovered by 5′ and 3′ RACE technology. These two sequences exhibited high similarity to the Arabidopsis CM genes, and were subsequently renamed CHORISMATE MUTASE1 (PhCM1) and CHORISMATE MUTASE2 (PhCM2), and were deposited in GenBank under accession numbers EU751616 and EU751617, respectively (Figure S1).

The predicted PhCM1 and PhCM2 proteins were 324 and 263 amino acids long, respectively. PhCM1 contains a predicted N-terminal chloroplast transit peptide (cTP) of 56 amino acids (using ChloroP 1.1, http://www.cbs.dtu.dk/services/chloroP/) and is therefore predicted to be plastid-localized (using Predator version 1.03, http://urgi.versailles.inra.fr/predotar/predotar.html/), but PhCM2 is probably located in the cytosol (Benesova and Bode, 1992). The predicted mature PhCM1 and PhCM2 proteins share 46.7% amino acid identity. When aligned with CM amino acid sequences from Arabidopsis thaliana, Fagus sylvatica, Solanum lycopersicum, Nicotiana tabacum, Oryza sativa, Vitis vinifera, Zea mays and Saccharomyces cerevisiae, common sequence features including a CM_2 superfamily domain in the N-terminal half of the predicted proteins and a conserved C-terminal domain of 19 amino acids were observed (Figure 2a). Additionally, an allosteric regulatory site (GS, marked by a red square in Figure 2a) was present in PhCM1, but not PhCM2, consistent with aromatic amino acid regulation of the plastidic PhCM1. Phylogenetic analysis demonstrated that the three solanaceous cytosolic CMs closely associate in an unrooted neighbor-joining tree (Figure 2b). PhCM1 associates with CMs from multiple species that contain both a predicted cTP and the allosteric regulatory site, and shares 62.2% identity with AtCM1.

Chloroplast import assay

To test the predicted subcellular localization of PhCM1 and PhCM2, both full-length coding sequences were cloned into a pGEM -T Easy vector, and in vitro transcribed and translated. The radiolabeled translation product was incubated with isolated chloroplasts (Pisum sativum) in a protein import assay (Figure 3). The radiolabeled PhCM2 translation product that associated with the chloroplast fraction was equal in size to the original translation product and was not protected from thermolysin protease treatment, indicating that PhCM2 did not enter the plastid. However, the PhCM1 translation product that associated with the chloroplast fraction was processed to a smaller size and was protected from thermolysin treatment, indicating that PhCM1 is imported into the plastid and processed to a mature size. Furthermore, the radiolabeled PhCM1 was associated with the stromal fraction of separated chloroplasts. Together with primary amino acid sequence features, these results demonstrate that PhCM1 is localized to the chloroplast stroma, while PhCM2 is most likely not located in the chloroplast.

PhCM1 and PhCM2 transcript abundance analysis

Because of the large drain on the free phenylalanine pool by the FVBP synthesis pathway, we hypothesized that a CM gene would be transcriptionally co-regulated with known FVBP genes. Three criteria of transcript accumulation: spatial, flower development and ethylene-treated were chosen for analysis by semi-quantitative RT-PCR and validated by quantitative RT-PCR (Figures 4 and S2). The spatial analysis included root, stem, stigma, anther, leaf, petal tube, petal limb and sepal tissues (Figures 4a and S2a). PhCM1 transcripts were detected at high levels in the petal limb and tube, and to a much lesser extent in the sexual organs, stem and root. PhCM2 transcripts were detected in all tissues examined, with relatively high levels in the petal tube and stem tissues. The MD flower development series consisted of whole flowers collected at 11 consecutive stages (for a detailed explanation, see Experimental procedures) from a small bud to floral senescence (Figures 4b and S2b). PhCM1 transcripts were detected at relatively low levels throughout

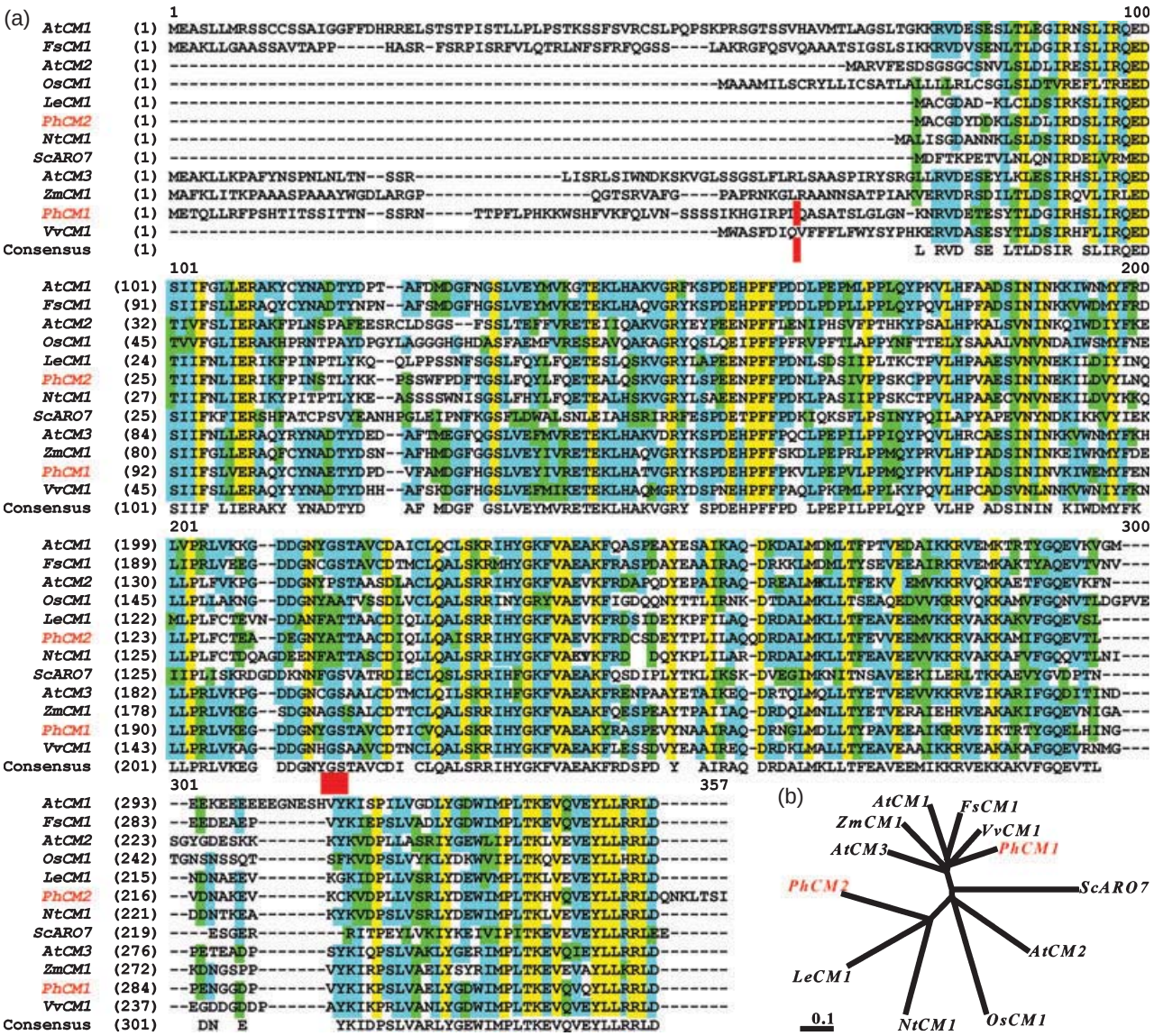

Figure 2. Predicted peptide sequence alignment and an unrooted neighbor-joining phylogenetic tree of CM proteins from various species.
Sequences represented are from Arabidopsis thaliana (accession numbers NP_566846, NP_196648, and NP_177096), Fagus sylvatica (accession number ABA54871), Solanum lycopersicum (accession number AAD48923), Nicotiana tabacum (accession number BAD26595), Oryza sativa (accession number NP_001061910), Petunia ı hybrida (accession numbers EU751616 and EU751617), Vitis vinifera (accession number CAO15322), Zea mays (accession number AY103806) and Saccharomyces cerevisiae (accession number NP_015385).
(a) Sequences were aligned using the AlignX program of the Vector NTI Advance software version 10.3.0 (Invitrogen, http://www.invitrogen.com/). Residues highlighted in blue represent consensus residues derived from a block of similar residues at a given position, those highlighted in green represent consensus residues derived from the occurrence of >50% of a single residue at a given position, and those highlighted in yellow represent consensus residues derived from a completely conserved residue at a given position. Petunia sequence names are highlighted in red on the left, the red vertical bar represents the beginning of the mature protein sequence used for PhCM1, and the red square indicates an allosteric regulatory site (GS).
(b) TreeView software with the nearest-joining method was used to create a phylogenetic tree. The scale bar represents distance in terms of the number of substitutions per site (i.e. 0.1 amino acid substitutions per site).

the closed bud stages of development (stages 1–5). Relatively high levels of PhCM1 transcripts were detected at anthesis (stage 6) and throughout all open flower stages of development examined (stage 7–10). The lowest level of PhCM1 transcripts was detected in observably senescing flower tissue (stage 11). PhCM2 transcripts were detected at similar levels throughout all stages examined except stage 11 (Figures 4b and S2b). The ethylene study used excised whole flowers from MD and an ethylene-insensitive (CaMV 35S::etr1-1) transgenic petunia line, 44568 (Wilkinson et al., 1997). All flowers were treated with air or ethylene (2 ll $L^{)1}$) for 0, 1, 2, 4 or 8 h starting at 12:00 h (Figures 4c

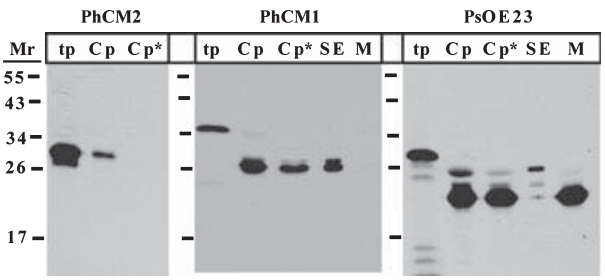

Figure 3. Plastid import assay.
Radiolabeled PhCM1, PhCM2 and PsOE23 were individually incubated with isolated pea chloroplasts. After import, the isolated chloroplasts were treated with thermolysin as indicated (Cp*). Proteolysis was terminated and the intact chloroplasts were then re-purified, washed, lysed, and fractionated. PsOE23 is a thylakoid lumen protein with a stromal intermediate, which was used as a positive control. The translation products (tp), chloroplasts (Cp), thermolysin-treated chloroplasts (Cp*), stromal extracts (SE) and total membranes (M) were analyzed by SDS–PAGE and fluorography. The positions of the molecular weight marker are shown on the left.

and S2c). PhCM1 transcript levels were reduced in MD flowers after 4 h of ethylene treatment compared to air treatments, but no change in PhCM1 transcript level was observed in experiments using 44568. In contrast, PhCM2 transcript levels were unchanged throughout the treatment in both genetic backgrounds. Together, these results indicate that the transcript accumulation profile for PhCM1 is similar to that of known FVBP genes and is therefore sufficient for FVBP production.

Total CM activity in petunia flowers

To investigate whether CM activity contributes to daily substrate pool oscillations (Underwood et al., 2005; Orlova et al., 2006) and concomitant rhythmic emission of FVBPs in MD (Verdonk et al., 2005), we developmentally staged MD flowers and collected whole corollas at three time points over the course of 24 h. Desalted crude protein extracts were obtained, and total CM activity was assayed for each time point, with close attention paid to non-enzymatic chorismic acid breakdown (Figure 5). Total CM activity was unchanged over the three time points, with an approximate mean specific activity of 0.07 nkat $mg^{)1}$. Not discounting the presence of a regulatory molecule in vivo that may be lost through the extraction process, the total CM activity in crude protein extracts from stage 9 and 10 MD corollas did not parallel the FVBP emission profiles.

Functional complementation and recombinant enzyme activity of PhCM1 and PhCM2

Despite the high homology with other CMs at the amino acid level, it was necessary to test the biochemical function of both PhCM1 and PhCM2. The coding sequences for both genes were cloned into a pET-32 vector and transformed into the CM-deficient Escherichia coli transformant KA12/pKIMP-UAUC (provided by Dr Peter Kast of the Swiss Federal Institute of Technology, Zurich, Switzerland). The KA12/pKIMP-UAUC system requires complementation of both phenylalanine and tyrosine auxotrophies under double antibiotic selection, and has been well characterized (Kast et al., 1996, 2000). Both pET-32–PhCM1 (without the cTP sequence) and pET-32–PhCM2 complemented KA12/pKIMP-UAUC when grown on minimal media without the addition of phenylalanine and tyrosine compared to all controls (Table S1). This result indicates that both PhCM1 and PhCM2 encode proteins that are sufficient for the enzymatic intramolecular conversion of chorismic acid to prephenic acid.

We then utilized the pET-32–CM vectors to transform E. coli strain BL21(DE3)pLysS with the aim of generating recombinant proteins for PhCM1 and PhCM2. PhCM1 and PhCM2 proteins were purified by Ni^{2+} affinity chromatography, and assayed for CM activity with and without addition of the aromatic amino acids (Figure 6). PhCM2 had a specific activity of approximately 5.0 nkat $mg^{)1}$ and was not affected by the presence of aromatic amino acids. However, the specific activity of PhCM1 was close to 2.2 nkat $mg^{)1}$, and increased approximately threefold in the presence of tryptophan. As is the case in Arabidopsis thaliana, Papaver somniferum (opium poppy) and Solanum lycopersicum (Benesova and Bode, 1992; Eberhard et al., 1996a,b; Mobley et al., 1999), the cytosolic PhCM2 is not allosterically regulated by the aromatic amino acids. In contrast to the allosteric regulation patterns found for plastidic CMs in Arabidopsis and opium poppy, phenylalanine and tyrosine had no effect on PhCM1 enzymatic activity, but the tryptophan regulation is similar in magnitude to that seen for AtCM1 (Eberhard et al., 1996b).

Suppression of PhCM1 by RNAi

Because the transcript accumulation profile for PhCM1 is similar to those of known FVBP genes (Figures 4 and S2) and the subcellular location for PhCM1 is the plastidial stroma (Figures 2a and 3), PhCM1 was chosen for RNAi-mediated gene silencing. A 213 bp fragment at the 3ï end of the PhCM1 coding sequence was used for the RNAi inducing fragment (Figure S3). As the PhCM1 RNAi fragment was <60% homologous to the corresponding region of PhCM2, we hypothesized that expression of PhCM2 and any other possible gene family members would be unaffected by the PhCM1 silencing construct, which was driven by a constitutive promoter (pFMV).

Fifty independent PhCM1 RNAi (ir-PhCM1) plants were generated by leaf disc transformation, and analyzed for reduced levels of PhCM1 transcripts compared to MD by semi-quantitative RT-PCR. Eight plants were chosen for further analysis, and were self-pollinated to produce T_1 seeds. Five transgenic T_1 ir-PhCM1 lines segregated in the expected 3:1 ratio for the transgene, and these lines were more extensively studied for gene transcript accumulation and FVBP emission differences compared to MD. Represen-

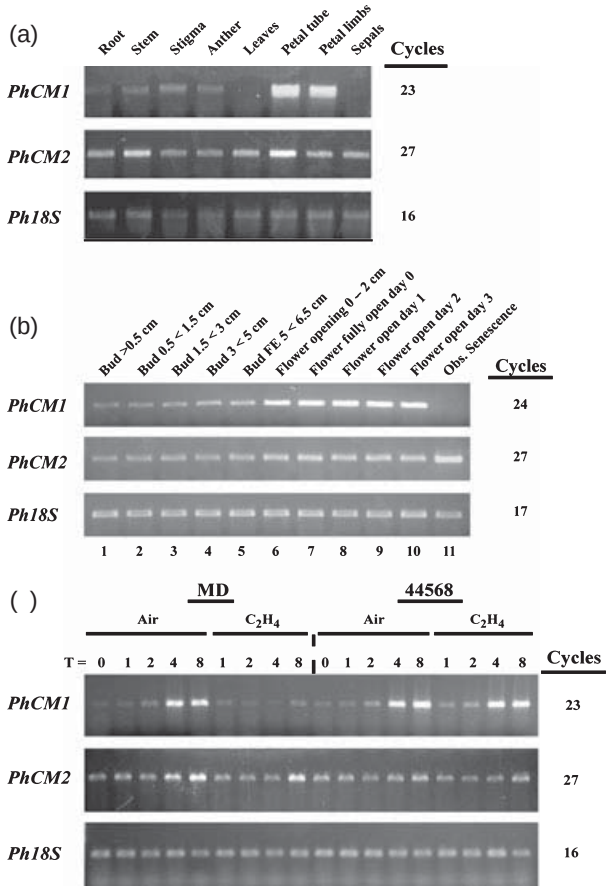

Figure 4. Semi-quantitative RT-PCR transcript accumulation analysis of PhCM1 and PhCM2 in petunia.
(a) Spatial analysis using root, stem, stigma, anther, leaf, petal tube, petal limb and sepal tissues of MD harvested at 16:00 h.
(b) Floral developmental analysis using MD flowers from 11 sequential stages at 16:00 h.
(c) Ethylene treatment analysis (2 ll $L^{)1}$) using excised MD and 44568 whole flowers treated for 0, 1, 2, 4 or 8 h.
The number of cycles used for amplification of each transcript is shown on the right. Ph18S was used as a loading control in all cases.

tative individuals from three independent T_1 ir-PhCM1 lines (2-4, 24-9 and 33-9) showed reduced PhCM1 transcript levels, but PhCM2 transcript levels were unchanged (Figure S4). Additionally, when transcript levels of other genes in the shikimate, phenylpropanoid and FVBP pathways were analyzed, no differences were observed (Figure S4).

All three selected T_1 ir-PhCM1 lines showed similar FVBP emission profiles. Using MD FVBP emission levels as a reference, phenylacetaldehyde was reduced 85–89% in the ir-PhCM1 lines (Figure 7). Emission of three volatile compounds derived from trans-cinnamic acid (benzaldehyde, benzyl benzoate and methyl benzoate) was reduced 73–84, 62–75 and 50–68%, respectively. Isoeugenol emission was slightly lower in the ir-PhCM1 lines compared to MD, but was not reduced as much as the rest of the major FVBPs analyzed here. Total FVBP emissions were reduced 33.5–40.9% in the T_1 ir-PhCM1 lines compared to MD (Figure 7). Taken together, these data suggest that the lower level of PhCM1 transcripts in the ir-PhCM1 lines results in lower levels of prephenic acid for phenylalanine synthesis, and concomitant reduced FVBP emission.

All T_1 ir-PhCM1 lines were self-pollinated, and T_2 generation plants were screened for homozygosity. The screen identified two homozygous T_2 ir-PhCM1 lines, 24-9 and 33-8 (Figure S5). Whole corollas from stage 9 MD, 24-9 and 33-8 plants were used for quantitative RT-PCR and quantitative total CM activity assays (Figure 8). Compared to MD, transcript accumulation for PhCM1 was reduced in lines 24-9 and 33-8 by 80–85%, but PhCM2 transcript accumulation was unaffected (Figure 8a). The total CM specific activity from desalted crude extracts from lines 24-9 and 33-8 was reduced by 81–84% compared to MD (Figure 8b). Together, these results indicate that reduction of PhCM1 transcript levels and subsequent total CM activity are sufficient for the reduction in total FVBP emissions in the ir-PhCM1 lines compared to MD.

Lines 24-9 and 33-8 were grown side-by-side with MD plants on numerous occasions, and no observable phenotypic differences were noted. There were no significant differences between MD, 24-9 and 33-8 in terms of seed germination, fresh weight, number of true leaves, aerial height (of 9-week-old plants) or stem lignin content (Figures S6 and S7).

DISCUSSION

CHORISMATE MUTASE (CM) has been extensively studied in prokaryotes and fungi, but comparatively less is known about CM in higher plants. The enzymatic reaction catalyzed by CM is the initial committed step in synthesis of the aromatic amino acids phenylalanine and tyrosine (Haslam, 1993), and MD corollas synthesize and emit large quantities of volatile benzenoid/phenylpropanoid compounds, which are derived from phenylalanine (Boatright et al., 2004). Therefore, we chose to investigate CM in petunia flowers through reverse genetic, molecular, biochemical and metabolic approaches. The results indicate that PhCM1 plays a major role in the production of FVBPs in petunia flowers.

In petunia, two CM cDNAs have been isolated. PhCM1 is plastid-localized based on the presence of a cTP sequence (ChloroP 1.1) and a chloroplast import assay (Figure 3) (Zybailov et al., 2008). Of the two putative plastidic Arabidopsis CMs, PhCM1 shares the highest identity to AtCM1 (Figure 2b). Transcript accumulation studies suggest that AtCM1 has a distinct role in the supply of phenylalanine and tyrosine under stressed conditions, while AtCM3 activity can produce requisite levels of prephenic acid under non-stressed growing conditions (Eberhard et al., 1996b; Mobley et al., 1999). PhCM2 is likely to be located in the cytosol due to the lack of a signal peptide (Figure 2a), inability to be imported into a chloroplast (Figure 3), and the lack of

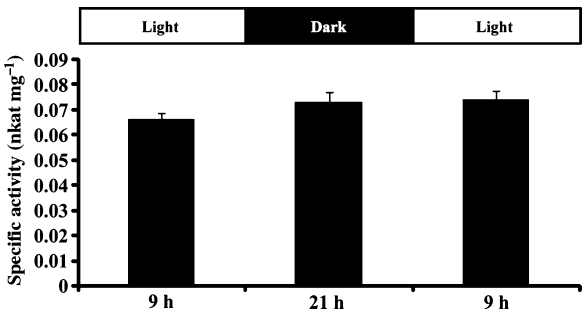

Figure 5. Total CM activity in desalted crude protein extracts from MD whole corollas starting at 9 h of stage 9 in flower development (means and SE; n = 6).

allosteric amino acid regulation (Figures 2a and 6), similar to the cytosolic isoforms in Arabidopsis thaliana, Papaver somniferum (opium poppy) and Solanum lycopersicum (AtCM2, LeCM1 and CM2 from poppy) (Benesova and Bode, 1992; Eberhard et al., 1996a,b). Recently, a subcellular localization study in Arabidopsis leaf tissue utilizing all six arogenate dehydratases and two arogenate dehydrogenases showed that these proteins, which are responsible for the ultimate production of phenylalanine and tyrosine (respectively), are plastidic proteins. Furthermore, pathway intermediates are confined to the plastid (Rippert et al., 2009). This indicates that cytosolic isoforms of CM are separated from substrate and other pathway proteins under normal growing conditions. Therefore, PhCM2 most likely does not play a major role in the production of prephenic acid during non-stressed growing conditions, as proposed for AtCM3. We searched extensively for additional CM sequences in petunia, but could not isolate a potential PhCM3. However, two lines of evidence support the existence of other plastidic CM family members in non-floral tissues of petunia and at the same time illustrate the biological specificity of PhCM1.

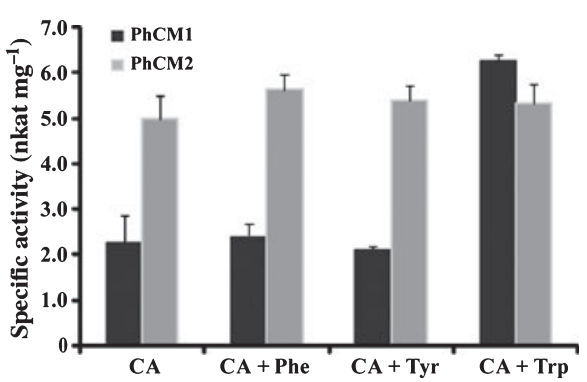

Figure 6. Enzyme activity and effects of aromatic amino acids on petunia CMs.
Recombinant protein was assayed for enzymatic activity in 50 mM KPO_4 buffer, pH 7.6, with 0.5 mM chorismic acid (CA) as a substrate and 50 lM phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) as allosteric effectors (means and SE; n = 4).

First, the ir-PhCM1 transgenic plants do not have observable impairments in vegetative growth compared to MD plants (Figures S6 and S7), but show a specific FVBP phenotype (Figures 7 and 8). Second, relative transcript accumulation for PhCM1 is extremely low in all tissues examined except the corolla (Figures 4a and S2a). However, production of the aromatic amino acids phenylalanine and tyrosine is essential for cellular processes throughout the plant.

The PhCM1 transcript accumulation profile is congruent with those of several other known FVBP genes in petunia. High levels of transcripts are detected in corollas from anthesis to senescence, and are reduced by ethylene exposure, which mimics the pollination event (Figure 4). PhCM2 transcript accumulation follows a more constitutive profile, with a noticeable exception in senescing floral tissue of MD (stage 11), in which its transcript accumulates to relatively high levels (Figures 4b and S2b). However, PhCM2 transcript accumulation does not appear to be affected by exogenous ethylene exposure (Figures 4c and S2c). This result implies that an increase in PhCM2 transcript abundance during senescence is not a direct effect of ethylene perceived at that developmental stage, and this may provide a favorable condition to examine the biological function of cytosolic CM isoforms in planta.

As FVBP emission is rhythmic, transcript accumulation from FVBP biosynthetic genes is rhythmic, and intermediate substrate pools in the FVBP pathway oscillate from low in the morning to high in the evening (Underwood et al., 2005; Verdonk et al., 2005; Orlova et al., 2006), it is reasonable to hypothesize that enzyme activity of one or more proteins in the FVBP pathway oscillates on a daily cycle. However, like PhBSMT activity (Kolosova et al., 2001), total CM activity was not significantly changed from morning to night in desalted crude extracts (Figure 5). In vitro, recombinant PhCM1 activity is increased in the presence of tryptophan (Figure 6), and so oscillations in the free tryptophan pool in vivo cannot be discounted as a regulatory mechanism affecting rhythmic FVBP emission.

AtCM1 and AtCM3 activities are allosterically down-regulated by phenylalanine and tyrosine (Eberhard et al., 1996b; Mobley et al., 1999), but recombinant PhCM1 is not affected by these aromatic amino acids (Figure 6). Petunia corolla limb tissue accumulates a large free phenylalanine pool in the evening, with a calculated concentration of 5.5 mM (Boatright et al., 2004; Kaminaga et al., 2006). Therefore, PhCM1 is sufficient to direct the flux of chorismic acid to the production of phenylalanine without feedback inhibition in the petunia flower. However, it must be noted that the phenylalanine concentration reported in petunia flowers is a whole-tissue measurement, and compartmentalization of the free phenylalanine pool and PhCM1 at a subcellular level remains a plausible mechanism allowing for the large phenylalanine pool. However, Arabidopsis thaliana tissues may not have a biological need for such a

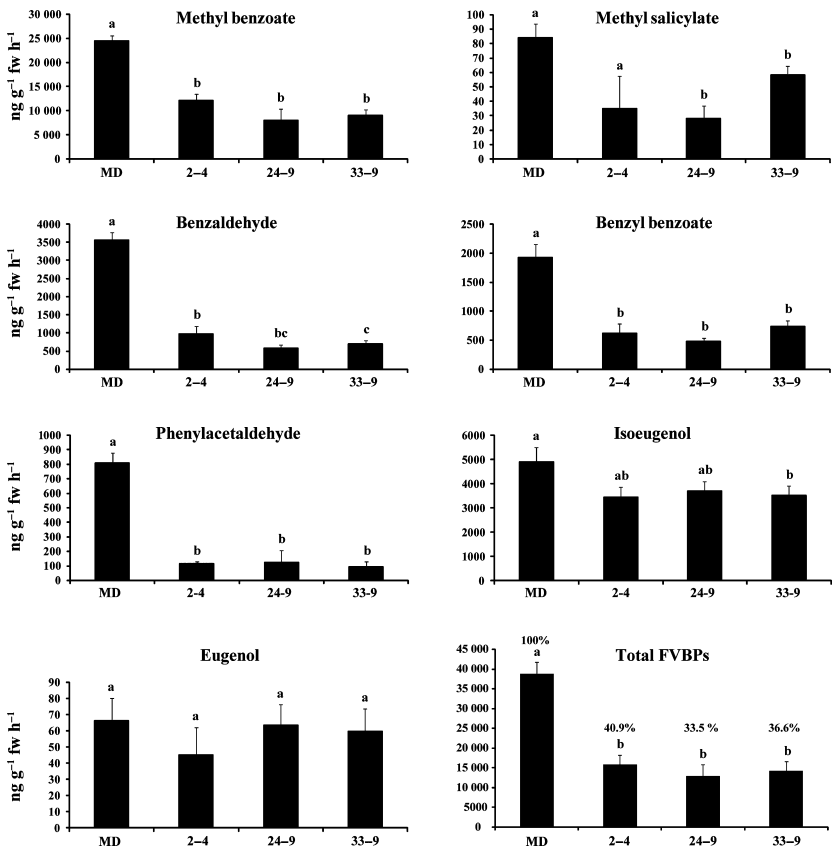

Figure 7. Floral volatile emission analysis from three independent T_1 PhCM1 RNAi lines (means and SE; n = 3).
Major volatile compounds from MD, ir-PhCM1 line 2-4, ir-PhCM1 line 24-9 and ir-PhCM1 line 33-9 flowers. Statistical analysis was performed using Duncan's multiple range test ($P < 0.05$) with SAS 9.2 software (http://www.sas.com/).

large phenylalanine pool, and it would be of interest to assay allosteric regulation of CMs from Arabidopsis lyrata ssp. Petraea, an out-crossing perennial that emits relatively high levels of benzaldehyde and phenylacetaldehyde from floral tissues (Abel et al., 2009).

RNAi-mediated gene silencing produced transgenic petunia plants (ir-PhCM1) that showed reduced levels of PhCM1 transcript, but not PhCM2 (Figure 8a), and reduced total CM enzyme activity (Figure 8b), with a concomitant reduction of FVBP emission (Figure 7). Therefore, PhCM1 has a central role in the production of FVBPs in a petunia flower. Interestingly, total FVBP emission is reduced in ir-PhCM1 lines by about 60% compared to MD, and recombinant PhCM1 activity is increased approximately threefold in the presence of tryptophan (Figure 6). Therefore, it is plausible diminished CM activity in vitro would be increased in vivo by a presumably high level of tryptophan in the flowers of the RNAi lines.

Metabolic analysis of the ir-PhCM1 lines (Figure 7) may illustrate the demand for substrate at each branch of the FVBP pathway. FVBPs derived directly from phenylalanine are the most affected by limiting substrate conditions, while the benzenoids formed from trans-cinnamic acid are affected significantly but to a lesser extent (Figure 7). In contrast, the phenylpropanoids derived from coniferyl acetate are the FVBPs that are least affected in the ir-PhCM1 lines. However, the regulation at each branch point of the pathway may consist of numerous variables, and therefore we can only speculate. PhCM1 RNAi lines in conjugation with metabolite labeling experiments may assist in delineating the flux through the FVBP pathway in the future.

EXPERIMENTAL PROCEDURES

Plant materials

Inbred Petunia ï hybrida cv. 'Mitchell Diploid' (MD) plants were utilized as a 'wild-type' control in all experiments. The ethylene-insensitive CaMV 35S:etr1-1 line 44568, generated in the MD genetic background (Wilkinson et al., 1997), was utilized as a negative control for ethylene sensitivity where applicable. MD, 44568 and PhCM1 RNAi plants were grown as previously described (Dexter et al., 2007). Ethylene treatments used 2 ll $L^{)1}$ of ethylene, with air treatment as a control.

cDNA isolation

Partial sequences from the Sol Genomics Network (http://solgenomics.net/) petunia EST database (Unigene: SGN-U208050) and from a petunia root EST collection (EST ID: dr001P0018N07_F.ab1), provided by Didier Reinhardt of the University of Fribourg, were used as references to obtain full-length cDNAs by 5ïand 3ïRACE with the SMART™ RACE cDNA amplification kit (Clontech Laboratories Inc., http://www.clontech.com/) according to the manufacturer's protocol. The resulting 1257 bp cDNA contained a 975 bp coding sequence (GenBank accession number EU751616) for a

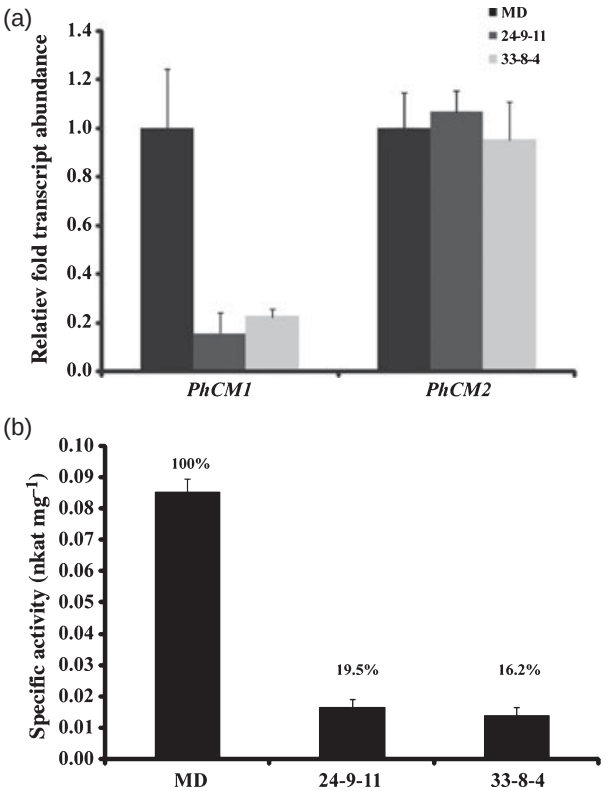

Figure 8. Comparative transcript analysis and total CM activity for MD and representative individuals from independent homozygous T_2 ir-PhCM1 lines.
(a) Quantitative RT-PCR was performed with two biological replicates and three technical replicates per biological replicate. The entire experiment was performed in duplicate, and analyzed by the DDC_t method with PhFBP1 and Ph18S as the internal references.
(b) Total CM activity in desalted crude protein extracts from whole corollas of MD and representative individuals from two independent homozygous T_2 ir-PhCM1 lines (24-9 and 33-8).
Values are means and SE.

predicted 324 amino acid protein and was termed PhCM1, while the 913 bp cDNA had a 792 bp coding sequence (GenBank accession number EU751617) for a predicted 263 amino acid protein, and was termed PhCM2. Both PhCM1 and PhCM2 coding sequences were amplified using Phusion™ Hot Start High-Fidelity DNA polymerase (New England Biolabs Inc., http://www.neb.com) and were cloned into a pGEM -T Easy vector (Promega Corp., http://www.promega.com/), and extensively sequenced and checked for errors. These constructs were used as template to clone the predicted mature PhCM1 and PhCM2 coding sequences into a pET-32 EK/LIC vector (Novagen, http://www.merck-chemicals.com/life-science-research).

Transcript accumulation analysis

All experiments were performed using at least two biological replicates, with equivalent results observed. In all cases, total RNA was extracted as previously described (Verdonk et al., 2003) and subjected to TURBO™ DNase treatment (Ambion Inc., http://www.ambion.com) followed by total RNA purification using the RNeasy Mini protocol for RNA clean-up (Qiagen, http://www.qiagen.com/). Total RNA was then quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, http://www.thermo.com), and 50 ng μl^{-1} dilutions were prepared and stored at)20 C.

Semi-quantitative RT-PCR was performed on a Veriti™ 96-well thermal cycler (Applied Biosystems, http://www.appliedbiosystems.com/). All semi-quantitative RT-PCR reactions used a Qiagen One-step RT-PCR kit with 50 ng total RNA template. To visualize RNA loading concentrations, samples were amplified using Ph18S (accession number AJ236020) primers and analyzed on an agarose gel. Gene-specific primers were designed and utilized for visualization of the relative transcript accumulation levels (Table S2).

The spatial transcript accumulation series consisted of total RNA isolated from root, stem stigma, anther, leaf, petal tube, petal limb and sepal tissues obtained from three individual MD plants at 16:00 h on multiple occasions over the course of a year. The developmental transcript accumulation series consisted of MD floral tissue collected at 11 different stages: floral bud <0.5 cm (stage 1); bud 0.5 to 1.5 cm (stage 2); bud 1.5 to 3.0 cm (stage 3); bud 3.0 to 5.0 cm (stage 4); bud fully elongated, 5.0 to 6.5 cm (stage 5); flower opening 0 to 2 cm limb diameter (anthesis) (stage 6); flower fully open days 0 (stage 7), 1 (stage 8), 2 (stage 9) and 3 (stage 10); observably senescing flower (flower open day 7 for MD) (stage 11). All tissues were collected at the same time and on the same day, and total RNA was isolated from all samples collected.

The exogenous ethylene series consisted of excised MD and 44568 stage 9 flowers (in tap water) placed into eight tanks, four for ethylene treatment and four for air treatment. Air and ethylene treatments were performed for 0, 1, 2, 4 and 8 h starting at 12:00 h. Immediately after treatment, flower samples were collected, stored at)80 C, and total RNA was isolated from all corolla tissues once all samples had been collected. The ethylene treatment experiment consisted of two biological replicates and was performed twice. For all tissue collections, individual samples consisted of three flowers.

Quantitative RT-PCR was performed and the results analyzed using a MyIQ real-time PCR detection system (Bio-Rad Laboratories Inc., http://www.bio-rad.com/). Stage 9 whole-corolla tissue was collected from MD plants and two independent homozygous T_2 PhCM1 RNAi lines at 16:00 h. Total RNA was isolated from all samples as described above, and transcript accumulation was initially analyzed by semi-quantitative RT-PCR. For subsequent quantitative RT-PCR analysis, the Power SYBR Green RNA-to-C_t™ 1-Step kit (Applied Biosystems) was used to amplify and detect the resulting products according to the manufacturer's protocol. Quantitative RT-PCR primers (Table S2) were designed using Primer Express software version 2.0 (Applied Biosystems), gene specificity during melt curve analysis was demonstrated, and then the primers were optimized.

Protein extraction, over-production and purification

Desalted crude protein extracts were obtained from whole-corolla tissue by grinding in a mortar and pestle with liquid nitrogen to a fine powder, addition of chilled extraction buffer [50 mM Bis/Tris/HCl pH 6.9, 10 mM b-mercaptoethanol, 5 mM $Na_2S_2O_5$, 1% polyvinylpyrrolidone, 1:100 protease inhibitor cocktail (P9599; Sigma, http://www.sigmaaldrich.com/), 10% glycerol], centrifugation at 4 C (15 min at 10 000 g) to separate cellular debris, and further separation of low-molecular-weight substances using a PD-10 desalting column (GE Healthcare, http://www.gehealthcare.com). Total crude protein concentration was determined by the Bradford method using BSA as the standard (Bio-Rad).

Biologically active pET-32–PhCM1 and pET-32–PhCM2 were expressed in E. coli BL21(DE3)/pLysS with induction using 1 mM isopropyl b-D-1-thiogalactopyranoside overnight at 37 C. Induction was analyzed for crude cellular extracts on a 10% polyacrylamide/Tris–HCl Ready Gel (Bio-Rad). Soluble protein was obtained from induced cells lysed using BugBuster protein extract reagent (Novagen) and affinity-purified by His-Band resin chromatography

(Novagen). The resulting recombinant proteins were then separated from any low-molecular-weight compounds and concentrated using a 30 000 nominal molecular weight limit Amicon Ultra-4 centrifugal filter device (Millipore, http://www.millipore.com/). Recombinant protein concentration was determined by the Bradford method using BSA as the standard.

Chorismate mutase enzyme activity assays

Specific activities in vitro were resolved by carefully following the absorbance of chorismic acid spectrophotometrically (SmartSpec™ 3000, Bio-Rad) at 274 nm (ε = 2630 M^{-1} cm^{-1}) (Gilchrist and Connelly, 1987; Kast et al., 1996). All assays were performed at 30 C with 0.5 mM chorismic acid (>90%; C1761, Sigma) in 50 mM KPO_4 buffer, pH 7.6. Where stated, 50 lM phenylalanine, tyrosine and tryptophan (P2126, T3754 and T0254, respectively; Sigma) were used to assay for allosteric regulation of enzyme activity. Non-enzymatic chorismic acid breakdown and inactive protein controls were used to normalize all data generated. Additionally, no activity was detected when purified tag fusion proteins from the empty pET-32 vector were used. Multiple biological replicates and corresponding technical replicates were used to generate all data shown.

Chloroplast import assay

Full-length coding sequences for PhCM1 and PhCM2 were cloned into a pGEM -T Easy vector (Promega) in the SP6 orientation. The chloroplast import assay was performed as described previously (Martin et al., 2009). Briefly, in vitro transcription and translation using wheat germ TNT (Promega) resulted in radiolabeled PhCM1, PhCM2 and PsOE23, which were individually incubated with isolated pea chloroplasts (Pisum sativum) for 15 min. After import, the isolated chloroplasts were treated with 100 lg ml^{-1} thermolysin for 40 min at 4 C. Proteolysis was terminated by addition of EDTA to a final concentration of 10 mM, and the intact chloroplasts were then re-purified by centrifugation (8 min at 3200 g) through 35% Percoll™ (GE Healthcare). Chloroplasts were washed, lysed, and fractionated into total membranes and stromal extracts by centrifugation for 18 min at 15 000 g. The translation products, chloroplasts, thermolysin-treated chloroplasts, stromal extracts and total membranes were analyzed by SDS–PAGE and fluorography. In Figure 3, the results for PhCM2 and PsOE23 are from a 20 h exposure, and those for PhCM1 are from a 4-day exposure. These results are from two different gels of the same samples loaded identically. The panels have been cropped and contrast-adjusted, but no other modifications have been made.

Volatile emission

For all volatile emission experiments, emitted floral volatiles from excised flowers were collected at 17:00 h and quantified as previously described (Underwood et al., 2005; Dexter et al., 2007).

Generation of ir-PhCM1 transgenic petunia

Generation of ir-PhCM1 transgenic plants was performed as described previously (Dexter et al., 2007), but with two fragments of the PhCM1 cDNA (Figure S3) amplified and ligated end-to-end in a sense/antisense orientation, with additional sequence information used for an inter-fragment intron (hairpin).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. PhCM1 and PhCM2 coding sequence alignment.

Figure S2. Quantitative RT-PCR transcript accumulation analysis of PhCM1 and PhCM2 in petunia.

Figure S3. Schematic representation and nucleotide comparison of the region used for production of petunia PhCM1 RNAi transgenic lines.

Figure S4. Semi-quantitative RT-PCR transcript accumulation analysis in floral tissues of three independent T_1 ir-PhCM1 lines.

Figure S5. Semi-quantitative RT-PCR transcript accumulation analysis in floral tissues of two independent homozygous T_2 ir-PhCM1 lines.

Figure S6. Physiological comparison between 9-week-old seedlings of MD and representative independent T_2 irPhCM1 homozygous lines 24-9 and 33-8.

Figure S7. Stem cross-sections from 9-week-old ir-PhCM1 petunia seedlings.

Table S1. Functional complementation of CM-deficient E. coli KA12/pKIMP-UAUC.

Table S2. Gene-specific primers used for the transcript accumulation analyses throughout this study.

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The GenBank accession numbers for the PhCM1 and PhCM2 sequences are EU751616 and EU751617, respectively.