CCoAOMT Down-Regulation Activates Anthocyanin Biosynthesis in Petunia[1]

Nur Fariza M. Shaipulah, Joëlle K. Muhlemann[2], Benjamin D. Woodworth, Alex Van Moerkercke[3], Julian C. Verdonk, Aldana A. Ramirez, Michel A. Haring, Natalia Dudareva, and Robert C. Schuurink*

Department of Plant Physiology, University of Amsterdam, Swammerdam Institute for Life Sciences, 1098 XH Amsterdam, The Netherlands (N.F.M.S., A.V.M., A.A.R., M.A.H., R.C.S.); Pusat Pengajian Sains Marin dan Sekitaran, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia (N.F.M.S.); Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907–2063 (J.K.M., B.D.W., N.D.); and Horticulture and Product Physiology, Plant Sciences Group, Wageningen University, Wageningen, the Netherlands 6700 AA (J.C.V.)

ORCID IDs: 0000-0002-1237-7951 (J.C.V.); 0000-0003-0777-7763 (N.D.).

Anthocyanins and volatile phenylpropenes (isoeugenol and eugenol) in petunia (*Petunia hybrida*) flowers have the precursor 4-coumaryl coenzyme A (CoA) in common. These phenolics are produced at different stages during flower development. Anthocyanins are synthesized during early stages of flower development and sequestered in vacuoles during the lifespan of the flowers. The production of isoeugenol and eugenol starts when flowers open and peaks after anthesis. To elucidate additional biochemical steps toward (iso)eugenol production, we cloned and characterized a caffeoyl-coenzyme A *O*-methyltransferase (PhCCoAOMT1) from the petals of the fragrant petunia 'Mitchell'. Recombinant PhCCoAOMT1 indeed catalyzed the methylation of caffeoyl-CoA to produce feruloyl CoA. Silencing of *PhCCoAOMT1* resulted in a reduction of eugenol production but not of isoeugenol. Unexpectedly, the transgenic plants had purple-colored leaves and pink flowers, despite the fact that cv Mitchell lacks the functional R2R3-MYB master regulator ANTHOCYANIN2 and has normally white flowers. Our results indicate that down-regulation of *PhCCoAOMT1* activated the anthocyanin pathway through the R2R3-MYBs PURPLE HAZE (PHZ) and DEEP PURPLE, with predominantly petunidin accumulating. Feeding cv Mitchell flowers with caffeic acid induced *PHZ* expression, suggesting that the metabolic perturbation of the phenylpropanoid pathway underlies the activation of the anthocyanin pathway. Our results demonstrate a role for PhCCoAOMT1 in phenylpropene production and reveal a link between PhCCoAOMT1 and anthocyanin production.

Phenylpropanoids represent an important class of widespread plant secondary metabolites derived from L-Phe. These essential compounds provide protection against UV light (flavonoids) and serve as structural components of cell walls (lignins). They also function in the defense against herbivores and pathogens, while floral pigments (anthocyanins) and scent compounds (volatile phenylpropanoids) mediate plant-pollinator interactions (Bhuiyan et al., 2009; Vanholme et al., 2010; Klahre et al., 2011; Falcone Ferreyra et al., 2012; Sheehan et al., 2012; Dudareva et al., 2013).

Petunia spp. are a model system in which to study anthocyanin and volatile phenylpropanoid biosynthesis due to the existence of cultivars with a variety of flower colors and scent bouquets (Tsuda, 2004; Quattrocchio et al., 2006; Schuurink et al., 2006). While most colored petunia varieties are scentless and used for the investigation of anthocyanin biosynthesis, the white varieties are fragrant and used to study the formation of volatile phenylpropanoids. Across *Petunia* spp., carbon allocation into scent or color compounds appears to be tightly regulated and tailored to attracting specific pollinators. For example, nocturnal hawkmoths are attracted to the fragrant white flowers of *Petunia axillaris*, bees to the slightly fragrant colored *Petunia integrifolia*, and hummingbirds to the brightly colored, scentless *Petunia exserta* (Ando et al., 2001; Hoballah et al., 2007; Klahre et al., 2011).

[1] This work was supported by the Malaysian Government (grant no. 810416115384 to N.F.M.S.).

[2] Present address: Department of Biology, Wake Forest University, 1834 Gulley Drive, Winston-Salem, NC 27109.

[3] Present address: Department of Plant Systems Biology, VIB, and Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium.

* Address correspondence to r.c.schuurink@uva.nl.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Robert C. Schuurink (r.c.schuurink@uva.nl).

N.F.M.S. performed most of the experiments and contributed to the writing; J.K.M. performed metabolite analyses and enzymatic assays; B.D.W. synthesized several compounds and analyzed enzymatic activity; A.V.M. cloned CCoAOMT1; J.C.V. performed proteome analysis; A.A.R. did the localization studies; M.A.H. cosupervised N.F.M.S.; N.D. conceived parts of the research plans and cowrote the article; R.C.S. conceived the project, supervised the work, and wrote the article with contributions from all the authors.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.01646

Petunia (*Petunia hybrida* 'Mitchell') has white flowers emitting high levels of Phe-derived volatiles: phenylacetaldehyde, phenylethylalcohol, methylbenzoate, benzylbenzoate, methylsalicylate, benzaldehyde, benzylalcohol, benzylacetate, phenylethylbenzoate, isoeugenol, eugenol, and vanillin. Based on the carbon side chain attached to the benzene ring, these compounds can be divided into three groups: benzenoids (C_6-C_1), phenylpropanoid-related compounds (C_6-C_2), and phenylpropenes (C_6-C_3). L-Phe acts as an immediate precursor for the C_6-C_2 compounds, whereas it is subjected to deamination by phenylalanine ammonia lyase (PAL) to form trans-cinnamic acid (t-CA), a starting metabolite for the biosynthesis of precursors for C_6-C_1 and C_6-C_3 compounds (Fig. 1). Formation of the benzenoids (C_6-C_1) from t-CA requires shortening of the side chain by a C_2 unit, which was shown to occur via the β-oxidative pathway (Van Moerkercke et al., 2009; Qualley et al., 2012). The volatile C_6-C_3 (phenylpropenes) compounds have been proposed to share the initial biosynthetic steps with the lignin biochemical pathway up to the formation of coniferyl alcohol. In this pathway, t-CA is first hydroxylated to *p*-coumaric acid, which is then converted to 4-coumaryl-CoA (Klempien et al., 2012), the branch point metabolite between anthocyanin and phenylpropene synthesis (Fig. 1; Boerjan et al., 2003; Dexter et al., 2007). While some phenylpropene-forming enzymes have been isolated and characterized from petunia, including PhCCR1, PhCFAT, PhEGS, and PhIGS (Fig. 1; Dexter et al., 2007; Koeduka et al., 2008, 2009; Muhlemann et al., 2014), there is still no genetic or biochemical evidence that all monolignol biosynthetic steps are shared with phenylpropene formation.

In anthocyanin biosynthesis, 4-coumaryl-CoA is converted by chalcone synthase (CHS) to chalcone, which undergoes further enzymatic modification to produce a variety of anthocyanins. The variations in

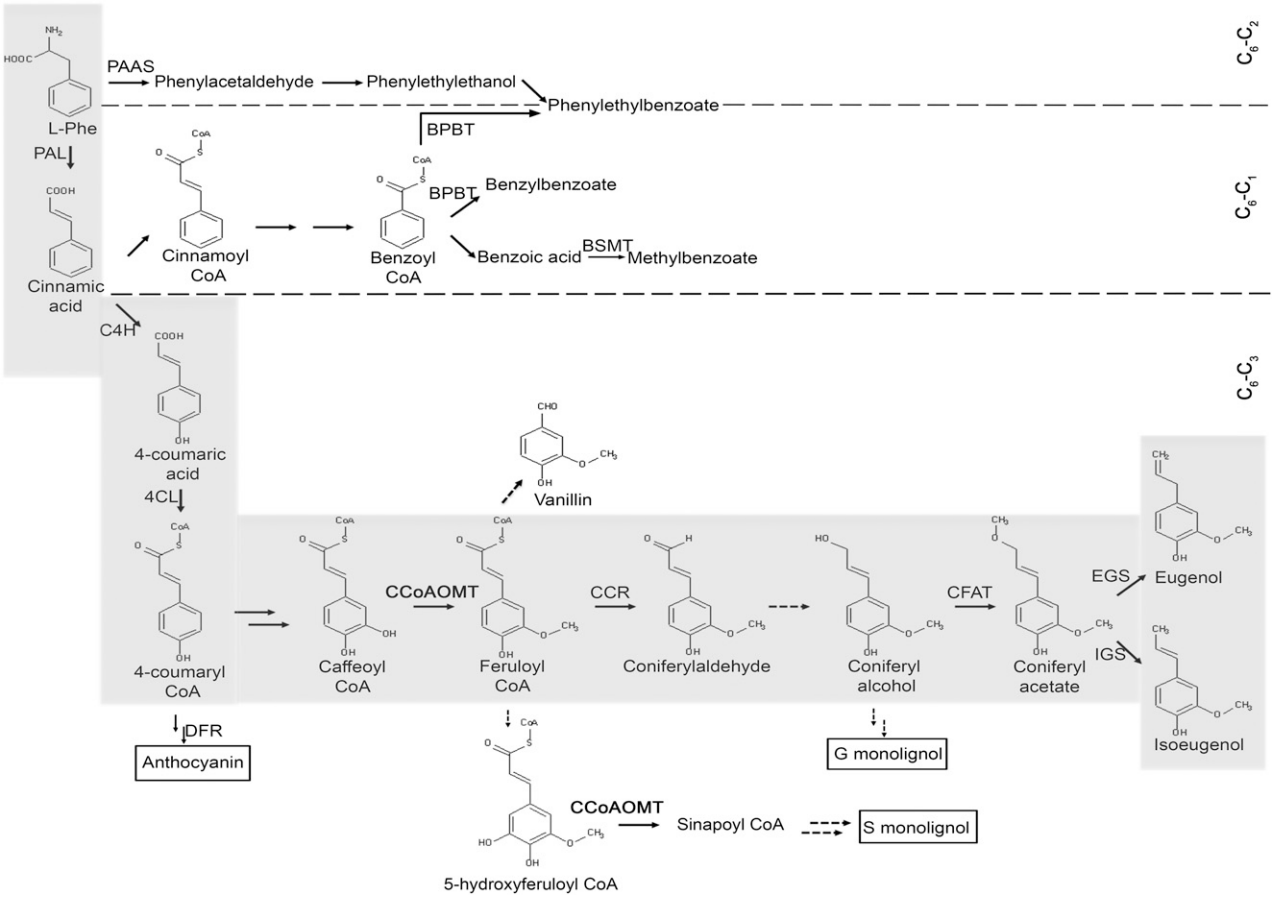

Figure 1. Metabolic pathways leading to volatile C_6-C_1, C_6-C_2, and C_6-C_3 compounds in petunia petals. The phenylpropene pathway is shaded gray, and the CCoAOMT reaction is illustrated in boldface letters. 4-Coumaryl-CoA is the precursor for flavonoid/anthocyanin biosynthesis. Solid arrows indicate established biochemical steps, while broken arrows represent hypothetical steps. Stacked arrows illustrate the involvement of multiple enzymatic reactions. 4CL, 4-Coumarate-CoA ligase; BPBT, benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase; BSMT, *S*-adenosyl-L-Met:benzoic acid/salicylic acid methyltransferase; C4H, cinnamate-4-hydrolase; CCR, cinnamoyl-CoA reductase; CFAT, coniferylalcohol acetyltransferase; DFR, dihydroflavonol reductase; EGS, eugenol synthase; IGS, isoeugenol synthase; PAAS, phenylacetaldehyde synthase.

color and scent between petunia cultivars suggest that anthocyanin and phenylpropene pathways are closely regulated (Cna'ani et al., 2015). Previous studies revealed that two R2R3-MYB transcription factors, ANTHOCYANIN2 (AN2) and ODORANT1 (ODO1), drive anthocyanin and volatile phenylpropanoid biosynthesis, respectively (Hoballah et al., 2007; Klahre et al., 2011). Interestingly, only a few studies have focused on interactions between phenylpropene and anthocyanin pathways in petunia (Verdonk et al., 2005; Ben Zvi et al., 2008; Cna'ani et al., 2015), leaving an open question about the carbon flux distribution between these two biosynthetic pathways and their coregulation.

Caffeoyl-coenzyme A *O*-methyltransferases (CCoAOMTs) have been studied in many plant species, including poplar (*Populus* spp.; Zhong et al., 2000), alfalfa (*Medicago sativa*; Marita et al., 2003), tobacco (*Nicotiana tabacum*; Zhong et al., 1998), and Arabidopsis (*Arabidopsis thaliana*; Do et al., 2007), as key enzymes in lignin biosynthesis. Here, we describe a role for PhCCoAOMT1 in volatile phenylpropene (C_6-C_3) formation in petunia 'Mitchell' and its link to the regulation of anthocyanin production. We show that, like the previously characterized CCoAOMTs, recombinant PhCCoAOMT1 catalyzes the methylation of caffeoyl-CoA to feruloyl-CoA and is involved in phenylpropene biosynthesis, specifically eugenol, with minor effects on lignin formation. Interestingly, *PhCCoAOMT1* down-regulation leads to the accumulation of anthocyanins in flowers and vegetative tissues, highlighting the metabolic connection between scent and color biosynthesis.

RESULTS

Functional Expression of PhCCoAOMT1

To extend the investigation of the contribution of the monolignol pathway to the biosynthesis of volatile phenylpropenes (Fig. 1; Muhlemann et al., 2014), we obtained from our petunia petal EST database one putative CCoAOMT with similarity to CCoAOMTs. This putative PhCCoAOMT1 encodes a protein with 85% and 90% similarity to the biochemically characterized CCoAOMTs from *Vitis vinifera* (Busam et al., 1997) and tobacco (Maury et al., 1999), respectively. Since the R2R3-MYB transcription factor ODO1 is one of the master regulators of phenylpropanoid/benzenoid volatile production in petunia (Verdonk et al., 2005; Van Moerkercke et al., 2012) and can up-regulate *CCoAOMTs* in tomato (*Solanum lycopersicum*; Dal Cin et al., 2011), we tested whether down-regulation of *ODO1* affected *PhCCoAOMT1* transcript levels. Indeed, the *PhCCoAOMT1* mRNA levels were significantly reduced in *ODO1*-silenced lines compared with the wild type (Supplemental Fig. S1), indicating that *PhCCoAOMT1* expression is ODO1 dependent and, thus, likely involved in scent production. To verify the enzymatic activity of PhCCoAOMT1, its coding region was cloned in the *Escherichia coli* expression vector pGEX-KG with an N-terminal glutathione *S*-transferase (GST) tag, which facilitated purification of the recombinant protein. The purified protein preferentially converted caffeoyl-CoA to feruloyl CoA, was able to methylate 5-hydroxyferuloyl-CoA, and no activity was detected with caffeic acid or 5-hydroxyferulic acid (Supplemental Table S1), consistent with the substrate specificities of previously characterized CCoAOMTs (Inoue et al., 1998; Martz et al., 1998; Maury et al., 1999). PhCCoAOMT1 is localized to the cytosol in planta (Supplemental Fig. S2), similar to the subcellular localization of other CCoAOMTs (Chen et al., 2000; Wang et al., 2013).

Down-Regulation of *PhCCoAOMT1* Expression Reduces Eugenol Biosynthesis

To investigate the role of PhCCoAOMT1 within the phenylpropanoid network, its expression was reduced via an RNA interference (RNAi) strategy. A hairpin construct of *PhCCoAOMT1* was generated, using the 3′ untranslated region under the control of a cauliflower mosaic virus 35S promoter, and used to transform cv Mitchell. Eighteen independent ir-*PhCCoAOMT1* lines were obtained. After characterization of the transcript levels of *PhCCoAOMT1* in petals from all T0 lines by quantitative reverse transcription (qRT)-PCR, three independent lines were brought to the next generations (line 10 to T1 and lines 4 and 13 to T3). All three lines showed a statistically significant reduction of approximately 40% in *PhCCoAOMT1* mRNA levels in petunia petals (Fig. 2).

To determine the effect of reduced *PhCCoAOMT1* transcript levels on volatile production and emission in transgenic lines, emitted volatiles and their internal pools were measured by gas chromatography-mass spectrometry (GC-MS). We focused on the

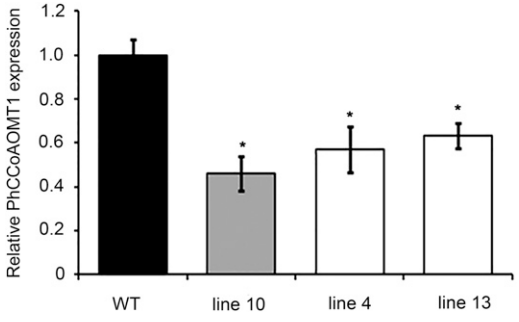

Figure 2. Down-regulation of *PhCCoAOMT1* in petunia flowers. *PhCCoAOMT1* transcript levels were determined by qRT-PCR. Petal limbs from three independent ir-*PhCCoAOMT1* lines (line 10 in the T1 generation and lines 4 and 13 in the T3 generation) and cv Mitchell as the wild-type control (WT) were collected from 2-d-old flowers. Expression values are depicted relative to the wild type, with its average value set to 1. Mean values ± SE of three biological replicas are shown, normalized for *FLORAL BINDING PROTEIN1* (*FBP1*) transcript levels. *, $P < 0.05$ as determined by Student's *t* test of each transgenic line compared with the wild type.

phenylpropenes (C_6-C_3), since we hypothesized that PhCCoAOMT1 is involved in the pathway leading to their production. Interestingly, internal pools of eugenol, but not of isoeugenol, were significantly decreased in all three transgenic lines compared with the wild type (Fig. 3A). The emission of eugenol showed a trend toward reduction, albeit not significantly, while isoeugenol emission was not affected in these ir-*PhCCoAOMT1* plants (Fig. 3B). Interestingly, the petals of lines 4 and 13 emitted more phenylacetaldehyde (C_6-C_2; Fig. 3B). In addition, line 13 emitted more phenylethylbenzoate (C_6-C_1/C_2) and produced more benzylbenzoate (C_6-C_1), which was not observed in the other two lines (Fig. 3B). Emission and internal pools of all other volatiles remained unaffected; thus, down-regulation of *PhCCoAOMT1* exclusively affects eugenol production in all three transgenic lines.

Down-Regulation of *PhCCoAOMT1* Expression Leads to the Coloration of Petunia Tissues

Unexpectedly, most of the 18 independent ir-*PhCCoAOMT1* lines had purple leaves already during earlier stages of the transformation process (e.g. during leaf initiation). In those lines, the floral buds were purple, whereas the petals had a pink blush upon opening that faded in older flowers. In addition, stems and leaves appeared purple (Fig. 4, A and B), and cross sections of stems displayed purple pigmentation in vascular bundles (Fig. 4D). This phenotype prompted us to examine whether *PhCCoAOMT1* transcript levels were affected in leaves of ir-*PhCCoAOMT1* lines. Indeed, *PhCCoAOMT1* expression in leaves was highly reduced compared with the wild type, with the strongest reduction in line 4 (80%), which was much stronger than in petals (Fig. 5).

PhCCoAOMT1 Belongs to a Small Gene Family

CCoAOMTs belong to a small gene family in other plant species like Arabidopsis and tobacco (Martz et al., 1998; Raes et al., 2003); thus, we searched the *P. axillaris* genome that has become available to the petunia community (http://flower.ens-lyon.fr/PetuniaPlatform/PetuniaPlatform.html) for more PhCCoAOMTs. This

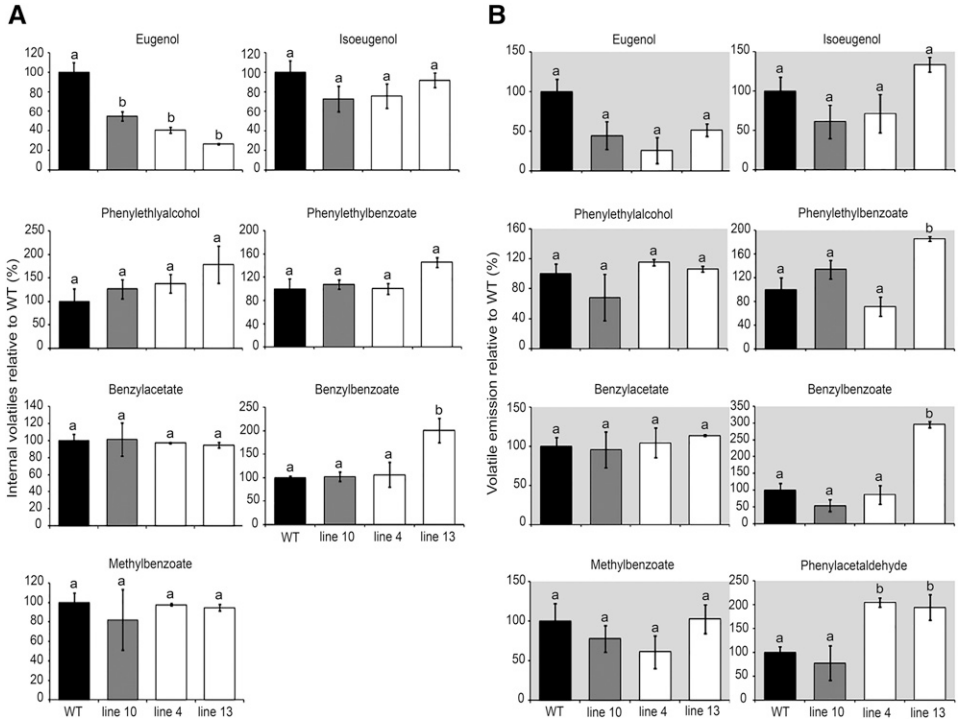

Figure 3. Effects of *PhCCoAOMT1* silencing on volatile production. A, Internal levels of volatile phenylpropanoid/benzenoid compounds in petal limbs of cv Mitchell wild-type control (WT) and ir-*PhCCoAOMT1* plants (line 10 in the T1 generation and lines 4 and 13 in the T3 generation). Three flowers were used for each biological replicate. Volatile levels in the wild type were set to 100%. Mean values ± SE of three biological replicates are shown, standardized using tetralin. B, Emission of volatile phenylpropanoid/benzenoid compounds from petal limbs of cv Mitchell and ir-*PhCCoAOMT1* plants. Three flowers were used for each biological replicate. Volatiles were collected from three detached flowers per biological replicate for 24 h ($n = 3$) and were analyzed by GC-MS. Emission of each volatile by the wild-type petals was set to 100%. Mean values ± SE of three biological replicates are shown, standardized using tetralin. Letters indicate significant differences among lines (ANOVA followed by Tukey's posthoc analysis, $P < 0.05$).

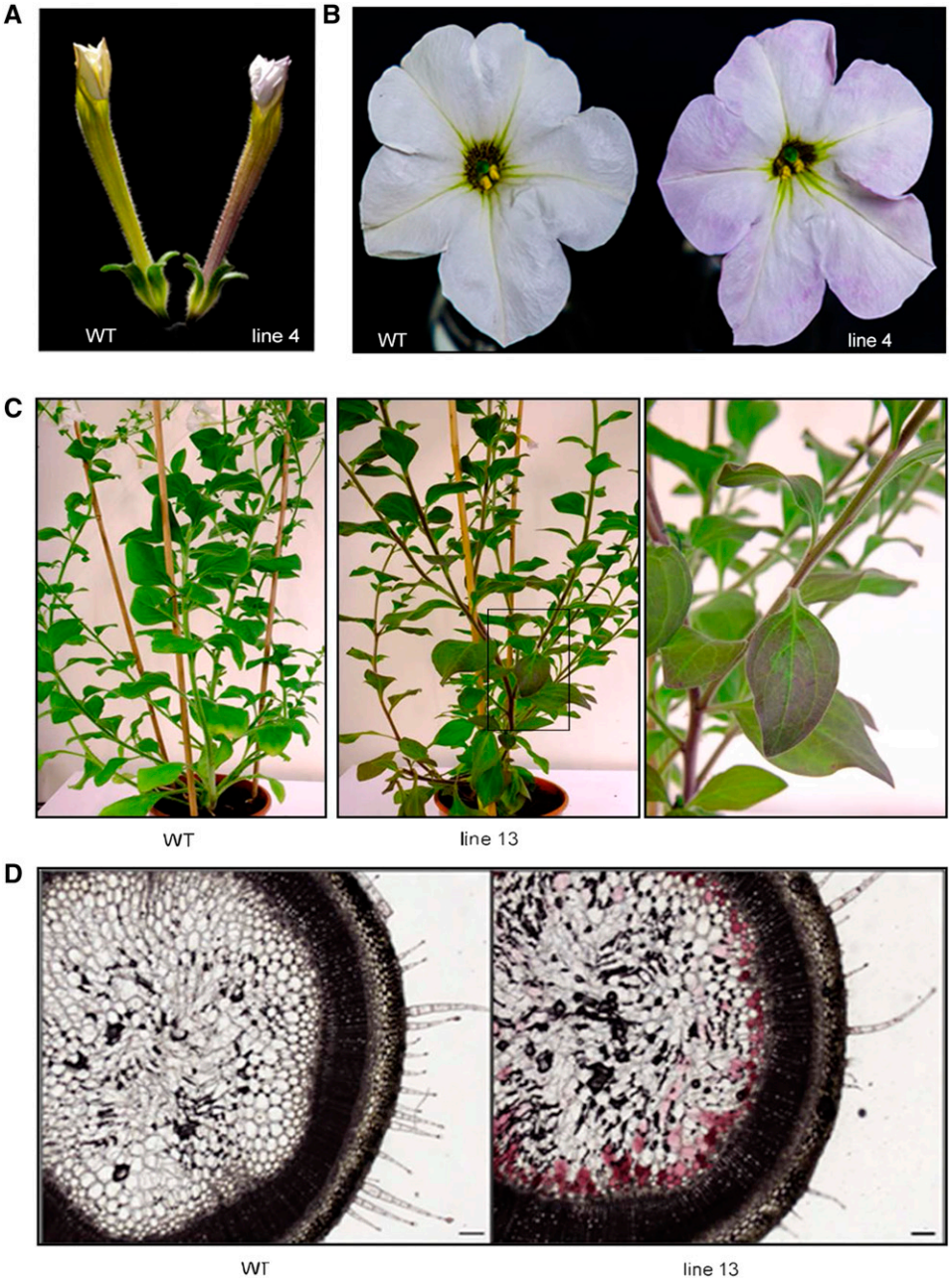

Figure 4. Phenotypes of ir-*PhCCoAOMT1* plants. A and B, Buds (A) and open flowers (B). The cv Mitchell wild-type control (WT) produces white (open) flowers, whereas the transgenic lines have purple-blushed flowers. C, Pigmentation in leaves and stems of wild-type and ir-*PhCCoAOMT1* plants. D, Unstained histological stem cross sections (300 μm) of cv Mitchell and *PhCCoAOMT1* line 13. The cross sections were made from 2-month-old plants. Bars = 25 μm.

search revealed two additional putative PhCCoAOMTs, designated PhCCoAOMT2 and PhCCoAOMT3. At the same time, we performed proteomics on cv Mitchell petals and detected several peptides (Supplemental Table S2) that matched the predicted amino acid sequence of either PhCCoAOMT2 or PhCCoAOMT3, indicating that these two putative CCoAOMTs are also expressed in petunia petals. Subsequently, the full-length complementary DNAs (cDNAs) of *PhCCoAOMT2* and *PhCCoAOMT3* were obtained from petal mRNA. The predicted amino acids sequences of PhCCoAOMT2 and PhCCoAOMT3 share 84.7% and 88.7% identity with PhCCoAOMT1, respectively. A homology tree with proteins from the plant *O*-methyltransferase A1 subfamily (Provenzano et al., 2014) shows that the three PhCCoAOMTs are highly related to characterized caffeoyl-CoA *O*-methyltransferases (Supplemental Fig. S3). Recombinant proteins of both PhCCoAOMT2 and PhCCoAOMT3 were able to convert caffeoyl-CoA to feruloyl-CoA and exhibited substrate specificities similar to PhCCoAOMT1 (Supplemental Table S1). Analysis of the *PhCCoAOMT2* and *PhCCoAOMT3* transcript levels in petals of ir-*PhCCoAOMT1* lines revealed that they were not affected (Supplemental Fig. S4), suggesting that the reduction in *PhCCoAOMT1* mRNA is responsible for the observed phenotypes in these lines.

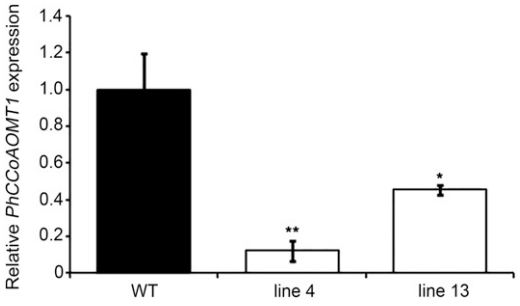

Figure 5. Transcript levels of *PhCCoAOMT1* in petunia leaves. Leaves from ir-*PhCCoAOMT1* and cv Mitchell wild-type control (WT) plants were collected from nodes 7 to 10, and transcript levels were determined by qRT-PCR. Expression values are depicted relative to the wild type, with its average value set to 1. Mean values ± SE of three biological replicas are shown, normalized for *ELONGATION FACTOR 1α* (*EF1α*). *, $P < 0.05$ and **, $P < 0.01$ as determined by Student's *t* test of each transgenic line compared with the wild type.

PhCCoAOMT1 Is Not Expressed Exclusively in the Flowers

PhCCoAOMT1 was highly expressed in petal limbs and tubes, and low levels of transcripts were also detected in stems, roots, and leaves (Figs. 5 and 6A). Thus, like the other known biosynthetic genes responsible for the formation of phenylpropanoid/benzenoid volatile compounds (Colquhoun et al., 2010), *PhCCoAOMT1* is most highly expressed in flowers. In petals, *PhCCoAOMT1* transcript levels were developmentally regulated: expression was low in closed floral buds, increased consistently throughout the developmental stages until anthesis (Fig. 6B), and then reached the lowest level upon senescence. Interestingly, the expression patterns of *PhCCoAOMT2* and *PhCCoAOMT3* resemble that of *PhCCoAOMT1* (Supplemental Fig. S5). Another hallmark of scent-related phenylpropanoid/benzenoid biosynthetic genes in petunia is a diurnal rhythmicity in their expression (Fenske et al., 2015). *PhCCoAOMT1* transcript levels peaked approximately 3 to 4 h before the onset of the dark period (Fig. 6C), like most of the scent biosynthetic genes (Colquhoun et al., 2010), thus preceding the production of (iso)eugenol (Verdonk et al., 2003). The expression of *PhCCoAOMT2* and *PhCCoAOMT3* also exhibited diurnal rhythms, similar to that of *PhCCoAOMT1* (Supplemental Fig. S5), with *PhCCoAOMT3* peaking later, just before the dark period.

Anthocyanin Biosynthesis Is Activated in ir-*PhCCoAOMT1* Plants

The ir-*PhCCoAOMT1* plants displayed a purple pigmentation in their flowers and vegetative tissues, which could be due to an increase in anthocyanin production. Liquid chromatography-mass spectrometry analysis of anthocyanin levels showed that petals, leaves, and stems of the ir-*PhCCoAOMT1* plants indeed accumulated delphinidin, malvidin, peonidin, and petunidin. In all anthocyanin-accumulating tissues, petunidin was the major compound, leading to the purple color of plants, with the highest levels in leaves and stems (Fig. 7A). These data further support that, in ir-*PhCCoAOMT1* plants, the metabolite pathway flux toward anthocyanin production is increased. To explore the molecular mechanisms of enhanced anthocyanin formation in ir-*PhCCoAOMT1* transgenic lines, we analyzed the transcript levels of *DIHYDROFLAVANOL 4-REDUCTASE* (*DFR*), *CHSA* and *CHSJ*, *FLAVONOID 3′5′ HYDROXYLASE* (*F3′5′H*), *GST* (*AN9*), and

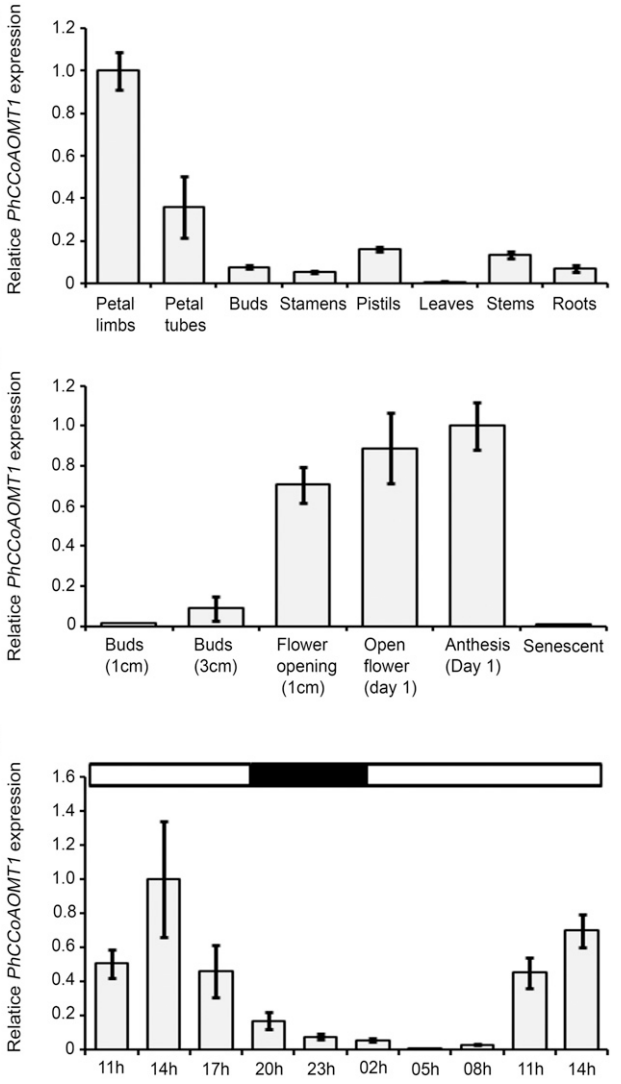

Figure 6. Expression profiles of *PhCCoAOMT1* in cv Mitchell. All transcript levels were determined by qRT-PCR. Mean values ± SE of three biological replicates are shown. A, Tissue-specific expression. The *PhCCoAOMT1* transcript levels were normalized for *EF1a* and are shown relative to levels in petal limbs. B, Developmentally regulated expression in petal limbs. The *PhCCoAOMT1* transcript levels were normalized for *FBP1* and are shown relative to levels at anthesis. C, Rhythmic expression in petal limbs. Petal limbs after anthesis were collected for every 3-h interval for 30 h. The *PhCCoAOMT1* transcript levels were normalized for *FBP1* and are shown relative to levels at 14 h on day 1.

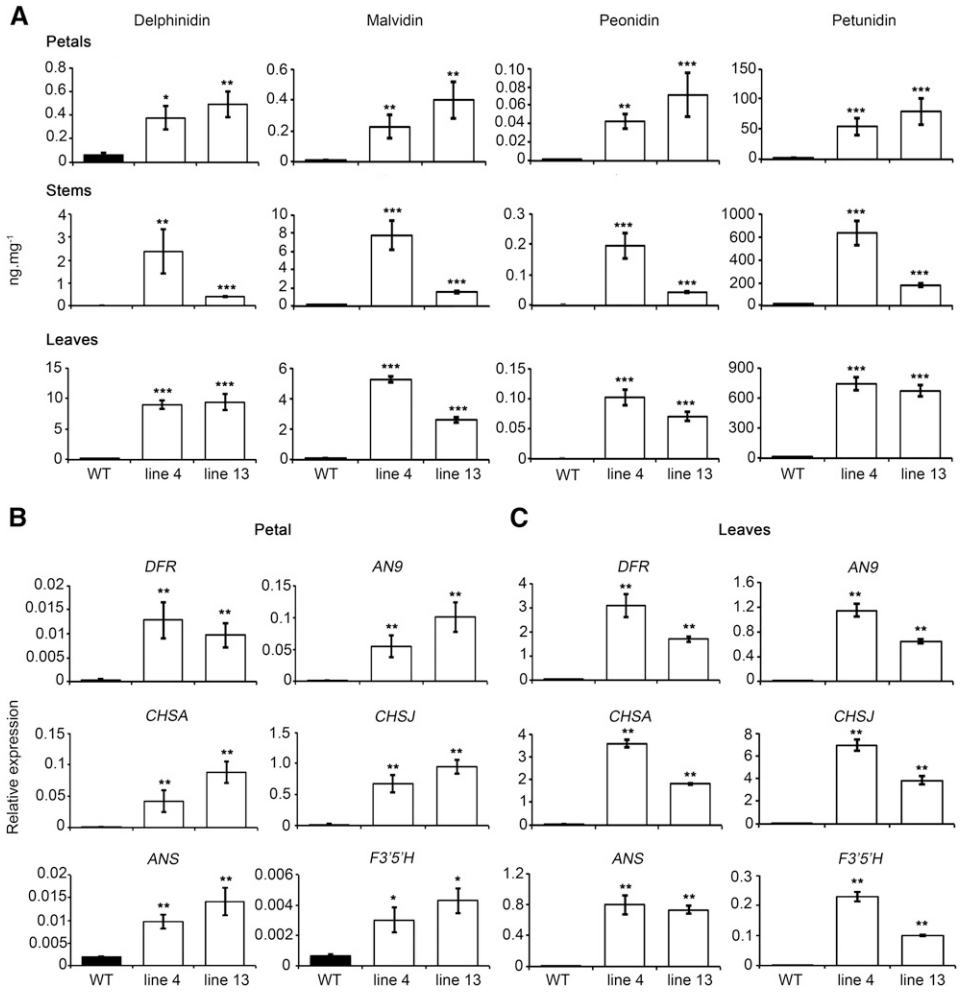

Figure 7. Accumulation of anthocyanin and expression of anthocyanin biosynthetic genes in ir-*PhCCoAOMT1* petals and vegetative tissues. A, Anthocyanin levels in petal limbs, leaves, and stems using liquid chromatography-mass spectrometry. B and C, Transcript levels of *DFR*, *CHSA*, *CHSJ*, *F3′5′H*, *GST* (*AN9*), and *ANS* in ir-*PhCCoAOMT1* petal limbs (B) and leaves (C), normalized with *FBP1* and *EF1α*, respectively. Mean values ± SE of three biological replicates are shown. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ as determined by Student's *t* test of each transgenic line compared with the wild type (WT).

ANTHOCYANIDIN SYNTHASE (*ANS*) in flowers and leaves. DFR is the first committed enzyme in the anthocyanin biosynthetic pathway in petunia flowers (Huits et al., 1994). Down-regulation of *PhCCoAOMT1* indeed resulted in elevated transcript levels of these six genes in both flowers and leaves (Fig. 7, B and C).

Silencing of *PhCCoAOMT1* Perturbs Gene Expression as Well as CoA Ester and Lignin Levels

To get more insight into the underlying metabolic perturbations in ir-*PhCCoAOMT1* plants that led to the production of anthocyanins, to lower levels of eugenol and higher production of phenylacetaldehyde, the levels of several CoA ester pathway intermediates were measured. Since CCoAOMT catalyzes the conversion of caffeoyl-CoA to feruloyl-CoA, feruloyl-CoA levels were expected to be lower in ir-*PhCCoAOMT1* than in wild-type control plants. However, *PhCCoAOMT1* down-regulation affected the levels of all four CoA esters measured in petunia petals (Fig. 8A). Unexpectedly, feruloyl-CoA levels were higher, albeit not significantly, in transgenic plants, whereas there was no accumulation of caffeoyl-CoA, as its level was undetectable, perhaps being incorporated in the anthocyanins (Griesbach et al., 1991; Provenzano et al., 2014). Upstream of caffeoyl-CoA, benzoyl-CoA levels increased and its precursor cinnamoyl-CoA decreased. 4-Coumaryl-CoA levels were much lower in the ir-*PhCCoAOMT1* plants, because it is the precursor of the anthocyanins and was likely depleted to sustain an elevated production of these compounds.

Overall, this metabolic analysis showed that the homeostasis of the CoA esters was severely perturbed in ir-*PhCCoAOMT1* transgenic lines. We also measured transcript levels of the genes downstream of CCoAOMT known to be involved in phenylpropene biosynthesis, including *PhCCR1*, which catalyzes the conversion of feruloyl-CoA to coniferylaldehyde (Muhlemann et al., 2014), *PhCFAT*, which is responsible for the acetylation of coniferyl alcohol (Dexter et al., 2007), and *PhIGS* and *PhEGS*, which are responsible for isoeugenol and eugenol formation, respectively (Koeduka et al., 2006, 2008). Interestingly, transcript levels of *PhCCR1* and *PhEGS* were significantly lower in

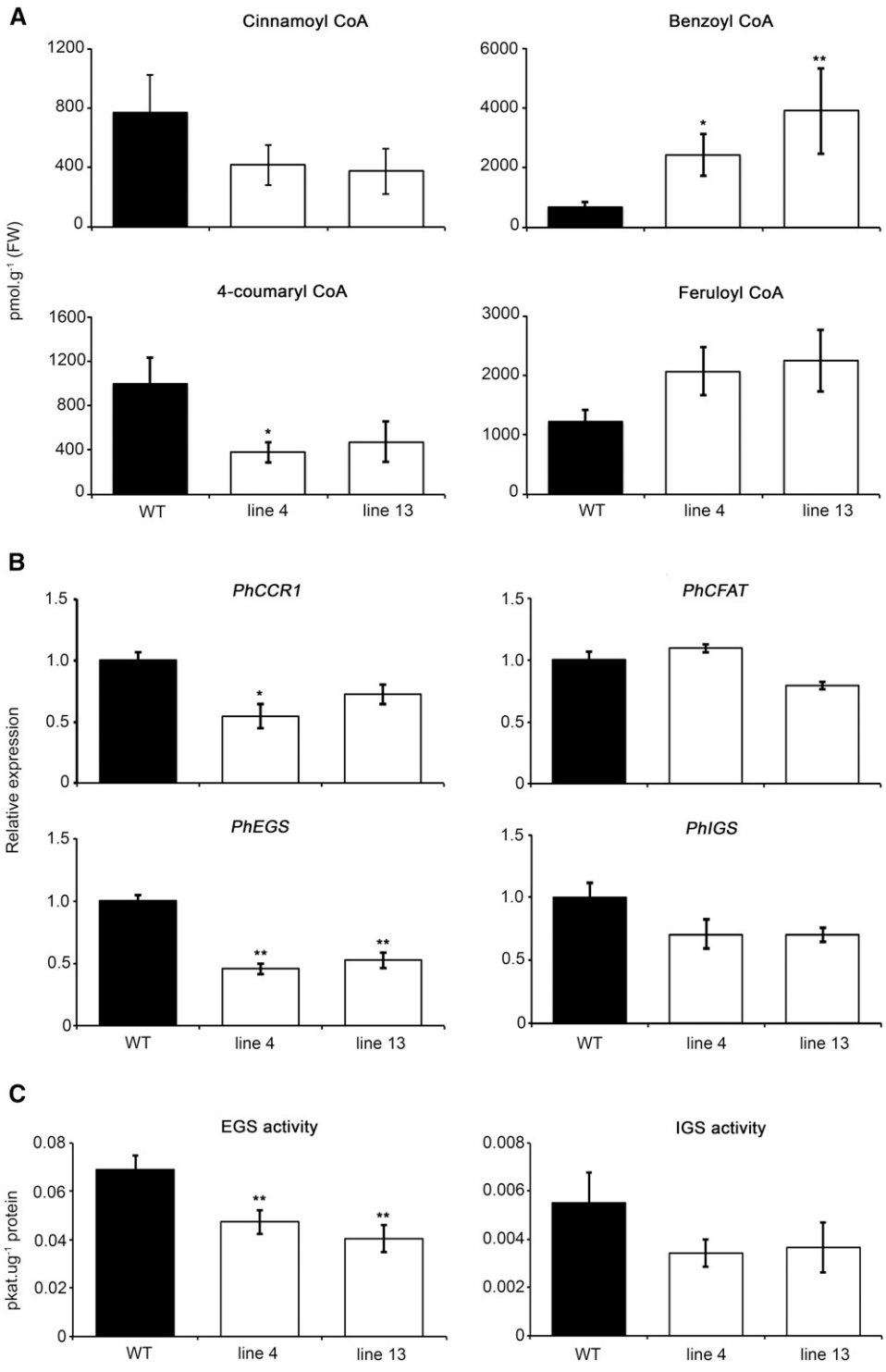

Figure 8. Quantification of CoA esters and transcript levels of phenylpropene biosynthesis-related genes in petunia petal limbs of the wild type (WT) and ir-*PhCCoAOMT1* lines. A, Hydroxycinnamoyl-CoA esters. Mean values ± SE of four biological replicates are shown. FW, Fresh weight. B, Transcript levels of PhCCR, PhCFAT, PhIGS, and PhEGS. Mean values ± SE of three biological replicates are shown, normalized for *FBP1* transcript level. Expression values are depicted relative to the wild type, with its average value set to 1. C, EGS and IGS enzymatic activities. Significant differences between transcript levels of the wild type and each transgenic line were determined by Student's *t* test: *, $P < 0.05$ and **, $P < 0.01$.

transgenic lines relative to the control (Fig. 8B). In contrast, *PhIGS* and *PhCFAT* mRNA levels were unchanged, although *PhIGS* mRNA appeared to be slightly but not significantly lower in transgenic lines. The lower *PhCCR1* transcript levels may explain the higher levels of feruloyl-CoA, the substrate for CCR, and the lower *PhEGS* transcript levels correlate with the reduced production of eugenol, the product of EGS. Consistent with the *PhEGS* transcript levels, EGS activity was significantly decreased in the ir-*PhCCoAOMT1* lines relative to the control (Fig. 8C). IGS activities were only slightly decreased in transgenic lines, in line with the *PhIGS* transcript level (Fig. 8C).

We also analyzed the expression of several other genes involved in benzenoid/phenylpropanoid volatile production. Transcript levels of *PhODO1* and *PhBSMT1* were similar in transgenic and wild-type petals, while *PhBPBT1* expression was considerably higher in line 13, correlating with higher phenylethylbenzoate and benzylbenzoate levels (Fig. 3; Supplemental Fig. S6). Transcript levels of *PhPAAS* were similar in transgenic and wild-type petals in spite of the fact that phenylacetaldehyde emission was higher in the transgenic lines (Supplemental Fig. S6). Since it has been shown that CCoAOMT is involved in lignin biosynthesis in plants (Vanholme et al., 2010), lignin levels were measured in petals and leaves as well. In spite of the fact that we did not see a phenotype related to lower lignin formation, the total lignin levels were indeed reduced significantly in both petals and leaves of the ir-*PhCCoAOMT1* line 13 (Fig. 9). In summary, silencing of *PhCCoAOMT1* perturbs the steady-state levels of the CoA esters, total lignin, and transcripts of several phenylpropene biosynthetic genes.

Regulation of Anthocyanin Biosynthesis in ir-*PhCCoAOMT* Lines

In colored petunia petal limbs, *DFR* expression is controlled by the R2R3-MYB transcription factor AN2 (Quattrocchio et al., 1999). However, cv Mitchell flowers lack pigmentation due to an AN2 mutation. Therefore, other MYB transcription factors are likely to be involved in anthocyanin regulation in the ir-*PhCCoAOMT1* lines. It was recently shown that ectopic overexpression in cv Mitchell of *PURPLE HAZE* (*PHZ*) or *DEEP PURPLE* (*DPL*), two *AN2* paralogs (Albert et al., 2011), leads to an activation of anthocyanin biosynthesis in flowers and vegetative tissues (Albert et al., 2011). Thus, transcript levels of *PHZ* and *DPL* were analyzed in cv Mitchell control and ir-*PhCCoAOMT1* lines. Up to 8-fold higher transcript levels of *PHZ* and *DPL* were observed in limbs of open petals of ir-*PhCCoAOMT1* plants relative to the control (Fig. 10A). Similarly, in leaves, *PHZ* and *DPL* transcript levels were elevated up to 30-fold relative to the control plants (Fig. 10B).

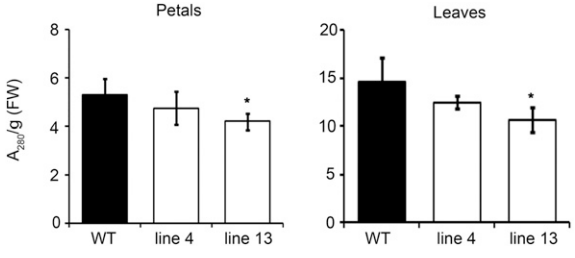

Figure 9. Effects of PhCCoAOMT1 silencing on lignin. Total lignin levels were quantified in petal limbs (A) and leaves (B) of wild-type control plants (WT) and transgenic lines 4 and 13. Mean values ± SE of four biological replicates are shown. *, $P < 0.05$ as determined by Student's *t* test of each transgenic line compared with the wild type. FW, Fresh weight.

We hypothesized that the induction of *PHZ* and/or *DPL* expression results from the metabolic perturbation of the phenylpropanoid network. For instance, higher levels of benzoyl-CoA (Fig. 8) or benzoic acid may induce anthocyanin biosynthesis, since it has been shown that benzoic acid induces *PAL* in cultured cells of *Chenopodium rubrum* (Ehness et al., 1997). To test this hypothesis, petunia flowers were fed with the organic acids that are intermediates in the phenylpropanoid pathways. Interestingly, feeding of flowers with caffeic acid induced *PHZ* expression but not *DPL*, while benzoic acid, ferulic acid, *p*-coumaric acid, and cinnamic acid (Fig. 10C; Supplemental Fig. S7) had no effect on *PHZ* transcript levels. Taken together, these results suggest that the purple phenotype observed in ir-*PhCCoAOMT1* transgenic lines is due to an activation of *PHZ* expression, possibly through caffeic acid, which, in turn, up-regulates the anthocyanin biosynthetic pathways leading to anthocyanin accumulation.

DISCUSSION

CCoAOMTs are widely distributed across all flowering plant species (Vanholme et al., 2010). They were mainly studied due to their role in lignin biosynthesis, as they provide the phenylpropanoid precursor feruoyl-CoA for monolignols (Barros et al., 2015). The monolignol pathway was shown to also contribute to the production of volatile phenylpropenes (Muhlemann et al., 2014), although the CCoAOMT involvement in their formation has not been shown. We show here that PhCCoAOMT1 indeed contributes to the volatile phenylpropene formation and that down-regulation of its expression leads to an activation of the biosynthesis of other phenylpropanoids (i.e. anthocyanins).

Despite the fact that two, also functional (Supplemental Table S1), PhCCoAOMT1 homologs are expressed in petunia petals (Supplemental Figs. S4 and S5), RNAi suppression of *PhCCoAOMT1* alone resulted in a visible phenotype (Fig. 4). Transgenic ir-*PhCCoAOMT1* plants had colored flowers accumulating anthocyanins (Fig. 7A), despite the lack of a functional AN2 transcription factor, the key regulator of anthocyanin biosynthesis (Quattrocchio et al., 1999; Hoballah et al., 2007). An increase in anthocyanin levels was also observed in leaves (even at a higher degree than in petals; Figs. 4 and 7), where the relative reduction in *PhCCoAOMT1* transcript levels by an RNAi construct under the control of the cauliflower mosaic virus 35S promoter was greater than in petals (Figs. 2 and 5). These results suggest that the molecular mechanism(s) responsible for the anthocyanin production is likely very similar in leaves and petals. Indeed, the expression of two R2R3-MYB transcription factors, *PHZ* and *DPL*, known to activate anthocyanin biosynthesis (Albert et al., 2011) was up-regulated in both leaves and petals (Fig. 10, A and B).

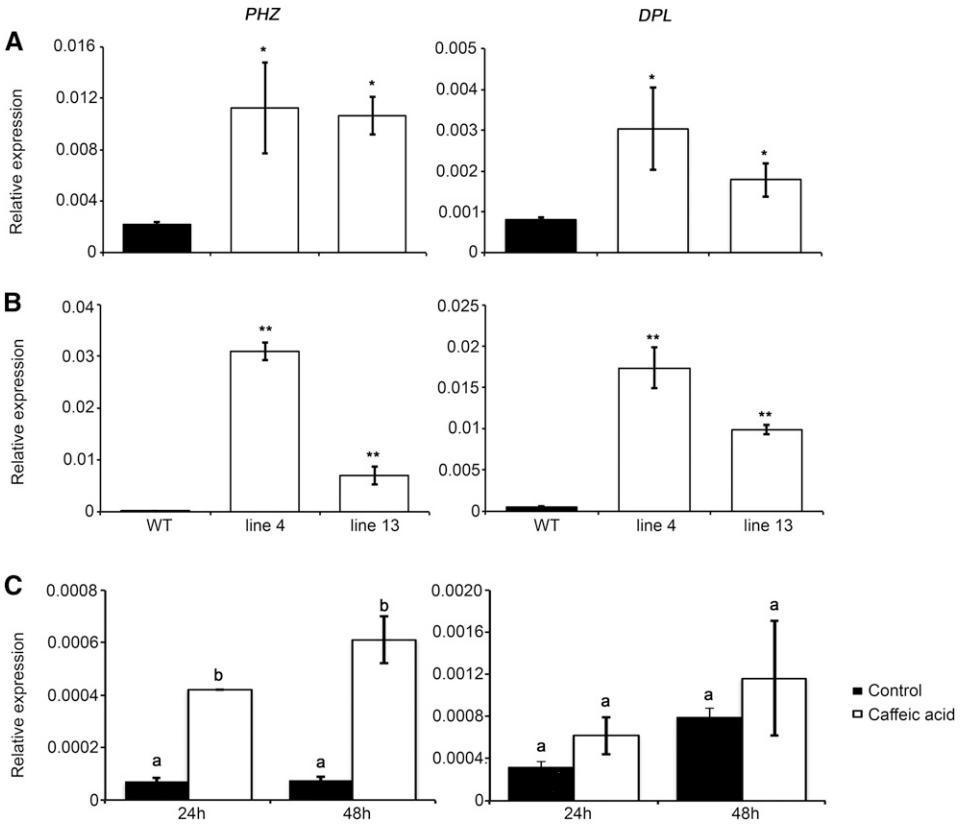

Figure 10. Effects of *PhCCoAOMT1* silencing on transcript levels of *PHZ* and *DPL*. A and B, Transcript levels were determined from petal limbs (A) and leaves (B) by qRT-PCR from ir-*PhCCoAOMT1* lines 4 and 13 and cv Mitchell as the wild-type control (WT). Mean values ± SE of three biological replicates are shown, normalized for *FBP1*. *, $P < 0.05$ and **, $P < 0.01$ as determined by Student's *t* test of each transgenic line compared with the wild type. C, Effects of caffeic acid on *PHZ* and *DPL* expression in cv Mitchell flowers. Two-day-old open flowers were detached from plants and fed with Suc with vitamin C added (control) or additionally caffeic acid for 24 and 48 h. Mean values ± SE of four biological replicas are shown, normalized for *FBP1* transcript levels. Letters indicate significant differences among lines (ANOVA followed by Tukey's posthoc analysis, $P < 0.05$).

The mechanisms up-regulating *PHZ* and *DPL* expression in leaves and petals remain undetermined. In petals, down-regulation of other members of the monolignol pathway, *PhCCR1* or *PhCFAT*, both acting downstream of CCoAOMT, or *COUMARATE-COENZYME A LIGASE*, acting upstream of CCoAOMT, did not affect anthocyanin biosynthesis (Dexter et al., 2007; Klempien et al., 2012; Muhlemann et al., 2014). Thus, it is possible that a metabolic imbalance in either the CoA esters or their acids can lead to the up-regulation of *PHZ* and *DPL* transcription factors. Comparative analyses of CoA esters in RNAi lines for which CoA esters have been measured, *PhCCR1*, *CINNAMATE-COENZYME A LIGASE*, *CINNAMOYL-COENZYME A HYDRATASE-DEHYDROGENASE*, and *PhCCoAOMT1* (Klempien et al., 2012; Qualley et al., 2012; Muhlemann et al., 2014), showed that the only difference between all these lines is the elevated benzoyl-CoA levels in the ir-*PhCCoAOMT1* plants. This suggests that benzoyl-CoA or, alternatively, benzoic acid might serve as an inducer of anthocyanin biosynthesis, as the latter has been shown to up-regulate *PAL* expression in cultured cells of *C. rubrum* (Ehness et al., 1997). However, when tested, benzoic acid did not, but caffeic acid did, induce *PHZ* expression (Fig. 10C). One plausible scenario is that perturbation of caffeic acid or caffeoyl-CoA levels at some point during flower development by *PhCCoAOMT1* down-regulation leads to an elevated *PHZ* expression and, subsequently, anthocyanin accumulation. This might occur in the flower buds when *PhCCoAOMT1* is already expressed at low levels (Fig. 6B).

PHZ and *DPL* expression in open petals of ir-*PhCCoAOMT1* plants is unusual, since, in general, anthocyanin biosynthesis in colored petunia flowers occurs early in development, before flower opening, and precedes floral volatile biosynthesis. In petunia, expression of the *AN2* transcription factor is turned off after anthesis, and as a consequence, anthocyanin production ceases (Verdonk et al., 2005; Albert et al., 2014). The cv Mitchell used in this study lacks the functional MYB transcription factors AN2 and AN4 (Quattrocchio et al., 1999), which regulate color production. Thus, in ir-*PhCCoAOMT1* flowers, *PHZ* and *DPL* are presumably responsible for anthocyanin formation, which takes place simultaneously with scent production. To date, there are other examples demonstrating a link between Phe-derived scent and color compounds. This connection has been shown in carnation (*Dianthus caryophyllus*), where the silencing of *FLAVONOID 3′,5′-HYDROXYLASE*, involved in anthocyanin biosynthesis, led to the redirection of carbon flux from anthocyanins to benzoates, resulting in a reciprocal increase in methylbenzoate production coupled to a decrease in pelargonidin (Zuker et al., 2002). In addition, the simultaneous enhancement of both pathways was achieved by introducing the Arabidopsis PRODUCTION OF ANTHOCYANIN PIGMENT1 (AtPAP1) MYB

transcription factor into petunia (Ben Zvi et al., 2008). The results obtained in this work are in line with previous studies and show that some metabolite intermediates in the scent pathway may regulate the transcript levels of transcription factors involved in color formation. The regulation of gene expression by a metabolite is not unique to ir-*PhCCoAOMT1* petunia plants. Another example of this type of regulation by a metabolite was shown in Arabidopsis, where methylerythritol cyclodiphosphate accumulation, related to the terpenoid precursor pathway, induced stress-related genes (Xiao et al., 2012).

In contrast to ir-*PhCCoAOMT1*, no increase in anthocyanin but an increase in flavonol glycosides production was observed in the *ccoaomt1* Arabidopsis mutant (Vanholme et al., 2012), suggesting that different modes of regulation for the phenylpropanoid network exist in Arabidopsis and petunia. Different modes of regulation were also discovered for GA_3, which activated anthocyanin biosynthesis in petunia petals (Weiss et al., 1995) but repressed it in Arabidopsis (Loreti et al., 2008). Many studies have shown that Suc can induce anthocyanin biosynthesis in leaves (Solfanelli et al., 2006; Qualley et al., 2012), although the underlying mechanisms are still unknown. Since Suc by itself cannot induce anthocyanin biosynthesis in petunia petals (Moalem-Beno et al., 1997) or *PHZ* expression (Fig. 10C), it is likely that different activation mechanisms are involved in petals and leaves. Conversely, caffeic acid induces *PHZ* expression in petals (Fig. 10C) but not in stems and leaves (data not shown); thus, the mechanism(s) in leaves remains to be resolved.

Down-regulation of *CCoAOMT* expression was also achieved in poplar, tobacco, and Arabidopsis. While no changes in coloration were observed in Arabidopsis and tobacco plants, poplar wood exhibited an orange color (Zhong et al., 2000). Rather than anthocyanins, as in petunia ir-*PhCCoAOMT1* plants, changes in coloration in poplar were due to the accumulation of hydroxycinnamic acids. Similarly, silencing of *CINNAMYL ALCOHOL DEHYDROGENASE*, acting downstream of CCoAOMT1 in the monolignol pathway, resulted in a reddish xylem in *Nicotiana attenuata* plants due to the accumulation of hydroxycinnamaldehydes (Kaur et al., 2012). Interestingly, more benzoic acid (C_6-C_1)-related compounds accumulated in poplar (*p*-hydroxybenzoate attached to lignin) and Arabidopsis (four benzenoic hexose esters) when *CCoAOMT* expression was reduced. Both lines 4 and 13 accumulated more benzoyl-CoA, but only line 13 produced more benzoyl-CoA-derived compounds (Figs. 3 and 8).

Despite the fact that ir-*PhCCoAOMT1* line 13 had reduced total lignin levels in flowers and leaves relative to control plants (Fig. 9), it did not exhibit a classical lignin-deficient phenotype: transgenic lines appeared equally sturdy as control plants. This is similar to the situation in alfalfa (Chen et al., 2006), poplar (Zhong et al., 2000), and tobacco (Zhong et al., 1998), where *CCoAOMT* knockdown/knockout had little to no effect on plant stature and development. Apparently, plants were able to cope successfully with reduced lignin, depending on which gene of the lignin biosynthesis pathway is perturbed (Vanholme et al., 2012).

To determine whether the ir-*PhCCoAOMT1* plants had to produce more Phe to accommodate both the bound anthocyanins and volatile phenylpropanoids/benzenoids biosynthesis, we made a rough estimation considering the carbon utilized in both pathways. The steady-state levels of volatiles were measured at one time point, and their glycosylated counterparts were not taken into account as they are not well known in petunia. For anthocyanin accumulation, petunidin is a dominant compound produced at approximately 65 $\mu g\ g^{-1}$ fresh weight (Fig. 7A). The total internal pools of volatiles constituted approximately 30 $\mu g\ g^{-1}$ fresh weight (Supplemental Table S3), while the emission was approximately 240 $\mu g\ g^{-1}$ fresh weight per 24 h (Supplemental Table S3). Thus, with these very simple assumptions, the anthocyanins, with flavonol levels slightly higher in petals (data not shown), require approximately 25% more Phe production. This is likely not a limitation for cv Mitchell plants, since the total amount of volatile production remains unchanged in ir-*PhCCoAOMT1* lines relative to control plants. Similarly, Phe production was not a limiting factor in a purple petunia (V26) expressing a feedback-insensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase. These transgenic plants produced considerably more Phe and volatiles but not more flavonoids or anthocyanins (Oliva et al., 2015), suggesting that volatile and color production are still differentially regulated in these plants. Previously, it was shown that, in petunia flowers, Phe levels oscillate during a daily light/dark cycle with a maximum at night, between 11 PM and 3 AM, positively correlating with volatile emission (Maeda et al., 2010). It remains to be determined when during the day/night cycle anthocyanins accumulate in the open flowers of the ir-*PhCCoAOMT1* lines. Interestingly, overexpression of the R2R3-MYB *AtPAP1* transcription factor in a colored and fragrant petunia variety led not only to an increase in pigmentation but also to a drastic increase in nocturnal volatile production. Feeding of these flowers with Phe increased otherwise negligible diurnal levels of volatiles to their nocturnal levels (Ben Zvi et al., 2008). These results raise the possibility that either all Phe produced during the day is directed to color formation or it is a limiting factor during the day and anthocyanin biosynthesis takes place at night.

The involvement of the monolignol pathway in the biosynthesis of volatile phenylpropenes in petunia has been proposed, first based on the discovery of PhCFAT (Dexter et al., 2007), later via the study of PhMYB4 and CINNAMATE-4-HYDROLASE (Colquhoun et al., 2011), and more recently with an investigation of the role of PhCCR1 (Muhlemann et al., 2014). It has been shown that transgenic petunia plants with reduced *PhCCR1* expression had decreased flux toward phenylpropenes, although internal and emitted pools of

these compounds remained unchanged. In contrast, our petunia lines with reduced *PhCCoAOMT1* expression produced less eugenol (Fig. 3). Interestingly, emission of eugenol was not significantly lower in the ir-*PhCCoAOMT1* lines, similar to the results obtained when EMISSION OF BENZENOIDS I was silenced (Spitzer-Rimon et al., 2012). This can be explained by an active emission process that lowers internal pools of eugenol while sustaining its emission level in petunia petals (Cna'ani et al., 2015; Widhalm et al., 2015).

In the ir-*PhCCoAOMT1* plants, expression of EGS and its enzymatic activity were also reduced (Fig. 8, B and C), making the effect of PhCCoAOMT1 on eugenol formation very complex. Although *IGS* transcript levels and enzymatic activity were only slightly, but not significantly, lower in the ir-*PhCCoAOMT1* lines, isoeugenol levels were not affected (Fig. 3). In cv Mitchell, isoeugenol levels are always much higher (up to 100-fold) than that of eugenol, in spite of minor differences between the K_m, V_{max}, and K_{cat} of IGS and EGS enzymes, suggesting some type of channeling of their common precursor coniferylacetate toward isoeugenol production (Koeduka et al., 2008). Taken together, our results show that *PhCCoAOMT1* down-regulation leads to metabolite perturbations that appear to result in the transcriptional up-regulation of anthocyanin transcription factors (*PHZ* and *DPL*) and down-regulation of *PhEGS* expression. While there is an apparent contribution of PhCCoAOMT1 to phenylpropene biosynthesis and the whole phenylpropanoid network, the role of CCoAOMT2 and CCoAOMT3 remains to be determined.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Petunia hybrida 'Mitchell' (*Petunia axillaris* × *P. hybrida* 'Rose of Heaven') was used as the genetic background to generate transgenic plants. Wild-type and transgenic plants were grown in a greenhouse under standard conditions (16-h photoperiod, 300–400 μmol m^{-2} s^{-1} light intensity, 60%–65% relative humidity, and 22°C/17°C day/night). Prior to tissue collection and volatile analysis, plants were transferred to a growth chamber (16-h photoperiod, 70% relative humidity, and 250–350 μmol m^{-2} s^{-1} light intensity at 21°C) at least 3 d prior to experiments.

Generation of the PhCCoAOMT1 RNAi Construct and Plant Transformation

To create a *PhCCoAOMT* hairpin construct, a 150-bp fragment of the 3′ untranslated region was amplified using forward 5′-ACAAGTTTGTACAAAAAAGCAGGCTTATCAACTCATTATTT-3′ and reverse 5′-ACCACTTTGTACAAGAAAGCTGGGTAAAGGAAATACAAGAT-3′ primers containing attB sites (underlined) for Gateway cloning. The fragment was cloned into pDONR207 vector (Invitrogen; www.lifetechnologies.com) and, subsequently, recombined with pK7gWIWG2(1) (Karimi et al., 2002), generating a *PhCCoAOMT1* hairpin driven by the cauliflower mosaic virus 35S promoter. Transgenic petunias were obtained with a leaf disc transformation protocol using *Agrobacterium tumefaciens* (strain GV3101; Horsch et al., 1985). The kanamycin-resistant transformants were screened for the presence of the *nptII* gene using the following primer pair: 5′-GCTTGGGTGGAGAGGCTATTC-3′ and 5′-CATCGCAAGACCGGCAACAG-3′. The plants were self pollinated, and selection for homozygous plants in the next generations was monitored on 50% Murashige and Skoog medium (pH 5.7) supplemented with 100 mg L^{-1} kanamycin.

RNA Isolation and qRT-PCR

Two-day-old flowers of petunia were collected 1 h before the onset of the dark period, unless specified otherwise. Leaves were harvested from nodes 7 to 10 from each plant. Total RNA was isolated from petal limbs and different organs using Tri Reagent and treated with TURBO DNA-free DNase (Ambion; https://www.lifetechnologies.com). One microgram of RNA was used to synthesize cDNA using RevertAid M-MuLV Reverse Transcriptase (Fermentas; https://www.lifetechnologies.com). qRT-PCR was performed using specific primers (Supplemental Table S4) and was normalized to transcript levels of FBP1 (Angenent et al., 1992) or EF1α (Mallona et al., 2010). Individual qRT-PCRs contained cDNA equivalent to 10 ng of RNA, 300 nM primers, and 4 μL of 5× HOTFIREPol EvaGreen qPCR Mix (Solis Biodyne; https://www.sbd.ee) in a total volume of 20 μL. Two-step qRT-PCR amplification (40 cycles of 95°C for 10 s followed by 60°C for 30 s) was performed using the ABI Prism 7000 real-time PCR detection system (Applied Biosystems; http://www.appliedbiosystems.com). A minimum of three biological replicates were used, and all experiments were performed at least twice.

Analysis of Volatiles

For headspace analysis, 2-d-old flowers from T3 transgenic lines and the cv Mitchell control were placed in desiccators as described by Verdonk et al. (2005). The volatiles in the headspace of the flowers were collected by trapping the outgoing air on 150 mg of Tenax TA for 24 h. Trapped volatiles were eluted using 1.5 mL of pentane:diethylether (4:1), containing 50 pg μL^{-1} tetralin (Sigma; https://www.sigmaaldrich.com) as an internal standard. To determine the internal pool of volatiles, approximately 200 mg of petal limbs was ground in liquid nitrogen to a fine powder. Samples were homogenized in 1 mL of pentane:diethylether (4:1), with 50 pg μL^{-1} tetralin. Water from the extracts was removed with anhydrous sodium sulfate before GC-MS analysis. Three replicates of each independent line were used for analysis.

The volatiles were analyzed using an Agilent 7890A gas chromatograph, coupled to an Agilent 7200 accurate-mass quadrupole time-of-flight mass spectrometer, operating in electron-impact mode. Splitless injection of a 1-μL sample was carried out at 50°C, with the injector port directly heated to 275°C at a rate of 240°C min^{-1}. The gas chromatograph oven temperature was kept at 40°C for 3 min and subsequently heated to 250°C at 15°C min^{-1}. Compounds were separated on a capillary HP-5ms column (30 m × 250 μm, with 0.25-μm film thickness; Agilent) with helium as the carrier gas at a flow rate of 1 mL min^{-1}. The quadrupole time-of-flight mass spectrometer was operated at 20 spectra s^{-1}, acquiring the mass-to-charge ratio 30 to 500 with detector voltage of 1,873 V. The time-of-flight mass spectrometer resolution was about 8,500 (fwhm) at mass-to-charge ratio 612. Identification of compounds was carried out by comparison of retention times and the mass spectra of authentic standards. At least three biological replicates were used per experiment, which was repeated at last two times. Fresh weight of the flowers was determined for each experiment.

Purification of Recombinant CCoAOMT Proteins and Proteomics

Full-length open reading frames of PhCCoAOMTs were amplified using gene-specific primers with restriction enzyme sites introduced (Supplemental Table S5). The PCR fragments were cloned into pGEX-KG expression vector with an N-terminal GST tag and confirmed by sequencing. PhCCoAOMT expression vectors were transformed into *Escherichia coli* (BL21 C41) to produce recombinant protein. A single colony was inoculated in 2× YT medium containing 50 μg mL^{-1} ampicillin. The next day, the culture was diluted 1:20 in 100 mL of 2× YT medium and grown at 37°C until the optical density at 600 nm reached approximately 0.6. Isopropyl β-D-thiogalactoside was added to 1 mM, and the culture was incubated at 18°C for 5 h. Cells were harvested by centrifugation at 5,000*g* for 15 min, frozen in liquid nitrogen, and stored at −80°C. The harvested cells were resuspended in extraction buffer (100 mM potassium phosphate buffer, pH 6.6, 0.5% Triton X-100, and 2 mM EDTA) containing 10 mM Complete Protease Inhibitor Cocktail (Roche Amersham; http://lifescience.roche.com). Cells were lysed by sonicating three times for 60 s, and crude protein extracts were centrifuged at 4,000*g* for 30 min. The supernatants were mixed with Glutathione-Sepharose 4G beads (GE Healthcare; www.gehealthcare.com/) and applied on a Bio-Spin chromatography column (Bio-Rad; http://www.bio-rad.com). GST::PhCCoAOMT proteins were eluted with 100 mM Tris-Cl, pH 7.6, 0.2 mM NaOH, and 20 mM reduced glutathione and

stored in 10% (v/v) glycerol at −80°C. Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard. Enzyme activity was analyzed as described previously by Zhong et al. (1998) with minor modifications in the following buffer: 100 mM Tris-Cl, pH 7.5, 0.2 mM $MgCl_2$, 2 mM dithiothreitol, half-strength Murashige and Skoog medium, and 0.1 mM phenylmethylsulfonyl fluoride. CCoAOMT assays were performed for 30 min at 30°C with 111 μM caffeoyl-CoA. The reaction was stopped with 5 μL of 50% TCA and analyzed by HPLC as described (Klempien et al., 2012).

For proteomic analysis, 10 g of petals was harvested at 5 PM and frozen in liquid N_2. Proteins were isolated according to Jones et al. (2009), concentrated, and sent to the Michigan State University Proteomics Facility for analysis as described (Verdonk et al., 2012). The obtained peptides were searched against petunia ESTs obtained from the SOL Genomics Network (http://sgn.cornell.edu/; Mueller et al., 2005).

Substrate Synthesis and Activity Assays with Recombinant CCoAOMTs

5-Hydroxyferulic acid was synthesized from 5-hydroxyvanillin according to Neish (1959). Caffeoyl-CoA and 5-hydroxyferuloyl-CoA were synthesized through acyl *N*-hydroxysuccinimide ester intermediates as described (Stöckigt and Zenk, 1975). The 45-μL reaction mixtures contained 2.5 μg of recombinant protein, 100 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 0.2 mM $MgCl_2$, 10% glycerol, 66 μM *S*-adenosyl-L-Met, and 45 μM substrate. Assays were allowed to proceed for 30 min at 30°C and subsequently quenched with TCA (5% final concentration). Assay products were determined by HPLC as described previously (Klempien et al., 2012).

Coupled IGS/EGS Assays

IGS and EGS activities were measured as described (Dexter et al., 2007) with slight modifications. Assay mixtures contained 1 mM coniferyl alcohol, 0.6 mM acetyl-CoA, 1 mM NADPH, 2 μg of purified recombinant PhCFAT, and 5 μg of desalted crude protein extract in a final volume of 150 μL (100 mM MES-KOH buffer, pH 6.5). Following a 5-h incubation at room temperature, hexane extracts of the reaction mixtures were concentrated under nitrogen and analyzed by GC-MS. Assays containing boiled protein or omitting purified PhCFAT did not show any production of (iso)eugenol. Results represent averages of three independent experiments.

Feeding Experiments with Organic Acids

Feeding experiments were performed for 8, 24, and 48 h in growth chambers under the conditions described above. Two-day-old flowers of cv Mitchell were detached from plants and kept in Erlenmeyer flasks supplied with 0.5 mM organic acids (Sigma) with or without 5% Suc. For caffeic acid, 1 mM vitamin C (Duchefa; https://www.duchefa-biochemie.com) was added. A minimum of three biological replicates were used, and the experiment was performed twice.

Extraction of Anthocyanin

For anthocyanin analysis, frozen petal limbs, leaves, and stems from wild-type and transgenic plants were used. Tissues were ground into a fine powder in liquid nitrogen. Fifty milligrams of powder in 500 μL of extraction buffer (1% formic acid in methanol) was used for each biological replicate. Lidocaine (Sigma) was added as an internal standard in extraction buffer. The samples were sonicated three times for 5 min and incubated overnight at 4°C. The samples were centrifuged at 20,000*g* for 15 min, and the supernatant was collected containing free and bound anthocyanins. To hydrolyze bound anthocyanins, 450 μL of hydrolysis buffer (5% hydrochloric acid in methanol) was added to 50 μL of the supernatant. Samples were boiled for 30 min followed by centrifugation at 20,000*g* for 15 min. The original and hydrolyzed supernatants were freeze dried overnight prior to anthocyanin analysis. Samples were analyzed by a 1260 Infinity HPLC device (Agilent) coupled to a QTRAP 5500 mass spectrometry system (AB Sciex; http://sciex.com) as described by Gerlach et al. (2014). Identification and quantification of peaks were performed by comparison with standards (cyanidin, delphinidin, malvidin, peonidin, and petunidin).

Stem Cross Sections

Stems from wild-type and transgenic lines were obtained from 2-month-old plants. Fresh stem cross sections (300 μm thickness) were obtained using a vibratome (Leica VT1000s) and put in 1× phosphate-buffered saline, pH 7.4. The cross sections were imaged using an upright microscope associated with a digital camera (Nikon E600FN).

Lignin and Hydroxycinnamoyl-CoA Ester Quantification

Total lignin was quantified using a thioglycolic acid method as described (Campbell and Ellis, 1992). Hydroxycinnamoyl-CoA esters were determined as described by Qualley et al. (2012).

PhCCoAOMT1, PhCCoAOMT2, and PhCCoAOMT3 have been deposited in GenBank with the accession numbers KT223506, KT223507, and KT223508, respectively.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Transcript levels of *PhCCoAOMT1* in *ODO1* silenced plants.

Supplemental Figure S2. *In planta* localization of PhCCoAOMT1.

Supplemental Figure S3. Phylogram of A1-type plant *O*-methyltransferases.

Supplemental Figure S4. Transcript levels of *PhCCoAOMT2/3* in ir-*PhCCoAOMT1* petals.

Supplemental Figure S5. Expression profiles of *PhCCoAOMT2/3*.

Supplemental Figure S6. Transcript levels of phenylpropanoid/benzenoid genes.

Supplemental Figure S7. Effect of organic acids on *PURPLE HAZE* expression.

Supplemental Table S1. Enzymatic activity of recombinant PhCCoAOMTs.

Supplemental Table S2. Proteomics data.

Supplemental Table S3. Quantified volatile compounds production.

Supplemental Table S4. List of qRT-PCR primers.

Supplemental Table S5. Primers for recombinant proteins.

ACKNOWLEDGMENTS

We thank Ludeck Tikovsky, Harold Lemeris, and Thijs Hendrix for excellent care of the plants, Michel de Vries for helping with the petunia transformations, and Jan den Blaauwen and Gideon Meerhoff for help with the stem cross sections.

Received October 22, 2015; accepted November 25, 2015; published November 30, 2015.

LITERATURE CITED

Albert NW, Davies KM, Lewis DH, Zhang H, Montefiori M, Brendolise C, Boase MR, Ngo H, Jameson PE, Schwinn KE (2014) A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. Plant Cell **26:** 962–980

Albert NW, Lewis DH, Zhang H, Schwinn KE, Jameson PE, Davies KM (2011) Members of an R2R3-MYB transcription factor family in Petunia are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. Plant J **65:** 771–784

Ando T, Nomura M, Tsukahara J, Watanabe H, Kokubun H, Tsukamoto T, Hashimoto G, Marchesi E, Kitching IJ (2001) Reproductive isolation

in a native population of Petunia sensu Jussieu (Solanaceae). Ann Bot (Lond) **88:** 403–413

Angenent GC, Busscher M, Franken J, Mol JN, van Tunen AJ (1992) Differential expression of two MADS box genes in wild-type and mutant petunia flowers. Plant Cell **4:** 983–993

Barros J, Serk H, Granlund I, Pesquet E (2015) The cell biology of lignification in higher plants. Ann Bot (Lond) **115:** 1053–1074

Ben Zvi MM, Negre-Zakharov F, Masci T, Ovadis M, Shklarman E, Ben-Meir H, Tzfira T, Dudareva N, Vainstein A (2008) Interlinking showy traits: co-engineering of scent and colour biosynthesis in flowers. Plant Biotechnol J **6:** 403–415

Bhuiyan NH, Selvaraj G, Wei Y, King J (2009) Role of lignification in plant defense. Plant Signal Behav **4:** 158–159

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol **54:** 519–546

Busam G, Junghanns KT, Kneusel RE, Kassemeyer HH, Matern U (1997) Characterization and expression of caffeoyl-coenzyme A 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L. Plant Physiol **115:** 1039–1048

Campbell MM, Ellis BE (1992) Fungal elicitor-mediated responses in pine cell cultures. I. Induction of phenylpropanoid metabolism. Planta **186:** 409–417

Chen C, Meyermans H, Burggraeve B, De Rycke RM, Inoue K, De Vleesschauwer V, Steenackers M, Van Montagu MC, Engler GJ, Boerjan WA (2000) Cell-specific and conditional expression of caffeoyl-coenzyme A-3-*O*-methyltransferase in poplar. Plant Physiol **123:** 853–867

Chen F, Srinivasa Reddy MS, Temple S, Jackson L, Shadle G, Dixon RA (2006) Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (Medicago sativa L.). Plant J **48:** 113–124

Cna'ani A, Spitzer-Rimon B, Ravid J, Farhi M, Masci T, Aravena-Calvo J, Ovadis M, Vainstein A (2015) Two showy traits, scent emission and pigmentation, are finely coregulated by the MYB transcription factor PH4 in petunia flowers. New Phytol **208:** 708–714

Colquhoun TA, Kim JY, Wedde AE, Levin LA, Schmitt KC, Schuurink RC, Clark DG (2011) PhMYB4 fine-tunes the floral volatile signature of Petunia × hybrida through PhC4H. J Exp Bot **62:** 1133–1143

Colquhoun TA, Verdonk JC, Schimmel BC, Tieman DM, Underwood BA, Clark DG (2010) Petunia floral volatile benzenoid/phenylpropanoid genes are regulated in a similar manner. Phytochemistry **71:** 158–167

Dal Cin V, Tieman DM, Tohge T, McQuinn R, de Vos RCH, Osorio S, Schmelz EA, Taylor MG, Smits-Kroon MT, Schuurink RC, et al (2011) Identification of genes in the phenylalanine metabolic pathway by ectopic expression of a MYB transcription factor in tomato fruit. Plant Cell **23:** 2738–2753

Dexter R, Qualley A, Kish CM, Ma CJ, Koeduka T, Nagegowda DA, Dudareva N, Pichersky E, Clark D (2007) Characterization of a petunia acetyltransferase involved in the biosynthesis of the floral volatile isoeugenol. Plant J **49:** 265–275

Do CT, Pollet B, Thévenin J, Sibout R, Denoue D, Barrière Y, Lapierre C, Jouanin L (2007) Both caffeoyl coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in Arabidopsis. Planta **226:** 1117–1129

Dudareva N, Klempien A, Muhlemann JK, Kaplan I (2013) Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol **198:** 16–32

Ehness R, Ecker M, Godt DE, Roitsch T (1997) Glucose and stress independently regulate source and sink metabolism and defense mechanisms via signal transduction pathways involving protein phosphorylation. Plant Cell **9:** 1825–1841

Falcone Ferreyra ML, Rius SP, Casati P (2012) Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front Plant Sci **3:** 222

Fenske MP, Hewett Hazelton KD, Hempton AK, Shim JS, Yamamoto BM, Riffell JA, Imaizumi T (2015) Circadian clock gene LATE ELONGATED HYPOCOTYL directly regulates the timing of floral scent emission in Petunia. Proc Natl Acad Sci USA **112:** 9775–9780

Gerlach N, Schmitz J, Polatajko A, Schlüter U, Fahnenstich H, Witt S, Fernie AR, Uroic K, Scholz U, Sonnewald U, Bucher M (2015) An integrated functional approach to dissect systemic responses in maize to arbuscular mycorrhizal symbiosis. Plant Cell Environ **38:** 1591–1612

Griesbach RJ, Asen S, Leonnarat BA (1991) Petunia hybrida anthocyanins acylated with caffeic acid. Phytochemistry **30:** 1729–1731

Huits HS, Gerats AG, Kreike MM, Mol JN, Koes RE (1994) Genetic control of dihydroflavonol 4-reductase gene expression in Petunia hybrida. Plant J **6:** 295–310

Hoballah ME, Gübitz T, Stuurman J, Broger L, Barone M, Mandel T, Dell'Olivo A, Arnold M, Kuhlemeier C (2007) Single gene-mediated shift in pollinator attraction in Petunia. Plant Cell **19:** 779–790

Horsch RB, Rogers SG, Fraley RT (1985) Transgenic plants. Cold Spring Harb Symp Quant Biol **50:** 433–437

Inoue K, Sewalt VJ, Murray GB, Ni W, Stürzer C, Dixon RA (1998) Developmental expression and substrate specificities of alfalfa caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase in relation to lignification. Plant Physiol **117:** 761–770

Jones AM, MacLean D, Studholme DJ, Serna-Sanz A, Andreasson E, Rathjen JP, Peck SC (2009) Phosphoproteomic analysis of nucleienriched fractions from Arabidopsis thaliana. J Proteomics **72:** 439–451

Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7:** 193–195

Kaur H, Shaker K, Heinzel N, Ralph J, Gális I, Baldwin IT (2012) Environmental stresses of field growth allow cinnamyl alcohol dehydrogenase-deficient *Nicotiana attenuata* plants to compensate for their structural deficiencies. Plant Physiol **159:** 1545–1570

Klahre U, Gurba A, Hermann K, Saxenhofer M, Bossolini E, Guerin PM, Kuhlemeier C (2011) Pollinator choice in Petunia depends on two major genetic loci for floral scent production. Curr Biol **21:** 730–739

Klempien A, Kaminaga Y, Qualley A, Nagegowda DA, Widhalm JR, Orlova I, Shasany AK, Taguchi G, Kish CM, Cooper BR, et al (2012) Contribution of CoA ligases to benzenoid biosynthesis in petunia flowers. Plant Cell **24:** 2015–2030

Koeduka T, Fridman E, Gang DR, Vassão DG, Jackson BL, Kish CM, Orlova I, Spassova SM, Lewis NG, Noel JP, et al (2006) Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via reduction of a coniferyl alcohol ester. Proc Natl Acad Sci USA **103:** 10128–10133

Koeduka T, Louie GV, Orlova I, Kish CM, Ibdah M, Wilkerson CG, Bowman ME, Baiga TJ, Noel JP, Dudareva N, et al (2008) The multiple phenylpropene synthases in both Clarkia breweri and Petunia hybrida represent two distinct protein lineages. Plant J **54:** 362–374

Koeduka T, Orlova I, Baiga TJ, Noel JP, Dudareva N, Pichersky E (2009) The lack of floral synthesis and emission of isoeugenol in Petunia axillaris subsp. parodii is due to a mutation in the isoeugenol synthase gene. Plant J **58:** 961–969

Loreti E, Povero G, Novi G, Solfanelli C, Alpi A, Perata P (2008) Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in Arabidopsis. New Phytol **179:** 1004–1016

Maeda H, Shasany AK, Schnepp J, Orlova I, Taguchi G, Cooper BR, Rhodes D, Pichersky E, and Dudareva N (2010) RNAi suppression of arogenate dehydratase1 reveals that phenylalanine is synthesized predominantly via the arogenate pathway in petunia petals. Plant Cell **22:** 832–849

Mallona I, Lischewski S, Weiss J, Hause B, Egea-Cortines M (2010) Validation of reference genes for quantitative real-time PCR during leaf and flower development in Petunia hybrida. BMC Plant Biol **10:** 4

Marita JM, Ralph J, Hatfield RD, Guo D, Chen F, Dixon RA (2003) Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. Phytochemistry **62:** 53–65

Martz F, Maury S, Pinçon G, Legrand M (1998) cDNA cloning, substrate specificity and expression study of tobacco caffeoyl-CoA 3-O-methyltransferase, a lignin biosynthetic enzyme. Plant Mol Biol **36:** 427–437

Maury S, Geoffroy P, Legrand M (1999) Tobacco *O*-methyltransferases involved in phenylpropanoid metabolism: the different caffeoyl-coenzyme A/5-hydroxyferuloyl-coenzyme A 3/5-*O*-methyltransferase and caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase classes have distinct substrate specificities and expression patterns. Plant Physiol **121:** 215–224

Moalem-Beno D, Tamari G, Leitner-Dagan Y, Borochov A, Weiss D (1997) Sugar-dependent gibberellin-induced chalcone synthase gene expression in petunia corollas. Plant Physiol **113:** 419–424

Mueller LA, Solow TH, Taylor N, Skwarecki B, Buels R, Binns J, Lin C, Wright MH, Ahrens R, Wang Y, et al (2005) The SOL Genomics Network: a comparative resource for Solanaceae biology and beyond. Plant Physiol **138:** 1310–1317

Muhlemann JK, Woodworth BD, Morgan JA, Dudareva N (2014) The monolignol pathway contributes to the biosynthesis of volatile phenylpropenes in flowers. New Phytol **204:** 661–670

Neish AC (1959) Preparation of caffeic and dihydrocaffeic acids by methods suitable for introduction of C14 into the beta-position. Can J Biochem Physiol **37:** 1431–1438

Oliva M, Ovadia R, Perl A, Bar E, Lewinsohn E, Galili G, Oren-Shamir M (2015) Enhanced formation of aromatic amino acids increases fragrance without affecting flower longevity or pigmentation in Petunia × hybrida. Plant Biotechnol J **13:** 125–136

Provenzano S, Spelt C, Hosokawa S, Nakamura N, Brugliera F, Demelis L, Geerke DP, Schubert A, Tanaka Y, Quattrocchio F, et al (2014) Genetic control and evolution of anthocyanin methylation. Plant Physiol **165:** 962–977

Qualley AV, Widhalm JR, Adebesin F, Kish CM, Dudareva N (2012) Completion of the core β-oxidative pathway of benzoic acid biosynthesis in plants. Proc Natl Acad Sci USA **109:** 16383–16388

Quattrocchio F, Verweij W, Kroon A, Spelt C, Mol J, Koes R (2006) PH4 of Petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. Plant Cell **18:** 1274–1291

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol J, Koes R (1999) Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower color. Plant Cell **11:** 1433–1444

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol **133:** 1051–1071

Schuurink RC, Haring MA, Clark DG (2006) Regulation of volatile benzenoid biosynthesis in petunia flowers. Trends Plant Sci **11:** 20–25

Sheehan H, Hermann K, Kuhlemeier C (2012) Color and scent: how single genes influence pollinator attraction. Cold Spring Harb Symp Quant Biol **77:** 117–133

Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P (2006) Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant Physiol **140:** 637–646

Stöckigt J, Zenk MH (1975) Chemical syntheses and properties of hydroxycinnamoyl-coenzyme A derivatives. Z Naturforsch C **30:** 352–358

Tsuda S, Fukui Y, Nakamura N, Katsumoto Y, Yonekura-Sakakibara K, Fukuchi-Mizutani M, Ohira K, Ueyama Y, Ohkawa H, Holton TA, et al (2004) Flower color modification of Petunia hybrida commercial varieties by metabolic engineering. Plant Biotechnol **21:** 377–386

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W (2010) Lignin biosynthesis and structure. Plant Physiol **153:** 895–905

Vanholme R, Storme V, Vanholme B, Sundin L, Christensen JH, Goeminne G, Halpin C, Rohde A, Morreel K, Boerjan W (2012) A systems biology view of responses to lignin biosynthesis perturbations in *Arabidopsis*. Plant Cell **24:** 3506–3529

Van Moerkercke A, Galván-Ampudia CS, Verdonk JC, Haring MA, Schuurink RC (2012) Regulators of floral fragrance production and their target genes in petunia are not exclusively active in the epidermal cells of petals. J Exp Bot **63:** 3157–3171

Van Moerkercke A, Schauvinhold I, Pichersky E, Haring MA, Schuurink RC (2009) A plant thiolase involved in benzoic acid biosynthesis and volatile benzenoid production. Plant J **60:** 292–302

Verdonk JC, Haring MA, van Tunen AJ, Schuurink RC (2005) ODORANT1 regulates fragrance biosynthesis in petunia flowers. Plant Cell **17:** 1612–1624

Verdonk JC, Hatfield RD, Sullivan ML (2012) Proteomic analysis of cell walls of two developmental stages of alfalfa stems. Front Plant Sci **3:** 279

Verdonk JC, Ric de Vos CH, Verhoeven HA, Haring MA, van Tunen AJ, Schuurink RC (2003) Regulation of floral scent production in petunia revealed by targeted metabolomics. Phytochemistry **62:** 997–1008

Wang Y, Chantreau M, Sibout R, Hawkins S (2013) Plant cell wall lignification and monolignol metabolism. Front Plant Sci **4:** 220

Weiss D, Van Der Luit A, Knegt E, Vermeer E, Mol J, Kooter JM (1995) Identification of endogenous gibberellins in petunia flowers (induction of anthocyanin biosynthetic gene expression and the antagonistic effect of abscisic acid). Plant Physiol **107:** 695–702

Widhalm JR, Jaini R, Morgan JA, Dudareva N (2015) Rethinking how volatiles are released from plant cells. Trends Plant Sci **20:** 545–550

Xiao Y, Savchenko T, Baidoo EE, Chehab WE, Hayden DM, Tolstikov V, Corwin JA, Kliebenstein DJ, Keasling JD, Dehesh K (2012) Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes. Cell **149:** 1525–1535

Zhong R, Morrison WH III, Himmelsbach DS, Poole FL II, Ye ZH (2000) Essential role of caffeoyl coenzyme A *O*-methyltransferase in lignin biosynthesis in woody poplar plants. Plant Physiol **124:** 563–578

Zhong R, Morrison WH III, Negrel J, Ye ZH (1998) Dual methylation pathways in lignin biosynthesis. Plant Cell **10:** 2033–2046

Zuker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, Itzhaki H, Forkmann G, Martens S, Neta-Sharir I, Weiss D, et al (2002) Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Mol Breed **9:** 33–41