# Supporting Information

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### SI Materials and Methods

**Generation of *PhCHD*-RNAi Lines.** A 364-bp fragment of *PhCHD* from nucleotide 821–1184 was amplified using the forward, 5′-CTCGAGTCTAGATCTTATCAGAGACATCAAAGGGT-3′ and reverse 5′-GGATCCGAATTCACAGCCTCTATGACCATATCCA-3′ primers. After verification by sequencing, the fragment was inserted into a modified pRNA69 vector in which the 35S promoter was replaced with *Clarkia breweri* petal-specific linalool synthase (*LIS*) promoter (a SacI–XhoI fragment) (1). The RNAi construct was generated by inserting the CHD fragment in opposite orientations separated by an intron in the pRNA69 vector. The section of this constructed plasmid containing the *LIS* promoter and the CHD inverted repeat was cut out and ligated into the binary vector p*ART27* (2) SacI and SpeI sites to obtain p*LIS-PhCHD*-RNAi plasmid.

**RNA Isolation and qRT-PCR Analyses.** Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen). For qRT-PCR analysis, total RNA was first treated with DNase I to eliminate genomic DNA using the TURBO DNA-free kit (Ambion) and 1 μg of RNA was subsequently reverse transcribed to cDNA in the total volume of 100 μL using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene-specific primer sets are shown in Table S2. For quantification of *PhCHD* transcript levels, the pCR4-TOPO carrying *PhCHD* was digested with EcoRI and the resulting fragment was purified from agarose gels using a QIAquick Gel Extraction Kit (Qiagen). After determining DNA concentration with NanoDrop 1000 Spectrophotometer (Thermo Scientific), the purified DNA fragment was diluted to 10 pg/mL and used to obtain standard curves in qRT-PCR with gene-specific primers. Individual qRT-PCR reactions were carried out in duplicates each containing 5 μL of the SYBR Green PCR Master mix (Applied Biosystems), 2 μL of 25 times diluted cDNA and 1.5 μL of 6 μM and 2 μM forward and reverse primers, respectively. Two-step qRT-PCR amplification (40 cycles of 95 °C for 3 s followed by 60 °C for 30 s) was performed using the StepOne Real-Time PCR system (Applied Biosystems). Based on the standard curves, absolute quantities of individual transcripts were calculated and expressed relative to the highest *CHD* expression in the dataset. Each data point represents the average of three to four independent biological samples.

**Preparation of Petunia Petal Crude Protein Extracts.** Petunia corollas (0.2 g) were harvested at 9:00 PM on day 2 postanthesis, flash frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The powder was transferred to a 1.5-mL microcentrifuge tube and thawed with 1 mL of freshly prepared 50 mM $KH_2PO_4$ pH 7.5, 10% (vol/vol) glycerol, 1% (wt/vol) polyvinylpolypyrrolidone, 10 mM β-mercaptoethanol, 5 mM $Na_2S_2O_5$, and 1 mM PMSF. Samples were centrifuged (16,000 × *g*; 10 min at 4 °C) to pellet debris. Supernatants were recovered, desalted on Sephadex G-50 fine resin column equilibrated with 50 mM $KH_2PO_4$ pH 7.5, 10% (vol/vol) glycerol, and the amount of protein was quantified.

**Heterologous Expression and Purification of Recombinant PhCHD.** To obtain the full-length cDNA of PhCHD, 5′ rapid amplification of cDNA ends (RACE) was performed according to the manufacturer's protocol (Life Technologies) with gene-specific (5′-ATGCTGAACCAAATTCTTTCCCTACAG-3′) and nested (5′- TGCTTTCCCAACAGCCATAAGGTCAA-3′) primers. For functional expression, the coding region of petunia CHD was obtained by PCR using the following forward primer, which introduced an NdeI site (5′-CATATGGCTCAAGTTAAGGTGAC-3′) and reverse primer, which introduced a BamHI site downstream of the stop codon (5′-GGATCCTTACATGCGTGACCTTGAAG-3′). After sequence verification, the amplified gene product was subcloned into the NdeI/BamHI sites of the pET-28a vector (Novagen) containing an N-terminal hexahistidine tag. Recombinant PhCHD was expressed in *Escherichia coli* and purified on Ni-NTA resin. Expression in *E. coli* Rosetta2 cells (Novagen), induction, harvesting, and crude extract preparation were performed as previously described (2) with the exception of the lysis buffer, which contained 100 mM K-phosphate, pH 7.5, 300 mM NaCl, 10% (vol/vol) glycerol, and 7 mM β-mercaptoethanol. Fractions with the highest protein concentration were purified by size-exclusion chromatography on HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare) using 50 mM K-phosphate buffer pH 7.5, 10% (vol/vol) glycerol as mobile phase. Protein purity was determined to be 99% by densitometry of SDS/PAGE gels after Coomassie Brilliant Blue staining. The purity of enzymes was taken into account for determination of $k_{cat}$ values.

**Enzyme Assays.** Analysis of reaction product was performed using HPLC-diode array detector (DAD) spectrophotometry at 260 nm (Agilent 1200 series LC with diode array detector; Agilent Technologies) and quantified on the basis of calibration curves generated with authentic benzoic acid (BA)-CoA. The reverse-phase column, Agilent ZORBAX SB-C18 (4.6 × 150 mm × 3.5 μm), was held at 40 °C and eluted at 1 mL/min with a solvent system consisting of 50 mM K-phosphate pH 7.0 (solvent A) and 100% methanol (solvent B). Solvent B was held at 10% for the first 5 min of the run, then stepped up to 25% B, followed by a linear gradient to 45% B from 5 to 8 min, held constant from 8 to 10 min, and stepped up to 60% B, where it was held from 10 to 12 min. The column was returned to 10% B and re-equilibrated for 5 min.

5,5′-dithiobis-(2-nitroBA) (DTNB) assays (100 μL) to measure BA-CoA thioesterase activity contained 50 mM $KH_2PO_4$ (pH 7.0), 100 μM substrate, and 34 μg of peroxisomal or crude extract protein. Reactions were incubated for 20–120 min at 30 °C and were then mixed with 100 μL of an aqueous solution of 400 μM DTNB. Changes in $Abs_{412}$ compared with blank samples were read after a 5-min incubation and compared with blank assays omitting either the substrate or protein.

**Product Verification by Liquid Chromatography (LC) TOF/MS.** To determine the product formed by CHD from cinnamic acid (CA)-CoA, reaction products produced without (3O3PP-CoA) and with PhKAT1 coupling (BA-CoA) were analyzed by LC TOF/MS. The chromatography was conducted on a Agilent 1100 HPLC system coupled to an Agilent 6210 mass-selective detector (MSD) time-of-flight mass spectrometer (Agilent Technologies). Separation was performed using a Luna C18 column (5 μm, 150 × 2 mm; Phenomenex), maintained at 40 °C with a flow rate of 0.3 mL/min. The mobile phase contained solvents A and B, where A was 20 mM ammonium acetate pH 9.0 and B was 100% methanol. The column was eluted with a linear gradient of 20–90% B over 0–25 min, held at 90% B from 25 to 33 min, repartitioned back to 20% B from 33 to 35 min and reequilibrated for 15 min (from 35 to 50 min). The electrospray ionisation (ESI) capillary voltage was 4.0 kV, nitrogen gas temperature was set to 350 °C, drying gas flow rate was 9.0 L/min, nebulizer gas pressure was 35 psig, fragmentor voltage was 155 V, skimmer was 60 V, and octopole

ion guide radio frequency (OCT) RF was 250 V. Mass data [from mass-to-charge ratios ($m/z$) of 100–1,700] were collected and analyzed using Agilent MassHunter B.02 software.

**Quantification of Organic Acids.** For analysis of organic acids, ~200 mg frozen petal tissues was spiked with 1.6 nmol of 4-chlorobenzoic acid and homogenized in 800 μL of 60% (vol/vol) methanol using a Bullet Blender Blue (Next Advance) followed by centrifugation at 16,000 × $g$ for 10 min at room temperature. Grinding/solvent extraction was repeated twice. Supernatants were concentrated to ~100 μL at 40 °C under a stream of dry nitrogen and analyzed by HPLC (Agilent 1200 series LC with diode array detection). The reverse-phase column, Agilent ZORBAX SB-C18 (4.6 × 150 mm × 3.5 μm), was held at 40 °C and eluted at 1 mL/min using a solvent system consisting of 0.1% formic acid in dd$H_2O$ (solvent A) and 0.1% formic acid in acetonitrile (solvent B). After 10 μL of sample injection, 10% B was held for 2 min, followed by a linear gradient to 25% B from 2 to 8 min, a linear gradient to 55% B from 8 to 22 min, a linear gradient to 90% B from 22 to 24 min, then 90% B held from 24 to 25 min followed by a linear gradient to 10% B from 25 to 27 min, and a 5 min equilibration back to 10% B. UV absorbances were monitored at 230 nm for BA, 238 nm for 4-chlorobenzoic acid, 278 nm for CA, 310 nm for *p*-CA, and 324 nm for caffeic and ferulic acids.

1. Cseke L, Dudareva N, Pichersky E (1998) Structure and evolution of linalool synthase. *Mol Biol Evol* 15:1491–1498.

2. Klempien A, et al. (2012) Contribution of CoA ligases to benzenoid biosynthesis in petunia flowers. *Plant Cell* 24:2015–2030.

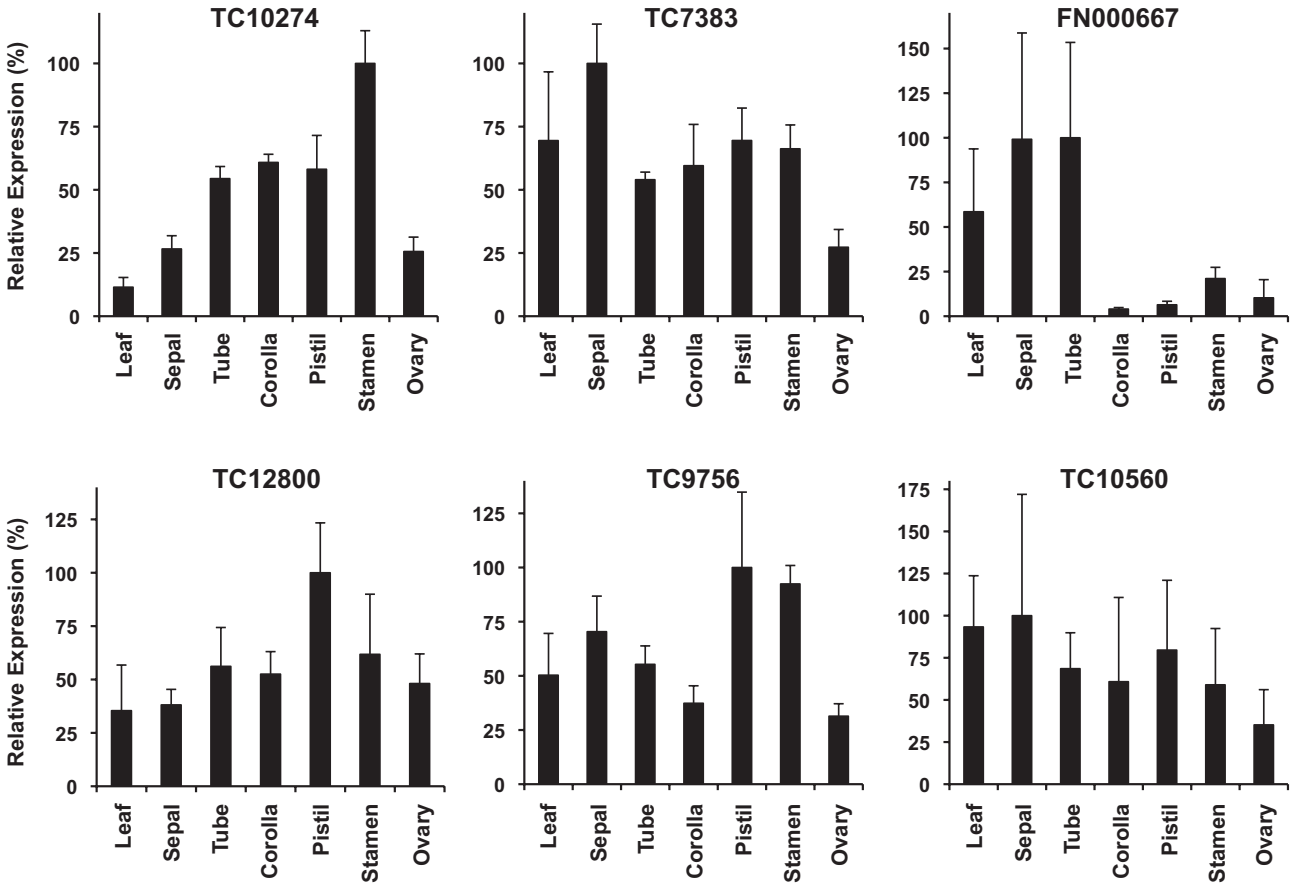


**Fig. S1.** Tissue-specific expression profiles of putative MFP candidates. Transcript levels were determined by qRT-PCR relative to the reference gene *elongation factor 1-α*, in leaves and floral tissues harvested at 3:00 PM day 2 postanthesis. Expression profiles are depicted relative to the tissue with the highest transcript level for each gene. All data are means ± SEM ($n$ = 3–4 biological replicates).

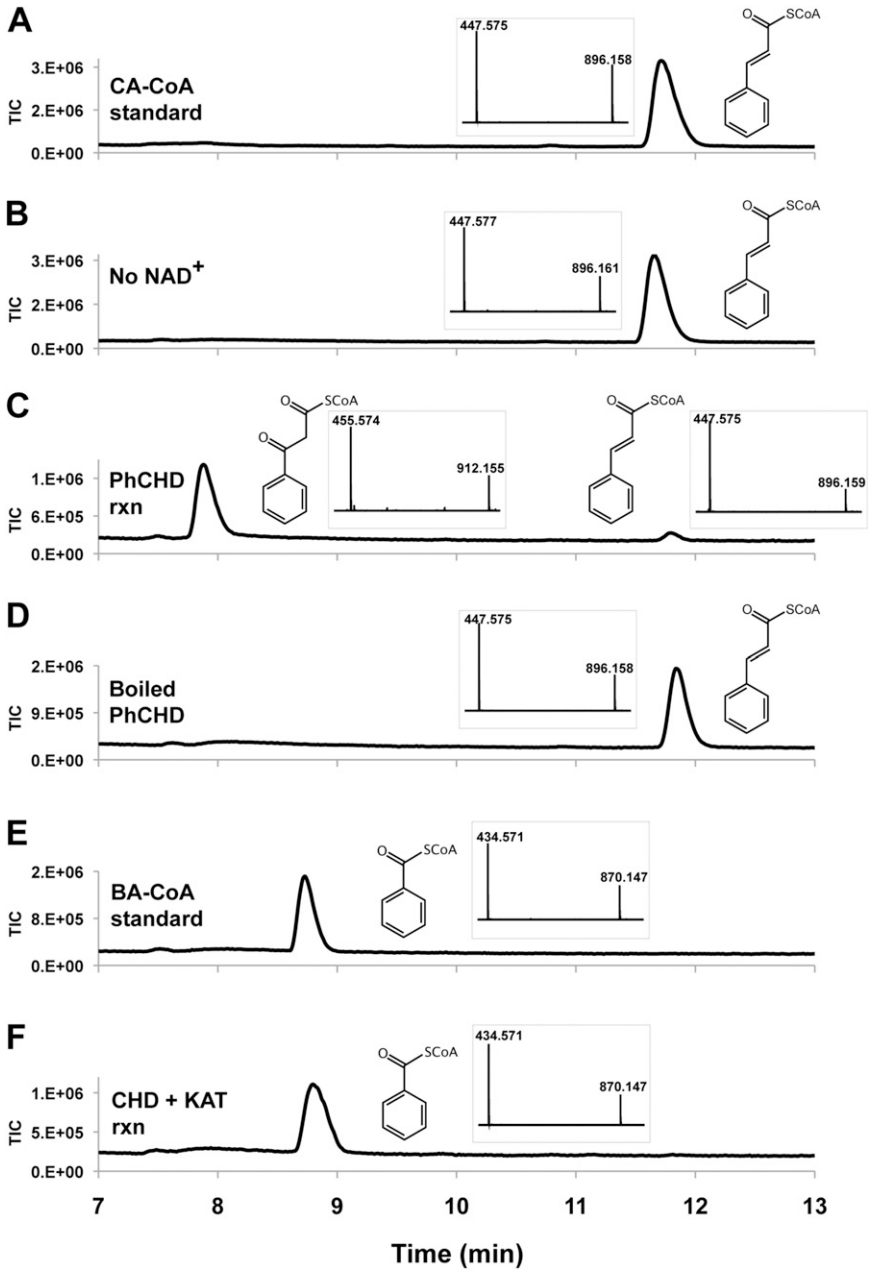


**Fig. S2.** LC MS analysis of product formed by recombinant PhCHD from cinnamoyl-CoA. Exact masses displayed are $(M-H)^-$. (*A*) Cinnamoyl-CoA standard. (*B*) Lack of product formation in the absence of $NAD^+$. (*C*) Product formed by PhCHD from cinnamoyl-CoA in the presence of $NAD^+$. (*D*) Lack of product formation in the presence of boiled PhCHD. (*E*) Authentic standard of benzoyl-CoA. (*F*) Product formed by PhCHD from cinnamoyl-CoA in the presence of $NAD^+$ and recombinant petunia keto-acyl thiolase 1 (PhKAT1) (1).

1. 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.

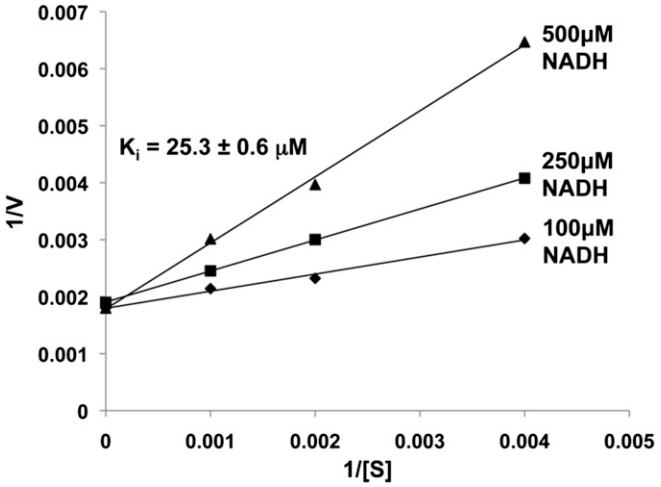


**Fig. S3.** Competitive nature of PhCHD inhibition by NADH. The inhibition constant ($K_i$) was determined as follows: $Ki = [Km]\cdot[I]/Km(app) - Km$.

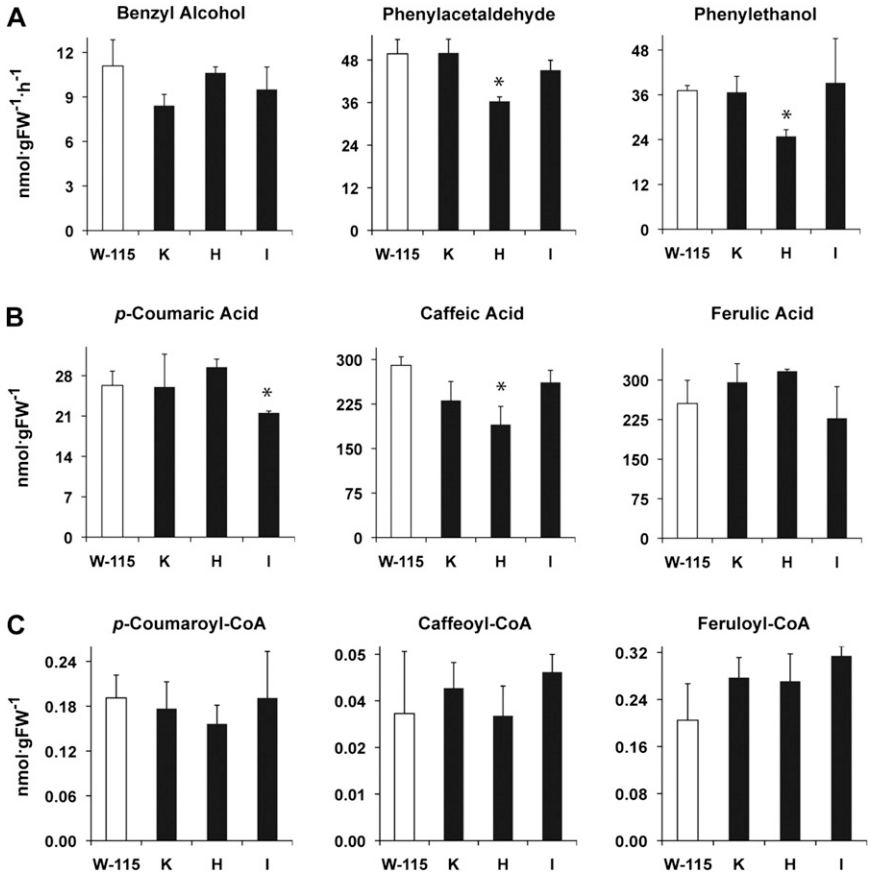


**Fig. S4.** Emitted benzenoid/phenylpropanoid compounds and internal pools of organic acids and CoA esters, in corollas of petunia flowers on day 2 post-anthesis that remain unaffected in *PhCHD*-RNAi lines. (*A*) Floral volatiles emitted from flowers from 4:00 to 10:00 PM. Rates are calculated hourly assuming uniform emission over 6 h. (*B*) Organic acid and (*C*) CoA-ester internal pool sizes at 9:00 PM. All data are means ± SEM ($n$ = 3 biological replicates). White bars represent wild-type petunia (W-115); black bars correspond to *PhCHD*-RNAi lines (K, H, and I). *$P < 0.05$ by analysis of variance (ANOVA). FW, fresh weight.

**Table S1. Substrate specificity of PhCHD with various aromatic and fatty acyl-CoAs, as well as $NADP^+$**

| Substrate | % |
|---|---|
| Cinnamoyl-CoA* | 100 |
| *p*-coumaroyl-CoA* | 9.5 |
| Caffeoyl-CoA* | ND |
| Feruloyl-CoA* | ND |
| Crotonoyl-CoA ($C_3$)* | ND |
| Hexanoyl-CoA ($C_6$)* | ND |
| Hexadecanoyl-CoA ($C_{16}$)* | ND |
| $NADP^{+\dagger}$ | ND |

ND, not detected.
*acyl-CoAs were at 0.4 mM with 1 mM $NAD^+$.
$^\dagger$[1 mM] in the presence of cinnamoyl-CoA at 0.4 mM.

**Table S2. Primer sets used for qRT-PCR analysis**

| Candidate | Forward primer | Reverse primer |
|---|---|---|
| *PhCHD* | 5′-GCATGTCGTGTTCTGGATGAA-3′ (900) | 5′-TCGGTAAGATGGGAAACTCATTC-3′ (300) |
| *TC10274* | 5′-CTTGTTGGTTTTGGTGTTGCA-3′ (300) | 5′-GAGCGGTATCAGCATTGATTTG-3′ (300) |
| *TC7383* | 5′-CTGAGGAGGGCAAGGAACTGGGT-3′ (900) | 5′-CAATGTCGAGTGCCCAGGACCG-3′ (50) |
| *FN000667* | 5′-CAAGAAAGGAAGCAAGCCAGAA-3′ (900) | 5′-TTTCCACCAGGAGCAATGTTG-3′ (900) |
| *TC12800* | 5′-CCCACTGTATTTTGGCAACCA-3′ (900) | 5′-GAGCCCCTATGATCCGATCTT-3′ (900) |
| *TC9756* | 5′-GTCTTTCTGCTCCGGTGTTGA-3′ (900) | 5′-ATTTGGGCTACCGGGTCAGT-3′ (900) |
| *TC10560* | 5′-CCCAAAGGCTTCCAAGGTTAG-3′ (900) | 5′-GTTCCTTGCCCTCCTCAGAAGT-3′ (900) |

Final concentrations used to achieve 95–100% efficiency are noted in parentheses in nanomoles. Primers were designed using the PrimerExpress software (Applied Biosystems).