

Floral Scent Production in *Clarkia breweri* (Onagraceae)¹

II. Localization and Developmental Modulation of the Enzyme *S*-Adenosyl-L-Methionine:(Iso)Eugenol *O*-Methyltransferase and Phenylpropanoid Emission

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We have previously shown (R.A. Raguso, E. Pichersky [1995] *Plant Syst Evol* 194: 55–67) that the strong, sweet fragrance of *Clarkia breweri* (Onagraceae), an annual plant native to California, consists of 8 to 12 volatile compounds, including 4 phenylpropanoids. Although some *C. breweri* plants emit all 4 phenylpropanoids (eugenol, isoeugenol, methyleugenol, and isomethyleugenol), other *C. breweri* plants do not emit the latter 2 compounds. Here we report that petal tissue was responsible for the bulk of the phenylpropanoid emission. The activity of *S*-adenosyl-L-methionine:(iso)eugenol *O*-methyltransferase (IEMT), a novel enzyme that catalyzes the methylation of the *para*-4'-hydroxyl of both eugenol and (iso)eugenol to methyleugenol and isomethyleugenol, respectively, was also highest in petal tissue. IEMT activity was absent from floral tissues of plants not emitting (iso)methyleugenol. A *C. breweri* cDNA clone encoding IEMT was isolated, and its sequence was shown to have 70% identity to *S*-adenosyl-L-methionine:caffeic acid *O*-methyltransferase. The protein encoded by this cDNA can use eugenol and isoeugenol as substrates, but not caffeic acid. Steady-state IEMT mRNA levels were positively correlated with levels of IEMT activity in the tissues, and no IEMT mRNA was observed in flowers that do not emit (iso)methyleugenol. Overall, the data show that the floral emission of (iso)methyleugenol is controlled at the site of emission, that a positive correlation exists between volatile emission and IEMT activity, and that control of the level of IEMT activity is exerted at a pretranslational step.

Flowers of many plants attract pollinators by producing and emitting a complex mixture of low-molecular-weight volatile compounds. Floral scents may function as both long- and short-distance attractants and nectar guides to a variety of animal pollinators (for review, see Dobson, 1993). Since insects are able to distinguish between complex floral scent mixtures, discriminatory visitation based on floral scent has important implications for population structure and reproductive isolation (Dodson et al., 1969; Galen and Kevan, 1983; Galen, 1985; Pellmyr, 1986).

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Despite the importance of floral scent to plant reproduction and evolution, the biochemical and genetic basis of scent production has received little attention. Previous reports have failed to identify and purify specific enzymes involved in the biosynthesis of scent components in flowers. Since many scent components are also found in floral tissues in bound, nonvolatile forms such as glycosides, it was originally hypothesized that scent compounds could possibly be synthesized elsewhere in the plant, bound into glycosides, and then transported to the emitting part of the flower, where they could be broken down to release the volatile components (Ackermann et al., 1989; Watanabe et al., 1993). However, direct and reproducible evidence of the transport of free scent constituents or their glycosides from vegetative tissue to floral tissue is lacking.

We have begun to investigate the biosynthetic pathways of scent components and their location in the plant by examining *Clarkia breweri* (Gray [Greene]; Onagraceae), an annual plant native to California. The strong, sweet fragrance of *C. breweri* consists of 8 to 12 different volatiles that fall into two groups: monoterpenoids and phenylpropanoids-benzenoids (Raguso and Pichersky, 1995). We have shown that linalool, an acyclic monoterpene, is synthesized in *C. breweri* petals and other floral tissues in a reaction catalyzed by LIS (Pichersky et al., 1994, 1995), and that the enzyme activity is regulated at a pretranslational level (Dudareva et al., 1996). A closely related species, *Clarkia concinna* (from which *C. breweri* is believed to have evolved [Raguso and Pichersky, 1995]), also possesses the gene encoding LIS, but the expression of this gene in this nonscented species is limited to the stigma, and the level of expression is much lower than in the stigma of *C. breweri*. Thus, linalool production in *C. breweri* flowers involves a change in regulation of an existing gene.

Here we report on the production of four phenylpropanoid components of the *C. breweri* scent, eugenol, isoeugenol, methyleugenol, and isomethyleugenol, during the lifespan of the flower. We show that methyleugenol and isomethyleugenol are produced from eugenol and isoeugenol, respectively, by the action of a single enzyme,

Abbreviations: CCOMT, *S*-adenosyl-L-Met:caffeoyl CoA *O*-methyltransferase; COMT, *S*-adenosyl-L-Met:caffeic acid *O*-methyltransferase; IEMT, *S*-adenosyl-L-Met:(iso)eugenol *O*-methyltransferase; LIS, *S*-linalool synthase; OMT, *O*-methyltransferase; SAM, *S*-adenosyl-L-Met.

IEMT. We have previously shown the existence of polymorphism in *C. breweri* for the production of methyleugenol and isomethyleugenol (Raguso and Pichersky, 1995). Here we show that the gene encoding IEMT exhibits flower-specific and temporal expression patterns in a line of *C. breweri* emitting methyleugenol and isomethyleugenol, but not in a line that does not emit these two compounds. Thus, the expression of specific biosynthetic genes in floral tissues may be a common phenomenon in scent production, and changes in the expression characteristics of such genes may lead to the evolution of new scents.

MATERIALS AND METHODS

Plant Material, Growth Conditions, Headspace Collection, and GC-MS Analysis

Details of the construction of true-breeding *Clarkia breweri* stocks, growing conditions, dynamic headspace collection on Tenax (Alltech Inc., Deerfield, IL) and activated charcoal sorbents, and chemical analyses via GC-MS were as described by Raguso and Pichersky (1995). All headspace collections were performed in a growth chamber (Conviron, Asheville, NC) under a 12-h light/12-h dark photoperiod. Temperature was set to 22°C during the light period and 18°C during the dark period. In all experiments, headspace collections from ambient air and from vegetative tissues were used as controls.

Time Course of Phenylpropanoid Production

Volatile phenylpropanoid production in individual flowers of four separate plants was monitored over a 6-d period beginning on the day before anthesis and continuing until floral abscission. Headspace volatiles were collected as described by Raguso and Pichersky (1995). The collections were made at 12-h intervals, corresponding to the dark and light periods in the growth chamber.

Localization and Quantitation of Phenylpropanoid Emission in Floral Parts

The specific floral parts responsible for scent emission were determined and the emission levels were quantified by headspace collection essentially as described by Raguso and Pichersky (1995). Headspace collection was made from attached, 2nd-d (hermaphroditic) intact flowers and from same-stage flowers in which floral organs had been systematically removed to leave only petals, only anthers, or only the pistil. To detect all volatiles emitted by a given flower part that could possibly emit different compounds at different times, a 24-h collection period was used.

IEMT Enzyme Extraction and Assay

Enzyme Extraction

A crude protein extract was prepared by macerating flower parts in a microcentrifuge tube in the presence of ice-cold buffer (10 volumes fresh weight) containing 50 mM

BisTris-HCl, pH 6.9, 10 mM 2-mercaptoethanol, 5 mM $\text{Na}_2\text{S}_2\text{O}_5$, 1% (w/v) PVP-40, and 10% (v/v) glycerol. The slurry was centrifuged for 10 min and the supernatant was transferred to a new tube. For each time point, flowers from three different plants were combined.

OMT Enzyme Assays and Product Analysis

Assay samples were prepared by adding to a 1.5-mL microcentrifuge tube: 10 μL of crude extract, 10 μL of assay buffer (250 mM Tris-HCl, pH 7.5, 10 mM DTT), 1 μL of 50 mM substrate (eugenol, isoeugenol, or other related compounds) in ethanol, 1 μL of S-[methyl- ^{14}C]adenosyl-L-Met (40–60 mCi/mmol) in 10 mM sulfuric acid:ethanol (purchased from NEN Research Products), and 28 μL of water to bring the assay volume to 50 μL . Assay samples were incubated at 30°C for 30 min in a heating block, after which 2.5 μL of 6 M HCl was added to stop the reaction. The radioactively labeled methylated product was extracted by the addition of 100 μL of ethyl acetate, and 20 μL of the organic phase (on top and clear in color) was transferred to a scintillation vial with 2 mL of nonaqueous scintillation fluid (Bio-Safe NA, Research Products International, Mount Prospect, IL) and counted in a liquid scintillation counter (model 2S6800, Beckman). The raw data (counts per minute) were converted to femtomoles of product produced per second based on the specific activity of the substrate, using the appropriate correction factors for counting efficiency.

To verify the identity of the products, organic extracts were analyzed in two ways. First, 20 μL was spotted on a 10 cm \times 20 cm silica gel 60 F₂₅₄ precoated TLC plate (EM Industries, Inc., Gibbstown, NJ), and 5 μL of a 5% (v/v) solution containing authentic methyleugenol or isomethyleugenol was spotted on the same plate as a standard. The plate was developed in a solvent system of 2:3:1 (v/v) benzene:acetic acid:water (De Carolis and Ibrahim, 1989). When the solvent was within 2 cm of the top edge of the plate, the plate was removed and allowed to dry. UV light revealed the elution points of the standards, from which retention-factor values were calculated.

TLC plates containing radioactively labeled product were analyzed by an imaging scanner (System 200, Bioscan, Inc., Washington, DC). Plates were scanned horizontally in lanes 0.6 cm in width for 5 min per lane by the Autochanger 3000 detector (Bioscan) under a steady flow of 90% argon/10% methane at a flow rate of 70 kPa (10 p.s.i.). Bioscan computer software produced full-color, two-dimensional images of TLC plates using a color scale to display the location of radioactivity, indicating the presence of radioactively labeled product. Comparison of the retention-factor values of radioactive spots and those of the nonradioactive standards tentatively determined the identities of radioactively labeled enzyme product.

In addition, "cold assays" with nonradioactive SAM were performed by scaling up the reaction to a total volume of 1000 μL and a final substrate concentration of 1 mM. The products were organically extracted and analyzed by GC-MS analysis.

Isolation and Purification of OMTs from *C. breweri*

Preparation of crude extract from *C. breweri* petals was performed as previously described (Pichersky et al., 1995). The enzyme activity was purified through successive chromatographic steps involving DE53 anion-exchange and hydroxyapatite columns as previously described (Pichersky et al., 1995). The last step of purification utilized a 5'-ADP affinity column from which the pyrophosphate group was removed with alkaline phosphatase as described by Attieh et al. (1995). A complete description of the purification protocol will appear elsewhere (J. Wang and E. Pichersky, unpublished data).

Protein Sequencing

The two proteins present in the final purified OMT preparation were separated on long SDS-PAGE and subjected to N-terminal sequencing in a protein sequencer (model 477, Applied Biosystems). In addition, the proteins were cleaved with cyanogen bromide, the digestion products were subjected to SDS-PAGE, and additional peptides were isolated and sequenced as previously described (Dudareva et al., 1996).

Isolation and Characterization of cDNA Clones

Since peptide sequence determination showed that the putative IEMT had substantial sequence similarity to COMT, a clone encoding aspen (*Populus tremuloides*) COMT (Bugos et al., 1991) was obtained from Dr. W. Campbell (Michigan Technological University, Houghton) and used to screen a *C. breweri* flower cDNA library (Dudareva et al., 1996). Clones were identified, isolated, and characterized as previously described (Dudareva et al., 1996).

RNA Isolation and RNA Gel-Blot Analysis

Total RNA was isolated from 0.1 g of frozen plant tissue as previously described (Dudareva et al., 1996). RNA samples (7 μ g per lane in blots to determine tissue-specific expression, 3 μ g per lane in blots to determine variation in expression in petals over the lifespan of the flower) were size-fractionated by electrophoresis under denaturing conditions in vertical urea-agarose gels at 4°C for 5 h at 20 W and transferred to Hybond-N⁺ membranes (Amersham). For IEMT blots, either a 1.3-kb IEMT cDNA fragment containing the coding region of the gene or a 0.3-kb fragment containing the 3' noncoding region of the gene (from nucleotide 1159 in Fig. 6 to the 3' end) was used as a probe. For COMT blots a 1-kb fragment containing the coding region of aspen COMT was used as a probe. Hybridizations were performed in 5 \times SSPE (0.75 M NaCl, 0.05 M NaH₂PO₄, pH 7.4, and 0.5 mM EDTA), 50% formamide, 5 \times Denhardt's solution, and 0.5% SDS at 37°C for at least 18 h. Membranes were washed at 37°C with 5 \times SSPE, 0.5% SDS, and twice with 2 \times SSPE at 65°C before being exposed to x-ray film. mRNA transcripts were quantified using a molecular imaging system (model GS-363, Bio-Rad). IEMT mRNA transcript levels were normalized to rRNA levels to overcome error in RNA quantitation by spectrophotome-

try. In addition, all gels contained a standard for equating signals among gels.

Expression of IEMT in *Escherichia coli*

The cDNA clone of IEMT was subcloned into the *Nde*I-*Bam*HI sites of the pET-T7-11a expression vector (Studier et al., 1990) by first amplifying by PCR the entire coding region of the clone with an oligonucleotide at the 5' end that introduced an *Nde*I site around the first ATG codon.

RESULTS

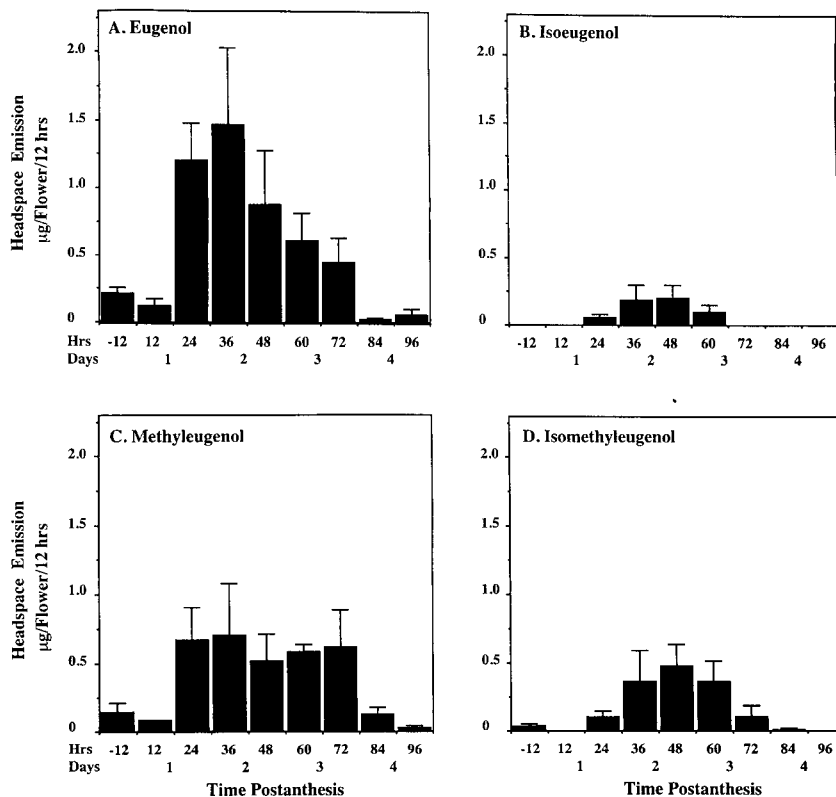
Temporal Variation in Scent Emission by Intact Flowers

We have previously shown (Raguso and Pichersky, 1995) that eugenol, isoeugenol, methyleugenol, and isomethyleugenol are constituents of the scent of *C. breweri* flowers, although some *C. breweri* plants were found that did not produce methyleugenol and isomethyleugenol (Raguso and Pichersky, 1995). To determine the amount of these compounds emitted at different stages of floral development, we performed time-course headspace collections at 12-h intervals, followed by GC-MS analysis, using an inbred line that emits all four compounds. We began headspace collection with buds on the evening before they opened and ended it 4 d later. Little or no emission was detected from unopened flowers (buds). The amounts emitted from buds and open flowers 1 to 4 d after anthesis are shown in Figure 1, A to D. Emissions of eugenol (Fig. 1A) and methyleugenol (Fig. 1C) both peaked on d 2 and declined thereafter. Emissions of isoeugenol (Fig. 1B) and isomethyleugenol (Fig. 1D) showed similar patterns, although peak emission occurred approximately 12 h later than the eugenol and methyleugenol peaks. Emission levels at peak time ranged from 0.22 μ g/flower for isoeugenol to 1.5 μ g/flower for eugenol per 12 h.

Localization and Quantification of Phenylpropanoid Emission from the Different Parts of the Flower

To determine the specific parts of the *C. breweri* flowers that emit these phenylpropanoids, we performed experiments in which living flowers were modified by selectively excising floral parts so that only one class of major floral organ (petals, stamens, or pistil) remained attached to the hypanthium and sepals. We then collected headspace volatiles from these modified flowers over a 24-h period. The data obtained were used to calculate the contribution of each part to the total emission of the flower (Fig. 2). These data revealed that the petals were the organs responsible for the majority of the phenylpropanoid emission. However, the emission of eugenol and isoeugenol in such flowers was greatly decreased (in the case of isoeugenol to below-detection levels). This observation suggests that tissues other than petals may be involved in controlling the flux of the pathway, perhaps by supplying precursors. Alternatively, the injury sustained by the flowers in these experiments may have influenced the outcome.

Figure 1. Emission of phenylpropanoids from *C. breweri* flowers as measured by headspace collection at 12-h intervals and GC-MS analysis. A, Emission of eugenol. B, Emission of isoeugenol. C, Emission of methyleugenol. D, Emission of isomethyleugenol.



IEMT and COMT Activities in Flowers

IEMT Activity in Flower Parts and Its Temporal Variation

The biochemical steps that lead to eugenol and isoeugenol synthesis, and their immediate precursors, are currently undetermined. However, it appeared likely that methyleugenol and isomethyleugenol could be synthesized from eugenol and isoeugenol, respectively, by the addition

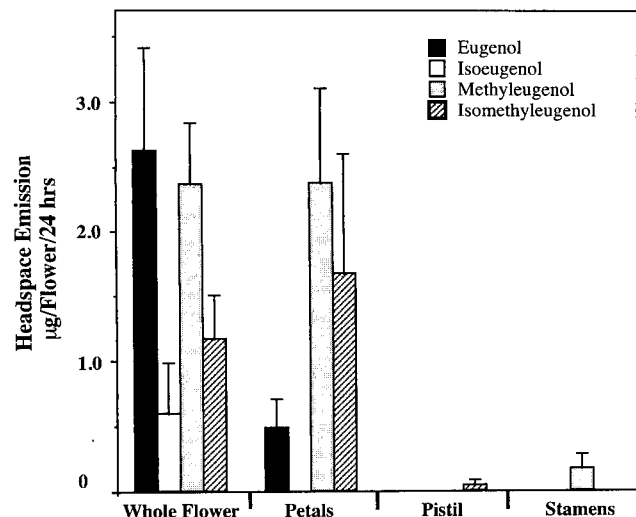


Figure 2. Emission of phenylpropanoids from *C. breweri* flowers and flower parts as measured by headspace collection at 12-h intervals and GC-MS analysis.

of a methyl group to the *p*-hydroxyl (4'-hydroxyl) moiety (Fig. 3). To our knowledge, a specific enzyme that methylates the 4'-hydroxyl of (iso)eugenol has not been previously described. However, methylation of the 3' hydroxyl of similar compounds has been found to be catalyzed by enzymes collectively termed OMTs that use SAM as the methyl donor (Attieh et al., 1995; Meng and Campbell, 1996). Therefore, we devised an enzymatic assay to test for the presence of IEMTs. The crude extracts were incubated with either eugenol or isoeugenol and [¹⁴C]SAM, and the product was extracted and analyzed. Products were identified by co-migration with standards on TLC plates and by GC-MS analysis.

C. breweri plants that did not emit methyleugenol and isomethyleugenol did not contain any IEMT activity in their floral parts. *C. breweri* plants that did emit these two compounds contained substantial IEMT in the petals, and some activity was also found in stamens and styles, with trace activity found in stigmata (Fig. 4, A and B). None of the remaining floral parts—sepals and ovaries—or leaves were found to contain any IEMT activity.

Whereas some IEMT activity was already found in mature buds just before anthesis, IEMT activity levels increased quickly after the flower opened and reached 90 to 100% of maximal levels on d 1 of anthesis (Fig. 4, A and B). Subsequent IEMT activity remained fairly constant. Interestingly, the patterns of changes in eugenol-OMT activity levels and isoeugenol-OMT activity levels were very similar (Fig. 4, A and B), with the ratio of the latter to the former ranging from 1.4 to 1.6 in all floral tissues and throughout the lifespan of the flower.

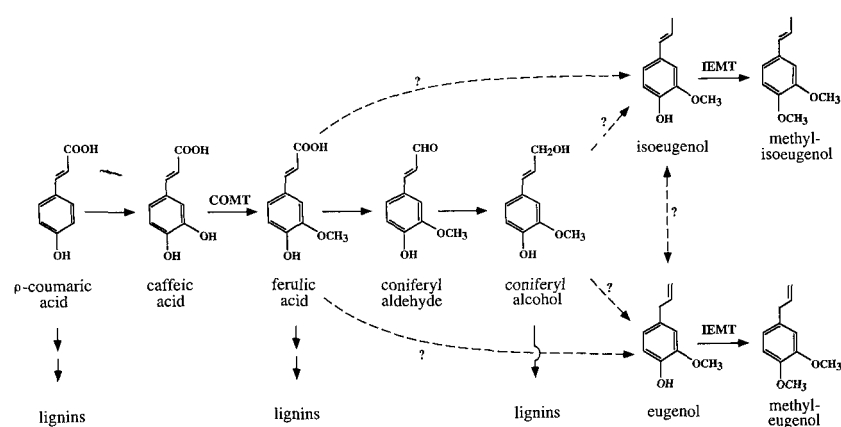


Figure 3. The reactions catalyzed by IEMT and the possible pathways leading to eugenol and isoegenol (based on Manitto et al., 1974; Senanayake et al., 1977). An alternative pathway involving caffeoyl acid CoA and CCOMT is not shown.

COMT Activity in Flower Parts and Its Temporal Variation

We also measured the activity in floral parts of COMT, an enzyme involved in lignin biosynthesis and possibly in (iso)eugenol biosynthesis as well. Similar to IEMT, COMT activity was highest in petals, but its pattern of gradual increase in activity over time was substantially different from the pattern for IEMT. Whereas IEMT increased quickly after anthesis and reached a maximum on the 2nd d after anthesis, COMT activity was relatively low at this time and increased only gradually to achieve a maximum on the 5th d after anthesis, at the end of flower's lifespan.

Isolation and Characterization of IEMT cDNA Clones

We purified IEMT from 1- to 4-d-old petal tissue in a procedure involving anion-exchange, hydroxyapatite, and adenosine-conjugated affinity column chromatography. The latter column is known to selectively bind a variety of OMTs (Attieh et al., 1995). This procedure yielded two proteins that migrated very close to each other on SDS-PAGE, each with an apparent molecular mass of 40 kD (Fig. 5, right lane). After separating the two proteins on long SDS-PAGE and subjecting them to N-terminal protein

sequencing, we obtained two very similar peptide sequences (from the top protein band: XTGNAETQLTP [X = unidentified]; from the bottom protein band: SPGNAEI-QIIP), both of which show some similarity to COMT N-terminal sequences of dicotyledonous plants in the data bank. In addition, several individual peptides were obtained from SDS-PAGE after cyanogen bromide cleavage of a mixture of the two proteins, and their sequences also showed significant similarity to COMT sequences. None of the peptide sequences showed any similarity to CCOMT, an enzyme that methylates caffeic acid bound to CoA in an alternative lignin biosynthesis pathway and that also has no significant similarity to COMT (Ye et al., 1994).

Since the N-terminal sequences of both proteins in the "purified" IEMT preparation showed similarity to COMT, we used a COMT cDNA clone from aspen (Bugos et al., 1991) as a probe in low-stringency hybridization screening of a *C. breweri* flower cDNA library. Several clones were isolated, and the nucleotide sequence of one of them, designated IEMT1 (see below), was determined for this study (Fig. 6). This clone contains 1486 nucleotides, not including the poly(A) tail, with an open reading frame of 368 codons, beginning with an ATG codon at positions 43 to 45. There

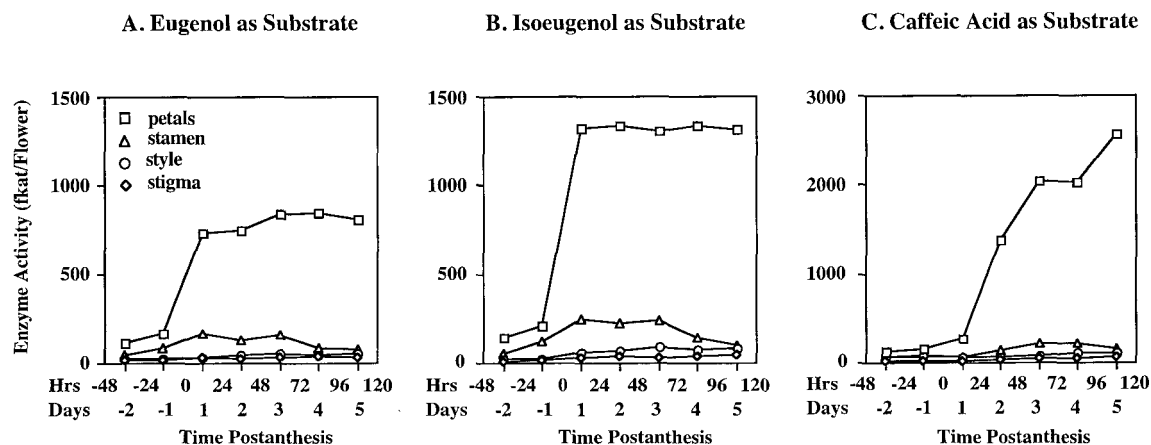


Figure 4. Levels of different OMT activities in different parts of the flower during the lifespan of the flower. A, Eugenol OMT activity. B, Isoegenol OMT activity. C, Caffeic acid OMT activity. Data are shown only for flower parts that contained detectable IEMT activity (fkcat = femtomoles of product per second).

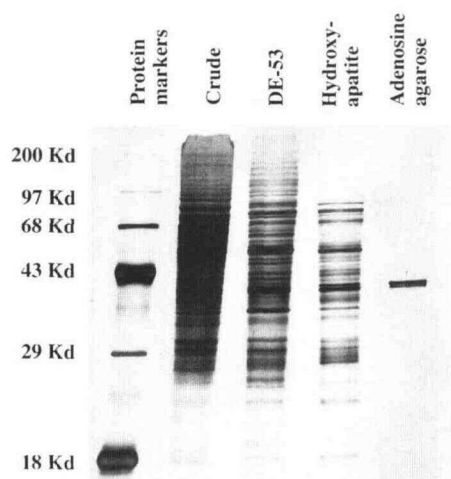


Figure 5. Steps in the purification of OMTs from *C. breweri* petals.

is one stop codon in-frame upstream of this ATG, suggesting that we have isolated a cDNA clone that contains the entire coding region. This conclusion is also supported by primer-extension experiments to determine the 5' end of the mRNA (data not shown). The molecular mass of the protein encoded by the open reading frame of IEMT1 is 40 kD, the same as that of the two proteins found in our purified OMT preparation (Fig. 5).

The N-terminal sequence of the protein encoded by IEMT1 does not match the N-terminal sequence of the higher-molecular-mass protein of the two present in the purified OMT preparation (3/10 mismatches). However, it did match in 9 of 10 positions the N-terminal sequence of the lower-molecular-mass protein (underlined sequences in Fig. 6). The one mismatch in the sequence may have been due to the difficulty in determining the correct N-terminal peptide sequence due to high background signals. The N-terminal sequence predicted from the open reading frame of IEMT1 is two amino acid residues longer than that of the N terminus experimentally determined, suggesting that some processing occurs either in vivo or during the purification procedure. In addition, the protein encoded by IEMT1 contains two of the internal peptide sequences determined experimentally, MLDRVLRLLASYSVVYTLRE and MFDGVPKGD AIFIK (Fig. 6). The IEMT1 protein is approximately 30% divergent from all available dicot COMT sequences. These COMTs vary among themselves by no more than 15%, with the exception of a *Zinnia elegans* sequence, designated COMT, the substrate specificity of which has not been extensively tested (Ye and Varner, 1995). IEMT1 shows sequence identity of 65% or less to several other types of plant OMTs in the data bank, and it contains the three conserved motifs (Fig. 6) identified by Kagan and Clarke (1994) and hypothesized by these authors to be involved in the binding of SAM.

To determine the enzymatic activity of the protein encoded by IEMT1, we cloned it into a pET-T7 (11a) expression system (Studier et al., 1990) and expressed it in *E. coli*. Cell lysates were prepared and assayed with the substrates eugenol and isoeugenol, as well as with several intermediates in the lignin biosynthesis pathway that are structurally

related to (iso)eugenol, including caffeic acid. We also tested the substrate specificity of cloned plant COMT expressed in *E. coli* (Meng and Campbell, 1996; J. Wang and E. Pichersky, unpublished data) and of our *C. breweri* affinity-purified OMT preparation (Fig. 5, right lane). The latter preparation showed high levels of OMT activity with eugenol, isoeugenol, caffeic acid, and 5-hydroxyferulic acid (Fig. 7A). However, lysates of *E. coli* expressing COMT had high activity only with caffeic acid and 5-hydroxyferulic acid, as previously described (Meng and Campbell, 1996), and essentially no activity with eugenol and isoeugenol (Fig. 7C). Lysates of *E. coli* expressing IEMT1 had high levels of activity with both eugenol and isoeugenol, but little or no activity with any of the other substrates tested (Fig. 7B). Moreover, the ratio of isoeugenol-OMT activity to eugenol-OMT activity in the IEMT-expressing *E. coli* lysates (1.4) was basically the same as that of the plant-purified OMT preparation and of the plant crude extracts as well (Fig. 4, A and B). Lysates of *E. coli* that contained a pET-T7 (11a) plasmid with no plant DNA insert had undetectable OMT activity with any of these substrates.

Since our results indicate that IEMT has only residual COMT activity (<3%) and that COMT has only residual IEMT activity, it appears that the substrate-specificity profile of the affinity-purified plant preparation (Fig. 7A) was obtained because this preparation has both COMT and IEMT activities, each contained on a separate protein. The lack of activity of COMT with eugenol and isoeugenol

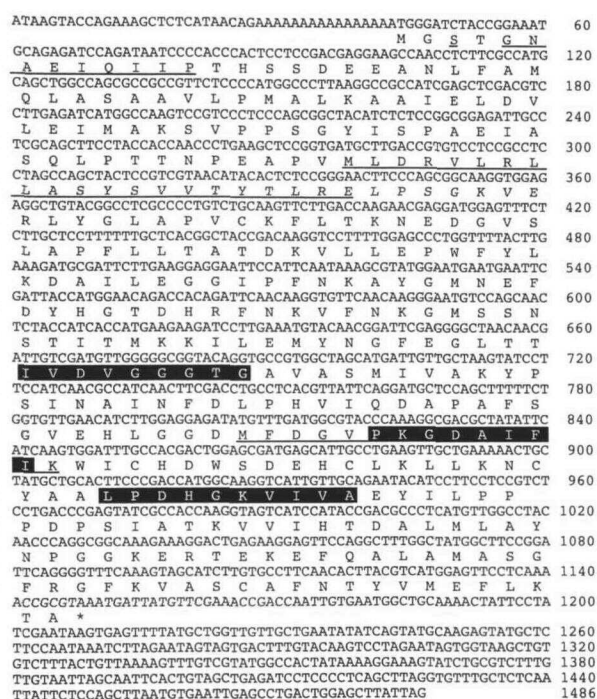


Figure 6. Nucleotide sequence of IEMT1 cDNA clone. The predicted protein sequence is shown below the nucleotide sequence. Numbers on right refer to the nucleotide sequence, and numbers on left refer to the protein sequence. Peptide sequences obtained experimentally are underlined. The three conserved motifs hypothesized to bind SAM (Kagan and Clarke, 1994) are shown in black boxes with white letters.

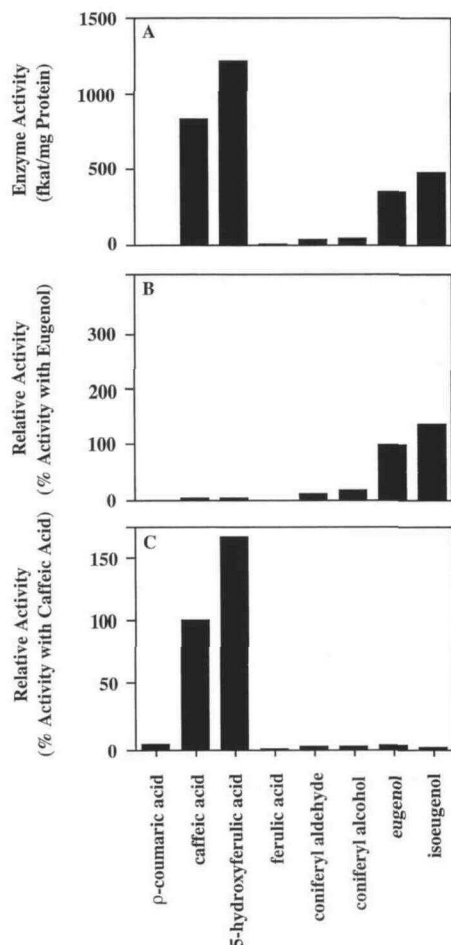


Figure 7. A, Enzymatic activity of the plant purified OMT preparation. Several related substrates in addition to eugenol and isoeugenol were tested as substrates (see text and Fig. 3). B, Activity of plant IEMT1 expressed in *E. coli*. Results are presented as a percentage of activity relative to activity with eugenol, which is arbitrarily set at 100%. C, Activity of plant COMT expressed in *E. coli*. Results are presented as a percentage of activity relative to activity with caffeic acid, which is arbitrarily set at 100%.

further indicates that the IEMT activity measurements shown in Figure 4 indeed represent only the activity of a distinct enzyme, i.e. IEMT. This enzyme was identified from the N-terminal sequence data as the lower-molecular-mass protein in our purified OMT preparation (Fig. 5).

Northern Analysis of IEMT in Flower Parts

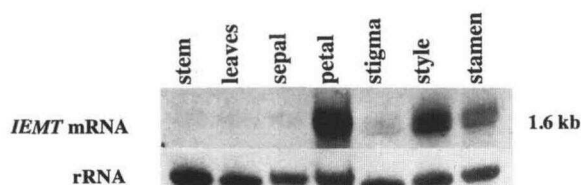
Levels of IEMT mRNA in the different tissues of (iso)methyleugenol emitters and nonemitters were determined by northern analysis (Fig. 8). mRNAs were extracted from the different tissues of flowers on the day of anthesis. In (iso)methyleugenol emitters, the highest levels of IEMT mRNA were observed in petals, followed by style and stamens. Little or no IEMT mRNA was observed in stigma and sepals, or in leaf and stem tissue (Fig. 8A). It should be pointed out that although IEMT mRNA levels are higher in style tissue than in stamens on a per cell basis, the style is a much smaller organ than stamens are, and as a conse-

quence there is much less total IEMT mRNA in the style than in stamens, consistent with the activity data shown in Figure 4, A and B. True-breeding plants that do not emit (iso)methyleugenol had little or no IEMT message in any of the tissues examined (Fig. 8B).

The size of the IEMT mRNA was estimated to be 1.6 kb, in agreement with the size of the cDNA clone. A broad band, and in many lanes two closely spaced bands, were observed, indicating some heterogeneity in message size. A probe obtained from the 3' noncoding region of IEMT1 also gave the same pattern (data not shown). The appearance of two distinct bands is most likely due to the fact that IEMT message fortuitously co-migrates with the abundant 18S rRNA, so that IEMT mRNA transcripts that are slightly larger than 18S rRNA and IEMT mRNA transcripts that are slightly smaller than 18S rRNA are separated by the 18S rRNA. This interpretation is also consistent with Southern analysis suggesting that (iso)methyleugenol-emitting *C. breweri* plants (as well as nonemitting plants) have a single IEMT gene (data not shown). With a probe derived from the coding region of COMT, a single band of 1.3 kb was observed (data not shown), indicating that under our stringent northern-blot conditions the IEMT probe does not cross-hybridize with COMT message but only with its own distinct message.

The variation in IEMT mRNA levels over the lifespan of the (iso)methyleugenol-emitting flowers was also examined. mRNA levels increased as the bud matured and

A (Iso)Methyleugenol-Emitting Plants



B (Iso)Methyleugenol-Nonemitting Plants

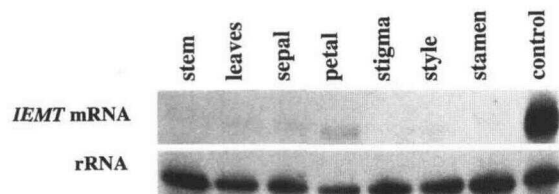


Figure 8. Expression of IEMT in flower parts. A, Northern-blot hybridization with mRNA from different tissues of an inbred line of plants that emit (iso)methyleugenol, using IEMT probe derived from the coding region of IEMT1. B, Northern-blot hybridization with mRNA from different tissues of an inbred line of plants that do not emit (iso)methyleugenol, using the same IEMT probe as in A. Lanes were loaded with 7 μ g of total RNA. Autoradiography was for 48 h. Control lane in B contained 7 μ g of total RNA from petals of (iso)methyleugenol-emitting flowers (i.e. sample identical to the petal RNA lane in A). Each blot was rehybridized with an 18S rDNA probe to standardize samples.

peaked just before anthesis. IEMT mRNA levels on the day of anthesis were about 75% of the levels just before anthesis, and remained relatively stable afterward (Fig. 9).

DISCUSSION

Temporal Variation in Scent Phenylpropanoids by Whole Flowers and Buds, and Localization of Emission to the Different Parts of the Flower

The strong, sweet floral scent of *C. breweri* is unique in its genus and is correlated with pollination by moths, a mode of reproduction that is novel among *Clarkia* species (MacSwain et al., 1973). Emission of the phenylpropanoid components of the scent begins at anthesis and reaches a peak on d 2 (Fig. 1). During the lifespan of the flower, marked variation in phenylpropanoid emission between the day and night periods was not observed. Our data also show that most of the floral emission of the four phenylpropanoids investigated in this study comes from petals, with the stamens contributing the remainder (Fig. 2).

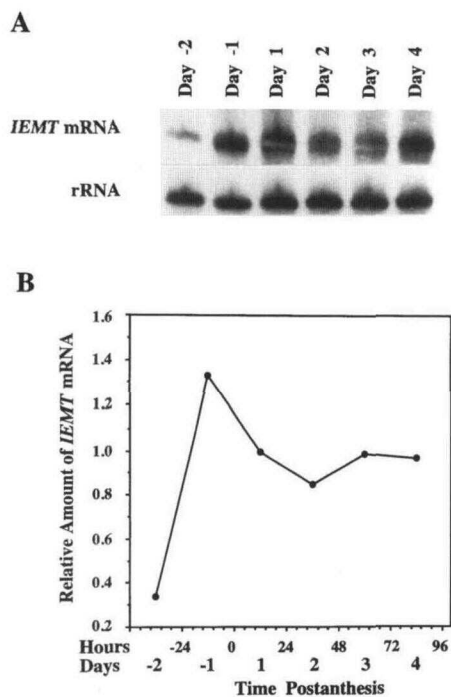


Figure 9. Expression of IEMT in petals during flower development. A, Representative northern-blot hybridization experiment with mRNAs extracted from petal tissue at different stages in the lifespan of (iso)methyleugenol-emitting flowers. Each lane contained 3 μ g of total RNA. Autoradiography was for 14 d. The blot was rehybridized with an 18S rDNA probe to standardize samples. B, Plot of the variation in levels of petal IEMT mRNA in (iso)methyleugenol-emitting flowers over time. Values were obtained by scanning blots with a phosphoimager. Each point is the average of four different experiments (including the one shown in A), and values were corrected by standardizing for 18S RNA levels.

Biosynthesis of Methyleugenol and Isomethyleugenol and the Expression of IEMT1 in Floral Tissues

Little is known about the enzymatic steps leading to eugenol and isoeugenol, although pulse-chase experiments with radioactive precursors have clearly shown that they are ultimately derived from Phe, with *p*-coumaric acid as an intermediate (Manitto et al., 1974; Senanayake et al., 1977). It is important to note that our investigation did not address the question of whether an early step in the biosynthesis of eugenol and isoeugenol involves the 3'-hydroxyl methylation of caffeic acid by COMT or, alternatively, the 3'-hydroxyl methylation of caffeoyl CoA by CCOMT (or neither) (Fig. 3). We have found that COMT enzyme levels do not parallel IEMT levels in petals, the emitting organ, but COMT may also be involved in other pathways in the petals, and we did not measure CCOMT activity levels. Rather, our investigation concentrated on the biosynthesis of (iso)methyleugenol via the action of IEMT.

To our knowledge, no enzymatic activity capable of converting eugenol and isoeugenol to methyleugenol and isomethyleugenol, respectively, has previously been reported from plants. Our data show the existence of a single enzyme, designated IEMT, which methylates both eugenol and isoeugenol to methyleugenol and isomethyleugenol, respectively, with high specificity (Fig. 7). Although this enzyme has high sequence similarity to COMTs, it cannot use caffeic acid as a substrate, nor can COMT use (iso)eugenol as a substrate. Levels of IEMT activity and mRNA in the different floral tissues of (iso)methyleugenol emitters strongly correlate with the production and emission of these two compounds by the same tissues, being highest in petals, followed by stamens, style, and stigma, and absent in sepals and in leaf and stem tissue (Fig. 8A). Moreover, nonemitting plants did not have IEMT activity or IEMT mRNA in any floral tissues (Fig. 8B), although they do contain the IEMT gene in their genome (data not shown). These results are very similar to those obtained for LIS, an enzyme that produces linalool, another floral scent compound in *C. breweri*. We observed strong positive correlation between levels of LIS enzyme activity, protein, and mRNA at the site of synthesis and emission of linalool in the flower (Pichersky et al., 1994; Dudareva et al., 1996).

However, whereas in the case of linalool levels of emission, enzyme activity, and mRNA in the petals all rose and fell in parallel (but with mRNA levels peaking 1–2 d ahead of enzyme activity and emission) until the end of the lifespan of the flower, the situation with IEMT was somewhat different. Isomethyleugenol and methyleugenol emission, IEMT activity, and mRNA levels in the petals all increased in parallel as the buds matured and the flowers opened (again, with mRNA levels peaking 1–2 d ahead of enzyme activity and emission). However, starting from the 3rd d of anthesis (1 d after the stigma becomes receptive and most pollination occurs), emission began to decline but IEMT activity remained relatively stable. IEMT mRNA levels actually went up a little after declining 25% from their peak on the day before anthesis (Figs. 4, A and B, and 9).

The consequence of high levels of IEMT activity without (iso)methyleugenol emission in old flowers is not known. It could be that methyleugenol and isomethyleugenol are still being made by the floral tissues but are being tied into nonvolatile compounds. Loughrin et al. (1992) have reported an increase in glycosidically bound scent compounds in tobacco floral tissues as the flowers aged. Although methyleugenol and isomethyleugenol cannot be directly linked to a sugar moiety, perhaps they are conjugated to other compounds. Alternatively, isomethyleugenol and methyleugenol biosynthesis may decline as the flower ages, even though IEMT levels remain high, because of changes in other factors involved (e.g. a decline in levels of other enzymes in the pathway, especially rate-limiting enzymes).

CONCLUSION

We show here that the initial synthesis of two phenylpropanoid scent compounds in *C. breweri* occur in the same part of the flower from which such compounds are emitted. Although evidence of enzymatic activity in flower tissue responsible for new synthesis of scent compounds is still scarce, this report contributes additional evidence that, at least in *C. breweri* flowers, scent compounds are produced de novo in the tissues from which they are emitted. We conclude that the difficulties encountered in the past in identifying scent-volatile-producing enzymes will be overcome as assays are developed to identify these enzymes, and are probably not due to the absence of such enzymes from floral tissues.

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