Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via reduction of a coniferyl alcohol ester

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Phenylpropenes such as chavicol, t-anol, eugenol, and isoeugenol are produced by plants as defense compounds against animals and microorganisms and as floral attractants of pollinators. Moreover, humans have used phenylpropenes since antiquity for food preservation and flavoring and as medicinal agents. Previous research suggested that the phenylpropenes are synthesized in plants from substituted phenylpropenols, although the identity of the enzymes and the nature of the reaction mechanism involved in this transformation have remained obscure. We show here that glandular trichomes of sweet basil (Ocimum basilicum), which synthesize and accumulate phenylpropenes, possess an enzyme that can use coniferyl acetate and NADPH to form eugenol. Petunia (Petunia hybrida cv. Mitchell) flowers, which emit large amounts of isoeugenol, possess an enzyme homologous to the basil eugenol-forming enzyme that also uses coniferyl acetate and NADPH as substrates but catalyzes the formation of isoeugenol. The basil and petunia phenylpropene-forming enzymes belong to a structural family of NADPH-dependent reductases that also includes pinoresinol–lariciresinol reductase, isoflavone reductase, and phenylocomaran benzyl ether reductase.

Fig. 1. Several phenylpropenol and their presumed phenylpropenol precursors. The carbon numbering system used in this study is also shown.

Given their historical importance to society and their continued high value as flavoring components, phenylpropenes have garnered the attention of plant biochemists interested in their means of biosynthesis in planta. Despite this interest, the biochemical pathways for the synthesis of eugenol, isoeugenol, and related compounds have not been completely elucidated to date. Previous work (1, 12) indicated that the initial biosynthetic steps are shared with the lignin/lignan biosynthetic pathway up to the phenylpropenol (monolignol) stage (Fig. 1). Regardless of which steps follow, the unusual nature of the reduction reaction to remove the oxygen functionality at C-9 has resisted elucidation, largely because of a lack of biochemical precedence. Moreover, the synthesis in planta of isomeric forms of the product of such a reaction, e.g., eugenol and isoeugenol, adds another layer of biochemical complexity.

Some basil (Ocimum basilicum) varieties synthesize and accumulate eugenol, chavicol, or their methylated derivatives in

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Abbreviations: PLR, pinoresinol–lariciresinol reductase; IFR, isoflavone reductase; PCBER, phenylocomaran benzyl ether reductase.

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the peltate glandular trichomes (glands) on the surface of their leaves (1, 13). In contrast, isoeugenol is one of three main volatiles emitted diurnally from the tube and corolla of the petunia (Petunia hybrida) flower (14). Because of the relatively high level of production of these compounds in these species, and because they are amenable to laboratory manipulation, they were chosen as model organisms to investigate the production of these compounds in planta. Here we show that a basil enzyme belonging to the PIP family of NADPH-dependent reductases [named for the first three enzymes identified in the family, pinoresinol–lacciresinol reductase (PLR), isoflavone reductase (IFR), and phenylcoumaran benzyl ether reductase (PCBER)] catalyzes the formation of eugenol. A closely related enzyme from petunia catalyzes the corresponding formation of isoeugenol. Notably, in both cases the substrate of these two enzymes is an ester derivative of coniferyl alcohol.

Results

Identification of Basil and Petunia cDNAs Encoding Enzymes Capable of Synthesizing Phenylpropanoids. We have previously constructed EST collections from basil glands and petunia flowers, which synthesize eugenol and isoeugenol, respectively (1, 13, 15). In our search for enzymes potentially capable of catalyzing their formation, we searched these databases and found that these collections of EST sequences contained >10 classes of oxidoreductase genes.

One such gene, encoding a protein with sequence similarity to “IFR-like” proteins (IRLs, a large group of PIP family proteins with no known function), was recently shown (16) to be florally expressed at a higher level in a petunia line in which expression of the R2R3 MYB-type transcription factor ODORANT1 is suppressed. Normally, flower-specific expression of ODORANT1 activates the metabolic pathway that leads to the synthesis of phenylpropanoid precursors of scent, such as phenylalanine. In addition to the observed upregulation of this IRL gene in ODORANT1-suppressed plants, expression of several other genes encoding some of the known enzymes involved in the last steps of the biosynthesis of floral volatiles (e.g., a methyltransferase that converts benzoic acid to methylbenzoate) was also higher in this line than in the wild type. The increase in the level of scent-producing enzymes appears to be the result of a mechanism that compensates for the decrease in the concentration of their substrates (16).

We obtained a full-length cDNA of this petunia PIP family member gene and determined its sequence. This gene, which we have now designated IGS1 (isoeugenol synthase 1), encodes a protein of 323 aa (calculated molecular mass 36.0 kDa) whose sequence is 38.7% identical to IFR from Medicago sativa, 45.7% identical to PCBER from Populus trichocarpa, and 42.5% identical to PLR from Thuya plicata (Fig. 2 and Fig. 7, which is published as supporting information on the PNAS web site). Using the petunia IGS1 protein sequence as a reference, we then searched these databases and found that these collections of EST sequences contained >10 classes of oxidoreductase genes.

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Concentration of 7.1 μg/mL. Similarly, the spent medium of E. coli cells expressing petunia IGS1 contained isoeugenol (Fig. 3B) at a concentration of 6.5 μg/mL. Control E. coli cells expressing an unrelated gene (MK51) (17) and cultured under identical conditions as those expressing EGS1 and IGS1 did not produce either eugenol or isoeugenol (data not shown). Expression of IGS1 and EGS1 in E. coli using a phIS-9 vector (a pET-based vector; see Materials and Methods) with an N-terminal His-tag extension gave similar results.

In Planta Tissue-Specific Expression of Petunia IGS1 and Basil EGS1 Correlates with Isoeugenol and Eugenol Biosynthesis. RNA transcripts of the petunia IGS1 gene were found only in floral tissue, mostly in the upper and lower parts of the petals (corolla and tube, respectively) (Fig. 4A). These tissues are responsible for most of the emission of floral volatiles, including isoeugenol (14). In basil, the EGS1 protein appears to be restricted to glands (Fig. 4B) where eugenol, chavicol, and their biosynthetic derivatives such as methyleugenol and methylevichicol are synthesized and stored (1).
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acetate. The pH optimum for both enzymes was determined to be pH 6.5. Both enzymes were stable for 30 min at 30°C but lost >95% activity after incubation for 30 min at 50°C. Divalent metals were generally only mildly inhibitory (<30% for Mg²⁺, Ca²⁺, Mn²⁺, and Fe²⁺), but Zn²⁺ and Cu²⁺ inhibited EGS1 by 45% and 85%, respectively, as well as IGS1 by 75% and 85%, respectively.

For IGS1, the $K_m$ value for coniferyl acetate was 1.6 mM, and the $K_m$ value for NADPH was 73 μM, whereas for EGS1 the corresponding values were 5.1 mM and 131 μM, respectively. For both enzymes, NADH could substitute for NADPH, but only at much higher concentrations (10-fold). The $V_{max}$ value of IGS1 was 7.1 nmol⁻¹·mg⁻¹·protein ($k_{cat} = 0.3$ s⁻¹), and the corresponding value for EGS1 was 19.9 nmol⁻¹·mg⁻¹·protein ($k_{cat} = 0.7$ s⁻¹). Thus, the catalytic efficiencies ($k_{cat}/K_m$) for these two enzymes with coniferyl acetate as substrate are 160 s⁻¹·M⁻¹ and 136 s⁻¹·M⁻¹. These values lie within the range of catalytic efficiencies determined for other members of the PIP reductase family (20, 21), with PLR being more and PCBER being less efficient than EGS1 or IGS1. Compounds related to the EGS1 and IGS1 substrates, such as coniferyl alcohol or cinnamaldehyde, did not inhibit the enzyme, but 4-bromo-cinnamaldehyde did inhibit the reaction catalyzed by EGS1 by 71% and the reaction catalyzed by IGS1 by 27% when present in equal molarity (0.5 mM) with coniferyl acetate.

**Discussion**

**Evolution and Function of EGS1 and IGS1.** Our data demonstrate that basil EGS1 and petunia IGS1, which catalyze the formation of eugenol and isoeugenol, respectively, are related to PLRs, IFRs, and PCBERs and are thus members of the PIP protein family of NADPH-dependent reductases (Fig. 2) (20–23). PLRs and PCBERs are involved in the biosynthesis of lignans throughout the plant kingdom, and IFRs are involved in the modification of some isoflavonoids (isoflavanoids occur mostly in legumes). Although the majority of compounds produced by PIP reductases are believed to function in plant defense (23), a subset of these compounds may have acquired additional roles. For example, both eugenol and isoeugenol are components of the floral scent of Clarkia breweri flowers (24, 25).

Sequence comparisons of EGS1 and IGS1 with a sampling of plant ESTs reveal that they share the highest percentage of identity (>40%) with a group of sequences from diverse plants that currently lack a definitive functional annotation (designated in the databases as IFR-like proteins, or IRLs). Because all of these sequences form a distinct clade in the large PIP gene/protein family (Fig. 2), it will be important to investigate the function of the encoded enzymes, including their possible role in phenylpropene biosynthesis or mechanistically similar reductive pathways in other plant species.

**The Substrate of EGS1 and IGS1 Is an Ester of Coniferyl Alcohol.** It had been shown that secretory glandular trichomes in the Lamiaceae family, to which basil belongs, are highly porous to small molecules, and such molecules, but not protein macromolecules, come out of the gland cells and are washed away during gland isolation procedures (1, 26). As a result, these glandular preparations can be used to test for the presence of enzymes and metabolic pathways with specific biochemical activities through the addition of various substrates and cofactors. Although coniferyl alcohol was converted to eugenol or isoeugenol, respectively, when added to growing E. coli cells expressing EGS1 or IGS1, isolated basil glands supplemented with coniferyl alcohol and NADPH did not produce either phenylpropene product. However, when acetyl-CoA, a substrate of acyltransferases that catalyze the formation of acetyl esters (27), was added as well to the glands, high levels of eugenol biosynthesis were observed (Fig. 5). Substituting acetyl-CoA with p-coumaroyl-CoA, a compound that is known to occur in basil glands both as an intermediate in the pathway to coniferyl alcohol and as a substrate of acyltransferases responsible for producing p-coumarate esters (28), resulted in greatly reduced synthesis of eugenol.

These results indicated that either coniferyl acetate or some related ester is the substrate of EGS1 and IGS1 (Fig. 6A). We therefore chemically synthesized coniferyl acetate and used it in...
in vitro assays against both EGS1 and IGS1. In these in vitro systems, high rates of product formation were observed, indicating that coniferyl acetate can serve as a favorable substrate for EGS1 and IGS1. The \( K_m \) values of both EGS1 and IGS1 with coniferyl acetate (5.1 mM and 1.6 mM, respectively) are relatively high but are comparable to the \( K_m \) values of several other enzymes recently identified as involved in the synthesis of scent and flavor compounds (e.g., ref. 29).

Although coniferyl acetate has been observed to occur in a variety of plants from both monocot and dicot branches of the angiosperms (30, 31), we have not yet been able to detect the presence of coniferyl acetate and measure its concentration in the relevant tissues of basil or petunia, possibly because of the instability of this compound. It therefore remains to be determined whether coniferyl acetate or a similar acylated form of coniferyl alcohol is the actual substrate in planta. However, petunia flowers in which the expression of a flower-specific gene that encodes an enzyme capable of acylating coniferyl alcohol is suppressed do not synthesize isoeugenol (R. Dexter, N.D., E.P., and D. Clark, unpublished observations), and a homologous enzyme that acylates coniferyl alcohol has been identified in sweet basil glands (M. Kim and D.R.G., unpublished observations), providing some indirect evidence in support of coniferyl acetate as the true substrate. The observation that the purified EGS1 and IGS1 enzymes cannot use coniferyl alcohol directly as a substrate but that E. coli cells expressing EGS1 or IGS1 and administered coniferyl alcohol produce eugenol or isoeugenol, respectively, is also of interest. This finding suggests that coniferyl alcohol is acylated by an endogenous E. coli enzyme before it can serve as a substrate for EGS1 or IGS1.

Proposed Mechanisms of EGS1 and IGS1. Some enzymes in the PIP reductase family, to which EGS1 and IGS1 belong: have been quite well studied (21, 32). The PLR, PCBER, and IFR members of this family characterized to date have all been shown to be regio-specific and/or enantio-specific in their reductions (21, 32). The mechanism(s) envisaged for these reductions include direct reduction of the secondary ethers or reduction of the corresponding quinone methide intermediates. Interestingly, with the PIP reductions there is a free phenolic group in all of the substrates that can potentially assist in the formation of transient (enzyme-bound) quinone methide intermediates before reduction (21). However, when the free phenolic moiety of PLR is absent (such as in sesamin with PLR), the reduction of the substrate furanofuran rings does not occur (S. G. Moinuddin, K. W. Kim, C. H. Kang, L. B. Davin, and N.G.L., unpublished observations).

Thus, EGS1 and IGS1 may catalyze the reductive reaction by using a concerted mechanism, with simultaneous reduction and acetate elimination (Fig. 6B), or by forming (in the active site) the quinone methide intermediate before reduction (Fig. 6C). Indeed, we were also able to indirectly test the requirement for formation of a quinone methide intermediate by using several analogs lacking a free p-hydroxyl group, including cinnamyl acetate and 4-bromo-cinnamyl acetate. These potential substrates did not undergo an NADPH-dependent reduction with either EGS1 or IGS1. Furthermore, cinnamyl acetate did not serve as an inhibitor during the conversion of coniferyl acetate to eugenol when added to assays, possibly because of failure to bind to the active site. On the other hand, the 4-bromo-cinnamyl acetate, which is sterically and electronically similar to the coniferyl acetate, did bind and acted as an effective inhibitor while not undergoing any measurable reduction. Given its close structural similarity to coniferyl acetate, one would expect that if a concerted mechanism were used by EGS1 and IGS1, then the bromine-containing analog with the Br substituent in place of the p-hydroxyl moiety of EGS/IGS substrates would have been reduced. Therefore, these initial results also suggest the involvement of a quinone methide intermediate in the reactions catalyzed by these phenylpropene-producing enzymes. Further spectroscopic studies (e.g., under rapid mixing conditions) will be needed to unequivocally demonstrate the involvement of this quinone methide intermediate in the reactions catalyzed by EGS1, IGS1, and other enzymes in the related PIP family. Determination of the three-dimensional structure of the active sites of these proteins will also contribute to an understanding of the reaction mechanism. Finally, work is needed to establish how the related metabolites, chavicol and t-anol, are formed (35) and whether this reaction utilizes the same enzyme(s) and/or related PIP-like homologues.

Materials and Methods

Additional detailed experimental procedures can be found in Supporting Text, which is published as supporting information on the PNAS web site.

EST Collections and Isolation of EGS1 and IGS1 cDNAs. The construction of EST collections and the resultant sequence databases from the petals of petunia and the peltate glands of basil have been reported (1, 15). The sequence of the oxidoreductase expressed at a high level in the ODORANT1-suppressed line (16) was used to identify petunia and basil homologues by BLAST searches at https://sativa.biology.lsa.umich.edu/blast/blast.html. Full-length cDNAs of EGS1 and IGS1 were obtained from the EST databases and fully sequenced.

Vector Construction. The full-length EGS1 and IGS1 cDNAs were amplified by PCR and ligated to the pCRT7/CT-TOPO TA expression vector (Invitrogen). IGS1 and EGS1 cDNAs were also recombined with the Gateway-compatible, T7 expression vector pHIS9 (P. O’Maille, personal communication) through a pENTR (Invitrogen) intermediate to produce a protein with an N-terminal His\(_9\) tag.

Heterologous Protein Expression in E. coli and Purification. The above constructs were transformed into BL21 Codon Plus cells (Invitrogen), and liquid cultures of E. coli harboring EGS1 or IGS1 expression constructs were induced with 0.5 mM isopropylthio-\(\beta\)-galactoside and grown at 18°C for 15 h. Cells were then harvested by centrifugation, resuspended in lysis buffer, and purified by using a Ni-NTA affinity column as described (17). Final yield was 16.4 \(\mu\)g of EGS1 protein per milliliter of culture and 11.6 \(\mu\)g of IGS1 protein per milliliter of culture.

Extraction and Analysis of Organic Compounds in Spent Medium. For determination of organic compounds in spent medium, hexane was added to the medium, vortexed briefly, and centrifuged to separate the phases. The hexane layers were concentrated and analyzed by GC-MS.

Enzyme Assays. IGS1 and EGS1 activities were typically assayed by incubating 1 \(\mu\)l of the purified enzyme solution (\(\approx 2 \mu\)g of protein) in a final volume of 200 \(\mu\)l of assay buffer containing 50 mM Mes-KOH (pH 6.5), 1 mM NADPH, and 1 mM substrate. After incubation at 28°C for 30 min, the reactions were stopped by extraction with 1 ml of hexane. Linalool was added as an internal standard (2 \(\mu\)g), and the resulting extract was concentrated and analyzed by GC-MS.

GC-MS Analysis. The details of the GC-MS analyses are described in Supporting Text.

Gland Preparation. Leaf glandular trichomes were isolated from young leaves of basil (var. SW) following the procedure described (1). The glands were used for incubation experiments, or a crude protein extract was prepared from them as reported (13).
Protein Blot Analysis. Polyclonal antibodies to basil EGS1 were made by Cocalico Biologicals (Reamstown, PA) from E. coli-expressed, affinity-purified, and gel-extracted protein. Antigen-EGS1 antibodies were used at a 1:2,000 dilution and incubated with the protein gel blots for 1 h. All other conditions of the protein gel blots were carried out as reported (17).

RNA Blot Analysis. Total RNA was isolated from petunia floral tissues and leaves and analyzed as described (15). The coding region of the IGS1 was used as a probe.

Synthesis of Stable Isotope-Labeled and Nonlabeled p-Coumaroyl and Coniferyl Alcohols and Their Esters. The details of these syntheses are described in Supporting Text.

Basil Gland Feeding Experiments. Peltate glandular trichome secretory cells were isolated from cultivar SW and purified as described by using the gland storage buffer for protein activity assays (1). After the glands were allowed to settle on ice for ~15 min, they were suspended in a larger volume of gland suspension buffer (1) at a concentration so that 2.5 μl of the original settled glands would be diluted to 50 μl for each of the experiments. Experiments were performed in standard 1.7-ml polypropylene microfuge tubes. Combinations of NADPH (1 mM final), acetyl-CoA (1 mM final), p-coumaroyl-CoA (1 mM final), and [8,9,13C]-coniferyl alcohol (2 mM) were added (50-μl final volume) as appropriate for each treatment, and the glands were incubated at room temperature with shaking at 200 rpm in Orbit shaker model 3520 (Lab-Line Instruments, Melrose Park, IL) for the designated time. Incubations were terminated by addition of 3 μl of 6 M HCl and 50 μl of ethyl acetate, followed by vortexing and removal of the upper (organic) phase for GC-MS analysis (3 μl per injection). Controls included incubations lacking one or all of the cofactors or the [8,9,13C]-coniferyl alcohol. None of the controls produced [8,9,13C]eugenol.

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