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

Takao Koeduka*†, Eyal Fridman*†‡, David R. Gang†§, Daniel G. Vassão¶, Brenda L. Jackson§, Christine M. Kish‖, Irina Orlova‖, Snejina M. Spassova**, Norman G. Lewis¶, Joseph P. Noel**, Thomas J. Baiga**, Natalia Dudareva‖, and Eran Pichersky*††

*Department of Molecular, Cellular, and Developmental Biology, University of Michigan, 830 North University Street, Ann Arbor, MI 48109-1048; §Department of Plant Sciences and Institute for Biomedical Science and Biotechnology, University of Arizona, Tucson, AZ 85721-0036; ¶Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340; ‖Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907; and **Howard Hughes Medical Institute, Jack H. Skirball Chemical Biology and Proteomics Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037

Communicated by Anthony R. Cashmore, University of Pennsylvania, Philadelphia, PA, May 5, 2006 (received for review March 31, 2006)

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 phenylcoumaran benzylic ether reductase.

floral scent | phenylpropanoids | phenylpropenes | plant volatiles | secondary compounds

The major groups of angiosperms synthesize various phenylpropenes, which include molecules such as the allylphenols chavicol and eugenol and the propenylphenols *t*-anol and isoeugenol (Fig. 1) and their derivatives (1, 2). Some monomeric phenylpropenes, including methyleugenol and methylchavicol, have also been reported from gymnosperms (3–5). These monomeric compounds are easily volatilized, and some are toxic to insects and microbes. Eugenol, for example, is a general acting antimicrobial and antianimal toxin with analgesic properties for humans (6–9). Plants often produce and store phenylpropenes in their vegetative parts as a defense against herbivores and parasitic bacteria and fungi. Moreover, phenylpropenes are sometimes emitted from flowers to attract insect pollinators, which detect the highly diluted airborne volatiles through their antennae (10).

Because of the antimicrobial properties of the phenylpropenes as well as their pleasing aromas and flavors, humans, since antiquity, have used plant material containing phenylpropenes to preserve and flavor their food. In early human history, spices were often harvested and packaged in warmer climates possessing favorable conditions for plant growth. Because of the long distances over which spices were traded and their essential role as food preservatives for people living in temperate zones who depended almost exclusively on stored food to survive through the winter, spices commanded steep prices. The food-preserving and analgesic properties of eugenol-rich cloves lured explorers like Ferdinand Magellan and Christopher Columbus across the seas in search of new routes to the Far East, which was, at the time, the sole source of cloves (11). Although Columbus never reached India, he did discover the Americas and, incidentally, found the source for a new spice, allspice (*Pimenta dioica*, Myrtaceae), which contains the beneficial phenylpropene compound isoeugenol.

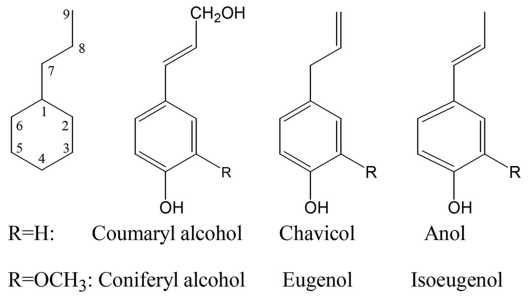

Fig. 1. Several phenylpropenes and their presumed phenylpropenol precursors. The carbon numbering system used in this study is also shown.

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

Conflict of interest statement: No conflicts declared.

Abbreviations: PLR, pinoresinol–lariciresinol reductase; IFR, isoflavone reductase; PCBER, phenylcoumaran benzylic ether reductase.

†T.K., E.F., and D.R.G. contributed equally to this work.

‡Present address: Smith Institute for Plant Sciences and Genetics, Faculty of Agriculture, Hebrew University, P.O. Box 12, Rehovot 76100, Israel.

††To whom correspondence should be addressed. E-mail: lelx@umich.edu.

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–lariciresinol reductase (PLR), isoflavone reductase (IFR), and phenylcoumaran benzylic 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 Phenylpropenes. 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 trichocorpa*, and 42.5% identical to PLR from *Thuja 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 identified a highly similar EST clone in the basil EST library. A full-length cDNA of this sequence, designated *EGS1* (eugenol synthase 1), encodes a 314-residue protein (molecular mass 35.6 kDa) that is 51.9% identical to IGS1 and is more closely related to it than to other members of the PIP family (Fig. 2).

The coding regions of *IGS1* and *EGS1* were each separately inserted into the *Escherichia coli* expression vectors pCRT7/CT-TOPO TA (without a His tag), and *E. coli* cells were transformed with these expression constructs. Cells were grown in LB medium supplemented with coniferyl alcohol at a concentration of 100 μg/ml. After induction with isopropylthio-β-galactoside, cells were allowed to grow for an additional 15-h period, after which the cells were isolated by centrifugation and the remaining culture medium (5 ml) was extracted one time with an equal volume of hexane. The spent medium of *E. coli* cells expressing basil *EGS1* contained eugenol (Fig. 3*A*) at a concentration of 7.1 μg/ml. Similarly, the spent medium of *E. coli* cells expressing petunia *IGS1* contained isoeugenol (Fig. 3*B*) at a concentration of 6.5 μg/ml. Control *E. coli* cells expressing an unrelated gene (*MKS1*) (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.

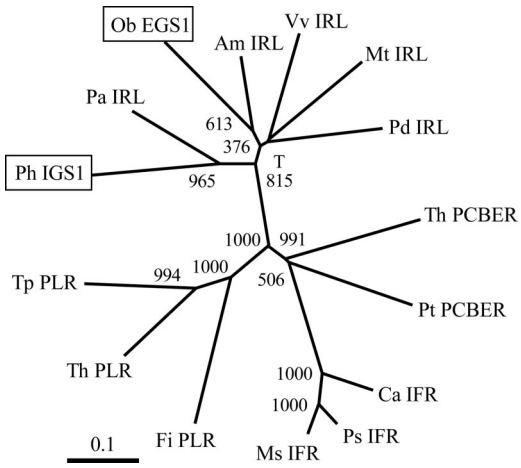

Fig. 2. IGS1 and EGS1 belong to the PIP family of reductases that includes PLR, IFR, and PCBER. Shown is an unrooted neighbor-joining phylogenetic tree of the protein sequences of basil EGS1 (Ob EGS1; GenBank accession no. DQ372812), petunia IGS1 (Ph IGS1; GenBank accession no. DQ372813), and related protein sequences from various plants. Proteins for which specific reductase activity has not been assigned have all been referred to as IRL (IFR-like). Am IRL is from *Antirrhinum majus* (snapdragon; GenBank accession no. AJ795463); Vv IRL is from *Vitis vinifera* (grapes; GenBank accession no. CB981554); Mt IRL is from *Medicago trancatula* (barrel medic; GenBank accession no. BF006345); Pd IRL is from *Populus deltoids* (eastern cottonwood; GenBank accession no. CV130547); Pa IRL is from *Persea americana* (avocado; GenBank accession no. CK755416); Th PCBER and Th PRL are from *Tsuga heterophylla* (western hemlock; GenBank accession nos. AAF64178 and AAF64184, respectively); Pt PCBER is from *Populus trichocarpa* (black cottonwood; GenBank accession no. CAA06707); Ca IFR is from *Cicer arietinum* (chickpea; GenBank accession no. Q00016); Ps IFR is from *Pisum sativum* (pea; GenBank accession no. P52576); Ms IFR is from *M. sativa* (alfalfa; GenBank accession no. AAC48976); Fi PLR is from *Forsythia intermedia* (border forsythia; GenBank accession no. AAC49608); Tp PLR is from *T. plicata* (red cedar; GenBank accession no. AAF63507). Sequence analysis was performed by using CLUSTAL X, and the nearest-joining method was used to create the phylogenetic tree. TREEVIEW was used to visualize the resulting tree. The scale indicates the average substitutions per site for each cladogram, and the numbers label the bootstrap value of each node (of 1,000 bootstrap trials). T, trichotomy.

***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. 4*A*). 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. 4*B*) where eugenol, chavicol, and their biosynthetic derivatives such as methyleugenol and methylchavicol are synthesized and stored (1).

Biosynthesis of Eugenol in Isolated Basil Glands. For the conversion of coniferyl alcohol to eugenol or isoeugenol to occur, the oxygenated moiety at C-9 must undergo cleavage. One possible way for this to occur would involve direct displacement of the hydroxyl group from coniferyl alcohol, although the latter is a

PLANT BIOLOGY

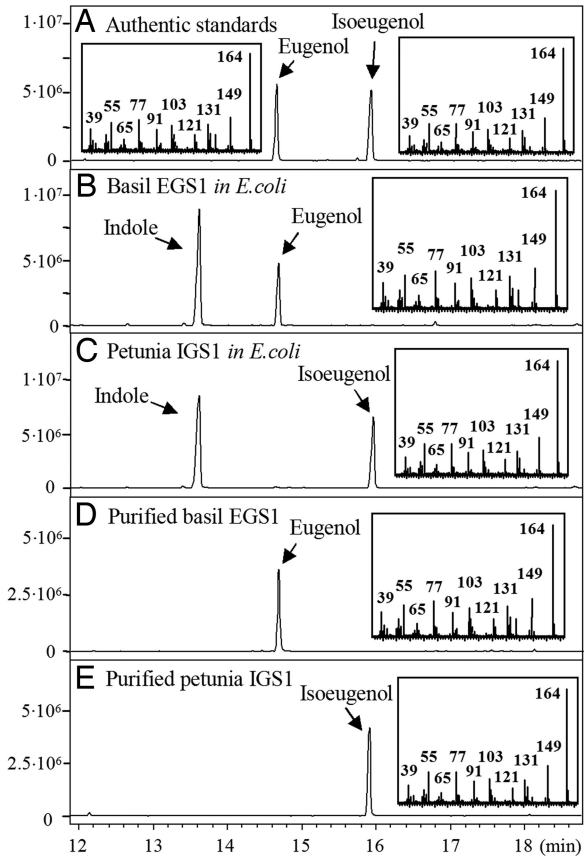

Fig. 3. *In vivo* and *in vitro* analyses of EGS1 or IGS1 activities. (*A*) GC-MS analyses of eugenol and isoeugenol standards. (*B*) GC-MS analysis of compounds found in the spent medium of *E. coli* cells expressing *EGS1* and grown in the presence of coniferyl alcohol. (*C*) GC-MS analysis of compounds found in the spent medium of *E. coli* cells expressing *IGS1* and cultured with coniferyl alcohol. (*D*) GC-MS analysis of compounds found in a reaction mixture containing purified EGS1, coniferyl acetate, and NADPH after 30 min of incubation. (*E*) GC-MS analysis of compounds found in a reaction mixture containing purified IGS1, coniferyl acetate, and NADPH after 30 min of incubation. All chromatograms are total ion chromatograms. Indole (labeled peak in *B* and *C*) is a volatile made by nontransformed as well as transformed *E. coli* cells during growth on standard media (33).

poor leaving group. However, in subsequent preliminary experiments we were not able to observe production of eugenol directly from coniferyl alcohol in *in vitro* assays using either purified EGS1 enzyme or production of isoeugenol using the IGS1 enzyme, nor was eugenol production observed in assays feeding [8,9-^{13}C]coniferyl alcohol to isolated basil glands (data

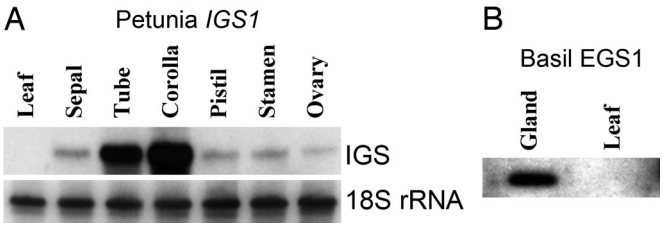

Fig. 4. Expression analyses of petunia IGS1 and basil EGS1. (*A Upper*) RNA gel blot probed with petunia *IGS1*. RNA samples (5 μg of total RNA per lane) from various flower tissues and leaves were analyzed. (*A Lower*) A duplicate blot probed with 18S rRNA as a control for equal loading. (*B*) A protein gel blot of total soluble protein extracts from leaves and purified glands (0.1 μg of protein per lane) probed with anti-EGS1 antibodies.

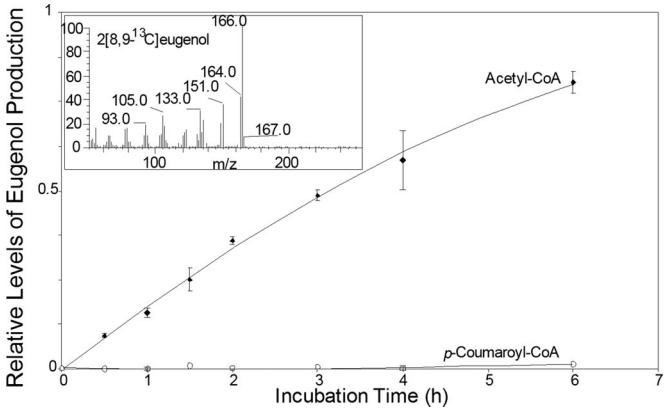

Fig. 5. Time course of the formation of [8,9-^{13}C]eugenol from [8,9-^{13}C]coniferyl alcohol in isolated glandular trichome secretory cells. Triplicate assays were performed with either acetyl-CoA or *p*-coumaroyl-CoA added to the trichomes in addition to the labeled coniferyl alcohol and NADPH. Relative levels of product formation were monitored by quantification of *m*/*z* 166 peak ([8,9-^{13}C]eugenol) in the GC-MS chromatogram. (*Inset*) Mass spectrum of eugenol peak from 6-h incubation. The molecular ion peak at *m*/*z* 164 is due to endogenous eugenol still present in the glandular trichomes of line SW after trichome isolation and purification.

not shown). Therefore, alternative mechanisms of eugenol/isoeugenol formation were considered. Although dihydroconiferyl alcohol (double bond in side chain reduced) could potentially undergo dehydration to yield eugenol, no such conversion was observed when this potential substrate was supplied to isolated gland secretory cells (data not shown). A third possible mechanism involves displacement of an organic acid from an acylated alcohol intermediate with subsequent/concomitant reduction. To examine this hypothesis, we incubated intact isolated basil glandular trichome secretory cell clusters obtained from cultivar SW (rich in eugenol) with several different assay mixtures containing [8,9-^{13}C]coniferyl alcohol and additional cofactors. We have previously shown that these secretory cells are biochemically active after removal from the leaf surface (18, 19). Products made by the isolated glands in these assays were analyzed by GC-MS.

In these experiments, efficient conversion of [8,9-^{13}C] coniferyl alcohol to [8,9-^{13}C]eugenol was observed when NADPH and acetyl-CoA were supplied. On the other hand, little [8,9-^{13}C]eugenol was formed when *p*-coumaroyl-CoA replaced acetyl-CoA (Fig. 5). Controls lacking NADPH or either of the CoA esters produced no detectable [8,9-^{13}C] eugenol. In glands supplemented with acetyl-CoA, >6% of the total [8,9-^{13}C]coniferyl alcohol was converted into eugenol after only 6 h. Assays for extended time periods (24 h), however, did show limited production of [8,9-^{13}C]eugenol when *p*-coumaroyl-CoA was added, but the rate of production with this potential cofactor was ≈20-fold lower than with acetyl-CoA. These experiments were repeated with two other basil cultivars with similar results.

Biochemical Characterization of Petunia IGS1 and Basil EGS1. Because the labeling experiments with the isolated basil glands suggested that an esterified form of coniferyl alcohol served as the substrate of EGS1, we next purified IGS1 and EGS1 from *E. coli* overexpression lines by Ni-NTA affinity chromatography and tested the proteins for activity by using an *in vitro* assay. In reactions containing coniferyl acetate and NADPH, EGS1 readily catalyzed the formation of eugenol, and IGS1 catalyzed the formation of isoeugenol (Fig. 3 *D* and *E*). Consistent with the results of the basil gland labeling experiments, no product was obtained when coniferyl alcohol was substituted for coniferyl

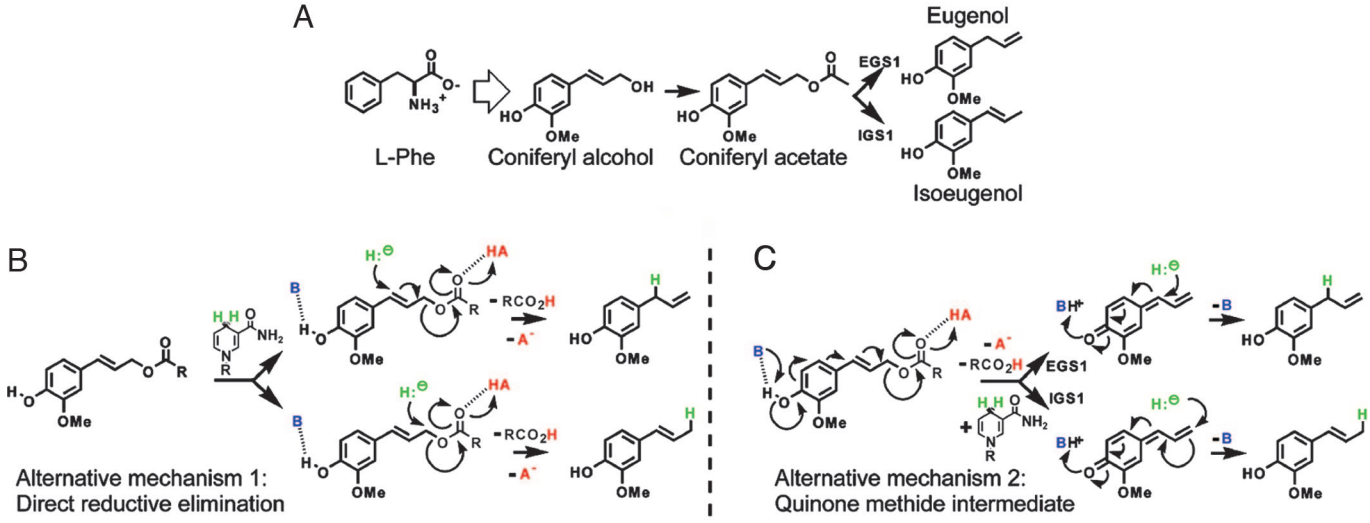

Fig. 6. Proposed biochemical pathway for the synthesis of eugenol and isoeugenol from phenylalanine and hypothetical reaction mechanisms for EGS1 and IGS1. (*A*) The multiple steps in the biosynthetic pathway from phenylalanine to coniferyl alcohol, an intermediate in the synthesis of lignin and lignans, were previously identified. (*B*) Hypothetical nucleophilic displacement mechanism, where reduction through hydride attack accompanies loss of an acyl leaving group possibly facilitated by stabilization of the negatively charged transition state of the acyl moiety through proton donation. B (in blue) represents a putative general base/hydrogen bond donor–acceptor hydrogen-bonded to the *p*-hydroxy group of coniferyl acetate. A (in red) represents a general acid/hydrogen bond donor–acceptor. (*C*) Hypothetical mechanism where formation of a quinone methide intermediate precedes reduction (34). It is not currently known which of the two hydrogens from the NADPH molecule (both shown in green) are transferred (in the form of a hydride) to the phenylpropanoid substrate. Hydrogen atoms not involved in the reaction are not shown.

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 Mg^{2+}, Ca^{2+}, Mn^{2+}, and Fe^{2+}), but Zn^{2+} and Cu^{2+} 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·s^{-1}·mg^{-1} protein (k_{cat} = 0.3 s^{-1}), and the corresponding value for EGS1 was 19.9 nmol·s^{-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 cinnamyl acetate, did not inhibit the enzyme, but 4-bromo-cinnamyl acetate 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 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 (isoflavonoids 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. 6*A*). 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 acetylating coniferyl alcohol is suppressed do not synthesize isoeugenol (R. Dexter, N.D., E.P., and D. Clark, unpublished observations), and a homologous enzyme that acetylates 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. 6*B*), or by forming (in the active site) the quinone methide intermediate before reduction (Fig. 6*C*). 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 spectrophotometric 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-β-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 μg of EGS1 protein per milliliter of culture and 11.6 μg 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 to 200 μl, and 3 μl was used for GC-MS analysis.

Enzyme Assays. IGS1 and EGS1 activities were typically assayed by incubating 1 μl of the purified enzyme solution ($\approx$2 μg of protein) in a final volume of 200 μl 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 μg), 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. Anti-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-^{13}C]coniferyl alcohol (2 mM) were added (56-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-^{13}C]coniferyl alcohol. None of the controls produced [8,9-^{13}C]eugenol.

This work was supported by National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service Grants 2001-35318-10006 and 2004-35318-14874 (to E.P.), 2005-35318-16207 (to N.D.), and ARZT-329100-G-25-532 (to D.R.G.); a grant from the Fred Gloeckner Foundation (to N.D.); National Science Foundation Grants 0210170 (to D.R.G.), 0236027 (to J.P.N.), and 0417291 (to N.G.L.); and National Institutes of Health Grant 5-R01-GM-066173-02 (to N.G.L.). E.F. was supported in part by Binational Agricultural Research and Development Fund Postdoctoral Fellowship Grant FI-328-2002. J.P.N. is an Investigator of the Howard Hughes Medical Institute.

1. Gang, D. R., Wang, J., Dudareva, N., Nam, K. H., Simon, J. E., Lewinsohn, E. & Pichersky, E. (2001) *Plant Physiol.* **125,** 539–555.
2. Moinuddin, S. G., Hishiyama, S., Cho, M. H., Davin, L. B. & Lewis, N. G. (2003) *Org. Biomol. Chem.* **1,** 2307–2313.
3. Brophy, J. J., Goldsack, R. G. & Rosenfelds, A. C. (2003) *J. Essent. Oil Res.* **15,** 217–220.
4. Huber, D. P. W., Gries, R., Borden, J. H. & Pierce, H. D. (2000) *Chemoecology* **10,** 103–113.
5. Krauze-Baranowska, M., Mardarowicz, M., Wiwart, M., Poblocka, L. & Dynowska, M. (2002) *Z. Naturforsch., C* **57,** 478–482.
6. Ahmed, M., Amin, S., Islam, M., Takahashi, M., Okuyama, E. & Hossain, C. F. (2000) *Pharmazie* **55,** 314–316.
7. Karapinar, M. (1990) *Int. J. Food Microbiol.* **10,** 193–200.
8. Moleyar, V. & Narasimham, P. (1992) *Int. J. Food Microbiol.* **16,** 337–342.
9. Sangwan, N., Vermin, B., Verma, K. & Dhindsa, K. (1990) *Pestic. Sci.* **28,** 331–335.
10. Raguso, R. A., Light, D. M. & Pichersky, E. (1996) *J. Chem. Ecol.* **22,** 1735–1766.
11. Le Couteur, P. M. & Burreson, J. (2004) in *Napoleon's Buttons*, ed. Tarcher, J. P. (Penguin, New York), pp. 9–35.
12. Klischies, M., Stockigt, J. & Zenk, M. H. (1975) *Chem. Commun.* **21,** 879–880.
13. Iijima, Y., Gang, D. R., Fridman, E., Lewinsohn, E. & Pichersky, E. (2004) *Plant Physiol.* **134,** 370–379.
14. Verdonk, J. C., Ric de Vos, C. H., Verhoeven, H. A., Haring, M. A., van Tunen, A. J. & Schuurink, R. C. (2003) *Phytochemistry* **62,** 997–1008.
15. Boatright, J., Negre, F., Chen, X., Kish, C. M., Wood, B., Peel, G., Orlova, I., Gang, D. R., Rhodes, D. & Dudareva, N. (2004) *Plant Physiol.* **135,** 1993–2011.
16. Verdonk, J. C., Haring, M. A., van Tunen, A. J. & Schuurink, R. C. (2005) *Plant Cell* **17,** 1612–1624.
17. Fridman, E., Wang, J., Iijima, Y., Froehlich, J. E., Gang, D. R., Ohlrogge, J. & Pichersky, E. (2005) *Plant Cell* **17,** 1252–1267.
18. Gang, D. R., Lavid, N., Zubieta, C., Chen, F., Beuerle, T., Lewinsohn, E., Noel, J. P. & Pichersky, E. (2002) *Plant Cell* **14,** 505–519.
19. Gang, D. R., Beuerle, T., Ullmann, P., Werck-Reichhart, D. & Pichersky, E. (2002) *Plant Physiol.* **130,** 1536–1544.
20. Dinkova-Kostova, A. T., Gang, D. R., Davin, L. B., Bedgar, D. L., Chu, A. & Lewis, N. G. (1996) *J. Biol. Chem.* **271,** 29473–29482.
21. Gang, D. R., Kasahara, H., Xia, Z. Q., Vander Mijnsbrugge, K., Bauw, G., Boerjan, W., Van Montagu, M., Davin, L. B. & Lewis, N. G. (1999) *J. Biol. Chem.* **274,** 7516–7527.
22. Fujita, M., Gang, D. R., Davin, L. B. & Lewis, N. G. (1999) *J. Biol. Chem.* **274,** 618–627.
23. Gang, D. R., Dinkova-Kostova, A. T., Davin, L. B. & Lewis, N. G. (1997) in *Phytochemical Pest Control Agents*, ed. Hedin, P. (Am. Chem. Soc., Washington, DC), pp. 58–89.
24. Raguso, R. A. & Pichersky, E. (1995) *Plant Syst. Evol.* **194,** 55–67.
25. Wang, J., Dudareva, N., Bhakta, S., Raguso, R. A. & Pichersky, E. (1997) *Plant Physiol.* **114,** 213–221.
26. Gershenzon, J., McCaskill, D., Rajaonarivony, J. I., Mihaliak, C., Karp, F. & Croteau, R. (1992) *Anal. Biochem.* **200,** 130–138.
27. Dudareva, N., D'Auria, J. C., Nam, K. H., Raguso, R. A. & Pichersky, E. (1998) *Plant J.* **14,** 297–304.
28. St-Pierre, B. & De Luca, V. (2000) in *Recent Advances in Phytochemistry Evolution of Metabolic Pathways*, eds. John, R. I., Romeo, T., Varin, L. & De Luca, V. (Elsevier Science, Oxford), pp. 285–315.
29. Wein, M., Lavid, N., Lunkenbein, S., Lewinsohn, E., Schwab, W. & Kaldenhoff, R. (2002) *Plant J.* **31,** 755–765.
30. del Rio, J. C., Gutiérrez, A. & Martinez, A. T. (2004) *Rapid Commun. Mass Spectrom.* **18,** 1181–1185.
31. Zdero, C., Bohlmann, F. & King, R. M. (1992) *Phytochemistry* **31,** 1703–1711.
32. Min, T., Kasahara, H., Bedgar, D. L., Youn, B., Lawrence, P. K., Gang, D. R., Halls, S. C., Park, H., Hilsenbeck, J. L., Davin, L. B., *et al*. (2003) *J. Biol. Chem.* **278,** 50714–50723.
33. Ross, J. R., Nam, K. H., D'Auria, J. C. & Pichersky, E. (1999) *Arch. Biochem. Biophys.* **367,** 9–16.
34. Chu, A., Dinkova, A., Davin, L. B., Bedgar, D. L. & Lewis, N. G. (1993) *J. Biol. Chem.* **268,** 27026–27033.
35. Vassão, D. G., Gang, D. R., Koeduka, T., Jackson, B., Pichersky, E., Davin, L. B. & Lewis, N. G. (2006) *Org. Biomol. Chem.*, 10.1039/b605407b.