Insect Biochemistry and Molecular Biology 186 (2026) 104441

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Independent evolution of geraniol-8-hydroxylase activity involved in iridoid formation in the Argentine ant (*Linepithema humile*)

Maithili Datta [a], Katrin Luck [a], Song Wu [a], Feng Chen [b], Yuko E. Ulrich [c], Sarah E. O'Connor [a,*], Tobias G. Köllner [a,**]

[a] Max Planck Institute for Chemical Ecology, Department of Natural Product Biosynthesis, 07745, Jena, Germany
[b] Department of Plant Sciences, University of Tennessee, TN, 37996, USA
[c] Max Planck Institute for Chemical Ecology, Independent Research Group Social Behavior, 07745, Jena, Germany

ARTICLE INFO

Keywords:
Iridoids
Cytochrome P450
Geraniol-8-hydroxylase
Evolution
Biosynthesis
Ants

ABSTRACT

Iridoids are bicyclic monoterpenoids that function as defense and signaling compounds in both plants and insects. Although recent research suggested that iridoids evolved independently in these two kingdoms of life, it remained unclear whether independent evolution of iridoid biosynthesis also occurred across different insect lineages. In order to gain insight into the evolution of iridoids in insects, we examined the first committed step of iridoid biosynthesis, the hydroxylation of geraniol to 8-hydroxygeraniol, in the Argentine ant (*Linepithema humile*) of the order Hymenoptera. This transformation is typically catalyzed by cytochrome P450 monooxygenases in both plants and insects. By integrating transcriptomic and metabolomic analyses across various body parts, we identified candidate P450 genes potentially involved in this reaction. The candidate genes were heterologously expressed in yeast, and enzyme activity was assessed by supplying geraniol to the yeast cultures. One candidate P450 enzyme displayed geraniol 8-hydroxylase (G8H) activity and was designated LhG8H. Phylogenetic analysis showed that LhG8H is evolutionarily distinct from previously characterized G8H enzymes in the pea aphid (*Acyrthosiphon pisum*) of the order Hemiptera and the mustard leaf beetle (*Phaedon cochleariae*) of the order Coleoptera. These results support the hypothesis that geraniol 8-hydroxylase activity, a key step in iridoid biosynthesis, evolved independently within the insect orders Hymenoptera, Hemiptera, and Coleoptera.

1. Introduction

Iridoids are a class of monoterpenoids defined by a characteristic cyclopentapyran (iridane) skeleton that are broadly distributed across both plants and insects. These compounds play important roles in chemical defense, mediating interspecific interactions, and supporting ecological adaptation (Beran et al., 2019). Iridoid biosynthesis involves a complex series of enzymatic transformations, starting from the universal monoterpenoid precursor geranyl pyrophosphate (GPP). GPP is converted to geraniol, which is then hydroxylated at C8 to produce the bis-alcohol 8-hydroxygeraniol. This intermediate is subsequently oxidized to the corresponding bis-aldehyde, 8-oxogeranial. Reduction of the C2–C3 double bond in 8-oxogeranial yields the reactive intermediate 8-oxocitronellyl enol/enolate, which can spontaneously cyclize to form iridodials or be further transformed, either enzymatically or non-enzymatically, into a variety of iridoids and iridoid-related compounds (Bergman et al., 2023; Thamm and Qu, 2016, Fig. 1).

Although the enzymes involved in iridoid biosynthesis have been extensively characterized in plants (Collu et al., 2001; Geu-Flores et al., 2012; Miettinen et al., 2014; Sherden et al., 2018), much less is known about their counterparts in insects. Recently, the complete iridoid biosynthetic pathway, including all associated enzymes, was elucidated in the pea aphid (*Acyrthosiphon pisum*) (Köllner et al., 2022). This study revealed that, despite the conservation of the underlying chemical transformations, the enzymes responsible for iridoid formation in plants and aphids are not homologous, supporting the hypothesis of independent evolutionary origins of iridoid biosynthesis in plants and insects. Additionally, the partial elucidation of the biosynthetic pathway leading to chrysomelidial, an iridoid-related monocyclic dialdehyde, in the mustard leaf beetle (*Phaedon cochleariae*) (Frick et al., 2013; Fu et al.,

* Corresponding author.
** Corresponding author.
E-mail addresses: mdatta@ice.mpg.de (M. Datta), kluck@ice.mpg.de (K. Luck), swu@ice.mpg.de (S. Wu), fengc@utk.edu (F. Chen), yulrich@ice.mpg.de (Y.E. Ulrich), oconnor@ice.mpg.de (S.E. O'Connor), koellner@ice.mpg.de (T.G. Köllner).

https://doi.org/10.1016/j.ibmb.2025.104441
Received 14 August 2025; Received in revised form 4 November 2025; Accepted 5 November 2025
Available online 6 November 2025

2019; Rahfeld et al., 2014) further suggests that iridoids may have evolved independently multiple times within insects.

The first committed step in the biosynthesis of iridoids and iridoid-related compounds is the hydroxylation of geraniol, a reaction typically catalyzed by cytochrome P450 monooxygenases in both plants and insects. P450s constitute a large and functionally diverse superfamily of heme-thiolate enzymes present across all domains of life, including archaea, bacteria, and eukaryotes (Nelson, 2018; Ngcobo et al., 2023). Although traditionally classified as cytochromes, these enzymes primarily function as monooxygenases, activating molecular oxygen and inserting one oxygen atom into their substrates (Rudolf et al., 2017). P450s are distinguished by their capacity to catalyze regio- and stereoselective oxidation reactions, which are essential for the biosynthesis and metabolism of a wide range of endogenous and xenobiotic compounds (Li et al., 2020). Cytochrome P450 enzymes that catalyze the hydroxylation of geraniol at the C8 position are referred to as geraniol 8-hydroxylases (G8Hs). While numerous G8Hs have been characterized in several plant species (e.g., *Catharanthus roseus* (Collu et al., 2001); *Swertia mussotii* (Wang et al., 2010); *Arabidopsis thaliana* (Höfer et al., 2013); *Lonicera japonica* (Zhang et al., 2025)), only two been identified in insects to date: ApG8H in *A. pisum* (Köllner et al., 2022) and PcG8H in *P. cochleariae* (Fu et al., 2019). Given the pivotal role of these enzymes in iridoid biosynthesis, characterization and phylogenetic analysis of G8H enzymes can provide valuable insights into the evolutionary trajectories of iridoid pathways across diverse biological lineages.

The term "iridoid" is a generic designation derived from the names of early-identified compounds, iridomyrmecin, iridodial, and iridolactone, originally found in the defensive secretions of ants from the genus *Iridomyrmex*. This ant genus is part of the Dolichoderinae subfamily and encompasses a highly diverse group of ants, many of which are ecologically dominant across various regions of the world (Ward et al., 2010). Due to their widespread presence and ecological success, Dolichoderines have long served as valuable models for investigating the chemical ecology of ants. One of the most extensively studied species within the Dolichoderinae subfamily is the Argentine ant, initially described as *Iridomyrmex humilis* and later reclassified as *Linepithema humile* following taxonomic revision (Shattuck, 1992). Native to regions of South America, particularly Brazil and Argentina, *L. humile* is now recognized as one of the most successful and widespread invasive ant species globally, having established populations in over 40 countries on

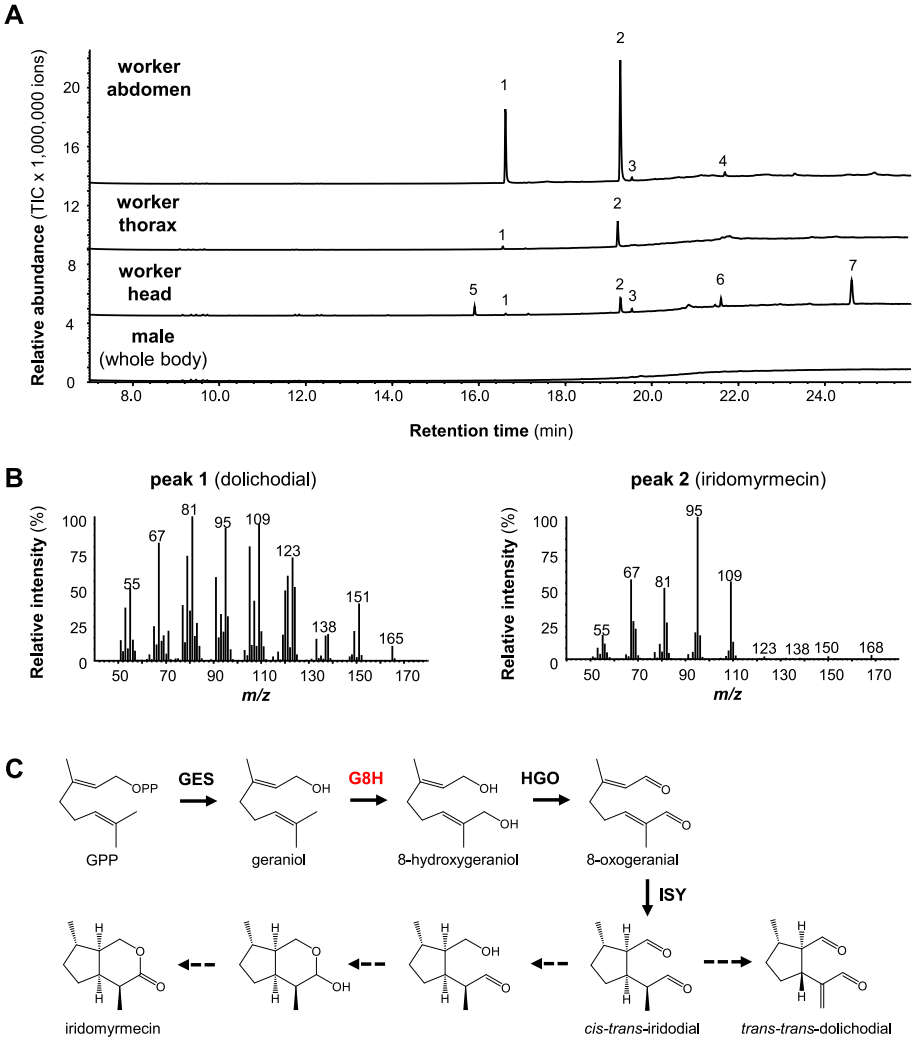

Fig. 1. Dolichodial and iridomyrmecin accumulate in the abdomen of *Linepithema humile* workers. (**A**) Ants were dissected and the various body parts of the workers and the entire body of the males were extracted with hexane. Extracts were analyzed using gas chromatography-mass spectrometry (GC-MS). Total ion chromatograms (TIC) are shown. 1, dolichodial; 2, iridomyrmecin; 3, heptadecane; 4, (*Z*)-7-hexadecenal; 5, 3-isopentyl-2,5-dimethylpyrazine; 6, 9-nonadecene; 7, (*Z*)-2,5-dimethyl-3-styryl-pyrazine. Compounds have been tentatively identified using the National Institute of Standards and Technology 3.0 mass spec library. (**B**) Mass spectra of peaks 1 (dolichodial) and 2 (iridomyrmecin). (**C**) Proposed pathway for the formation of dolichodial and iridomyrmecin in *L. humile*. GES, geraniol synthase; G8H, geraniol-8-hydroxylase; HGO, hydroxygeraniol oxidase; ISY, iridoid synthase. The enzyme identified in this study, G8H, is shown in red. Dotted arrows indicate reactions not yet been reported in the literature.

six continents (Angulo et al., 2024; Wetterer et al., 2009; Wild, 2007). Like many invasive ants, *L. humile* displays secondary polygyny and can establish large colonies consisting of millions of workers, often distributed across multiple interconnected nests. As omnivorous insects, Argentine ants feed on a variety of prey, including other insects and small vertebrates, while also collecting plant material, nectar, and insect honeydew (Angulo et al., 2024). Outside their natural habitat, *L. humile* invades both urban and agricultural environments, where they aggressively displace native ant species, disrupting local community structures and ecosystem functions. Moreover, as a major household and crop pest, Argentine ants impose substantial economic costs and are difficult to manage due to their flexible nesting habits, seasonal polydomy, and rapid reinvasion after disturbance (Angulo et al., 2024). These characteristics, coupled with their unique chemical communication systems and versatile foraging strategies, make *L. humile* an excellent model for studying invasion biology, social organization, and chemical ecology in ant societies (Angulo et al., 2024). Chemical analyses of *L. humile* workers have revealed that they produce substantial amounts of iridomyrmecin, along with the iridoid-related compound *trans,trans*-dolichodial (Pavan, 1949; Fusco et al., 1955; Cavill et al., 1976). Both of these compounds have been identified as defensive allomones (Welzel et al., 2018) and are also known to be components of the trail pheromone in this species (Choe et al., 2012).

In this study, we report the identification of an enzyme with geraniol 8-hydroxylase activity from *L. humile*. Using transcriptomic data, we identified candidate P450 genes expressed in the abdomen, the primary site of iridoid biosynthesis, and characterized the enzyme activity through heterologous expression in yeast. Enzyme function was assessed by substrate feeding, followed by product analysis using gas chromatography-mass spectrometry (GC-MS). Phylogenetic analysis was performed to understand the evolutionary relatedness of G8Hs from different insects. Our findings provide new insights into the evolution of geraniol 8-hydroxylase activity in insects.

2. Materials and methods

2.1. Collection and extraction of L. humile

Workers and winged males of *L. humile* were collected on the campus of the University of Tennessee, Knoxville, in summer 2023. Heads, thoraces, and abdomens from 10 ants were separately pooled and extracted with 100 μL hexane for 2 h. One μL of the hexane extracts was analyzed by gas chromatography-mass spectrometry (GC-MS) as described below.

2.2. Transcriptome sequencing and gene identification

For RNA extraction from *L. humile* workers, 20 heads, 20 thoraces, and 20 abdomens were dissected and pooled. Three independent samples were prepared for every body part. Since only a limited number of winged males were found, we only prepared two samples, each containing three whole ants. The twelve samples were each supplemented with 1 mL of TRIzol reagent and three stainless steel beads, then homogenized using a TissueLyser II (Qiagen) for three cycles of 2 min each. Total RNA was extracted using the Qiagen RNeasy Mini Kit following the manufacturer's protocol, eluted in 20 μL of RNase-free water, and sent to Novogene for RNA-seq library preparation (poly-A enrichment) and sequencing (NovaSeq PE150 platform; paired-end reads; 6 GB of raw data per sample). The resulting reads were quality-trimmed and mapped to the *L. humile* reference transcript set version 1.2 (Gene bank accession number, GCF_040581485.1-RS_2024_08) using CLC Genomics Workbench (Qiagen Bioinformatics) (Mapping parameters: mismatch cost, 2; insertion cost, 3; deletion cost, 3; length fraction, 0.8; similarity fraction, 0.9; maximum number of hits, 20). Empirical analysis of digital gene expression (EDGE) implemented in the software CLC Genomics Workbench was used for gene expression analysis. P-Values are given as false discovery rate (FDR)-adjusted values. To identify cytochrome P450 genes potentially involved in iridoid biosynthesis, we first selected those genes annotated as such. We further refined this list by selecting genes expressed at least tenfold more in the abdomen, the site of iridoid production, than in any other tissue, with a minimum expression threshold of 10 RPKM. This approach identified five potential candidate genes, *P450-2* through *P450*-6. However, when these genes were expressed in yeast, the resulting proteins did not exhibit G8H activity. Consequently, we broadened the search criteria to consider a less stringent fold change of around two between the abdomen and other tissues. This resulted in the identification of three additional genes (*P450*-7 to *P450*-9). One of these genes (*P450*-9) was subsequently characterized as LhG8H. RNAseq data were stored in the EDMOND data archive of the Max Planck Society and are available at https://doi.org/10.17617/3.ACNJA0.

2.3. Gene amplification and cloning

The complete open reading frames (ORFs) of the 8 P450 candidate genes identified as described above as well as a gene annotated as P450 reductase (LhRed) were amplified from cDNA prepared from the abdominal tissue of *L. humile* worker ants using the primers shown in Supplemental Table 1. cDNA synthesis was performed using 1 μg of total RNA, which was first treated with ezDNase (Thermo Fisher Scientific) to eliminate genomic DNA contamination. Reverse transcription was carried out using SuperScript IV reverse transcriptase following the manufacturer's protocol (Thermo Fisher Scientific). ORFs were subsequently cloned into the pESC-Leu-2d yeast expression vector along with the P450 reductase gene *LhRed* using two distinct multiple cloning sites that enabled the co-expression of both the respective P450 gene candidate and *LhRed*. Gene sequences amplified from cDNA are given in supplemental dataset 1. The sequences of LhG8H and LhRed can be found in the National Center for Biotechnology Information (NCBI) reference nucleotide sequence database under the accessions XM_012376963.2 and XM_012375434.2, respectively.

2.4. Heterologous expression of P450 candidates in Saccharomyces cerevisiae

Expression constructs were transferred into the *Saccaromyces cerevisiae* strain INVSc1 (Thermo Fisher) using the S.c. EasyComp Transformation Kit (Invitrogen) according to the manufacturer's instructions. A starter culture of 30 mL Sc-Leu minimal medium (6.7 g/L yeast nitrogen base without amino acids, but with ammonium sulfate; 100 mg/L of each L-adenine, L-arginine, L-cysteine, L-lysine, L-threonine, L-tryptophan, and uracil; 50 mg/L of each L-aspartic acid, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-tyrosine, L-valine; 20 g/L d-glucose) was inoculated with single yeast colonies and grown overnight at 28 °C and 180 rpm. The main cultures were grown using 100 mL yeast peptone glucose agar (YPGA) (Glc) full medium (10 g/L yeast extract, 20 g/L bactopeptone, 74 mg/L adenine hemisulfate, 20 g/L d-glucose), inoculated with one-unit OD600 of the starter cultures and incubated under the same conditions for 30–35 h. The overnight cultures were centrifuged (5000×*g*, 16 °C, 5 min), followed by an induction of candidate gene expression by resuspending the cells in 100 mL YPGA (Gal) medium (see above, but including 20 g/L galactose instead of D-glucose) and grown for another 8h at 25 °C and 160 rpm.

2.5. In vivo assays

For measuring G8H activity, 500 μL of induced culture (in galactose) containing the cassette of *LhRed* along with each of the P450 candidate genes were grown for 8 h. After adding the substrate geraniol (1 mM, solved in dimethyl sulfoxide) and the cofactor NADPH (3 mM), the culture was incubated for 16 h at 28 °C and 180 rpm. Following incubation, each culture was overlaid with 100 μL of ethyl acetate and

shaken for an additional 1 h to facilitate the extraction of hydrophobic reaction products into the organic phase. To ensure efficient phase separation, samples were vortexed vigorously for 1 min, centrifuged at 3000 rpm to pellet any particulate debris, and subsequently flash-frozen in liquid nitrogen. The upper organic phase, containing the extracted enzyme products, was carefully collected by pipette. A 1 μL aliquot of this phase was then injected into the gas chromatography–mass spectrometry (GC-MS) system for analysis and identification of enzymatic products (see below for GC-MS conditions and settings).

2.6. Gas Chromatography–Mass spectrometry analysis

Hexane extracts of body parts and P450 enzyme products were analyzed using an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973 quadrupole mass selective detector (Agilent technologies). For compound separation, a Phenomenex ZB-5 column (30 m × 0.25 mm × 0.25 μm) was used. Samples (1 μL) were injected without split at an initial oven temperature of 60 °C. The temperature was held for 2 min and then increased to 250 °C with a gradient of 10 °C min^{-1}, and then further increased to 310 °C with a gradient of 100 °C min^{-1} and a hold of 2 min (carrier gas flow, 1.1 mL min^{-1}; solvent delay, 7 min). Compounds were identified by comparison of retention times and mass spectra to those of authentic standards or by comparison with reference spectra in the Wiley and National Institute of Standards and Technology libraries.

2.7. Phylogenetic analysis

Gene sequences annotated as cytochrome P450 genes were obtained from the genomes of *A. pisum*, *P. cochleariae*, and *L. humile*, all available in the NCBI genome database. Amino acid sequences longer than 450 residues were considered full-length and aligned using the MUSCLE algorithm (cost matrix, BLOSUM; gap open, −2.90; gap extend, 0.00; hydrophobicity multiplier, 1.20; cluster method, UPGMA) implemented in MEGA 11 (Tamura et al., 2021). Tree reconstruction was done with IQTREE (Minh et al., 2020) using a maximum likelihood algorithm (model/method; LG model; substitutions type, amino acids; rates among sites, uniform rates; gaps/missing data treatment, partial deletion; site coverage cutoff; 0.0). Bootstrap resampling analyses with 1000 replicates were performed to evaluate the tree's topologies.

2.8. Structure modelling

The three-dimensional structures of PcG8H, ApG8H, LhG8H, CrG8H (*Catharanthus roseus*), SmG8H (*Swertia mussotii*), LjG8H (*Lonicera japonica*), AtG8H (*Arabidopsis thaliana*), and three non-G8H clan 3 P450s including XP_001119981.4_Amel (*Apis mellifera*), ADL59603.1_Mper (*Myzus persicae*), and AFI45040.1_Dpon (*Dendroctonus ponderosae*) were predicted using AlphaFold3 (https://alphafoldserver.com). The top-ranked prediction ("model_0/ranked_0″) was selected for each protein. Then, structures were superposed in PyMOL v2.x (Schrödinger LLC, www.pymol.org) using a pairwise structural alignment.

3. Results

3.1. Iridomyrmecin and dolichodial accumulate in the abdomen of L. humile workers, but not in males

Previous studies have reported that iridomyrmecin and dolichodial accumulate exclusively in the abdomens of *L. humile* workers (Cavill and Houghton, 1974a). To confirm these results with our collected ants, we analyzed hexane extracts from their heads, thoraces, and abdomens using gas chromatography-mass spectrometry (GC-MS). Consistent with previous reports, our analysis revealed that both iridomyrmecin and dolichodial accumulated predominantly in the abdomen (Fig. 1). The small amounts detected in the head and thorax extracts are likely due to sectional cross-contamination caused by the release of the defensive secretion during dissection. Along with iridoids, we tentatively identified minor quantities of heptadecane and (*Z*)-9-hexadecenal, the latter previously proposed as a trail pheromone component in *L. humile* (Cavill et al., 1979; Choe et al., 2012), within the abdominal extracts (Supplemental Fig. 1). Supporting earlier findings (Cavill and Houghton, 1974b), two pyrazines, 5-(isopentyl)-2,3-dimethylpyrazine and (Z)-2, 5-dimethyl-3-styrylpyrazine, as well as 9-nonadecene, were found exclusively in the head region (Fig. 1A; Supplemental Fig. 1). Notably, iridoids were not detectable in whole-body extracts of winged males (Fig. 1A).

3.2. Identification of G8H candidate genes

To identify G8H candidates, we sequenced the transcriptomes of heads, thoraces, and abdomens of worker ants, as well as the whole-body transcriptome of winged males. We then searched for cytochrome P450 genes that were predominantly expressed in the abdomen of worker ants, the presumed site of iridoid biosynthesis. In addition to five genes that showed near-exclusive expression in the worker abdomen, three others with markedly higher expression in the abdomen relative to other body regions were also selected for further analysis due to their overall high expression levels (Table 1; Supplemental Fig. 2). A putative P450 reductase gene, designated *LhRed*, also exhibited elevated expression in the abdomen and was chosen as the redox partner for in vivo functional characterization of the candidate G8H genes.

3.3. Characterization of LhG8H in vivo

To functionally characterize the eight candidate P450 enzymes, each gene was individually co-expressed with the cytochrome P450 reductase gene *LhRed* in *S. cerevisiae*. The resulting yeast strains were fed with geraniol and NADPH as a substrate, and the resulting products were extracted using ethyl acetate and analyzed by GC-MS. Among the tested P450s, only one exhibited catalytic activity with geraniol, producing (*E*, *E*)-8-hydroxygeraniol, and was therefore named LhG8H (Fig. 2, Supplemental Fig. 3). In addition to (*E,E*)-8-hydroxygeraniol, LhG8H also produced minor amounts of an unidentified monoterpenoid, likely an isomer of 8-hydroxygeraniol, as well as a monoterpenoid tentatively identified as 8-hydroxycitronellol. No LhG8H-related products were detected in yeast cultures expressing only LhRed or harboring an empty vector. Aside from LhG8H, none of the other candidate enzymes showed activity under the tested conditions. Notably, citronellol, citronellyl acetate, geranic acid, and geranyl acetate were detected in all yeast cultures supplemented with geraniol, regardless of *G8H* expression (Fig. 2), indicating that endogenous yeast enzymes can convert geraniol into these compounds. A multiple sequence alignment of LhG8H and other characterized insect and plant G8Hs revealed that LhG8H possesses all the structural motifs required for cytochrome P450 activity, such as the A-helix, the K-helix, the PERF motif, and the heme-binding loop with the highly conserved cysteine residue (Fig. 3). In addition, structural alignments showed that insect and plant G8H proteins all possess the highly conserved P450 fold (Supplemental Fig. 4, Supplemental Table 2).

3.4. Phylogenetic analysis of LhG8H with known insect G8Hs

G8H enzymes have previously been identified in only two insect species: *A. pisum* (Köllner et al., 2022) and *P. cochleariae* (Fu et al., 2019). To explore the evolutionary context of *L. humile* LhG8H, we performed a phylogenetic analysis using full-length cytochrome P450 sequences from these two species, along with all annotated full-length P450s from *L. humile*. The resulting Maximum Likelihood tree (Fig. 4, Supplemental Figs. 5–7) grouped the P450s from *A. pisum*, *P. cochleariae*, and *L. humile* into four major clans: clan 2, clan 3, clan 4, and the mitochondrial clan (clan MITO). Clan 3 contained the largest number of P450s, followed by clans 4, 2, and MITO. While clans 2, 4, and MITO

Table 1
Expression of candidate P450 genes in *Linepithema humile*. The expression data are shown as mean RPKM values obtained by RNA-seq. The mean values for the workers were obtained from three replicates of 20 individuals each, and the mean values for the males were obtained from two replicates of three individuals each.

Gene ID	Locus	Name	Worker (head)	Worker (thorax)	Worker (abdomen)	Male (whole body)
XM_012375434.1	LOC105677070	LhRed	16.1	12.6	31.0	29.1
XM_012369097.1	LOC105673453	P450-2	0.2	0.2	33.8	0.5
XM_012361707.1	LOC105668957	P450-3	0.1	0.3	15.2	0.3
XM_012364418.1	LOC105670731	P450-4	0.2	1.7	67.8	0.4
XM_012367457.1	LOC105672486	P450-5	0.9	0.2	25.4	2.1
XM_012375165.1	LOC105676933	P450-6	4.0	0.2	65.3	6.3
XM_012375356.1	LOC105677043	P450-7	23.6	11.7	130.7	54.7
XM_012369082.1	LOC105673448	P450-8	18.6	36.7	74.0	19.4
XM_012376963.1	LOC105678007	LhG8H	57.2	48.4	101.5	19.9

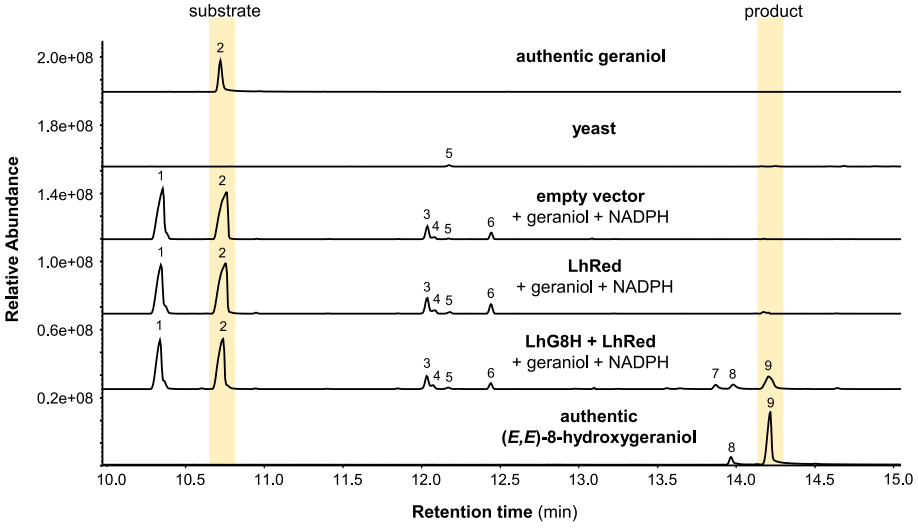

Fig. 2. Characterization of LhG8H. Candidate P450 genes were co-expressed with the P450 reductase gene *LhRed* in *Saccharomyces cerevisiae* and the yeast cells were supplemented with geraniol as substrate. Reaction products were extracted with ethyl acetate and analyzed using gas chromatography-mass spectrometry (GC-MS). Total ion chromatograms for LhG8H, the negative controls, and the authentic standards geraniol and (*E,E*)-8-hydroxygeraniol are shown. 1, citronellol*; 2, geraniol*; 3, citronellyl acetate; 4, geranic acid; 5, n-decanoic acid; 6, geranyl acetate; 7, 8-hydroxycitronellol; 8, unidentified monoterpenoid; 9, (*E,E*)-8-hydroxygeraniol*. Compounds marked with an asterisk were identified using authentic standards. All other compounds have been tentatively identified using the National Institute of Standards and Technology 3.0 mass spec library.

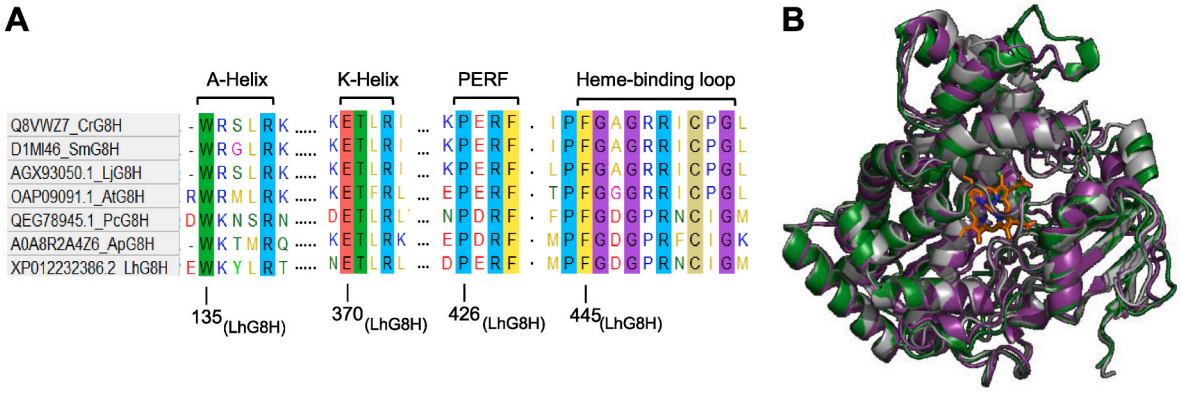

Fig. 3. Conserved motifs and domains of G8Hs from *Linepithema humile* (LhG8H), *Acyrthosiphon pisum* (ApG8H), *Phaeton cochleariae* (PcG8H), and characterized G8Hs from plants. (A) Multiple sequence alignment of PcG8H, ApG8H, LhG8H, and characterized plant G8Hs. Conserved motifs and residues crucial for cytochrome P450 activity are indicated. CrG8H, *Catharanthus roseus*; SmG8H, *Swertia mussotii*; LjG8H, *Lonicera japonica*; AtG8H, *Arabidopsis thaliana*. Amino acids are numbered according to LhG8H. **(B)** Structural alignment of PcG8H (grey), ApG8H (green), and LhG8H (violet). The heme group in the active site is shown in orange.

shared a common branch, clan 3 formed a distinct lineage. Despite representing different insect orders (Hymenoptera, Hemiptera, and Coleoptera), P450s from all three species were distributed across all clans. Notably, the G8H enzymes from *A. pisum*, *P. cochleariae*, and *L. humile* clustered together within clan 3, suggesting a shared ancestral origin of their amino acid sequences. However, sequence identity between LhG8H and its counterparts was relatively low, 31.4 % with ApG8H and 31.9 % with PcG8H, while ApG8H and PcG8H shared a

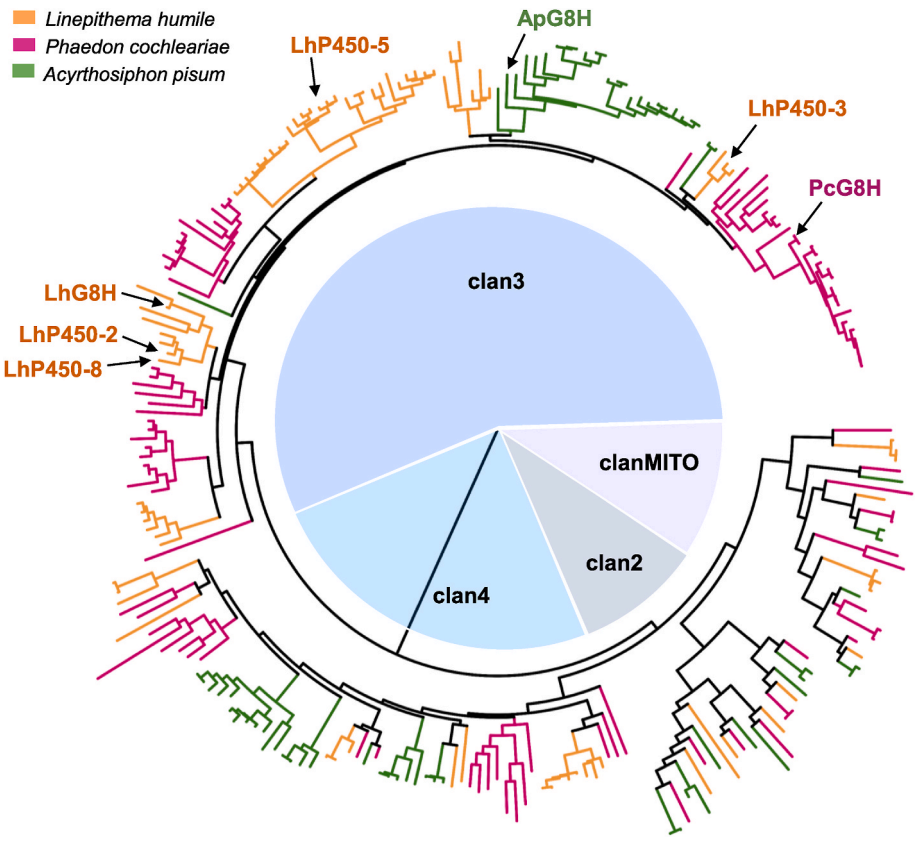

Fig. 4. Cladogram analysis of P450 proteins from *Acyrthosiphon pisum*, *Phaeton cochleariae*, and *Linepithema humile*. Full-length P450 gene sequences (n = 243) were retrieved from the genomes of each species, and the corresponding protein sequences were aligned. A phylogenetic tree was constructed using the Maximum Likelihood method. Known insect G8H enzymes are highlighted. Note, subtrees for the individual clans including accession numbers are given in Supplemental Material.

higher identity of 55.4 %. The LhG8H, ApG8H, and PcG8H sequences were consistently grouped with those of other P450 enzymes from the same species, while neighboring clades containing sequences from the other species did not include known G8Hs. Furthermore, LgG8H clustered with P450-2 and P450-8 (Supplemental Fig. 7), which both did not exhibit G8H activity in our assays (Supplemental Fig. 3). These observations suggest that G8H activity evolved independently within Coleoptera, Hymenoptera, and Hemiptera from different ancestral clan 3 P450 enzymes. Including functionally characterized clan 3 enzymes from other insects (information about these enzymes are given in Supplemental Table 3) in the phylogenetic analysis could further strengthen the potential scenario of parallel evolution of G8H activity. As shown in Supplemental Fig. 8, PcG8H grouped closer to CYP6DJ1 (AFI45040.1) from the mountain pine beetle (*Dendroctonus ponderosae*), a P450 involved in monoterpene detoxification (Chiu et al., 2019), than to G8Hs from *A. pisum* and *L. humile*. ApG8H, however, showed higher similarity to the nicotine-detoxifying CYP6CY3 (ADL59603.1; Bass et al., 2013) from the aphid *Myzus persicae* than to LhG8H or PcG8H (Supplemental Fig. 8). Moreover, the structural similarity among insect G8Hs was no greater than the similarity between insect G8Hs and other enzymes in the P450 clan 3 (Supplemental Fig. 4D, Supplemental Table 2), suggesting that there are no G8H-specific structural features.

4. Discussion

In this study, we identified LhG8H from the Argentine ant L. *humile*, a P450 enzyme that catalyzes the hydroxylation of geraniol, which is the first committed step of the iridoid pathway. Phylogenetic analysis revealed that all known insect G8H enzymes, LhG8H (this study), ApG8H (Köllner et al., 2022), and PcG8H (Fu et al., 2019), belong to the same P450 clan but show low sequence similarity and distinct evolutionary trajectories (Fig. 4, Supplemental Figs. 7 and 8). Despite catalyzing the same reaction, their divergence across Hymenoptera, Hemiptera, and Coleoptera suggests independent evolutionary origins. According to the classification by Weng and Noel (2013), this pattern fits "parallel evolution", where enzymes with the same fold and function arise from ancestors with different functions. This contrasts with "convergent evolution", where enzymes evolve similar activities despite having unrelated structures. Thus, the emergence of G8H activity in these three insect orders likely represents parallel evolution within the P450 family. Although still relatively rare, examples of independently evolved enzymes involved in natural product biosynthesis in insect and other Arthropodes are growing. Terpene synthases (TPSs) are among the best studied examples, having been shown to have repeatedly evolved from isoprenyl diphosphate synthases (IDS). In Coleoptera, Hemiptera, and Diptera, TPSs derive from farnesyl diphosphate synthases (Beran et al., 2016; Lancaster et al., 2018; Rebholz et al., 2023a, 2023b; Ducker et al., 2024), while in Lepidoptera, TPSs appeared to have evolved from geranylgeranyl diphosphate synthases (Darragh et al., 2021). These cases suggest that TPS activity also arose from parallel evolution from ancestral IDS enzymes. Another parallel evolutionary event is the biosynthesis of cyanogenic compounds in the burnet moth *Zygaena filipendulae* (Lepidoptera) and cyanogenic millipedes (Diplopoda). In both, a cytochrome P450 enzyme independently evolved to catalyze the formation of α-hydroxynitriles, the final step in cyanogenesis (Jensen et al., 2011; Yamaguchi et al., 2017). Together, these examples highlight the recurring emergence of similar enzymatic functions across divergent insect and arthropod lineages through parallel evolution.

Cytochrome P450 enzymes in insects constitute a large and functionally diverse superfamily of heme-thiolate proteins (Feyereisen,

1999). While these enzymes are widely recognized for their roles in xenobiotic detoxification, processing insecticides, plant allelochemicals, and other environmental toxins (Lu et al., 2021), many P450s are also critical for endogenous processes, such as the biosynthesis and degradation of ecdysteroids and juvenile hormones, which regulate development, molting, and reproduction (Daimon and Shinoda, 2013; Iga and Kataoka, 2012). This dual role in both detoxification and biosynthesis highlights the evolutionary versatility of insect P450s, allowing insects to adapt to chemically diverse environments. Geraniol, a monoterpenoid commonly found in essential oils, is known for its insecticidal and repellent effects across a wide range of insects, including mosquitoes, ticks, and cockroaches (Chen and Viljoen, 2010; Nasiou and Giannakou, 2018). Given its toxicity, it is plausible that some insects have evolved biochemical strategies to modify and detoxify geraniol, allowing them to tolerate or exploit environments dominated by geraniol-producing plants. Interestingly, all known insect G8Hs belong to P450 clan 3 (Fig. 4), which is primarily associated with xenobiotic detoxification (Feyereisen, 1999; Yu et al., 2015). Furthermore, our phylogenetic analysis revealed that PcG8H is closely related to CYP6DJ1, a P450 enzyme from the mountain pine beetle (*Dendroctonus ponderosae*) that detoxifies cyclic monoterpenes such as limonene and terpinolene by converting them into epoxides and alcohols (Chiu et al., 2019) (Supplemental Fig. 8). This raises the possibility that G8H activity in iridoid biosynthesis may have evolved from ancestral P450s originally involved in monoterpene detoxification. Notably, LhG8H shows highest expression in the abdomen, where iridoids are produced, but is also expressed in the thorax and head (Table 1; Supplemental Fig. 2). This suggests that LhG8H may play a role in both iridoid biosynthesis and terpene detoxification. In fact, such overlapping functions in terpene detoxification, pheromone biosynthesis, and even olfaction have already been discussed for P450 enzymes in pine beetles (Blomquist et al., 2021). Future research should investigate P450 enzymes closely related to G8Hs in the pea aphid, mustard leaf beetle, and Argentine ant to assess their potential roles in terpene hydroxylation.

All known iridoid pathways begin with the hydroxylation of geraniol to form 8-hydroxygeraniol (Fig. 1C). LhG8H catalyzes this reaction, which is the first committed step of iridoid biosynthesis. However, the downstream steps leading to iridomyrmecin and dolichodial, the primary iridoids found in *L. humile*, remain unresolved. We propose that 8-hydroxygeraniol is oxidized to 8-oxogeranial, which is in turn converted by an iridoid synthase into *cis-trans*-iridodial, which then undergoes a series of transformations: an enzyme-catalyzed reduction, spontaneous cyclization, and a final oxidation step to yield iridomyrmecin (Fig. 1C). A second, stereochemically distinct reduction of *cis-trans*-iridodial may lead to the formation of *trans-trans*-dolichodial. In plants, iridoid synthases are members of the short-chain dehydrogenase/reductase (SDR) family (Alagna et al., 2016; Geu-Flores et al., 2012) while in aphids, the iridoid synthase has been identified as a membrane-bound enzyme likely derived from a polyprenol reductase (Köllner et al., 2022). Either protein class could plausibly fulfill the role of iridoid synthase in *L. humile*. Similarly, candidate enzymes for the final oxidation and reduction steps may include SDRs or, as demonstrated in the oxidation of nepetalactol to nepetalactone in *A. pisum*, oxidases from the glucose-methanol-choline (GMC) family.

The patchy distribution of iridoids across insect lineages could, in principle, be explained by either independent evolution or the repeated loss of the biosynthetic pathway. Our results suggest that G8H activity likely arose independently through parallel evolution within the orders Hymenoptera, Hemiptera, and Coleoptera. However, it remains unclear whether all steps of this complex pathway share the same evolutionary history. Future work will build on the tools established in this study to identify and characterize the remaining enzymes involved in iridoid biosynthesis in *L. humile*, which will provide deeper insights into the evolutionary origins of this pathway in insects.

CRediT authorship contribution statement

Maithili Datta: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Katrin Luck:** Writing – review & editing, Methodology, Investigation. **Song Wu:** Writing – review & editing, Investigation. **Feng Chen:** Writing – review & editing, Investigation, Conceptualization. **Yuko E. Ulrich:** Writing – review & editing, Investigation, Conceptualization. **Sarah E. O'Connor:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Tobias G. Köllner:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Competing interest statement

The authors declare no competing interests.

Acknowledgements

We thank Maritta Kunert and Sarah Heinicke for help and assistance with GC-MS analysis. This work was supported by the Max Planck Society.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2025.104441.

Data availability

Data will be made available on request.

References

Alagna, F., Geu-Flores, F., Kries, H., Panara, F., Baldoni, L., et al., 2016. Identification and characterization of the iridoid synthase involved in oleuropein biosynthesis in olive (*Olea europaea*) fruits. J. Biol. Chem. 291 (11), 5542–5554.

Angulo, E., Guénard, B., Balzani, P., Bang, A., Frizzi, F., et al., 2024. The Argentine ant, *Linepithema humile*: natural history, ecology and impact of a successful invader. Entomol. Gen. 44 (1), 41–61.

Bass, C., Zimmer, C.T., Riveron, J.M., Wilding, C.S., Wondji, C.S., Kraussmann, M., Field, L.M., Williamson, M.S., Nauen, R., 2013. Gene amplification and microsatellite polymorphism underlie a recent insect host shift. Proc. Natl. Acad. Sci. U.S.A 110 (48), 19460–19465.

Beran, F., Köllner, T.G., Gershenzon, J., Tholl, D., 2019. Chemical convergence between plants and insects: biosynthetic origins and functions of common secondary metabolites. New Phytol. 223 (1), 52–67.

Beran, F., Rahfeld, P., Luck, K., Nagel, R., Vogel, H., et al., 2016. Novel family of terpene synthases evolved from *trans*-isoprenyl diphosphate synthases in a flea beetle. Proc. Natl. Acad. Sci. U.S.A 113 (11), 2922–2927.

Bergman, M.E., Franks, A.E., Phillips, M.A., 2023. Biosynthesis, natural distribution, and biological activities of acyclic monoterpenes and their derivatives. Phytochem. Rev. 22 (2), 361–384.

Blomquist, G.J., Tittger, C., MacLean, M., Keeling, C.I., 2021. Cytochromes P450: terpene detoxification and pheromone production in bark beetles. Curr. Opin. Insect Sci. 43, 97–102.

Cavill, G.W.K., Houghton, E., 1974a. Volatile constituents of the Argentine ant, *Iridomyrmex humilis*. J. Insect Physiol. 20, 2049–2059.

Cavill, Houghton, E., 1974b. Some pyrazine derivatives from the Argentine ant, *Iridomyrmex humilis*. Aust. J. Chem. 27 (4), 879–889.

Cavill, G.W.K., Houghton, E., McDonald, F.J., Williams, P.J., 1976. Isolation and characterization of dolichodial and related compounds from the Argentine ant, *Iridomyrmex humilis*. Insect Biochem. 6, 483–490.

Cavill, G.W.K., Robertson, P.L., Davies, N.W., 1979. An Argentine ant aggregation factor. Experientia 35, 989–990.

Chen, W., Viljoen, A.M., 2010. Geraniol - a review of a commercially important fragrance material. South Afr. J. Bot. 76 (4), 643–651.

Chiu, C.C., Keeling, C.I., Henderson, H.M., Bohlmann, j, 2019. Functions of mountain pine beetle cytochromes P450 CYP6DJ1, CYP6BW1 and CYP6BW3 in the oxidation of pine monoterpenes and diterpene resin acids. PLoS One 14 (5), e0216753.

Choe, D.H., Villafuerte, D.B., Tsutsui, N.D., 2012. Trail pheromone of the Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: formicidae). PLoS One 7 (9), e45016.

Collu, G., Unver, N., Peltenburg-Looman, A.M.G., Van der Heijden, R., Verpoorte, R., Memelink, J., 2001. Geraniol 10-hydroxylase, a cytochrome P450 enzyme involved in terpenoid indole alkaloid biosynthesis. FEBS Lett. 508 (2), 215–220.

Daimon, T., Shinoda, T., 2013. Function, diversity, and application of insect juvenile hormone epoxidases (CYP15). Biotechnol. Appl. Biochem. 60 (1), 82–91.

Darragh, K., Orteu, A., Black, D., Byers, K.J.R.P., Szczerbowski, D., et al., 2021. A novel terpene synthase controls differences in anti-aphrodisiac pheromone production between closely related *Heliconius* butterflies. PLoS Biol. 19 (1), e3001022.
Ducker, C., Baines, C., Guy, J., Euzébio Goulart Santana, A., Pickett, J.A., Oldham, N.J., 2024. A diterpene synthase from the sandfly *Lutzomyia longipalpis* produces the pheromone sobralene. Proc. Natl. Acad. Sci. U.S.A 121 (12), e2322453121.
Feyereisen, R., 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44, 507–533.
Frick, S., Nagel, R., Schmidt, A., Bodemann, R.R., Rahfeld, P., et al., 2013. Metal ions control product specificity of isoprenyl diphosphate synthases in the insect terpenoid pathway. Proc. Natl. Acad. Sci. U.S.A 110 (11), 4194–4199.
Fu, N., Yang, Z.L., Pauchet, Y., Paetz, C., Brandt, W., et al., 2019. A cytochrome P450 from the mustard leaf beetles hydroxylates geraniol, a key step in iridoid biosynthesis. Insect Biochem. Mol. Biol. 113, 103212.
Fusco, R., Tr, Va, 1955. Ricerche sull'iridomirmecina, l'insetticida naturale secreto dalla *Iridomyrmex humilis* Mayr. La Chimica e l'Industria. 37 (4), 251–259.
Geu-Flores, F., Sherden, N.H., Glenn, W.S., O'Connor, S.E., Courdavault, V., et al., 2012. An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis. Nature 492 (7427), 138–142.
Höfer, R., Dong, L., André, F., Ginglinger, J., Lugan, R., et al., 2013. Geraniol hydroxylase and hydroxygeraniol oxidase activities of the CYP76 family of cytochrome P450 enzymes and potential for engineering the early steps of the (seco) iridoid pathway. Metab. Eng. 20, 221–232.
Iga, M., Kataoka, H., 2012. Recent studies on insect hormone Metabolic pathways mediated by cytochrome P450 enzymes. Biol. Pharm. Bull. 35 (6), 838–843.
Jensen, N.B., Zagrobelny, M., Hjernø, K., Olsen, C.E., Houghton-Larsen, J., et al., 2011. Convergent evolution in biosynthesis of cyanogenic defence compounds in plants and insects. Nat. Commun. 2 (1), 273.
Köllner, T.G., David, A., Luck, K., Beran, F., Zhou, J.-J., et al., 2022. Biosynthesis of iridoid sex pheromones in aphids. Proc. Natl. Acad. Sci. U.S.A 119 (42), e2211254119.
Lancaster, J., Khrimian, A., Young, S., Lehner, B., Luck, K., et al., 2018. De novo formation of an aggregation pheromone precursor by an isoprenyl diphosphate synthase-related terpene synthase in the harlequin bug. Proc. Natl. Acad. Sci. U.S.A 115 (37), e8634–e8641.
Li, Z., Jiang, Y., Guengerich, F.P., Ma, L., Li, S., Zhang, W., 2020. Engineering cytochrome P450 enzyme systems for biomedical and biotechnological applications. J. Biol. Chem. 295 (3), 833–849.
Lu, K., Song, Y., Zeng, R., 2021. The role of cytochrome P450-mediated detoxification in insect adaptation to xenobiotics. Curr. Opin. Insect Sci. 43, 103–107.
Miettinen, K., Dong, L., Navrot, N., Schneider, T., Burlat, V., et al., 2014. The seco-iridoid pathway from *Catharanthus roseus*. Nat. Commun. 5, 3606.
Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., Haeseler, A., Lanfear, R., 2020. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37 (5), 1530–34.
Nasiou, E., Giannakou, I.O., 2018. Effect of geraniol, a plant-based alcohol monoterpene oil, against *Meloidogyne javanica*. Eur. J. Plant Pathol. 152 (3), 701–710.
Nelson, D.R., 2018. Cytochrome P450 diversity in the tree of life. Biochim. Biophys. Acta, Proteins Proteomics 1866 (1), 141–154.
Ngcobo, P.E., Nkosi, B.V.Z., Chen, W., Nelson, D.R., Syed, K., 2023. Evolution of cytochrome P450 enzymes and their redox partners in archaea. Int. J. Mol. Sci. 24 (4), 4161.
Pavan, M., 1949. Ricerche sugli antibiotica di origine animale. Ric. Sci. 19, 1011–1017.
Rahfeld, P., Kirsch, R., Kugel, S., Wielsch, N., Stock, M., et al., 2014. Independently recruited oxidases from the glucose-methanol-choline oxidoreductase family enabled chemical defences in leaf beetle larvae (subtribe *Chrysomelina*) to evolve. Proc. Biol. Sci. 281 (1788), 20140842.
Rebholz, Z., Lancaster, J., Larose, H., Khrimian, A., Luck, K., et al., 2023a. Ancient origin and conserved gene function in terpene pheromone and defense evolution of stink bugs and hemipteran insects. Insect Biochem. Mol. Biol. 152, 103879.
Rebholz, Z., Shewade, L., Kaler, K., Larose, H., Schubot, F., et al., 2023b. Emergence of terpene chemical communication in insects: evolutionary recruitment of isoprenoid metabolism. Protein Sci. 32 (5), 1–20.
Rudolf, J.D., Chang, C.Y., Ma, M., Shen, B., 2017. Cytochromes P450 for natural product biosynthesis in: *streptomyces*: sequence, structure, and function. Nat. Prod. Rep. 34 (9), 1141–1172.
Shattuck, S.O., 1992. Review of the dolichoderine ant genus *Iridomyrmex* mayr with descriptions of three new genera (Hymenoptera: formicidae). Aust. J. Entomol. 31 (1), 13–18.
Sherden, N.H., Lichman, B., Caputi, L., Zhao, D., Kamileen, M.O., et al., 2018. Identification of iridoid synthases from *Nepeta* species: iridoid cyclization does not determine nepetalactone stereochemistry. Phytochemistry 145, 48–56.
Tamura, K., Stecher, C., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38, 3022–3027.
Thamm, A.M.K., Qu, Y., 2016. Discovery and metabolic engineering of iridoid/secoiridoid and monoterpenoid indole alkaloid biosynthesis. Phytochem. Rev. 15 (3), 339–361.
Wang, J., Liu, Y., Cai, Y., Zhang, F., Xia, G., Xiang, F., 2010. Cloning and functional analysis of geraniol 10-hydroxylase, a cytochrome p450 from *Swertia mussotii* Franch. Biosci. Biotechnol. Biochem. 74 (8), 1583–1590.
Ward, P.S., Brady, S.G., Fisher, B.L., Schultz, T.R., 2010. Phylogeny and biogeography of dolichoderine ants: effects of data partitioning and relict taxa on historical inference. Syst. Biol. 59 (3), 342–362.
Welzel, K.F., Lee, S.H., Dossey, A.T., Chauhan, K.R., Choe, D.H., 2018. Verification of Argentine ant defensive compounds and their behavioral effects on heterospecific competitors and conspecific nestmates. Sci. Rep. 8 (1), 1477.
Weng, J.K., Noel, J.P., 2013. Chemodiversity in *Selaginella*: a reference system for parallel and convergent metabolic evolution in terrestrial plants. Front. Plant Sci. 4, 1–17.
Wetterer, J.K., Wild, A.L., Suarez, A.V., Roura-Pascual, N., Espadaler, X., 2009. Worldwide spread of the Argentine ant, *Linepithema humile* (Hymenoptera: formicidae). Myrmecol News 12, 187–194.
Wild, A.L., 2007. Taxonomic Revision of the Ant Genus *Linepithema* (Hymenoptera: Formicidae), 126. University of California *Publications in Entomology*, pp. 1–151.
Yamaguchi, T., Kuwahara, Y., Asano, Y., 2017. A novel cytochrome P450, CYP3201B1, is involved in (*R*)-mandelonitrile biosynthesis in a cyanogenic millipede. FEBS Open Bio 7 (3), 335–347.
Yu, L., Tang, W., He, W., Ma, X., Vasseur, L., et al., 2015. Characterization and expression of the cytochrome P450 gene family in diamondback moth, *Plutella xylostella* (L.). Sci. Rep. 5, 8952.
Zhang, S., Liu, Z., Li, J., Liu, Q., Zhang, Y., 2025. Cloning and functional verification of geraniol-10-hydroxylase gene in *Lonicera japonica*. PeerJ, e18832.