Novel family of terpene synthases evolved from *trans*-isoprenyl diphosphate synthases in a flea beetle

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Sesquiterpenes play important roles in insect communication, for example as pheromones. However, no sesquiterpene synthases, the enzymes involved in construction of the basic carbon skeleton, have been identified in insects to date. We investigated the biosynthesis of the sesquiterpene (6*R*,7*S*)-himachala-9,11-diene in the crucifer flea beetle *Phyllotreta striolata*, a compound previously identified as a male-produced aggregation pheromone in several *Phyllotreta* species. A (6*R*,7*S*)-himachala-9,11-diene–producing sesquiterpene synthase activity was detected in crude beetle protein extracts, but only when (*Z*,*E*)-farnesyl diphosphate [(*Z*,*E*)-FPP] was offered as a substrate. No sequences resembling sesquiterpene synthases from plants, fungi, or bacteria were found in the *P. striolata* transcriptome, but we identified nine divergent putative *trans*-isoprenyl diphosphate synthase (*trans*-IDS) transcripts. Four of these putative *trans*-IDSs exhibited terpene synthase (TPS) activity when heterologously expressed. Recombinant *Ps*TPS1 converted (*Z*,*E*)-FPP to (6*R*,7*S*)-himachala-9,11-diene and other sesquiterpenes observed in beetle extracts. RNAi-mediated knockdown of *Ps*TPS1 mRNA in *P. striolata* males led to reduced emission of aggregation pheromone, confirming a significant role of *Ps*TPS1 in pheromone biosynthesis. Two expressed enzymes showed genuine IDS activity, with *Ps*IDS1 synthesizing (*E*,*E*)-FPP, whereas *Ps*IDS3 produced neryl diphosphate, (*Z*,*Z*)-FPP, and (*Z*,*E*)-FPP. In a phylogenetic analysis, the *Ps*TPS enzymes and *Ps*IDS3 were clearly separated from a clade of known coleopteran *trans*-IDS enzymes including *Ps*IDS1 and *Ps*IDS2. However, the exon–intron structures of *IDS* and *TPS* genes in *P. striolata* are conserved, suggesting that this TPS gene family evolved from *trans-IDS* ancestors.

isoprenyl diphosphate synthase | sesquiterpene synthase | (6*R*,7*S*)-himachala-9,11-diene | aggregation pheromone | flea beetle

Terpenes play important roles in insect communication and defense, especially the C_{15} sesquiterpenes, which often act as sex, alarm, or aggregation pheromones or protection against enemies (1–3). To understand more about the biological function and evolution of sesquiterpenes in insects, it would be helpful to have more knowledge of their biosynthetic origins. Sesquiterpenes are biosynthesized from three C_5 isopentenoid units supplied by the mevalonate pathway, which are then joined sequentially via the action of enzymes known as *trans*-isoprenyl diphosphate synthases (*trans*-IDS) to produce the linear C_{15} intermediate (*E*,*E*)-farnesyl diphosphate (FPP). *Trans*-IDS enzymes have been identified and characterized in a number of insect species (4–10). The huge diversity of sesquiterpene carbon skeletons are formed from FPP in the next step by the catalysis of terpene synthases (TPSs). Numerous TPSs have been identified in plants, fungi, and bacteria based on sequence similarity (11–13). However, no homologs of known terpene synthases have been reported from available insect genomic and transcriptomic sequences (3, 7). A unique bifunctional enzyme producing the C_{10} intermediate geranyl diphosphate (GPP) as well as the linear monoterpene myrcene in the bark beetle *Ips pini* represents the only insect terpene synthase known to date (14).

The reactions catalyzed by TPSs involve the generation of a highly reactive carbocation intermediate, which can undergo a wide array of different cyclizations, hydride shifts, and other rearrangements. The reaction cascade is either initiated by a metal ion-dependent ionization of the diphosphate moiety or a protonation of the substrate, and can be terminated by proton abstraction or water addition (11, 13, 15). Because the reaction cascade may be branched and termination may occur at multiple levels, many TPSs are multiproduct enzymes forming complex mixtures of compounds (16–19). Moreover, some TPSs also accept multiple substrates to produce monoterpenes, sesquiterpenes, and diterpenes (11).

Among sesquiterpene-producing insects are several genera in the leaf beetle subfamily Galerucinae in which males emit species-specific volatile sesquiterpene blends comprised mainly of himachalene-type compounds (20–23). In the flea beetles *Phyllotreta cruciferae*, *Phyllotreta striolata*, and *Phyllotreta vittula*, (6*R*,7*S*)-himachala-9,11-diene is a major male-produced sesquiterpene, and this compound was shown to be a key aggregation pheromone component (23–25).

Significance

Whether insect sesquiterpenes are synthesized de novo, derived from plant precursors, or produced by symbionts is often unknown. We identified an evolutionarily novel terpene synthase gene family in the striped flea beetle, a notorious pest of *Brassica* crops in North America and Asia, and one of these genes was shown to be directly involved in the biosynthesis of the male-specific sesquiterpene aggregation pheromone. Phylogenetic and gene structure analyses indicate that an expansion of the *trans*-isoprenyl diphosphate synthase gene family in the ancestor of the subfamily Galerucinae enabled functional diversification toward this terpene synthase gene family. These insights into how flea beetles synthesize their aggregation pheromones may lead to new approaches for pest management.

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We investigated the biosynthesis of the sesquiterpene aggregation pheromone in *P. striolata*, an important pest of crucifer crops in North America and Southeast Asia (26). Here, we report the identification of an evolutionarily novel family of terpene synthases in this flea beetle. No transcripts with similarity to plant or microbial terpene synthases were evident in a transcriptome of this species. However, a remarkably high number of genes were present that were predicted to encode enzymes similar to insect *trans*-IDSs, which produce geranyl diphosphate [(*E*)-GPP] and/or farnesyl diphosphate [(*E*,*E*)-FPP] from the C_5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (5, 9, 10). Functional characterization of the recombinant IDS-like enzymes led to the discovery of four TPS enzymes which formed a separate clade in a phylogenetic analysis of coleopteran *trans*-IDS and *trans*–IDS-like enzymes. We investigated the role of these TPS in the aggregation pheromone biosynthesis of *P. striolata* as well as their putative evolutionary origin.

Results

TPS Activity Is Present in *P. striolata* Crude Protein Extracts. To search for TPS activity in *P. striolata*, we incubated crude protein extracts prepared from male or female adults with (*E*,*E*)-FPP, the canonical substrate for sesquiterpene synthases; however, no activity was observed. Because some plant sesquiterpene synthases use other FPP stereoisomers, we conducted further incubations and found to our surprise that both sexes converted (*Z*,*E*)-FPP to the aggregation pheromone compound (6*R*,7*S*)-himachala-9,11-diene and other known flea beetle sesquiterpenes (Fig. 1*A*), whereas no sesquiterpene products were detected when (*Z*,*Z*)-FPP was used as a substrate (Fig. 1*A* and Fig. S1*A*). A search for FPP synthase (FPPS) activity in the same crude protein extracts using DMAPP and IPP as substrates revealed (*E*,*E*)-FPPS as well as (*Z*,*E*)-FPPS activity (Fig. 1*B* and Fig. S1*B*). Both TPS and (*Z*,*E*)-FPPS activity were much higher in crude extracts from male *P. striolata* compared with females (11- and 23-fold, respectively; $n = 3$) as would be expected if these activities were associated with the formation of the male-specific aggregation pheromone (6*R*,7*S*)-himachala-9,11-diene (Fig. 1*C*).

Identification and Functional Characterization of *IDS-like* Genes from *P. striolata*. We searched for candidate genes encoding TPS enzymes in a *P. striolata* transcriptome database (27) based on amino acid sequence similarity, but no sequences homologous to known plant, fungal, or bacterial TPS were found. However, nine transcripts were predicted to encode *trans*-IDS enzymes (i.e., GPPS or FPPS). Full-length ORFs of these genes were obtained using rapid amplification of cDNA ends–PCR (RACE-PCR) (Dataset S1). An alignment of the corresponding amino acid sequences (Fig. S2) revealed considerable sequence divergence as amino acid identities ranged from 13.5% to 72.8%. The active site of *trans*-IDS enzymes generally contains two aspartate-rich motifs (DDxxD), which are critical for catalytic activity (28), but only three out of the nine proteins possessed both motifs. In five proteins, the second aspartate-rich motif was altered, and in one protein both motifs were modified (Fig. S2).

To determine the enzymatic activities of the *P. striolata* IDS-like gene products, the ORFs lacking the putative signal peptides (Fig. S2) were heterologously expressed as N-terminal His-tag fusions in *Escherichia coli*. Eight out of nine candidate genes were successfully expressed with this approach. Gene names were assigned according to the functional characterization of the corresponding recombinant proteins and the results of a phylogenetic analysis of *P. striolata* IDS-like enzymes with known *trans*-IDS from Coleoptera (Fig. 2).

Interestingly, only two recombinant enzymes, *Ps*IDS1 and *Ps*IDS3, showed IDS activity in assays with DMAPP and IPP as substrates. *Ps*IDS1 produced (*E*,*E*)-FPP (Fig. S3*A*), but *Ps*IDS3, unprecedented for a *trans*-IDS, generated *cis* double bonds synthesizing neryl diphosphate [NerylPP = (*Z*)-GPP; Fig. S3*B*] as well as (*Z*,*Z*)-FPP (Fig. S4*C*). When (*E*)-GPP and IPP were provided as substrates, *Ps*IDS3 synthesized (*Z*,*E*)-FPP (Fig. S3*C*).

To analyze potential TPS activities of the *P. striolata* IDS-like proteins, assays were performed with the substrates (*E*)-GPP, (*E*,*E*)-FPP, (*Z*,*E*)-FPP, (*Z*,*Z*)-FPP, and (*E*,*E*,*E*)-GGPP. Although *Ps*IDS1, *Ps*IDS2, *Ps*IDS3, and *Ps*IDS-like showed no detectable activity with the tested potential substrates, recombinant *Ps*TPS1, *Ps*TPS2, *Ps*TPS3, and *Ps*TPS4 all demonstrated TPS activity. *Ps*TPS1 converted (*Z*,*E*)-FPP into a mixture of sesquiterpenes with (6*R*,7*S*)-himachala-9,11-diene, *trans*-α-himachalene,

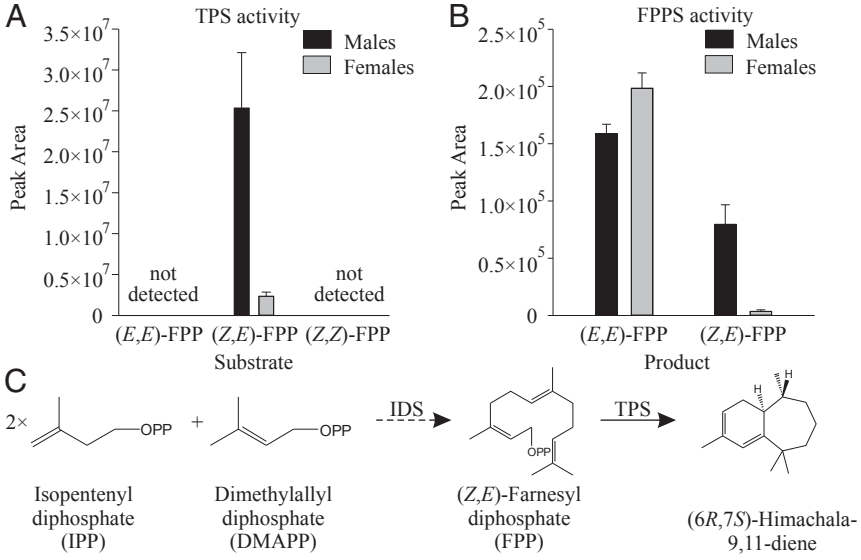

Fig. 1. Sesquiterpene synthase (TPS) activity (*A*) and farnesyl diphosphate synthase (FPPS) activity (*B*) in crude protein extracts of male and female *Phyllotreta striolata* adults. Crude protein extracts were incubated with the substrates (*Z*,*E*)-FPP, (*E*,*E*)-FPP, and (*Z*,*Z*)-FPP (*A*), or with IPP and DMAPP (*B*), respectively. TPS enzyme products were collected using a solid-phase microextraction (SPME) fiber and analyzed with GC-MS. The peak areas for the four products (6*R*,7*S*)-himachala-9,11-diene, *trans*-α-himachalene, (6*R*,7*S*)-2,2,6-trimethyl-10-methylenebicyclo[5.4.0]undec-1(11)-ene, and γ-cadinene were summed up to calculate the relative TPS activity. FPPS enzyme products were analyzed using LC-MS/MS. Means and SEs are shown ($n = 3$). A proposed pathway for the formation of sesquiterpenes from IPP and DMAPP in *P. striolata* is shown in *C*.

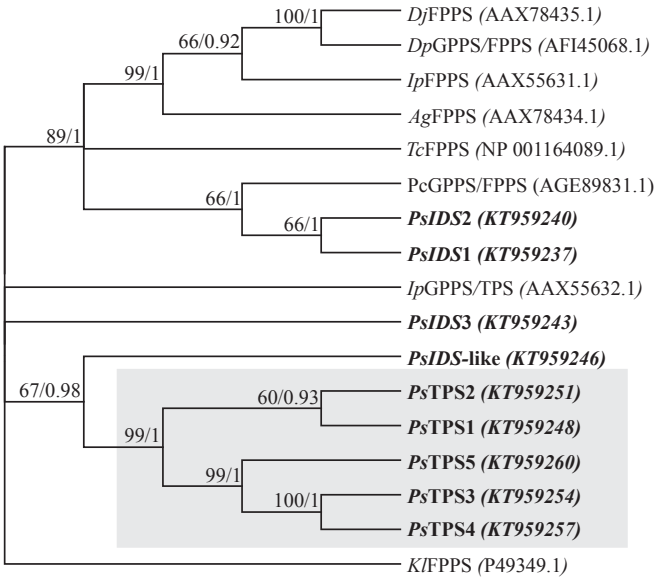

Fig. 2. Majority-rule cladogram inferred from maximum-likelihood analysis of IDS and TPS enzymes from *P. striolata* (shown in bold) along with other coleopteran *trans*-IDSs, and GPPS/TPS from *Ips pini*. The shaded box highlights the clade of evolutionarily novel TPS enzymes. The tree was rooted using a fungal FPPS from *Kluyveromyces lactis* (*Kl*). Bootstrap values (1,000 replicates) and posterior probability values from a Bayesian analysis using the same data set are shown next to each node. Sequences included in the analysis are as follows: Coleoptera: *Ag*, *Anthonomus grandis*; *Dj*, *Dendroctonus jeffreyi*; *Dp*, *Dendroctonus ponderosae*; *Ip*, *Ips pini*; *Pc*, *Phaedon cochleariae*; *Ps*, *Phyllotreta striolata*; *Tc*, *Tribolium castaneum*.

(6*R*,7*S*)-2,2,6-trimethyl-10-methylenebicyclo[5.4.0]undec-1(11)-ene, and γ-cadinene being the predominant products (Fig. 3). The enzyme was also able to accept (*Z*,*Z*)-FPP as substrate, converting it into (*R*)-β-bisabolene and an unidentified sesquiterpene hydrocarbon (Fig. S4*A*). In contrast, enzyme assays with (*E*,*E*)-FPP, (*E*)-GPP, and (*E*,*E*,*E*)-GGPP revealed no detectable product formation for *Ps*TPS1 (Fig. S4*A*). The N-terminal His-tag had no influence on *Ps*TPS1 product specificity, but expression of the full-length *Ps*TPS1 with its putative N-terminal signal peptide was not successful (Fig. S4*B*). Because divalent metal cofactors often influence the activity and product specificity of terpene synthases (29, 30), we tested *Ps*TPS1 in the presence of different concentrations of magnesium and manganese. Although enzyme activity increased with higher magnesium and manganese ion concentrations, the product specificity of *Ps*TPS1 was not influenced by these metal cofactors (Fig. S5).

Like *Ps*TPS1, *Ps*TPS2 also showed sesquiterpene synthase activity. (*E*,*E*)-FPP was converted into β-elemene, a thermal rearrangement product formed from germacrene A upon injection in the heated inlet of the GC, and an unidentified sesquiterpene alcohol (Fig. S4*C*). Catalysis of (*Z*,*E*)-FPP and (*Z*,*Z*)-FPP resulted in complex sesquiterpene mixtures consisting mainly of unidentified compounds (Fig. 3 and Fig. S4). In contrast to *Ps*TPS1, *Ps*TPS2 also accepted (*E*)-GPP as substrate and produced several monoterpenes with linalool being the predominant one (Fig. S4*A*). Diterpene formation from (*E*,*E*,*E*)-GGPP could not be observed for *Ps*TPS2.

*Ps*TPS3 and *Ps*TPS4 accepted all tested prenyl diphosphates as substrates and produced mainly acyclic alcohols resulting from the initial ionization of the substrate and a subsequent capture of the carbocation formed by addition of a water molecule. (*E*)-GPP was converted into linalool, (*E*,*E*)-FPP and (*Z*,*E*)-FPP were converted into (*E*)-nerolidol, (*Z*,*Z*)-FPP was converted into (*Z*)-nerolidol, and (*E*,*E*,*E*)-GGPP was converted into geranyllinalool (Fig. 3 and Fig. S4*A*).

Two recombinant proteins, *Ps*IDS2 and *Ps*IDS-like, showed neither IDS, nor TPS activity under our assay conditions. However, the aspartate-rich motifs required for catalysis were modified in both proteins (Fig. S2), which might explain the lack of enzyme activity with these substrates.

Enzyme activity of the putative terpene synthase *Ps*TPS5 was not tested because this protein could be expressed neither in *E. coli* nor in insect cells (*SI Materials and Methods*).

PsTPS1 Is Involved in Aggregation Pheromone Biosynthesis. Because recombinant *Ps*TPS1 converted (*Z*,*E*)-FPP to the major component of the male-produced aggregation pheromone, (6*R*,7*S*)-himachala-9,11-diene, as observed in *P. striolata* crude protein extracts, we used RNAi to examine the role of *Ps*TPS1 in aggregation pheromone biosynthesis in vivo. Males that were injected with *PsTPS1*-derived dsRNA showed significantly reduced *PsTPS1* transcript abundance after 11 d compared with *gfp*-injected or noninjected adults (by >77%; $P < 0.001$; Fig. 4*A*). Correspondingly, *PsTPS1*-injected males also emitted significantly fewer male-specific sesquiterpenes compared with controls (by >56%; $P < 0.01$; Fig. 4*B*), confirming a significant role of *Ps*TPS1 in aggregation pheromone biosynthesis.

Transcript Levels of *PsIDS*, *PsIDS-like*, and *PsTPS* Genes in Male and Female *P. striolata*. Expression levels of *PsIDS*, *PsIDS*-like, and *PsTPS* genes in male and female *P. striolata* were compared by quantitative RT-PCR (qRT-PCR). Most genes, including *PsIDS1*, *PsIDS3*, and *PsTPS1*, were significantly more expressed in males compared with females (Fig. 5). However, with *PsTPS1* transcript abundance in females corresponding to 72.3% of that in males, expression levels were similar in both sexes. Expression of *Ps*IDS3, on the other hand, was about 20 times higher in males than in females.

Evolution of *P. striolata TPS* and *cis-IDS* Genes. The evolutionary relationship between the nine *P. striolata trans*–IDS-like enzymes and *trans*-IDS from Coleoptera as well as the GPPS/TPS enzyme from *I. pini* was inferred in a maximum-likelihood analysis. *Ps*IDS1 and *Ps*IDS2 clustered together with a GPPS/FPPS from the leaf beetle *Phaedon cochleariae* in a clade with all known coleopteran *trans*-IDS enzymes included in the dataset (Fig. 2). The four TPS enzymes formed a separate clade including *Ps*TPS5 (not expressed), which was supported by high bootstrap and posterior probability values (99/1). *Ps*IDS3 as well as the *I. pini* GPPS/TPS were separated from both clades (Fig. 2). The evolutionary

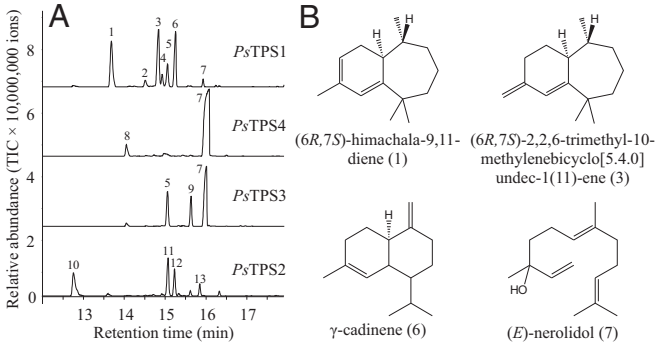

Fig. 3. Biochemical characterization of TPS enzymes from *Phyllotreta striolata*. (*A*) Genes were overexpressed in *Escherichia coli*, and purified recombinant proteins were incubated with (*Z*,*E*)-FPP. Enzyme products were collected using SPME and analyzed with GC-MS. 1, (6*R*,7*S*)-himachala-9,11-diene; 2, *trans*-α-himachalene; 3, (6*R*,7*S*)-2,2,6-trimethyl-10-methylenebicyclo[5.4.0]undec-1(11)-ene; 4, (*Z*)-α-bisabolene; 5, β-bisabolene; 6, γ-cadinene; 7, (*E*)-nerolidol; 8, (*E*)-β-farnesene; 9, (*E*)-α-bisabolene; 10, unidentified sesquiterpene hydrocarbon; 11–13, unidentified sesquiterpene hydrocarbons. (*B*) Structures of major *Ps*TPS products.

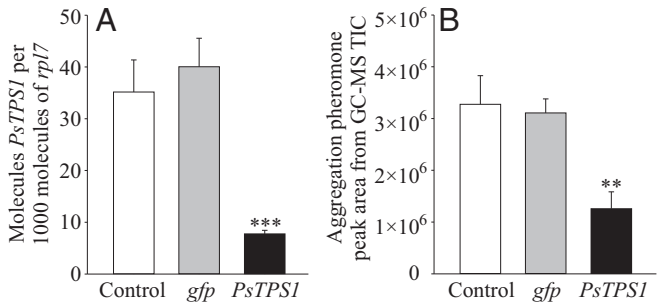

Fig. 4. Knockdown of *PsTPS1* in male *P. striolata* adults by RNAi. (*A*) *PsTPS1* mRNA expression levels in *P. striolata* males 11 d after dsRNA injection of *PsTPS1* or *gfp* and in uninjected adults (control). Copy number estimates are given per 1,000 copies of mRNA for the reference gene *rpl7* ($n = 6$, +SEM). (*B*) Aggregation pheromone emission of control, *gfp*-, and *PsTPS1* dsRNA-injected male *P. striolata*. The GC-MS peak areas of four male-specific sesquiterpenes, (6*R*,7*S*)-himachala-9,11-diene, *trans*-α-himachalene, γ-cadinene, and *ar*-himachalene were summed up ($n = 6$, +SEM). $^{**}P < 0.01$; $^{***}P < 0.001$.

origin of the *P. striolata cis*-IDS and TPS enzymes from *trans*-IDS was confirmed in a broader phylogenetic analysis including coleopteran GGPPS as well as *trans*-IDS enzymes from plants. All *P. striolata* enzymes clustered with insect *trans*-IDS and plant FPPS, and were separated from plant GPPS and plant and insect GGPPS (Fig. S6*A*).

To investigate the evolution of the *IDS/TPS* gene family in the leaf beetle subfamily Galerucinae, we identified putative members in the transcriptomes of two related species, the horseradish flea beetle, *Phyllotreta armoraciae*, and the cabbage stem flea beetle, *Psylliodes chrysocephala*. From both beetle species, eight transcripts were obtained as full-length sequences using RACE-PCR, except for one partial sequence from *P. chrysocephala* (*Pc*TPS3). The corresponding amino acid sequences were aligned with *Ps*IDS, *Ps*IDS-like, *Ps*TPS sequences to conduct a phylogenetic analysis (Fig. S6*B*). The resulting phylogenetic tree revealed three putative IDS enzymes in *P. armoraciae* as well as in *P. chrysocephala* clustering with *Ps*IDS1, *Ps*IDS2, and *Ps*IDS3 according to the species phylogeny, suggesting that they are orthologs. On the other hand, we found species-specific sequence diversification in the TPS clade. For example, two putative TPS from *P. armoraciae*, *Pa*TPS1 and *Pa*TPS2, clustered together with *Ps*TPS1 and *Pc*TPS1 (Fig. S6*B*).

We analyzed whether different selection pressures act on the ancestral coleopteran *trans*-IDS (GPPS/FPPS) clade compared with the *Ps*TPS clade, *Ps*IDS3, and *Ps*IDS-like, respectively. The result indicates different strengths of purifying selection acting on the different branches/genes (Fig. S6*C*). The *trans*-IDS clade representing the ancestral function and the *cis*-IDS *Ps*IDS3 are under strong purifying selection ($\omega \leq 0.065$), whereas the *Ps*TPS clade, and *Ps*IDS-like, which showed neither IDS nor TPS activity in our experiments, appear to be under more relaxed constraints ($\omega = 0.234$ and $\omega = 0.5345$, respectively; Fig. S6*C*).

To further test the hypothesis that *PsTPS* and *PsIDS3* genes evolved from insect *trans-IDS* genes, we analyzed the exon–intron structures of *PsIDS1*, *PsIDS3*, and *PsTPS1*, and compared them to the structures of coleopteran (*Tribolium castaneum* and *Dendroctonus ponderosae*), dipteran (*Drosophila melanogaster*), and lepidopteran (*Bombyx mori*) *trans-IDS* genes. The number of introns per gene ranged from three introns present in genes of *P. striolata* and *T. castaneum* to seven introns in the *GPPS/FPPS* gene of *D. ponderosae* (8) (Fig. S7). The positions and corresponding intron phases of the three introns found in *P. striolata* genes were conserved in all analyzed insect *IDS* genes, except for the third conserved intron, which was not present in the putative *FPPS* gene from *D. melanogaster* but is otherwise conserved. We assume that the poor alignment of the first intron position in *PsIDS3* and *PsTPS1* is caused by an inaccurate sequence correspondence in the N-terminal region due to extensive sequence diversification.

Discussion

***P. striolata* Possesses an Evolutionarily Novel Class of TPS That Likely Originated from Insect *trans*-IDSs.** Although TPSs are well known in plants, fungi, and bacteria, sesquiterpene synthases have not been described in insects so far. In our attempt to study the molecular basis of sesquiterpene pheromone production in *P. striolata*, we identified and characterized a small gene family encoding enzymes with TPS activity. We found that *P. striolata* contains at least four different TPS enzymes, and biochemical characterization of the recombinant enzymes revealed broad substrate and product specificity (Fig. 3 and Fig. S4*A*). The identified *P. striolata* enzymes shared no amino acid sequence similarity with other TPSs from plants, fungi, and bacteria, but were similar to insect *trans*-IDS enzymes (Figs. S2 and S6*A*). In contrast to *P. striolata* TPSs, which showed no IDS activity in our enzyme assays, the bark beetle *I. pini* possesses a bifunctional enzyme that synthesizes (*E*)-GPP as well as the monoterpene myrcene (14).

Although insects usually contain up to three *trans*-IDS enzymes producing (*E*,*E*)-FPP or both (*E*)-GPP and (*E*,*E*)-FPP (7, 9, 10, 31, 32), we identified at least eight genes similar to insect *trans*-IDS in the transcriptomes of each of the three galerucine flea beetle species studied. Several predicted enzymes from *P. armoraciae* and *P. chrysocephala* also cluster in the TPS clade; however, whether these species also produce sesquiterpenes is unknown.

A comparison of the exon–intron structures of *P. striolata* TPS and *trans*-IDS genes with known insect *trans*-IDS genes revealed three conserved intron positions to be present in all analyzed coleopteran *trans*-IDS genes (Fig. S7). Altogether, this indicates that *P. striolata* TPSs evolved from an insect *trans*-IDS ancestor, thus representing an evolutionarily novel class of TPS enzymes.

***Ps*TPS1 Is Responsible for Sesquiterpene Pheromone Production in *P. striolata* Using the Unusual (*Z*,*E*)-FPP Isomer as Its Substrate.** The majority of characterized terpene synthases from plants, fungi, and bacteria accept exclusively *all-trans*-prenyl diphosphates as substrates in vivo. However, several plant sesquiterpene synthases from the Solanaceae were recently reported to convert (*Z*,*Z*)-FPP into sesquiterpenes in planta (33, 34), and one report indicates that (*Z*,*E*)-FPP might act as a TPS substrate in plants (35). Here, we demonstrated that one of the characterized terpene synthase enzymes from *P. striolata* enzymes, *Ps*TPS1, converted

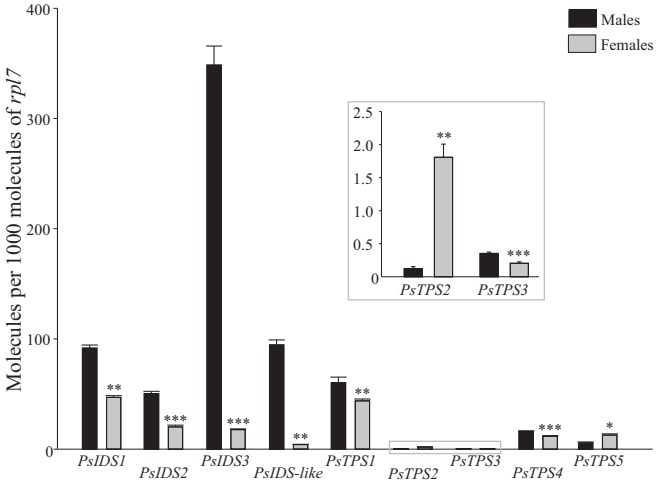

Fig. 5. mRNA expression levels of *IDS*, *IDS-like*, and *TPS* genes in male and female *P. striolata* as determined by RT-qPCR. Copy number estimates are given per 1,000 copies of mRNA for reference gene *rpl7* ($n = 5$, +SEM). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(*Z*,*E*)-FPP into a mixture of sesquiterpene olefins matching most of the major sesquiterpenes emitted by adult male *P. striolata* (23) and detected in enzyme activity assays using crude protein extracts from male *P. striolata* (Fig. 3 and Fig. S1*B*). Although the recombinant enzyme was also able to accept (*Z*,*Z*)-FPP as a substrate (Fig. S4*A*), the product derived from this compound was not detected in the volatile blend of the beetles. Moreover, crude protein extracts from adult male beetles showed *Ps*TPS1 activity only when provided with (*Z*,*E*)-FPP (Fig. 1*A*), suggesting that the enzyme accepts this uncommon (*Z*,*E*)-isomer as its native substrate to produce the aggregation pheromone. Indeed, silencing of *PsTPS1* using RNAi in male beetles resulted in a significantly reduced pheromone emission (Fig. 4*B*), which indicates a crucial role of *Ps*TPS1 in pheromone production in *P. striolata*. Because sesquiterpene emission is restricted to males (23), we were surprised to find similar expression levels of *PsTPS1* in males and females (Fig. 5). However, sesquiterpene synthase activity was ∼11-fold higher in crude protein extracts prepared from males compared with females when (*Z*,*E*)-FPP was used as substrate, suggesting that sesquiterpene pheromone production might be regulated at a posttranscriptional level.

A proposed reaction mechanism for the formation of the (*Z*,*E*)-FPP–derived enzyme products is provided in Fig. S8. The cofactor-dependent initial ionization of (*Z*,*E*)-FPP results in the formation of the cisoid farnesyl carbocation, which then might undergo sequential 11,1-cyclization, a 1,3-hydride shift, and a 6,1-cyclization leading to the himachalane skeleton, followed by another 1,3-hydride shift, and further rearrangements and proton abstraction to give the end products (6*R*,7*S*)-himachala-9,11-diene, (6*R*,7*S*)-2,2,6-trimethyl-10-methylenebicyclo[5.4.0]undec-1(11)-ene, and *trans*-α-himachalene. γ-Cadinene is likely formed by an alternative 10,1-cyclization of the initial farnesyl carbocation followed by a 1,3-hydride shift and a 6,1-cyclization. Notably, *Ps*TPS1 was not able to accept (*E*,*E*)-FPP as a substrate, suggesting that either the binding of this FPP isomer is impaired by steric constraints in the active site or that the isomerization of the ionization-derived transoid farnesyl carbocation into the cisoid form, which is required for subsequent 6,1-cyclization, cannot be catalyzed by this enzyme.

Although several *Ps*TPS enzymes also showed monoterpene, sesquiterpene, and diterpene synthase activity in vitro (Fig. 3 and Fig. S4*A*), the respective enzyme products were not observed in the *P. striolata* volatile blend. Furthermore, in the sesquiterpene synthase activity assays conducted with crude protein extracts and the three FPP isomers, none of the other TPS products was detectable (Fig. S1). These findings might be due to the overall low expression levels of *PsTPS2*, *PsTPS3*, and *PsTPS4* in *P. striolata* adults (Fig. 5). Moreover, it is conceivable that *PsTPS2*, *PsTPS3*, and *PsTPS4* are expressed only in specific cells of adults with limited substrate availability, or in other developmental stages of the beetle. Further studies are needed to elucidate the biological role of the other TPS enzymes in *P. striolata*.

Both *trans*- and *cis*-IDSs Are Present in *P. striolata*. To determine the origin of the unusual TPS substrate, (*Z*,*E*)-FPP, in *P. striolata*, we analyzed IDS activity in the beetle. It was not possible to detect prenyl diphosphates by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in crude *P. striolata* extracts, but (*E*,*E*)-FPPS activity was detected in vitro in crude protein extracts of both sexes. This finding is expected because (*E*,*E*)-FPP is the precursor for insect juvenile hormone biosynthesis and is required for basic processes such as prenylation of proteins and ubiquitin biosynthesis in all insect orders studied (4). What was unexpected in the context of previous work was our detection of (*Z*,*E*)-FPPS activity in crude beetle protein extracts, which was present at ∼23-fold higher levels in males than females (Fig. 1*B*).

Two out of the three *P. striolata IDS* genes, *PsIDS1* and *PsIDS2*, encode enzymes with high sequence identity (>48%) to characterized FPPS/GPPS from other Coleoptera. *Ps*IDS1 was shown to produce (*E*,*E*)-FPP (Fig. S3*A*) and its position in the phylogenetic tree (Fig. 2) suggests that this enzyme is the *P. striolata* representative of the ubiquitous (*E*,*E*)-FPP synthase involved in the formation of juvenile hormone, ubiquitin, and prenylated proteins in insects. In *Ps*IDS2, the second aspartate-rich motif is altered to KDxxN, which might explain the lack of activity in our assays. Interestingly, *Ps*IDS3 converted IPP and DMAPP into NerylPP (Fig. S4) and (*Z*,*Z*)-FPP, but produced the *Ps*TPS1 substrate (*Z*,*E*)-FPP when supplied with (*E*)-GPP and IPP (Fig. S4*C*). The production of (*Z*,*E*)-FPP is not unprecedented. However, previous reports implicate a separate IDS family in this catalysis, the *cis*-IDSs. A *cis*-IDS from *Myobacterium tuberculosis* was described to generate (*Z*,*E*)-FPP from (*E*)-GPP and IPP, which in turn is used as substrate by a decaprenyl diphosphate synthase to synthesize components of the cell wall (36, 37). All *cis*-IDS enzymes known to date exhibit large sequence and structural differences from *trans*-IDS (28, 38). However, *Ps*IDS3 shares no sequence similarity with known *cis*-IDS enzymes and possesses both aspartate-rich motifs characteristic of *trans*-IDS (Fig. S2), which are absent in *cis*-IDS enzymes. Thus, *Ps*IDS3 apparently represents (to our knowledge) the first *cis*-IDS that evolved from a *trans*-IDS.

Because we could not detect any (*Z*,*Z*)-FPPS activity in the beetle (Fig. S1*B*), and *PsIDS3* was significantly more expressed in males than in females (about 15-fold; $n = 5$, t test, $t = 28.9$, $P < 0.001$), we hypothesize that *Ps*IDS3 might provide the (*Z*,*E*)-FPP substrate for sesquiterpene pheromone production in *P. striolata* by catalyzing a head-to-tail condensation of (*E*)-GPP and IPP. However, none of the other *P. striolata trans*-IDS enzymes identified in this study produced significant amounts of (*E*)-GPP under our assay conditions. It was previously shown that product chain length or activity of plant and insect IDSs can be influenced by heterooligomerization, which may also play a role in (*E*)-GPP and (*Z*,*E*)-FPP synthesis in *P. striolata* (9, 39–41). Moreover, IDS product chain length may also be influenced by the IPP/DMAPP ratio (8), and different metal ion cofactors as recently demonstrated in the mustard leaf beetle, *Phaedon cochleariae* (5). The putative role of *Ps*IDS and *Ps*IDS-like enzymes in the biosynthesis of (*Z*,*E*)-FPP are subjects of ongoing research.

Outlook. The male-specific aggregation pheromones of *Phyllotreta* spp. mediate mass attacks on economically important crucifer crops, and pheromone-baited traps or lures may prove an effective approach to reducing the damage caused by these major insect pests. In *P. striolata*, the full pheromone blend consists of several compounds (22, 23) that may require the activity of additional enzymes besides *Ps*TPS1, such as monooxygenases. However, because the *Ps*TPS1 product (6*R*,7*S*)-himachala-9,11-diene was shown to act as the major aggregation pheromone component of several *Phyllotreta* species (23, 24), use of this substance may have promise in pest control. Because chemical synthesis of (6*R*,7*S*)-himachala-9,11-diene is quite complex (42, 43), recombinant *Ps*TPS1 may offer a viable method for producing large quantities of this sesquiterpene. Further progress in understanding pheromone biosynthesis and its regulation in *Phyllotreta* spp. could open up other ways for controlling these important pest species.

Materials and Methods

Crude protein extracts were prepared from male and female *P. striolata* adults and analyzed for TPS and IDS activity. For TPS activity assays, protein extracts were incubated with different FPP isomers, and assay products were detected by solid-phase microextraction coupled with gas chromatography–mass spectrometry (GC-MS). IDS activity assays using IPP and DMAPP as substrates were analyzed with LC-MS/MS. Nine putative *trans-IDS* transcripts were identified in the *P. striolata* transcriptome, and the corresponding full-length sequences were obtained using RACE-PCR. The candidate genes were amplified as N-terminally truncated ORFs from cDNAs, cloned into the expression vector pET100/D-TOPO or pET200/D-TOPO, and expressed as N-terminal His-tag fusions in *Escherichia coli*. Recombinant proteins were partially purified via the

His-tag and tested for TPS and IDS activity. *PsIDS1*, *PsIDS3*, and *PsTPS1* were additionally amplified from genomic DNA of *P. striolata* and sequenced to determine intron positions. The function of *Ps*TPS1 in vivo was investigated in RNAi experiments by injecting male *P. striolata* adults with dsRNA of *PsTPS1* or *gfp* (control), followed by quantitative RT-PCR and quantification of sesquiterpenes emitted by feeding males. Details on organisms, phylogenetic analyses, experiments, analytical procedures, and data analyses are provided in *SI Materials and Methods*. All sequences reported in this manuscript have been deposited in the GenBank database (accession nos. KT959237–KT959261).

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