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SHORT COMMUNICATION

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Midgut tissue of male pine engraver, *Ips pini*, synthesizes monoterpenoid pheromone component ipsdienol de novo

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Abstract For over three decades the site and pathways of bark beetle aggregation pheromone production have remained elusive. Studies on pheromone production in *Ips* spp. bark beetles have recently shown de novo biosynthesis of pheromone components via the mevalonate pathway. The gene encoding a key regulated enzyme in this pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG-R*), showed high transcript levels in the anterior midgut of male pine engravers, *Ips pini* (Say) (Coleoptera:Scolytidae). *HMG-R* expression in the midgut was sex, juvenile hormone, and feeding dependent, providing strong evidence that this is the site of acyclic monoterpenoid (ipsdienol) pheromone production in male beetles. Additionally, isolated midgut tissue from fed or juvenile hormone III (JH III)-treated males converted radiolabeled acetate to ipsdienol, as assayed by radio-HPLC. These data support the de novo production of this frass-associated aggregation pheromone component by the mevalonate pathway. The induction of a metazoan *HMG-R* in this process does not support the postulated role of microorganisms in ipsdienol production.

Introduction

The acyclic monoterpene alcohols ipsenol and ipsdienol, isolated from the bark beetle *Ips paraconfusus* Lanier, were the first coleopteran pheromones identified (Silverstein et al. 1966; Wood et al. 1968). In *Ips pini*, feeding by adult males stimulates the production of juvenile hormone III, which in turn stimulates the de novo biosynthesis of ipsdienol (Tillman et al. 1998). Ipsdienol attracts both sexes for the "mass attack" involved in colonizing trees (Wood 1982). The association of monoterpene synthesis with conifers (Croteau 1981), the ubiquity of the precursor myrcene in host pines (Gershenzon and Croteau 1991), and the demonstration that deuterated myrcene was converted to ipsenol and ipsdienol (Hendry et al. 1980) led to the widely accepted concept that *Ips* spp. bark beetles, in contrast to many other insects (Tillman et al. 1999), obtain the pheromone components ipsenol and ipsdienol by a simple modification of the dietary precursor myrcene (Hughes 1974; Renwick et al. 1976; Byers 1989; Vanderwel 1994).

This model of bark beetle pheromone production, however, has been brought into question by five significant lines of evidence supporting the de novo synthesis of ipsdienol via the mevalonate pathway. First, myrcene may not be present in sufficient quantities in some host trees to account for the amount of pheromone produced (Byers 1981; Byers and Birgersson 1990). Second, myrcene-treated male *I. pini* from California produce a relatively non-attractive racemic mixture of ipsdienol [45–61% of the (–)-enantiomer], while unfed, JH III-induced male *I. pini* produce ipsdienol with an 87–96% (–)-enantiomeric composition (F. Lu et al., unpublished data). The natural enantiomeric composition of ipsdienol produced by California *I. pini* feeding on pine phloem is 95–98% (–) (Seybold et al. 1995a). Third, when treated with the *HMG-R* inhibitor, compactin, the related beetle *I. duplicatus* showed a marked reduction in ipsdienol synthesis (Ivarsson et al. 1993). Fourth, the incorporation of labeled acetate and mevalonate into ipsdienol has been directly demonstrated (Seybold et al. 1995b; Tillman et al. 1998; F. Lu et al., unpublished data). Finally, topical treatment of unfed male *I. pini* with JH III resulted in an approximately 20-fold increase in *HMG-R* transcript levels (Tillman et al., unpublished data).

Localizing the site of pheromone biosynthesis in bark beetles has proven to be a formidable task. Recent studies with *I. paraconfusus* have implicated tissue in the metathorax by assaying various body regions for incor-

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poration of radiolabeled acetate into pheromone precursors and by northern analyses of *HMG-R* mRNA (Ivarsson et al. 1998). The following work provides the first definitive demonstration that the aggregation pheromone component ipsdienol is produced de novo in anterior midgut tissue of the male pine engraver, *I. pini*.

Materials and methods

Insects

Ips pini were collected as immatures in infested pine logging debris from the Lassen National Forest, California and reared to the adult stage as previously described (Browne 1972; Seybold et al. 1995b). Some beetles were also collected from the University of Nevada Whittell Forest (Little Valley, 30 km south of Reno, Nevada, at 39°15′ 00″ N, 119°52′30″ W). Insects were treated with JH III (Sigma-Aldrich) and fed on Jeffrey pine, *Pinus jeffreyi*, phloem as previously described (Seybold et al. 1995b; Tillman et al. 1998). The JH III (10 µg/0.5 µl) in acetone was applied topically, and the incubation period was 16 h. Insects were fed on pine phloem for 20 h.

Cloning and sequencing *HMG-R*

3′ RACE (40 cycles: 94°C/40 s–56°C/60 s–72°C/150 s) utilizing a degenerate primer PARAHMD and CSX (Tittiger et al. 1999) yielded a 1.2 kb PCR product from polyadenylated RNA prepared (Pharmacia) from JH III-treated male *I. pini*. This product (clone 13) was utilized as a probe to screen a λ phage cDNA library constructed from JH III-treated male *I. pini* yielding an additional 0.4 kb upstream (clone λ3A). A primer (λ3AREV; CCTCTGTTGGTGCTAGCAACC) from the 5′ end of λ3A and an upstream primer from *I. paraconfusus* (770F1; TGCCACACTTACCCTTACAG) yielded a 1.7 kb product (clone L9) by PCR (40 cycles: 94°C/40 s–55°C/60 s–72°C/210 s). The 5′ end of *HMG-R* from *I. pini* was isolated by PCR (40 cycles: 94°C/40 s–58°C/60 s–72°C/90 s) using template DNA from a second λ phage cDNA library, T3 vector primer, and the gene specific primer (SP8; GGTGAATAGTCCCGCAATGCC) from the 5′ end of clone L9. All PCR products were cloned into PT7Blue and double-pass sequenced (Applied Biosystems Prism 310) using dRhodamine dye terminators (Perkin-Elmer). The composite sequence (confirmed by RT-PCR amplification using primers at 5′ and 3′ ends) was deposited in Genbank (AF304440). Sequence analysis was done using Vector NTI Suite (V.6; InforMax).

Northern blots

Adult beetles were dissected into approximate head, thoracic, and abdominal body regions. The dissections were approximate because the external morphology obscures the true borders between body regions. The tissues were frozen at –80°C until total RNA was isolated using TRIZOL Reagent (Life Technologies). Triplicate samples of five beetles per sample were used. RNA was separated on glyoxal agarose gels (Sambrook et al. 1989), transferred to Hybond N membranes (Amersham), and fixed by UV crosslinking. Membranes were hybridized with the mouse β-actin (Stratagene) or *HMG-R* cDNA. Both probes were labeled with [^{32}P]dCTP by PCR using appropriate primers. Membranes were washed and imaged with a BioRad molecular imager, and densitometry was done with Molecular Analyst software (BioRad).

In situ hybridization

An ‘exposed whole mount’ technique was devised and used for in situ hybridization experiments because traditional slide-based methods were inadequate. After treatment with JH III or feeding, males were embedded in TBS (Fisher), frozen, and sectioned sagittally to varying depths of approximately 0.1 mm. These ‘partial exposed whole mounts’ were removed from TBS by shaking in 4% paraformaldehyde (PFA) at room temperature and then fixed in 4% PFA at 4°C for 5 h. Specimens were washed twice in phosphate buffered saline (PBS) and allowed to prehybridize in a humidified chamber at 70°C for 1 h. All samples were then incubated for 18 h in a 300-fold dilution of a 0.8 kb digoxigenin (DIG)-labeled antisense *HMG-R* riboprobe synthesized (Wilkinson 1992) from the center portion of clone 13 (representing base pairs 2,473 to 3,200 of Genbank AF304440). A high stringency (0.2X SSC) wash at 70°C for 2 h was followed by a room temperature 4 h incubation in TBST + 20% sheep serum as a blocking agent. Anti-DIG alkaline phosphatase-conjugated Fab fragments (Roche) were incubated on sectioned male beetles for 1 h with slow shaking. Specimens were then washed twice in 0.1 M Tris pH 7.5/0.15 M NaCl to remove unbound antibody and their pH equilibrated to 9.5 by washing in 0.1 M Tris pH 9.5/0.1 M NaCl/0.05 M $MgCl_2$. Hybridized probes were visualized colorimetrically using alkaline phosphatase substrate 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Roche). This reaction was stopped with 4% PFA upon proper development. Alimentary canals from both sexes were processed in the same manner as ‘exposed whole mounts’ except that tissues were dissected from live beetles 16 h after topical JH III application or the initiation of feeding on pine phloem. The tissues were then placed directly in PFA for 5 h before prehybridization as described above.

In vitro incubations with labeled acetate

Midgut tissue samples from 3–5 beetles were pooled and incubated for 30 min with 5 µCi of [1-^{14}C]acetate in a phosphate buffer (Tittiger et al. 1999) and then extracted with 300 µl pentane: diethyl ether (1:1) followed by three 300 µl pentane extracts. The combined extracts were reduced in volume, and an aliquot was assayed by liquid scintillation counting (LSC) and then by radio-HPLC as previously described (Seybold et al. 1995b) using an Econosil silica 10-micron column. Fractions were assayed by LSC. Unlabeled ipsdienol standard was added to the sample prior to chromatography.

Results

To determine the exact site of pheromone synthesis, we first isolated and sequenced multiple cDNA clones comprising the full length *HMG*-R cDNA from JH III-treated male *I. pini*. The 3.2 kb full-length cDNA (Genbank AF304440) contained a 2,598 bp open reading frame encoding a protein of 866 amino acids with a high degree of similarity to other known HMG-R proteins (data not shown). Reasoning that *HMG-R* expression would be greatly elevated in the tissue(s) producing the pheromone, a 0.5 kb portion of this clone was used as a probe in Northern analysis of RNA isolated from heads, thoraces, or abdomens of JH III-treated insects (Fig. 1). The majority of *HMG-R* expression was observed in the male thorax, consistent with biochemical and molecular data in *I. paraconfusus* (Ivarsson et al. 1998; Tittiger et al. 1999). A smaller, yet significant, amount of expression was observed in the abdomen, with only a basal amount in the head.

In situ hybridization was performed to localize *HMG-R* transcript more precisely. JH III-treated and acetone-treated (control) male insects were sagittally sec-

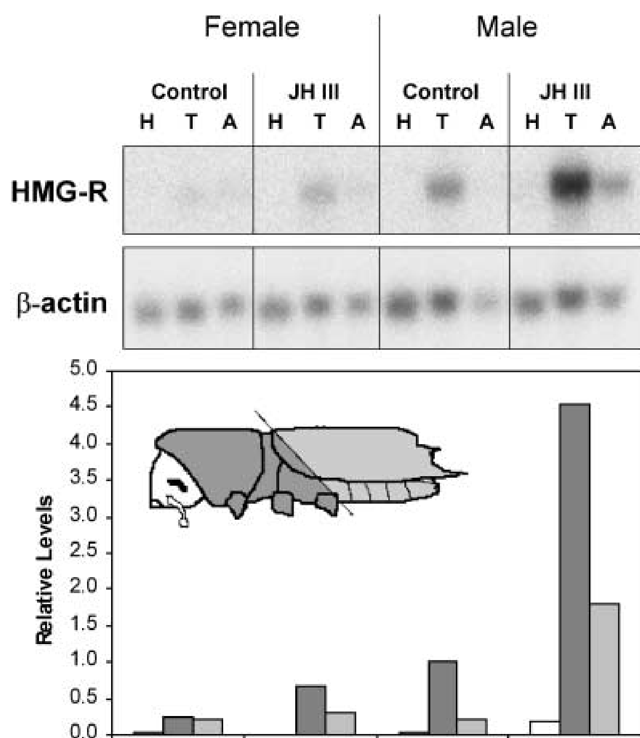

Fig. 1 Tissue localization of *HMG-R* expression in *Ips pini*. *Top* Northern blot analysis of RNA isolated from approximate head (*H*), thorax (*T*), and abdomen (*A*) body regions. The same membrane was probed with *HMG-R* and β-actin cDNA. *Bottom* Relative *HMG-R* mRNA levels. The *inset* shows the approximate orientation of the thoracic-abdominal cut for dissection

tioned to varying depths and the exposed tissue probed for *HMG-R* expression. Repeated experiments demonstrated obvious signal in an area of the alimentary canal residing predominantly within the thorax, with a smaller portion in the abdomen (Fig. 2a). This region was subsequently identified as the anterior midgut. Acetone-treated (control) male beetles did not exhibit signal in the anterior midgut (Fig. 2b).

To rule out other portions of the alimentary canal as pheromone-producing tissue, intact alimentary tissue was isolated posteriorly to the proventriculus from control and JH III-treated male and female beetles and probed for *HMG-R* expression in situ. The anterior midgut in JH III-treated male beetles had dark and succinct signal compared to the remainder of the alimentary canal (Fig. 2c). Alimentary canals of control male beetles were probed in an identical manner, resulting in only a faint background signal (Fig. 2d), indicating that JH III-treatment was sufficient to raise *HMG-R* transcript levels in the anterior midgut. JH III-treated males, hybridized with the *HMG-R* sense strand (control), showed an expected background-level of signal (Fig. 2e). Non-pheromone-producing female beetles showed only faint signal in the anterior midgut, even after treatment with JH III (Fig. 2f).

Although JH III induces pheromone production in *I. pini* (Tillman et al. 1998), we further addressed the possibility that the signal seen in the male midgut could be an artifact of topical JH III application. To rule out this possibility, male beetles were fed on host tree phloem with no other treatment for 20 h and their alimentary canals were subsequently isolated and subjected to in situ hybridization. Identical to the JH III-treated males, the midgut showed intense, localized signal (Fig. 2g), whereas only background signal was seen in unfed males (Fig. 2h).

To unambiguously demonstrate that midgut tissue synthesizes pheromone, isolated alimentary canals from fed and JH III-treated males (Tillman et al. 1998) were dissected into proventriculus, midgut, and hindgut, incubated with radiolabeled acetate, extracted, and analyzed by radio-HPLC. Most of the radioactivity in the pentane:ether extract from midgut tissue was associated with the peak corresponding to an ipsdienol standard (Fig. 3). Proventriculus and hindgut tissue incorporated very little radioactivity, and no radioactivity was recovered in ipsdienol from a fat body tissue control (data not shown). To verify that acetate was incorporated into ipsdienol, the material recovered in the radioactive peak was converted to its camphanate derivative (Seybold et al. 1995b) which co-migrated with an ipsdienol–camphanate standard upon rechromatography (data not shown). To exclude microbial involvement, midgut tissue from JH III-treated males was cut open, washed with buffer, and then incubated with radiolabeled acetate. Washed midgut tissue readily incorporated radioactivity into ipsdienol (data not shown).

Discussion

Here we have shown a specific and substantial up-regulation of anterior midgut-localized *HMG-R* expression in response to both JH III application and feeding in male *I. pini.* We have also shown that midgut tissue vigorously incorporates radiolabeled acetate into ipsdienol. These observations demonstrate that the midgut is the site of pheromone production in *I. pini* and further support the previously demonstrated de novo synthesis of ipsdienol (Seybold et al. 1995b; Tillman et al. 1998). In addition, our data provide evidence against an alternative model of ipsdienol production involving gut-tract bacteria (Borden 1985). Since an insectan *HMG-R* was induced by JH III, and this enzyme is not part of the deoxyxylulose–phosphate (DOX-P) pathway utilized by many prokaryotes for isoprenoid synthesis, de novo ipsdienol production by gut bacteria is unlikely (Rohmer et al. 1993; Lichtenthaler et al. 1997).

A role for the diet in the origin of lepidopteran pheromones was once considered (Hendry et al. 1975; Hendry 1976) and rejected (Miller et al. 1976), and a current review of research on pheromone biosynthesis concludes that most insects produce pheromone components de novo (Tillman et al. 1999). Until recently, however, dietary precursors played a prominent role in our understanding of bark beetle pheromone production, particu-

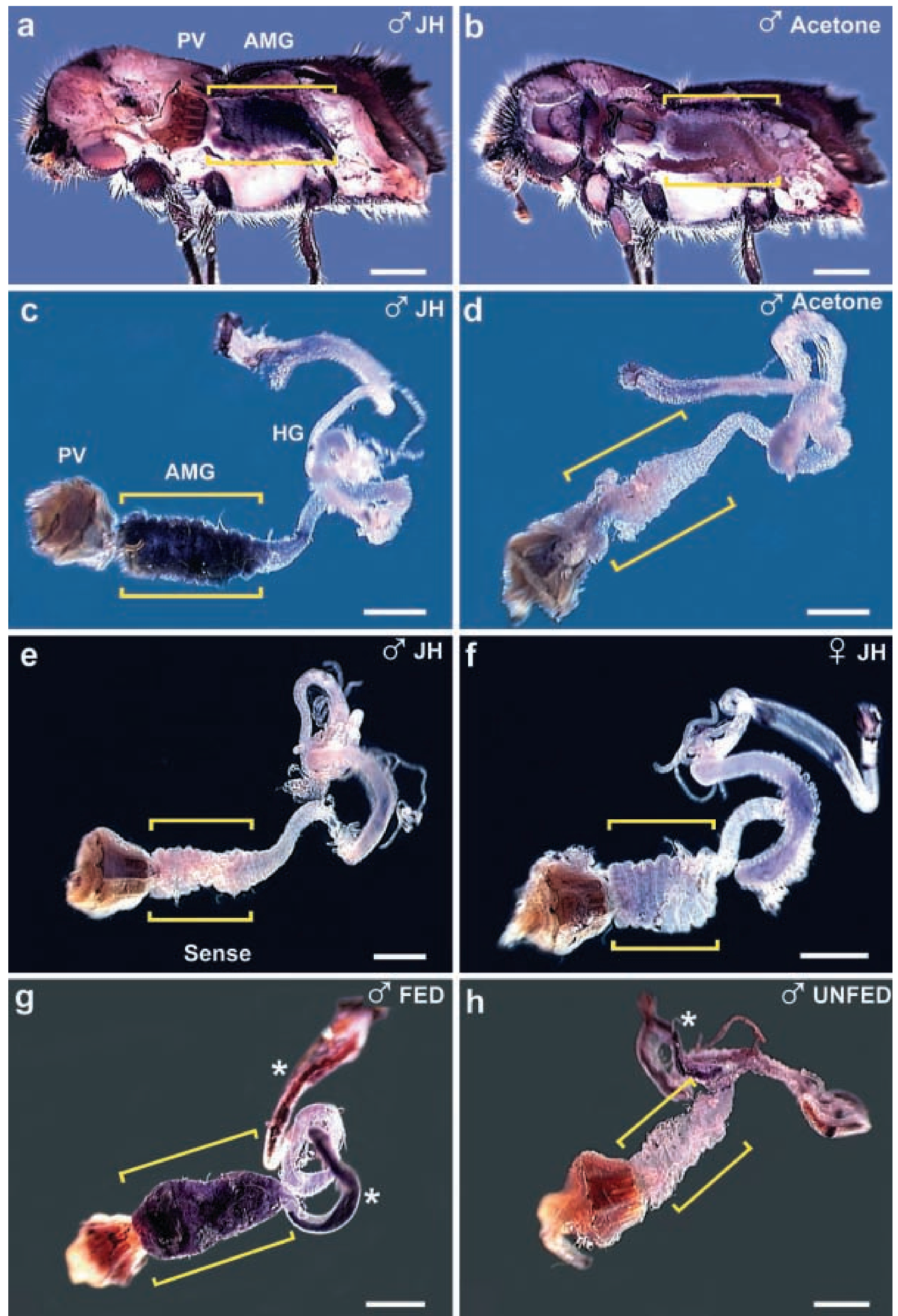

Fig. 2a–h Anatomical mapping of JH III- and feeding-induced *HMG-R* expression in *Ips pini* via in situ hybridization. **a, b** Exposed sagittal view revealing proventriculus and anterior midgut of a JH III-treated male (**a**) and an acetone-treated male control (**b**). **c–h** Isolated alimentary canals of JH III-treated male (**c**), acetone-treated male control (**d**), JH III-treated male sense control (**e**), JH III-treated female control (**f**), fed male (**g**), and unfed male control (**h**). *Yellow brackets* denote the anterior midgut region in all panels. *JH* denotes topical treatment with juvenile hormone III. *PV* Proventriculus, *AMG* anterior midgut, *HG* hindgut. The *asterisks* in **g** and **h** denote nonspecific staining of fecal material in gut tract. *Scale bars* 0.5 mm

larly ipsdienol. It will be of interest to determine the relative role of dietary precursors versus de novo synthesis in the production of other terpenoid and non-terpenoid bark beetle pheromone components.

When combined with recent work demonstrating de novo production of pheromone components in *Ips* spp. (Ivarsson et al. 1993; Seybold et al. 1995b; Tillman et al. 1998), our data establish a new paradigm for bark beetle pheromone production. Synthesis of pheromone components by midgut tissue and subsequent movement through the alimentary canal comprise a logical and efficient process that agrees with data from *I. paraconfusus* showing that ipsdienol and ipsenol accumulate in the hindgut (Byers 1983) before being excreted into the frass with the fecal pellet. To our knowledge, this is the first time that midgut tissue has been shown to produce pheromone in an insect. We therefore postulate the existence of highly modified alimentary canal cells: veritable

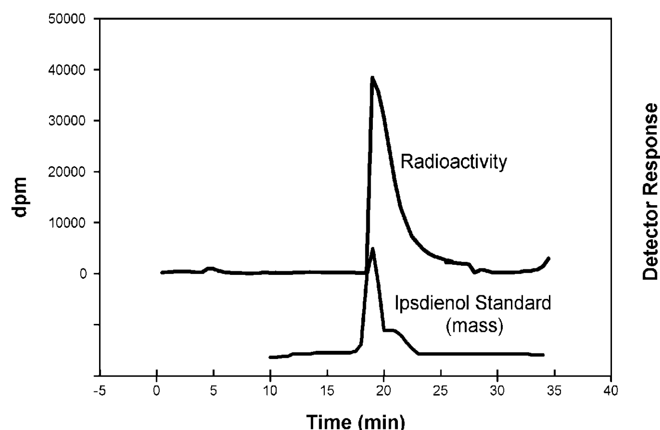

Fig. 3 Radio-HPLC of pentane-diethyl ether extract of midgut tissue from five fed male *Ips pini* incubated with 5 μCi [1-^{14}C]acetate as described in the text

'pheromone factories' whose specialized machinery uniquely responds to increased levels of JH III with rapid and copious production of pheromone components.

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