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Functional expression of a bark beetle cytochrome P450 that hydroxylates myrcene to ipsdienol ☆

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Abstract

The final steps in the pheromone-biosynthetic pathway of the pine engraver beetle, *Ips pini* (Say) (Coleoptera: Scolytidae) are unknown, but likely involve myrcene (7-methyl-3-methylene-1,6-octadiene) hydroxylation to produce the aggregation pheromone component, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol). We have isolated a full-length *I. pini* cDNA encoding a cytochrome P450, *CYP9T2*. The recovered cDNA is 1.83 kb and the open reading frame encodes a 532 amino acid protein. *CYP9T2* is regulated by the same physiological factors that induce pheromone production. Quantitative real-time PCR experiments showed that feeding on host phloem induced *CYP9T2* expression in males, but not females, and that basal expression levels are highest in male midguts, similar to other *I. pini* pheromone-biosynthetic genes. Microsomes prepared from Sf9 cells co-expressing baculoviral-mediated recombinant CYP9T2 and housefly (*Musca domestica*) NADPH-cytochrome P450 reductase converted myrcene to ipsdienol. The product identified by coupled GC-MS was mostly (4*R*)-(−)-ipsdienol, an important aggregation pheromone component for western North American *I. pini*. These results are consistent with *CYP9T2* encoding a myrcene hydroxylase that functions near the end of the pheromone-biosynthetic pathway.

Keywords: P450; Bark beetle; Pheromone biosynthesis; Detoxification; Functional expression; Monoterpene; Baculovirus

1. Introduction

Pine bark beetles are intimately associated with monoterpenoids both as host resin defense components (Raffa, 2001; Trapp and Croteau, 2001; Langenheim, 2003) and as semiochemicals (Seybold et al., 2000; Santos et al., 2006). The monoterpene, myrcene (7-methyl-3-methylene-1,6-octadiene), is of particular significance to the pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *I. pini* encounters host-produced myrcene when individuals infest weakened or fallen trees and branches from a number of species (Anderson, 1969; Kegley et al., 2002). Male *I. pini* exposed to myrcene vapors produce racemic ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Quilici, 1997), consistent with the suggestion that successful colonization involves metabolism of host monoterpenes (Byers, 1995; Phillips and Croteau, 1999).

Male *I. pini* also produce myrcene de novo (Martin et al., 2003) as a probable intermediate in the biosynthesis of aggregation pheromone. The aggregation pheromone of western North American populations is a blend of mostly (4*R*)-(−)-ipsdienol (Birch et al., 1980; Seybold et al., 1995a, b; Miller et al., 1997). It is produced by males de novo (Seybold et al., 1995a, b) in the anterior midgut (Hall et al., 2002) and is regulated by feeding and juvenile hormone (JH) III (Tillman et al., 1998; Seybold et al., 2000). The metabolic pathway diverts from the mevalonate pathway at geranyldiphosphate (GPP) through the activity of GPP synthase (GPPS) (Fig. 1) (Gilg et al., 2005). The intermediate steps from GPP to ipsdienol are unclear, but likely include myrcene as a metabolic intermediate (Hughes, 1974; Hendry et al., 1980; Seybold et al., 1995a, b; Martin et al., 2003). Thus, both diet and endogenous metabolism are sources of myrcene in *I. pini*.

☆ *Data deposition:* The sequence reported in this paper has been deposited in GenBank, accession no. DQ676820.

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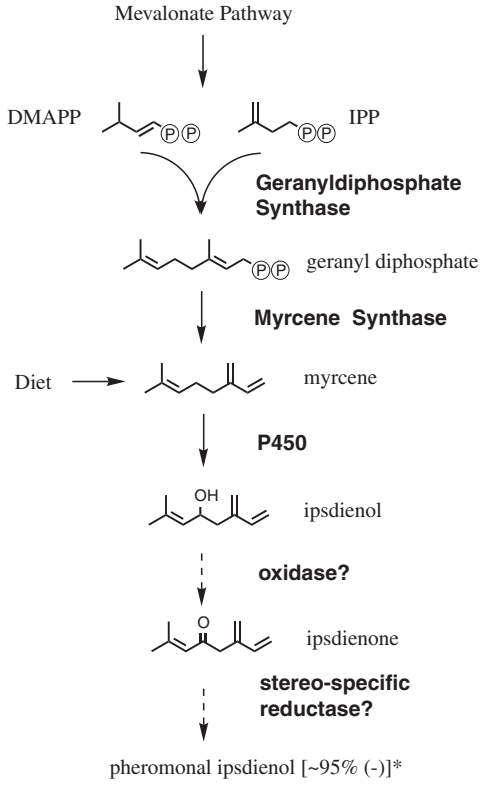

Fig. 1. Monoterpene metabolism in *I. pini*. Myrcene is ingested in the diet and produced *de novo*, and converted to ipsdienol. Hypothesized reactions involving ipsdienone are shown with dashed arrows. Circled "P"s designate phosphates. DMAPP, dimethylallyldiphosphate; IPP, isopentenyldiphosphate. *The enantiomeric composition for "pheromonal ipsdienol" is noted for western North American *I. pini* populations.

Vanderwel and Oehlschlager (1987) predicted that a cytochrome P450 enzyme likely hydroxylates myrcene to ipsdienol. Insect cytochromes P450 are physiologically important for biosynthesis and degradation of endogenous compounds, including pheromones, and also catalyze metabolic detoxification of insecticides and host plant chemicals (Wen et al., 2003; Feyereisen, 2005). The different enantiomeric blends of ipsdienol produced from detoxification and pheromone biosynthesis in *I. pini* suggests either stereo-selective modification to increase the enantiomeric excess of (4*R*)-(−)-ipsdienol (Fig. 1), as is implied in *Ips paraconfusus* (Fish et al., 1984), or that enzymes with differing stereo-specific product profiles may hydroxylate myrcene in *I. pini*.

Of nearly 200 identified coleopteran P450 sequences, most are from the recently completed *Tribolium* genome project (http://www.bioinformatics.ksu.edu/BeetleBase) and none have been functionally characterized. Here we report the cDNA isolation and functional expression of *CYP9T2*, a putative pheromone-biosynthetic P450 from *I. pini*. It is the most highly transcribed P450 gene in pheromone-biosynthetic *I. pini* midguts and has an expression pattern consistent with other pheromone-biosynthetic genes (Keeling et al., 2004, 2006). Functional assays show that CYP9T2 hydroxylates myrcene specifically to produce ipsdienol, and that the western *I. pini* pheromone component, (4*R*)-(−)-ipsdienol, is produced in ~fourfold excess over the non-pheromonal (4*S*)-(+)-enantiomer. This first example of an insect pheromone-biosynthetic P450 confirms the prediction that ipsdienol production is catalyzed by a P450 (Vanderwel and Oehlschlager, 1987; Hunt and Smirle, 1988) and suggests an explanation for the presence of the putative pheromone precursor ipsdienone in male *I. pini*.

2. Materials and methods

2.1. Reagents and chemicals

Hink's TNM-FH Medium 1x (Supplemented Grace's Medium) and Grace's Insect Basal Medium 1x were from Mediatech, Inc. (Herndon, VA) and FBS was from Atlas Biologicals (Fort Collins, CO). Grace's Insect Medium 2x, 4% Agarose Gel, and Pluronic F-68 were from Gibco (Grand Island, NY). The Sf9 cells were a gift from G. Pari (U. Nevada, Reno) and the housefly reductase baculoviral clone (Andersen, 1997; Wen et al., 2003) was kindly provided by M. Schuler (U. Illinois at Urbana-Champaign). [4-^{2}H]myrcene and the ipsdienol standard were gifts from D. Vanderwel (U. Winnipeg) and C. Oehlschlager (ChemTika International, San Jose, Costa Rica), respectively. Unlabeled myrcene and hemin were from Sigma-Aldrich (St. Louis, MO). All oligonucleotides were from Integrated DNA Technologies (Coralville, IA).

2.2. Insects

Immature *I. pini* were obtained from infested Jeffrey pine (*Pinus jeffreyi*) bolts collected from the Sierra Nevada in California and Nevada, USA and reared to adults as per Browne (1972). Emerged adult *I. pini* were collected daily, separated by sex according to Wood (1982), and stored for up to 2 weeks at 4 °C in moist paper towels. Larvae were removed from under the bark and dissected the same day.

2.3. cDNA isolation

Clustering analysis of *I. pini* ESTs suggested that cluster 128 (12 EST) represented the most highly expressed cytochrome P450 in the midgut (Eigenheer et al., 2003). The longest cDNA in the cluster, *IPG002G06* (**CB408249**), was selected for further sequencing by primer walking (Table 1) of purified plasmid template DNA (Qiagen, QIAprep Spin Miniprep Kit, Valencia, CA) using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1. The reactions were run on an ABI3730 DNA Analyzer at the Nevada Genomics Center (UNR) and the sequences were analyzed using Vector NTIv.9 software (Informax, N. Bethesda, MD).