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# Myrcene Hydroxylases do not Determine Enantiomeric Composition of Pheromonal Ipsdienol in *Ips* spp.

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**Abstract** Myrcene (7-methyl-3-methylene-1,6-octadiene) hydroxylation is likely one of the final reactions involved in the production of the *Ips* spp. (Coleoptera: Scolytidae) aggregation pheromone components, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) and ipsenol (2-methyl-6-methylene-7-octen-4-ol). To gain insight into the evolution of pheromone production, we isolated a full-length cDNA from the pinyon ips, *Ips confusus* (LeConte), that encodes a pheromone-biosynthetic cytochrome P450, *I. confusus* CYP9T1 (IcCYP9T1). The recovered cDNA is 1.70 kb, and the open reading frame encodes a 532 amino acid protein. IcCYP9T1 is 94% identical to the pine engraver, *Ips pini* (Say), CYP9T2 ortholog that hydroxylates myrcene. Quantitative real-time PCR experiments showed that *IcCYP9T1*, as does *CYP9T2*, has an expression pattern similar to other pheromone-biosynthetic genes in *I. pini*. Basal expression levels were higher in males than females, and expression was significantly induced in male, but not in female, anterior midguts by feeding on host phloem. Microsomes, prepared from Sf9 cells co-expressing baculoviral-mediated recombinant IcCYP9T1 and house fly (*Musca domestica*) NADPH-cytochrome P450 reductase, converted myrcene to ~85%-(*R*)-(−)-ipsdienol. These results are consistent with *IcCYP9T1* encoding a myrcene hydroxylase that functions near the end of the pheromone-biosynthetic pathway. Since the *I. confusus* pheromone blend contains >90%-(*S*)-(+)-ipsdienol, these results confirm further that *Ips* spp. myrcene hydroxylases do not control the final ipsdienol enantiomeric blend. Other enzymes are required following myrcene hydroxylation to achieve the critical quantity and enantiomeric composition of pheromonal ipsenol and ipsdienol used by different *Ips* spp.

**Keywords** *Ips* · Bark beetle · Pheromone · P450 · Pheromone biosynthesis · Monoterpene · Functional expression

Sequences

The IcCYP9T1 cDNA sequence has been deposited in Genbank, accession number EU915209

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## Introduction

Pine bark beetles rely on aggregation pheromones to coordinate efforts to colonize host trees. *Ips* spp. (Coleoptera: Scolytidae) pheromones include the chiral monoterpenoid alcohols, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) and/or ipsenol (2-methyl-6-methylene-7-octen-4-ol). There is considerable variation in the quantity and enantiomeric composition of these compounds produced among species, within different populations, and even among individuals within a population (Lanier et al. 1980; Byers 1989; Miller et al. 1989). For example, the principal pheromone component of western populations of the pine engraver, *Ips pini*, consists of ~95% (*R*)-(−)-ipsdienol, whereas eastern populations produce much greater amounts of (*S*)-(+)-ipsdienol (Miller et al. 1997; Domingue et al. 2006).

The mechanism by which the ratio of ipsdienol enantiomers is controlled is not clear. Ipsdienol is synthe-

sized de novo via the mevalonate pathway (Seybold et al. 1995) in the anterior midguts of male *I. pini* (Hall et al. 2002), *Ips confusus* (Ginzel et al., unpublished data), and probably also in other *Ips* spp. Although early steps in the pathway are well understood in *I. pini*, final steps are still being characterized. The ipsdienol biosynthetic pathway branches from the main mevalonate pathway via the activity of a dual function enzyme, geranyldiphosphate synthase/myrcene synthase (GPPS/MS) (Gilg et al. 2005, Unpublished data). Achiral myrcene, whether produced endogenously (Martin et al. 2003) or ingested with the phloem, is then hydroxylated to ipsdienol by CYP9T2 (Sandstrom et al. 2006). Since CYP9T2 from a western *I. pini* population produces ipsdienol in vitro in a ~4:1 (*R*)-(−):(*S*)-(+) ratio, other enzymes must act to increase the proportion of (*R*)-(−)-ipsdienol before it is released. Indeed, Vanderwel (unpublished data) proposed that an oxidoreductase system oxidizes ipsdienol to ipsdienone and then stereo-selectively reduces it to (*R*)-(−)-ipsdienol, and a similar mechanism was proposed to determine the final enantiomeric composition of ipsdienol in *Ips paraconfusus* (Fish et al. 1984) (Fig. 1). Genetic analyses of eastern and western *I. pini* populations suggest that, while a single locus contributes significantly to the observed distribution of enantiomers (Domingue et al. 2006), other factors are involved, and the stereoselectivity of the cytochrome P450 may be important in some instances (Domingue and Teale 2008).

Although significant progress has been made in understanding pheromone biosynthesis and regulation in *I. pini*, studies that involve species with different pheromone ratios would be useful to investigate the mechanism(s) determining pheromonal ipsdienol composition, and to provide insight into the evolution of pheromone systems. To this end, we have begun studies of pheromone biosynthesis in the pinyon ips, *I. confusus*. *I. confusus* is reproductively isolated from *I. pini* by both its host preference and pheromone system. *I. confusus* infests and kills pinyon pines (*Pinus monophylla* and *Pinus edulis*) (Furniss and Carolin 1977), whereas *I. pini* in the southwestern USA largely infests weakened or fallen trees and branches of *Pinus ponderosa* (ponderosa pine), *Pinus contorta* (lodgepole pine), and *Pinus jeffreyi* (Jeffery pine) (Kegley et al. 2002). The aggregation pheromone blend emitted by *I. confusus* consists of about 99%-(S)-(−)-ipsenol and 95%-(*S*)-(+)-ipsdienol (Young et al. 1973), very different from the approximately 95%-(*R*)-(−)-ipsdienol produced by the western North American *I. pini* (Miller et al. 1997).

Here, we report the isolation and characterization of a CYP9T2 ortholog, IcCYP9T1, from *I. confusus* in order to assess its role in pheromone biosynthesis and in determining the enantiomeric ratio of the key pheromone component, ipsdienol.

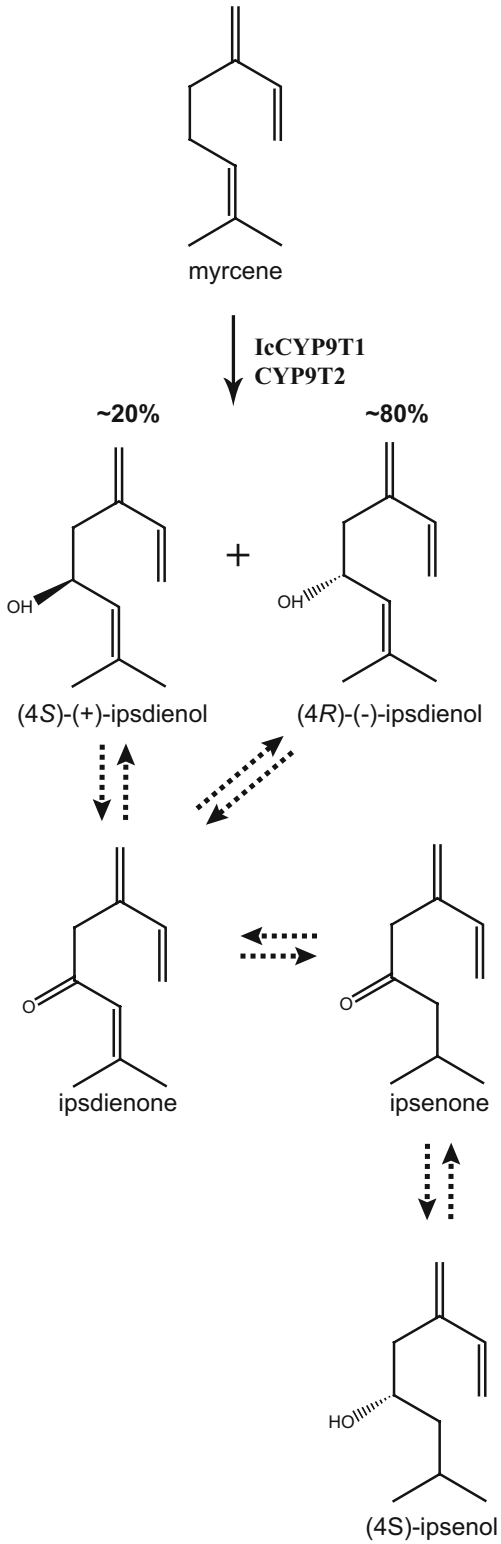


**Fig. 1** Pheromone production in *Ips pini* and *Ips confusus*. Recombinant IcCYP9T1 and CYP9T2 are able to convert myrcene, which is ingested in the diet and produced de novo, to a ~5:1 mixture of (*R*)-(−):(*S*)-(+)-ipsdienol. Hypothesized pheromone-biosynthetic reactions, to achieve the crucial enantiomeric compositions of mostly (−)-ipsdienol for western North American *I. pini* populations and mostly (−)-ipsenol and (+)-ipsdienol for *I. confusus*, are shown as *dashed lines*

## Methods and Materials

*Insects* Immature *I. confusus* were obtained from infested *P. edulis* bolts collected from the Bureau of Land Management (BLM), land east of Carson City, Nevada, USA. The insects were reared in a greenhouse to adults as per Browne (1972) and emerged adults (i.e., those that exited the brood tree) were collected daily. Adults were separated by sex according to Lanier and Cameron (1969) and were stored for up to 2 weeks at 4°C in moist paper towels.

*Experimental* All reagents and chemicals, cloning procedures, cell culture, recombinant protein production, microsome preparation, and enzyme assays were performed essentially as described previously (Sandstrom et al. 2006), with the following modifications:

*cDNA isolation* Two male *I. confusus* were inserted into separate small holes drilled into the phloem of fresh *P. edulis* bolts, secured with wire mesh, and allowed to feed for 23 h before their midguts were dissected and flash frozen in liquid nitrogen. The midguts were kept overnight at −80°C, and RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen). To obtain a cDNA homologous to CYP9T2, first-strand cDNA synthesis and 3′ and 5′-Rapid amplification of cDNA ends (RACE) was achieved with the Clontech SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). *CYP9T2*-specific primers ("Ic9T2F1", GTGGCAAAAACTAACACCGCTGAAGAC; "Ic9T2R1", GTCTTCAGCGGTGTTAGTTTTTGCCAC, and "Ic9T2R2", CCGATTCATAGGTGACTTTGTTCCC), and the provided anchor primer (10× Universal Primer A Mix) were used as described in the manufacturer's instructions. Amplified products from the RACE PCR reactions were visualized on agarose gels, cloned into pST-Blue-1 AccepTor™ Vector (Novagen, San Diego, CA, USA), and transformed in *E. coli* NovaBlue Singles™ Competent Cells (Novagen). Recombinants were identified by colony PCR as described by the AccepTor™ Vector Kit protocol. Purified PCR products were sequenced by primer walking by using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 on an ABI3730 DNA Analyzer at the Nevada Genomics Center (UNR). All sequence analysis was done with Vector NTIv.9 software (Informax, N. Bethesda, MD, USA). The full-length cDNA was assembled from the overlapping RACE products and confirmed by PCR amplification of first strand cDNA with primers flanking the putative open reading frame (ORF). The complete deduced amino acid sequence was submitted to the P450 Nomenclature Committee (David Nelson, personal communication) and was given the name *Ips confusus CYP9T1* (IcCYP9T1).

*Expression Analysis* mRNA levels were determined by quantitative Real Time RT-PCR (qRT-PCR). cDNA templates for the fed male and female anterior midgut time-course studies were prepared previously (J. Bearfield, unpublished). Briefly, beetles were fed for 4, 8, 16, or 32 h, while unfed controls were held in plastic cups in the dark. There were six biological replicates that each included five beetles. For the tissue-distribution study, males and females were either allowed to feed on the phloem of *P. edulis* bolts or kept as unfed controls in plastic cups in the dark for 26 h and then dissected in water under a stereomicroscope. Tissues from the head, anterior midgut, posterior midgut, hindgut, fat body, and carcasses of eight insects were pooled and frozen in $N_2$(l). There were four male and female biological replicates.

Real time PCR primers with minimal potential for primer–dimer formation were identified by using Vector NTI (Version 7.1, Invitrogen) among primers suggested by Primer Express software (Applied Biosystems, Foster City, CA, USA). Relative gene expression was determined with the ΔΔCT method (Livak and Schmittgen 2001). The results were normalized with an internal control gene, *cytoplasmic actin*, which is unaffected by feeding in *I. confusus* (J. Bearfield, unpublished).

*Cloning* The *IcCYP9T1* ORF was amplified by PCR by using Ic9T1F1 (GCACCATGGTGGTCGGGTTGGTT) and IcCYP9T1R1 (tagged; GCCTCGAGGGTTCAAATG CAAGGT) primers, directionally cloned into the *NcoI* and *Xho*I sites of pENTR4 (Invitrogen) by standard methods (Sambrook et al. 1989), and transformed into DH5α cells. The reverse primer ensured that the construct would have a C-terminal extension containing a V5 epitope and the polyhistidine tag (encoded by the vector). An untagged version was not constructed because products and their relative abundance were statistically identical in assays of both the untagged and tagged versions of CYP9T2 (Sandstrom et al. 2006). The recombinant plasmid, pENTR4-IcCYP9T1V5H6, was confirmed by sequencing.

*Recombinant IcCYP9T1 Protein Detection* IcCYP9T1 production was determined by western blotting using 1:10,000 Anti-V5 primary antibody (Invitrogen), 1:20,000 Goat Anti-Mouse secondary antibody (Biorad, Hercules, CA, USA), and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

*Enzyme Assays* Recombinant IcCYP9T1 assays were performed as described by Sandstrom et al. (2006).

*Gas Chromatography–Mass Spectrometry* A Thermo Finnigan Polaris Q ion trap coupled with a Trace gas chromatograph was used for the gas chromatography–

mass spectrometry (GC-MS) analyses. The GC was fitted with a 60 m×0.25 mm i.d. DB-5 capillary column (J&W Scientific, Palo Alto, CA, USA) and was programmed from 50 (1 min hold) to 200°C at 5°C $min^{-1}$, and then to 320°C at 10°C $min^{-1}$. Split injection (100:1 ratio) with a constant column flow of 1.5 ml $min^{-1}$ of helium was used. The enantiomeric composition of ipsdienol was determined by chiral separation by using the same instruments with a CycloSil-B (30 m×0.25 mm i.d., 0.25 μm film thickness) column (J&W Scientific) at 100°C and with a helium flow rate of 1.3 ml $min^{-1}$.

## Results

*DNA Isolation and Expression* The cDNA for the CYP9T2 ortholog from *I. confusus* was isolated by PCR techniques. The 1,699 bp cDNA contained a 1,596 nt ORF encoding a 532 amino acid (a.a.) protein flanked by 74 nt and 26 nt 5′ and 3′ UTRs, respectively, and is therefore likely full-length. The predicted translation product has a molecular mass of 61 kDa and a pI of 8.9 (Gasteiger et al., 2003). It was designated *I. confuses* CYP9T1 (IcCYP9T1) by the P450 Nomenclature Committee (David Nelson, personal communication). The primary structure has an N-terminal membrane anchor and typical P450 conserved residues, including the WxxxR, ExxR, and PxxFxPERF ('PERF') motifs, and the canonical heme-binding domain (PFxxGxRxCxG) surrounding the heme-cysteine ligand (Cys475; Fig. 2) (Feyereisen 2005). BLAST searches (Altschul et al. 1990) indicated IcCYP9T1 is most similar to coleopteran CYP9s (not shown). It has a 98% a.a. identity (7 a.a. different) with the functionally uncharacterized partial length CYP9T1 from *I. paraconfusus* (IpCYP9T1) (Huber et al. 2007) and is 94% identical (34 a.a. different) to the *I. pini* myrcene hydroxylase CYP9T2 (Sandstrom et al. 2006) (Fig. 2).

Message levels were determined by qRT-PCR by using *cytoplasmic actin* as a normalizing gene. *IcCYP9T1* mRNA levels in males increased substantially over time upon feeding, with a maximal induction of approximately 400-fold at 32 h (Fig. 3a). In contrast, *IcCYP9T1* mRNA levels in females were repressed at 4, 8, and 16 h and only minimally induced at 32 h by feeding (Fig. 3a). *IcCYP9T1* mRNA localized predominantly to the anterior midgut of fed males (Fig. 3b). Levels in females were substantially lower than those in males, with no clear induction in any tissue upon feeding (Fig. 3b).


**Fig. 2** Alignment of the deduced a.a. sequences of IcCYP9T1, IpCYP9T1 (ABF06554) and CYP9T2 (ABG74909). Conserved residues are indicated by a *dash*. The missing portion of IpCYP9T1 is indicated by a *gap* (a.a. 1–20). The membrane anchor domain is *underlined*. Conserved WxxxR, ExxR, and PxxF motifs are *boxed*. The heme-binding domain (PFxxGxRxCxG) is *shaded*

*Enzyme Assays and Product Formation* A baculoviral system was used to produce sufficient enzyme to analyze IcCYP9T1 functionally. The V5-epitope was used to follow recombinant protein production, as an antibody for IcCYP9T1 was unavailable. Western blot analysis indicated high expression of an approximately 65 kDa protein at 4 and 5 days post-infection (not shown). CO-difference absorption spectra of microsomes from Sf9 cells producing IcCYP9T1 had the characteristic peak at 450 nm, while microsomes from Sf9 cells without recombinant protein did not (not shown).

Assays with microsomes from Sf9 cells that co-express recombinant IcCYP9T1 and housefly P450 reductase were analyzed by GC-MS for myrcene hydroxylation products. Selective ion monitoring (SIM) analysis at *m/z*=85 (for ipsdienol) of microsomes incubated with unlabeled myrcene yielded a product with a retention time and mass spectrum identical to an ipsdienol standard (Fig. 4a). Products from reactions incubated with deuterium-labeled myrcene had the same retention time (not shown), but with a mass spectrum of appropriate *m/z* diagnostic fragments that was 1 amu larger. A time course of 5, 10, and 15-min reactions with unlabeled myrcene showed a linear increase in ipsdienol production relative to the *n*-octanol standard (not shown). Ipsdienol was not detectable by SIM analysis of products from reactions run in the absence of substrate (not shown), boiled microsomes (Fig. 4b), or microsomes infected with housefly P450 reductase baculovirus construct only (not shown). No other hydroxylated versions of myrcene (amitinol, *E*-myrcenol (2-methyl-6-methylene-2,7-octadien-1-ol), linalool (2,6-dimethylocta-2,7-dien-6-ol), or geraniol (2,6-dimethyl-2,6-octadien-8-ol)) were detected in any sample.

GC-MS analysis with a CycloSil-B column, of extracts of recombinant IcCYP9T1/P450 reductase, incubated with either unlabeled or deuterium-labeled myrcene, produced two major peaks with retention times corresponding to (*R*)-(−)-ipsdienol and (*S*)-(+)-ipsdienol. Reactions using unlabeled myrcene as substrate yielded enantiomeric percentages that averaged 85%-(*R*)-(−)-ipsdienol (Fig. 4c).

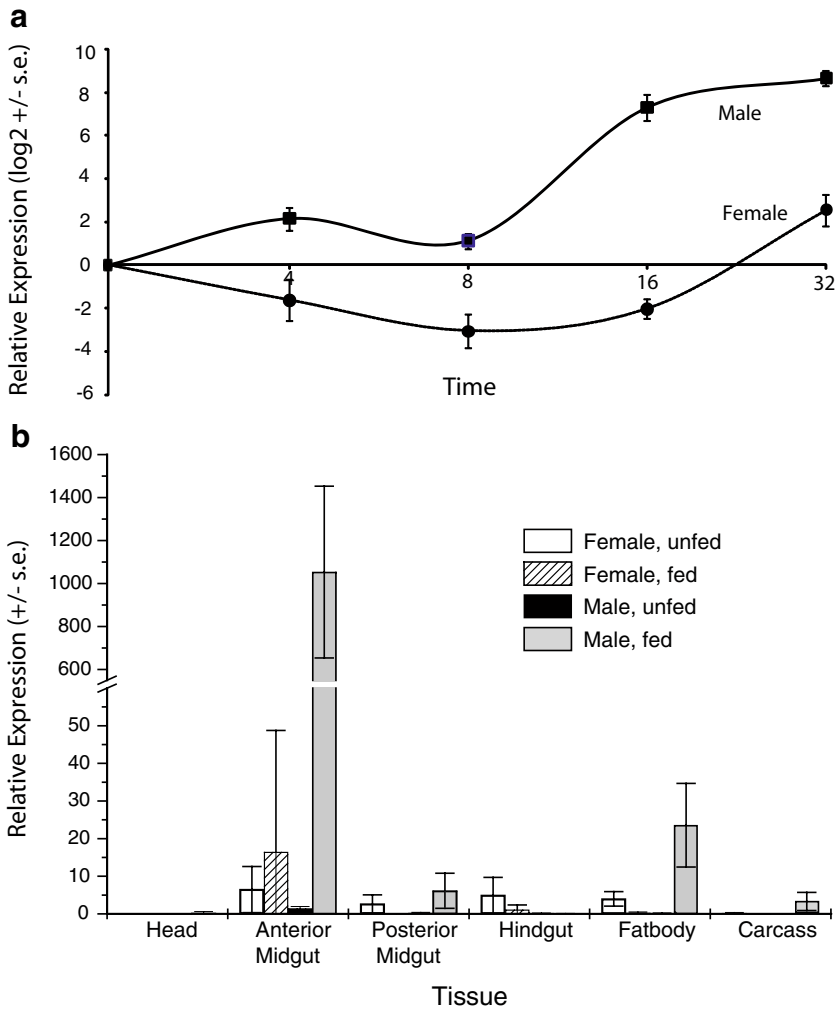


**Fig. 3** Real time quantitative RT-PCR analysis of IcCYP9T1 mRNA levels. **a** Time course of midgut expression ($log_2$) comparing fed males and females. **b** Relative *IcCYP9T1* mRNA levels (+/− standard error) in various tissues of starved or fed (26 h) males and females

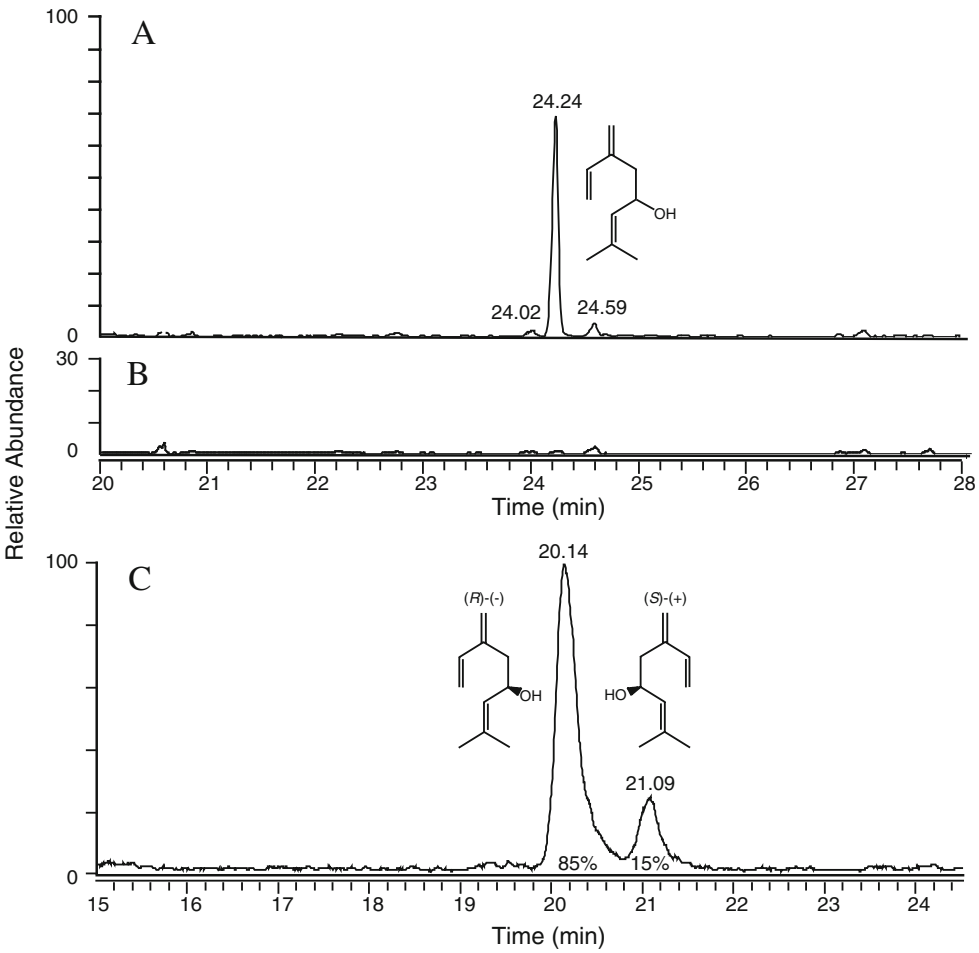


**Fig. 4** Gas chromatography–mass spectrometry (GC-MS) analysis of ipsdienol formed from myrcene by recombinant IcCYP9T1. Selected ion monitoring (*m/z*=85 for ipsdienol) using a DB-5 column for reactions containing **a** unlabeled myrcene (identical to the ipsdienol standard, not shown) or **b** boiled microsomes (negative control). **c** Enantiomeric ratio of reaction products, determined by chiral GC-MS

## Discussion

We report the isolation and functional expression of a monoterpene-oxidizing cytochrome P450 cDNA, IcCYP9T1, which produces the pheromone component, (*R*)-(−)-ipsdienol. The predicted translation product has a hydrophobic N-terminal target sequence and many motifs, including the classic heme-binding domain, common to P450s (Fig. 2). A CYP9T1 partial cDNA was first isolated and characterized from the California five-spined ips, *I. paraconfusus*, although its biochemical function was not determined (Huber et al. 2007). *I. paraconfusus* and *I. confusus* are sibling species, and once were considered a single species. It is, therefore, not surprising that the IcCYP9T1 and IpCYP9T1 orthologs share such high (98%) sequence identity (Fig. 2). The seven non-identical amino acids are nevertheless conserved, and appear located in positions that do not contribute to the active site (not shown), suggesting that the activities of the two enzymes should be similar.

Bark beetle pheromone-biosynthetic genes are typically up-regulated by feeding (Keeling et al. 2004), transcribed predominantly in the anterior midgut (Tillman et al. 2004; Gilg et al. 2005; Bearfield et al. 2006), and have basal expression levels that are higher in the pheromone-producing sex (Keeling et al. 2004, 2006). IcCYP9T1 mRNA levels increased substantially in male, but not in female, anterior midguts in response to feeding (Fig. 3a,b). *IcCYP9T1* mRNA was predominantly localized to the anterior midgut of pheromone-producing male *I. confusus* (Fig. 3b), and there was a fivefold basal level difference between male and female expression. These data correlate well with expression patterns of other *I. confusus* (Bearfield et al., unpublished), *I. paraconfusus* (Ivarsson et al. 1998; Tittiger et al. 1999; Huber et al. 2007) and *I. pini* pheromone-biosynthetic genes (Keeling et al. 2006), and strongly support a role for *IcCYP9T1* in male-specific aggregation pheromone biosynthesis.

Interesting differences in transcript accumulation patterns exist between *CYP9T2, IcCYP9T1*, and *IpCYP9T1*. Feeding induced an approximately 28-fold *CYP9T2* mRNA expression in male *I. pini* midguts (Sandstrom et al. 2006), over 400-fold expression in male *I. confusus* midguts (Fig. 3a,b), and an astonishing 85,000-fold expression in male *I. paraconfusus* whole bodies (Huber et al. 2007).

There are also differences in the timing of the up-regulation, with male *I. paraconfusus* achieving high levels within only 8 h of feeding (Huber et al. 2007), while the highest expression in *I. confusus* (Fig. 3a) and *I. pini* (Sandstrom et al. 2006) occurred by 32 h. Finally, while the difference between male and female basal levels of expression was not reported for *I. paraconfusus*, male *I. confusus* had an approximately five-fold higher expression level than females (not shown), which was much less than the ~500-fold difference in basal CYP9T2 mRNA levels in observed in male and female *I. pini* (Keeling et al. 2006). It should be emphasized that the study by Huber et al. (2007) investigated whole bodies, and the tissue distribution of IpCYP9T1 is unknown. Furthermore, IpCYP9T1 levels in that study were normalized to *CYP4G27*, rather than *cytoplasmic actin*. We analyzed the stability of expression of four candidate normalizing genes, *CYP4G27*, *cytoplasmic actin*, *β-tubulin*, and *ubiquitin C* in *I. confusus* by using qBase (Hellemans et al. 2007) and found that *CYP4G27* and *cytoplasmic actin* were similarly stable (A. Griffith, unpublished data). We chose to continue to use *cytoplasmic actin* as a normalizing gene in order to maintain continuity with previous studies. Thus, while quantitative comparisons between our studies and that of Huber et al. (2007) may have reduced value, the general trends among the three species appear qualitatively similar.

Similarities in sequence and expression patterns with the myrcene hydroxylase CYP9T2 (Sandstrom et al. 2006) suggested myrcene as a logical IcCYP9T1 substrate. A baculovirus system was used to produce sufficient enzyme for functional assays. Sf9 microsomes that contain recombinant IcCYP9T1 readily hydroxylated myrcene to ipsdienol (Fig. 4). The amount of ipsdienol produced increased linearly with incubation time, and myrcene hydroxylation activity was abolished when microsomes were heat-denatured prior to the reaction (Fig. 4b), confirming that ipsdienol is an enzymatic product. Furthermore, microsomes from cells that were infected with recombinant housefly P450 reductase baculovirus (without recombinant IcCYP9T1) did not hydroxylate myrcene (not shown). Thus, ipsdienol production was due to IcCYP9T1 and not to an endogenous activity of Sf9 cells. Other possible myrcene hydroxylation products were not detected, suggesting high product specificity.

Recombinant IcCYP9T1 produced ~85%-(−)-ipsdienol, similar to the ~81%-(−)-ipsdienol produced by recombinant CYP9T2 (Sandstrom et al. 2006). This enantiomeric excess is nearly antipodal to the ~90%-(+)-ipsdienol found in the *I. confusus* pheromone blend. While the enantiomeric ratios of ipsdienol from recombinant enzyme assays may be affected by experimental conditions (i.e., the reactions were done with Sf9 microsomes, not purified recombinant enzyme), the observed excess of (*R*)-(−)-ipsdienol is assumed to be biologically relevant. Our data strengthen the observation that myrcene hydroxylases contribute little to the final enantiomeric blend of pheromonal ipsdienol (Sandstrom et al. 2006), and further support the suggestion that enzymes downstream from myrcene hydroxylation are required to achieve the crucial enantiomeric compositions of pheromone used by different species. The terminal steps in ipsenol and ipsdienol pheromone biosynthesis in scolytids likely involve oxidases and/or reductases that catalyze the interconversion between ipsdienol, ipsdienone, ipsenone, and ipsenol (Fish et al. 1979; Ivarsson 1997; Vanderwel, personal communication). There may be situations, such as those that can occur in hybrid zones, in which downstream enzymes with different product profiles may compete, or not be active, and the enantiomeric composition would then be determined by the P450 (Domingue and Teale 2008).

Functional expression of IcCYP9T1 provides insight into P450 evolution and control of *Ips* spp. pheromone production. The enantiomeric ratios of ipsdienol produced by IcCYP9T1 and CYP9T2 are similar and apparently unrelated to the final pheromone blend. Even though a single a.a. change can alter P450 activity (Feyereisen 2005), the 94% a.a. identity between IcCYP9T1 and CYP9T2 appears to be sufficient to confer similar activity. The conservation of activities can be taken to indicate a common ancestral function, most likely host-resin detoxification. However, both genes appear to have acquired, or else inherited from a common ancestor, regulatory motifs that coordinate their transcription with other pheromone-biosynthetic genes. Indeed, co-ordinate regulation would almost be necessary given the large amounts of toxic myrcene produced endogenously during pheromone biosynthesis. In this respect, these P450s still function as detoxification enzymes in pheromone-biosynthetic midguts. Thus, they may serve as an example of how enzymes acquire new biological roles by variations in their corresponding gene expression, despite retaining their original activities. This is consistent with the application of pre-existing metabolic activities to conserve resources and develop regulated pheromone component production. Future investigation will determine the substrate specificity of IcCYP9T1 and CYP9T2. The study of these proteins and additional homologues in different *Ips spp*. or populations will lead to a better understanding of the origin and regulation of bark beetle pheromone biosynthesis, thus allowing for the development of new pest management tools.

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