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CYP341B14: A cytochrome P450 involved in the specific epoxidation of pheromone precursors in the fall webworm *Hyphantria cunea*

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ABSTRACT

Two of the four sex pheromone components in the fall webworm *Hyphantria cunea* (Lepidoptera: Arctiidae), *cis*-9,10-epoxy-(3*Z*,6*Z*)-3,6-henicosadiene and *cis*-9,10-epoxy-(3*Z*,6*Z*)-1,3,6-henicosatriene, possess an epoxy ring within their molecules. These compounds have been suggested to be biosynthesized from dietary linolenic acid via the following enzymatic reactions; chain elongation, terminal desaturation (in the case of the latter component), decarboxylation, and epoxidation. The last step of this biosynthesis, epoxidation, is known to occur specifically in the sex pheromone gland of females. We identified the enzyme involved in the epoxidation of pheromone precursors by focusing on cytochromes P450, which are known to catalyze the oxidation of various compounds. Three P450-like sequences (*Hc_epo1*, *Hc_epo2*, and *Hc_epo3*) were identified in the cDNA library prepared from the sex pheromone gland of *H. cunea*. Among these clones, only *Hc_epo1* was specifically expressed in the pheromone gland. The full-length sequence of *Hc_epo1* contained an ORF of 1527 bp, which encoded a protein of 509 amino acids with a predicted molecular weight of 57.9 kDa. The deduced Hc_epo1 amino acid sequence possessed the characteristics of P450. A phylogenetic analysis of the sequence indicated that Hc_epo1 belonged to the CYP341B clade in the CYP341 family. Therefore, it was named CYP341B14. A subsequent functional assay using Sf-9 cells transiently expressing CYP341B14 demonstrated that this P450 protein was able to specifically epoxidize a (*Z*)-double bond at the 9th position in the pheromone precursor, (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene.

1. Introduction

The chemical structures of the sex pheromones utilized by moths are remarkably diverse (Ando, 2014; El-Sayed, 2014). Since sex pheromones play important roles in premating reproductive isolation between species, the diversification of sex pheromones may be closely related to the diversification of moth species (Lassance et al., 2013; Symonds and Elgar, 2008). Moth sex pheromones have been classified into type I (75%), type II (15%), and a miscellaneous type (10%) based on their chemical structures (Ando et al., 2004). Type-I pheromones consist of unsaturated aliphatic compounds with a C_{10}–C_{18} straight chain and a terminal functional group such as hydroxyl, acetoxyl, or formyl group. On the other hand, type-II pheromones are predominantly composed of C_{17}–C_{23} hydrocarbons with two or three *cis* double bonds at the 3, 6, or 9 positions, and their corresponding epoxy derivatives (Ando et al., 2004; Millar, 2000, 2010).

Type-I and type-II pheromones are known to be produced from different starting compounds. Type-I sex pheromones are derived from de novo synthesized saturated fatty acids, typically palmitic acid (Jurenka, 2004), whereas type-II pheromones are biosynthesized from dietary linoleic or linolenic acid (Jurenka, 2004; Millar, 2000; Wei et al., 2003). Thus, the double bonds characteristic to type-II compounds originate from dietary polyunsaturated fatty acids.

The fall webworm *Hyphantria cunea* is a serious polyphagous defoliator. The female moths of *H. cunea* produce the following four compounds as sex pheromone components (Senda et al., 1991): (9*Z*,12*Z*)-9,12-octadecadienal [Z9,Z12-18:Ald], (9*Z*,12*Z*,15*Z*)-9,12,15-octadecatrienal [Z9,Z12,Z15-18:Ald], *cis*-9,10-epoxy-(3*Z*,6*Z*)-3,6-henicosadiene [Z3,Z6,epo9-21:H], and *cis*-9,10-epoxy-(3*Z*,6*Z*)-

Abbreviations: GC–MS, gas chromatograph coupled to a mass spectrometer; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

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1,3,6-henicosatriene [1,Z3,Z6,epo9-21:H]. Of these components, the former two aldehydes are exceptional in that they possess the characteristics of both type I (a terminal functional group) and type II (2 *cis* double bonds at the 3 and 6 positions). Tracing experiments using deuterium-labeled compounds suggested that these unusual compounds are produced in the pheromone gland from linoleic or linolenic acids via their alcohol derivatives (Fig. S1; Kiyota et al., 2011). The latter two epoxides are characterized by the epoxy ring in their molecules. The biosynthetic pathways of these type-II compounds have been suggested as follows (Fig. S1 and references therein). First, the pheromone precursor hydrocarbons, (3*Z*,6*Z*,9*Z*)-3,6,9-henicosatriene [Z3,Z6,Z9-21:H] and (3*Z*,6*Z*,9*Z*)-1,3,6,9-henicosatetraene [1,Z3,Z6,Z9-21:H], are synthesized from dietary linolenic acid in specialized cells called oenocytes via chain elongation, terminal desaturation (in the case of 1,Z3,Z6,Z9-21:H), reduction, and oxidative decarbonylation (Fig. S1; Qiu et al., 2012). These precursors are subsequently transported to the pheromone gland via the hemolymph, and the final step in pheromone biosynthesis, the epoxidation of alkenes, occurs specifically in this gland. The substrate specificities of the epoxidase in *H. cunea* have been characterized using various synthetic substrates (Kiyota et al., 2011); however, the molecular entity of the epoxidase has yet to be clarified.

Cytochromes P450 (CYPs or P450s) are heme proteins present in a wide range of organisms from microorganisms to plants and animals, and are known for their ability to transfer one oxygen atom into various substrates (monooxygenase activities) (Omura, 2010). However, P450s actually catalyze remarkably diverse reactions (Feyereisen, 2012; Mansuy, 1998; Omura, 2013). In insects, CYPs are involved in many physiological functions such as the metabolism of hormones (Daimon et al., 2012; Helvig et al., 2004; Niwa et al., 2004), adaptation to host plants (Niu et al., 2011; Schuler, 2011; Schuler and Berenbaum, 2013), and resistance to insecticides (Daborn et al., 2007; Feyereisen, 2012).

In the present study, we investigated the possible involvement of P450 in the epoxidation of pheromone precursors in *H. cunea*. We herein described the cloning of *Hc_epo1* (CYP341B14), a gene encoding the pheromone gland-specific P450, which was able to catalyze the regio-specific epoxidation of the sex pheromone precursor, Z3,Z6,Z9-21:H.

2. Materials and methods

2.1. Insect and cell line

H. cunea larvae were collected in Moriya, Ibaraki prefecture, Japan (35.56°N, 139.57°E) and reared on mulberry (*Morus bombycis*) leaves at 24 °C under a photoperiod of 16-h light and 8-h dark. The insect cell culture (Sf9) was maintained in TC-100 medium (Life Technologies) with 10% fetal bovine serum.

2.2. Construction of a cDNA library

To construct a cDNA library, pheromone glands were dissected from 170 virgin adult females (1–3 day old), and immediately stored at −80 °C. Total RNA was extracted from the pheromone glands using the RNAiso reagent (Takara Bio Inc.), and mRNA was isolated using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Life Technologies). A plasmid cDNA library was constructed using the cDNA Library Construction Kit (Takara Bio) according to the manufacturer's instructions.

To examine cDNA inserts, 288 colonies were randomly selected and PCR was performed with the T7 and T3 primers (Table 1) using Ex Taq DNA polymerase (Takara Bio) under the following conditions: 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, and finally 72 °C for 7 min. The PCR products were analyzed by electrophoresis on a 1.0% agarose gel for size and quality. The clones showing a single band were sequenced from the 5′ end using the T7 primer (Table 1). A search for homologous genes was performed in each clone using tblastx (NCBI: http://www.ncbi.nlm.nih.gov/).

2.3. Tissue distribution analysis of candidate genes

Flight muscles, legs, eggs, fat bodies, epidermis, and pheromone glands were dissected from 1- to 3-day-old virgin female moths in PBS (2.5 mM KCl, 141 mM NaCl, 8.1 mM Na_2HPO_4, and 2.5 mM KH_2PO_4, pH = 7.0). mRNA was isolated from these samples as described above. mRNA (100 ng) was reverse-transcribed using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio) and the oligo-dT primer supplied in the kit. RT-PCR was performed with gene-specific primer pairs for the target genes (Table 1) and Ex Taq DNA polymerase (Takara Bio) under the following conditions; 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 1 min, and finally 72 °C for 7 min.

2.4. Cloning of Hc_epo1 cDNA

Total RNA prepared from 20 pheromone glands of *H. cunea* were used for 3′- and 5′- RACE. The full-length cDNA of *Hc_epo1* was obtained using the GeneRacer® Kit (Invitrogen) with the gene-specific primer sets (Table 1). To verify the connection between the central, 3′-end, and 5′-end regions, PCR was performed using first strand cDNA and a pair of gene-specific primers (Table 1) designed to amplify the entire sequence.

2.5. Transient expression of Hc_epo1 and Western blotting

The InsectSelect™ Glow System (Invitrogen) was used to transiently express His-tagged recombinant proteins. We first examined the time course of gene expression by transfecting 2 μg of empty pIZT/V5-His vector into Sf9 insect cells with Cellfectin® II reagent (Invitrogen) according to the manufacturer's instructions. The fluorescence of GFP was detected every 12 h by the FLoid™ Cell Imaging Station (Life Technologies).

Table 1
Primers used in this study.

Name	Sequence (5′→3′)	Remarks
T7 primer	TAATACGACTCACTATAGGG	For
T3 primer	ATTAACCCTCACTAAAGGGCG	colony PCR
P450-1F	GTTATCCTTTGATTGGG	For
P450-1R	CATGTAGTTCCAAACAGGTTGCG	RT-PCR
P450-2F	ATGCTTACAAAAGACCG	
P450-2R	CCGCTATGAAATATGGC	
P450-3F	GTGTCAACAATACTTAAGCC	
P450-3R	GAATCCTTAATATTCCAGTCCC	
5′Primer	CGACTGGAGCACGAGGACACTGA	For RACE
5′Nested Primer	GGACACTGACATGGACTGAAGGAGTA	
3′Primer	GCTGTCAACGATACGCTACGTAACG	
3′Nested Primer	CGCTACGTAACGGCATGACAGTG	
P450-1R	CGCTCAGCTATTAGTGATGAAACGATC	
P450-1R nest	CGCTCCGTTCCCTTCGACTGCCTTAAG	
P450-1F	CGCGAAGCTATTAAAAATGGCGGAATG	
P450-1F nest	AAATATTATATGGTTGCAGTAGATGCTG	
Hc_epo1F	GAAAATCTTAACTGTGCCTTCAGCG	Amplify
Hc_epo1R	TAAACAACTTTATCAGTGTGGTGGC	Hc_epo1 ORF
Hc_epo1-pIZT-F	**GAGCTC**ATGTTGTTACTAGTATTACTAGTA	For transient
Hc_epo1-pIZT-R	**GCGGCCGC**CAGTACATTTGATGTTATTTTTCCT	expression

Bold characters in Hc_epo1-pIZT-F and Hc_epo1-pIZT-R indicate *Sac* I and *Not* I sequences, respectively.