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Functional characterization of the epoxidase gene, *Li_epo1* (*CYP341B14*), involved in generation of epoxyalkene pheromones in the mulberry tiger moth *Lemyra imparilis*

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ABSTRACT

Epoxidation of alkenes derived from essential fatty acids is a key step in the biosynthesis of sex pheromones in moth species that utilize alkenyl sex pheromones. The position of the epoxy ring in the pheromone molecule differs depending on the species, thereby conferring diversities on sex pheromones. To date, only one pheromone gland (PG)-specific epoxidase, Hc_epo1 (CYP341B14), has been reported. Hc_epo1, which was identified from an arctiid moth *Hyphantria cunea*, catalyzes the epoxidation of a double bond at position 9 of the triene, Z3,Z6,Z9-21:H. In the present study, we investigated the PG-specific epoxidase from another arctiid, the mulberry tiger moth *Lemyra imparilis*, in order to verify whether cytochrome P450 in the CYP341B subfamily, to which Hc_epo1 belongs to, is responsible for the epoxidation of pheromone precursors at position 9 in moths other than *H. cunea*. A fragment of the *Hc_epo1* homolog was amplified from cDNA prepared from the PG of *L. imparilis* by PCR with degenerate primers. The deduced amino acid sequence of the subsequently cloned homolog, Li_epo1, showed 88.5% identity to Hc_epo1. A functional assay using the Sf9 insect cell line–baculovirus expression system showed that Li_epo1 exhibited epoxidase activity with high selectivity to the double bond at position 9 of two trienes, Z3,Z6,Z9-21:H and Z3,Z6,Z9-23:H, precursors of epoxy diene sex pheromone components in *L. imparilis*.

1. Introduction

Moths constitute a large group in insects, comprising approximately 160,000 species (https://www.si.edu/spotlight/buginfo/moths). Sex pheromones play an essential role in premating reproductive isolation between moth species, which is underlain by the marked diversity of chemical structures of female sex pheromone molecules (see Ando and Yamamoto, 2018; El-Sayed, 2014). Moth sex pheromones are chemically classified into type I, type II, and others, with the former two accounting for more than 80% (Ando et al., 2004; Ando and Yamakawa, 2011; Millar, 2000). Moth species in Geometridae and Erebidae families often utilize type-II sex pheromones, which are long-chain hydrocarbons with two or three (*Z*) double bonds at the 3, 6, or 9 positions, and epoxy derivatives thereof (Arn et al., 1997). Most type-II sex pheromones are biosynthesized from dietary linoleic acid (LA) or α-linolenic acid (ALA), typically via chain elongation, decarboxylation, and subsequent epoxidation (Millar, 2000; Rule and Roelofs, 1989; Wei et al., 2003). LA and ALA, ingested from diet during the larval stage, are initially transformed to alkene sex pheromone precursors in oenocytes in the abdomen of adult female moths. These precursors are then loaded onto hemolymph lipophorin, transported to the sex pheromone gland (PG) located at the abdominal tip, and secreted without further modifications or after epoxidation (Fujii et al., 2007, 2015; Matsuoka et al., 2006; Millar, 2000; Miyamoto et al., 1999; Wei et al., 2003).

The optional epoxidation of alkenes, which occurs in the last step of pheromone biosynthesis, confers great diversity on sex pheromones because epoxidation occurs in different combinations of double bonds in a pheromone molecule in a species-specific manner. According to the database of lepidopteran sex pheromones (Ando and Yamamoto, 2018), non-epoxidized and mono-epoxidized alkenes account for approximately 39% and 54% of type-II pheromones, respectively (Table 1). Di-epoxidized alkenes are rare and tri-epoxidized alkenes have not been reported to date. Regarding the position of epoxy ring in mono-epoxidized pheromone components, position 9 is most abundant (45%), followed by position 6 (32%) and position 3 (23%) (Table 1).

The first PG-specific epoxidase, Hc_epo1 (CYP341B14), which specifically catalyzes the epoxidation of a double bond at position 9 of the triene (3Z,6Z,9Z)-3,6,9-henicosatriene (Z3,Z6,Z9-21:H) was identified from an arctiid moth *Hyphantria cunea* (Rong et al., 2014). Hc_epo1 is a cytochrome

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Table 1
Distribution of the position of epoxy rings in Type-II sex pheromones [a].

Superfamily	Family	Subfamily	Position of the epoxy rings									non-epo
			mono-epo						di-epo		unknown[b]	
			3epo	6epo	9epo	4epo[c]	7epo[c]	11epo[c]	3,6	6,9		
Geometroidea	Geometridae		30	29	21	1					2	48
Noctuoidea[d]	Noctuidae[d]				1							
	Erebidae[d]	Erebinae		1	13							13
		Calpinae	1	5	3				1			5
		Hypeninae			4							
		Herminiinae		8			1				1	
		Lymantriinae[e]		1	2		8	1	1	1		6
		Arctiinae[e]			17		1					21
Pyraloidea	Crambidae						1					
Tischerioidea	Tischeriidae											1
Yponomeutoidea	Lyonetiidae											4
Total			31	44	61	1	11	1	2	1	3	98

[a] As of September 2018 (excerpted from https://lepipheromone.sakura.ne.jp/lepi_phero_list).
[b] The epoxy ring position in the pheromone component is unidentified.
[c] Atypical type-II pheomone components, which are not derived from essential fatty acids.
[d] Some subfamiles formerly placed in Noctuidae have been transferred to the family Erebidae (Regier et al., 2017; Zahiri et al., 2012).
[e] These two subfamilies were formerly treated as two independent families (Lymantridae and Arctiidae) in the superfamily Noctuoidea.

P450 (CYP) belonging to the CYP341B subfamily. Among the CYP341B sequences deposited in public databases, three CYPs: CYP341B1 of the silkmoth *Bombyx mori* (Bombycidae), CYP341B2 of the corn earworm *Helicoverpa armigera* (Noctuidae), and CYP341B3 of the Egyptian cotton leafworm *Spodoptera littoralis* (Noctuidae), have attracted our attention because none of these species produce type-II pheromones and, thus, epoxidation is not involved in sex pheromone biosynthesis. Before considering the functions of CYPs in the subfamily CYP341B, more information on the epoxidases involved in type-II pheromone biosynthesis is needed because only a single sequence of the PG-specific epoxidase, Hc_epo1, has been reported to date.

The mulberry tiger moth *Lemyra imparilis*, which belongs to the same Arctiinae subfamily as *H. cunea*, is a serious polyphagous defoliator in Japan. *L. imparilis* utilizes cis-9,10-epoxy-(3Z,6Z)-3,6-henicosadiene (Z3,Z6,epo9-21:H), one of the components of the *H. cunea* sex pheromone, as its main sex pheromone component (Ando et al., 2004). In addition to this, cis-9,10-epoxy-(3Z,6Z)-3,6-tricosadiene (Z3,Z6,epo9-23:H) is reported as a minor component of sex pheromone in *L. imparilis* (Ando et al., 2004). These compounds are considered to be produced by the epoxidation of the triene precursors, Z3,Z6,Z9-21:H and Z3,Z6,Z9-23:H, in the PG. In the present study, we cloned a *CYP341B* gene from *L. imparilis* using degenerate primers designed to amplify the fragments of *CYP341B*, and performed a functional assay to investigate whether CYP341B in this species also exhibits Z9-specific epoxidase activity for triene precursors.

2. Materials and methods

2.1. Insect and cell line

L. imparilis larvae were collected in Tottori prefecture, Japan (35.503°N, 134.132°E) and reared on SilkMate™ 2M (Nosan Corp., Yokohama, Japan) at 24 °C under a 16-h light and 8-h dark photoperiod. The Sf9 insect cell culture was maintained in TC-100 medium (AppliChem, Munich, Germany) with 10% fetal bovine serum as described previously (Katsuma et al., 2006).

2.2. Chemicals

Authentic Z3,Z6,Z9-21:H and Z3,Z6,Z9-23:H were provided by Prof. Emeritus Tetsu Ando (Tokyo University of Agriculture and Technology). Epoxides of Z3,Z6,Z9-21:H and Z3,Z6,Z9-23:H were obtained as follows. Z3,Z6,Z9-21:H or Z3,Z6,Z9-23:H (10 mg, 0.03 mmol) and m-chloroperoxybenzoic acid (mCPBA; > 70%, 10 mg, 0.06 mmol) were dissolved in dry CH_2Cl_2 (1 ml) and stirred on ice for 2 h and then at room temperature for 1 h. The reaction was monitored every 1 h by thin-layer chromatography using 10% diethyl ether in hexane as the developing solvent. After neutralization and subsequent removal of residual mCPBA on a silica gel column, a mixture of positional isomers of alkene epoxides was eluted with 10% diethyl ether in hexane.

2.3. Tissue collection and cDNA preparation

Flight muscles (FM), midgut (MG), Malpighian tubule (MT), legs (Leg), eggs (Egg), fat bodies (FB), epidermis (Ep), and PG 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). Total RNA was extracted from each tissue type using the RNAiso reagent (Takara Bio Inc., Kusatsu, Japan). Total RNA (ca. 1 μg) was reverse-transcribed using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Bio) and the oligo-dT primer supplied in the kit.

2.4. Cloning of Li_epo1 cDNA

cDNA prepared from the PG was used as a template for PCR. Degenerate primers (Table S1) were designed based on the sequences of CYP341B1 (*B. mori*; AB436841), CYP341B2 (*H. armigera*; KM016716), and *Hc_epo1* (Rong et al., 2014). PCR was performed with Ex Taq DNA polymerase (Takara Bio) under the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 1 min, and finally 72 °C for 7 min. Amplicons produced by contamination from exogenous sources were distinguished by parallel PCR performed with the same components, excluding the cDNA, forward, or reverse primers (Fig. S2). The full-length cDNA of *Li_epo1* was obtained by 3′- and 5′-RACE using the GeneRacer® Kit (Invitrogen, Tokyo, Japan) with gene-specific primer sets (Table S1). In order 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 S1) designed to amplify the entire sequence.

2.5. Tissue distribution analysis of Li_epo1

Total RNA (ca. 1 μg) obtained from each tissue type 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 gene (Table S1) and Ex Taq DNA

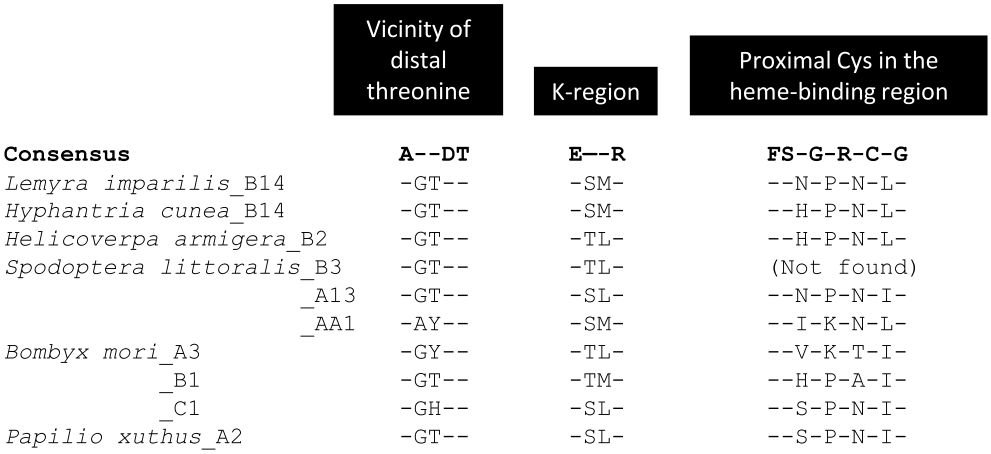

Fig. 1. Homology comparison of three motifs (distal threonine, K-region, and proximal Cys in the heme-binding region) of P450s in CYP341 families. Consensus sequences are shown in bold characters.

polymerase (Takara Bio) under the following conditions: 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min, and finally 72 °C for 7 min.

2.6. Recombinant expression system

The coding region of *Li_epo1* with or without the His-tag at the C-terminus was amplified by PCR with a pair of gene-specific primers which contained restriction enzyme sites (Table S1). Recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPVs) expressing Li_epo1 with or without the His-tag, Li_epo1-His-AcNPV and Li_epo1-AcNPV, respectively, were constructed using the Bac-to-Bac Baculovirus Expression System (Invitrogen), as described previously (Fujii et al., 2011). Two micrograms of Li_epo1-His-AcNPV, Li_epo1-AcNPV, or Bac1-AcNPV (negative control) were transfected into Sf9 cells with Cellfectin® II reagent (Invitrogen) according to the manufacturer's instructions. The infection of Sf9 cells with recombinant AcNPVs was performed using passage 2 (P2) viral stock.

The expression of the Li_epo1 protein in Sf9 cells was confirmed by a western blotting analysis. The time course of gene expression by infecting with the Li_epo1-His-AcNPV recombinant virus was investigated every 12 h to assess expression kinetics. Sf9 cells infected by Li_epo1-His-AcNPV, Li_epo1-AcNPV, or Bac1-AcNPV, as well as medium, were then collected 48 h post-infection by centrifugation at 1,000 × *g* for 10 min. Cells were rinsed three times with PBS and homogenized in PBS containing cOmplete™ Mini protease inhibitor cocktail (4% v/v of the final volume; Roche Applied Science, Penzberg, Germany). After the addition of SDS sample buffer, samples prepared from cells and medium were separated on a 10% gel by SDS-PAGE, and transferred to an Immobilon-P membrane (Millipore, Tokyo, Japan). The membrane was incubated with a mouse anti-His antibody (MBL, 1:5,000 dilution) for 1 h and subsequently with a goat anti-mouse IgG-HRP conjugate (1:10,000 dilution) for 1 h. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used according to the instructions of the manufacturer, and chemiluminescence was detected using ImageQuant 400 (GE Healthcare Biosciences, Chicago, USA). The same samples were also analyzed by SDS/PAGE and stained with Coomassie Brilliant Blue (CBB) as loading controls.

2.7. Functional assay of recombinants

Sf9 cells seeded on a 60-mm cell culture dish containing 4 mL of serum-free TC-100 medium were infected with Li_epo1-His-AcNPV, Li_epo1-AcNPV, or Bac1-AcNPV. Medium was replaced 48 h post-infection with that containing 40 μg of an alkene substrate (Z3,Z6,Z9-21:H or Z3,Z6,Z9-23:H) and 1.5 mg of NADPH (Oriental Yeast Co., LTD., Tokyo, Japan), and then incubated for a further 12 h. Cells were subsequently rinsed with PBS three times, and resuspended in 100 μL of distilled water. The products from the alkene substrate were then extracted from cells with 500 μL of hexane at room temperature for 1 h and analyzed as described below.

2.8. GC–MS analyses

A gas chromatograph equipped with a capillary column (DB-Wax, 30 m × 0.25 mm i.d.; 0.25 μm film thickness; J & W Scientific, Folsom, USA) coupled with a mass spectrometer (QP2010 SE GC–MS; Shimadzu, Kyoto, Japan) was used for the analysis. Detection threshold of octafluoronaphthalene using this GC-MS was 1 pg (data provided by the manufacturer; *m/z* 272, S/N > 200). The column oven temperature was maintained at 120 °C for the first 2 min, then increased at 12 °C/min to 180 °C, and at 5 °C/min to 240 °C (Rong et al., 2014). The ionization voltage was 70 eV, and the flow rate of the carrier gas (Helium) was 1.0 ml/min. The epoxydiene (Z3,Z6,epo9-21:H or Z3,Z6,epo9-23:H) was detected at *m/z* 79 (Ando and Yamakawa, 2011). The study by Ando and Yamakawa (2011) was referred to for the mass fragmentation pattern of these compounds.

2.9. Phylogenetic analysis

We used an online service provided by the DNA Data Bank of Japan (DDBJ; http://clustalw.ddbj.nig.ac.jp/index.php?lang=en) for the phylogenetic analysis of Li_epo1. Amino acid sequences were aligned using Clustal W, and the phylogenetic tree was constructed by the neighbor-joining method with the DISTANCE option set to "Kimura." The bootstrap analysis was performed with 1,000 resamplings. CYP15A1 (*Diploptera punctata*, AY509244) was used as an outgroup.

2.10. Data deposition

The nucleotide sequence of *Li_epo1* (*CYP341B14*) has been submitted to DDBJ (http://www.ddbj.nig.ac.jp/) with Accession No. LC326250.

3. Results

3.1. Sequence analysis of Li_epo1 cDNA

A fragment of the *L. imparilis* homolog of *Hc_epo1* was obtained by PCR using degenerate primers (Table S1). The full-length sequence of the homolog (*Li_epo1*) was obtained in subsequent 3′- and 5′-RACE experiments (Fig. S1). *Li_epo1* contained an open reading frame of 1,530 bp, which encoded a protein of 510 amino acid residues with an estimated molecular mass of 57.9 kDa (Fig. S1). Its amino acid identity to Hc_epo1 was 88.5%. The Li_epo1 sequence contained motifs known to be conserved in P450s, such as a heme-binding region (amino acid positions at 432–441) including the proximal cysteine residue (amino acid position at 439), a distal

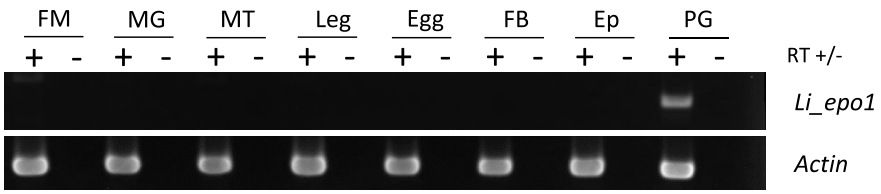

Fig. 2. Distribution of *Li_epo1* transcripts in various tissues of *L. imparilis*. FM: flight muscles, MG: midgut, MT: Malpighian tubule, Leg: legs, Egg: eggs, FB: fat bodies, Ep: epidermis, and PG: pheromone glands.

threonine residue (amino acid position at 305) with its upstream sequence (amino acid positions at 301–304), and a K-region (amino acid positions at 359–362) located between the proximal cysteine and distal threonine residue (Fig. 1; Fig. S1).

3.2. Tissue distribution of Li_epo1 transcripts

The tissue distribution pattern of *Li_epo1* was investigated by RT–PCR using *Li_epo1*-specific primers (Table S1). *Li_epo1* transcripts (621 bp) were specifically detected in the PG (Fig. 2).

3.3. In vitro functional assay of Li_epo1

The product of the Li_epo1-His-AcNPV recombinant virus was detected 36 h post-infection, but was markedly degraded 60 h or 72 h post-infection (Fig. 3A). A western blot analysis performed 48 h post-infection showed that Li_epo1 was not degraded and localized in cells (Fig. 3A and B). Based on these results, the following functional assays on Li_epo1 were performed using Sf9 cells 48 h post-infection. Since the apparent molecular weight of His-tagged Li_epo1, approximately 58.9 kDa, was close to the value estimated from its amino acid sequence, Li_epo1 was indicated to be expressed without any post-translational modifications (Fig. 3B).

In assays using Z3,Z6,Z9-21:H as the substrate, GC–MS analyses of the hexane extracts of Sf9 cells infected with Li_epo1-His-AcNPV or Li_epo1-AcNPV detected the presence of a chemical product, the retention time of which was close to that of authentic Z3,Z6,epo9-21:H (Fig. 4A). This product was not detected in Sf9 cells infected with the negative control, Bac1-AcNPV (Fig. 4A). The product was identified as Z3,Z6,epo9-21:H by comparisons of its retention time and mass spectra with those of three epoxy-ring positional isomers (epo3,Z6,Z9-21:H, Z3,epo6,Z-21:H, and Z3,Z6,epo9-21:H) as follows. Comparisons of the retention time of the product (14.92 min) with those of Z3,epo6,Z9-21:H (14.70 min), epo3,Z6,Z9-21:H (≈14.9 min), and Z3,Z6,epo9-21:H (≈14.9 min) indicated it to be either epo3,Z6,Z9-21:H or Z3,Z6,epo9-21:H (Fig. 4A). Since the mass spectrum of the product was identical to that of authentic Z3,Z6,epo9-21:H (Fig. 4B and C), and lacked diagnostic ions (m/z 234 [M^+-72], 248 [M^+-58]) derived from an epoxy ring at position 3 (Fig. 4B and C), it was concluded to be Z3,Z6,epo9-21:H. A detailed analysis of the mass chromatograms of Li_epo1-His-AcNPV and Li_epo1-AcNPV extracts indicated that production of epo3,Z6,Z9-21:H was negligible (Fig. S3). Collectively, these results indicated that Li_epo1, regardless of the absence/presence of the His-tag at the C terminus, catalyzed the specific epoxidation of a (*Z*)-double bond at position 9 of the triene precursor, Z3,Z6,Z9-21:H.

We next examined whether Li_epo1 catalyzes the epoxidation of Z3,Z6,Z9-23:H, a triene with different carbon chain length. GC–MS analyses of the hexane extract of Sf9 cells infected with Li_epo1-AcNPV detected the presence of a product, the retention time of which was close to that of authentic Z3,Z6,epo9-23:H (Fig. 5A). This product was not detected in the extract of Sf9 cells infected with the negative control, Bac1-AcNPV (Fig. 5A). This product was identified as Z3,Z6,epo9-23:H by comparisons of its retention time and mass spectral characteristics with those of three epoxy-ring positional isomers, epo3,Z6,Z9-23:H, Z3,epo6,Z-23:H, and Z3,Z6,epo9-23:H (Fig. 5A, B, C; Fig. S4).

3.4. Phylogenetic analysis of Li_epo1

A BlastP search of public databases with the deduced amino acid sequence of Li_epo1 as a query indicated that this protein was most similar to CYP341B14 (*H. cunea*; Accession No. AB795798; amino acid identity to Li_epo1 was 88.5%), followed by CYP341B3 (*S. littoralis*; Accession No. JX310095; 48.6%), CYP341B2 (*H. armigera*; KM016716; 45.6%), and CYP341B1 (*B. mori*; AB436841; 34.5%). Reconstruction of the phylogenetic tree of insect CYPs confirmed that Li_epo1 belongs to the CYP341 family together with Hc_epo1 (Fig. 6). CYP341B14 was assigned to *Li_epo1*.

4. Discussion

We previously and for the first time identified an epoxidase (Hc_epo1) involved in the biosynthesis of type-II sex pheromones (Rong et al., 2014). In the present study, we functionally characterized the PG-

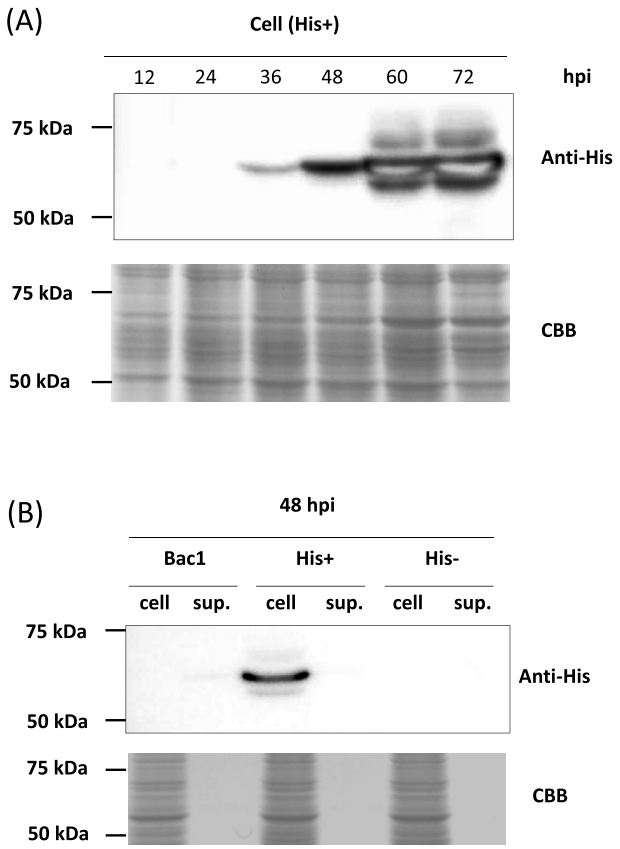

Fig. 3. Expression of the recombinant Li_epo1 protein using a baculovirus expression system. (A) Time course of the expression of the Li_epo1 protein in Sf9 cells infected with Li_epo1-His-AcNPV. (B) The expression of the Li_epo1 protein in Sf9 cells 48 hpi with Bac1-AcNPV, Li_epo1-His-AcNPV, and Li_epo1-AcNPV. Bac1-AcNPV: negative control. Li_epo1-His-AcNPV and Li_epo1-AcNPV: AcNPV constructs expressing the Li_epo1 protein with and without the His-tag at the C terminus, respectively. The molecular weight of Li_epo1 with a histidine tag was estimated to be 58.9 kDa. The same samples were also analyzed by SDS/PAGE and stained with CBB. hpi: hours post-infection.

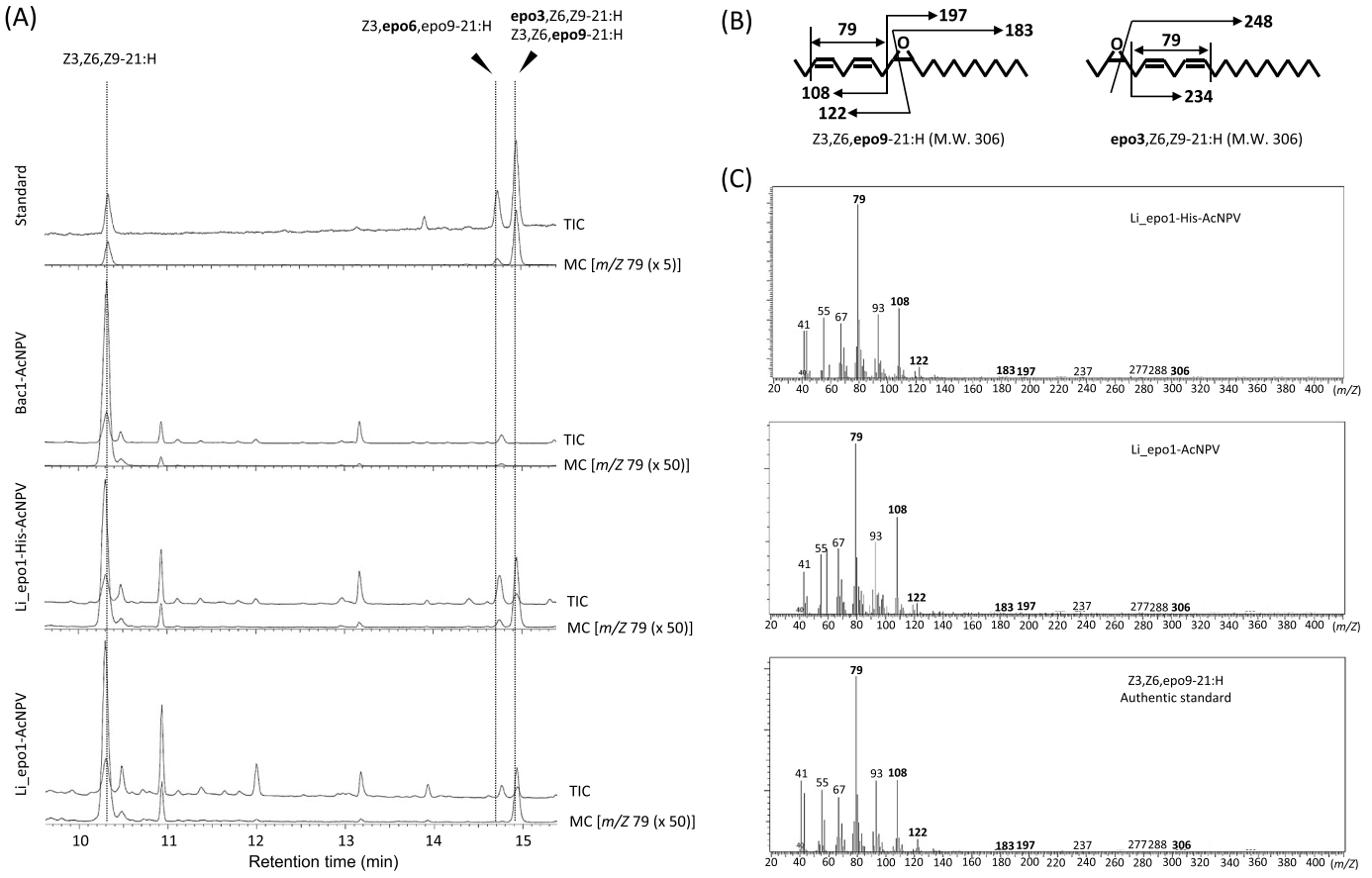

Fig. 4. Results of the functional assay of Li_epo1 using Z3,Z6,Z9-21:H as substrate. (A) Total ion chromatograms (TIC) and mass chromatograms (*m/z* 79; base ion peak of the substrate and product in this study) of a mixture of Z3,Z6,Z9-21:H and its epoxide isomers epo3,Z6,Z9-21:H, Z3,epo6,Z9-21:H, and Z3,Z6,epo9-21:H standards (upper traces), an extract from Sf9 cells infected with Bac1-AcNPV (middle traces; control), and an extract from Sf9 cells infected with Li_epo1-His-AcNPV or Li_epo1-AcNPV (lower traces). (B) Diagnostic fragment ions of the epoxy ring positional isomers of Z3,Z6,Z9-21:H (Ando and Yamakawa, 2011). Two isomers have the diagnostic ion m/z 79 in common. (C) Mass spectra of the products of Li_epo1-His (upper) and Li_epo1 (middle), and that of synthetic Z3,Z6,epo9-21:H (lower). Bold figures indicate diagnostic fragment ions of Z3,Z6,epo9-21:H.

specific epoxidase *Li_epo1* in another arctiid moth *L. imparilis*. Li_epo1 was shown to take two trienes, Z3,Z6,Z9-21:H and Z3,Z6,Z9-23:H, as substrates and produce Z3,Z6,epo9-21:H and Z3,Z6,epo9-23:H, two epoxy diene pheromone components of *L. imparilis*. However, in addition to Z3,Z6,epo9-21:H and Z3,Z6,epo9-23:H, *L. imparilis* produces two other type-II components: an epoxy triene 1,Z3,Z6,epo9-21:H and an epoxy monoene Z6,epo9-23:H (Ando et al., 2004). It currently remains unclear whether Li_epo1 is involved in the production of these components, namely, whether Li_epo1 takes their precursors, i.e., a tetraene 1,Z3,Z6,Z9-21:H and a diene Z6,Z9-23:H, as substrates. The involvement of epoxidases other than Li_epo1 in the production of pheromone components in *L. imparilis* also needs to be carefully investigated.

Stereochemistry of epoxy-rings in the pheromone molecules is crucial for the pheromonal activity. For example, males of the Israeli strain of *Ascotis selenaria* are attracted to the (3S,4R)-isomer of (Z,Z)-6,9-cis-3,4-epoxynonadecadiene, whereas males of the Japanese strain are attracted to its (3R,4S)-isomer (Becker et al., 1983, 1990; Cossé et al., 1992; Ando et al., 1997). The major sex pheromone component of *H. cunea* is cis-9,10-epoxy-(3Z,6Z)-3,6-henicosadiene. *H. cunea* females exclusively produce the (9S,10R)-isomer, which only is biologically active (Tóth et al., 1989). Stereochemistry of epoxy-rings in the pheromone components of *L. imparilis* is currently unknown since isomers of epoxydienes cannot be separated by GC under the conditions used in this study. Enantioselectivity of Li_epo1 remains to be studied in the future.

A phylogenetic analysis indicated that *Li_epo1* and *Hc_epo1* belong to the same CYP341B subfamily (Fig. 6). Although *B. mori*, *S. litura*, and *H. armigera* do not utilize type-II pheromones and, thus, epoxidation is not included in their pheromone biosynthetic pathways, all of them possess CYP341B genes (Fig. 6). Since these CYP341B sequences are deduced from genome sequences, the expression of these genes in the PG and other tissues in the 3 species needs to be investigated. Moreover, whether the proteins encoded by these genes exhibit epoxidase activity is of great interest. Since moths belonging to relatively recently diverged groups (Geometridae and Erebidae) have been shown to produce type-II pheromones, the production of type-II pheromones and, thus, the utilization of epoxidase in pheromone production, are considered to be derived traits. Information on the roles of CYP341B genes in non type-II pheromone producers will provide an insight into how *CYP341B* genes became involved in the biosynthesis of type-II pheromones.

To date, more than 140 moth species have been reported/suggested to utilize epoxyalkene pheromones (Table 1; Ando et al., 2008). Epoxy rings in type-II pheromones occur not only at position 9, but also at a combination of positions 3, 6, and 9. In the subfamily Arctiinae, however, all the epoxyalkene components in the pheromones examined to date carry an epoxy ring at position 9, except for one species (Table 1). The single exceptional species within the Arctiinae utilizes atypical type-II pheromone components, which is not derived from essential fatty acids. The exclusive use of 9epo compounds in Arctiinae may suggest that homologs of Hc_epo1 and Li_epo1 are exclusively involved in the epoxidation of sex pheromone precursors in this subfamily.

The sequences and functions of CYPs in insects are extremely diversified by repeated duplication events and the subsequent

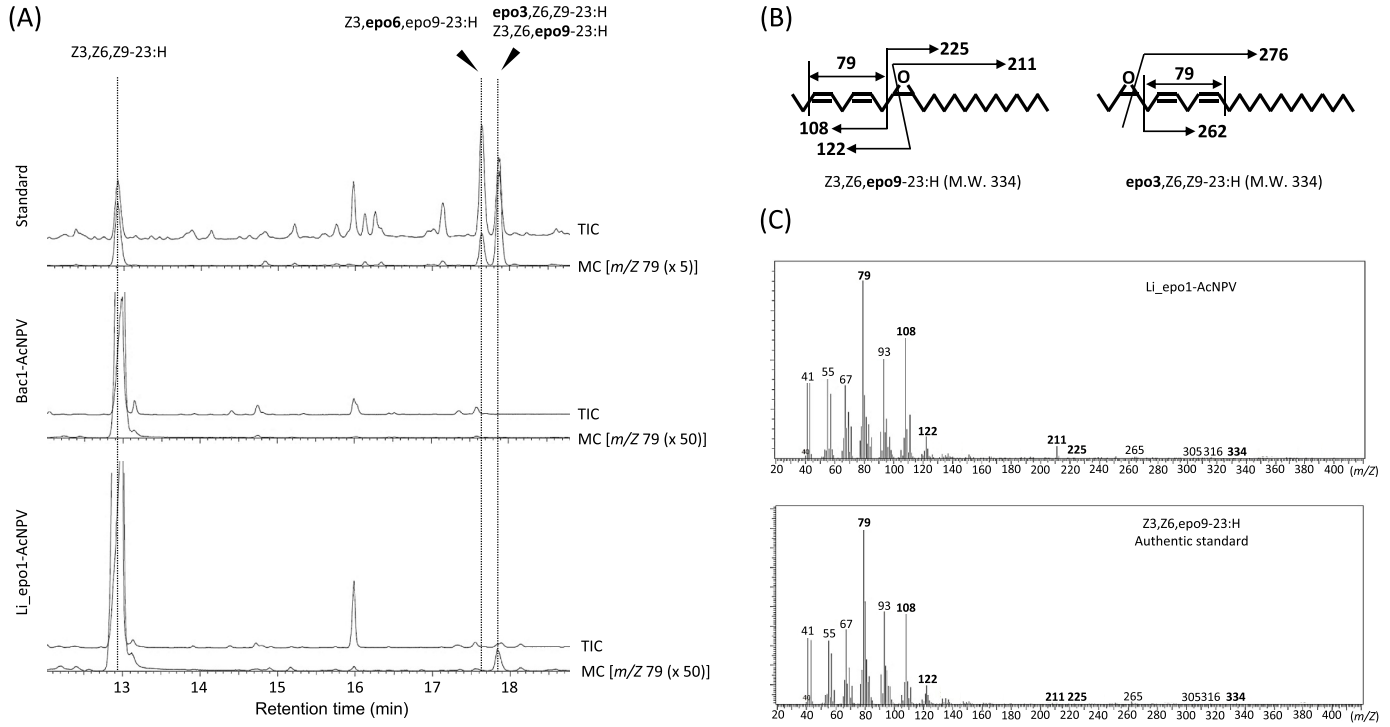

Fig. 5. Results of the functional assay of Li_epo1 using Z3,Z6,Z9-23:H as substrate. (A) Total ion chromatograms (TIC) and mass chromatograms (*m/z* 79; base ion peak of the substrate and product in this study) of a mixture of Z3,Z6,Z9-23:H and its epoxide isomers epo3,Z6,Z9-23:H, Z3,epo6,Z9-23:H, and Z3,Z6,epo9-23:H standards (upper traces), an extract from Sf9 cells infected with Bac1-AcNPV (middle traces; control), and an extract from Sf9 cells infected with Li_epo1-AcNPV (lower traces). (B) Diagnostic fragment ions of two epoxide isomers of Z3,Z6,Z9-23:H (Ando and Yamakawa, 2011). These two isomers have the diagnostic ion m/z 79 in common. (C) Mass spectra of the products of Li_epo1 (upper) and synthetic Z3,Z6,epo9-23:H (lower). Bold figures indicate diagnostic fragment ions of Z3,Z6,epo9-23:H.

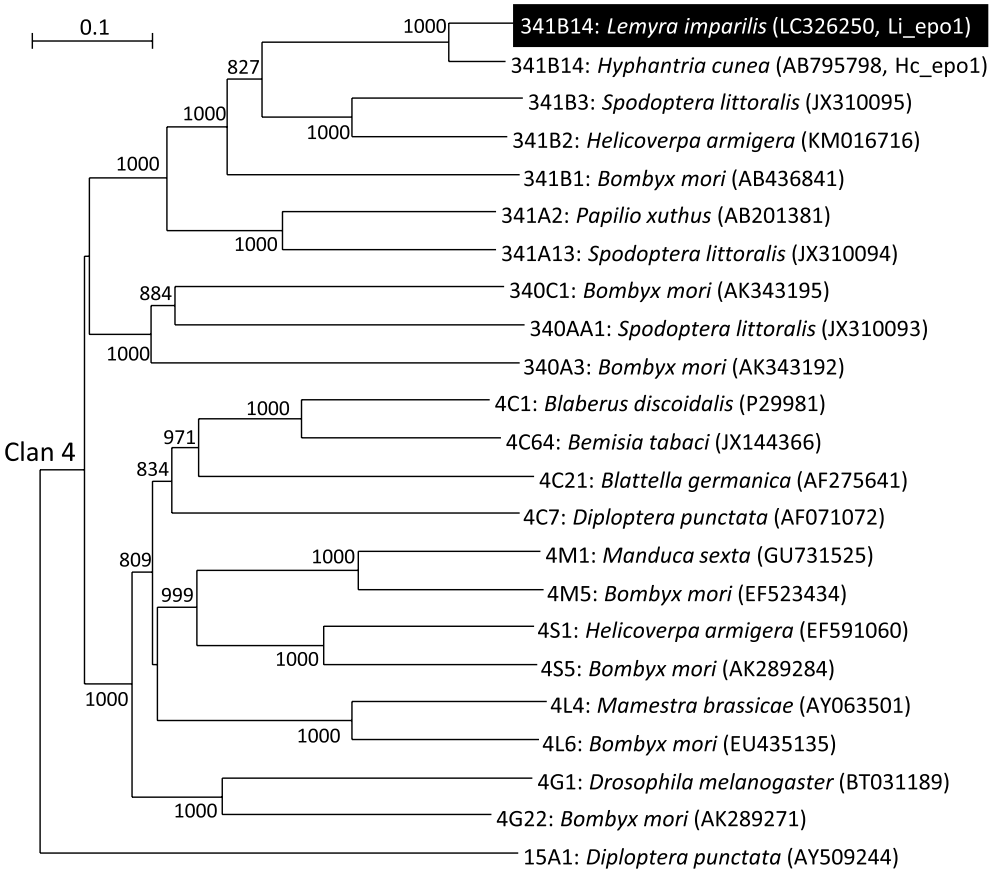

Fig. 6. Phylogenetic relationships of Li_epo1 and related insect cytochrome P450 (CYP). The phylogenetic tree was reconstructed using the neighbor-joining method. CYP15A1 of the Pacific beetle cockroach *Diploptera punctata* (AY509244) was used as an outgroup. The GenBank accession numbers and species names are shown next to the names of CYP genes. Bootstrap values after 1,000 replications are shown near the branches. The branch length is drawn to the genetic distance.

acquirement of new functions by these duplicated genes (Calla et al., 2017; Feyereisen, 2012). We demonstrated that 2 CYPs in the CYP341B subfamily are involved in the epoxidation of the Z9 double bond in the alkenes, Z3,Z6,Z9-21:H and Z3,Z6,Z9-23:H. Further studies are warranted in order to clarify whether CYPs in the same subfamily form an epoxy ring at positions other than 9. Since even a single amino acid substitution may affect the substrate specificity or catalytic activity of CYP (Ortiz de Montellano, 2015), CYPs in the same subfamily may have different regio-specificities in terms of epoxidation. If CYPs in CYP341B are specialized for the epoxidation of a double bond at position 9, then the strategy of cloning genes involved in epoxidation of a double bond at positions 3 and 6 using degenerate primers may not be applicable considering the low identity of sequences between CYPs in different subfamilies/families. In this case, a transcriptome analysis using RNA-seq will be an alternative strategy for investigating epoxidases that work on double bonds at other positions. A comprehensive analysis of CYPs expressed in the PG of moths utilizing type-II pheromones with an epoxy ring at positons 3 or 6 will enable the efficient identification of CYP genes involved in sex pheromone production.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.02.001.

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