Insect Biochemistry and Molecular Biology 41 (2011) 22–28

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Cloning and functional characterization of a fatty acid transport protein (FATP) from the pheromone gland of a lichen moth, *Eilema japonica*, which secretes an alkenyl sex pheromone

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ARTICLE INFO

Article history:
Received 22 July 2010
Received in revised form
15 September 2010
Accepted 17 September 2010

Keywords:
Fatty acid transport protein (FATP)
Sex pheromone
Eilema japonica
Free fatty acid

ABSTRACT

Sex pheromones of moths are largely classified into two types based on the presence (Type I) or absence (Type II) of a terminal functional group. While Type-I sex pheromones are synthesized from common fatty acids in the pheromone gland (PG), Type-II sex pheromones are derived from hydrocarbons produced presumably in the oenocytes and transported to the PG via the hemolymph. Recently, a fatty acid transport protein (BmFATP) was identified from the PG of the silkworm *Bombyx mori*, which produces a Type-I sex pheromone (bombykol). BmFATP was shown to facilitate the uptake of extracellular fatty acids into PG cells for the synthesis of bombykol. To elucidate the presence and function of FATP in the PG of moths that produce Type-II sex pheromones, we explored *fatp* homologues expressed in the PG of a lichen moth, *Eilema japonica*, which secretes an alkenyl sex pheromone (Type II). A *fatp* homologue cloned from *E. japonica* (*Ejfatp*) was predominantly expressed in the PG, and its expression is upregulated shortly after eclosion. Functional expression of EjFATP in *Escherichia coli* enhanced the uptake of long chain fatty acids (C_{18} and C_{20}), but not pheromone precursor hydrocarbons. To the best of our knowledge, this is the first report of the cloning and functional characterization of a FATP in the PG of a moth producing a Type-II sex pheromone. Although EjFATP is not likely to be involved in the uptake of pheromone precursors in *E. japonica*, the expression pattern of *Ejfatp* suggests a role for EjFATP in the PG not directly linked to pheromone biosynthesis.

1. Introduction

Females of many moth species secrete species-specific sex pheromones that mediate sexual communication for mate finding. To date, sex pheromones of more than 570 lepidopteran species have been chemically identified (Ando, 2006). Sex pheromones are classified into two major types (Type I and II) based on whether the sex pheromone compound contains a terminal functional group (Type I) or not (Type II) (Millar, 2000; Ando et al., 2004). Type-I sex pheromones are composed of C_{10}–C_{18} unsaturated acyclic aliphatic compounds with functional groups such as aldehyde, alcohol, and acetate ester (Jurenka, 2003). On the other hand, Type-II sex pheromones are predominantly composed of C_{17}–C_{23} hydrocarbons with two or three (*Z*)-double bonds at the 3-, 6-, or 9- positions, and epoxy derivatives thereof (Arn et al., 1992, 1997).

Type-I pheromones are synthesized in the pheromone gland (PG), a functionally differentiated organ located close to the terminal abdominal segment of female moths (Bjostad et al., 1987). Pheromone biosynthesis in the silk moth *Bombyx mori*, which produces the Type-I pheromone bombykol, (*E,Z*)-10,12-hexadecadien-1-ol, has been studied extensively (Matsumoto et al., 2007). Bombykol is produced *de novo* from palmitate via specific desaturation and reduction (Bjostad et al., 1987; Ando et al., 1988). Pheromone gland cells of *B. mori* are characterized by abundant lipid droplets (LDs), which are composed of various triacylglycerols with the bombykol precursor (Matsumoto et al., 2002). A rapid increase of LDs in *B. mori* shortly before the eclosion of female adults suggests that the LDs act as store of pheromone precursors (Fónagy et al., 2000, 2001; Matsumoto et al., 2007).

In contrast, the Type-II pheromones originate from long chain hydrocarbons produced outside PGs (Schal et al., 1998; Subchev and Jurenka, 2001; Jurenka et al., 2003; Wei et al., 2004; Matsuoka et al., 2006). Being synthesized in oenocytes or abdominal epidermal cells, precursors of Type-II pheromones (hydrocarbons) are transported to the PG by lipophorin in the hemolymph

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doi:10.1016/j.ibmb.2010.09.008

(Schal et al., 1998; Wei et al., 2004; Matsuoka et al., 2006). For example, the Japanese giant looper *Ascotis selenaria* secretes *cis*-3,4-epoxy-(*Z,Z*)-6,9-nonadecadiene (epo3,Z6,Z9-19:H) and (*Z,Z,Z*)-3,6,9-nonadecatriene (Z3,Z6,Z9-19:H) as sex pheromone components (Ando et al., 1997). It was confirmed that Z3,Z6,Z9-19:H injected into the hemolymph was incorporated into the PG, and converted into epo3,Z6,Z9-19:H, suggesting that Z3,Z6,Z9-19:H in the hemolymph is the direct precursor of the sex pheromone (Wei et al., 2003). Hydrocarbon pheromone precursors have been found in the hemolymph of several other moths that produce Type-II sex pheromones (Schal et al., 1998; Subchev and Jurenka, 2001; Jurenka et al., 2003; Wei et al., 2003).

Fatty acid transport protein (FATP) is an evolutionarily conserved membrane-bound protein that facilitates the uptake of extracellular long chain fatty acids into the cell. FATP homologues are widely found in many organisms from mycobacteria to humans. In humans and mice, six isoforms of FATP (FATP1–FATP6) have been identified. The tissue-specific expression of these isoforms suggests that each plays a distinct role in lipid metabolism through the uptake of specific fatty acids (Hirsch et al., 1998; Hall et al., 2003). Recently, a fatty acid transport protein (BmFATP) was identified in the PG of *B. mori*, and shown to have an essential role in bombykol synthesis through the uptake of extracellular fatty acids (Ohnishi et al., 2009). *Bmfatp* is predominantly expressed in the PG, and its level of expression increased sharply one day before eclosion. RNAi-mediated gene silencing of BmFATP resulted in a significant reduction in bombykol production (Ohnishi et al., 2009).

Although FATP homologues have been identified in several insect species other than *B. mori* (Hirsch et al., 1998; Doege and Stahl, 2006), they have not been found in moths that produce Type-II sex pheromones. The precursors (hydrocarbons) of Type-II sex pheromones in the hemolymph must be incorporated into the PG, but there is no information on the mechanism of incorporation. Recently, we identified the sex pheromone of a lichen-feeding moth, *Eilema japonica* (Lepidoptera; Arctiidae), as a mixture of dienyl and trienyl hydrocarbons (Z6,Z9-21:H, Z3,Z6,Z9-21:H, Z6,Z9-22:H, and Z3,Z6,Z9-22:H; Fujii et al., 2010). In the present study, to obtain a clue as to the mechanism of hydrocarbon uptake by the PG, we used this insect to check for the presence of *fatp* homologues in moths that produce Type-II pheromones. For comparison, we also explored *fatp* homologues from the PGs of *A. selenaria*, which produces a Type-II sex pheromone, and *Ostrinia scapulalis*, which produces a Type-I sex pheromone (Huang et al., 1997).

2. Materials and methods

2.1. Insects

Female moths of *E. japonica* and *A. selenaria* were collected at Bunkyo-ku, Tokyo, Japan (35.4°N, 139.4°E) in 2009. The adzuki bean borers, *Ostrinia scapulalis,* used in this study were offspring of female moths collected at Matsudo, Chiba, Japan (35.5°N, 139.6°E) in 2008. Larvae of the three species were reared on an artificial diet, Silkmate™ 2 M (Nosan Corp., Yokohama, Japan), under a temperature of 24 °C, 50–70% relative humidity, and a 16 h light:8 h dark cycle.

2.2. Cloning of FATP genes and rapid amplification of cDNA ends (RACE)

Total RNA was isolated from the PGs of 1- or 2-day-old virgin female moths of *E. japonica*, *A. selenaria*, or *O. scapulalis*, using an RNeasy mini kit (Qiagen, Valencia, CA) with RNase-free DNase (Qiagen). First-strand cDNA was synthesized using an RNA PCR kit (Takara-bio, Osaka, Japan) with 300 μg of total RNA. The central region of the *fatp* homologue was amplified using a set of degenerate primers, DGF and DGR (Table 1), designed based on the conserved regions of FATP proteins of *B. mori*, *Apis mellifera*, *Anopheles gambiae*, and *Drosophila melanogaster* (GenBank accession nos: BAG68297, XP624496, XP321320 and NP723597, respectively). PCR was performed with ExTaq DNA polymerase (Takara-bio) under the following conditions: 94 °C for 2 min, followed by 5 cycles of 94 °C for 1 min, 46 °C for 1 min, 72 °C for 1 min, then followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The products were subcloned into a pGEM-T Easy vector (Promega, Madison, WI), and sequenced using an ABI PRISM 377 Sequencer (Applied Biosystems, Carlsbad, CA).

The 5′ and 3′ end nucleotide sequence information of *Ejfatp*, *Asfatp,* and *Osfatp* cDNA was obtained using a GeneRacer Kit (Invitrogen, Carlsbad, CA) with the gene-specific primers listed in Table 1. Full-length cDNA sequences were obtained by combining the central region and 3′- and 5′- RACE fragments. The full-length cDNAs including open reading frame (ORF) were subcloned into pGEM-T easy vector (Promega), and sequenced to verify the entire sequence.

2.3. Phylogenetic analysis

The deduced amino acid sequences of putative FATPs were aligned using the Clustal W program (Thompson et al., 1994), and a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). The following sequences, which showed high scores in a BlastP search of the NCBI database with EjFATP (GenBank accession no. AB561865) as a query, were used for this analysis: *Anopheles gambiae*, XP321320; *Apis mellifera*,

Table 1
Primers used in this study.

Degenerate primers
DGF: 5′-AYATHGGNGARATGTG-3′
DGR: 5′-GTNGCNCCRTARAAYTC-3′
Gene-specific primers (GSP) for RACE
EJF: 5′-GTCGTCGAAACCTTCACCTACAGATAA-3′
EJFnest: 5′-CGGACAGTTTATGGCAATGGAATGAGA-3′
EJR: 5′-CCATTGCCATAAACTGTCCGAACTTTGT -3′
EJRnest: 5′-GGTGAAGGTTTCGACGACAGGACATAT-3′
ASF: 5′-CACGGACCGCCAGCACAAAGTTA-3′
ASFnest: 5′-CGGAAATGGAATGAGACCTACGATTTG-3′
ASR: 5′-AAATCGTAGGTCTCATTCCATTTCCGTAGA-3′
ASRnest: 5′-GGGCGGCGTGGATAGCACGTA-3′
OSF: 5′-GGCGACTCCTCCATCGGCTACT-3′
OSFnest: 5′-GTTCGCGTAGTGTACGGAAATGGAAT-3′
OSR: 5′-GGCCAGATCGCTTGTCTCATTCCATTT-3′
OSRnest: 5′-GCCGATGGAGGAGTCGCCAAAATA-3′
Primers for RT-PCR
RTOSf: 5′-GCCTGATTGTACTAGCCATACAG-3′
RTOSr: 5′-TTCCTCATACTCGGCGCCATAG-3′
RTEJf: 5′-TCCAGGACTGGCAGACT-3′
RTEJR: 5′-TGTAGGTGAAGGTTTCGAC-3′
RTASF: 5′-GTACCGTGGCCATCAGAA-3′
RTASR: 5′-CGCCGGTATAATACGAGAC-3′
Primers for qPCR
QEJF: 5′-TCTCGGCGTCTAGTTACTTCCCTG-3′
QEJR: 5′-CTGTAGGTGAAGGTTTCGACGACAG-3′
QactinF: 5′-CACACCTTCTACAACGAGCTGCG-3′
QactinR: 5′-GAGAGCACGGCCTGGATGGC-3′
Primers for construction of as pCold-EjFATP
HG8-F: 5′-GCCGGAGCTCATGACTGCCAGCGAGTTTTC-3′
HG8-R: 5′-GCCGGGATCCCAGTCTGACTCTTCCAGATA-3′

Y = C or T; N = A,G, C or T; R = A or G; M = A or C; V = A,C or G.