Comparative studies on the sex pheromone biosynthesis in moths with a focus on lipid metabolism in the pheromone gland

（ガ類の性フェロモン生合成に関する比較研究－フェロモン腺における脂質代謝に注目して－）

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0. General introduction

0.1. Lepidopteran sex pheromone

Pheromones are utilized by a wide variety of organisms for communication within the species. Pheromones are classified into several types such as sex pheromones, aggregation pheromones and marking pheromones based on the behaviors evoked by pheromone stimulation. Females of many moth species secrete species-specific sex pheromone compounds that mediate sexual communication for mate finding. The first description of long-distant sexual communication in lepidopteran species was made by Fabre in the 19th century in his memorial work, "Souvenirs entomologiques". However, we had to wait until 20th century for the first chemical identification of a sex pheromone, (*E,Z*)-10,12-hexadecadien-1-ol (bombykol) from the silk moth, *Bombyx mori* (Butenandt et al. 1959). Since then sex pheromones have been chemically identified from more than 600 lepidopteran species (Ando, 2010; El-Sayed, 2010).

These sex pheromones of moths are classified into two major types (Type I and Type 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, or acetate ester (Jurenka 2004), and about 75% of the known lepidopteran sex pheromones fall into this class (Ando et al. 2004). 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, which are utilized by a number of species in the families Geometridae, Arctiidae and Lymantriidae (Millar 2000; Ando et al. 2004).

0.2. Biosynthetic pathway of lepidopteran sex pheromones

Type I pheromones are synthesized in the pheromone gland (PG), a functionally differentiated tissue located close to the terminal abdominal segment of female moths (Bjostad et al. 1987). Type I pheromones are biosynthesized *de novo* from saturated fatty acids, which are supplied via common fatty acid synthesis pathways, by a series of desaturation and chain elongation/shortening steps that attribute pheromone components with specific chain lengths, double bond positions, and double bond geometries. The terminal functional group of the sex pheromone molecules is finally modified by reduction, oxidation, or acetylation to produce alcohols, aldehydes, or acetates, respectively (Tillman et al. 1999).

The silkmoth, *Bombyx mori*, has been used as a model of moths producing Type I sex pheromone compounds. The biosynthesis of its sex pheromone, bombykol, has been elucidated at the molecular level (Matsumoto 2007). Bombykol is synthesized via *de novo* from acetyl-CoA through palmitate, which is stepwise converted to bombykol by Δ11 desaturation, Δ10,12 desaturation, and fatty-acyl reduction (Fig. 0.2.A) (Rafaeli 2002).

In contrast, the Type II sex pheromones originate from long chain hydrocarbons produced outside PGs (Schal et al. 1998; Subchev and Jurenka 2001; Jurenka et al. 2003; Wei et al. 2003; Wei et al. 2004; Matsuoka et al. 2006). After being synthesized in oenocytes or abdominal epidermal cells, the precursors of Type II pheromones (hydrocarbons) are transported to the PG by lipophorin, a transporter protein in the hemolymph (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 (Fig. 0.2.B) (Ando et al. 1997). The epoxy pheromone compound is formed

from the corresponding polyunsaturated hydrocarbon, Z3,Z6,Z9-19:H, in the PG (Miyamoto et al. 1999). The direct precursor Z3,Z6,Z9-19:H was detected in the hemolymph of the females only, and the deuterium-labeled triene injected into the hemocoel of the females was shown to be converted to the epoxy pheromone after incorporation into the PGs (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; Wei et al. 2003).

In many species of moths, female pheromone biosynthesis is under circadian fluctuation due to the photoperiodic release of a peptide hormone, pheromone-biosynthesis-activating neuropeptide (PBAN), which is secreted from the suboesophageal ganglion (SOG) (Rafaeli 2005). In *B. mori*, PBAN is released into the hemolymph after eclosion and acts directly on the PG to stimulate sex pheromone biosynthesis (Arima et al. 1991; Fónagy et al. 1992; Hull et al. 2005). The effect of PBAN on the biosynthesis of sex pheromones has been investigated in several species, and it turned out that the biochemical step regulated by PBAN is different among moths (Rafaeli 2005). In bombykol biosynthesis, fatty acyl reduction is regulated by PBAN (Fig. 0.2.A) (Rafaeli 2005). In *A. selenaria*, on the other hand, pheromone biosynthesis is regulated by PBAN at the step of precursor uptake by the PGs (Fig. 0.2.B) (Wei et al. 2004). However, pheromone biosynthesis in not all moth species is regulated by this peptide (Rafaeli and Jurenka, 2003). In the case of the cabbage looper, *Trichoplusia ni*, PBAN is secreted but does not regulate pheromone production (Tang et al. 1989).

0.3. Lipid droplets in the PG implicated for involvement in sex pheromone biosynthesis

Accumulation of lipid droplets within the cytoplasm is a common morphological feature of the PG cells of many lepdopteran species; to date, LDs in about 10 moth species have been described in the literature (Percy J. E. 1974; Percy R. et al. 1975; Bjostad and Roelofs 1983). However, only lipid droplets in the PG of *B. mori* have been chemically identified, and it has been proved that the lipid droplets play an important role in sex pheromone synthesis, i.e., storage and release of pheromone precursors (Fig. 0.2.A) (Fónagy et al. 2000; Fónagy et al. 2001; Matsumoto et al. 2007). Shortly before the eclosion of a female silkmoth, bombykol-producing cells can be characterized by the abundant lipid droplets within the cytoplasm (Fónagy et al. 2000; Fónagy et al. 2001). These abundant lipid droplets begin to accumulate one or two days prior to the eclosion, and the number of lipid droplets increase sharply on the day of eclosion. Then the number of lipid droplets decreases after elosion, along with the release of sex pheromone in response to stimulation by PBAN. The chemical analysis demonstrated that the lipid droplets are composed of various triacylglycerols (TGs) that comprise the precusor of bombykol, Δ10,12-hexadecadienoate, as a moiety (Fónagy et al. 2000; Fónagy et al. 2001). In contrast to the wealth of information available on the lipid droplets in the moths using Type I sex pheromones, at present, no information is available on the composition and the role of lipid droplets in the moths using Type II sex pheromones.

0.4. Fatty acid transport protein

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. As discussed in the previous section, lipid droplets play an important role in the sex

pheromone biosynthesis of some moths. Recently, a fatty acid transport protein (BmFATP) was identified in the pheromone gland (PG) of *B. mori*. BmFATP was shown to have an essential role in bombykol synthesis through the uptake of extracellular fatty acids (Fig. 0.2.A; Ohnishi et al. 2009).

When I started the present study, there was no information on the presence of FATP in the PG of moths that produce Type II pheromones. In contrast to moths that produce Type I sex pheromone, a FATP may be unnecessary for moths that produce Type II sex pheromones because the pheromone components in these moths originate from long chain hydrocarbons produced outside PGs, not fatty acids (Fig. 0.2.B) (Jurenka et al. 2003; Wei et al. 2004; Matsuoka et al. 2006).

0.5. Overview of this thesis

Although lipid droplets in the PG appear to play an important role in the sex pheromone biosynthesis, only the roles in *B. mori* have been studied in depth at the molecular level. In chapter 1 of this thesis, the presence of lipid droplets in the PGs of *Eilema japonica* (Fig. 0.1.B) and *A. selenaria*, which produce Type II pheromones, was investigated in comparison with *B. mori* and *Ostrinia scapulalis* (adzuki bean borer) (Fig. 0.1.A), which produce Type I pheromones.

The hydrocarbon precursor of Type II sex pheromones in the hemolymph must be incorporated into the PG, but there is no information on the mechanism of incorporation. In chapter 2 of this thesis, to obtain a clue as to the mechanism of hydrocarbon uptake by the PG of moths using Type II pheromones, I explored the presence of *fatp* homologues in *E. japonica*. For comparison, I also explored *fatp* homologues in the PGs of *A. selenaria*, which produces a Type II sex pheromone, and *O. scapulalis*, which produces a

Type I sex pheromone (E11-14:OAc and Z11-14:OAc) (Huang et al. 1997). The tissue expression profiles of three FATP homologues and fluctuations of *Ejfatp* expression were also investigated in this chapter.

In the following chapter (chapter 3), function of EjFATP was investigated using an *Escherichia coli* expression system. The role of OsFATP in pheromone biosynthesis was subsequently investigated by RNAi mediated gene silencing.

In general discussion (chapter 4), the significance of the findings in this thesis for understanding the evolution of Type I and Type II sex pheromones was argued.

Fig. 0.1.

A B

Fig. 0.1. Adults of *O. scapulalis* (A) and *E. japonica* (B). Male is left one and female is right one in both figures.

Fig. 0.2.

A

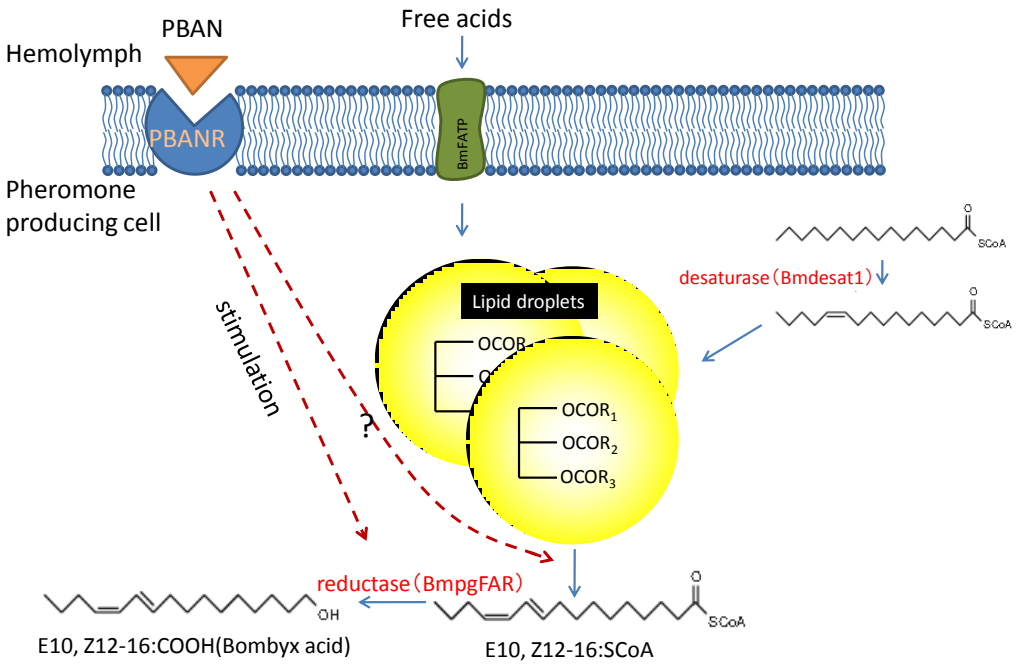


Fig. 0.2. A. Sex pheromone biosynthetic pathway proposed for *B. mori* that produces Type I pheromones. All steps of pheromone biosynthesis occur in the pheromone gland in this insect.

B

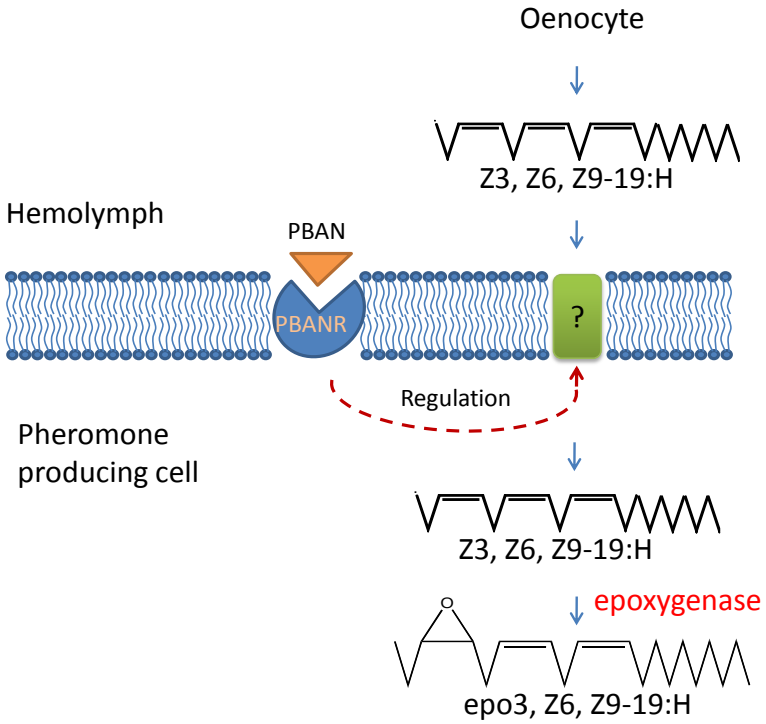


Fig.0.2.B. Sex pheromone biosynthetic pathway proposed for *A. selenaria* that produces Type II pheromone. Only the final epoxidation step occurs in the pheromone gland.

Chapter 1. The presence of lipid droplets in the pheromone gland *of Eilema japonica* and *Ostrinia scapulalis*

1.1. Introduction

Accumulation of lipid droplets within the cytoplasm is a common morphological feature of the pheromone gland (PG) cells of many lepdopteran species, for example, in *Trichoplusia ni,* the lipid droplets in PG are the source of the immediate precursors to the sex pheromone (Bjostad and Roelofs 1983). To date, lipid droplets in the PGs of several species of moths such as *Orgyia leucostigma,* and *Choristoneura spp.* have been investigated (Feng and Roelofs 1977; Percy J. and George 1979).

Lipid droplets in the PG of *Bombyx mori* have been studied in depth. *B. mori*, which secretes Type I sex pheromone, is a good model for understanding the mechanism of sex pheromone production at the molecular level. The most obvious characteristic feature of bombykol-producing cells is the abundance of lipid droplets within the cytoplasm. The lipid droplets begin to accumulate 1 or 2 days prior to eclosion, and the number of lipid droplets increases sharply on the day of eclosion (Fónagy et al. 2000). After eclosion, they decrease in size and number over the course of the day when females are actively releasing bombykol. The lipid droplets then re-accumulate at night when females remain inactive (Foster 2001).

However, the knowledge on the lipid droplets in the PG of moths that use Type II sex pheromone has been limited. Only a geometrid moth, *Ascotis selenaria*, was investigated for lipid droplets, but no droplet has been detected in the PG of this species (Fujii et al. 2007).

Because there are significant differences between the biosynthetic pathways for production of Type I and Type II pheromones, in order to understand the difference on lipid droplets of moths producing Type I and Type II sex pheromone compounds, in this chapter, the presence of lipid droplets in the PGs of *Eilema japonica*, which secretes 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) as sex pheromone (Fujii et al. 2010), and *O. scapulalis*, which produces a Type I sex pheromone (E11-14:OAc and Z11-14:OAc) (Huang et al. 1997) was investigated using *B. mori* and *A. selenaria* as controls.

1.2. Materials and methods

1.2.1. Insects

Female moths of *E. japonica* and *A. selenaria* were collected at Bunkyo-ku, Tokyo, Japan (35.4 °N, 139.4 °E) in 2008. *O. scapulalis* used in this study were offspring of larvae collected at Matsudo, Chiba, Japan (35.5 °N, 139.6 °E) in 2008. All offspring were reared on an artificial diet, Silkmate^{TM} 2M (Nosan Corp.) under conditions of 24 °C, 50–70% relative humidity, and a 16 h light : 8 h dark cycle. Pupae were separated by sex based on the morphology of the terminal abdominal segment, and maintained under the same environmental conditions as for rearing. Adult female and male were separately transferred to new plastic cups and maintained until use with a supply of sugar water. Adult females of *B. mori* (P50) were provided by Laboratory of Insect Genetics and Bioscience, the University of Tokyo.

1.2.2. Microscopy

The lipid droplets in the PGs of the three species were investigated by Nile Red staining according to the method of Fónagy (2000). Briefly, the PGs dissected from each species of female moths at eclosion were trimmed and then fixed with 4% formaldehyde. The staining was performed by 1/20 volume of Nile Red (Molecular probes) saturated acetone stock solution, and incubated in the dark for 10 min. Processed PGs were

washed with PBS, placed on a slide, sealed with a cover slip, and subjected to microscopic observation. The observations and microphotography were performed with an Olympus BX-60 system equipped with a PM-30 exposure unit and a BH20-RFL-T3 light source (400 × magnification). Nile Red was seen at the following spectral settings: 330-385 nm band pass exciter filter, 400 nm dichroic mirror and 420 nm long pass barrier filter (Olympus cube WU).

1.3. Result

Detection of lipid droplets by fluorescent microscopy

One of the prominent morphological features of the PG cells of *B. mori* is the abundance of large lipid droplets in the cytoplasm, and these lipid droplets are clearly visualized by a phenoxazine dye, Nile Red (Greenspan et al. 1985). Staining experiments with Nile Red was performed using the PGs of *O. scapulalis*, *E. japonica*, *B. mori* (a positive control), and *A. selenaria* (a negative control). After staining, abundant large lipid droplets were observed in the PG cells of *B. mori* (Fig. 1.1.). A few lipid droplets were found in the PG cells of *O. scapulalis* and *E. japonica* (Fig. 1.1.). In accordance with the results of observation by Fujii (2007), no lipid droplets were detected in the PG cells of *A. selenaria* (Fig. 1.1.).

1.4. Discussion

Although accumulation of lipid droplets within the cytoplasm is said to be a common feature of the PG cells of many lepidopteran species, not many moths were actually investigated for the presence of lipid droplets in the PG (Feng and Roelmone 1977; Percy 1979; Percy-Cunningham and MacDonald 1987; Fónagy et al. 2000, 2001).

In *B. mori*, a rapid decrease of lipid droplets is induced by pheromone-biosynthesis-activating neuropeptide (PBAN). The disappearance of lipid droplets after eclosion could be prevented by decapitation, which removed the source of PBAN, and the disappearnce was resumed by a PBAN injection into the hemocoel. By HPLC and GC-MS analyses, it was confirmed that the bombykol precursor, Δ10,12-hexadecadienoate is the major moiety in triacylglycerols (TGs), which compose the lipid droplets (Matsumoto et al. 2002; Ohnishi et al. 2006). These results demonstrate that the lipid droplets play a role in storing the bombykol precursor in the form of TGs and releasing it for bombykol production in response to PBAN stimulus. In the genus *Ostrinia*, the presence of lipid droplets has been checked only in the PG of *O. nubilalis*. In the present study, a few lipid drops were observed in the PGs of *O. scapulalis* and *E. japonica* (Fig. 1.1.). Because *O. nubilalis* and *O. scapulalis* use biosynthetic pathway of bombykol to produce sex pheromone, so it is possible that the lipid droplets in the PG of *O. scapulalis* play a role in storing sex pheromone precursor in TGs as that of *B. mori*. Interestingly, lipid droplets were also found in the PG of *E. japonica*, which produces Type II sex pheromone (Fig. 1.1.). Moths using Type II sex pheromone do not utilize fatty acids as material for pheromone biosynthesis in the PG (Fig. 1.2.), and lipid droplets were not detected in the PG of *A. selenaria* (Fujii et al. 2007). Therefore, the role or function of lipid droplets formed in the PG of *E. japonica* is an interesting question to be addressed.

B. mori produces a large amount of sex pheromone (≈1200 ng/female at its maximum), in comparison with *O. scapulalis* and *E. japonica* (*O. scapulalis*, 5–15 ng/female; *E. japonica*, 20–200 ng/female) (Kaissling and Kasang 1978; Ando et al. 1996; Huang et al. 2002; Fujii et al. 2010). So the abundance of lipid droplets in the PGs

of *B. mori* might reflect the scale of sex pheromone production. The silkworm is a relatively large-sized insect that has been selected in sericulture for thousands of years to increase silk production and for easier rearing, and this could have made *B. mori* to produce a huge amount of sex pheromone, and hence abundant lipid droplets.

Fig. 1.1.

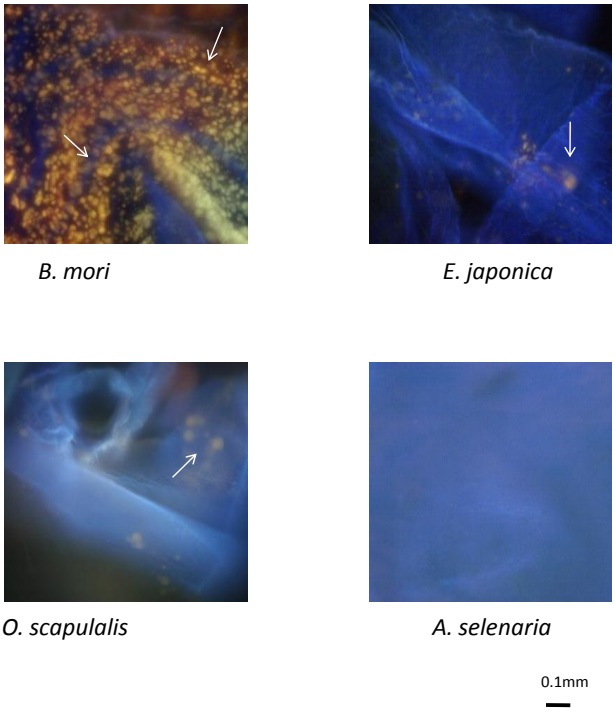


Fig 1.1. Photographs of the pheromone gland of *B. mori*, *E. japonica*, *O. scapulalis*, and *A. selenaria* stained with Nile red. Arrow indicates the position of lipid droplets. Scale bar is 100 μm.

Chapter 2. Cloning of a gene encoding fatty acid transport protein (FATP) in the pheromone glands of *Ostrinia scapulalis*, *Eilema japonica* and *Ascotis selenaria*

2.1. Introduction

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, and can be characterized by two highly conserved sequences, the ATP/AMP and FATP/VLACS signature motifs. As membrane-bound protein, FATPs have some trans-membrane domains on the N-terminal (e.g., murine FATP1 has one transmembrane domain and BmFATP has two) (Lewis et al. 2001; Obermeyer et al. 2007). In humans and mice, six isoforms of FATP (FATP1–FATP6) have been identified. The tissue-specific expression of these isoforms suggested that each plays a distinct role in lipid metabolism through the uptake of specific fatty acids (Hirsch et al. 1998; Hall et al. 2003). For example, FATP4 strongly expressed in the small intestine is essential for absorption of dietary lipids (Stahl et al. 1999).

As discussed in chapter 1, lipid droplets play an important role in the sex pheromone biosynthesis of some moths. In *B. mori*, lipid droplets serve as the storage of pheromone precursors, which are released in response to pheromone-biosynthesis-activating neuropeptide (PBAN) stimulus (Fig. 0.2.A) (Fónagy et al. 2000; Fónagy et al. 2001). Recently, a fatty acid transport protein (BmFATP) was identified in the pheromone gland (PG) of *B. mori*. BmFATP was 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).

However, a *fatp* homologues gene of FATP as found in *B. mori* seems unnecessary for moths that produce Type II sex pheromone, because the pheromone components originate from long chain hydrocarbons produced outside PGs (Fig. 0.2.B) (Schal et al. 1998; Subchev and Jurenka 2001; Jurenka et al. 2003; Wei et al. 2004; Matsuoka et al. 2006). The precursors of Type II pheromones (hydrocarbons) generated in oenocytes or abdominal epidermal cells are transported to the PG by lipophorin via the hemolymph (Schal et al. 1998; Jurenka et al. 2003; Wei et al. 2004; Matsuoka et al. 2006). In the Japanese giant looper *Ascotis selenaria*, a model moth producing Type II sex pheromones, direct pheromone precursors (Z3,Z6,Z9-19:H) in hemolymph are transported by lipophorin and specifically incorporated into the PG (Wei et al. 2003; Matsuoka et al. 2006). Lipophorin is a nonspecific shuttle for hydrocarbons including the pheromone precursors (Matsuoka et al. 2006). The specific delivery system for pheromone precursor has also been found in several other moths that produce Type II sex pheromones (Schal et al. 1998; Subchev and Jurenka 2001; Jurenka et al. 2003). However, little is known about the mechanism of delivery and uptake of hydrocarbon precursor by the PG.

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 identified in moths that produce Type II sex pheromones. The hydrocarbon precursor of Type II sex pheromones in the hemolymph must be incorporated into the PG, but there is no information on the mechanism of incorporation. In this chapter, to obtain a clue as to the mechanism of hydrocarbon uptake by the PG, we used *Eilema japonica*, which secretes 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) as sex pheromone (Fujii et al. 2010), to explore the presence of *fatp* homologues in moths that produce Type II pheromones. For comparison, we also

explored *fatp* homologues in the PGs of *A. selenaria*, which produces a Type II sex pheromone, and *Ostrinia scapulalis* (adzuki bean borer moth), which produces a Type I sex pheromone (E11-14:OAc and Z11-14:OAc) (Huang et al. 1997).

2.2. Materials and methods

2.2.1. Insect

Cultures of *E. japonica* and *A. selenaria* were established from females collected from Bunkyo-ku, Tokyo, Japan (35.4 °N, 139.4 °E), and females of *O. scapulalis* collected at Matsudo, Chiba, Japan (35.5 °N, 139.6 °E). All rearing conditions were the same as previously (see section 1.2.1).

2.2.2. Cloning of FATP genes

Total RNA was isolated from the PGs of females (1- to 2-day-old moths) using an RNeasy mini kit and RNase-free DNase (Qiagen). First-strand cDNA synthesis was performed using an RNA PCR kit (AMV) ver 2.1 (Takara) with 500 ng of total RNA. The central region of *fatp* homologous genes were amplified using two degenerate primers, DGF and DGR (Table2.1), designed based on conserved regions of FATP proteins (or putative FATP proteins) of several insect species including *Bombyx mori*, *Apis mellifera*, *Anopheles gambiae* and *Drosophila melanogaster* (GenBank accession nos. AB451529, XP_624496, XP_321320 and NP_723597). RT-PCR was performed under the following conditions: 94 ℃ for 2 min, followed by 5 cycles of 94 ℃ for 1 min, 46 ℃ for 1 min, 72 ℃ for 1 min, then followed by 25 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. The amplification products were excised from 2% agarose gels, ligated into a linearized pGEM®-T Easy

vector (Promega), and used to transform JM109 competent cells (Promega). The inserts were sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 377HN Sequencer (Applied Biosystems).

2.2.3. Rapid amplification of cDNA ends (RACE)

The 3′ and 5′ ends of the *Osfatp*, *Ejfatp* and *Asfatp* cDNA were obtained by RACE (Rapid amplification of cDNA ends) method using a GeneRacer™ Kit (Invitrogen). The first-strand cDNA of *Osfatp*, *Ejfatp* and *Asfatp* were synthesized from the total RNA extracted from the PGs of females (1- to 2-day-old). The first PCR in the 5′ RACE was performed using the GeneRacer 5′ Primer (Invitrogen) and gene-specific primers EJF, ASF, and OSF (Table 2.1), respectively. The nested PCRs were performed using the GeneRacer 5′ Nested Primer (Invitrogen) and gene-specific primers, EJFnest, ASFnest, and OSFnest (Table 2.1), respectively. The first PCR in the 3′ RACE was performed using the GeneRacer 3′ Primer (Invitrogen) and gene-specific primers, EJR, ASR, and OSR (Table 2.1), respectively. The nested PCRs were performed using the GeneRacer 3′ Nested Primer (Invitrogen) and gene-specific primers, EJRnest, ASRnest, and OSRnest (Table 2.1), respectively. The resultant cDNAs obtained in the 5′ and 3′ RACE were subcloned using pGEM-T easy vector system I (Promega), and sequenced to check the insert.

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.

2.2.4. 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) (http://clustalw.ddbj.nig.ac.jp/top-j.html). 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*, XP624496; *Ascotis selenaria* (AsFATP), AB561867; *Bombyx mori* (BmFATP), BAG68297; *Drosophila melanogaster*, CG3394, CG30194, and NP723597; *Homo sapiens*, EAW84611; *Mus musculus*, AAH23114 and AAH28937; *Ostrinia scapulalis* (OsFATP), AB561866; and *Saccharomyces cerevisiae*, NP009597. The KAIKO base (http://sgp.dna.affrc.go.jp/Kaikobase/) was explored for *Ejfatp* homologues in the *Bombyx* genome.

2.2.5. Tissue distribution profiles of *fatps*

PGs and other tissues were dissected in PBS, and total RNA was isolated from these tissues using an RNeasy mini kit and RNase-free DNase (Qiagen). The first-strand cDNA synthesis was performed using an RNA PCR kit (AMV) ver 2.1 (Takara) with 500 ng of total RNA. PCR was performed using Ex-Taq polymerase (Takara) and primers designed based on the nucleotide sequences of *Osfatp*, *Ejfatp*, and *Asfatp* (Table 2.1). The conditions of the PCR were 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. Amplified products were checked by electrophoresis on 2% agarose gel.

2.2.6. Quantitative PCR (qPCR) analysis for *Ejfatp*

Total RNA was isolated from the PGs of *E. japonica* at different developmental stages, and reverse transcribed into cDNA as described above (see section 2.2.2), and diluted to 1/4. qPCR was performed with 2 × Power SYBR Green PCR master mix (Applied Biosystems) with an ABI PRISM 7700 thermal cycler (Applied Biosystems). The conditions for the PCR were 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 30 s. The primers used in this experiment are listed in Table 2.1. Amounts of *Ejfatp* mRNA were normalized with those of endogenous *β*-actin.

2.2.6. Semi-Quantitative RT-PCR analysis for *Osfatp*

Total RNA was isolated from the PGs of *O. scapulalis* at different developmental stages, and reverse transcribed into cDNA as described above (see section 2.2.2). PCR was performed using Ex-Taq polymerase (Takara) and primers designed based on the nucleotide sequence of *Osfatp* (Table 2.1). The conditions of the PCR were 94 ℃ for 2 min, followed by 25 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. Amplified products were checked on 2% agarose gel by electrophoresis. Endogenous *β*-actin was used as a control.

2.3. Results

2.3.1. Cloning and characterization of *fatp* homologues

Twenty clones were picked up random each species and sequenced. In *E. japonica,* 17 of 20 clones contained the same fragment. In *A. selenaria,* 15 of 20 clones contained the same fragment. And in *O. scapulalis,* 18 of 20 contained the same fragment. According to these fragments, the open reading frames of FATPs from *E. japonica*, *A. selenaria*, and *O. scapulalis* encoded amino acid (aa) sequences of 700 aa (EjFATP), 572 aa (AsFATP), and 650 aa (OsFATP), respectively. The EjFATP, AsFATP and OsFATP sequences showed a high degree of identity to the BmFATP sequence (70.3%, 77.9%, and 72.6%, respectively) (Fig. 2.1). These FATPs contained two highly conserved motifs, the ATP/AMP signature motif common to the adenylate-forming enzymes, and the FATP/VLACS signature motif conserved in all FATPs reported to date (Fig. 2.1). A secondary structural analysis of the three homologues with the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) suggested that EjFATP contained three possible N-terminal transmembrane domains (amino acid positions 44–66, 69–91, and 103–128), and OsFATP contained one (position 11–33). However, AsFATP contained no trans-membrane domains.

2.3.2. Phylogenetic analysis of FATP homologues

To investigate the evolutionary relationship between the homologues characterized in the present study and other FATP proteins, a phylogenetic tree was constructed with the proteins in public databases showing homology to EjFATP. Three homologues of BmFATP (BGIBMGA006187, BGIBMGA006189, and BGIBMGA014057) found in the *Bombyx* genome were also included in the analysis. The phylogenetic analysis showed

that EjFATP, OsFATP, AsFATP, and BmFATP (BGIBMGA006185) form a clade, and these are most closely related to BGIBMGA006187 (Fig. 2.2.). EjFATP, OsFATP, AsFATP, and BmFATP did not split into two groups corresponding to the type of sex pheromone (Type I or Type II). BGIBMGA006189 and BGIBMGA014057 are dispersed in the insect FATP clade, but the latter is interesting in that it formed a distinct clade with CG3394 of *D. melanogaster*.

2.3.3. Tissue distribution of three *fatp* homologues

The expression profiles of *Ejfatp*, *Asfatp*, and *Osfatp* were investigated by RT-PCR. As shown in Fig. 2.3, the *Ejfatp* transcript was predominantly expressed in the PG and midgut, with substantially lower levels in the ovary, flight muscle, Malpighian tubule, and epidermis (Fig. 2.3.A). The *Osfatp* transcript was predominantly expressed in the midgut, with low-level expression in other tissues (Fig. 2.3.A). In contrast, the *Asfatp* transcript was expressed in most tissues examined at similar levels (Fig. 2.3A). In the larval stage, *Ejfatp* was predominantly expressed in the midgut, and at substantially lower levels in the head (Fig. 2.3B).

2.3.4. Fluctuations of *Ejfatp* expression in PGs

To check fluctuations of the *Ejfatp* expression level, a qPCR analysis was performed with cDNA prepared from PGs sampled at different times. The relative expression level of *Ejfatp* was low 2 days before eclosion, but increased 4.96 folds upon eclosion (Fig. 2.4.A). No difference in the expression level of *Ejfatp* was found between the photophase and scotophase (Fig. 2.4.B).

2.3.5. Fluctuations of *Osfatp* expression in PGs

Semi-Quantitative RT-PCR was used for the analysis of fluctuations in *Osfatp* expression. The expression of *Osfatp* was low at 2 days before eclosion, and so was 1 day after the eclosion. It increased sharply at 2 days after the eclosion (Fig. 2.5.). The expression of *β*-actin in the PGs (control) was at a similar level from the pupal stage to 7 days after eclosion (Fig. 2.5.).

2. 4. Discussion

Although the presence of *fatp* is reported for several moth species, only BmFATP of *B. mori* has been functionally characterized (Ohnishi et al. 2009). In this study, three FATP homologues (EjFATP, AsFATP and OsFATP) were isolated from the PGs of three moth species, *E. japonica*, *A. selenaria*, and *O. scapulalis* (Fig. 2.1). While the expression of BmFATP was practically limited to the PG, the three homologues were expressed in multiple tissues with different expression patterns (Fig. 2.3A). The differences in tissue specificity may suggest that these FATPs play discrete roles in lipid metabolism.

FATP homologues are widely found in many organisms from mycobacterium to human. In human and mice, six isoforms of FATP (FATP1 to FATP6) have been identified (Schaffer and Lodish 1994; Lewis et al. 2001; Stahl 2004). A search for FATP gene homologues in the KAIKO base (http://sgp.dna.affrc.go.jp/Kaikobase/) revealed four FATP homologues (Fig. 2.2). It is suggested that insects also have multiple FATP isoforms as in mammals.

FATPs have a common feature that they have transmembrane domains in their N-terminal (Lewis et al. 2001; Obermeyer et al. 2007; Ohnishi et al. 2009). By the analysis of membrane protein structure using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/), EjFATP contained three possible N-terminal transmembrane domains, and OsFATP contained one. However, AsFATP lacked the transmembrane domain (Fig. 2.1.), suggesting that AsFATP might be nonfunctional. Interestingly, a few putative lipid droplets (LDs) were found in the PG cells of *O. scapulalis* and *E. japonica*, while no lipid droplet was found in the PG cells of *A. selenaria* (Fujii et al. 2007) (Fig. 1.1.). Taken these results altogether, I considered that FATPs might play a role in the accumulation of lipid droplets in the pheromone gland.

Enzymes involved in sex pheromone biosynthesis in moths show common features such as specific expression in the PG and up-regulation of expression shortly before or after adult emergence (Roelofs et al. 2002; Moto et al. 2003; Liu et al. 2004; Moto et al. 2004; Ohnishi et al. 2006; Antony et al. 2009). The predominant expression of *Ejfatp* in the PG (Fig. 2.3.A) and up-regulation of *Ejfatp* expression shortly after emergence (Fig. 2.4.A; Fig. 2.5) suggest the involvement of EjFATP in sex pheromone biosynthesis. BmFATP from the PG of *B. mori* was shown to play an important role in the sex pheromone synthesis by stimulating the uptake of fatty acids (Ohnishi et al. 2009). However, Type II sex pheromones are not biosynthesized through modified fatty acid biosynthetic pathways. Type II pheromone precursors (hydrocarbons) are synthesized in oenocytes or abdominal epidermal cells, transported by lipophorin in hemolymph, and then specifically taken up into PG (Schal et al. 1998; Wei et al. 2004; Fujii et al. 2007). So despite the expression profile suggests EjFATP involved in sex pheromone

biosynthesis, further investigations are needed to explain the function of EjFATP in this biosynthesis.

Although *Osfatp* was not predominantly expressed in the PG, the expression of *Osfatp* sharply increased on the 2nd day after eclosion. The result of sex pheromone titer of sex pheromone of *O. scapulalis* show a periodicity, i.e., low in the photophase and high in the scotophase. However, sex pheromone titer of 1-day-old *O. scapulalis* female moth did not show this fluctuation, and remained at a low level (Murata 2008). So the fluctuation of *Osfatp* expression was consistent with the fluctuation of sex pheromone (Fig. 2.3.A), suggesting the involvement of OsFATP in sex pheromone biosynthesis.

A previous study indicated that FATP4 of mammals strongly expressing in the small intestine is essential for dietary lipid absorption (Stahl et al. 1999). In insects, some similar observations were reported. BmFATP expressed in a high level in the larval stage (Ohnishi et al. 2009). A FATP homolog in *D. melanogaster* was localized in the midgut of the larvae (Stahl et al. 1999). In the present study, *Ejfap* and *Osfatp* were found strongly expressed in the midgut (Fig. 2.3.). These results suggest FATPs are possibly involved in lipids absorption of midgut.

Table 2.1.

Primers used in this chapter

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′

Fig. 2.1.

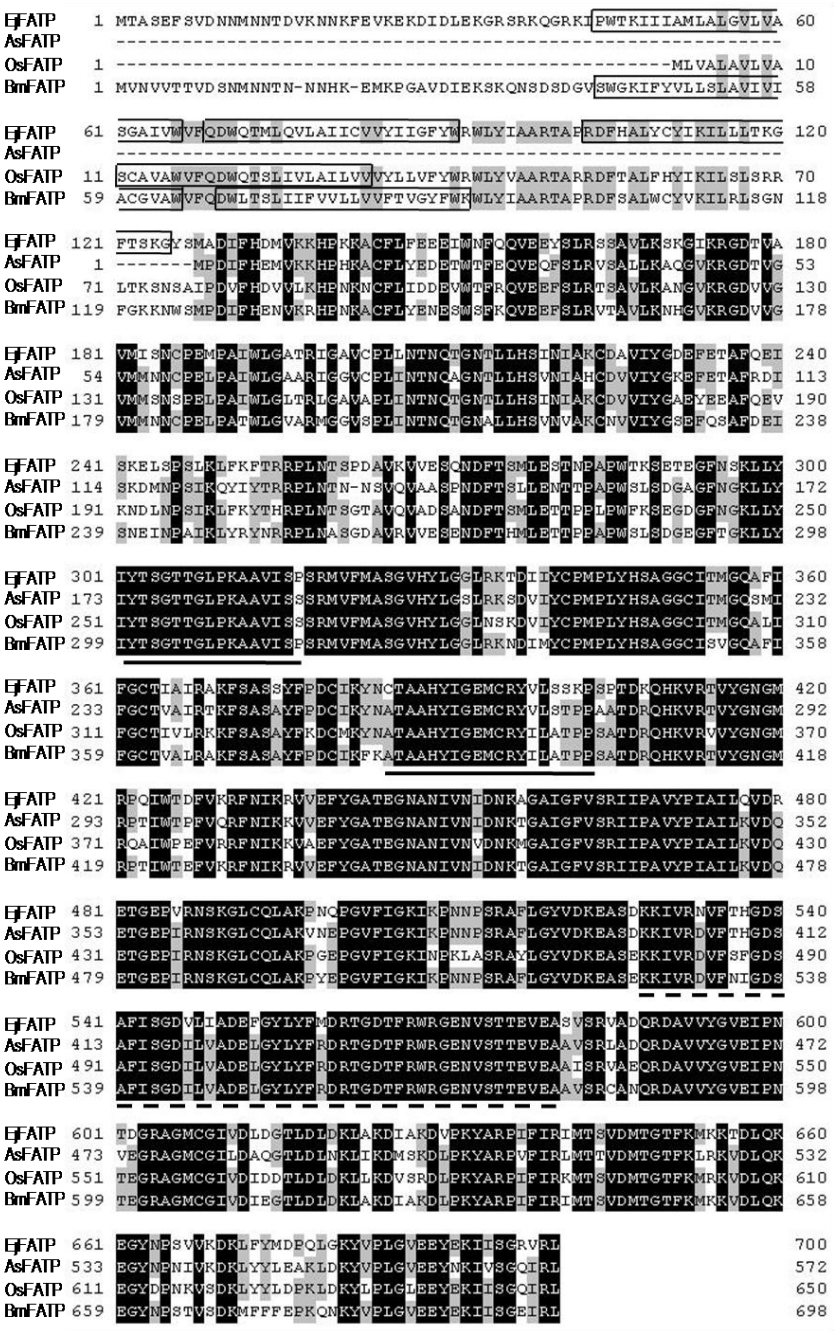


Fig. 2.1. Multiple alignment of EjFATP, AsFATP, OsFATP, and BmFATP. Deduced amino acid sequences of *Ejfatp*, *Asfatp*, and *Osfatp* are aligned with *Bmfatp* (GenBank accession number: BAG68297). Identical amino acids and those shared in more than three sequences are highlighted by black and gray, respectively. The ATP/AMP signature motif consisting of two highly conserved regions is underlined. The FATP/VLACS signature motif is underscored with a dotted line. The amino acids enclosed by squares compose the trans-membrane domains predicted by the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/).

Fig. 2.2.

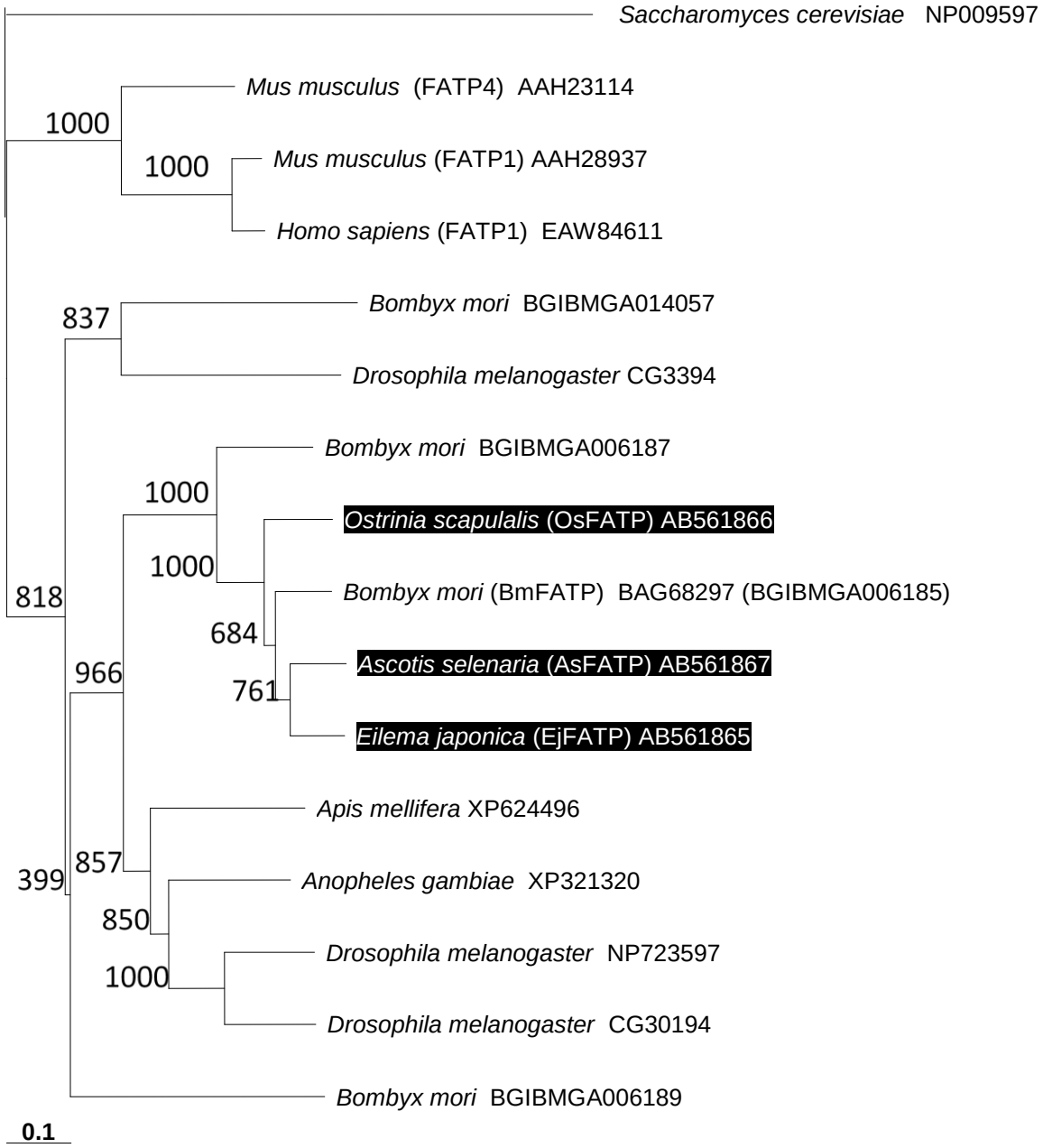
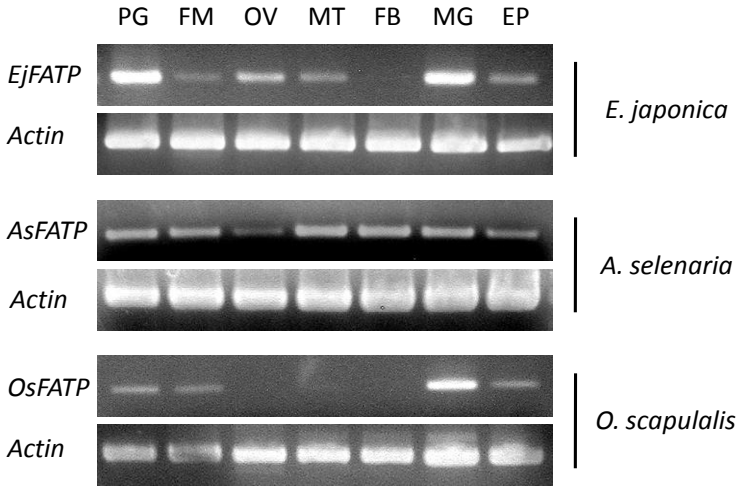


Fig. 2.2. Phylogenetic analysis of lepidopteran FATPs and homologues. Neighbor-joining tree of lepidopteran FATPs and sequences showing a high degree of identity to EjFATP. Genbank accession numbers of the sequences are shown beside the species name. Bootstrap values with 1000 resamplings are shown near the branches, and the genetic distance is drawn to scale.

Fig. 2.3.

A



B

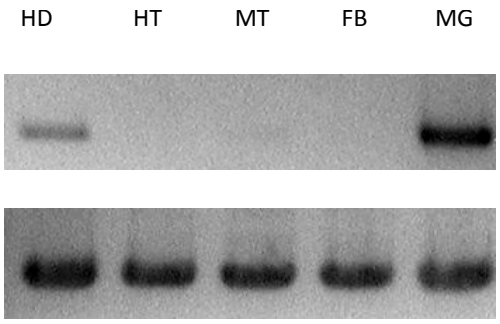
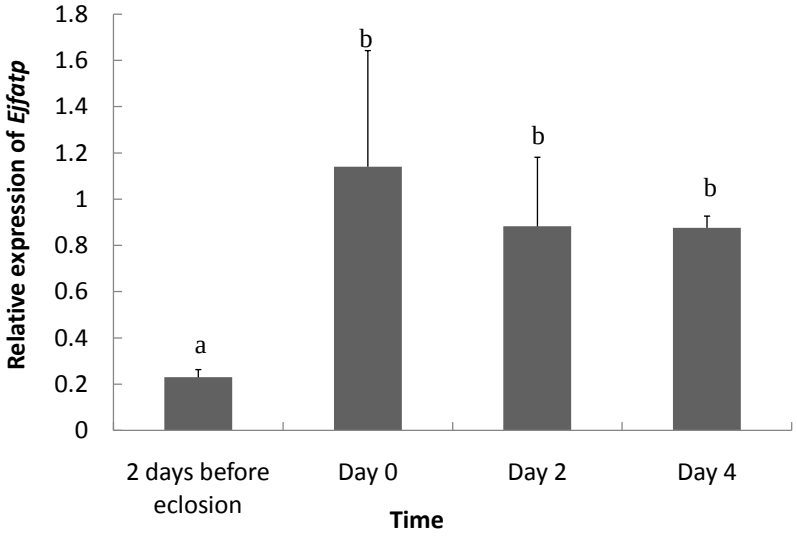


Fig. 2.3. Tissue distribution pattern of *fatp* homologues. (A) RT-PCR analysis was performed with cDNA prepared from total RNA of 2- to 3-day-old adult virgin females. PG: pheromone gland. FM: flight muscle. Ov: ovary. MT: Malpighian tubule. FB: fat body. MG: midgut. EP: epidermis. The β-actin gene was used as a positive control. (B) A RT-PCR analysis was performed with cDNA prepared from total RNA of larvae of *E. japonica*. HD: head. HT: heart. MT: Malpighian tubule. FB: fat body. MG: midgut. The β-actin gene was used as a positive control.

Fig. 2.4.

A



B

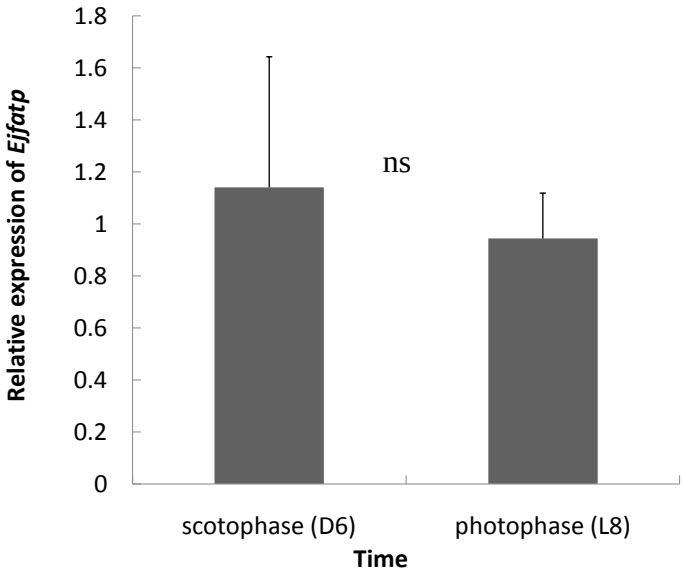


Fig. 2.4. Comparative quantitative real-time PCR analysis of *Ejfatp*. (A) Relative expression of *Ejfatp* at different ages (2–3 days before eclosion, 0 day, 2 days, and 4 days after eclosion). Means with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$). (B) Relative expression of *Ejfatp* in the scotophase and photophase. Values are expressed as the mean ± SD ($n \geq 3$). Ns: not significantly different (student's t-test, $P > 0.05$). The data are normalized with reference to the control (β-actin).

Fig. 2.5.

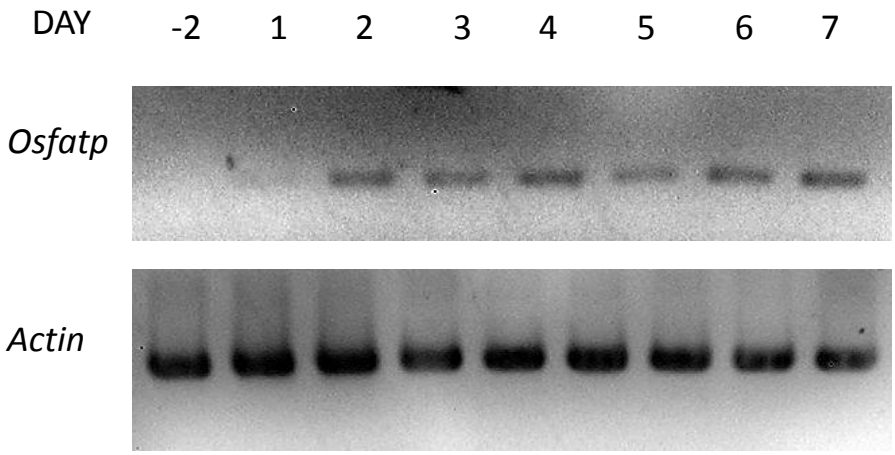


Fig. 2.5. Semi-Quantitative RT-PCR was used for analysis of fluctuations of *Osfatp* expression. DAY: the days after eclosion (-2 : 2 days before eclosion). The β-actin gene was used as a positive control.

Chapter 3. Functional assay of EjFATP and OsFATP

3.1 Introduction

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. The first FATP, FATP1, was characterized from rat by Schaffer and Lodish (1994). Overexpressed FATP1 in 3T3-L1 cells (derived from mammalian adipose tissue) results in 2.6-fold increase in the internalization of palmitic acid (C16), oleic acid (C18), and arachidonic acid (C20), suggesting that FATP1 does not have a specific preference for any of these long chain fatty acids (Schaffer and Lodish 1994). Later, FATP homologues have been widely found in many organisms from mycobacteria to humans (Hall et al. 2003, Hirsch et al. 1998). FATP from *Mycobacterium tuberculosis* expressed in *Escherichia coli* expression system showed a increase in the rate of palmitic acid uptake, suggesting the function of FATP gene family is conserved throughout the evolution (Hirsch et al. 1998). The function of FATPs have been extensively studied using various methods like *in vitro* loss-function (e.g., in mutant yeast), gain-of-function (e.g., transfectants in cultured mammalian cells), transgenic and knockout mouse model systems (Doege and Stahl 2006).

Although FATP homologues have been reported in several insect species, only BmFATP from *Bombyx mori* had been functionally characterized. BmFATP was shown to have an essential role in bombykol synthesis through the uptake of extracellular fatty acids to forming LDs in the PG cells (Ohnishi et al. 2009). RNAi-mediated gene silencing of BmFATP resulted in a significant reduction in bombykol production (Ohnishi et al. 2009). In chapter 2, three FAPT homologues was isolated from the PGs of three different moths, and two of them, EjFATP and OsFATP were speculated as functional genes by results of RT-PCR analysis (see Fig. 2.3.). *Ejfatp* was discovered

from pheromone gland (PG) of *Eilema japonica* which produces Type II sex pheromone, and the expression profiles of *Ejfatp* showed that it might be involved in sex pheromone biosynthesis. In this chapter, function of EjFATP was characterized using *Escherichia coli* expression system. Although *Osfatp* was not predominantly expressed in PGs, the fluctuation of *Osfatp* was consistent with fluctuation of sex pheromone, which suggests the involvement in sex pheromone biosynthesis as the BmFATP. Because the similar biosynthesis pathway of sex pheromone in *B. mori* and *O. scapulalis*, function of OsFATP was characterized using RNAi mediated gene silencing according to the protocol of functional characterization of BmFATP by RNAi treatment. .

3.2 Materials and methods

3.2.1 Expression of recombinant EjFATP protein by *E. coli*

The coding region of *Ejfatp* was amplified by PCR from the cDNA template prepared from the PGs of *E. japonica*, using HG8-F and HG8-R primers (Table 3.1). The PCR product was subcloned to pGEM-T easy vector system I (Promega) and sequenced to check the insert. Vector containing the gene was extracted from *E. coli*, digested with *Sac* I and *Bam*H I (Takara-bio), and ligated to the pCold I vector (Takara-bio). The expression vector constructed (pCold-His-EjFATP) was introduced into competent *E. coli* BL21 (DE3) cells (Takara-bio), and expression of the recombinant EjFATP was induced by 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The transformants were cultured at 15 ℃ for about 3 h until the OD_{600} of the *E. coli* culture reached 0.3, and the cells were collected by centrifugation at 6000 × *g* for 5 min.

3.2.2 SDS-PAGE and Western blotting

To examine the expression of EjFATP, the cell pellets obtained as described in 3.2.1 were resuspended in 100 µl of cell lysis buffer (50 mM Tris, 150 mM NaCl, and 1% Nonidet P-40, pH 7.8), and incubated at 37 ℃ for 10 min. The cell lysate was centrifuged at 12000 $\times g$ for 10 min at 4 ℃, and the supernatant was collected.

After the addition of an SDS sample buffer (0.125M Tris, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue), the samples were separated on 10% polyacrylamide gels, and blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Billerica, MA) using a Trans-Blot SD cell (Bio-Rad, Hercules, CA). After blocking with 2% skim milk in TNT (10 mM Tris–HCl pH 8.0, 0.05% Tween-20, 150 mM NaCl) at room temperature for 2 h, the membrane was incubated with the anti-His antibody (Qiagen, 1: 3000 dilution), and subsequently with goat anti-mouse IgG-HRP conjugate (Bio-Rad, 1: 3000 dilution). The recombinant protein was visualized with the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL), and recorded with Image Quant Imager 400Lumi (GE Healthcare BioScience, Tokyo, Japan).

To examine whether EjFATP forms inclusion bodies in the *E. coli* expression system, the cell pellets obtained as described in 3.2.1 were resuspended in 100 µl of phosphate-buffered saline (PBS, 2.5mM KCl, 141mM NaCl, and 8.1 mM Na_2HPO_4, pH 7.0), then sonicated for 2 min to obtain the cell lysate. The cell lysate was centrifuged at 600 $\times g$ for 10 min at 4 ℃, and the supernatant was collected. The pellets were resuspended in 100 µl PBS. After the addition of an SDS sample buffer (0.125M Tris, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue), the samples were separated by SDS-PAGE and the expression of recombinant protein was examined by subsequent Western blot analysis as described above.

3.2.3 Characterization of overexpressed recombinant EjFATP function

The analysis of fatty acid incorporation was performed essentially as described by Knoll et al. (1995). *E. coli* cells were collected by centrifugation at 12000 × *g* for 10 min at 4 ℃, washed with PBS, and resuspended in PBS at a final density of 3×10^9 cells/ml, which was estimated using the standard curve of OD_{600}. One milliliter of the cell suspension was pre-incubated at 37 ℃ for 10 min, and the assay was initiated by the addition of 100 nM mixture of lauric acid, myristic acid, palmitic acid, stearic acid, or eicosanoic acid. All authentic fatty acids were obtained from Sigma-Aldrich (St. Louis, MO), and prepared as 4 mM ethanolic stocks as described previously (Faergeman et al. 1997). After incubation for 5 min, cells were collected by centrifugation at 6000 × *g* for 5 min, and the cell pellets were washed three times with PBS.

Fatty acids in the *E. coli* cells, both free and esterified, were converted to methyl esters for the analysis with GC, as follows. Total lipids were extracted from the cells with 500 μl of a 2:1 (v/v) mixture of chloroform: methanol. The esterified fatty acids in the extract were transesterified by alkaline methanolysis according to the method of Foster (2001). Free fatty acids were subsequently methyl esterified by a conventional method using diazomethane. A QP5050 GC-mass analyzer system (Shimadzu, Kyoto, Japan) equipped with a capillary column (DB-Wax, 30 m×0.25 mm i.d., J & W Scientific, Folsom, CA) was used for the analysis of fatty acid methyl esters. The oven temperature was first maintained at 120 ℃ for 2 min, then raised at 12 ℃/min to 180 ℃, and finally raised at 5 ℃/min to 240 ℃. Helium was used as the carrier gas at a flow rate of 1 ml/min.

The amounts of fatty acid methyl esters were estimated based on the peak areas of a total ion chromatogram.

3.2.4. Synthesis and Injection of double strand RNA (dsRNA)

The templates for synthesis of dsRNA corresponding to OsFATP were prepared using gene-specific primers containing T7 polymerase recognition sites (RI-F 1342-1360bp and RI-R 1689-1709bp nucleotide sequences corresponding to the T7 promoter region are underlined) (Table 3.1). PCR was performed under the conditions of 6 cycles of 94 ℃ for 30 s, 56.5 ℃ for 30 s, 68 ℃ for 90 s followed by 30 cycles of 94 ℃ for 30 s, 66 ℃ for 30 s, 68 ℃ for 90 s using KOD-Plus-(Toyobo, Osaka, Japan). The products were purified using Wizard SV Gel and PCR Clean-Up kit (Promega).

Two microgram PCR product was used as templates, and dsRNA was generated using the MEGAscript RNAi Kit (Ambion) according to the manufacturer's instructions. After synthesis, the dsRNA was diluted with diethyl pyrocarbonate(DEPC)-treated H_2O, and the RNA concentration was measured (A_{260}). The products were analyzed by gel electrophoresis to verify annealing of the RNA strands. Samples were diluted to defined concentrations (1 and 5 μg/μl) and injected into hemocoeal near the abdominal tip of 2-day-old pupae with a 10-μl microsyringe (Hamilton). Control pupae were injected with 2 μl of DEPC-treated H_2O alone. After the injection, pupae were maintained under usual conditions until adult emergence.

3.2.5. Evaluation of *Osfatp* down-regulation by RT-PCR

PGs were dissected in PBS, and total RNA was isolated from these tissues using a RNeasy mini kit and RNase-free DNase (Qiagen). The first-strand cDNA was

synthesized from 500 ng of total RNA using an RNA PCR kit (AMV) ver 2.1 (Takara). PCR was performed using Ex-Taq polymerase (Takara) and the primers designed based on the nucleotide sequence of *Osfatp* (Table 1.1). The conditions of PCR were 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. After PCR, the PCR products were electrophoresed on a 1.5% agarose gel in TAE buffer.

3.2.6. Effect of *Osfatp* down-regulation on sex pheromone titer

PGs of 2-day-old moths were dissected at D4. The PGs were immersed individually in hexane (10 μl/female; residual pesticide analysis grade, Wako Pure Chemicals, Kyoto). The PGs were removed after 30 min, and the extract was kept at -20^{o}C prior to analysis. A gas chromatograph GC-17A (shimadzu, Kyoto, Japan) equipped with a flame ionization detector, and a DB-Wax column (J & W Scientific, CA) was used for the analysis of extracts. The oven temperature was first maintained at 120 ℃ for 2 min, then raised at 12 ℃/min to 180 ℃, and finally raised at 5 ℃/min to 240 ℃. Helium was used as the carrier gas at a flow rate of 1 ml/min. The amounts of sex pheromone were estimated based on the peak areas of a total ion chromatogram.

3.3 Results

4.3.1 Expression of recombinant EjFATP

To examine the function of EjFATP, a recombinant protein was produced using the *E. coli* expression system. Expression of the recombinant EjFATP was verified by SDS-PAGE and Western blotting (Fig. 3.1., Fig. 3.2.). Inclusion body formation in *E. coli* was

occasionally observed when it expresses proteins from other organism (Martinez-Alonso et al. 2009). In this research, it was confirmed that EjFATP did not form inclusion bodies in the *E. coli* expression system (Fig. 3.1.). Western blotting with the anti-His antibody showed that a His-tagged EjFATP of approximately 72 kDa was produced (Fig. 3.2.). No expression of EjFATP was observed in the negative control, in which IPTG was not added (Fig. 3.2.).

3.3.2 Functional characterization of recombinant EjFATP

To investigate the function of EjFATP, *E. coli* cells expressing EjFATP were incubated in PBS for 5 min in the presence of 100 nM lauric acid, myristic acid, palmitic acid, stearic acid, or eicosanoic acid. In comparison with the control *E. coli* cells, cells expressing EjFATP showed an enhanced ability (47.8%, 49.1%) to take up stearic acid or eicosanoic acid, but not lauric acid and myristic acid (Fig. 3.3.A). To examine the involvement of EjFATP in sex pheromone biosynthesis, we examined the ability of EjFATP to take up one of sex pheromone component (Z3,Z6,Z9-21:H), its analogs and candidate precursors. Expression of EjFATP had no effect on the uptake of the tested hydrocarbons (Fig. 3.3.B).

3.3.3 Silencing of endogenous *Osfatp* by RNAi

To elucidate the *in vivo* function of the OsFATP in pheromone biosynthesis, double-stranded RNA (dsRNA) corresponding to the open reading frame of OsFATP was synthesized, injected and evaluated its inhibitory effect on sex pheromone production *in vivo* by according to RNAi protocol used on *B. mori* (Ohnishi et al. 2009). When we injected 2 μg and 10 μg of dsRNA for OsFATP into 2-day-old female pupae, OsFATP

mRNA levels in the PG after eclosion were dose-dependently reduced as compared with the control pupae injected with DEPC-treated H_2O (Fig. 3.4.).

To assess the effects of OsFATP knockdown on sex pheromone production, the terminal abdominal segments of female moths were cut off, and the amount of sex pheromone in the PGs was measured (see section 3.2.6). As shown in Fig. 3.6, no significant dose-dependent reduction in sex pheromone production was observed.

3.4 Discussion

In this chapter, to understand the function of EjFATP cloned from the PG of *E. japonica*, we used *E. coli* expression system to express EjFATP. *E. coli* cells expressing EjFATP showed an enhanced ability to take up stearic acid or eicosanoic acid, but not lauric acid and myristic acid (Fig. 3.3.A). The uptake of palmitic acid by the cells expressing EjFATP might not be detected due to the abundance of endogenous palmitic acid (Fig. 3.3.A). These results indicate that EjFATP indeed plays a role in the uptake of extracellular fatty acids into cells. In the chapter 1, a few lipid drops (LDs) was observed in the PGs of *E. japonica*, the results of functional characterization of EjFATP expressed in *E. coli* suggests the conjunction with the lipid droplets and EjFATP. But the chemical components of LDs are still an important evidence remaining to understand.

The precursors (hydrocarbons) of Type II sex pheromones are thought to be specifically incorporated into the PG from the hemolymph (Schal et al. 1998; Wei et al. 2004; Matsuoka et al. 2006; Fujii et al. 2007). However, the mechanism of the specific incorporation has still remained unknown. Therefore, we considered the possibility that EjFATP in *E. japonica* enhances the transport of hydrocarbons, but the results of functional assay did not support our hypothesis (Fig. 3.3.B).

In *B. mori*, injection of dsRNA corresponding to BmFATP elicited 50% reduction in bombykol produce (Ohnishi et al. 2009). Using RNAi mediated gene knockdown, we examined the *in vivo* functional role of OsFATP in the biosynthesis of sex pheromone. Injecting pupae 2 day after the larval-pupal molt with water, 2, and 10 µg of dsRNAs corresponding to the ORFs of *Osfatp*, resulted in a dose-dependent knockdown of the corresponding gene transcripts (Fig. 3.5.). However, the reduction in sex pheromone production was not observed (Fig. 3.6.). To produce abundant bombykol, abundant LDs in PG of *B. mori* play the role of store and release bombykol precursor. Bombykol production dependent on the size and number of LDs in PGs of *B. mori*. Comparing to *B. mori*, the number of LDs in pheromone gland of *O. scapulalis* was few. Sex pheromone production of *O. scapulalis* was much less than that of *B. mori*, and I speculated it is low dependent on the number of LDs in PGs of *O. scapulalis*. This might be the reason dsRNAs corresponding to the *Osfatp* did affect the sex pheromone production significantly.

Table 3.1.

Primers used in this chapter

Primers for construction of as pCold-EjFATP
HG8-F: 5′-GCCGGAGCTCATGACTGCCAGCGAGTTTTC-3′
HG8-R: 5′-GCCGGGATCCCAGTCTGACTCTTCCAGATA-3′
Primers for synthesis ds RNA for OsFATP
RI-F:5′- CCTAATACGACTCACTATAGGGCGGCCGGGTGAGCCTGGAGTGT -3′
RI-R:5′- CCTAATACGACTCACTATAGGGCGGTCCAGGTCTAGCGTGTCGTC -3′
Primers for RT-PCR for OsFATP
RTOSf: 5′-GCCTGATTGTACTAGCCATACAG-3′
RTOSr: 5′-TTCCTCATACTCGGCGCCATAG-3′

Fig. 3.1.

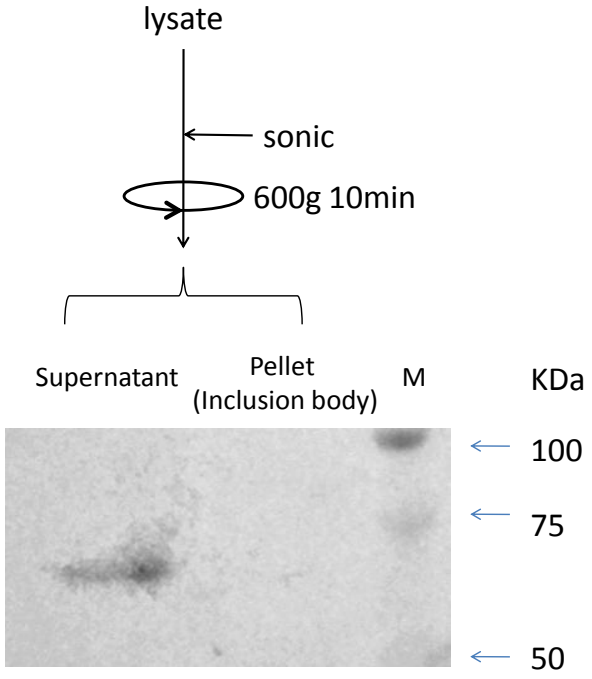


Fig. 3.1. Preliminary expression using *E. coli* to check recombinant EjFATP protein form. The lysates prepared from *E. coli* EjFATP were separated on a 10% acrylamide gel. A Western blot analysis was performed with anti-His antibody. The recombinant protein (72 kDa) is indicated by an arrow on the left.

Fig. 3.2.

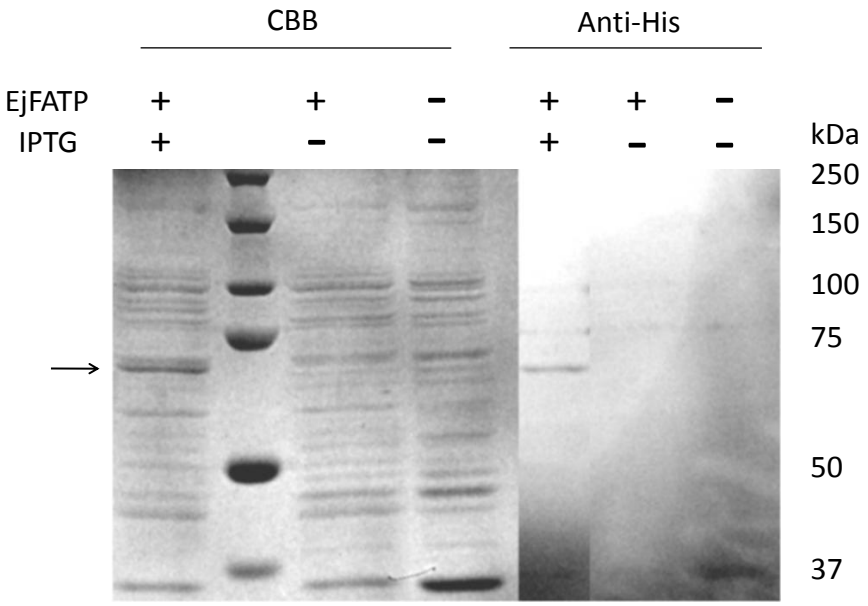


Fig. 3.2. Expression of a recombinant EjFATP protein using the *E. coli* expression system. The lysates prepared from the control *E. coli* cells and those expressing EjFATP were separated on a 10% acrylamide gel, and stained with Coomassie brilliant blue (CBB, left panel). A Western blot analysis was performed with anti-His antibody (right panel). The recombinant protein (72 kDa) is indicated by an arrow on the left.

Fig. 3.3.

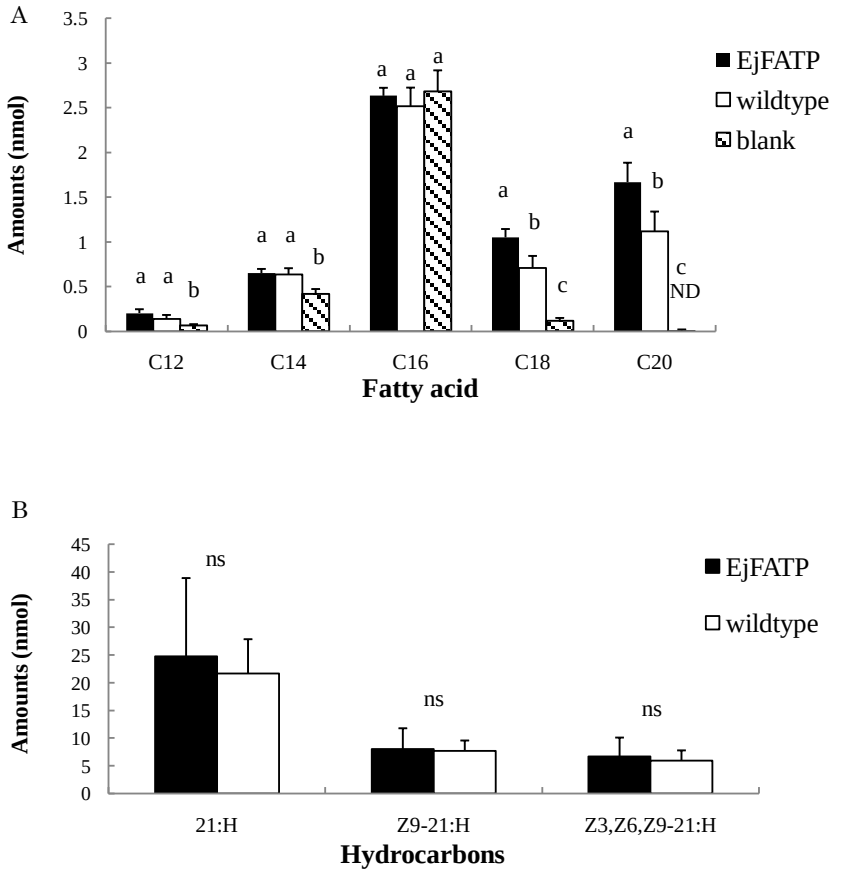


Fig. 3.3. Functional assay of the recombinant EjFATP protein. (A) Fatty acid transport analysis of EjFATP. Black, white, and shaded bars indicate the fatty acid uptake by BL21 *E. coli* expressing EjFATP, BL21 *E. coli* (control), and intrinsic fatty acids in BL21 *E. coli*, respectively. C12: lauric acid. C14: myristic acid. C16: palmitic acid. C18: stearic acid. C20: eicosanoic acid. ND: not detected. Values are expressed as the mean ± SD ($n \geq 4$). Means in each experimental group with the same letter are not significantly different (Tukey-Kramer test, $P > 0.05$). (B) Uptake of the sex pheromone precursor of *E. japonica* and its analogs by BL21 *E. coli* expressing EjFATP and untransformed BL21 *E. coli*. 21:H: heneicosane. Z9-21:H: (*Z*)-9-henicosaene. Z3,Z6,Z9-21:H: (*Z,Z,Z*)-3,6,9-henicosatriene. Values are expressed as the mean ± SD ($n = 4$). Ns: means in each experimental group are not significantly different (student's t-test, $P > 0.05$).

Fig. 3.4.

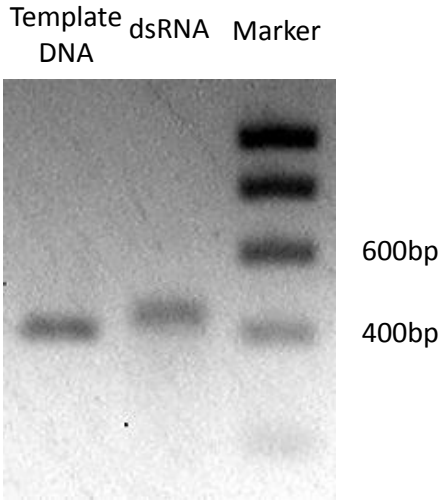


Fig. 3.4. dsRNAi production was examined on 1% agarose gel by electrophoresis. Two microgram of template DNA and dsRNA was used in this examination. The size of marker was indicated on the right.

Fig. 3.5.

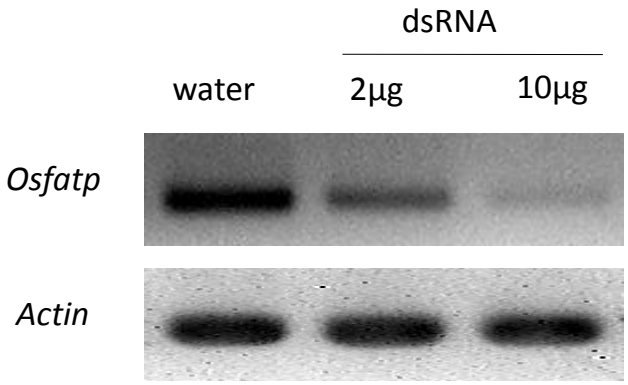


Fig. 3.5. Suppression of transcript level of endogenous *Osfatp* by RNAi.

Transcript analysis was performed using cDNAs prepared from the total RNA of pupae injected with water, 2 μg or 10 μg dsRNAs corresponding to ORF in *Osfatp*.

Fig. 3.6.

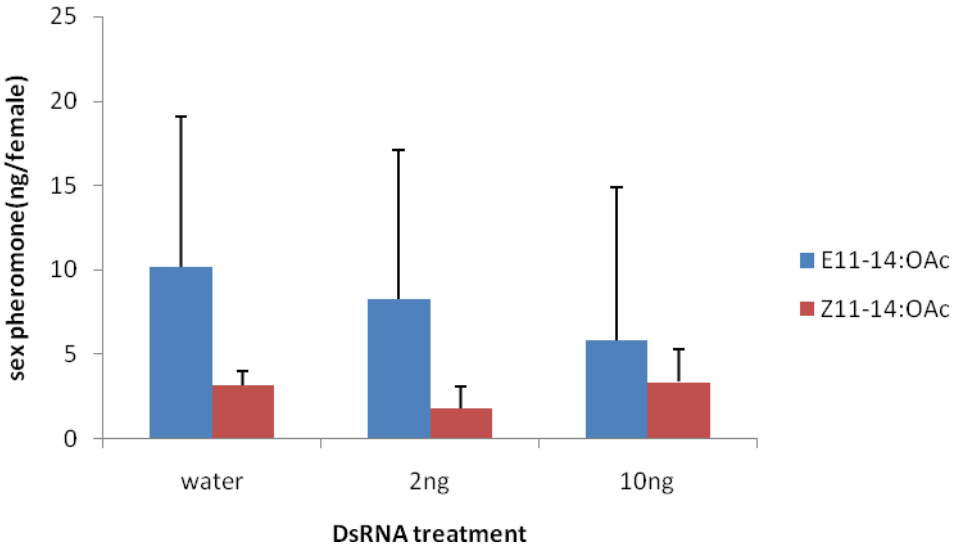


Fig. 3.6. Effects of *Osfatp* knockdown on sex pheromone production.

Two-day-old pupae were injected with water, 2, and 10 µg of dsRNA corresponding to *Osfatp*. Bars represent mean values ± SD (n => 3).

4. General discussion

4.1. Lipid droplets and fatty acid transport proteins (FATP)

Sex pheromones of lepidopteran moths are derived from fatty acids (Jurenka 2004). In this study, I focused on lipid metabolism in the sex pheromone biosynthesis using the moths *Ostrinia scapulalis* and *Eilema japonica*, which produce Type I and Type II sex pheromones, respectively.

Accumulation of lipid droplets in the cytoplasm is considered as a common feature of the pheromone producing cells in many lepidopteran species (Percy-Cunningham and MacDonald 1987). In chapter 1, the presence of lipid droplets was investigated in the PG of two moths species. Surprisingly, a few lipid droplets were observed in the PGs of both moths (Fig. 1.1.). Since *B. mori*, the model moth that uses Type I sex pheromone, utilizes abundant lipid droplets in the PG as the storage to control its sex pheromone release, it might suggest that the lipid droplets in the PG of *O. scapulalis* also act as the storage of sex pheromone. However, the knowledge on the lipid droplets in the PG of moths using type II sex pheromone has been limited. The model moth using Type II sex pheromone, *Ascotis selenaria* was proved have no lipid droplets in its PG cells described as Fujii et al. previously (Fig. 1.1.). So it became interesting to know about the formation and function of lipid droplets in PG of *Eilema japonica* producing Type II sex pheromones.

Subsequently, in chapter 2, three FATP homologues (OsFATP, EjFATP and AsFATP) were cloned from the PG of *O. scapulalis*, *E. japonica* and *A. selenaria*, respectively. In chapter 3, the function of EjFATP was proved using an *Escherichia coli* expression system. In comparison with the control *E. coli* cells, cells expressing EjFATP showed an enhanced ability to take up stearic acid or eicosanoic acid, but not lauric acid and myristic acid (Fig. 3.3.A).

BmFATP, the first FATP gene characterized from lepidopteran species, was shown to have an essential role in bombykol synthesis through the uptake of extracellular fatty acids (Ohnishi et al. 2009). The three FATP homologues cloned in this study have high degree of identity to BmFATP, which suggest they have a similar function to that of BmFATP. However, AsFATP might be an exception because it loss trans-membrane domains which find in all other FATPs. Interestingly, no LDs were detected in PG cells of *A. selenaria* (Fujii et al. 2007) (Fig. 1.1.). Given these results altogether, it possibly suggested that FATPs might play a role of the accumulation of LDs in pheromone gland.

4.2. How are FATPs involved in sex pheromone biosynthesis?

BmFATP was shown to have an essential role in bombykol synthesis through the uptake of extra-cellular fatty acids (Ohnishi et al. 2009). RNAi-mediated gene silencing of BmFATP resulted in a significant reduction in bombykol production (Ohnishi et al. 2009). In this study, EjFATP was found predominantly expressed in the PG, which suggests it was involved in sex pheromone biosynthesis.

However, there are several questions to be addressed about how FATPs involved in sex pheromone biosynthesis.

In Type I pheromones biosynthesis, sex pheromones are synthesized in the PG (Bjostad et al. 1987). For example, bombykol is produced *de novo* from palmitate via specific desaturation and reduction in the PG cells (Bjostad et al. 1987; Ando et al. 1988). So in this case, why BmFATP should transport fatty acids into PG cells? Another question is about the RNAi-mediated gene silencing. RNAi-mediated gene silencing was utilized very successfully in study the proteins in biosynthesis pathway of bombykol (Ohnishi et al. 2006). Injection of dsRNA corresponding to pgACBP, pgdesat1, or

pgFAR elicited a large reduction in bombykol production (>90% reduction with 10 μg injections) (Ohnishi et al. 2006). However, injection of dsRNA corresponding to BmFATP elicited only 50% reduction in bombykol production. It may suggest that BmFATP is a little different with pgACBP, pgdesat1, and pgFAR. Expressions of these genes also support this point of view. Expression of pgACBP, pgdesat1, and pgFAR is specific to PG in the adult females (Ohnishi et al. 2006), but *BmFATP* transcript is dominantly expressed in PG with a less intense signal detectable in Malpighian tubules, fatty body and midgut (Ohnishi et al. 2009). These results suggest genes like pgACBP, pgdesat1, and pgFAR are evolved specifically for sex pheromone biosynthesis, but BmFATP is not.

On the other hand, in the biosynthesis of Type II sex pheromone, the role of FATP is more difficult to understand, since the pheromone precursor, hydrocarbons must be incorporated into the PG from the hemolymph, but not fatty acids (Fig. 0.2.B) (Schal et al. 1998; Subchev and Jurenka 2001; Jurenka et al. 2003; Wei et al. 2004; Matsuoka et al. 2006). Hence, it was a surprise that a fatp homologue, *Ejfatp*, was expressed in the PG of *E. japonica*, which produces Type II sex pheromone. The function of EjFATP was characterized using an *E. coli* expression system. Overexpression of EjFATP by *E. coli* enhanced the uptake of long chain fatty acids (C_{18} and C_{20}), but not pheromone precursor hydrocarbons.

Considering such questions in an integrated manner, the fatty acids incorporated by FATPs into the PG might not be converted to sex pheromones directly both in moths using Type I and Type II sex pheromone. One evidence supporting this point of view is that TG species alteration by RNAi-mediated knockdown of BmFATP transcripts. Silencing BmFATP brought about a striking reduction in specific TG peaks which are

exclusively comprised of equivalents of unsaturated C_{18} fatty acyls. Because these fatty acids are dietary fatty acids, it suggests that the role of BmFATP is the uptake of extracellular fatty acid, but not affect the sex pheromone biosynthesis in PG cells. So it is possible that BmFATP and EjFATP are involved in the production of sex pheromone by taking up fatty acids into the PG to meet the high demand of energy, because PG is one of the most active glands during mating period (Fig.4.1.).

4.3. Sex pheromone precursor transport system in the moths producing Type II sex pheromones

Lipophorin is a kind of reusable shuttle that transports lipids via the hemolymph from one tissue to another (Chino et al. 1981; Tsuchida and Wells 1988). Except diacylglycerol and other lipids such as cholesterols and carotenoids (Chino et al. 1981), epicuticular hydrocarbons and contact sex pheromones were also transported by lipophorin to the target tissues (Katase and Chino 1984; Gu et al. 1995; Pho et al. 1996; Fan et al. 2004; Matsuoka et al. 2006). In moths producing Type II sex pheromones, lipophorin transports sex pheromones from the oenocytes to a pheromone gland (Jurenka and Subchev 2000; Subchev and Jurenka 2001; Jurenka et al. 2003). In *E. japonica*, sex pheromone was found in its hemolymph, which indicates the transport of sex pheromones by lipophorin (personal communication with Dr. Fujii).

However, the mechanism of precursor delivery and subsequent uptake system at the pheromone gland has never been fully understood. At least, the possibility of specialization in lipophorin binding with sex pheromones was denied in *Ascotis selenaria* (Matsuoka et al. 2006). So it maybe that lipophorin specifically unloads the pheromone precursor at the gland. It would be interesting to determine whether or not the FATP is

specialized for the lipophorin or the hydrocarbons. Therefore, I considered the possibility that EjFATP in *E. japonica* enhances the transport of hydrocarbons. I tested EjFATP expressed by *E. coli* by sex pheromone of *E. japonica* and its homologs, but the results did not support our hypothesis (Fig. 3.3.B; Fig. 4.2.).

For future research, lipophorin receptor is a candidate for binding or endocytosing lipophorin on the membrane of PG cells. In some insect species, lipophorin receptor was found expressed only in specific developmental stage and specific tissues for lipid components (Dantuma et al. 1999; Cheon et al. 2001; Gopalapillai et al. 2006; Ciudad et al. 2007). It suggests the expression of lipophorin was met the demand of lipids of specific tissues.

4.4. Evolution of Type I and Type II sex pheromones

The two major types of sex pheromones (Type I and Type II) are biosynthesized through different pathways (Millar 2000; Ando et al. 2004). Until quite recently, all moths examined were found to produce either Type I or Type II pheromone. However, a moth that produce both Type I and Type II was discovered by Wang et al. (2010). The navel orangeworm, *Amyelois transitella* has been reported to secrete a mixture of Type I and Type II sex pheromones [(*Z*,*Z*)-11,13-hexadecadienal, (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-tricosapentaene, and (*Z*,*Z*,*Z*,*Z*,*Z*)- 3,6,9,12,15-pentacosapentaene] (Wang et al. 2010). It indicates that two systems for the production of both types of sex pheromones are not exclusive. In this final section, I will discuss the evolution of the biosynthetic pathways for Type I and Type II pheromones.

The biosynthetic pathway for the production of Type I pheromone was derived from the one for the production of common fatty acids (Bjostad et al. 1987). As already

described, the sex pheromone of *B. mor* is produced *de novo* from palmitate via specific desaturation and reduction (Bjostad et al. 1987; Ando et al. 1988; Matsumoto et al. 2007). On the other hand, Although biosynthesis of Type II sex pheromone is not well understood as compared with that of Type I, I think that the synthetic pathway is derived from the one for the production of cuticular hydrocarbons (cHC). The following findings may support my hypothesis. Firstly, Type II sex pheromones and cHC are long chain hydrocarbons, both of which are thought to be produced by the oenocytes. Secondly, they use the same transport system; Type II sex pheromones are transported by lipophorin and specifically taken up by the PG. The cHC are also transported by lipophorin and specifically taken up by the epidermal cells. In some insect groups other than lepidoptera, cHCs are known to act as pheromones. For example, females of *Drosophila melanogaster* produce sex-specific dienes on its cuticle to promote mating (Billeter et al. 2009). In many social insects, such as *Formica exsecta*, cHCs play an important role in nestmate recognition (Martin and Drijfhout 2009). Taken together, it is reasonable to consider that Type II sex pheromones of moths evolved from cuticular hydrocarbons.

Fatty acid biosynthesis and hydrocarbon biosynthesis are fundamental metabolisms in insects including moths. The evolution of Type I and Type II pheromone biosynthesis should have been accompanied by inventions of new enzymes specifically involved in pheromone biosynthesis. For example, the evolution of the desaturase and fatty acyl reductase in the biosynthetic pathways for Type I pheromones should have enabled production of a far greater variety of sex pheromones (Roelofs et al. 2002; Lassance et al. 2010). However, the knowledge on the enzymes involved in the pheromone production, especially those involved in the biosynthesis of Type II sex pheromones, is still limited.

Molecular cloning and functional analysis of these genes are essential for understanding the evolution of Type I and Type II sex pheromones.

Fig. 4.1.

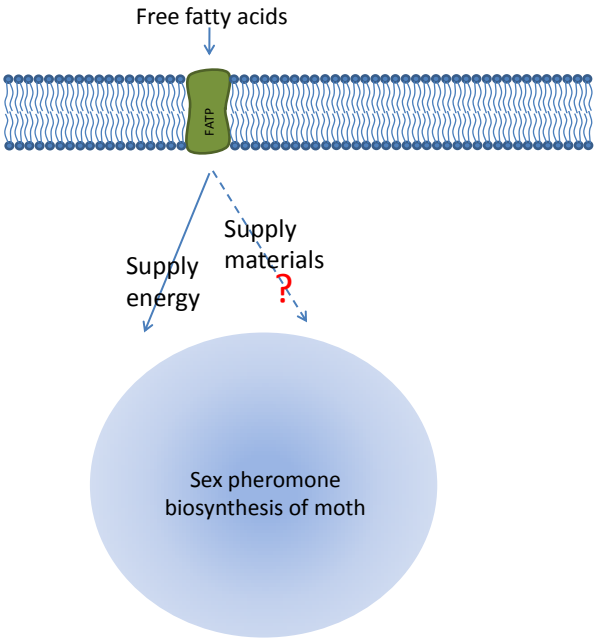


Fig. 4.1. Common functions of FATP in the two types of moths producing Type I and Type II sex pheromones.

Fig. 4.2.

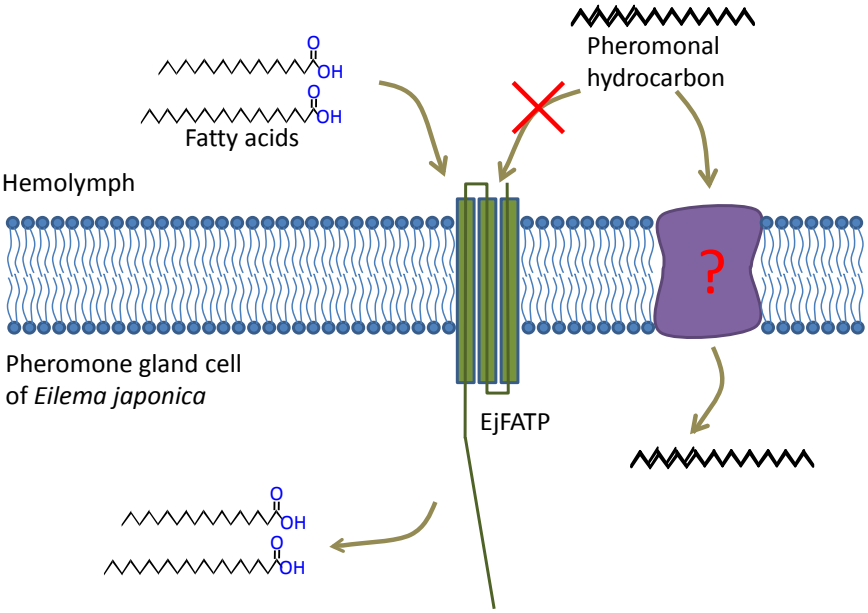


Fig. 4.2. Estimated function of EjFATP in the pheromone gland of *Eilema japonica*.

Fig. 4.3.

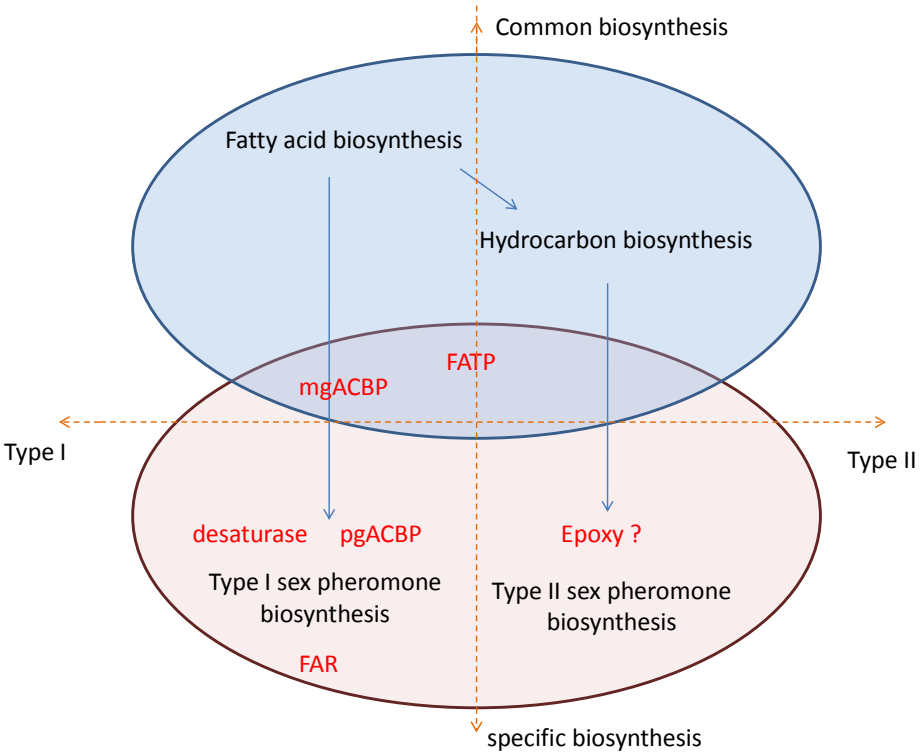


Fig. 4.3. A hypothesis on the evolution of Type I and Type II sex pheromones, and the proteins (red) involved in their production. FATP: fatty acid transport protein. mgACBP: midgut acyl-CoA-binding protein. FAR: fatty acyl reductase. pgACBP: pheromone gland cyl-CoA-binding protein.

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論文の内容の要旨

生産環境生物学専攻

平成１９年度博士課程入学

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Comparative studies on the sex pheromone biosynthesis in moths with a focus on lipid metabolism in the pheromone gland

（ガ類の性フェロモン生合成に関する比較研究ーフェロモン腺における脂質代謝に注目してー）

生物は個体間の情報伝達において視覚や聴覚を利用すると同時に、化学物質による刺激を重要な伝達手段の一つとして利用しており、その中でも昆虫は、化学物質による情報伝達を高度に発達させた。

ガ類は、多くの場合メスが性フェロモンを放出し、その性フェロモンは一般に複数の揮発性化合物の混合である。性フェロモンは交配前生殖隔離の重要な要因であるため、化学物質を正しく生産する仕組みは種の維持に不可欠である。ガ類における性フェロモンは遠方より同種オス個体を選択的に誘引する強力な生理活性を示す事から、現在600を超える種で同定されている。ガ類性フェロモン化合物は、メス成虫の腹部末端に位置するフェロモン腺よばれる節間膜で生合成されるが、末端官能基を含むグループ（Type I）と末端官能基の無いグループ（Type II）に大別される。これらType IとType II性フェロモン化合物は何れも脂肪酸代謝により生合成されるが、その過程の反応経路には大きな違いがある。

Type I性フェロモンは直鎖状の炭素骨格と1-3個の2重結合、そしてアルコール、アセテート、アルデヒドに代表される官能基を持つ。これらの成分は、フェロモン腺において、体内に普遍的なパルミチン酸（palmitic acid）やステアリン酸（stearic acid）などの飽和脂肪酸が炭素鎖の短縮や不飽和化、還元といった酵素反応を受けることにより合成される。その

一方、Type II性フェロモンは、食物由来のLinolenic acidから合成した炭化水素が原料であると考えられている。それらの成分は腹部にあるエノサイトにより生合成され、フェロモン腺へ運ばれ、そこでエポキシ化等の最終的な化学的修飾が施される事が示されている。

ガ類性フェロモン腺では他の組織より肥大した油滴が観察される事があるが、化学成分や油滴そのものの形成のメカニズムが明らかとされているのはカイコガに限られる。カイコガのフェロモン腺には、数多くの油滴顆粒が存在し、その中には性フェロモン前駆体となるトリアシルグリセロールが豊富に含まれている。その油滴は羽化2日前より急激に増大し、羽化後は性フェロモンであるボンビコールの産生に伴って、その数とサイズが日周性をもって劇的に変動する。

FATPは哺乳類では6種類のホモログが存在していることが明らかとされており、それぞれが異なった組織で異なった機能を果たしている。しかし、昆虫では、FATPに関する知見が限られている。近年、カイコガの性フェロモン腺からfatty acid transport protein (FATP)が単離され、BmFATPと名付けられた。この遺伝子をRNAi法によりBmfatp発現を抑制した結果、ボンビコール量の半減が認められている。更に、BmFATPは細胞外遊離脂肪酸のフェロモン腺細胞内への取り込み、ボンビコール前駆体の貯蔵体である脂肪滴の形成を促進することにより、ボンビコールの産生を調節することが結論づけられた。

本研究では、アズキノメイガ（*Ostrinia scapulalis*）とキマエホソバ（*Eilema japonica*）を用いて、Type IとType Ⅱ性フェロモン化合物の脂肪酸代謝に注目し、FATPを中心としたガ類の性フェロモン生合成に関する比較研究を行った。

1. フェロモン腺中の油滴の観察

Type I性フェロモンを利用するアズキノメイガとType Ⅱ性フェロモンを利用するキマエホソバを用いて、性フェロモン腺における油滴をNile Redにより染色し暗視野顕微鏡下で観察した。この時、陽性対照として用いたカイコガでは、フェロモン腺で多くの油滴顆粒が観察されたが、アズキノメイガとキマエホソバでは僅かな油滴顆粒が存在する事を明らかとした。この結果は、ガ類フェロモン腺でType IやType Ⅱ生合成経路と関係なく、油滴が存在する事を示している。Type Ⅱ性フェロモンを利用するガ類はフェロモン腺において遊離脂肪酸が生合成系に関与しない。従って、キマエホソバのフェロモン腺からの油滴の検出は、その役割が興味深い。

2. FATPのクローニング

本章ではアズキノメイガ、キマエホソバとヨモギエダシャク（*Ascotis selenaria*）それぞれの性フェロモン腺からOsFATP、EjFATPとAsFATPをコードする遺伝子をクローニングし、それら系統関係や発現パターンを解析した。系統分析の結果は、3種類のFATPがカイコガのBmFATPと高い相同性を示し（OsFATP 72.6%；EjFATP 77.9%；AsFATP 70.3%）、同一のグループとなる事を示唆した。Type Ⅱ性フェロモンを利用しているキマエホソバから単離したEjfatpは、Real time PCR法により性フェロモン腺で高発現し、発現量の時間変動が蛹期に低く、羽化後に約5倍まで増加した。アズキノメイガ由来の*Osfatp*はフェロモン腺で高い発現量を示さなかったが、発現量の時間変動はフェロモン分泌の変動と一致した。これらの結果から現段階では、EjFATPとOsFATPはカイコガ同様フェロモン生合成に関わっている可能性が示唆された。一方、アミノ酸配列の高次構造を解析ソフト（SOSUI）により予測した結果、AsFATPでは、他のFATPでN末側に保存されている膜貫通領域が認められないため機能が無いと考えられる。ヨモギエダシャクフェロモン腺より油滴が観察されていない報告と合わせて、ガ類フェロモン腺で発現するFATPと油滴の形成の関わりが示唆された。

3. EjFATPとOsFATPの機能解析

Type II性フェロモンを利用するガ類は腹部にあるエノサイトにより生合成されたフェロモン前駆体炭化水素をフェロモン腺へ運搬すると考えられている。従って、フェロモン生合成に直接関与するFATPがフェロモン腺に存在することは考えられない。その為、大腸菌過剰発現系を用いて、キマエホソバ性フェロモン腺由来のEjFATPの機能解析を行った。EjFATPが発現した大腸菌は野生株と比較してステアリン酸（stearic acid）とアラキジン酸（eicosanoic acid）をそれぞれ1.48倍と1.49倍取り込む事がわかった。この結果より、Type II性フェロモンを利用するガ類もまた、Type I性フェロモン化合物を利用するガ類と同様のFATPを利用していることが分かった。

アズキノメイガはカイコガと同様にType Iフェロモン化合物を分泌するガ類昆虫である理由からカイコガと同様にRNAi法により、Osfatpのフェロモン生合成への関与を評価した。RNAi処理したアズキノメイガにおけるOsfatpの発現は、二本鎖Osfatp注射量と依存して減少したが、それに伴うノックダウン個体のフェロモン量の減少は認められなかった。

結論

以上、本研究課題ではガ類フェロモン腺における脂質代謝に注目し、Type IとType Ⅱを生産する性フェロモン生合成に関する比較研究を行った。Type IとType Ⅱ性フェロモン生合成経路は大きく異なるが、何れのTypeを生産するフェロモン腺からも油滴を発見した。また、FATPホモログが性フェロモンのTypeと関係なく、発現していることを明らかとした。これらの結果から、フェロモン腺における脂質代謝の根幹が類似している事を示唆している。

更に、大腸菌発現系とRNAi法を用いて、EjFATP、OsFATPの機能を評価した。大腸菌で発現したEjFATPは、フェロモン腺由来のFATPが細胞外脂肪酸をフェロモン腺細胞内に取り込み、フェロモン腺内の油滴の形成を促進すると思われる。RNAi法でOsFATPの結果から、OsFATPsが性フェロモン性合成と関係ない事が分かった。これらの結果から、FATPはフェロモン生合成反応に直接関わっていない事が示されたが、フェロモン腺で必要されるエネルギー供給に関する動きを担うのではないかと考えられる。