J Chem Ecol (2014) 40:439–451
DOI 10.1007/s10886-014-0433-1

Putative Pathway of Sex Pheromone Biosynthesis and Degradation by Expression Patterns of Genes Identified from Female Pheromone Gland and Adult Antenna of *Sesamia inferens* (Walker)

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Received: 15 December 2013 / Revised: 17 March 2014 / Accepted: 21 April 2014 / Published online: 10 May 2014

Abstract The general pathway of biosynthesis and degradation for Type-I sex pheromones in moths is well established, but some genes involved in this pathway remain to be characterized. The purple stem borer, *Sesamia inferens*, employs a pheromone blend containing components with three different terminal functional groups (Z11-16:OAc, Z11-16:OH, and Z11-16:Ald) of Type-I sex pheromones. Thus, it provides a good model to study the diversity of genes involved in pheromone biosynthesis and degradation pathways. By analyzing previously obtained transcriptomic data of the sex pheromone glands and antennae, we identified 73 novel genes that are possibly related to pheromone biosynthesis (46 genes) or degradation (27 genes). Gene expression patterns and phylogenetic analysis revealed that one *desaturase* (*SinfDes4*), one *fatty acid reductase* (*SinfFAR2*), and one *fatty acid xtransport protein* (*SinfFATP1*) genes were predominantly expressed in pheromone glands, and clustered with genes involved in pheromone synthesis in other moth species. Ten genes including five *carboxylesterases* (*SinfCXE10, 13, 14, 18,* and *20*), three *aldehyde oxidases* (*SinfAOX1*, *2* and *3*), and two *alcohol dehydrogenases* (*SinfAD1* and *3*) were expressed specifically or predominantly in antennae, and could be candidate genes involved in pheromone degradation. *SinfAD1* and *3* are the first reported alcohol dehydrogenase genes with antennae-biased expression. Based on these results we propose a pathway involving these potential enzyme-encoding gene candidates in sex pheromone biosynthesis and degradation in *S. inferens*. This study provides robust background information for further elucidation of the genetic basis of sex pheromone biosynthesis and degradation, and ultimately provides potential targets to disrupt sexual communication in *S. inferens* for control purposes.

Keywords Purple stem borer · Transcriptome analysis · Sex pheromone gland · Sex pheromone biosynthesis · Degradation

Abbreviations

PG	Pheromone glands
FAR	Fatty acid reductases
AO	Alcohol oxidase
ACT	Acetyltransferase
FATP	Fatty acid transport proteins
ACBP	Acyl-CoA binding proteins
OR	Odorant receptor
ODE	Odorant degrading enzyme
CXE	Carboxylesterase
AOX	Aldehyde oxidase
AD	Alcohol dehydrogenase
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription PCR
qPCR	Quantitative real-time PCR
cDNA	Complementary DNA
RACE	Rapid amplification of cDNA End
CoA	Coenzyme A
OAc	Acetate ester

Electronic supplementary material The online version of this article (doi:10.1007/s10886-014-0433-1) contains supplementary material, which is available to authorized users.

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OH	Alcohol
Ald	Aldehyde
SE	Standard error

Introduction

Species-specific sex pheromones play a key role in sex communication and subsequent mating of most moth species (Vogt 2005). To date, sex pheromones of more than 640 lepidopteran species have been reported (Ando et al. 2004). Moth pheromones usually are a blend of two or more components, and are generally divided into two types based on the presence (Type-I) or absence (Type-II) of a terminal functional group in the components (Ando et al. 2004; Millar 2000). Type-I pheromones are used by most moths, and composed of C10–C18 unsaturated acyclic aliphatic compounds with a functional group such as a formyl, hydroxyl, or acyloxyl group (Ando et al. 2004; Witzgall et al. 2004). Type-II pheromones mainly are composed of C17-23 hydrocarbons with 2 or 3 double bounds at the 3, 6, or 9 positions, and their corresponding epoxy derivatives (Arn et al. 1997). Moth pheromones especially the Type-I pheromones are produced and released by specialized pheromone glands (PG) located along the inter-segmental membrane between abdominal segments 8th and 9th of females (Raina et al. 2000; Tillman et al. 1999).

A general pathway of biosynthesis for Type-I pheromones has been established starting with a palmitic or stearic acid synthesized *de novo* in PG through modifications of fatty acid biosynthetic pathway (Ando et al. 2004). By combinations of several enzymatic reactions (i.e., desaturation, chain-shorting reaction, reduction, acetylation, and oxidation), the palmitic or stearic acids are converted step-wise to the final pheromone components (Moto et al. 2004; Park et al. 2008; Tillman et al. 1999). So far, classes of essential enzymes involved in moth pheromone synthesis have been identified biochemically or/and molecularly. They are: 1) Desaturases, to introduce double bonds into pheromone precursors, are the most intensively studied class of enzymes involved in sex pheromone biosynthesis. Many desaturase genes acting on certain carbon chain positions have been functionally characterized, including Δ5 (Foster and Roelofs 1996; Hagstrom et al. 2014), Δ6 (Wang et al. 2010), Δ9 (Liu et al. 1999; Park et al. 2008), Δ11 (Fujii et al. 2011; Jeong et al. 2003), Δ10-12 (Moto et al. 2004), and Δ14 desaturases (Roelofs and Rooney 2003). 2) Fatty acid reductases (FAR), responsible for reducing fatty acids to alcohols, also have been functionally identified in some moth species, including *pgFAR-Z/E* of *Ostrinia nubilalis* (Lassance et al. 2010) and *pgFAR* of *Bombyx mori* (Moto et al. 2003). 3) Alcohol oxidase (AO) and acetyltransferase (ACT) have been suggested by biochemical studies to oxidize alcohols into the corresponding aldehyde components (Teal and Tumlinson 1987; Wang et al. 2010) and to convert alcohols into acetate esters (Jurenka and Roelofs 1989), respectively. However, none of the genes encoding these two enzyme classes has been functionally characterized. In addition, fatty acid transport proteins (FATP) and acyl-CoA binding proteins (ACBP) have been found to play roles in the production of bombykol in *B. mori* by RNA interference (Ohnishi et al. 2006, 2009). Although the subject has been studied intensely, unknown classes of genes that might be involved in pheromone production are still likely to be elucidated, for example, *acetyl-CoA carboxylases* (*ACC*), *acyl-CoA oxidases* (*ACO*), *aldehyde reductases* (*ALR*), *elongation of fatty acids proteins* (*ELO*), and *short-chain dehydrogenases* (*SCD*) (Vogel et al. 2010). These enzymes are potentially important in moth sex pheromone biosynthesis. Exhaustively identifying candidate genes from different species is important to better understand pheromone biosynthesis and speciation in moths.

In the males, three major classes of protein are involved in the perception of female sex pheromones. They are pheromone binding proteins (PBPs), odorant receptors (ORs), and pheromone degrading enzymes (PDEs). PBPs are thought to bind hydrophobic pheromone molecules and transport them through the aqueous sensillum lymph to reach the membrane surface of the sensory neuron where ORs are situated (Field et al. 2000; Leal 2013; Pelosi et al. 2006; Vogt 2005). The binding of odorant molecule alone or in complex with PBPs activates ORs and initiates a signal transduction cascade leading to an electrical signal. After OR activation, the pheromone molecules are thought to be rapidly inactivated to restore the sensitivity of ORs for receiving new chemical signals (Vogt 2003, 2005; Vogt and Riddiford 1981). Pheromone inactivation is crucial for maintaining high sensitivity of pheromone reception, and the process has been demonstrated to be accomplished by enzymatic degradation in the sensillar lymph (Ferkovich et al. 1980; Kasang 1971; Leal 2013; Mayer 1975; Vogt and Riddiford 1981). However, only a few *PDE* genes have been functionally characterized (Chertemps et al. 2012; Choo et al. 2013; Durand et al. 2010, 2011; Ishida and Leal 2005, 2008), and the inactivation mechanisms are not well understood.

The purple stem borer, *Sesamia inferens* (Lepidoptera: Noctuidae), is a polyphagous insect pest found in many Asian countries (Chai and Du 2012). It damages various crops, including rice, corn, and sugarcane, and since the 1990s has become one of the major rice pests in China (Gao et al. 2010; Xu et al. 2011). The female sex pheromone of *S. inferens* is a blend of (*Z*)-11-hexadecenyl acetate (Z11-16:OAc), (*Z*)-11-hexadecenol (Z11-16:OH), and (*Z*)-11-hexadecenal (Z11-16:Ald) (Zhu et al. 1987), which are molecules containing three distinct terminal functional groups typically found in Type-I sex pheromones. Thus *S. inferens* serves as a good model to study enzymes involved in the biosynthesis, as well as the degradation of sex pheromones.

In the present study, we took advantage of previously obtained transcriptomic data from adult antenna and female sex pheromone gland of *S. inferens* (Zhang et al. 2013) and identified 73 putative genes that might be involved in sex pheromone biosynthesis and degradation. Tissue expression patterns of these genes were investigated using qPCR, and phylogenetic analyses were performed in an effort to predict gene function. The analysis revealed that some identified genes are specifically or highly expressed in antennae or in the female sex pheromone glands. Based on the results, we proposed a putative pathway of sex pheromones biosynthesis and degradation in *S. inferens* and highlighted gene candidates for further functional studies.

Methods and Materials

Insects and Tissue Collection Larvae of the purple stem borer, *S. inferens*, originally were collected from a rice field at the Jiangsu Provincial Academy of Agricultural Sciences, Nanjing, China, and reared on fresh wild rice stems in glass bottles (diam = 7 cm, height = 11 cm) until pupation and sexing (Zhang et al. 2012). Rearing conditions were 28 ± 1 °C, 70–80 % RH and a 14:10, L, D photoperiod. Adults were provided with a cotton swab dipped in 10 % honey solution, and renewed daily. All tissues were collected from 3-d-old virgin male and female adults at 5–7 h into the scotophase, and immediately stored at−70 °C until use. Sex pheromone glands were extracted from female abdomens.

RNA Isolation and cDNA Synthesis

RNA was isolated from antennae, legs, and wings of 20–30 individuals; abdomens and PGs of 15–20 individuals; and fat bodies, thoraxes, and epidermises of 10–15 individuals. Total RNA was extracted using the SV 96 Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's instructions. RNA quality was checked with a spectrophotometer (NanoDropTM 1000, Thermo Fisher Scientific, Waltham, MA, USA). Single-stranded cDNA templates were synthesized from 1 μg of total RNA from various tissue samples using the PrimeScript™ RT Master Mix (TaKaRa, Dalian, China).

Sequence Retrieval, RACE Amplification and Sequence Analysis All putative genes were retrieved from previously obtained transcriptomic data (Zhang et al. 2013) that was reassembled using Trinity (v2012-10-05) (Novogene, Beijing, China). The SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) was used to amplify the 5′ and 3′ regions of target genes following the manufacturer's instructions. The RACE PCR products were subcloned into the pEASY-T3 cloning vector (TransGene, Beijing, China) and positive clones were sequenced by GenScript (Nanjing, China). Full-length sequences were determined by assembling the cDNA fragments and the sequences obtained from the 5′ and 3′ RACE PCR. The RACE primers (Table S1) were designed using Primer Premier 5.0 (PREMIER Biosoft International, CA, USA).

Open reading frames (ORFs) of the putative genes were predicted using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The similarity searches were performed using the NCBI-BLAST network server (http://blast.ncbi.nlm.nih.gov/).

Phylogenetic Analysis Phylogenetic trees were constructed by phylogenetic analyses of SinfDess, SinfFARs, SinfCXEs and SinfAOXs, based on the amino acid sequences of these genes and sequences of other insects. The desaturase (Des) data set contained six sequences from *S. inferens*, and 53 from other insects. The FAR data set contained three sequences from *S. inferens*, and 54 from other insects. The CXE data set contained 15 sequences from *S. inferens* (amino acids >80 aa), and 69 from other insects. The AOXs data set contained three sequences from *S. inferens*, and 11 from other insects. The protein names and accession numbers of the genes used for phylogenetic tree construction are listed in Table S2. Amino acid sequences were aligned using ClustalX 2.0, and unrooted trees were constructed by MEGA5.0 using the Neighbor-joining method, with Poisson correction of distances. Node support was assessed by bootstrap using 1,000 bootstrap replicates.

Reverse Transcription-PCR Analysis Gene-specific primers across ORFs of predicted chemosensory genes were designed using Primer Premier 5.0 (PREMIER Biosoft International, CA, USA) and were listed in Table S1. PCR experiments including negative controls (no cDNA template) were carried out in a MyCycler™ (Bio-Rad, USA) under the following conditions: 94 °C for 4 min; 28–35 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 40 sec, with a final incubation for 10 min at 72 °C. The reactions were performed in 25 μl with 15 ng of single-stranded cDNA, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.4 μM for each primer and 1.25 U r*Taq* DNA polymerase (TaKaRa, Dalian, Liaoning, China). PCR products were analyzed by electrophoresis on 1.5 % w/v agrose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA), and the resulting bands were visualized with ethidium bromide. The gene encoding *S. inferens* glyceraldehyde-3-phosphate dehydrogenase (*SinfGAPDH*) was used as a reference gene for checking the integrity of the cDNA template and expression quantification of the target genes. To check the repeatability of the tissue expression measurements, 15 genes were randomly chosen to perform a second biological replicate.

Quantitative Real-Time PCR Quantitative Real-Time PCR (qPCR) was performed on an ABI 7500 (Applied Biosystems, Foster City, CA, USA) using a mixture of 10 μl 2× SYBR Green PCR Master Mix (TaKaRa, Dalian, Liaoning, China), 0.4 μl of each primer (10 μM), 2.5 ng of sample cDNA, and 6.8 μl sterilized ultrapure H_2O. The reaction programs were 30 sec at 95 °C, 40 cycles of 95 °C for 5 sec, and 60 °C for 34 sec. The results were analyzed using the ABI 7500 analysis software SDS 1.4. The qPCR primers (Table S1) were designed using Beacon Designer 7.7 (PREMIER Biosoft International, CA, USA). Amplification was followed by the measurement of fluorescence during a 55 to 95 °C melting curve in order to detect whether a single gene-specific peak was observed and to check for the absence of primer dimer peaks. A single and discrete peak was detected for all primers tested. Negative controls were reactions without templates (replacing cDNA with H_2O).

Expression levels of 46 putative sex pheromone biosynthesis genes were calculated relatively to the reference gene using the Q-Gene method in the Microsoft Excel-based software of Visual Basic (Muller et al. 2002; Simon 2003). To ensure the reliability of data analysis, we chose *SinfGAPDH* and *Sinf28SrRNA* as reference genes. Each sample had three biological replicates each with three technique replicates.

Statistical Analysis Data (mean ± SE) from various samples were subjected to one-way nested analysis of variance (ANOVA) followed by a least significant difference test (LSD) for mean comparison. Two-sample analysis was performed by Student *t*-test using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Identification of Putative Genes Involved in Sex Pheromone Biosynthesis and Degradation By blast analysis of the transcriptome data of *S. inferens* (Zhang et al. 2013) using the reported genes of other moth species in NCBI as queries (Gu et al. 2013; Strandh et al. 2008; Vogel et al. 2010), we identified a total of 46 genes encoding proteins putatively involved in sex pheromone biosynthesis. These included six Dess, three FARs, five FATPs, three ACBPs, five ACTs, seven ACCs, four ACOs, six ALRs, four ELOs, and two SCDs (Table 1). A total of 27 genes encoding putative odorant-degrading enzymes were identified, including 18 carboxylesterases (CXEs), three aldehyde oxidases (AOXs), and six alcohol dehydrogenases (ADs) (Table 2).

Among the 73 newly identified genes, three *Dess*, one *FAR*, two *FATPs*, one *ACBP*, ten *CXEs*, three *AOXs*, and four *ADs* contained complete open reading frames (ORF) (Tables 1 and 2), which were obtained either by transcriptome analysis (17 genes) or by RACE methodology (seven *CXEs*).

Phylogenetic Analysis In order to assign putative functions to the 73 genes, phylogenetic analyses were conducted for each group of the enzymes. A phylogenetic tree of the desaturases (Fig. S1) showed that four *S. inferens* desaturases clearly clustered in four different groups of insect desaturases; including the Δ11-desaturases (SinfDes4), Δ9-desaturases (18C > 16C) (SinfDes2), Δ9-desaturases (16C > 18C) (SinfDes3), and Δ9-desaturases (14C-26C) (SinfDes5). In the FAR phylogenetic tree, all three *S. inferens* FARs clustered within the lepidopteran pgFAR group, which contains previously identified FARs involved in moth sex pheromone biosynthesis (Hagstrom et al. 2012) (Fig. S2).

According to Durand et al. (2010b), a set of currently available insect carboxylesterase sequences was used to construct a CXE phylogenetic tree. The tree revealed that the 15 *S. inferens* CXEs divided into three different groups; an intracellular clade (A–C) (five genes), an extracellular clade (D–G) (nine genes) and a neurosignalling clade (K–M) (one gene) (Fig. S3). All three *S. inferens* AOXs clustered within the lepidopteran antennal AOX group. Within this group, two aldehyde oxidase, MbraAOX and AtraAOX2, have been reported to be involved in the degradation of aldehyde pheromone components (Choo et al. 2013; Merlin et al. 2005) (Fig. S4).

Tissue Expression Profile of the Putative Sex Pheromone Biosynthesis Genes qPCR analyses of the expression patterns of 46 putative sex pheromone biosynthesis genes showed that only three genes, *SinfDes4*, *SinfFAR2*, and *SinfFATP2*, displayed PG-predominant or biased expression (Fig. 1). The others were expressed at relatively higher levels in fat body, leg or thorax.

Tissue Expression Profile of the Putative Sex Pheromone Degradation Genes The expression patterns of all 27 putative ODE genes characterized by RT-PCR showed ten genes with antennae-specific or biased expression (Fig. 2 and Fig. S5). *SinfCXE10* was expressed specifically in the antennae of both sexes. *SinfCXE13*, *14*, *18*, and *20* also had highly antennae-biased expression. Further qPCR tests indicated that these five *S. inferens CXE* genes all had similar expression levels between male and female antennae.

Among the three *S. inferens AOX* genes, *SinfAOX1*, and *SinfAOX2* were specifically expressed in antennae and male-biased (significantly higher in males than in females), whereas *SinfAOX3* was expressed equally in the antennae of both sexes and male abdomens (Figs. 2a, c, and Fig. S5). Of the six *S. inferens AD* genes, *SinfAD1* and *SinfAD3* were expressed at higher levels in antennae than in other tissues (antenna bias), but the expression levels were similar between sexes (Figs. 2a, d, and Fig. S5).

Table 1 The best blastx matches of putative sex pheromone biosynthesis genes of *Sesamia inferens*

Gene name	Acc. number	ORF length (aa)	Complete ORF	Best blastx match				
				Name	Acc. number	Species	E value	Identity (%)
Desaturase (*Des*)								
Des1	KF960738	140	No	acyl-CoA Delta(11) desaturase-like	XP_004925564.1	[*Bombyx mori*]	5.00E-93	77
Des2	KF960739	354	Yes	acyl-CoA desaturase NPVE	AGR49313.1	[*Agrotis ipsilon*]	0	92
Des3	KF960740	353	Yes	acyl-CoA delta 9 desaturase	AGR49311.1	[*Agrotis ipsilon*]	0	95
Des4	KF960741	324	No	acyl-CoA desaturase HvirLPAQ	AGO45839.1	[*Heliothis virescens*]	0	85
Des5	KF960742	37	No	delta-9 desaturase 14-26	AFO38465.1	[*Spodoptera exigua*]	4.00E-52	94
Des6	KF960743	372	Yes	acyl-CoA desaturase HsubKSVE	AGO45848.1	[*Heliothis subflexa*]	0	84
Fatty-Acyl Reductase (*FAR*)								
FAR1	KF960747	163	No	fatty-acyl reductase	EHJ64478.1	[*Danaus plexippus*]	8.00E-61	60
FAR2	KF960748	456	Yes	fatty acid reductase	AFD04726.1	[*Heliothis subflexa*]	0	74
FAR3	KF960749	80	No	fatty acid reductase	AFD04726.1	[*Heliothis subflexa*]	1.00E-23	66
Acetyltransferase (*ACT*)								
ACT1	KF960764	103	No	putative acetyl transferase	NP_001182381.1	[*Bombyx mori*]	2.00E-70	94
ACT2	KF960765	258	No	acetyltransferase 1	AGQ45622.1	[*Agrotis ipsilon*]	1.00E-133	86
ACT3	KF960766	105	No	acetyltransferase 1	BAH03386.1	[*Ostrinia scapulalis*]	3.00E-60	81
ACT4		36	No	acetyltransferase	AGQ45624.1	[*Agrotis ipsilon*]	6.00E-27	98
ACT5	KF960767	140	No	acetyltransferase	AGQ45624.1	[*Agrotis ipsilon*]	5.00E-114	95
Alcohol oxidase (*AO*)								
AO	KF960775	63	No	salicyl alcohol oxidase precursor	CAQ19344	[*Chrysomela populi*]	5.00E-08	39
Acyl-CoA Oxidase (*ACO*)								
ACO1	KF960760	276	No	putative acyl-CoA oxidase	EHJ63526.1	[*Danaus plexippus*]	2.00E-106	61
ACO2	KF960761	158	No	probable peroxisomal acyl-coenzyme A oxidase 1-like	XP_004932403.1	[*Bombyx mori*]	1.00E-80	72
ACO3	KF960762	65	No	probable peroxisomal acyl-coenzyme A oxidase 1-like	XP_004932404.1	[*Bombyx mori*]	3.00E-44	91
ACO4	KF960763	83	No	peroxisomal acyl-coenzyme A oxidase 3-like	XP_004930485.1	[*Bombyx mori*]	4.00E-38	66
Acetyl-CoA Carboxylase (*ACC*)								
ACC1		47	No	acetyl-CoA carboxylase-like	XP_004930758.1	[*Bombyx mori*]	2.00E-30	98
ACC2	KF960754	53	No	acetyl-coA carboxylase	AGR49308.1	[*Agrotis ipsilon*]	2.00E-41	99
ACC3	KF960755	34	No	acetyl-coA carboxylase	AGR49308.1	[*Agrotis ipsilon*]	1.00E-40	95
ACC4	KF960756	87	No	acetyl-coA carboxylase	AGR49308.1	[*Agrotis ipsilon*]	6.00E-49	94
ACC5	KF960757	133	No	acetyl-coA carboxylase	AGR49308.1	[*Agrotis ipsilon*]	8.00E-84	98
ACC6	KF960758	147	No	acetyl-CoA carboxylase-like	XP_004930758.1	[*Bombyx mori*]	9.00E-106	100
ACC7	KF960759	291	No	acetyl-coA carboxylase	AGR49308.1	[*Agrotis ipsilon*]	1.00E-172	95

Table 1 (continued)

Gene name	Acc. number	ORF length (aa)	Complete ORF	Best blastx match				
				Name	Acc. number	Species	E value	Identity (%)
Aldo-Ketose Reductase (*ALR*)								
ALR1	KF960768	216	No	aldose reductase-like	XP_004933320.1	[*Bombyx mori*]	1.00E-101	72
ALR2	KF960769	310	No	putative aldose reductase	EHJ78631.1	[*Danaus plexippus*]	2.00E-127	57
ALR3	KF960770	134	No	aldo-keto reductase 2E	BAL70378.1	[*Bombyx mori*]	5.00E-73	78
ALR4	KF960771	104	No	aldo-ketose reductase 1	AGQ45611.1	[*Agrotis ipsilon*]	1.00E-63	82
ALR5	KF960772	195	No	aldo-ketose reductase 1	AGQ45611.1	[*Agrotis ipsilon*]	3.00E-121	82
ALR6	KF960773	269	No	aldo-ketose reductase 1	AGQ45611.1	[*Agrotis ipsilon*]	1.00E-163	84
Elongation of Very Long Chain Fatty Acids Protein (*ELO*)								
ELO1		53	No	elongation of very long chain fatty acids protein	XP_004931952.1	[*Bombyx mori*]	4.00E-26	88
ELO2		51	No	elongation of very long chain fatty acids protein 2-like	XP_004921851.1	[*Bombyx mori*]	1.00E-18	68
ELO3		42	No	elongase	BAM19709.1	[*Papilio xuthus*]	5.00E-32	100
ELO4		56	No	elongation of very long chain fatty acids protein	XP_004924776.1	[*Bombyx mori*]	3.00E-15	64
Short-Chain Dehydrogenase (*SCD*)								
SCD1		44	No	short-chain dehydrogenase/reductase	YP_005366760.1	[*Corallococcus coralloides*]	2.00E-10	50
SCD2	KF960774	75	No	short chain type dehydrogenase	BAM18031.1	[*Papilio xuthus*]	6.00E-22	51
Fatty Acid Transport Protein (*FATP*)								
FATP1	KF960750	551	No	fatty acid transport protein	BAJ33523.1	[*Eilema japonica japonica*]	0	85
FATP2	KF960751	501	No	long-chain fatty acid				
transport protein 4-like	XP_004929240.1	[*Bombyx mori*]	0	78				
FATP3	KF960752	661	Yes	FATP	ACT22576.1	[*Manduca sexta*]	0	79
FATP4	KF960753	643	Yes	long-chain fatty acid				
transport protein 4-like	XP_004929241.1	[*Bombyx mori*]	0	82				
FATP5	KF960745	95	No	fatty acid transport protein	EHJ68760.1	[*Danaus plexippus*]	3.00E-60	80
Acyl-CoA Binding Protein (*ACBP*)								
ACBP1	KF960744	90	Yes	Acyl-CoA-binding protein homolog	P31824.1	[*Manduca sexta*]	5.00E-38	76
ACBP2		69	No	acyl-CoA binding protein	ABK29477.1	[*Helicoverpa armigera*]	1.00E-29	86
ACBP3	KF960746	255	No	acyl-CoA-binding domain-containing				
protein 6-like	EHJ65203.1	[*Danaus plexippus*]	3.00E-115	91				

Note: Genes without accession numbers were those fragments that were less that 200 bp in length, as fragments less than 200 bp are unable to be deposited in the GenBank, thus no accession numbers were available for these genes).

Table 2 The best blastx matches of putative sex pheromone degradation genes of *Sesamia inferens*

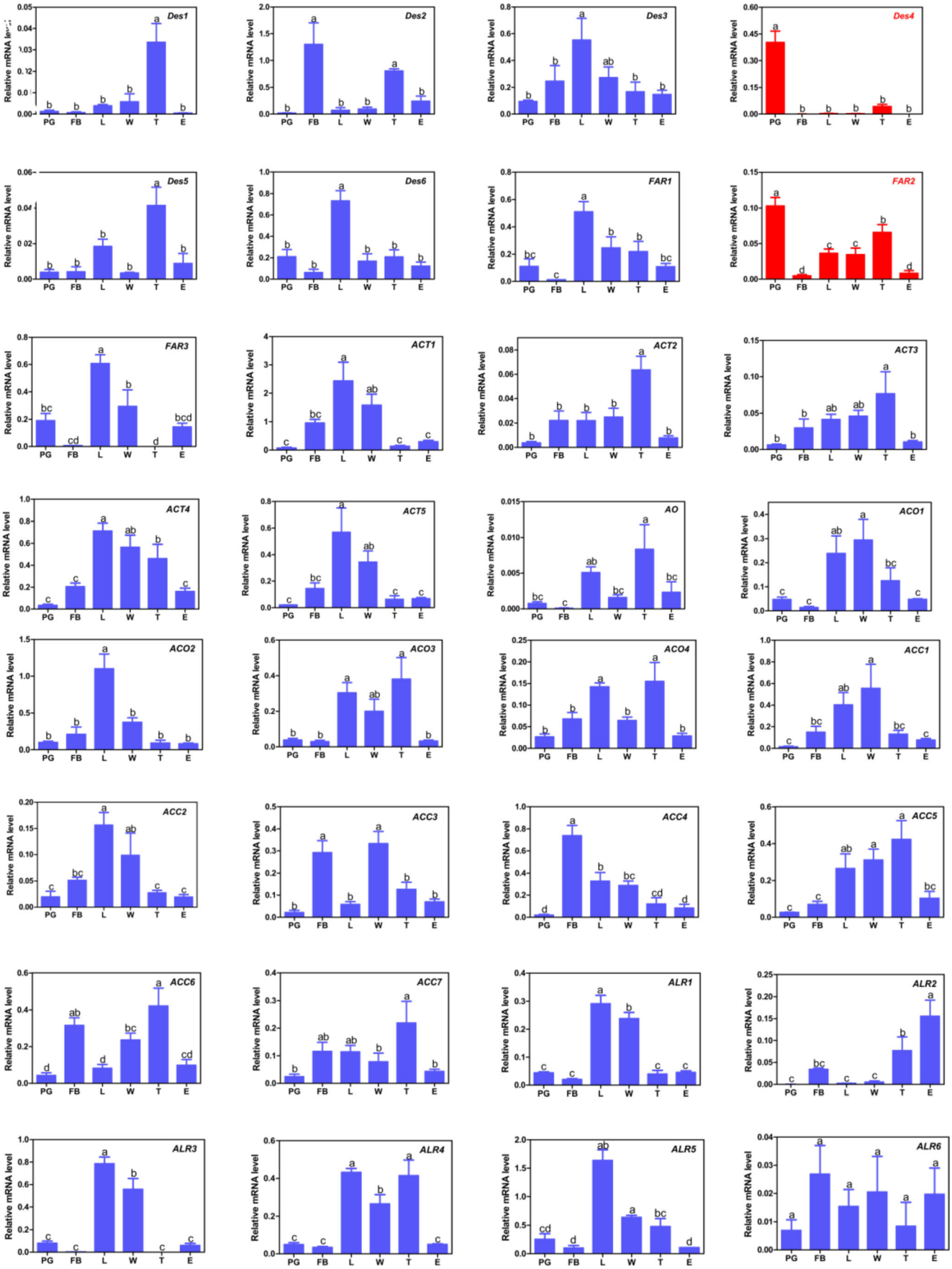
Gene name	Acc. number	ORF length (aa)	Complete ORF	Best blastx match				
				Name	Acc. number	Species	E value	Identity (%)
Carboxylesterase (*CXE*)								
CXE1	KF960776	564	Yes	esterase (EST1)	ABH01081.1	[*Spodoptera littoralis*]	0	83
CXE2	KF960777	80	No	antennal esterase CXE2	ACV60229.1	[*Spodoptera littoralis*]	2.00E-30	75
CXE3	KF960778	535	Yes	antennal esterase CXE3	ACV60230.1	[*Spodoptera littoralis*]	0	76
CXE5	KF960779	575	Yes	antennal esterase CXE5	ADR64702.1	[*Spodoptera exigua*]	0	83
CXE6	KF960780	518	No	antennal esterase CXE6	ACV60233.1	[*Spodoptera littoralis*]	0	67
CXE9	KF960781	553	Yes	antennal esterase CXE9	ACV60236.1	[*Spodoptera littoralis*]	0	77
CXE10	KF960782	537	Yes	antennal esterase CXE10	ACV60237.1	[*Spodoptera littoralis*]	0	59
CXE11	KF960783	541	Yes	antennal esterase CXE11	ACV60238.1	[*Spodoptera littoralis*]	0	72
CXE12	KF960784	357	No	antennal esterase CXE12	ACV60239.1	[*Spodoptera littoralis*]	1.00E-160	69
CXE13	KF960785	584	Yes	antennal esterase CXE13	ACV60240.1	[*Spodoptera littoralis*]	0	83
CXE14	KF960786	558	Yes	antennal esterase CXE14	ACV60241.1	[*Spodoptera littoralis*]	0	78
CXE16	KF960787	520	No	antennal esterase CXE16	ACV60243.1	[*Spodoptera littoralis*]	0	80
CXE18	KF960788	544	Yes	antennal esterase CXE18	ADR64698.1	[*Spodoptera litura*]	0	83
CXE19	KF960789	360	No	antennal esterase CXE19	ACV60246.1	[*Spodoptera littoralis*]	0	95
CXE20	KF960790	548	Yes	antennal esterase CXE20	ACV60247.1	[*Spodoptera littoralis*]	0	75
CXE26	KF960791	437	No	carboxylesterase CXE26	AEL33699.1	[*Spodoptera littoralis*]	0	81
CXE28	KF960792	302	No	carboxylesterase CXE28	AEL33701.1	[*Spodoptera littoralis*]	0	66
CXE30	KF960793	180	No	carboxylesterase CXE30	AEL33703.1	[*Spodoptera littoralis*]	4.00E-109	91
Aldehyde Oxidase (*AOX*)								
AOX1	KF960794	1283	Yes	aldehyde oxidase 1	NP_001103812.1	[*Bombyx mori*]	0	66
AOX2	KF960795	1288	Yes	aldehyde oxidase 2	NP_001103811.1	[*Bombyx mori*]	0	67
AOX3	KF960796	1267	Yes	antennae-specific aldehyde oxidase 2	AGQ43599.1	[*Amyelois transitella*]	0	63
Alcohol Dehydrogenase (*AD*)								
AD1	KF960797	270	No	alcohol dehydrogenase	AGQ45609.1	[*Agrotis ipsilon*]	1.00E-98	90
AD2	KF960798	376	Yes	alcohol dehydrogenase	NP_001040507.1	[*Bombyx mori*]	0	95
AD3	KF960799	347	Yes	alcohol dehydrogenase	NP_001040423.1	[*Bombyx mori*]	1.00E-180	73
AD4	KF960800	356	Yes	putative alcohol dehydrogenase	EHJ73729.1	[*Danaus plexippus*]	0	74
AD5	KF960801	324	Yes	alcohol dehydrogenase	XP_004921850.1	[*Bombyx mori*]	2.00E-174	77
AD6	KF960802	136	No	alcohol dehydrogenase	AGQ45608.1	[*Agrotis ipsilon*]	1.00E-105	80

To check the repeatability of the tissue expression, 15 genes were randomly selected to do a second measurement using different cDNA templates, and the results were consistent with those from the first measurement (Fig. S5).

Discussion

The present study has identified 73 new genes, including 46 putative genes involved in sex pheromone biosynthesis and 27 in pheromone degradation, through analysis of the transcriptomic data of adult antennae and female sex pheromone glands of *S. inferens* (Zhang et al. 2013). Phylogenetic analyses and tissue expression profiling highlighted two pheromone biosynthesis genes and ten pheromone degradation genes that are good candidates for further functional studies and could potentially be used as target genes for pest control purposes.

The three sex pheromone components (Z11-16:OAc, Z11-16:OH, and Z11-16:Ald) of *S. inferens* (Zhu et al. 1987) all contain an Δ11-double bond. The biosynthesis pathway of Δ11-containing sex pheromone has been studied in several moth species. The defined pathway involves a step of Δ11-desaturation catalyzed by a Δ11-desaturase (Fujii et al. 2011; Hao et al. 2002; Liu et al. 2002b; Roelofs et al. 2002; Wu et al.


Des1
Des2
Des3
Des4
Des5
Des6
FAR1
FAR2
FAR3
ACT1
ACT2
ACT3
ACT4
ACT5
AO
ACO1
ACO2
ACO3
ACO4
ACC1
ACC2
ACC3
ACC4
ACC5
ACC6
ACC7
ALR1
ALR2
ALR3
ALR4
ALR5
ALR6
Relative mRNA level
PG FB L W T E

◀ **Fig. 1** Expression patterns of putative sex pheromone biosynthesis genes in different tissues of female *Sesamia inferens*. PG, female pheromone glands; FB, fat bodies; L, legs; W, wings; T, thoraxes and E, epidermis

1998). It is likely that a Δ11-desaturase is responsible for the introduction of the Δ11-double bond in the pheromone components of *S. inferens*. It is also possible, although rarely reported (Liu et al. 2002a), that a Δ9-desaturase can participate in the production of Δ11-containing pheromone component by introducing a Δ9-double bond at 14:CoA, followed by a carbon chain elongation to Δ11-16:CoA. In the present study, six desaturase genes were obtained, but only one (*SinfDes4*) displayed a PG-predominant expression pattern. Further phylogenetic analysis showed that *SinfDes4* is clearly assigned to the Δ11-desaturase group, closely related to *MbraZ11* of *Mamestra brassicae* (GenBank accession no. ABX90049). *SinfDes5* is assigned to the Δ9-(14-26C)-desaturase group, while the remaining three genes belong to other desaturase groups. Therefore, *SinfDes4* is likely involved in the desaturation of 16C saturated acids to the unsaturated acids, with a double bond introduced at 11th position of the carbon chain. However, the involvement of *SinfDes5* in this process can not be ruled out, with further studies required.

Previous research revealed that in the process of sex pheromone biosynthesis, once the specific unsaturated fatty acid precursors are produced, they will be converted into corresponding alcohols by FARs (Hagstrom et al. 2012; Lassance et al. 2010; Lienard et al. 2010; Moto et al. 2003). In *S. inferens*, we identified three *FARs*, but only *SinfFAR2* is expressed at significantly higher levels in PG than in other tissues, suggesting a role in the biosynthesis of Z11-16:OH. The other two FAR-encoding genes that were identified might not be involved in this process, as their expressions in PGs were not higher than in other tissues, even though they were clustered in the moth pgFAR group in the phylogenetic analysis. The third gene class showing high levels of expression in PG are the *SinfFATP* genes, of which *SinfFATP1* was the most highly expressed. FATPs have been functionally confirmed to bind and transport fatty acids across insect hemolymph into PG cells for pheromone biosynthesis in *B. mori* (Ohnishi et al. 2009) and *Eilema japonica* (Qian et al. 2011). *SinfFATP1* may play a similar role in pheromone biosynthesis of *S. inferens*.

Alcohol oxidases (AOs) and acetyltransferases (ACTs) are essential in the biosynthesis of formyl and acyloxyl components, respectively, although no such gene encoding such an activity has been identified. In our study, we found one *AO*

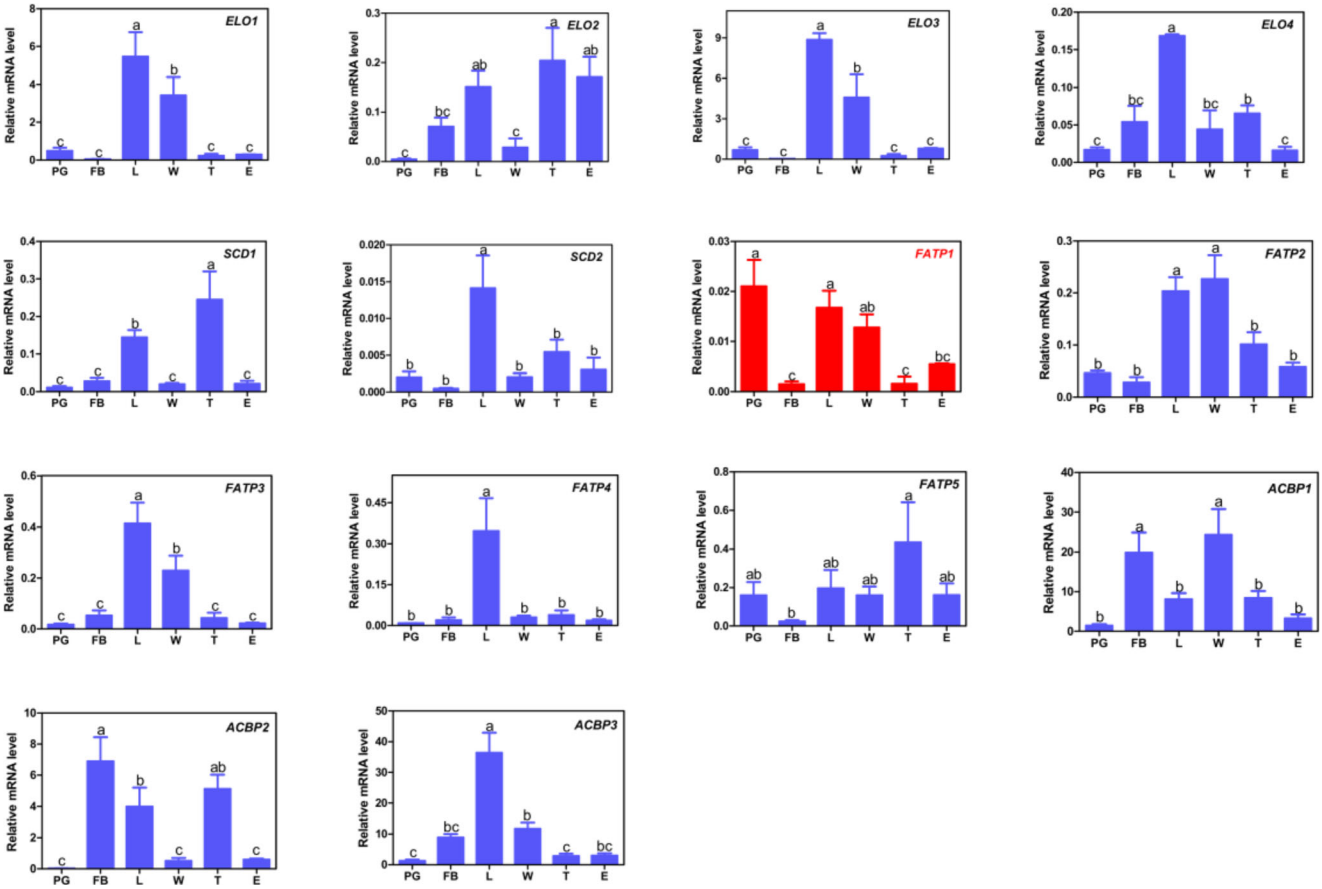


Fig. 1 (continued)

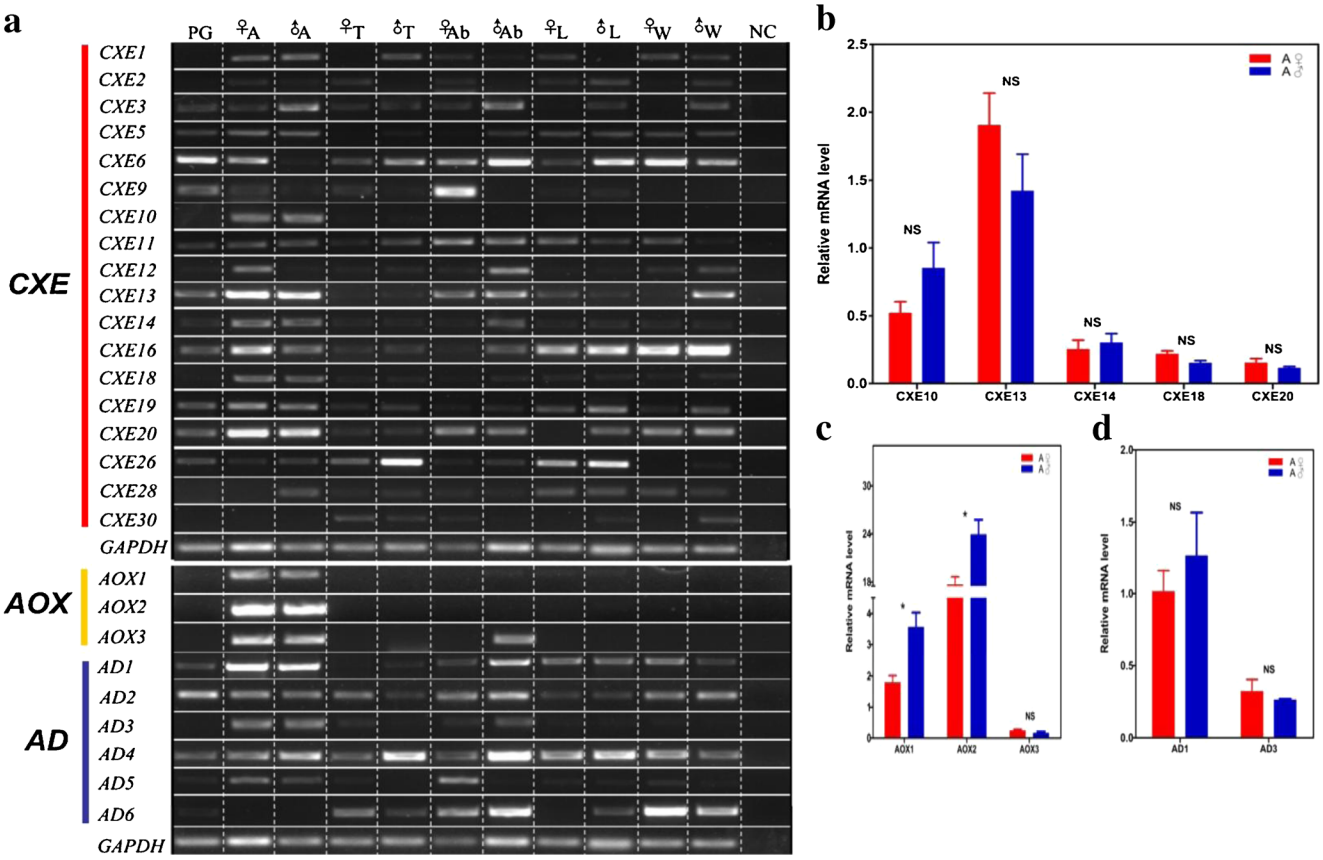


Fig. 2 Expression patterns of putative odorant-degrading enzyme (ODE) genes in *Sesamia inferens*. (**a**) Expression of all ODE genes by RT-PCR. GAPDH gene was used as a positive control and NC (no cDNA template) as a negative control. PG, female pheromone glands; A, antennae; T, thoraxes; Ab, abdomens (female without PG); L, legs; W, wings. (**b**–**d**) Relative expression levels of ODE genes in adult antennae of both sexs. ♀, female; ♂, male. An asterisk indicates a significant difference between male and female expression levels ($P<0.05$, Student *t*-test), whereas "NS" indicates no significant difference ($P>0.05$, Student *t*-test)

and five *ACT* genes, but none of them showed PG-predominant expression. However, expression patterns alone cannot exclude the possibility that these genes might be involved in sex pheromone biosynthesis, as they may also play roles in other physiological processes. Another possibility is that genes with PG-specific or biased expression patterns might not be revealed by transcriptome sequencing due to very low expression levels. In addition, mRNA levels are not always consistent with protein levels (Lee et al. 1999; Newman et al. 2006). There might be genes being PG-specific or biased at protein level that do not show such patterns at the mRNA level. Alternative approaches are needed to detect candidates of *AO* and *ACT* genes that are specifically expressed in PGs.

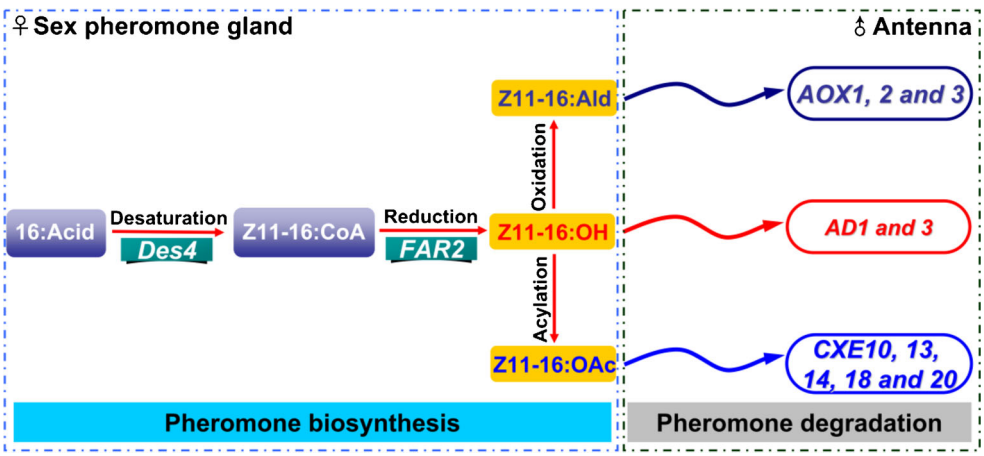


Fig. 3 A proposed biosynthesis and degradation pathway for the sex pheromone components of *Sesamia inferens* [adapted from (Jurenka 2004; Moto et al. 2003; Tillman et al. 1999; Vogt 2005)]

In general, insect ODEs have the ability to attack specific functional groups, such as acetate esters, aldehydes, and alcohols (Vogt 2005). *S. inferens* should have ODEs of all three subclasses in the antennae as the sex pheromone contains components of all three functional groups. In the present study, 18 *CXEs* were identified, of which *SinfCXE10* was antennae-specific and another four *SinfCXEs* were antennae-biased (*SinfCXE13*, *14*, *18*, and *20*), suggesting that they may play a role in the degradation of Z11-16:OAc or plant volatile esters. To date, only a single *AOX* gene has been functionally characterized in insects from *Amyelois transitella* (Choo et al. 2013). Of the three *SinfAOX* candidates identified in the current study, *SinfAOX1* and *SinfAOX2* were antennae-specific and *SinfAOX3* was predominantly expressed in antennae. *SinfAOX1* and *SinfAOX2* also were significantly male biased in their expression, which strongly suggests a role in the degradation of the Z11-16:Ald component. As for *ADs* acting in the degradation of alcohol pheromone components, no gene has been reported, although the existence of *AD* genes was suggested by a biochemical study two decades ago (Kasang et al. 1989). We obtained six *SinfADs* from *S. inferens* antennae, and among those, *SinfAD1* and *SinfAD3* were predominantly expressed in antennae, but not male biased in their expression. It is possible that those two genes have functions in degradation of plant volatiles or of both plant volatiles and sex pheromones. The ability of the same enzyme to degrade both sex pheromones and plant volatiles may constitute an efficient system that could participate to maintain the high sensitivity of the sex pheromone detection system, as illustrated by SlitCXE7 from *S. littoralis* (Durand et al. 2011) and AtraAOX2 from *A. transitella* (Choo et al. 2013).

Finally, based on the general moth sex pheromone biosynthesis pathway suggested in previous studies (Albre et al. 2012; Choi et al. 2005; Jurenka 2004; Ohnishi et al. 2006; Roelofs et al. 2002; Tillman et al. 1999), and the results obtained in the current study, we propose a putative biosynthesis and degradation pathway of the sex pheromone in *S. inferens* (Fig. 3). In the diagram, several candidate genes for some key steps are suggested and in our opinion they should be considered first for further functional studies in the future. For other steps where no candidate gene is suggested, more studies with alternative approaches are required.

Acknowledgments We thank Master students Rong Jin, Guan-Heng Zhu, He-Tan Chang, and Si-Bao Wang (Nanjing Agricultural University, China) for assistance in collecting the insects. We also thank Dr. Peng He (Guizhou University, China) and two anonymous reviewers for critical suggestions in writing of the manuscript. This work was supported by a Special Fund for Agro-scientific Research in the Public Interest (201303017) and a grant from the National Natural Science Foundation (31372264) of China.

Conflict of Interest The authors declare no conflict of interest.

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