Odorant degrading carboxylesterases regulate larva foraging and adult mating in Grapholita molesta (Busck)

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Abstract

Insect antennal carboxylesterases (CXEs) play key roles in the process of ester odor degradation. In this study, 23 candidate CXEs were identified by transcriptome analysis of Grapholita molesta. The GmolCXE1 and GmolCXE5 were highly expressed in the antennae of adults and significantly up-regulated in the antennae of male moths after exposure with odors from female moths. GmolCXE7, 10, 13, 14 and 20-22 were abundantly expressed in the larval heads. The relative expression of GmolCXE13, 14 and 21 were significantly up-regulated after stimulation with odors from ripe fuji apples and crown pears. RNA interference assays demonstrated that the GmolCXE1, 5 and GmolCXE14, 21 may involve in regulating the adults' mating and larval foraging respectively. Our study suggested that GmolCXE1 and GmolCXE5 could degrade the sex pheromone component (Z/E)-8-dodecenyl acetate to its product (Z/E)-8-dodecenol with evidence from EAG responses and GC-MS analysis. GmolCXE14, 21 could degrade odorant volatiles (Ethyl butanoate/ ethyl hexanoate) of ripe crown pears by GC-MS analysis. These GmolCXEs mediated foraging and mating would be potential and effective molecular targets to develop behavioral antagonists against larvae and adults of G. molesta.

Keywords

Carboxylesterases, ester sex pheromones, ester odorant volatiles, degradation, behavioral antagonists

1 INTRODUCTION

Olfaction plays incredibly important roles for insects to search for food, mate, spawning locations and avoid predators by detecting molecule clues in their environment such as host plant volatiles, sex pheromones and alarm pheromones (Vogt, 2005; Pelosi et al., 2006; Dweck et al., 2013; Li and Liberles, 2015). During the reception of odor molecules, many key proteins are involved, including odorant binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptor (ORs), odorant degrading enzymes (ODEs), ionotropic receptors (IRs), and sensory neuron membrane proteins (SNMPs) (Rützler and Zwiebel, 2005; Leal, 2013). Among these proteins, ODEs act as a rapid odorant inactivator to maintain a low stimulus level within antennal sensilla in the process of odorant signal reception (Vogt et al., 1985). Once olfactory receptor neurons (ORNs) were stimulated by odor molecules, they must be rapidly removed by ODEs to prevent the accumulation of stimulus and restore the sensitivity of the sensory neuron (Leal, 2013). There are a few insect ODEs that have been functional characterized to date, such as carboxylesterases (CXEs), cytochrome P450s, glutathione S-transferases (GSTs), UDP-glycosyltransferases (UGTs), aldehyde oxidases, epoxide hydrolases, and alcohol dehydrogenases (Vogt, 2005; Leal, 2013).

Insect carboxylesterases belong to the carboxyl/cholinesterase family, a branch of the α/β –hydrolase fold superfamily. The enzymes in this superfamily hydrolyze ester bonds, which are present in many insect and

plant odors, insect pheromones and hormones as well as pesticides (Vogt, 2005; Durand et al., 2010a; He et al., 2015). The first insect ODE was an antennae-specific esterase (ApolSE) identified from the sensillar fluid of male silkworm *Antheraea Polyphemus* antennae (Vogt and Riddiford, 1981). It was characterized as a pheromone degrading enzyme (PDE) that can rapidly degrade sex pheromone [(*6E* ,*11Z*)-hexadecadienyl acetate, Z11-16: Ac] *in vivo* and*in vitro* (Vogt and Riddiford, 1981; Vogt et al. 1985; Ishida and Leal, 2005). The other antennae-specific pheromone-degrading esterase, PjapPDE, was identified from antennae of the Japanese beetle*Popillia japonica* (Ishida and Leal 2008). Kinetic studies showed that PjapPDE is involved in rapid inactivation of sex pheromone (R)-japonilure. In *Drosophila* , one male antennal specific expressed esterase, Esterase-6 has been proved to be an odorant-degrading enzyme involving in modulating sex pheromone response (Chertemps et al. 2012). Additionally, many antennal CXEs have been identified from the moths (Maïbèche-Coisne et al., 2004; Merlin et al. 2007; Jordan et al., 2008; Durand et al., 2010b; He et al. 2014a, b, c), honey bee (Kamikouchi et al., 2004), and fruit fly (Younus et al, 2014). Recent years, with more advanced sequencing technologies, more*CXEs* were identified in olfactory organs by antennal transcriptome analysis (Merlin et al., 2007; Liu et al., 2015; Zhang et al., 2016; Sun et al., 2017; Zhang et al., 2017b). However, the molecular mechanisms of the CXEs involved in pests foraging, mating and selecting oviposition sites are not fully investigated.

The oriental fruit moth or peach moth, *Grapholita molesta*(Busck), is a notorious fruit pest throughout the world (Cardé et al., 1979; Zhang et al., 2018). The larvae prefer eating shoot and pulp inside of fruits, which causes extensive fruit damage and leads to serious economic losses if not controlled (Myers et al., 2007). Many odorant molecules that *G. molesta* uses for host plant location, oviposition and mating are ester compounds, such as (*Z*)-8-dodecenyl acetate (Z8-12:Ac) and (*E*)-8-dodecenyl acetate (E8-12:Ac) belonging to critical sex pheromone components released by female moths of *G. molesta* (Allred, 1995), and butyl acetate, hexyl acetate, hexyl hexanoate and butyl hexanoate that are volatiles of host plants (pear, apple and peach) (Zhao et al., 2013; Tian et al., 2018). Therefore, we hypothesize that *G. molesta*antennal CXEs may play important roles in plant odor or insect pheromone degradation and are potentially involved in modulating the foraging and mating behavior of *G. molesta* .

In the present study, putative *GmolCXEs* were identified and analyzed from the transcriptomic analysis of *G. molesta* larva heads. Then the expression patterns of *GmolCXE* s of adults and larvae were examined under normal feeding conditions and after exposure with odors from female moths or ripe fuji apples and crown pears. Five*GmolCXEs* were selected for further functional studies by combining RNA interference (RNAi), behavior, electrophysiological and chromatographic studies. The proposed research provides a new insight into functions of odorant degrading esterases in modulating adult and larval behavior of *G. molesta* that potentially can be good targets to control this notorious agricultural pest.

2 METHODS

2.1 Insect rearing

The larvae and adults of *G. molesta* were obtained from infested fruits of pear orchard in Changli, Hebei, China in July 2018. The adults were fed with a 10% honey solution. The larvae were fed with fuji apples. The next generations were reared in an artificial climate cabinet (PXZ-430B, Ningbo Jiangnan Instrument Factory, China) at the College of Plant Protection, China Agricultural University (Beijing, China). Rearing conditions were 25 ± 0.5°C with a photoperiod of 14 L: 10 D (Light, Dark) and 70% relative humidity.

2.2 Identification and classification of candidate*GmolCXEs*

2.2.1 Sequence analysis

Candidate *GmolCXEs* were identified through keyword screening of the BLASTX annotations of transcriptomic data from *G. molesta*larva heads (fourth instar larvae, fifth instar larvae for 1-2 days and fifth instar larvae for 6-7 days). The open reading frames (ORFs) of all genes were predicted using the NCBI ORF finder. Similarity searches were performed with the NCBI-BLAST network server (*http://blast.ncbi.nlm.nih.gov/*). Putative N-terminal signal peptides were predicted using the SignalP

5.0 server (*http://www.cbs.dtu.dk/services/SignalP/*). The theoretical isoelectric points and molecular weights of the deduced proteins were calculated using the ExPASy tool (*http://web.expasy.org/compute_pi/*). Catalytic residues were predicted by searching the NCBI Conserved Domain Database (*http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml*).

2.2.2 Phylogenetic tree construction

The amino acid sequences of *GmolCXEs* and *CXEs* from other species were aligned using ClustalX 2.0 (Larkin et al., 2007). A neighbor-joining tree was constructed by the program MEGA 8.0 using the neighbor-joining method with a p-distance model and a pairwise deletion of gaps (Tamura et al., 2013; Tan et al., 2018). Node support was assessed using a bootstrapping procedure with 1,000 replicates. And the Fig tree 4.3 is used as the auxiliary software to modify the tree (Wei et al., 2017). The protein names and accession numbers corresponding to the genes used for construction of the phylogenetic tree are listed in Table S1.

2.3 Spatial expression of putative *GmolCXEs* in larvae and adults

2.3.1 Tissue collection for gene expression

The head and abdomen of 15 fourth instar larvae were collected for three biological replicates. The antennae, head, thorax, abdomen, legs and wings for both sexes at 3 days after adult eclosion were collected and kept in different microcentrifuge tubes (1.5 mL, no RNAase). Three biological replicates (100 individuals per replicate) were performed for adult samples. All samples were frozen and stored in liquid nitrogen until the RNA extraction.

2.3.2 RNA Extraction, cDNA Synthesis and quantitative real-time PCR (qRT-PCR)

The total RNA of each sample was extracted using the Invitrogen TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA quantity and quality were determined by a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, USA). First-strand cDNA was synthesized with a FastQuant RT Kit (with gDNase) (Tiangen Biotech, Beijing, China). The cDNA was serially diluted (3-fold), and the dilutions were used to analyze the qRT-PCR efficiency of the primers (Table S2). The *β-actin* and *EF1-a* genes of *G. molesta* were used as the internal controls (Chen et al., 2014; Cao et al., 2015). The qRT-PCR mixture was prepared as instructed by the SYBR ® Premix Ex TaqTM (TliRNase H Plus) manual (Takara, Dalian, China). The reactions were performed on a StepOne thermocycler (ABI, Carlsbad, USA) using the two-step method and were analyzed with a melting curve analysis program. The specificity of qRT-PCR primers was confirmed by the melting curve and sequencing of the qRT-PCR products. The data were analyzed by using the $2^{-\Delta\Delta^{\sim}T}$ method (Livak et al., 2001).

2.4 *GmolCXE* gene expression after odor-stimulation

2.4.1 Male adults stimulated by female adults

The new emerged virgin male and female adults (100 individuals per replicate) were separately kept in two different artificial climate cabinets (PXZ-430B, Ningbo Jiangnan Instrument Factory, China) for 3 days since eclosion. When male and female moths were at their mating peak after 3 days, they were placed with 1:1 ratio in the same artificial climate cabinet with a breathable barrier avoiding mating (Fang, 2017) (Figure S1 A). After 2 h, the antennae of male adults were collected and quickly frozen in the liquid nitrogen. Expression levels of *GmolCXEs* in male antennae before and after female stimulation were measured by qRT-PCR. The adults were fed with a 10% honey solution. Three biological replicates were performed.

2.4.2 Larvae stimulated by fresh ripe fuji apples or crown pears

Fourth instar larvae were obtained that had been starved for 1 day. For each experiment, fresh ripe fuji apples (Yantai, Shandong, China) or crown pears (Baoding, Hebei, China) were put in the corners of two different plexiglas cages (50 × 50 × 50 cm) , and 60 starved larvae were released in the center of plexiglas cages and isolated with a container to avoid feeding (Figure S1 B). After 2 h, the head of odor stimulated and unstimulated larvae were collected and quickly frozen in the liquid nitrogen. Expression levels of *GmolCXEs*

in head of odor stimulated and unstimulated larvae were measured by qPCR. Three biological replicates were performed.

2.5 RNAi-mediated gene silencing

2.5.1 double-stranded RNA (dsRNA) synthesis and injection

dsRNAs of five target esterase genes (*dsCXE1* , *dsCXE5* ,*dsCXE13* , *dsCXE14* and *dsCXE21*) were synthesized with the MEGAscript^{TM} T7 high yield transcription kit (Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's instructions. The cDNA of larva heads and adult antennae was used as the templates. The primers used for dsRNA synthesis are shown in Table S2. *ds* EGFP (GenBank number: ACY56286) was used as a control in all RNAi experiments. The concentration of dsRNAs dissolved in DEPC-treated water was determined by a NanoDrop 2000 spectrophotometer, and its integrity was analyzed on 1.0% agarose gels. Then, 5 μg dsRNA were microinjected into each individual (injection sites: the base of thorax foot of fourth instar larvae and anterior thorax of male adults) with a microINJECTOR System MINJ-1 (Tritech Research, Los Angeles, CA, USA).

2.5.2 Y-tube assays

Behavioral assays are performed with a glass Y-tube olfactometer (10 cm body length; 8 cm arm length; 1.5 cm inner diameter; 60 degree for the angle of two arms). Air aspirated with a vacuum pump (Qianxi Air Company, Beijing, China) was filtered through an activated-charcoal filter and a sorbent cartridge (Porapak Q, 50 mg, 80/100 mesh, Supelco, Bellefonte, PA, USA) with a 0.3 L/min flow rate. Experimental samples (fresh ripe fuji apples or crown pears / 30 virgin female adults for 3 days after eclosion / 10^{-3} M ethyl butanoate and 10^{-3} M ethyl hexanoate from crown pear volatiles) and control samples (DEPC water/ 10^{-3} M HPLC purity grade hexane) were placed into the two arm sides respectively. Each individual larva or male adult was placed at the entrance of the olfactometer. The number of larvae or male adults in each arm exceeded 2 cm and stayed for 30s were recorded within 5 min. If larva or male adult made no choice within 5 min, it was not counted. For each experiment, 30 male adults or 30˜60 larvae were tested in the Y-tube olfactometer. All assays were performed in the behavior room at 25 ± 0.5 and 70% relative humidity. Behavioral assays of male adults were operated under red light (0.61 lux). The olfactometer was washed with detergent and water before each experiment. Each experiment was repeated for three times.

2.5.3 Electroantennogram (EAG) recordings

Physiologically active antenna, cutting along the basal segment, was immediately placed between two glass electrodes that attached to an electrically conducting gel (Spectra 360 Electrode Gel) (Zhang et al., 2017). And 20 μL odor stimulus source was added on a filter paper strips (0.5 × 5 cm) that placed inside pasteur pipettes. The humidified air stream was supplied by a Syntech stimulus controller (CS-55 model, Syntech, Germany) at 50 cm/sec and maintained at a pulse time of 2 sec. The EAG responses of male antennae to odor compounds were recorded. After each sample was tested, hexane was used for testing as the control. Three biological replicates were performed for each odor compound combination in the experiment. Odor stimulus sources, sex pheromone mix components (Z8-12:Ac: E8-12:Ac = 9:1), were diluted with hexane to a final concentration of 1000 ppm. The proportion of mix components (Z8-12:Ac: E8-12:Ac = 9:1) was designed based on previous studies (Li et al., 2011).

EAG recordings were also used for evaluating functions of GmolCXE1 and GmolCXE5 in odor degradation. The purification of GmolCXE1 and GmolCXE5 was described in 'method 2.6.1'. Odor stimulus sources included two ester sex pheromone components (Z8-12:Ac and E8-12:Ac), mix components (Z8-12:Ac: E8-12:Ac = 9:1) and their treatment samples. The ester sex pheromone components were diluted with hexane to a final concentration of 1000 ppm. Treatment samples showed that sex pheromone components were treated with 50 μg purified GmolCXE1 or GmolCXE5 for 1 h at 28 in 50 mM Tris-HCl (pH 7.4), then the reactions were extraction with 500 μl hexane. Three biological replicates were performed.

2.6 Degradation analysis of GmolCXEs with free ester compounds

2.6.1 Protein expression and purification

The target GmolCXE proteins were expressed and purified as described in previous studies (Wei et al., 2019; Zhu et al., 2017). In brief, cDNAs were amplified using specific primers with enzyme recognition sites (Table S2). After digestion with restriction enzymes, the target gene fragments were inserted into expression vector pET30a (+) digested with the same restriction enzymes. The recombinant plasmids with target GmolCXE genes were induced in *Escherichia coli* Transetta (DE3) cells with 1 mM I isopropyl-β-D-thiogalactoside (IPTG) at 15 for 12 h. After sonication and centrifugation, recombinant proteins of GmolCXE1, 14 were mainly present as inclusion bodies, and GmolCXE5, 21 major expressed in supernatant. The inclusion bodies were dissolved in 6 M Guanidine Hydrochloride and purified with Tris buffer containing 8 M urea by Ni ion affinity chromatography (GE Healthcare Biosciences, Uppsala, Sweden). The supernatant were purified directly by the Ni ion affinity chromatography Then the purified proteins were refolded by extensive dialysis against 20 mM Tris-HCl buffer (PH=7.40).

2.6.2 Collection and detection of Volatile organic compounds (VOCs) from fresh ripe crown pears

A push-pull system was used to collect headspace VOCs as described in previous study (Lu et al. 2012). Briefly, fresh ripe crown pears (ca. 1,500 g) were placed into a glass jar (2000 mL) for extraction. Air aspirated with a vacuum pump (Qianxi Air Company, Beijing, China) was filtered through an activated-charcoal filter and a sorbent cartridge (Porapak Q, 50 mg, 80/100 mesh, Supelco, Bellefonte, PA, USA) with a 400 mL/min flow rate. The sorbents were held between plugs of glass wool in a glass tube (10 cm long, 0.5 cm inner diam). Samples were collected at 24±1 °C and 65–70 % RH for 8 h. Odor volatiles were eluted with 500 μL hexane (HPLC grade, Sigma-Aldrich) at room temperature. Sample volumes were condensed to 50 μL by using a slow stream of nitrogen. 0.5 μg benzaldehyde (99 %, Fluka, Switzerland) was added to each sample as an internal standard. Samples were analyzed by GC-MS. Identification of VOCs was verified by comparison with authentic samples. Three biological replicates were set in the experiments.

2.6.3 Gas chromatography–mass spectrometry (GC-MS)

Compounds were detected by GC-MS with Agilent 7890B/7200 (30 m × 0.25 mm ID, 0.25 μM film, J&W Scientific Inc., Folsom, CA, USA). For electron impact (EI) mass spectra, the ionization voltage was 70 eV, and the emission current was 34.6 μA (Lu et al., 2012). The temperatures of the ion source and interface were 230 °C and 280 °C, respectively (Lu et al. 2012). Ten μg of each freshly purified GmolCXE was incubated with each ester substrate (final concentration 50 μM) in 20 μL of 20 mM Tris buffer (pH = 7.4) at 28 °C for 1 h. Then the reaction was extracted with 50 μL hexane. The processed samples were measured under the same test conditions by GC-MS. The percentage of conversion was calculated by the product amount of the corresponding alcohol or ester compounds (with two independent replicates) (He et al., 2015).

2.7 Statistical analysis

All statistic comparison was determined using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Data multiple comparison was assessed by ANOVA following Turkey's HSD multiple comparison test ($P < 0.05$, a, b, c, d and e). The statistical significance of the difference between two treatments was analyzed using a pairwise Student's t test ('*', $0.01 < P < 0.05$, '**', $0.001 < P < 0.01$, '***', $P < 0.001$, 'NS', $P > 0.05$). The statistical significance of RNAi-mediated knockdown assays are analyzed by chi-square test ("*", $0.01 < P < 0.05$, "**", $0.001 < P < 0.01$, "***", $P < 0.001$, 'NS',$P > 0.05$).

3 RESULTS

3.1 Identification and phylogenetic analysis of candidate *GmolCXE*s

Based on the bioinformatic analysis, 23 candidate *GmolCXEs* were identified in the larval head transcriptome of *G. molesta* . These sequences were named *GmolCXE1-23* according to the similarity to known Lepidopteran CXEs and numbers of amino acids in open reading frames (ORFs) (Table 1). Among them, 15 *GmolCXEs* contained full-length ORFs, and the rest 8 sequences were partial. The 15 full-length *GmolCXEs*

contain a conserved motif pattern including an oxyanion hole (GGA, etc.), a catalytic triad (S-Xa-E-Xb-H), and a conserved Ser active site (G-X-S-X-G) (Figure S2 and Table 2). The predicted theoretical isoelectric points of these esteases are ranged from 5.22 to 9.27, and calculated molecular masses varies from 58.79 kDa to 74.82 kDa (Table 2). Phylogenetic analysis showed that 23 putative *GmolCXEs* are closely clustered into 7 different clades (Figure 1). These clades include moth antennal esterases (*GmolCXE1* ,*2* , *14* , *15* , *17* , *18* , *20*), cuticular and antennal esterases (*GmolCXE5*), ß-esterases and pheromone esterases (*GmolCXE13* , *21*), lepidopteran juvenile hormone esterases (JHE) (*GmolCXE7*), mitochondrial and cytosolic esterases (*GmolCXE3a* , *3b* , *4* , *6* , *10* ,*12* , *16*), α-esterases (*GmolCXE8* , *9* ,*11* , *19*) and neuroligins (*GmolCXE22*).*GmolCXE1* and *GmolCXE13* are closely clustered with *ApolODE* and *ApolPDE* of *Antheraea Polyphemus* , respectively (Figure 1). All sequence information of these 23 *GmolCXEs* has been released in the GenBank database (Table 1).

3.2 Screening of five target *GmolCXEs* involved in odor reception of foraging and mating

In male and female antennae of *G. molesta* adults, the relative expression levels of *GmolCXE1* and *GmolCXE5* were significantly higher than other esterase genes (Figure 2 A). The *GmolCXE1* and *GmolCXE5* were widely expressed in all tissues of adults in both male and females, whereas their expression in antennae were higher than other tissues (Figure 2 B and C). After female adult stimulation, the expressions of *GmolCXE1* and *GmolCXE5* in the male antennae was induced by about 2- folds (Figure 2 D).

In the 4th instar *G. molesta* larvae, the expressions of seven esterase genes (*GmolCXE7* , *10* ,*13* , *14* , *20-22*) were significantly higher in the head than in the abdomen (Figure 3 A). Among these genes, the expressions of *GmolCXE13* , *GmolCXE14* and *GmolCXE21* in the larva head were significantly up-regulated as much as twice after fresh ripe fuji apples and crown pears stimulations than those in the control (Figure 3 B).

3.3 GmolCXE1 and GmolCXE5 regulated the mating behaviors of male adults in *G. molesta*

Male adults were disturbed in the search for female adults by GmolCXEs silencing

After RNAi for 24 h, the expression levels of *GmolCXE1 and GmolCXE5* decreased significantly (Figure 4). The expression of *GmolCXE1 and GmolCXE5* decreased by 56.46% and 47.62%, respectively (Figures 4 A and B). The number of male adults choosing female adults had a significant reduction by *GmolCXE1* silencing, while no difference was observed by *GmolCXE5* silencing ($P > 0.05$) (Figure 4 C). The male adults injected with *dsCXE1* and *dsCXE5* spent much time and exhibited more frequencies of turning back during the searching period in the Y-tube compared to male adults injected with *dsEGFP* as control (Figure 4 D and E). EAG amplitudes in response to sex pheromone mix components (Z8-12: Ac**:** E8-12: Ac = 9**:** 1) released by female moths were significantly reduced when *GmolCXE1* or *GmolCXE5* were knocked down in male adults by RNAi (Figure 4 F).

GmolCXEs degraded free ester sex pheromone components in mating

We firstly expressed GmolCXE1 and GmolCXE5 proteins using *E. coli* Transetta (DE3) cells. Then we purified proteins by Ni ion affinity chromatography (Figure S3). The sex pheromone mixtures were incubated with each purified esterase protein for 1 h before submitted to EAG analysis. As shown in Figure 5, the antennae of male moths had a strong EAG responses to these two ester sex pheromone compounds Z8-12:Ac, E8-12:Ac, and mix (Z8-12:Ac: E8-12:Ac=9:1) under normal conditions. However, after incubated with the purified GmolCXE1 or GmolCXE5 proteins, the EAG amplitudes in response to sex pheromone compounds Z8-12:Ac, E8-12:Ac, and mix were significantly reduced in male moths (Figure 5 A-C).

GC-MS results showed that after Z/E8-12:Ac treated with GmolCXE1 or GmolCXE5, the metabolites Z/E8-12:OH were detected (Figure 6 A-C and E-G). And degradation percentage of Z8-12:Ac were 100% (CXE1) and 35.52% (CXE5) (Figure 6D), and degradation percentage of E8-12:Ac were 64.99% (CXE1) and 33.23% (CXE5) (Figure 6 H).

3.4 GmolCXE14 and GmolCXE21 but not GmolCXE13 regulated the food foraging behaviors in larvae of *G. molesta*

Larvae were disturbed in the search for food and ester volatiles by GmolCXEs silencing

The relative expression levels of *GmolCXEs* decreased mostly by 50.83% for 24 h (*GmolCXE13*), 75.23% for 48 h (*GmolCXE14*) and 58.33% for 24 h (*GmolCXE21*) after injected with 5 μg corresponding dsRNAs of *GmolCXEs* (Figure 7 A-C).

Our behavior assays showed that the numbers of larvae choosing ripe fuji apples or crown pears declined significantly after *GmolCXE14 and GmolCXE21* silencing respectively (Figure 7 E and F). Besides, the larvae injected with *dsCXE14* and *dsCXE21* spent much time finding food compared to larvae injected with *dsEGFP* (Figure 7 H and I). However, after knocking down *GmolCXE13* , there was no significant change on larvae searching for food compared to control group (knocking down *GmolEGFP*) (Figure 7 D and G).

Ethyl butanoate and ethyl hexanoate were detected from ripe crown pear by GC-MS analysis (Figure S4). The 4th instar larvae of *G. molesta* exhibited attracting behavior response to these two ester volatiles (Figure 8 A). However, the attraction of two ester volatiles to larvae decreased significantly after the *GmolCXE14* or *GmolCXE21* silencing (Figure 8 B and C).

GmolCXEs degraded free ester volatiles in foraging

After purified by Ni ion affinity chromatography (Figure S3), proteins of GmolCXE14 and GmolCXE21 were incubated with ethyl butanoate or ethyl hexanoate for 1 h and the products were submitted to GC-MS analysis. GC-MS results showed peak areas of two ester compounds treated with purified GmolCXE14 and GmolCXE21 were significantly reduced compared to the control groups (Figure 8 D-I). The degradation percentage of ethyl butanoate were 94.03% (CXE14) and 87.23% (CXE21) (Figure 8 J), and degradation percentage of ethyl hexanoate were 50.32% (CXE14) and 16.88% (CXE21) (Figure 8 K).

Discussion

The remarkable sensitivity of insect olfactory system has intrigued scientists for decades. The behavioral responses of moths to odor molecules reveal that insects have a highly sensitive olfactory system (Kaissling, 1987; Ishida and Leal, 2005). ODEs acting as rapid odorant inactivators play the major roles in insect behavior (Leal, 2013). However, the molecular mechanism of ODEs involved in the odorant reception of insects is not fully understood. Here, we identified the *G. molesta* CXEs and demonstrated functions of several target GmolCXEs in regulating the adult and larva behavior by metabolizing sex pheromone components or food ester volatiles in *G. molesta* .

In the present study, we identified 23 candidate *GmolCXE* genes by the larval heads' transcriptome analysis. The number of the *GmolCXEs* identified in *G. molesta* was similar to the identified species in Lepidoptera from *C. suppressalis* (19 CXEs) (Liu et al., 2015), *S. inferens* (20 CXEs) (Zhang et al., 2014),*S. littoralis* (20 CXEs) (Durand et al., 2010b), *S. litura* (24 CXEs) (Zhang et al., 2016). Our *GmolCXE1 and GmolCXE5* were widely distributed in the olfaction associated organs of adults and highly expressed in antennae, which was similar to the reported finding in *S. litura* (Zhang et al., 2016) and *Ectropis obliqua* (Sun et al., 2017). Besides expression in adults, seven genes (*GmolCXE7* , *10* , *13* , *14* and *20 -22*) were highly expressed in larval heads. However, *GmolCXE1* , *5* , *13* , *14* and 21 expression of *G. molesta* were significantly up-regulated after fresh fruits (fuji apples and crown pears) and female adult stimulation. No publications reported the screening of CXEs involved in behaviors (foraging, mating, etc.) via by odor-stimulation experiments. GmolCXE1 and GmolCXE13 in the evolutionary tree were along with ApolODE/ PDE that degraded the pheromone of *A. Polyphemus* in vitro (Ishida & Leal, 2005). Consequently, we inferred the *GmolCXE1* , *5* and *GmolCXE13* , *14* , *21* might be involved in the process of odor molecule reception during mating and foraging of *G. molesta* respectively.

Previous studies demonstrated that male moths of Lepidoptera insect showed 0.5-1.0 sec behavioral responses to sudden changes of pheromone concentration in the environment during this pheromone-oriented flight (Kramer, 1975; Vogt et al., 1985). The purified ApolCXE degraded the *A. polyphemus* pheromone with a half-life of 15 msec (Vogt et al., 1985). In this study, the male moths of *G. molesta* displayed a weak responses to ester sex pheromones of female moths when *GmolCXE1* and *GmolCXE5* were knocked down

by RNAi. Similarly, foraging of the larvae were weaken and the responses of larvae to ethyl butanoate and ethyl hexanoate were decreased after the *GmolCXE14* and*GmolCXE21* silencing. CXEs acting as ODEs can rapidly inactivate ester odor molecules (sex pheromones, odorant volatiles) in order to restore the sensitivity of the sensory neuron (Vogt et al., 1985; Leal, 2013). Once the CXEs were knocked down, odorant molecules would continuously stimulate the olfactory receptor neurons so that restore the sensitivity of the sensory neuron could not cannot be restored and reset. That's why *G. molesta* spent much time looking for food or mates after *GmolCXEs* silencing. No studies were reported the behavioral experiments of *CXEs* knocked down by RNAi. Nevertheless, other molecules (OBPs, ORs, etc.) associated with odor recognition were widely reported that male moths reduced the responses to sex pheromone components and plant volatiles after these genes silencing (Dong et al., 2017; Zhang et al., 2017a). The above mentioned results verified the *GmolCXE1* and*GmolCXE5* involved in regulating the courtship and mating behavior of male adults, and the *GmolCXE14* and *GmolCXE21* involved in regulating the foraging behavior of larvae in *G. molesta* . Therefore, make clear that these GmolCXEs degrades the corresponding ester odor molecules in the process of odor recognition is the question we're going to solve.

The calculated molecular masses of GmolCXE1, 5 14, 21 (59.30˜64.79 kDa) were similar to that of purified GmolCXE1, 5, 14, 21 (59 kDa < MW< 65 kDa) measured by gel filtration (Figure S2). (*Z*)-8-dodecenyl acetate and (*E*)-8-dodecenyl acetate are major ester sex pheromone components of *G. molesta* (Cardé et al., 1979). Ethyl butanoate and ethyl hexanoate were verified to be major ester odorant volatiles of ripe crown pears in this paper. Odorant volatiles of host plants and sex pheromones, plays critical roles in the foraging, courtship and mating of insect (Leal, 2013; Li and Liberles, 2015). Previous studies had demonstrated ApolCXE could degrade sex pheromone component Z11-16: Ac of *A. Polyphemus* by electrophysiological test (Ishida and Leal, 2005), and SexiCXE10 could degrade ester sex pheromones (Z11-14:Ac, Z9-16:Ac and Z11-16:Ac) and plant volatiles ((Z)-3-hexenyl acetate) by GC (He et al., 2015). Here, by EAG, GC-MS and fluorescence binding assays, we verified that the GmolCXE1 and GmolCXE5 degraded the (*Z/E*)-8-dodecenyl acetate to produce (*Z/E*)-8-dodecenol respectively. Study has indicated that PjapPDE degrade sex pheromones of *Popillia japonica* , and the degradation products are inactive into the sensillar lymph of pheromone-detecting sensilla on the *P. japonica* antennae (Ishida and Leal, 2008). Meanwhile, we also demonstrated ethyl butanoate and ethyl hexanoate were degraded by GmolCXE14 and GmolCXE21 respectively. Our results supported the report that the ApolCXE rapidly degraded the sex pheromones of *A. polyphemus*under conditions where there were no competing pheromone-protein interactions (Vogt et al., 1985).

There is a clear sense that CXEs are an important part in the process of odor signal (Leal, 2013). Many studies researched on that adult CXEs could degrade ester sex pheromone components (Ishida and Leal, 2008; Chertemps et al., 2015; He et al., 2015), few studies showed adult CXEs could degrade ester plant volatiles (Durand et al., 2010a; He et al., 2015). Here, not only did we show that adult' GmolCXE1 and GmolCXE5 degraded ester sex pheromones helping male adults to find female ones for mating, but we also showed that larval GmolCXE14 and GmolCXE21 degraded ester plant volatiles helping them forage host plants (crown pear).

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Data Accessibility

Nucleotide sequences of 23 identified *GmolCXEs* had been uploaded to NCBI Genbank (Table S1). The accession numbers of reference genes were KF022227.1 (*β-αϛτιν*), KT363835.1 (*EΦ1-α*) in GenBank.

Author Contributions

S.H.W., Q.S.T. and X.X.L. conceived and designed the experiments. S.H.W. performed the experiments. S.H.W. and Q.S.T. analyzed data. Z.L. and J.C.L. contributed reagents/materials/analysis tools. S.H.W.,

Q.S.T., Z.L., W.M.T., F.Z. and X.X.L. revised the manuscript. All authors discussed the results and approved the final version.

Supplementary Information

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51.

TABLES

Table 1. Best BLAST hits for candidate CXEs identified in the chemosensory organs of *C. molesta* .

Table 2. Motif analysis and biochemical characteristics of the 15 putative*GmolCXEs* with full-length sequences.

E: Glutamate; H: Histidine; SP: signal peptide; MW: molecular weight; PI: isoelectric point; Y: Yes; N: No.

FIGURES

Figure 1. Phylogenetic tree analysis of candidate GmolCXEs in the *C. molesta*. The tree was constructed with MEGA 8.0 using the neighbor-joining method. The values at the nodes are the results of bootstrapping with 1,000 replicates. *GmolCXE* genes are shown in red. Different color regions represent different categories of CXEs in phylogenetic tree. All the candidate GmolCXEs distributed into the designate clades of phylogenetic tree according to the classification system in reported references (Oakeshott et al., 2005; Durand et al., 2010).The accession numbers in GenBank and protein names of other species CXEs involved in tree are listed in Table S1.

Figure 2. qRT-qPCR analysis of *GmolCXEs* in male/female antennae or male antennae after female moth stimulation. A: Relative mRNA levels of 23 *GmolCXE* s in male and female antennae. B and C: The spatial expression patterns of*GmolCXE1* (B) and *GmolCXE5* (C) in different tissues of male and female adults. D: Relative expression analysis of 23 *GmolCXE* s in male antennae after female adults stimulation.

Figure 3. qRT-PCR analysis of *GmolCXE*s in larva head/ abdomen or larva head after fresh fruit stimulation. A: Relative mRNA expressions of 23 *GmolCXE* s in the head and abdomen of 4th instar larvae. B: Relative mRNA expressions of 23 *GmolCXE* s in the 4th instar larvae after fresh fuji apples and crown pears stimulation by qRT-PCR.

Figure 4. Behavioral assays after *GmolCXE1* and *GmolCXE5* of male adults knocked down by RNAi. A and B: The relative expression within 72 hours of *GmolCXE1* (A) and *GmolCXE5* (B) in male antenna after injected with the corresponding dsRNAs. C: Comparison of the numbers of individuals that find female adults. D: Time spent finding female moths by *GmolCXE1* and *GmolCXE5* silencing respectively. E: Frequency of male moths turning back in Y-tube searching for female adults. F: EAG response to sex pheromone mix components of female moths. "n" showed the number of larvae that did not respond to female moths or water.

Figure 5. Electrophysiological assays analysis for esterase treated sex pheromone components. A, B and C: Male moths' EAG responses to two sex pheromone components and mix components treated with purified GmolCXE1 and GmolCXE5 respectively. Z8-12:Ac**:**(*Z*)-8-dodecenyl acetate. E8-12:Ac**:**(*E*)-8-dodecenyl acetate. Mix: Z8-12:Ac: E8-12:Ac=9:1.

Figure 6. Degradation analysis of GmolCXE1 and GmolCXE5 with sex pheromone components by GC-MS. A and E: Control group. B and F: The two ester sex pheromone components treated with purified GmolCXE1. C and G: The two ester sex pheromone components treated with purified GmolCXE5. D and H: Percent of degradation on two ester sex pheromone components treated with purified GmolCXE1 and GmolCXE5 respectively. RT: retention time. PA: peak area. Percent of degradation (%) = (1- peak area of alcohol/ peak area of control)*100

Figure 7. Behavioral assays after *GmolCXE13*,*GmolCXE14* and *GmolCXE21* of larvae knocked down by RNAi.A, B and C: The relative expression within 72 hours of *GmolCXE13* ,*GmolCXE21* and *GmolCXE14* after larvae injected with the corresponding dsRNA. D and F: Comparison of the numbers of individuals that find fresh fruits after *GmolCXE13* , *GmolCXE21* and *GmolCXE14* silencing respectively. G and I: Time spent searching for food after *GmolCXE13, GmolCXE14* and *GmolCXE21* silencing respective. "n" showed the number of larvae that did not respond to food or water.

Figure 8. Behavioral assays of larvae responses to two ester volatiles from ripe crown pears and degradation analysis of GmolCXE14 and GmolCXE21 with two ester volatiles by GC-MS. A: Behavioral assays of larvae responses to ethyl butanoate and ethyl hexanoate by Y-tube. B and C: Behavioral assays of larvae responses to ethyl butanoate and ethyl hexanoate after *GmolCXE14* and *GmolCXE21* knocked down respectively. D and G: Control group. E and H: The two ester sex pheromone components treated with purified GmolCXE14. F and I: The two ester sex pheromone components treated with purified GmolCXE21. J and K: Percent of degradation on two ester volatiles treated with purified GmolCXE14 and GmolCXE21 respectively. RT: retention time. PA: peak area. Percent of degradation (%) = (peak area of ester volatiles-treatment / peak area of ester volatiles-control)*100

Figure S1. Diagrammatic drawing of fourth instar larvae or male adults by odor-stimulation. A: The fourth instar larvae were stimulated by ripe fuji apples or crown pears. B: The male moths were stimulated by female moths.

Figure S2. The protein sequences analysis of 15 full-length *GmolCXEs* by ClustalX 2.0. The shaded labeled region in the figure were the conserved motif of 15 full-length GmolCXEs. The conserved motif-pattern with the motif order Oxyanion-Ser active site-Glu-His.

Figure S3. Expression and purification of GmolCXEs were testedusing SDS-PAGE analysis. A, B, C and D: Expression and purification of GmolCXE1, GmolCXE5, GmolCXE14 and GmolCXE21 respectively. M: *TransBlue Plus* ™ Protein Marker. 1: Expression of proteins without IPTG. 2: Expression in the supernatant of proteins with 1.0 mM IPTG after ultrasonic crushing. 3: Expression in the inclusion body of proteins with 1.0 mM IPTG after ultrasonic crushing. 4: Purified proteins with His-tag.

Figure S4. Chromatogram and mass spectrogram of two eater volatiles from ripe crown pears were analyzed by GC-MS. A: Collection time analysis of odor volatiles from ripe crown pears. B and C: Chromatogram and mass spectrogram of ethyl butanoate respectively. D and E: Chromatogram and mass spectrogram of ethyl hexanoate respectively.

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