Putative carboxylesterase gene identification and their expression patterns in *Hyphantria cunea* (Drury)

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The olfactory system of insects is important for behavioral activities as it recognizes internal and external volatile stimuli in the environment. Insect odorant degrading enzymes (ODEs), including antennal-specific carboxylesterases (CXEs), are known to degrade redundant odorant molecules or to hydrolyze important olfactory sex pheromone components and plant volatiles. Compared to many well-studied Type-I sex pheromone-producing lepidopteran species, the molecular mechanisms of the olfactory system of Type-II sex pheromone-producing *Hyphantria cunea* (Drury) remain poorly understood. In the current study, we first identified a total of ten CXE genes based on our previous *H. cunea* antennal transcriptomic data. We constructed a phylogenetic tree to evaluate the relationship of HcunCXEs with other insects' CXEs, and used quantitative PCR to investigate the gene expression of *H. cunea* CXEs (HcunCXEs). Our results indicate that HcunCXEs are highly expressed in antennae, legs and wings, suggesting a potential function in degrading sex pheromone components, host plant volatiles, and other xenobiotics. This study not only provides a theoretical basis for subsequent olfactory mechanism studies on *H. cunea,* but also offers some new insights into functions and evolutionary characteristics of CXEs in lepidopteran insects. From a practical point of view, these HcunCXEs might represent meaningful targets for developing behavioral interference control strategies against *H. cunea*.

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Abstract

The olfactory system of insects is important for behavioral activities as it recognizes

internal and external volatile stimuli in the environment. Insect odorant degrading

enzymes (ODEs), including antennal-specific carboxylesterases (CXEs), are known to

degrade redundant odorant molecules or to hydrolyze important olfactory sex

pheromone components and plant volatiles. Compared to many well-studied Type-I sex

pheromone-producing lepidopteran species, the molecular mechanisms of the olfactory

system of Type-II sex pheromone-producing *Hyphantria cunea* (Drury) remain poorly

understood. In the current study, we first identified a total of ten CXE genes based on our

previous *H. cunea* antennal transcriptomic data. We constructed a phylogenetic tree to

evaluate the relationship of HcunCXEs with other insects' CXEs, and used quantitative

PCR to investigate the gene expression of *H. cunea* CXEs (HcunCXEs). Our results

indicate that HcunCXEs are highly expressed in antennae, legs and wings, suggesting a

potential function in degrading sex pheromone components, host plant volatiles, and

other xenobiotics. This study not only provides a theoretical basis for subsequent

olfactory mechanism studies on *H. cunea,* but also offers some new insights into

functions and evolutionary characteristics of CXEs in lepidopteran insects. From a

practical point of view, these HcunCXEs might represent meaningful targets for

developing behavioral interference control strategies against *H. cunea*.

Introduction

43 A complete insect olfactory process requires the participation and cooperation of various

44 olfaction-related proteins (*Scott et al., 2001*; *Vogt, 2003*; *Leal, 2013*). During the process,

45 external liposoluble odor molecules first pass through the polar pores on the sensillum

46 surface, then enter the lymph under the integument where they further combine with

47 odorant binding proteins (OBPs) before being transferred to the dendritic membrane of

48 olfactory receptor neurons (ORNs) (*Tegoni, Campanacci & Cambillau, 2004*; *Leal, 2013*;

49 *Pelosi et al., 2018*). The molecule-bound odorant receptors (ORs) then convert the chemical

50 signals into electrical signals that are transmitted to the central nervous system through

51 axons of the ORNs (*Song et al., 2008*). This whole process guides insects to make

52 relevant physiological responses and behavioral decisions. Once the signal transmission

53 is completed, redundant odorant molecules need to be degraded or inactivated by

54 odorant degrading enzymes (ODEs) in the antennal sensilla; otherwise, the odorant

55 receptors will remain in a stimulated state, which may lead to poor spatio-temporal

56 resolution of the odor signal, and pose fatal hazards to the insects (*Vogt & Riddiford,*

57 *1981*; *Steinbrecht, 1998*; *Durand et al., 2010b*; *Leal, 2013*). ODEs degrade redundant

58 odorant molecules in the lymph of antennal sensilla and within the cells (*He et al., 2014a*).

59 Traditionally, ODEs can be divided into five categories based on the structural difference

60 of various target substances: carboxylesterase (CXE), cytochrome P450 (CYP), alcohol

61 dehydrogenase (AD), aldehyde oxidase (AOX) and glutathione *S*-transferase (GST)

62 (*Rybczynski, Reagan & Lerner, 1989*; *Ishida & Leal, 2005*; *Pelletier et al., 2007*; *Durand*

63 *et al., 2010a*). However, ODEs of different categories have been shown to catalytically

64 interact with odor molecules of the same type and structure. It is currently believed that

65 the different enzyme families of ODEs may work together in degradation and clearing of

66 the same type of odor molecule (*Steiner et al., 2019*).

67 As primary metabolic enzymes, CXEs are widely distributed among insects, microbes

68 and plants (*Guo & Wong, 2020*). The active site contains several conserved serines,

69 which promote the cleavage and formation of ester bonds (*Bornscheuer, 2002*) and play

70 an important role in the metabolism of heterologous substances, pheromone

71 degradation, neurogenesis, developmental regulation and many other functions (*Yu et*

72 *al., 2009*). In addition to the metabolism and detoxification of endobiotics and xenobiotics,

73 another important role of CXEs is to maintain the sensitivity of ORNs. The CXEs enable

74 rapid degradation of stray odors and prevent vulnerable ORNs from being continuously

75 invaded by harmful volatile xenobiotics (*Li et al., 2013*). So far, a large number of genes

76 encoding CXEs have been identified and their functions in insect olfaction have also

77 been investigated in various insects, including *Drosophila melanogaster, Mamestra*

78 *brassicae, Antheraea polyphemus, Sesamia nonagrioides*, *Popillia japonica*, *Spodoptera*

79 *littoralis, Epiphyas postvittana, Agrilus planipennis*, *S. litura, S. exigua*. (*Vogt, Riddiford &*

80 *Prestwich, 1985*; *Maïbèche-Coisne et al., 2004*; *Ishida & Leal, 2005*; *Merlin et al., 2007*;

81   *Ishida & Leal 2008*; *Jordan et al., 2008*; *Durand et al., 2010b*; *Mamidala et al., 2013*; *He*

82   *et al., 2014a*; *He et al., 2014b*; *He et al., 2014c*; *He et al., 2015; Chertemps et al., 2015*).

83   For instance, the *A. polyphemus* pheromone-degrading enzyme CXE (*Apol*PDE) was

84   shown to effectively degrade its sex pheromone acetate component (*Maïbèche-Coisne*

85   *et al., 2004*; *Ishida & Leal, 2005*). In *P. japonica* and *D. melanogaster*, the purified native

86   or recombinant antennal CXEs were found to degrade their sex pheromone constituents

87   (*Ishida & Leal, 2008*; *Younus et al., 2014*). In addition, some of CXEs from *S. exigua*, *S.*

88   *littoralis* and *S. litura* were also found to degrade both their sex pheromones and plant

89   volatiles, as well as hydrolyze volatile esters released from their natural food sources

90   *(Gomi, Inudo & Yamada, 2003; Durand et al., 2011; Chertemps et al. 2015).*

91   The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera; Erebidae), native to North

92   America, is a worldwide quarantine pest insect. This moth has now spread to most

93   European countries (except the Nordics), South Korea, North Korea and China, and

94   lately to Central Asia (*Itô & Miyashita, 1968; Gomi, 2007*). As an invasive pest, *H. cunea*

95   was first found in Dandong (Liaoning province, China) and has rapidly spread to Hebei

96   and adjacent provinces in China (*Gomi, 2007; Yang et al., 2008; Tang, Su & Zhang,*

97   *2012*). In 2012, the State Forestry Administration's Forest Pest Inspection and

98   Identification Center identified the first outbreak of *H. cunea* in Sanshan district, Wuhu

99   City, Anhui Province, which was the southernmost known outbreak of *H. cunea*. Its

100 invasion has caused serious damage to local forests, agricultural crops and

101 landscaping/ornamental trees, resulting in great economic and ecological losses. Thus,

102 effective quarantine programs and environmentally safe pest management solutions are

103 needed to combat this serious invasive pest insect. More importantly, a better

104 understanding of its chemical ecology may facilitate more effective pest management

105 strategies. Previous studies have described four sex pheromone components, including

106 two straight chain aldehydes, (9*Z*,12*Z*)-octadecadienal (Z9, Z12-18Ald) and (9*Z*,12*Z*,15*Z*)-

107 octadecatrienal (Z9, Z12, Z15-18Ald), and two epoxides, (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-

108 3,6-heneicosadiene (Z3, Z6-9S, 10R-epoxy-21Hy) and (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-

109 1,3,6-heneicosatriene (1, Z3, Z6-9S, 10R-epoxy-21Hy), which are produced by female *H.*

110 *cunea* (*M. et al., 1989*). There are two major groups of moth sex pheromones: Type I

111 pheromones and Type II pheromones (*M. et al., 1989*; *Millar, 2000; Ando et al., 2004*).

112 Type I pheromones mostly contain C_{10}-C_{18} unsaturated hydrocarbons and a terminal

113 functional group (>75% moth species). Type II pheromones lack a terminal functional

114 group and contain C_{17}-C_{23} unsaturated hydrocarbons and epoxy derivatives (*Millar, 2000,*

115 *Ando et al., 2004*). Compared to many well-studied Type-I sex pheromone-producing

116 moth species, the molecular mechanisms of olfaction in the Type-II sex pheromone-

117 producing *H. cunea* are poorly understood. In the current study, a total of 10 CXE genes

118 were identified based on our previous *H. cunea* antennal transcriptomic data (*Zhang et*

119 *al., 2016*). To understand the potential physiological roles of these HcunCXEs, we

120 constructed a phylogenetic tree to evaluate the relationship of HcunCXEs with other

121 insects' CXEs, and used reverse transcription-quantitative PCR (RT-qPCR) and reverse

122 transcription PCR (RT-PCR) to investigate the expression of these genes. We found that

123 HcunCXEs displayed either antennae- or leg/wing-biased expression. The differential

124 expression pattern of HcunCXEs suggests a potential function in degrading pesticides

125 and/or other xenobiotics.

126

127

128 **Materials and Methods**

129 **Insect rearing and tissue collection**

130 *H. cunea* pupae were collected from a first-generation population at Baimao Town,

131 Jiujiang District, Wuhu City, Anhui province. Insect cages were used for rearing *H. cunea*

132 pupae at 25°C, 70-80% RH and 14L:10D hour photoperiod. After eclosion, adults were

133 provided with 1% honey water. In the fourth hour of the second dark period, antennae,

134 thoraxes, abdomens, legs, and wings of virgin males and females were dissected under

135 the microscope and pooled by sex and body part. Male and female pupae and fourth

136 instar larvae were also sampled. Five samples were taken for each body part with the

137 exception of antennae, of which 30 pairs were collected by pulling out from the base of

138 the antennae with tweezers. Dissected body parts or whole-body samples were flash

139 frozen in liquid nitrogen and stored at -80℃ until use.

140

Gene annotation

141

142 The *H. cunea* antennal transcriptome (PRJNA605323) (*Zhang et al., 2016*) was used as

143 a reference sequence for mapping clean reads for each tested sample. Gene annotation

144 was carried out using Nr (NCBI non-redundant protein sequences), Nt (NCBI nucleotide),

145 Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of

146 proteins/enKaryotic Ortholog Groups), Swiss-Prot (A manually annotated and reviewed

147 protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes) and

148 GO (Gene Ontology) databases (Fig. S1-4). Based on the results of gene annotation and

149 BLAST comparison, the candidate genes of HcunCXE were determined and named

150 according to the identification order from the antennal transcriptomic data.

151

152 **Homologous search and sequencing analysis of CXE genes in *H. cunea***

153 The *H. cunea* CXE genes were identified according to the BLAST results on NCBI. The

154 Open Reading Frame finder (OFR Finder) (https://www.ncbi.nlm.nih.gov/orffinder/) was

155 used to search for the open reading frame of these CXE genes. An ExPASy tool

156 (http://web.expasy.org/compute_pi/) (*Petersen et al., 2011*) was used to calculate their

157 theoretical isoelectric points (pI) and molecular weights (MW) of the full-length

158 HcunCXEs gene candidates, and SignalP-5.0

159 (https://services.healthtech.dtu.dk/service.php?SignalP) was used to predict signal

160 peptides of the CXE genes (*Petersen et al., 2011*).

161

Phylogenetic analysis of CXE genes in *H. cunea*

163 Genes related to the CXEs of *H. cunea* and other reported insects (*Seasamia inferens,*

164 *Spodoptera littoralis, Spodoptera exigua, Cnaphalocrocis medinalis, Bombyx mori,*

165 *Drosophila melanogaster, Tribolium castaneum, Mamestra brassicae* and *Antheraea*

166 *polyphemus*) were subjected to multi-sequence alignment with MAFFT (*Wong et al.,*

167 *2008*). Amino acid sequences were automatically aligned by the MAFFT program

168 version 7 (http://mafft. cbrc.jp/alignment/software/algorithms/algorithms.html), using L-

169 INS-i strategy (*Katoh & Standley, 2013*). The phylogenetic tree was constructed using

170 MEGA-X (*Tamura et al., 2011*) and maximum likelihood method (1000 bootstrap

171 repetitions) for systematic evolution analysis. The adopted model was LG-G+I, and all sites

172 were used for Gap/Missing Data Treatment. Lastly, the phylogenetic tree was edited on the

173 website iTOL (https://itol.embl.de/). The genes of insect ODEs required for the

174 phylogenetic tree are shown in Supplementary Table S1.

175

RNA extraction and synthesis of the first-strand cDNA

177 The sampled body tissues were ground using a Tissue-Tearor which rapidly

178 homogenized the samples in DEPC-treated sterile water. TRIzol reagent (Invitrogen, USA)

179 was used for extraction and purification of total RNA from each sample according to the

180 manufacturer's instructions. The degradation and contamination of RNA was monitored on

181   1% agarose gels, and purity was checked using a NanoPhotometer® spectrophotometer

182   (IMPLEN, CA, USA). First-stranded cDNA templates were synthesized using 1 µg of

183   RNA template with the PrimeScript™ RT reagent Kit according the manufacturer's

184   instructions (TaKaRa, Japan).

185

186   **RT-qPCR and RT-PCR analysis**

187   Expression profiles of the identified *H. cunea* CXE genes in different body parts of adults

188   and two other life stages were analyzed. Tissues included antenna of 30 adults of each

189   sex, legs of 5 adults of each sex, wings of 5 adults of each sex, thoraxes and abdomens

190   of 5 adults of each sex, 5 whole pupae of each sex and 5 larvae (fourth instar).

191   The RT-qPCR and RT-PCR assays were employed for production of multiple copies of

192   DNA. RT-qPCR reaction was conducted in a 25µL reaction mixture system containing 12.5µL of

193   SYBR® Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Japan), 1µL of each primer, 2µL of

194   sample cDNA, and 8.5µL of sterilized H_2O.

195   The RT-qPCR cycles were set at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec,

196   60°C for 30 sec. Each experiment was carried out in a CFX96 real-time PCR detection

197   instrument (Bio-rad, USA) using 8-strip PCR tubes (Bio-rad, USA). The reaction data were

198   recorded, and the dissolution curves were appended. Both Elongation factor-1 alpha (EF1-a) and

199   glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal reference. Three

200   biological replicates were performed, and the reproducibility confirmation of each RT-qPCR

201   reaction was replicated three times for each sample (Table S2) (*Xu et al., 2018*).

202   The variability of each gene expression in different body tissues was tested by using the Q-

203   Gene method (*Muller et al., 2002*; *Simon, 2003*). The relative expression of mRNA of each gene

204   (mean ± SD) was analyzed using one-way ANOVA (SPSS22.0 for Windows, IBM, USA),

205   followed by LSD and Duncan's tests at α = 0.05. GraphPad Prism v5.0 Software (GraphPad

206   Software Inc, CA, USA) was used for graphical plotting/mapping.

207   RT-PCR analysis was performed as follows: 94°C for 2 min of initiation, and 29 cycles of

208   94°C for 30 sec, 52°C for 30 sec, 72°C for 15 sec, and 2 min at 72°C for final extension.

209   Elongation factor-1 alpha (EF1-a) of *H. cunea* was used as an internal reference. In addition,

210   instead of template cDNA, RNase-free water was used as the blank control. The reaction mixture

211   contained 12.5μL of 2x Ex Taq MasterMix (CWBIO, China), 1μL of each primer, 1μL of sample

212   cDNA, and H$_2$O to bring the total to 25μL. A 10μL aliquot of each reaction product was used for

213   gel electrophoresis. The RT-PCR primer sequences of CXE genes in *H. cunea* are listed in

214   Supplementary Table S3.

215

216   **Results**

217   **Identification of CXE genes from *H. cunea***

218   Based on a comparative analysis of the *H. cunea* antennal transcriptome using BLASTX

219   databases (*Zhang et al., 2016*), a total of 10 HcunCXE genes were identified. BLASTX

220   comparison showed that these 10 HcunCXE genes have high homology with CXE genes

221   of *S. inferens*. Six HcunCXEs (HcunCXE1, HcunCXE3-5 and HcunCXE7-8) had

222   complete ORFs (Table 1). The molecular weights of these HcunCXEs ranged from 10.52

223   to 62.23 kDa (Table 2). Only HcunCXE7 and HcunCXE9 have predicted signal peptide

224   sites (Table 2).

225

226   **Phylogenetic analysis of *H. cunea* CXEs**

227   To evaluate the relationship of HcunCXEs with other insects' CXEs, a phylogenetic tree

228   was constructed (Fig. 1). The HcunCXEs genes could be divided into two subclasses:

229   extracellular gene subclass (generally secreted enzymes, substrates include hormone

230   and pheromones) and generally intracellular enzymes, dietary metabolism/ detoxification

231   functions (Fig. 1). Three HcunCXEs (HcunCXE1, 7 and 9) were clustered in the

232   generally secreted enzymes subclass. The other 7 HcunCXEs including HcunCXE2-6,

233   HcunCXE8 and HcunCXE10 fell into the intracellular gene subclass. In addition, the

234   clade of intracellular gene subclass formed by HcunCXEs was most closely related to

235   those formed by *S. inferens, C. medinalis, S. exigua* and *S. littoralis* CXEs. Sequence

236   alignments showed that the amino acid identities of HcunCXE1 and SinfCXE18,

237   HcunCXE9 and SinfCXE1, HcunCXE7 and SinfCXE13, HcunCXE7 and CmedCXE5

238   were 73.9%, 71.3%, 74.6% and 65%, respectively (Fig. S5). These results suggest that

239   the intracellular CXEs in *H. cunea* shared a more recent common ancestor with the

240 CXEs in *S. inferens, C. medinalis, S. exigua* and *S. littoralis* than with the CXEs in other

241 insect species.

242

243 **Tissue distribution of HcunCXEs**

244 We next examined the expression of HcunCXE genes in adult female and male

245 antennae, legs and wings using RT-qPCR with primers specific for each of the 10

246 HcunCXEs genes (Table S2). All HcunCXEs were expressed in the antennae (Fig. 2 and

247 Fig. S6). Among which, three HcunCXEs (HcunCXE4, 5, 8) were highly expressed in the

248 antennae (Fig. S6 C and D). Two HcunCXEs (HcunCXE1 and 3) were female-biased

249 (Fig. 2 A and C) and two HcunCXEs (HcunCXE 9 and 10) were male-biased (Fig. 2 I and

250 J); although the sex-biased expression is not statistically significant, there is a clear

251 numerical difference between expression level in the sexes. These results indicate that the

252 most abundant CXE genes in the antenna are not extracellular CXEs that likely participate in

253 volatile odorant degradation. The most abundant CXEs are likely involved in primary metabolic

254 activities and it would thus seem logical that their expression is much higher than for the other

255 specialized CXEs in the antenna. The other HcunCXEs, however, were equally expressed

256 in both sexes. Comparing expression across tissues, five HcunCXEs (2, 3, 5, 7 and 8)

257 were highly expressed in the legs and wings (Fig. S6 A and B). Expression of HcunCXE2

258 and HcunCXE7 was higher in the legs or wings than that in the antennae (Fig. 2 B and

259 G).

260   To investigate whether these HcunCXEs are also expressed in the other body parts

261   or life stages, a RT-PCR experiment was carried out using total RNA samples taken from

262   *H. cunea* adults and other life stages (pupae and larvae). Gel electrophoresis bands

263   were generated from HcunCXE2 products from the adult thoraxes and abdomens (Fig.

264   3). In addition, faint/light bands of HcunCXE7 and HcunCXE8 were detected in both

265   thoraxes and abdomens, as well as the pupae. Interestingly, nine out of 10 HcunCXEs

266   (HcunCXE1-5 and 7-10) were also detected in the larvae, indicating that HcunCXEs are

267   widely expressed in the larval stage.

268

269   **Discussion**

270   In the current study, 10 putative CXE genes were identified based on our previous *H.*

271   *cunea* antennal transcriptomic data (*Zhang et al., 2016*). All 10 *H. cunea* CXE genes

272   showed high homology to the CXE genes identified in *S. inferens* (identity ≥59%, Fig. 1

273   and Table 1). We speculated that some of these *H. cunea* CXE genes mainly degrade

274   sex pheromone components and host plant volatiles. Unlike many well-studied Type-I

275   sex pheromone-producing lepidopteran insects (>75% moth species), the *H. cunea* sex

276   pheromone is comprised of Type II pheromone components (*Ando & Inomata, 200*4). At

277   present, most of the published moth ODEs are from the Type I sex pheromone

278   producing lepidopterans; thus, our study represents the first report of ODE genes from a

279   Type II sex pheromone-producing moth species. *H. cunea* is an extremely polyphagous

280   species with high fecundity (several hundred eggs/female) and dispersal capacity. *H. cunea*

281   larvae are generalists, capable of feeding on over 170 species of host plants, including

282   many broad-leaved tree species. To cope with such diverse host plant species, this moth

283   must have developed a series of olfactory receptor neurons to recognize diverse plant

284   volatiles (*Zhang et al., 2016*). The number (n=10) of CXE genes we identified from *H.*

285   *cunea* was lower than those of other reported lepidopterans species: 19 in *Chilo*

286   *suppressalis*, 35 in the tea geometrid *Ectropis obliqua* Prout and 76 in *B. mori* (*Yu et al.,*

287   *2009*; *Liu et al., 2015*; *Sun et al., 2017*). These results suggest that *H. cunea* does not

288   seem to require more CXEs, since the other ODEs including CYP, AD, AOX and GST

289   are likely involved in odorant degradation in olfactory processes. On the other hand, the

290   difference in number of CXEs in various species might result from differences in sample

291   preparation and sequencing method/depth. In addition, the ecological/evolutionary

292   differences across species may also be a reason. Insects have to adapt to their external

293   environment; different environments lead to the formation of different physiological and

294   behavioral characteristics.

295   The phylogenetic tree analysis showed that HcunCXE1, 7 and 9 belong to the

296   extracellular gene subclass, including the secretory enzymes that likely act on hormones

297   and pheromones (Fig. 1). The remaining 7 CXE genes fell into the intracellular gene

298   subclass (Fig. 1), including intracellular enzymes that mostly play roles in dietary

299   metabolism and detoxification. Chertemps et al. (2012) demonstrated that an

300 extracellular CXE of *D. melanogaster*, esterase-6 (EST-6), is responsible in or related to

301 the sensory physiological and behavioral responses to its pheromone. A subsequent

302 study found that EST-6 was able to degrade various volatile esters in vitro and function

303 as expected for an ODE which plays a role in the response of the flies to esters

304 (*Chertemps et al., 2012*). Thus, these *H. cunea* CXE genes (HcunCXE2, 3, 4, 5, 6, 8 and

305 10) may also affect the mating and courtship competitions in *H. cunea* through

306 degradation of some ester kairomones or plant allelochemicals. On the other hand,

307 based on the omnivorous nature of *H. cunea* and its species-specific sex pheromone,

308 these CXE genes may be a unique category of *H. cunea* which degrade odor substances.

309   Antennal-specific or highly expressed esterases belong to the CXE type in the

310 carboxy/cholinesterases (CCEs) family. The first ODE was identified form *A.*

311 *polyphemus* (ApolSE) as an antenna-specific esterase, with a high ability to degrade the

312 acetate component (*E6Z*11-16: AC) of its pheromone blend (*Vogt & Riddiford, 1981*).

313 Since then, antennal-specific esterases have been cloned from *A. polyphemus* (*Ishida &*

314 *Leal, 2002*) and *Mamestra brassicae* Linnaeus (*Maïbèche-Coisne et al., 2004*). Recent

315 studies show that many insect CXEs are expressed specifically in antennae, and their

316 major functions in olfactory process are to degrade odor molecules. Interestingly, the

317 expression of some HcunCXEs in the legs and wings were found to be higher than those

318 in the antennae (HcunCXE2, 3 and 7). The ten *H. cunea* CXEs genes we identified

319 through the gene expression analysis had a low level of expression in different body

320   tissues of *H. cunea* adults (Fig. 2 and Fig. S6). However, they were widely expressed in

321   the larvae, which may be related to their extremely broad host plant range that needs

322   more CXEs to degrade large amount of carboxylic acid esters. Our quantitative PCR

323   results (Fig. 2 and Fig. S6) indicated that some HcunCXEs genes were highly expressed

324   in both male and female antennae. HcunCXE1 and HcunCXE9 belong to the same

325   subclass as ApolPDE and MbraCXE (Fig. 1). Previous studies have shown that

326   ApolPDE and MbraCXE function as pheromone degradation enzymes (*Maïbèche-*

327   *Coisne et al., 2004; Ishida and Leal, 2005*). These HcunCXEs are likely for degradation

328   of sex-pheromones and/or plant volatiles both from hosts or non-hosts. However, the

329   HcunCXEs genes that were highly expressed in the legs and wings might be related to

330   the degradation of non-volatile substances for contact signals. In addition, a previous

331   study of SexiCXE14 and SexiCXE15 (antennae-enriched carboxylesterase genes in

332   *Spodoptera exigua*) showed that antenna bias expression plays a role in the degradation

333   of volatile substances and sex pheromones in plants (*He et al., 2015*). However, the

334   expression of SexiCXE11 was much higher in abdomen and wings, and its activity in

335   hydrolyzing plant volatile substances was stronger than that in degrading ester sex

336   pheromones (*He et al., 2019*). In the current study, HcunCXE1, 3, 4, 5, 6, 8, 9, and 10

337   showed antenna-biased expression, while the expression of HcunCXE2 and 7 in legs

338   and wings was higher than that in antennae. These results suggested that HcunCXEs

339   have different functions and may participate in the degradation of host plant volatiles

340   and/or other xenobiotics.

341   CXEs play multiple key roles in the hydrolysis of carboxylic acids esters. CXEs also

342   include some metabolic enzymes that are associated with insecticide resistance (*Li,*

343   *Schuler & Berenbaum, 2007*). Many previous studies in insect CXEs focused on their

344   functions in mediating insecticide resistance (*Hemingway & Karunaratne, 1998*; *Li,*

345   *Schuler & Berenbaum, 2007*). In contrast, the mechanisms underlying degradation of

346   plant allelochemicals are still unclear. It has been shown that phenolic glycosides can

347   induce expression of *Papilio canadensis* CXEs (*Lindroth, 1989*). Moreover, in *Lymantria*

348   *dispar*, the activities of CXEs were positively correlated with the larval survival, indicating

349   that these esterases might be involved in the glycoside metabolism (*Lindroth, 1989*;

350   *Lindroth & Weisbrod, 1991*). In the current study, nine out of 10 HcunCXEs were

351   expressed in the larvae (Fig. 3), indicating that the activities of HcunCXEs may positively

352   correlate with survival of *H. cunea* larvae. Although the gene expression of HcunCXEs in

353   *H. cunea* midgut and some other tissues are still unknown, based on these previous

354   findings, it is reasonable to speculate that HcunCXEs might also play multiple functions

355   in *H. cunea* physiology and metabolism. In addition, a significant increase of CXE activity

356   in the midgut of *S. litura* was observed during uptake of the plant glycoside rutin

357   (*Ghumare, Mukherjee & Sharma, 1989*). The CXEs in *Sitobion avenae* have been

358   suggested to participate in gramine detoxification (*Cai et al., 2009*). Quercetinrutin and 2-

359   tridaconone were also found to induce the activities of CXEs in *Helicoverpa Armigera*

360   (*Gao et al., 1998*; *Mu, Pei & Gao, 2006*). Understanding the specific function of

361   HcunCXEs will require further analyses using in vitro and in vivo methods.

362   Little is known about *H. cunea* olfaction mechanisms at the molecular level,

363   especially concerning how CXEs degrade various semiochemicals in its chemical

364   communication system. Further research is needed to 1) understand the functions of

365   antennal-specific CXEs in *H. cunea* via cloning, expression and purification of these

366   CXEs and enzymatic kinetic analysis; 2) determine the locations/distributions of related

367   CXEs by *in-situ* hybridization; 3) evaluate the potential correlations between CXE

368   transcription levels and their corresponding electrophysiological and behavioral

369   responses by silencing CXEs via RNA interference (*Caplen, 2004*), and 4) ultimately

370   discover the mode of action or functionality of CXEs in the olfactory signal conduction

371   (signal inactivation).

372

373   **Conclusions**

374   In summary, we identified 10 CXE genes in *H. cunea* by analyzing its antennal

375   transcriptomic data. These HcunCXEs displayed an antennae-or leg/wing-biased

376   expression. The ubiquitous expression of these HcunCXEs in different tissues and life

377   stages suggest that they have multiple roles, *i.e*, degradation of odor molecules,

378   metabolism and detoxification of dietary and environmental xenobiotics. Our findings

379 provide a theoretical basis for further studies on the olfactory mechanism of *H. cunea*

380 and offer some new insights into functions and evolutionary characteristics of CXEs in

381 lepidopteran insects. From a practical point of view, these HcunCXEs might represent

382 meaningful targets for developing behavioral interference control strategies against *H.*

383 *cunea.*

384

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389

390 **Competing Interests**

391 Dr. Qing-He Zhang is an employee of Sterling International, Inc., Spokane, WA, USA.

392

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601

602

603   **Figure legends**

604   **Figure 1 Molecular phylogeny comparing HcunCXEs with CXEs from other insect**

605   **species.** 10 CEXs (HcunCXE1-10) from *H. cunea* (Hcun) and CXEs from *S. exigua*

606   (Sexi), *C. medinalis* (Cmed), *B. mori* (Bmor), *D. melanogaster* (Dmel), *T. castaneum*

607   (Tcas), *S. inferens* (Sinf), *S. littoralis* (Slit) were used to construct the phylogenetic tree.

608   The phylogenetic tree was aligned by MAFFT, and constructed by MEGA-X using

609   maximum likelihood method. The adopted model is LG-G+I, and the model value is

610   shown in table S4 of additional materials. 1000 bootstraps were used to create the tree.

611   A: Extracellular gene subclass (Generally secreted enzymes, substrates include

612   hormone and pheromones); B: Generally intracellular enzymes, dietary metabolism/

613   detoxification functions; C: juvenile hormone esterase (JHE); D: Nerouligins; E:

614   acetylcholinesterases (AChE).

615

616   **Figure 2 Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues.** (A-J)

617   HcunCXEs (HcunCXE1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). FA, female antennae; MA, male

618   antennae; L, legs; W, wings. The relative mRNA levels were normalized to those of the

619   *EF1-a* gene and analyzed using the Q-gene method. All values are shown as the mean ±

620   SEM. The data were analyzed by the least significant difference (LSD) test after one-way

621   analysis of variance (ANOVA). Different letters indicate significant differences between

622   means ($P < 0.05$).

623

624   **Figure 3 RT-PCR analysis of HcunCXEs gene expression in tissues taken from *H.***

625   ***cunea* adults and other life stages.** EF1-a was used as an internal control; NC,

626   negative control with no template in the reaction.

627

628

629 **Figure. S1 Homology analysis of *H. cunea* unigenes.** (A) E-value distribution. (B)

630 Similarity distribution. (C) Species distribution. All unigenes that had BLASTX

631 annotations within the NCBI nr database with a cutoff *E*-value of 10^{-5} were analyzed.

632 The first hit of each sequence was used for analysis.

633

634 **Figure. S2 Gene ontology (GO) assignment of *H. cunea* unigenes.** The GO

635 classification map was done by uploading the GO ID numbers of genes for their

636 involvement in biological processes, cellular components, and molecular functions.

637

638 **Figure. S3 Clusters of Orthologous Groups (KOG) classification of *H. cunea*.** The

639 letters along the *x*-axis represents the name of 26 groups of KOG, and the y-axis is the

640 percentage of the number of genes annotated to the group to the total number of genes

641 annotated.

642

643 **Figure. S4 Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of *H.***

644 ***cunea* unigene.** The *x*-axis indicates the percentage of annotated genes, and the y-axis

645 indicates the KEGG categories. The capital letters against the colored bars indicate five

646 main categories: (A) cellular processes, (B) environmental information processing, (C)

647 genetic information processing, (D) metabolism, and (E) organism systems.

648

649   **Figure S5. Comparison of the amino acid sequences of HcunCXEs with CXEs**

650   **proteins from different species.** A, HcunCXE1 with SinfCXE18; B, HcunCXE9 and

651   SinfCXE1; C, HcunCXE7 with SinfCXE13 and CmedCXE5. The percentages on the right

652   represent the amino acid identities.

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654   **Figure S6. Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues.** The

655   relative mRNA levels were normalized to those of the *EF1-a* gene and analyzed using

656   the Q-gene method. All values are shown as the mean ± SEM. The data were analyzed

657   by the least significant difference (LSD) test after one-way analysis of variance (ANOVA).

658   Different letters indicate significant differences between means ($P < 0.05$). FA, female

659   antennae; MA, male antennae; L, legs; W, wings.

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Figure 1

Molecular phylogeny comparing HcunCXEs with CXEs from other insect species.

10 CEXs (HcunCXE1-10) from *H. cunea* (Hcun) and CXEs from *S. exigua* (Sexi), *C. medinalis* (Cmed), *B. mori* (Bmor), *D. melanogaster* (Dmel), *T. castaneum* (Tcas), *S. inferens* (Sinf), *S. littoralis* (Slit) were used to construct the phylogenetic tree. The phylogenetic tree was aligned by MAFFT, and constructed by MEGA-X using maximum likelihood method. The adopted model is LG-G+I, and the model value is shown in table 4 of additional materials. The Bootstrap value of this tree is 1000, which is to integrate the branch length tree with the Bootstrap value tree and then beautify it. A: Extracellular gene subclass (Generally secreted enzymes, substrates include hormone and pheromones); B: Generally intracellular enzymes, dietary metabolism/ detoxification functions; C: juvenile hormone esterase (JHE); D: Nerouligins; E: acetylcholinesterases (AChE).

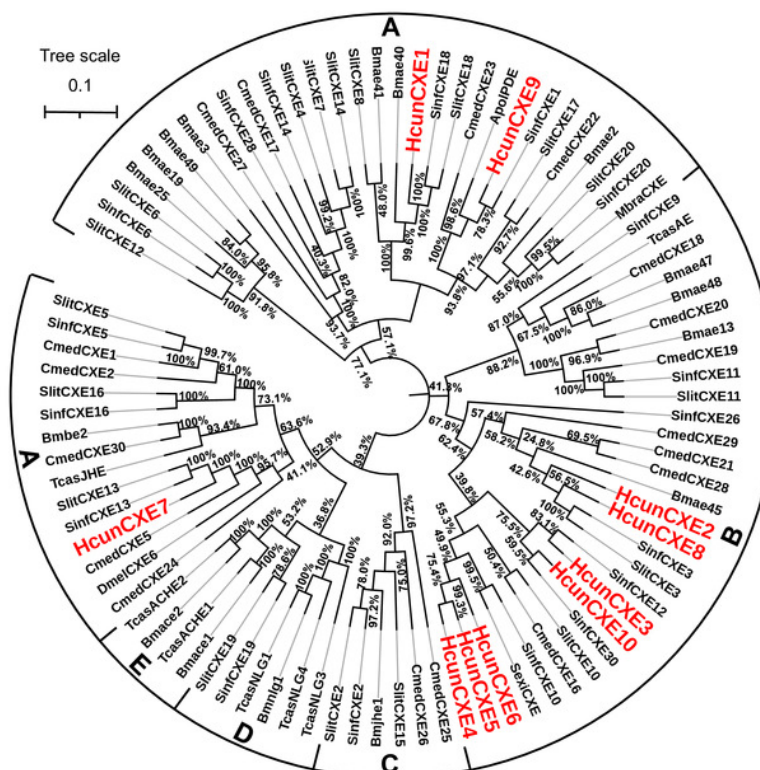



Figure 2

Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues.

(A-J) HcunCXEs (HcunCXE1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). FA, female antennae; MA, male antennae; L, legs; W, wings. The relative mRNA levels were normalized to those of the *EF1-a* gene and analyzed using the Q-gene method. All values are shown as the mean ± SEM. The data were analyzed by the least significant difference (LSD) test after one-way analysis of variance (ANOVA). Different letters indicate significant differences between means (*P* < 0.05).

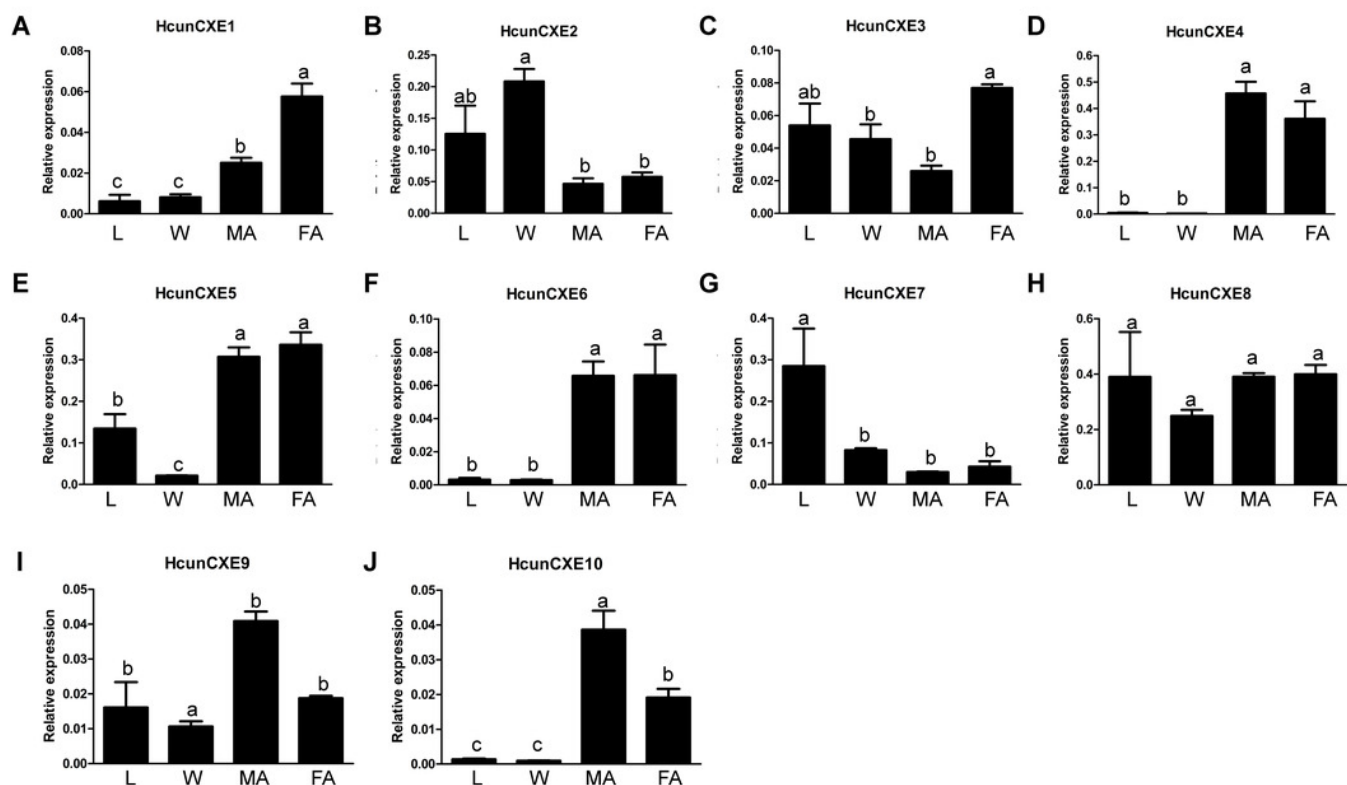
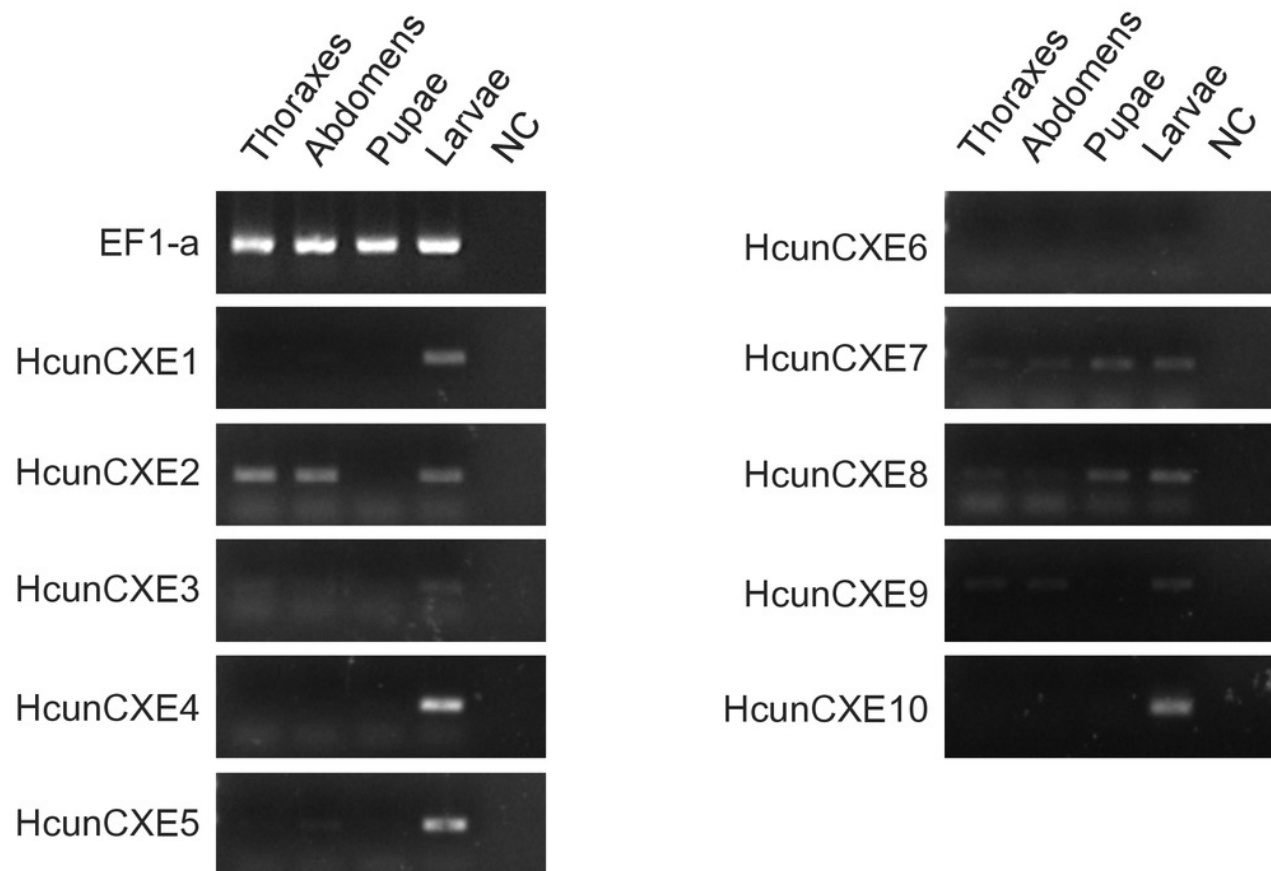



Figure 3

RT-PCR analysis of HcunCXEs gene expression in tissues taken from *H. cunea* adults and other life stages.

EF1-a was used as an internal control; NC, negative control with no template in the reaction.

Gene name, information of open reading frame and Blastx match of the 10 putative
HcunCXEs identified in this study.

Note: ORF, open reading frame . *S. inferens, Sesamia inferens.*

2 **Table 1:**

3 **Gene name, information of open reading frame and Blastx match of the 10 putative**

4 **HcunCXEs identified in this study.**

Gene Name	ORF Length (bp)	Complete ORF	FPKM value	Best Blastx Match			
				Species	Acc.number	E - value	Identity (%)
HcunCXE1	1668	YES	4.9	*S. inferens*	AII21990.1	0.0	73
HcunCXE2	777	NO	3.77	*S. inferens*	AII21980.1	3e-135	73
HcunCXE3	375	YES	3.26	*S. inferens*	AII21980.1	2e-105	60
HcunCXE4	1389	YES	61.01	*S. inferens*	AII21984.1	0.0	59
HcunCXE5	1593	YES	143.14	*S. inferens*	AII21984.1	0.0	62
HcunCXE6	1161	NO	17.04	*S. inferens*	AII21984.1	4e-174	62
HcunCXE7	1677	YES	13.18	*S. inferens*	AII21987.1	0.0	75
HcunCXE8	1608	YES	12.64	*S. inferens*	AII21980.1	0.0	66
HcunCXE9	1653	YES	6.13	*S.inferens*	AII21978.1	0.0	71
HcunCXE10	273	NO	21.32	*S. inferens*	AII21984.1	8e-39	64

5 Note: ORF, open reading frame. *S. inferens, Sesamia inferens.*

Gene name and characteristics including molecular weight, isoelectric point and signal peptide of the 10 putative HcunCXEs with open reading frames.

Note: SP, signal peptide; pI, isoelectric point; MW, Molecular weight.

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Table 2:
Gene name and characteristics including
molecular weight, isoelectric point and
signal peptide of the 10 putative
HcunCXEs with open reading frames.

Gene Name	MW (Kda)	PI	SP
HcunCXE1	62.23	7.56	NO
HcunCXE2	28.44	5.67	NO
HcunCXE3	13.98	4.85	NO
HcunCXE4	52.2	5.31	NO
HcunCXE5	59.52	5.41	NO
HcunCXE6	43.17	5.09	NO
HcunCXE7	61.71	6.32	1-17
HcunCXE8	60.68	5.75	NO
HcunCXE9	62.18	8	1-16
HcunCXE10	10.52	8.89	NO

Note: SP, signal peptide; pI, isoelectric point; MW, Molecular weight.