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Identification of Putative Carboxylesterase and Glutathione *S*-transferase Genes from the Antennae of the *Chilo suppressalis* (Lepidoptera: Pyralidae)

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ABSTRACT. In insects, rapid degradation of odorants in antennae is extremely important for the sensitivity of olfactory receptor neurons. Odorant degradation in insect antennae is mediated by multiple enzymes, especially the carboxylesterases (CXEs) and glutathione *S*-transferases (GSTs). The Asiatic rice borer, *Chilo suppressalis*, is an economically important lepidopteran pest which causes great economic damage to cultivated rice crops in many Asian countries. In this study, we identified 19 putative *CXE* and 16 *GST* genes by analyzing previously constructed antennal transcriptomes of *C. suppressalis*. BLASTX best hit results showed that these genes are most homologous to their respective orthologs in other lepidopteran species. Phylogenetic analyses revealed that these *CXE* and *GST* genes were clustered into various clades. Reverse-transcription quantitative polymerase chain reaction assays showed that three *CXE* genes (*CsupCXE8*, *CsupCXE13*, and *CsupCXE18*) are antennae-enriched. These genes are candidates for involvement in odorant degradation. Unexpectedly, none of the *GST* genes were found to be antennae-specific. Our results pave the way for future researches of the odorant degradation mechanism of *C. suppressalis* at the molecular level.

Key Words: *Chilo suppressalis*, odorant-degrading enzyme, phylogenetic analysis, expression pattern

Carboxylesterases (CXEs) and glutathione *S*-transferases (GSTs) are two major families of metabolic enzymes widely distributed in animals, plants, insects, and microbes (Hayes et al. 2005, Oakeshott et al. 2005). In insects, CXEs and GSTs have a broad range of functions. They play essential roles not only in the metabolism of a variety of physiologically important endogenous compounds, but also in the detoxification of a diversity of harmful exogenous compounds, such as plant allelochemicals and insecticides, leading to rapid adaptation of insects to host plants and insecticide resistance (Enayati et al. 2005, Li et al. 2007).

Apart from the functions of metabolism and detoxification of endobiotics and xenobiotics, insect CXEs and GSTs also play critical roles in the olfaction that maintains the sensitivity of the olfactory receptor neurons (ORNs) by rapidly degrading stray odorants and protect the vulnerable ORNs against harmful volatile xenobiotics (Leal 2013). These were also called odorant-degrading enzymes (ODEs) (Vogt 2003). To date, an ever-growing number of genes encoding CXEs and GSTs have been identified in the antennae of various insect species, including *Antheraea polyphemus* (Vogt and Riddiford 1981, Vogt et al. 1985, Ishida and Leal 2005), *Manduca sexta* (Rogers et al. 1999), *Mamestra brassicae* (Maïbèche-Coisne et al. 2004), *Sesamia nonagrioides* (Merlin et al. 2007), *Popillia japonica* (Ishida and Leal 2008), *Epiphyas postvittana* (Jordan et al. 2008), *Spodoptera littoralis* (Merlin et al. 2007, Durand et al. 2010a), *Agrilus planipennis* (Mamidala et al. 2013), *Spodoptera litura* (He et al. 2014a), *Spodoptera exigua* (He et al. 2014a,b,c; He et al. 2015), *Bombyx mori* (Tan et al. 2014), and *Drosophila melanogaster* (Chertemps et al. 2012, Younus et al. 2014), and their functions in olfaction were investigated. For example, the first characterized *A. polyphemus* pheromone-degrading enzyme (ApolPDE) is a CXE. This enzyme could effectively degrade the acetate component of the sex pheromone in a series of in vitro studies (Vogt and Riddiford 1981, Vogt et al. 1985, Ishida and Leal 2005). Other studies also revealed that the purified native or recombinant antennal CXEs from *P. japonica* and *D. melanogaster* could degrade sex pheromone constituents (Ishida and Leal 2008, Younus et al. 2014). Furthermore, antennal CXEs from *Sp. littoralis*, *Sp. exigua* and *Sp. litura* seem involved not only in the degradation of sex pheromones but also plant volatiles (Durand et al. 2010b, 2011; He et al. 2014a,c). GSTs have also been implicated in odorant degradation. For instance, in *M. sexta*, a GST (GST-msolf1) is localized in the pheromone-sensitive sensilla and inactivates volatile plant aldehydes (Rogers et al. 1999). In *B. mori*, a GST (BmGSTD4) is specifically distributed in the sensillum lymph of male antennae, indicating the enzyme might be involved in pheromone degradation (Tan et al. 2014).

To date, benefiting from expressed sequence tag (EST) and transcriptome sequencing approaches, more and more odorant-processing genes from insect antennae have become recognized (Grosse-Wilde et al. 2011, Gu et al. 2011, Leal et al. 2013, Cao et al. 2014, Gong et al. 2015). However, these studies mainly focused on binding proteins (including odorant-binding proteins and chemosensory proteins) and chemoreceptors (including olfactory receptors and ionotropic receptors) and identification of genes implicated in odorant degradation has not yet been performed. So far, the *CXE* and *GST* repertoires have only been identified in the antennae of five insect species: *E. postvittana* (Jordan et al. 2008), *Sp. littoralis* (Legeai et al. 2011), *S. nonagrioides* (Glaser et al. 2013), *Ag. planipennis* (Mamidala et al. 2013), and *D. melanogaster* (Younus et al. 2014). Consequently, additional insect species need to be investigated.

The Asiatic rice borer, *Chilo suppressalis* (Lepidoptera: Pyralidae), is a severe lepidopteran rice pest in many Asian countries, causing great economic losses to rice crops (Su et al. 2014). The establishment of two antennal transcriptomes of *C. suppressalis* by Cao et al. (2014) have provided a great opportunity for systematically identifying antennal *CXE* and *GST* genes and assessing their roles in moth species. In this article, we report: (1) identification and sequence analyses of 19 *CXE* and 16 *GST* genes from the *C. suppressalis* antennal transcriptome; (2) phylogenetic analyses of these genes; (3) investigation of tissue-specific expression patterns of these genes. We found three *CXE* genes were specifically expressed in the antennae of *C. suppressalis*. These genes are excellent candidates for involvement in odorant degradation.

Materials and Methods

Insect Rearing and Tissue Collection. The *C. suppressalis* colony used in this study originated from a field population collected in an experimental plot of Anhui Agricultural University, Hefei, Anhui, China in June, 2014. Larvae were reared on rice variety Taichung Native 1 (TN1, susceptible to almost all herbivores of rice) in an insectary at 26 ± 1°C, 75% relative humidity under a 16:8 (L:D) h photoperiod. Adults were provided with 10% (weight/volume) honey solution on sterile cotton swabs. Male antennae, female antennae, abdomens, and legs were dissected from 3-d-old unmated adults, immediately frozen in liquid nitrogen, and stored at −80°C before use.

Homology Searches. Previously, two antennal transcriptome datasets of *C. suppressalis* were released (NCBI accession numbers: SRX497236 and SRX497239, Cao et al. 2014). Here, we use the basic local alignment search tool (BLAST, Altschul et al. 1997) to identify putative *CXE* and *GST* genes from the two datasets. The annotated CXE and GST protein sequences from representative insect species, such as *D. melanogaster*, *B. mori* and *Sp. littoralis*, were retrieved from GenBank and used as queries for TBLASTN searches. The cutoff E-value of TBLASTN algorithm was set as 1×10^{-5}.

Bioinformatic Analyses. The open reading frames (ORFs) of genes were predicted using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Theoretical isoelectric points and molecular weights of deduced proteins were calculated using an ExPASy tool (http://web.expasy.org/compute_pi/). Signal peptide and transmembrane domain were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Homology searches were performed using BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi). Catalytic residues were predicted by searching the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml). The protein sequences of CXEs and GSTs from *C. suppressalis* and other insect species were aligned using Clustal Omega (http://www.ebi.ac.uk/tools/msa/clustalo/). Phylogenetic trees were constructed by MEGA 5.05 software using the neighbor-joining method with 1,000 bootstrap replications (Tamura et al. 2011). The GenBank accession numbers of sequences used are listed in Supp Table 1 (online only).

RNA Isolation and cDNA Synthesis. Total RNA was isolated from different tissues (male antennae, female antennae, abdomens, and legs) of *C. suppressalis* adults using RNAiso Plus (TaKaRa, Dalian, China) following the manufacturer's protocol. Each RNA sample was treated with the RNase-free DNase I (TaKaRa, Dalian, China) to eliminate potential contamination of genome DNA. RNA integrity was confirmed by gel electrophoresis and RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Each RNA sample was reverse-transcribed to first-strand cDNA using the PrimeScript First Strand cDNA Synthesis Kit (Takara, Dalian, China).

Reverse-Transcription Quantitative Polymerase Chain Reaction. Primer pairs for quantitative polymerase chain reaction (qPCR) were designed using BatchPrimer3 (http://probes.pw.usda.gov/batchprimer3/) and are listed in Supp Table 2 (online only). The housekeepers *actin A1* and ribosomal protein 49 (*rp49*) were used as reference genes (Teng et al. 2012). qPCR assays were performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). 20 µl reaction volumes contained 10 µl iTaq Universal SYBR Green Supermix, 0.4 µl (0.2 µM) of each primer, 1 µl (10 ng) cDNA template, and 8.2 µl nuclease-free water. qPCRs were run on a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA) with the following parameters: one cycle of 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 25 s. To confirm that only one single gene was detected by fluorescence dye, a heat-dissociation protocol was added at the end of thermal cycle, and the amplified products were analyzed by gel electrophoresis. In addition, each product was sequenced to verify the amplification of correct target. A no-template control and a no-reverse transcriptase control were both included on each reaction plate to detect possible contamination. Three independent biological replicates were performed, approximately 150 male antennae, 150 female antennae, 30 abdomens (15 from male and 15 from female, pooled together), and 240 legs (120 from male and 120 from female, pooled together) were used in each replicate. These replicates were tested on multiple reaction plates and on each plate two reference genes (*actin A1* and *rp49*) were all included.

Quantification of gene expression was conducted according to a modified version of the ΔCq method (Pfaffl 2001; Hagström et al. 2014). The steps are briefly described as follows: the efficiency (E) of a primer pair in a sample was determined from the slope of the log-linear portion of the calibration curve: $E = 10^{[-1/\text{slope}]}$. Then the formula

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{Cq}_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{ref}})^{\Delta \text{Cq}_{\text{ref}}(\text{control}-\text{sample})}}$$

was used to calculate the relative expression ratio for a particular gene in a sample versus a control in comparison to reference genes. In the formula, E_{target} is the efficiency of target gene transcript; E_{ref} is the efficiency of reference gene transcript; $\Delta \text{Cq}_{\text{target}}$ is the Cq deviation of control–sample of the target gene transcript; and $\Delta \text{Cq}_{\text{ref}}$ is the Cq deviation of control–sample of the reference gene transcript. Because two reference genes (*actin A1* and *rp49*) were used in qPCR, here $(E_{\text{ref}})^{\Delta \text{Cq}_{\text{ref}}}$ is the average value for *actin A1* and *rp49* in each sample.

Data Statistics. Statistical analyses of data were carried out using Data Processing System (DPS) software v9.5 (Tang and Zhang 2013). The relative expression levels from three independent biological replicates were log-transformed and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test. The level of significance was set at $P < 0.05$.

Results and Discussion

Identification of CXE Genes from *C. suppressalis* Antennae. So far, genome-wide analyses of *CXE* genes have been performed for model insect species, such as *D. melanogaster*, *Anopheles gambiae*, *B. mori*, *Apis mellifera*, *Nasonia vitripennis*, and *Tribolium castaneum*. From these 35, 51, 76, 24, 41, and 49 *CXE* genes were identified, respectively (Yu et al. 2009, Oakeshott et al. 2010). However, researches on the large-scale identification of the *CXE* gene families in insect antennae are limited. In this study, a total of 19 sequences with high identities and low E-values to *CXE* genes from known insects were identified from the antennal transcriptome of *C. suppressalis* (Table 1, Supp Fig. 1 [online only]). These sequences were named as *CsupCXE1* to *CsupCXE19* (Table 1). Of these, 18 sequences had full-length ORFs while one sequence (*CsupCXE8*) was an incomplete cDNA with truncations in both 5′- and 3′-regions. The GenBank accession numbers and lengths of all the 19 *CsupCXE* genes are presented in Table 1. The lengths of the 18 deduced full-length CsupCXE proteins ranged from 524 to 709 amino acid residues. These had predicted theoretical isoelectric points ranging from pH 4.66 to 8.28, and predicted molecular weights ranged from 59.61 to 78.36 kDa (Table 1). The numbers of antennal *CXE* genes in *C. suppressalis* are more than those obtained from *E. postvittana* (five genes, Jordan et al. 2008), but less than those from *Sp. littoralis* (20 genes, Durand et al. 2010a), *D. melanogaster* (26 genes, Younus et al. 2014), and *Ag. planipennis* (56 genes, Mamidala et al. 2013). There is a possibility that other *CsupCXE* genes may have been undetected by this transcriptomic approach but still be expressed in the antennae.

N-terminal signal peptides were predicted for 11 of the 18 CsupCXE protein sequences, except for CsupCXE8 due to its incomplete N-terminal region (Table 1). The presence of a signal peptide indicated that the corresponding enzyme could be secreted. BLASTX best hits in GenBank are all sequences from Lepidopteran species with sequence identities from 52 to 73% and E-values from 0.0 to 3×10^{-169} (Table 1). Multiple sequence alignments showed that all the

Table 1. Key genetic features of the *Chilo suppressalis* antennal CXEs

Gene name	GenBank ID	ORF (aa)	SP	pI	Mw (kDa)	BLASTX best hit			
						Gene name and species	GenBank ID	Identity (%)	E-value
CsupCXE1	KP938884	583	Yes	5.31	66.07	putative odorant-degrading enzyme [*Antheraea polyphemus*]	AAM14415	61	0.0
CsupCXE2	KP938886	577	Yes	6.58	63.37	juvenile hormone esterase precursor [*Manduca sexta*]	AAG42021	55	0.0
CsupCXE3	KP938882	524	Yes	6.59	59.73	carboxylesterase [*Cnaphalocrocis medinalis*]	AJN91194	55	0.0
CsupCXE4	KP938875	531	No	5.38	60.41	esterase B1-like [*Bombyx mori*]	XP_004926539	61	0.0
CsupCXE5	KP938876	526	No	5.91	60.41	esterase FE4-like [*B. mori*]	XP_004933870	52	0.0
CsupCXE6	KP938877	709	No	4.66	78.36	carboxyl/choline esterase CCE014a [*Helicoverpa armigera*]	ADF43475	64	0.0
CsupCXE7	KP938878	578	No	8.28	66.08	carboxylesterase [*Cn. medinalis*]	AJN91204	59	0.0
CsupCXE8	KP938879	>485	–	–	–	antennal esterase CXE17 [*Danaus plexippus*]	EHJ64436	53	3e-169
CsupCXE9	KP938880	561	Yes	5.49	62.52	carboxylesterase [*Cn. medinalis*]	AJN91198	58	0.0
CsupCXE10	KP938872	544	No	4.95	61.45	carboxylesterase-like [*B. mori*]	XP_004925500	55	0.0
CsupCXE11	KP938874	533	No	5.58	59.61	antennal esterase CXE11 [*Spodoptera littoralis*]	ACV60238	61	0.0
CsupCXE12	KP938883	527	Yes	7.16	59.91	carboxylesterase [*Cn. medinalis*]	AJN91194	54	0.0
CsupCXE13	KP938887	557	Yes	6.87	60.91	carboxylesterase [*Cn. medinalis*]	AJN91196	73	0.0
CsupCXE14	KP938889	552	Yes	6.11	62.38	carboxylesterase [*Cn. medinalis*]	AJN91201	60	0.0
CsupCXE15	KP938873	535	No	5.91	59.98	carboxylesterase 6 [*Plutella xylostella*]	ADX30519	61	0.0
CsupCXE16	KP938881	559	Yes	6.68	62.11	carboxylesterase [*Cn. medinalis*]	AJN91193	70	0.0
CsupCXE17	KP938888	551	Yes	6.48	62.18	antennal esterase CXE17 [*Da. plexippus*]	EHJ64436	54	0.0
CsupCXE18	KP938885	548	Yes	6.25	62.25	carboxylesterase [*Cn. medinalis*]	AJN91199	71	0.0
CsupCXE19	KP938890	561	Yes	6.86	63.24	carboxylesterase [*Cn. medinalis*]	AJN91192	66	0.0

19 CsupCXE proteins, included the truncated CsupCXE8, displayed a conserved sequence motif, such as the conserved pentapeptide Gly-X-Ser-X-Gly and oxyanion hole residues (Gly, Gly and Ala), although these residues in some sequences were variable (Table 2). These residues are essential for the enzymatic activity of CXE proteins (Oakeshott et al. 2005). In addition to the serine residues, all the CsupCXEs possess the conserved glutamate and histidine residues of the catalytic triad (Table 2) and therefore might be active enzymes.

Phylogenetic Analysis of Insect CXEs. Based on the sequence similarity and substrate specificity, insect CXEs can be divided into three major clades: intracellular catalytic, secreted catalytic, and neurodevelopmental. They can also be subdivided into eight classes: α-esterases, β-esterases, juvenile hormone esterases (JHEs), acetylcholinesterases (AChEs), gliotactins, neurotactins, neuroligins, and glutactins (Oakeshott et al. 2005). Here, we performed a phylogenetic analysis for the 19 antennal CXEs from *C. suppressalis* together with CXEs from *D. melanogaster*, *Ap. mellifera*, *Sp. Littoralis*, and some other related species (Fig. 1). The topology of the neighbor-joining tree was very similar to those obtained in previous studies (Yu et al. 2009, Durand et al. 2010a), and clearly some of the CXEs are the putative orthologs, such as CsupCXE11, SlitCXE11 and SnonCXE11, and CsupCXE13, ApolPDE, SlitCXE13, SnonCXE13 and SexiCXE13 (Fig. 1).

In this tree, insect CXEs can be divided into eight different clades (Fig. 1). Of these, eight CsupCXEs (CsupCXE1, 3, 8, 9, 12, 14, 17, and 18) with signal peptides were clustered into a clade together with moth α-esterases and antennal esterases: the former are well known for their detoxification functions to degrade insecticides (Li et al. 2007). In some insects, α-esterases are associated with resistance to organophosphorus (OP) insecticides (Jackson et al. 2013, Li et al. 2013). CsupCXE1 fell into a branch together with the *A. polyphemus* ODE (ApolODE, Ishida and Leal 2002) and *M. brassicae* odorant-degrading esterase (MbraEST, Maïbèche-Coisne et al. 2004). The two CXEs are specifically expressed in antennae and possibly involved in odorant degradation. CsupCXE14 fell into a branch together with the SexiCXE4 and SexiCXE14 of *Sp. exigua* (Fig. 1). The two enzymes have function in the degradation of plant volatiles and sex pheromones (He et al. 2014b,c). The SlitCXE7 of *Sp. littoralis* also fell into this branch (Fig. 1). The gene encodes an enzyme which can efficiently hydrolyze pheromone compounds as well as plant volatiles (Durand et al. 2011).

CsupCXE16 and CsupCXE19 were clustered into the 'integumental esterases' clade (Fig. 1). This clade includes the *A. polyphemus* integumental esterase (ApolIE, Ishida and Leal 2002). ApolIE is expressed throughout the body of adult males and females, and may be involved in the clearance of pheromones from the integument (Vogt and Riddiford 1986). CsupCXE13 was clustered into the 'β-esterases and pheromone-degrading esterases' clade (Fig. 1). This clade includes a well-characterized pheromone-degrading enzyme of *A. polyphemus* (ApolPDE). The enzyme is male antennae-specific and involved in the rapid inactivation of sex pheromones (Ishida and Leal 2005).

Seven CsupCXEs (CsupCXE4, 5, 6, 7, 10, 11, and 15) lack signal peptides were clustered into the 'α-esterases' clade (Fig. 1). In this clade, CsupCXE10 fell into a branch together with two well-characterized ODEs: SlitCXE10 and SexiCXE10 (Durand et al. 2010b; He et al. 2015), suggested these CXEs are orthologues and may have similar functions in odorant degradation. CsupCXE2 fell into a clade together with lepidopteran JHEs (Fig. 1). JHEs have critical roles in regulating larval to adult transition in insects and other arthropods (Oakeshott et al. 2005). The phylogenetic diversity of these *C. suppressalis* CXEs suggested that they might play different roles in the metabolism of odorants, endobiotics or xenobiotics.

Identification of Antennal GSTs from *C. suppressalis*. To date, genome-wide identification of *GST* genes has been performed for several model insect species, such as *D. melanogaster*, *An. gambiae*, *B. mori*, *Ap. mellifera*, *N. vitripennis*, and *T. castaneum*, from these 37, 28,

Table 2. Catalytic motifs of the *C. suppressalis* CXEs

Gene name	GxSxG version	E	H	Oxyanion hole version
CsupCXE1	GESAG	+	+	GGA
CsupCXE2	GQSAG	+	+	GGA
CsupCXE3	GESYG	+	+	GGY
CsupCXE4	GCSAG	+	+	GGA
CsupCXE5	GESAG	+	+	GGA
CsupCXE6	GESGG	+	+	GGG
CsupCXE7	GESAG	+	+	GGA
CsupCXE8	GESAG	+	+	GGA
CsupCXE9	GYSAG	+	+	GGA
CsupCXE10	GESAG	+	+	GGA
CsupCXE11	GFSAG	+	+	GGA
CsupCXE12	GESYG	+	+	GGY
CsupCXE13	GCSAG	+	+	GGA
CsupCXE14	GYSAG	+	+	GGA
CsupCXE15	GESAG	+	+	GGA
CsupCXE16	GCSAG	+	+	SGA
CsupCXE17	GESAG	+	+	GGA
CsupCXE18	GESAG	+	+	GGA
CsupCXE19	GCSAG	+	+	PGA

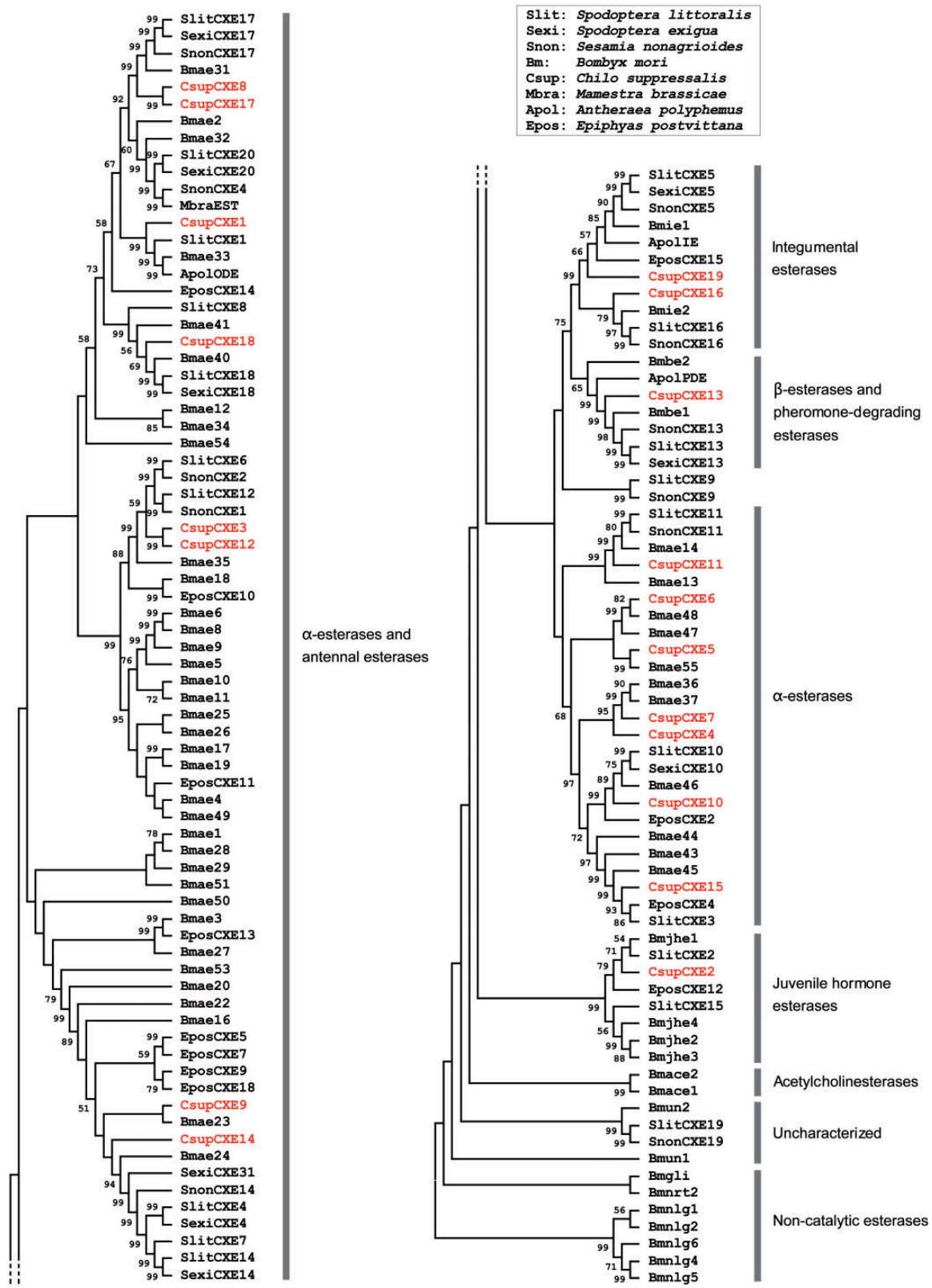

Fig. 1. Phylogenetic analysis of CXEs from various insect species including *Antheraea polyphemus*, *Bombyx mori*, *Chilo suppressalis*, *Epiphyas postvittana*, *Mamestra brassicae*, *Spodoptera exigua*, *Spodoptera littoralis*, and *Sesamia nonagrioides*. Bootstrap values above 50% are shown for each node. The *C. suppressalis* CXEs are highlighted in red.

23, 8, 19, and 35 *GST* genes were isolated, respectively (Yu et al. 2008, Oakeshott et al. 2010). In this study, we identified a total of 16 cDNA fragments encoding putative GSTs from the *C. suppressalis* antennal transcriptome (Table 3, Supp Fig. 1 [online only]). Of these, 14 sequences had complete ORFs while two sequences (*CsupGSTd4* and *CsupGSTe2*) were incomplete cDNAs with truncated 3′-regions (Table 3). These *C. suppressalis* antennal *GST* genes were named *CsupGSTd1* to *CsupGSTu1* according to the classification system and

Table 3. Clade and genomic data for the *C. suppressalis* antennal GSTs

Clade	Gene name	GenBank ID	ORF(aa)	pI	Mw(kDa)	BLASTX best hit			
						Gene name and species	GenBank ID	Identity (%)	E-value
Delta	*CsupGSTd1*	KP938857	246	6.21	27.88	glutathione S-transferase [*Amyelois transitella*]	ACX47897	73	1e-132
	CsupGSTd2	KP938858	218	6.91	24.30	glutathione S-transferase [*Antheraea pernyi*]	ACB36909	81	1e-129
	CsupGSTd3	KP938859	221	5.08	24.27	glutathione S-transferase [*Pl. xylostella*]	AHW45900	71	2e-105
	CsupGSTd4	KP938860	>161	–	–	glutathione S-transferase delta 4 [*Spodoptera litura*]	AIH07597	98	3e-93
Epsilon	*CsupGSTe1*	KP938861	228	8.18	26.68	glutathione S-transferase epsilon 2 [*Cn. medinalis*]	AIL29311	69	1e-114
	CsupGSTe2	KP938862	>222	–	–	glutathione S-transferase epsilon 3 [*Cn. medinalis*]	AIL29312	64	9e-99
	CsupGSTe3	KP938863	228	8.18	26.68	glutathione S-transferase epsilon 2 [*Cn. medinalis*]	AIL29311	69	1e-114
Omega	*CsupGSTo1*	KP938864	254	6.54	28.78	glutathione S-transferase omega 1 [*Cn. medinalis*]	AIL29316	89	4e-168
	CsupGSTo2	KP938865	269	8.11	31.01	glutathione S-transferase omega 2 [*B. mori*]	NP_001037406	61	7e-104
	CsupGSTo3	KP938866	240	7.68	28.66	glutathione S-transferase omega 3 [*Cn. medinalis*]	AIZ46903	90	6e-162
	CsupGSTo4	KP938867	284	6.10	32.43	glutathione S-transferase omega 2 [*Cn. medinalis*]	AIL29317	60	8e-122
Sigma	*CsupGSTs1**	GU453917	204	5.71	23.23	glutathione S-transferase sigma [*C. suppressalis*]	ADD14027	100	4e-145
	CsupGSTs2	KP938868	205	8.51	23.80	glutathione S-transferase [*Choristoneura fumiferana*]	AAF23078	67	8e-96
Theta	*CsupGSTt1*	KP938869	228	8.84	26.42	glutathione S-transferase theta 1 [*Da. plexippus*]	EHJ70012	65	3e-113
Zeta	*CsupGSTz1*	KP938870	214	8.07	24.51	glutathione S-transferase zeta 1 [*B. mori*]	NP_001037418	94	1e-149
Unclassified	*CsupGSTu1*	KP938871	233	5.97	26.87	glutathione S-transferase unclassified 1 [*B. mori*]	NP_001108462	82	2e-141

*This sequence is identical to a previously reported sigma class GST from *C. suppressalis* (Huang et al. 2011a).

order of discovery (Table 3). These sequences have been deposited into GenBank, the accession numbers listed in Table 3. The length of the 14 deduced full-length proteins ranged from 204 to 284 amino acid residues and had predicted theoretical isoelectric points ranging from pH 5.08 to 8.84, with predicted molecular weights between 23.23 and 32.43 kDa (Table 3). The numbers of the *C. suppressalis* antennal *GST*s are more than those identified from the antennae of *E. postvittana* (11 genes, Jordan et al. 2008), *Sp. littoralis* (14 genes, Legeai et al. 2011), and *Ag. planipennis* (13 genes, Mamidala et al. 2013), but fewer than those isolated from *D. melanogaster* (31 genes, Younus et al. 2014). We cannot rule out that transcriptome sequencing may not be powerful enough to discover all the *GST* genes, especially those transcripts with extremely low abundance in the antennae.

BLASTX best hit results of all the 16 *GST*s was shown in Table 3. All of the *GST*s had high identities (60–98% identities) and low E-values (4×10^{-168} to 3×10^{-93}) to their respective orthologs from Lepidopteran species. Of these, *CsupGSTd1* showed 73% identity to the previously identified antennal *GST* (*AtraGST*) of *Amyelois transitella* (Leal et al. 2009) (Table 3, Supp Fig. 2 [online only]). *CsupGSTd1* also showed 70 and 69% identities to the olfactory-specific *GST* (*GST-msolf*) from *M. sexta* (Rogers et al. 1999) and a delta class *GST* (*BmorGSTd1*) from *B. mori*, respectively (Supp Fig. 2 [online only]). *CsupGSTs1*, is identical to a sigma class *GST* gene (*CsGSTsigma*) previously identified in *C. suppressalis* by Huang et al. (2011a) (Table 3).

Cytosolic GST proteins have two domains in their secondary structure: an N-terminal thioredoxin-like domain, responsible for glutathione GSH binding (G-site), and a C-terminal domain, which contains a pocket of hydrophobic substrate binding site (H-site) (Ketterman et al. 2011). Multiple sequence alignment analyses showed that, for all the deduced CsupGST proteins, a conserved G-site can be found in the N-terminal domain and a more variable H-site can be observed in the C-terminal domain (Supp Fig. 3 [online only]). Variations of the H-site enables GSTs to accommodate various electrophilic substrates (Lerksuthirat and Ketterman 2008).

Phylogenetic Analysis of Insect GSTs. The insect cytosolic GSTs can be classified into six groups (delta, epsilon, omega, sigma, theta, and zeta) based on sequence similarity, genomic organization, and biochemical properties (Ketterman et al. 2011). GSTs which could not be classified by the nomenclature system were assigned as an unclassified subgroup (Ketterman et al. 2011). To better understand the classification and phylogenetic relationships of the CsupGSTs, a phylogenetic tree was constructed (Fig. 2). In this tree, the 16 CsupGSTs were placed into seven clades, including six cytosolic classes: delta (four CsupGSTs), epsilon (three), omega (four), sigma (two), theta (one) and zeta (one), and the 'unclassified' clade (one) (Fig. 2).

CsupGSTd1, CsupGSTd2, CsupGSTd3, and CsupGSTd4 fell into the delta class and CsupGSTe1, CsupGSTe2, and CsupGSTe3 fell into the epsilon class (Fig. 2). Delta and epsilon are two most common classes in insects (Enayati et al. 2005). Members from these two classes are well-known for their detoxification functions and usually related to insecticide resistance (Enayati et al. 2005, Li et al. 2007). Delta class GSTs are also involved in the degradation of odorants. Two well-characterized olfactory-related GSTs in *M. sexta* and *B. mori* both grouped with the delta class (Rogers et al. 1999, Tan et al. 2014). In addition, a delta class GST (AtraGST) was found highly expressed in the antennae of *Am. transitella*, indicating its possible involvement in inactivating odorants (Leal et al. 2009). In the tree, CsupGSTd1 was clustered into a branch together with GST-msolf and AtraGST (Fig. 2), suggesting that they might be orthologs and play similar roles in the odorant inactivation. CsupGSTs1 and CsupGSTs2 were clustered into the sigma class (Fig. 2). Members belonging to the sigma class are considered to have housekeeping roles. For example, in *D. melanogaster*, *B. mori* and *Sp. litura*, sigma class GSTs could act as antioxidants, conjugating lipid peroxidation products (Singh et al. 2001, Yamamoto et al. 2006), or as signaling molecules recognizing invasive pathogens (Huang et al. 2011b). However, in *Locusta migratoria*, sigma class GSTs are also involved in the detoxification of insecticides (Qin et al. 2012, 2013).

GSTs belonging to the omega, theta and zeta classes and the unclassified subgroup are involved in the detoxification of xenobiotics. For instance, in *Anopheles cracens*, an omega class GST (AcGSTO1-1) bound but did not metabolize the organophosphate temephos ('Abate') (Wongtrakul et al. 2010). Also in *An. cracens*, a theta-class GST functions not only as peroxidase but also as binding protein for organophosphates (Wongtrakul et al. 2010). A zeta-class GST (bmGSTZ) in *B. mori* can metabolize the neurotoxin permethrin and may contribute to permethrin resistance (Yamamoto et al. 2009). Furthermore, in *L. migratoria*, an unclassified GST (*LmGSTu1*) is responsible for the detoxification of the organophosphate insecticide chlorpyrifos (Qin et al. 2013). However, olfactory-related functions of GSTs belonging to these classes have not yet been determined.

Expression Patterns of CXE and GST Genes. Tissue-specific expression patterns of the 19 *CsupCXE* and 16 *CsupGST* genes were evaluated by qPCR in various tissues, including male and female antennae, abdomens, and legs. The results showed that most *CsupCXE* genes were highly expressed in the nonolfactory tissues (Fig. 3). Only three genes were mainly expressed in antennae: *CsupCXE8*, *CsupCXE13*, and *CsupCXE18*. In addition, *CsupCXE8* and *CsupCXE13* were almost

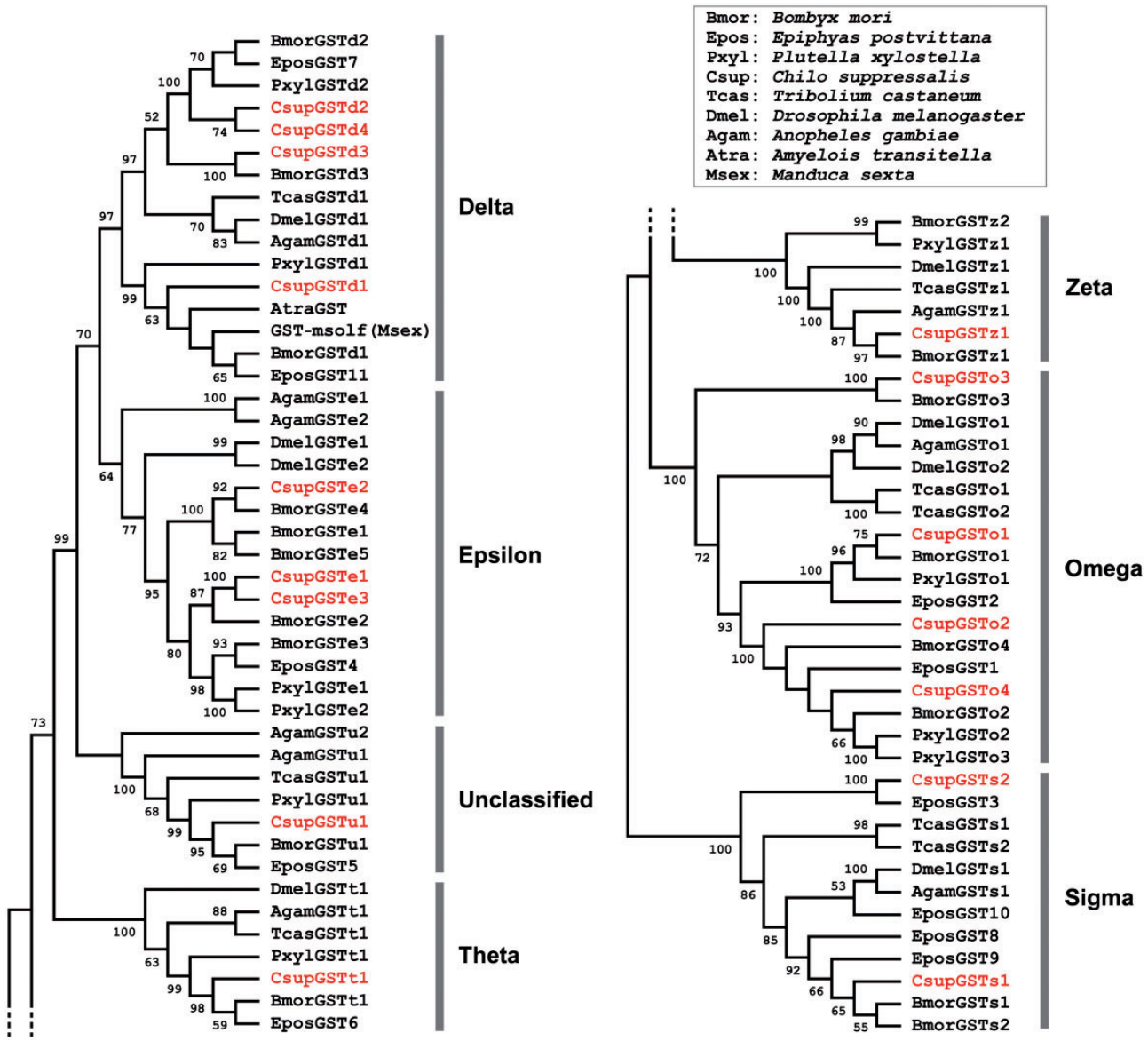

Fig. 2. Phylogenetic relationships of GSTs from *An. gambiae*, *Amyelois transitella*, *B. mori*, *C. suppressalis*, *Drosophila melanogaster*, *E. postvittana*, *Manduca sexta*, *Plutella xylostella*, and *Tribolium castaneum*. These GSTs are grouped into six major clades (delta, epsilon, omega, sigma, theta, and zeta), and the unclassified subgroup. Bootstrap values above 50% are shown for each node of the tree. The *C. suppressalis* GSTs are highlighted in red.

equally expressed in male and female antennae, while the *CsupCXE18* transcripts were more abundant in male antennae than female antennae (Fig. 3). The deduced proteins encoded by these genes all had signal peptides (Table 1), suggesting that these enzymes could be secreted into the sensillar lymph and possibly degrade odorants or pheromones. Indeed, a number of CXEs restricted to insect antennae showed capacity to degrade sex pheromone constituents and plant volatiles (Ishida and Leal 2005; Durand et al. 2010b, 2011; He et al. 2014b,c; He et al. 2015). Interestingly, the expression profile of *CsupCXE13* (antennae-enriched) is intermediate between its orthologs: *ApolPDE* of *A. polyphemus* (male antennae-specific, Ishida and Leal 2005), *SlitCXE13* of *Sp. littoralis* (ubiquitous, Durand et al. 2010a), and *SexiCXE13* (ubiquitous, He et al. 2014a). This phenomenon is possibly due to the within-species variation.

The key role of CXEs is hydrolyze esters of carboxylic acids. However, the pheromone blend produced by female *C. suppressalis* was identified as a mixture of Z-11-hexadecenal (Z-11-16Ald), Z-13-octadecenal (Z-13-18Ald), and Z-9-hexadecenal (Z-9-16Ald) (Tatsuki et al. 1983): none of these compositions are esters. There is a possibility that *CsupCXE8*, *CsupCXE13*, and *CsupCXE18* might be involved in the degradation of volatiles emitted by rice crops, i.e., methyl jasmonate or methyl salicylate (Bi et al. 2007). Apart from rice volatiles, acetate pheromones from sympatric insect species could also be substrates for these antennae specific CXEs. For example, the sex pheromone blend of the rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), consists of (Z)-11-hexadecenyl acetate and (Z)-13-octadecenyl acetate (Kawazu et al. 2009) and could possibly be degraded by antennal CXEs of *C. suppressalis*.

Unexpectedly, we found that none of the *CsupGST* genes was restricted to antennae (Fig. 4). Most of the *CsupGST* genes were mainly expressed in the abdomen, or ubiquitously expressed in both antennae and nonolfactory tissues (Fig. 4). That none of the *CsupGST*s were antennae-specific is somewhat surprising given that olfactory-specific GSTs have been isolated from other moth species, such as *M. sexta* and *B. mori* (Rogers et al. 1999, Tan et al. 2014). We found *CsupGSTd1* was ubiquitous in antennae, abdomen, and legs (Fig. 4). Considering

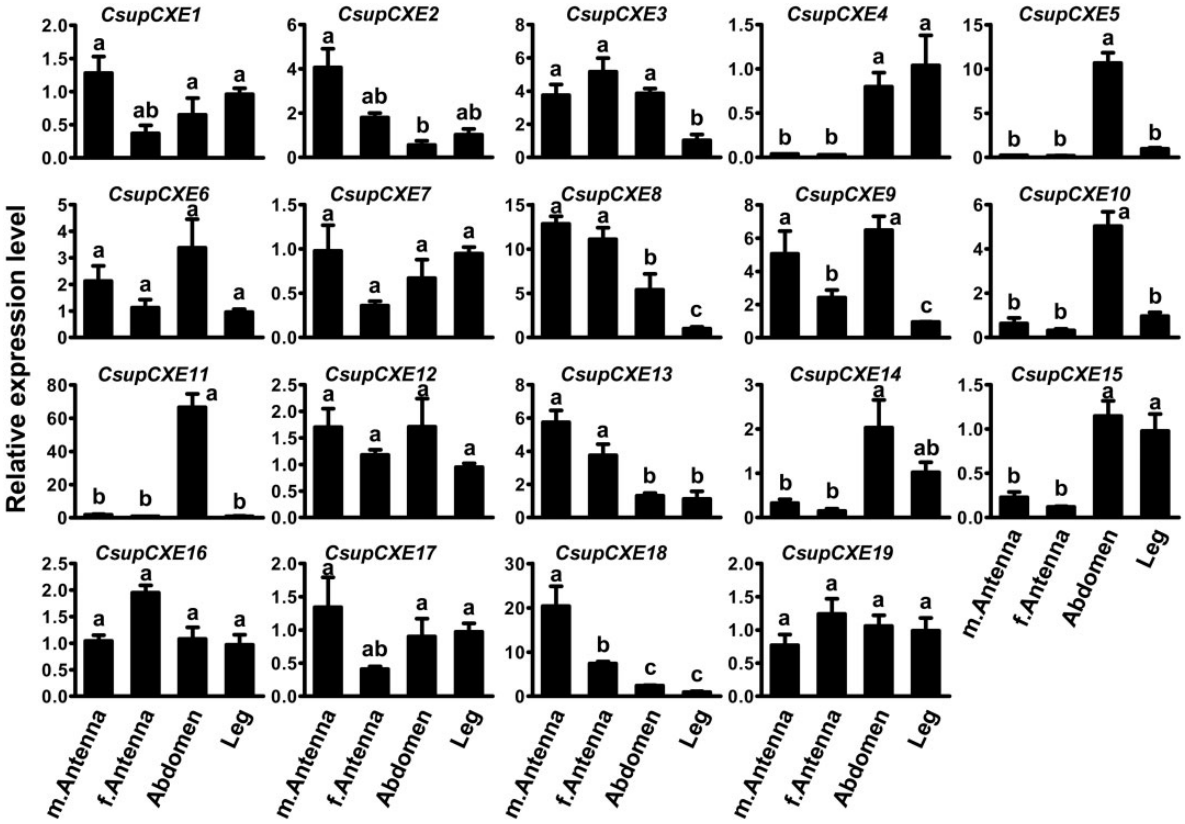

Fig. 3. Relative expression levels of *CsupCXE* genes in different tissues of *C. suppressalis* adults. Levels of gene expression were normalized relative to that in leg (onefold). Different lowercase letters indicate significant differences (one-way ANOVA with Tukey's test, $P < 0.05$).

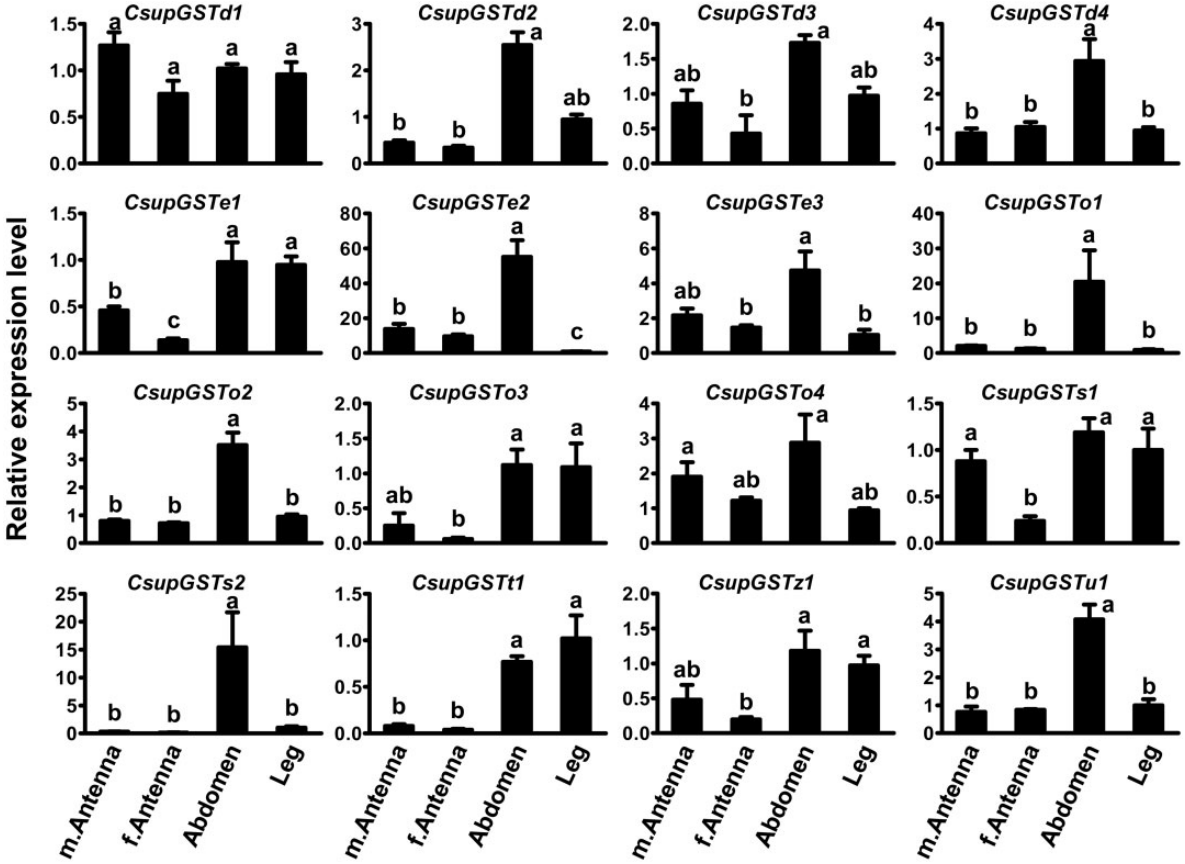

Fig. 4. Relative expression levels of *CsupGST* genes in different adult tissues. Levels of gene expression were normalized relative to that in leg (onefold). Different lowercase letters indicate significant differences (one-way ANOVA with Tukey's test, $P < 0.05$).

that delta class GSTs are commonly associated with xenobiotic detoxification (Li et al. 2007), and that *CsupGSTd1* is the orthologous locus of the olfactory-related *GST-msolf1* of *M. sexta* (Rogers et al. 1999), we hypothesized that CsupGSTd1 might play a dual role in degradation of odorants and detoxification of xenobiotics. However, whether CsupGSTd1, as well as other antennal GSTs, have a role in olfaction remains to be investigated.

In conclusion, this is the first study of genes involved in the degradation of odorants in the lepidopteran species *C. suppressalis*. We identified 19 putative *CXE* and 16 *GST* genes in the antennal transcriptome of the moth, analyzed their phylogenetic relationships and investigated their expression profiles in different tissues. We found three *CXE* genes are enriched in antennae, indicating their potential involvement in odorant degradation. This study suggests that our transcriptome analysis approach is efficient in identifying divergent genes and the results also make it possible for future research of these antennal degradation enzymes in inactivating diverse chemicals.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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