Bulletin of Entomological Research

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Chemosensory genes in the head of *Spodoptera litura* larvae

Lu-Lu Li[1], Ji-Wei Xu[1], Wei-Chen Yao[1], Hui-Hui Yang[1], Youssef Dewer[2], Fan Zhang[3], Xiu-Yun Zhu[1] and Ya-Nan Zhang[1]

[1]College of Life Sciences, Huaibei Normal University, 100 Dongshan Road, Huaibei 235000, China; [2]Bioassay Research Department, Central Agricultural Pesticide Laboratory, Agricultural Research Center, 7 Nadi El-Seid Street, Dokki 12618 Giza, Egypt and [3]Key Laboratory of Animal Resistance Research, College of Life Science, Shandong Normal University, 88 East Wenhua Road, Jinan 250014, China

Research Paper

Cite this article: Li L-L, Xu J-W, Yao W-C, Yang H-H, Dewer Y, Zhang F, Zhu X-Y, Zhang Y-N (2021). Chemosensory genes in the head of *Spodoptera litura* larvae. *Bulletin of Entomological Research* **111**, 454–463. https://doi.org/10.1017/S0007485321000109

Received: 2 September 2020
Revised: 19 December 2020
Accepted: 18 January 2021
First published online: 26 February 2021

Keywords:
Chemosensory genes; feeding mechanism; larval head; *Spodoptera litura*

Author for correspondence:
Xiu-Yun Zhu,
Email: xyzhuhbnu@163.com;
Ya-Nan Zhang,
Email: ynzhang_insect@163.com

Abstract

The tobacco cutworm *Spodoptera litura* (Lepidoptera: Noctuidae) is a polyphagous pest with a highly selective and sensitive chemosensory system involved in complex physiological behaviors such as searching for food sources, feeding, courtship, and oviposition. However, effective management strategies for controlling the insect pest populations under threshold levels are lacking. Therefore, there is an urgent need to formulate eco-friendly pest control strategies based on the disruption of the insect chemosensory system. In this study, we identified 158 putative chemosensory genes based on transcriptomic and genomic data for *S. litura*, including 45 odorant-binding proteins (OBPs, nine were new), 23 chemosensory proteins (CSPs), 60 odorant receptors (ORs, three were new), and 30 gustatory receptors (GRs, three were new), a number higher than those reported by previous transcriptome studies. Subsequently, we constructed phylogenetic trees based on these genes in moths and analyzed the dynamic expression of various genes in head capsules across larval instars using quantitative real-time polymerase chain reaction. Nine genes–*SlitOBP8*, *SlitOBP9*, *SlitOBP25*, *SlitCSP1*, *SlitCSP7*, *SlitCSP18*, *SlitOR34*, *SlitGR240*, and *SlitGR242*–were highly expressed in the heads of 3- to 5-day-old *S. litura* larvae. The genes differentially expressed in olfactory organs during larval development might play crucial roles in the chemosensory system of *S. litura* larvae. Our findings substantially expand the gene inventory for *S. litura* and present potential target genes for further studies on larval feeding in *S. litura*.

Introduction

Insects recognize a large number of odor volatile compounds in the environment primarily via their chemosensory systems that induce corresponding behavioral responses. Compound perception is mediated by a series of proteins, including odorant-binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), sensory neuron membrane proteins, and odorant-degrading enzymes (ODEs) (Field *et al.*, 2000; Leal 2013; He *et al.*, 2020).

In insects, odor perception is initiated in their olfactory organs, and the initial step of odor detection is the binding of odor molecules to OBPs or CSPs (Getahun *et al.*, 2016). OBPs and CSPs, produced in the lymph of the chemosensilla, bind to chemical cues in the external environment and transmit them to ORs via the sensillum lymph, thereby activating signal transduction (Jacquin-Joly and Merlin, 2004; Leal 2013; Chen *et al.*, 2018; Pelosi *et al.*, 2018; He *et al.*, 2019a, 2019b). Electrostatic interactions between the ligand and transmembrane segments of the olfactory receptor and Van Der Waals interactions between the ligand and hydrophobic pocket of the receptor are responsible for receptor activation (Vogt, 2003; Clark and Ray, 2016). Eventually, odor molecules are inactivated by ODEs to maintain receptor activity by preventing persistent binding to the receptor (Durand *et al.*, 2011; He *et al.*, 2014, 2015).

OBPs are water-soluble small-molecule proteins and are present at high concentrations in the aqueous sensillum lymph (Zhou, 2010; Pelosi *et al.*, 2018). They can be divided into different types in Lepidoptera, including general odorant-binding proteins (GOBPs), which are used by all insects to recognize plant volatile substances or environmental odors and are distributed within the bascionic sensillum, and pheromone-binding proteins (PBPs), which primarily exist in the antennae of adult moths and are distributed in the trichoid sensillum (Picimbon and Gadenne, 2002; Guo *et al.*, 2012; Liu *et al.*, 2015b). CSPs are widely distributed in various chemoreceptor lymphatic fluids of insects. They are acidic water-soluble proteins with molecular weights of 12–14 kDa, transport odor molecules to corresponding receptors, and serve as carriers of chemical irritants (Maleszka *et al.*, 2007). Genome and transcriptome sequencing have led to the identification of CSPs in several insect species (Liu *et al.*, 2014b; Zhang *et al.*, 2014; Cheng *et al.*, 2015; Ingham *et al.*, 2020).

Insect ORs, with a seven-transmembrane domain (Hopf *et al*., 2015) are located on the dendritic membrane of odorant receptor neurons (ORNs) and are generally composed of 400 amino acids. In addition to variable odorant specificity subunits, the odorant receptor co-receptor (Orco) subunit required for conventional ORs in every insect is broadly present in ORNs and plays a role in the detection of odorant substances by ORs (Larsson *et al*., 2004). For example, the deletion of Orco results in a loss of response to sex pheromones in *Bombyx mori* and greatly influences mating behavior (Liu *et al*., 2017); knockdown of Orco resulted in the inability of rice planthoppers to seek or locate host plants (He *et al*., 2018). Furthermore, Orco is a highly conserved gene and it is important to examine its evolution and functional flexibility (Soffan *et al*., 2018). Similar to ORs, GRs have a seven-transmembrane domain and are also a key part of the chemosensory system in Lepidoptera (Agnihotri *et al*., 2016). They are often co-expressed in single ORNs and detect different environmental stimuli (Mang *et al*., 2016). The first insect GRs were identified in *Drosophila melanogaster* and since then, many GRs have been identified in other insects such as *Anopheles gambiae*, *B. mori*, and *Heliothis virescens* (Hallem *et al*., 2006). Insect GRs can be sugar-, bitter-, fructose-, and carbon dioxide-related (Xu *et al*., 2012; Raad *et al*., 2016). Moreover, insect GRs have additional functions, such as *BmGR8* in *B. mori*, a sugar receptor subfamily that has a specific response to inositol and could prolong the larval duration (Zhang *et al*., 2011), and *DmGR5a* in *D. melanogaster* that serves as a trehalose-specific receptor (Chyb *et al*., 2003; Dahanukar *et al*., 2007). These findings suggest that ORs and GRs are ubiquitous in insects and the chemosensory and non-chemosensory functions they mediate are complex and diverse. However, there is a lack of studies on the exact role of ORs and GRs in the larval feeding process of serious agricultural pests such as *Spodoptera litura*.

S. litura (Lepidoptera: Noctuidae) is a polyphagous insect in tropical and subtropical regions worldwide (Dinesh-Kumar *et al*., 2018; Li *et al*., 2018). It can harm more than 389 economically important plants such as corn, peanut, and cabbage, and has become one of the most destructive agricultural pests in Asia (Ahmad *et al*., 2013; CABI 2018). This pest has a high capacity for reproduction and development (Wu *et al*., 2018). *S. litura* larvae feed on a wide range of plants; they eat the whole leaf, buds, fruit, and flowers (Smith *et al*., 1997). These findings indicate that *S. litura* larvae have a highly selective and sensitive chemosensory system, which is crucial for finding food sources, development, and avoiding dangerous or unsuitable habitats and hosts. There is an urgent need for safe management strategies for the control of *S. litura*. One novel alternative to chemical pesticides, which lead to serious environmental problems, is based on the disruption of the insect chemosensory system (Xu *et al*., 2010; Ingham *et al*., 2020). In this study, we successfully identified 158 putative chemosensory genes, including 45 OBPs (nine were new), 23 CSPs, 60 ORs (three were new), and 30 GRs (three were new), in *S. litura* based on genomic and transcriptomic data. We then constructed phylogenetic trees based on these genes in moths and analyzed the dynamic expression levels of various genes in larval head capsules during development using quantitative real-time polymerase chain reaction (qRT-PCR). The chemosensory genes identified in this study are potential targets for future functional studies aimed at the development of novel behavioral disturbance agents to control *S. litura* populations.

Materials and methods

Insects and tissue sample collection

S. litura individuals were raised under laboratory conditions as follows: temperature of 27 ± 1°C, relative humidity of 75 ± 5%, and photoperiod of 14L:10D. The antennae were collected separately from 3-day-old virgin female and male adults and sent to Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) for transcriptome sequencing. For gene expression analyses, new fresh eggs (Egg) (50 for each replicate), heads of 1- to 6-day-old larvae (LH) (the 1- to 2-day-old larvae, 50 for each replicate; and the 3- to 6-day-old larvae, 20 for each replicate), and antennae of 3-day-old virgin male (MA) and female (FA) adults (25 pairs for each replicate) were collected and placed in nuclease-free centrifuge tubes. Each sample type included three biological replicates, and all samples were preserved at −80°C until RNA extraction.

qRT-PCR

In accordance with our previously described methods (Zhang *et al*., 2015b; Zhang *et al*., 2016), total RNAs of all samples were extracted, and high-quality RNA was used to synthesize corresponding cDNAs. Subsequently, the cDNA was used as templates for qRT-PCR analyses. The qRT-PCR primers are listed in Table S1 and amplification was executed following the manufacturer's instructions, on a LightCycler 96 Multiwell Plate. *SlitGAPDH* and *SlitEF* were used as internal reference genes (Zhang *et al*., 2016) to calculate relative expression levels. The data were analyzed using Q-Gene (Muller *et al*., 2002; Simon, 2003). Each biological sample had three technical replicates.

Transcriptome analysis

The purity of total RNA was determined using the NanoDrop instrument (based on the OD260/280 ratio). Subsequently, cDNA synthesis, library construction, sequencing, de novo assembly, and annotation of unigenes were performed following our previously described procedures (Zhang *et al*., 2015b; Zhang *et al*., 2016).

Reference sequence alignment

HISAT (2.0.4) was used to analyze the genomic location of the filtered sequences. HISAT effectively compares spliced reads with RNA sequencing data (RNA-seq). It is currently the most accurate alignment software. In this study, default parameters were used. If an appropriate reference genome is selected and the data are not contaminated, the percentage of mapped reads or fragments generated by a laboratory is typically greater than 70%. The percentage of multiple mapped reads or fragments assigned to multiple locations does not typically exceed 10% (Mortazavi *et al*., 2008).

Analysis of expression abundance

The expression abundance of each unigene was calculated following the Fragments Per Kilobase per Million (FPKM) method. This method can eliminate the influence of differences in length and number of sequences on the expression abundance. In this study, HTSeq (v0.6.1) was used to evaluate the expression levels.

https://doi.org/10.1017/S0007485321000109 Published online by Cambridge University Press

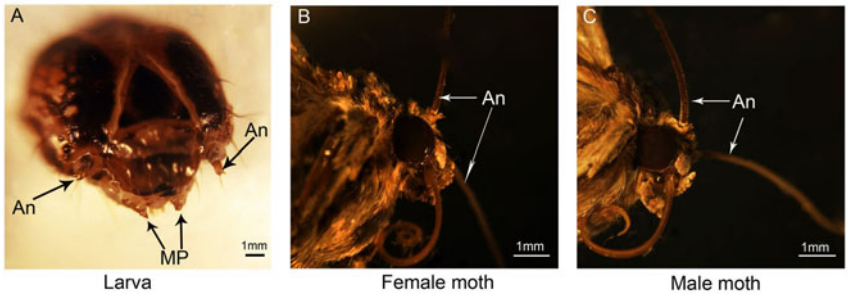

Fig. 1. Chemosensory organs of *S. litura*. (a) larva, (b) Female moth, (c) Male moth. An, antenna; MP, maxillary palps.

Table 1. The quality of *S. litura* transcriptomic data

Sample name	SlitEgg	SlitLH	SlitFA	SlitMA
Raw reads	37,661,368	39,848,092	70,642,568	73,142,860
Clean reads	36,447,774	38,709,312	70,642,568	73,142,860
Clean bases (Gb)	3.68	3.91	10.6	10.97
Error rate (%)	0.02	0.02	0.02	0.02
Q20 (%)	98.67	98.76	96.49	96.78
Q30 (%)	95.94	96.06	91.78	92.3
GC content (%)	44.01	45.79	44.46	43.87

FPKM values of 0.1 and 1 were used as thresholds to determine whether a gene is expressed.

Phylogenetic analysis

Phylogenetic trees based on amino acid sequences were constructed using OBPs, CSPs, ORs, and GRs of *S. litura* and other Lepidoptera species including *B. mori*, *Manduca sexta*, *Helicoverpa armigera*, *Dendrolimus punctatus*, *Epiphyas postvittana*, *Spodoptera littoralis*, and *Danaus plexippus*. This method is more convenient when analyzing the evolutionary relationship of chemosensory genes between *S. litura* and other Lepidoptera species and also helps to infer the possible functional characteristics of some chemosensory genes of *S. litura*. Signal peptide analysis, sequence alignment, and phylogenetic tree construction were performed following our previously reported procedures (Zhang *et al.*, 2015b; Zhang *et al.*, 2017).

Statistical analysis

Cycle threshold (Ct) values were obtained using qRT-PCR and used to determine the mean normalized expression value for each gene using Q-Gene 96 method. The expression levels of chemosensory genes were compared using one-way analysis of variance in SPSS 21.0 (SPSS Inc., Chicago, IL, USA); values of $P < 0.05$ indicated a significant difference.

Results and discussion

Overview of transcriptomic data

We assembled and analyzed the transcriptomes of four tissues of *S. litura,* based on the Illumina sequencing data, as follows: eggs (referred to as SlitEgg; reads from National Center for Biotechnology Information [NCBI] server ID: DRX080341), larval heads (referred to as SlitLH; reads from NCBI server ID: DRX080340), female antennae (referred to as SlitFA), and male antennae (referred to as SlitMA) (fig. 1). In total, we obtained 3,766,1368 raw reads from SlitEgg, 39,848,092 from SlitLH, 70,642,568 from SlitFA, and 73,142,860 from SlitMA. After removing low-quality and adaptor-containing reads, 38,709,312, 37,661,368, 70,642,568, and 73,142,860 clean reads were obtained from the four tissues, respectively. The GC contents and Q30 values were over 40 and 90%, respectively (table 1), suggesting that the transcriptomic data were of good quality and could be used for subsequent analyses.

The proportion of total reads mapped to the reference genome was greater than 70%, with less than 2.5% of reads mapped to multiple locations, and spliced reads accounted for less than 30% of the total reads (Table S2), indicating the absence of contamination and appropriate reference genome selection. In an RNA-seq analysis, gene expression levels could be estimated by obtaining counts in genome regions or reads in gene exons. The read count is positively correlated with gene length and sequencing depth in addition to the true gene expression level. FPKM values are usually used to compare the expression levels of different genes. We found that the distributions of FPKM values in the four tissue types of *S. litura* at each interval value were similar, and the highest proportion of reads had an FPKM value of 3–60 (Table S3). This indicates that the differentially expressed genes are common to the different tissues of *S. litura*, providing a basis for the identification of key differentially expressed genes for further functional analyses.

Characteristics of chemosensory genes in S. litura

Some chemosensory genes belonging to *S. litura* have been obtained using RNA-seq analyses exclusively on adult antennae

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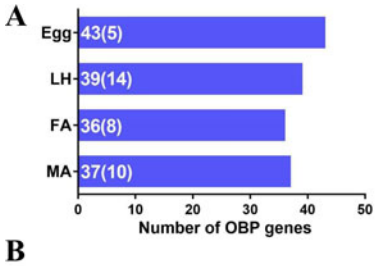

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Gene	Egg	LH	FA	MA	Gene	Egg	LH	FA	MA
PBP1	0.53	0.00	15976.43	62555.83	OBP19	1.19	4.31	0.22	0.11
PBP2	1.03	0.14	33684.89	29633.85	OBP20	2.71	0.15	5.14	4.47
PBP3	2.42	0.00	6348.86	2094.87	OBP21	0.74	1.49	7286.28	4134.22
GOBP1	0.71	0.00	22929.90	14408.62	OBP22	240.87	344.33	798.02	205.93
GOBP2	9.57	35.04	43291.59	22823.16	OBP23	14.15	12.37	42.97	47.36
OBP1	3.98	8.45	12.23	14.10	OBP24	22.07	3.46	0.00	0.00
OBP2	0.58	2.33	0.11	0.31	OBP25	3.62	18770.39	795.72	584.87
OBP3	85.43	17.18	0.00	0.00	OBP26#	23.92	3.45	616.57	447.16
OBP4	0.69	0.00	0.10	0.00	OBP27#*	6.40	28.55	561.32	593.30
OBP5	0.20	17.22	10044.22	15971.77	OBP28#*	0.29	0.00	35536.33	12144.72
OBP6	8.15	281.62	716.41	1236.79	OBP29#*	0.77	0.00	0.14	0.41
OBP7	15.00	42.18	0.42	0.71	OBP30#*	0.87	41.26	0.00	0.00
OBP8	13.25	279.46	0.00	0.00	OBP31#	0.20	4.15	0.00	0.00
OBP9	0.00	1646.73	0.77	1.19	OBP32#*	1.96	6.61	0.00	0.00
OBP10	5.84	16.00	2.10	1.19	OBP33#	0.38	5.18	0.00	0.00
OBP11	6.64	6.09	0.00	0.20	OBP34#	3.27	2.31	6111.35	16828.10
OBP12	14.90	20.38	0.31	0.21	OBP35#*	2.70	2261.96	5465.34	7043.16
OBP13	1.48	2.67	0.00	0.40	OBP36#*	10.31	55.47	251.45	150.31
OBP14	2.63	12.75	1396.66	956.10	OBP37#	6.15	3.00	76.91	71.12
OBP15	4.38	4.35	269.65	144.01	OBP38#	1.40	30.91	623.82	736.00
OBP16	0.24	0.38	2610.52	1388.02	OBP39#*	5.76	1.22	6.91	2.77
OBP17	0.00	2.70	1173.31	327.35	OBP40#*	0.20	6.19	1.23	3.38
OBP18	2.34	7.35	19.15	7.75					

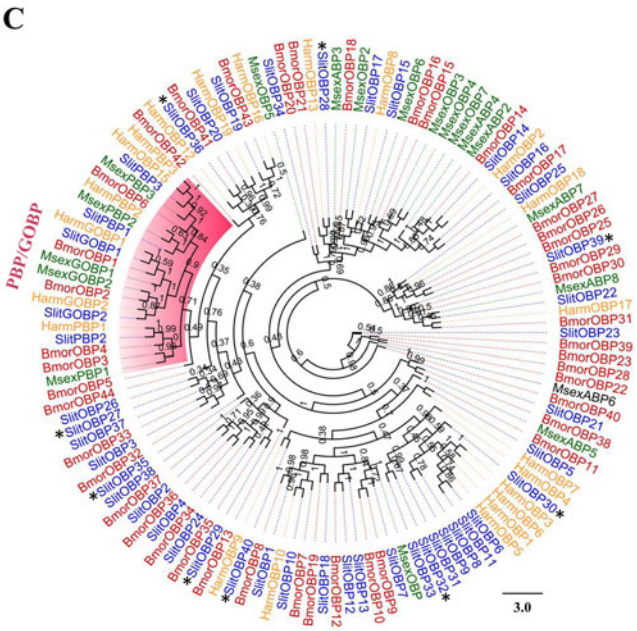

Fig. 2. The OBP genes of *S. litura*. (a) The number of OBP genes in SlitEgg, SlitLH, SlitFA, and SlitMA. The digits in the histogram represent number of OBPs that are highly expressed in different samples; (b) The FPKM values of OBPs are in different samples. Orange represents a highly expressed gene and the red represents the FPKM values of these genes and are in the top 3 of all LH expression genes. '#' representing the OBP was not found in the previous genomic study, '*' representing the OBP is newly discovered gene in this study; (c) Phylogenetic tree of OBPs from *S. litura* and other moths. The *S. litura* OBPs are shown in blue. Bmor, *Bombyx mori*; Msex, *Manduca sexta*, and Harm, *Helicoverpa armigera*. '*' representing the OBP is a newly discovered gene in this study.

or pheromone glands. Gu *et al.* (2015) reported 38 OBPs obtained from adult antennae, and Feng *et al.* (2015) reported 74 (26 ORs, 21 OBPs, 18 CSPs) obtained from adult antennae. In addition, we previously obtained 25 OBPs and 14 CSPs from pheromone glands of female moths (Zhang *et al.*, 2015b). However, the type and number of chemosensory genes identified in these studies are less comprehensive than those based on genome analyses (Cheng *et al.*, 2017). A total of 45 IRs have been identified and analyzed based on the genomic and transcriptomic data by Zhu *et al.* (2018). Using phylogenetic analysis and extensive-expression profiles, they demonstrated that SlitIRs have diverse functional roles in olfaction, taste, and reproduction. Therefore, in this study, we conducted a more in-depth analysis based on the results of genome analyses. According to the sequence similarity and characteristics of insect chemosensory genes, 158 putative genes (45 OBPs, 23 CSPs, 60 ORs, and 30 GRs) were identified from the transcriptomic and genomic data (Tables S4 and S5), which included 15 new genes (nine OBPs, three ORs, and three GRs), a number higher than that of genes identified in other moth species such as *H. armigera* (122 genes) (Liu *et al.*, 2014a), *H. assulta* (147 genes) (Xu *et al.*, 2015), and *Plutella xylostella* (116 genes) (Yang *et al.*, 2017). The large amount of transcriptome data suggests that *S. litura* is a useful taxon for understanding chemosensory genes in insects.

OBPs

We obtained 45 different unigenes encoding putative OBPs in *S. litura*, including 15 that have not been reported in previous genomic studies of the species (Cheng *et al.*, 2017) and nine that were newly identified. Sequence analysis showed that all sequences had full-length open reading frames (ORFs) and ten SlitOBPs did not have signal peptide regions. Furthermore, based on the FPKM results, 43, 39, 36, and 37 SlitOBPs were found in the Egg, LH, FA, and MA samples, respectively, and SlitOBPs were most frequent in the LH (14 genes, including three new SlitOBPs), followed by MA (ten genes, including two new SlitOBPs), FA (eight genes, including three new SlitOBPs), and Egg (five genes, including one new SlitOBP) samples (fig. 2), indicating that these genes of larval and adult moths play important roles in the perception of external odorants involving in development and hatching or host searching by newly hatched larvae.

The phylogenetic analysis showed that 36 SlitOBPs and nine new SlitOBPs were widely distributed along various branches. SlitPBP1, SlitPBP2, SlitPBP3, SlitGOBP1, and SlitGOBP2 formed a group including the PBP/GOBP subfamily (fig. 2c), suggesting that SlitOBPs have functions similar to those of other moth OBPs (Poivet *et al.*, 2012; Jeong *et al.*, 2013; Pelosi *et al.*, 2014; Liu *et al.*, 2015b). Notably, seven SlitOBPs (SlitOBP6, SlitOBP8, SlitOBP9, SlitOBP11, and SlitOBP31–33, SlitOBP32 identified as new OBP) formed a cluster, indicating that these genes have species-specific functions, which should be evaluated in the future.

CSPs

Twenty-three putative genes encoding CSPs were obtained in *S. litura* based on the transcriptomic data, consistent with previous counts based on the *S. litura* genome (Cheng *et al.*, 2017). All SlitCSPs had full-length ORFs with four conserved cysteines and

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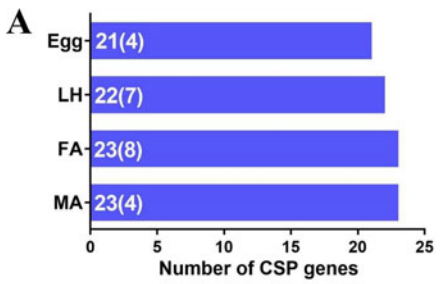

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Gene	Egg	LH	FA	MA
CSP1	3.46	5918.29	11.64	8.26
CSP2	8.58	20.00	65.81	143.31
CSP3	37.59	105.99	33.96	97.52
CSP4	231.02	3.93	0.15	0.59
CSP5	178.19	0.76	28.02	31.37
CSP6	5.02	176.34	0.26	0.26
CSP7	46.51	1279.97	0.39	3.19
CSP8	2.74	19.58	0.25	0.61
CSP9	96.40	67.73	36.26	58.11
CSP10	11.00	66.20	0.79	5.14
CSP11	0.00	255.26	7646.75	5399.60
CSP12	586.29	9.59	1.05	2.83
CSP13	17.75	38.23	32948.88	16752.61
CSP14	12.92	0.38	631.61	1553.74
CSP15	1.15	1.11	2666.72	1637.72
CSP16	125.35	312.13	3690.87	4509.51
CSP17	13.76	66.81	686.07	414.86
CSP18	13.90	13923.45	8561.24	1333.47
CSP19	51.09	8422.62	16124.02	6348.95
CSP20	35.26	2585.05	19437.54	6551.45
CSP21	259.10	3910.99	5493.25	2061.32
CSP22	0.00	0.17	6708.93	4029.95
CSP23	2.48	0.00	12.49	15.20

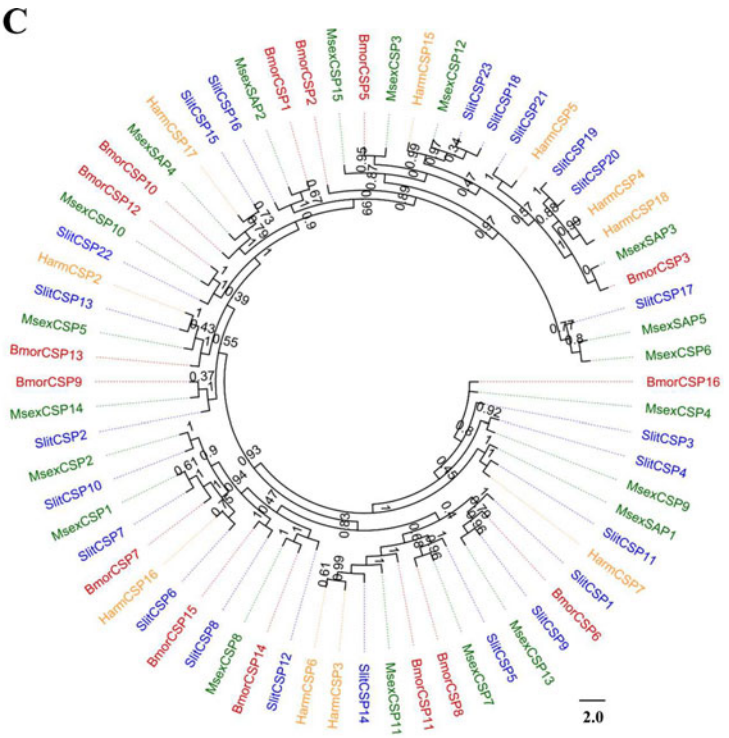

Fig. 3. The CSP genes of *S. litura*. (a) The number of CSP genes in SlitEgg, SlitLH, SlitFA, and SlitMA. The digits in the histogram represent the number of CSPs that are highly expressed in different samples; (b) The FPKM values of CSPs are in different samples. Orange represents a highly expressed gene and the red representing the FPKM values of these genes are in the top 4 of all LH expression genes; (c) Phylogenetic tree of CSPs from *S. litura* and other moths. The *S. litura* CSPs are shown in blue. Bmor, *Bombyx mori*; Msex, *Manduca sexta*, and Harm, *Helicoverpa armigera*.

21 SlitCSPs were predicted to have signal peptides at their N termini. Based on the FPKM results, 21, 22, 23, and 23 SlitCSPs were found in the Egg, LH, FA, and MA tissues, respectively, and eight genes were abundantly expressed in FA, four in MA, and seven in LH. Similarly, the number of *SlitCSPs* enriched in the LH was higher than that in the Egg (fig. 3). *SlitCSPs* might play a key role in physiological and behavioral activities in male adults and larvae.

A phylogenetic tree of moth CSPs was constructed using various CSPs of *S. litura*, *B. mori*, *M. sexta*, and *H. armigera*. Most branches were highly supported (bootstrap values greater than 70%), and most SlitCSPs formed a cluster on the same branch with BmorCSPs, MsexCSPs, and HarmCSPs (fig. 3c). Based on these findings, these SlitCSPs may have chemosensory or other physiological functions similar to those of CSPs in other moths (Pelosi *et al.*, 2005; Celorio-Mancera Mde *et al.*, 2012; Zhang *et al.*, 2014).

ORs

Sixty unigenes encoding putative ORs were acquired by analyzing the transcriptome data for *S. litura*, of which four were not found in previous *S. litura* genomic studies (Cheng *et al.*, 2017) and three were novel SlitORs (SlitOR74, 75, 77). Except for SlitOR74 and SlitOR75, other SlitORs were predicted to have full-length ORFs that encode 385–473 amino acids with 3–8 transmembrane domains (TMDs). The FPKM analyses indicated 31, 22, 59, and 58 SlitORs in the Egg, LH, FA, and MA tissues, respectively, and SlitORs were most frequently expressed in MA (30 genes, including a new gene SlitOR75), followed by FA (24 genes, including the new gene SlitOR74), Egg (three genes, including the new gene SlitOR77), and LH (2 genes) (fig. 4). These results indicate that SlitORs are involved in the chemosensory process of adult moths and have different functions similar to ORs of other insects (Carraher et al. 2015, Yan et al. 2020), and the OR genes expressed in eggs may help the newly hatched *S. litura* larvae to search for the host in time.

A phylogenetic tree was constructed based on the amino acid sequences of moth ORs from *S. litura*, *B. mori*, *H. armigera*, and *D. punctatus*, *E. postvittana*, *S. littoralis*, and *D. plexippus*, and each cluster had at least one orthologue in another moth taxon (fig. 4c). All the three new SlitOR genes were clustered with the OR genes of other moths (SlitOR74-BmorOR67, SlitOR75-HarmOR35, and SlitOR77-DpleOR9/DpleOR10/DpleOR11/DpleOR42/BmorOR32), indicating that these genes may have similar physiological functions. A total of six SlitORs (SlitOR1, 3, 5, 6, 7, and 57) clustered with the moth PR subfamily, the number is more than that in previous reported (Zhang *et al.*, 2015a). According to the recent study of a novel candidate pheromone receptors in moths (Bastin-Heline *et al.*, 2019), four SlitORs (SlitOR11, SlitOR13, SlitOR14, and SlitOR53) in *S. litura* were clustered in novel lineage of candidate PRs. In the future, we plan to conduct in-depth functional analysis of these traditional and novel candidate PRs to help better reveal the exact molecular mechanism of sex pheromone perception in *S. litura*. As expected,

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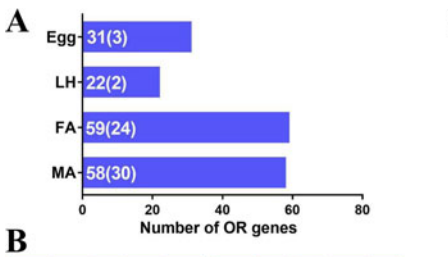


B

Gene	Egg	LH	FA	MA	Gene	Egg	LH	FA	MA
OR1	2.63	0.00	0.04	6.93	OR40	0.20	0.00	27.00	24.28
OR2	0.30	0.37	246.39	497.23	OR42	0.00	0.00	1.22	2.24
OR3	0.00	0.00	10.54	23.98	OR43	0.18	0.07	12.51	13.53
OR5	0.00	0.10	8.54	0.08	OR44	0.00	0.00	17.97	24.83
OR6	0.00	0.00	0.48	5.06	OR45	0.00	0.00	10.82	8.97
OR7	0.00	0.00	7.59	30.48	OR47	0.00	0.00	2.21	4.89
OR9	0.08	0.02	15.83	20.03	OR49	0.07	0.00	6.15	4.56
OR10	6.68	2.03	0.04	0.00	OR51	0.13	0.00	29.03	27.24
OR11	0.07	0.00	8.11	10.05	OR53	1.82	1.19	13.59	6.93
OR13	0.00	0.00	8.47	2.32	OR54	0.49	0.86	25.95	30.17
OR14	0.06	0.00	5.28	0.21	OR55	0.13	0.08	9.93	8.94
OR15	0.19	0.00	0.10	2.39	OR56	0.23	0.13	12.94	24.83
OR16	0.00	0.08	2.81	4.37	OR57	0.00	0.00	49.36	116.06
OR17	4.35	0.45	5.39	5.17	OR58	0.00	0.00	3.71	3.51
OR18	0.06	0.05	14.91	18.00	OR60	0.00	0.00	21.47	16.35
OR20	0.09	0.00	1.20	0.84	OR61	0.00	0.04	8.81	9.87
OR21	0.00	0.00	2.22	2.66	OR63	0.00	0.00	0.05	0.16
OR23	0.00	0.00	7.84	0.05	OR64	0.00	0.00	2.85	6.52
OR24	0.00	0.00	2.52	3.57	OR65	0.20	0.47	0.36	0.46
OR25	0.00	0.00	2.52	2.03	OR66	0.00	0.22	2.52	3.55
OR26	0.44	0.00	9.95	17.36	OR67	0.18	0.00	0.00	0.05
OR27	1.84	0.30	2.30	5.85	OR68	0.12	0.00	2.24	6.98
OR28	2.59	0.33	29.75	29.60	OR69	0.00	0.06	12.23	17.99
OR30	0.00	0.00	1.85	0.08	OR71	0.32	0.00	5.20	9.26
OR32	0.18	0.00	8.26	0.07	OR72	0.00	0.00	8.27	8.52
OR34	30.42	132.25	72.02	119.95	OR73	0.00	0.00	3.25	3.12
OR35	0.00	0.00	50.82	31.67	OR74*	0.00	0.00	0.06	0.00
OR36	0.00	0.00	5.55	7.86	OR75*	0.80	0.28	2.40	2.41
OR37	0.00	0.00	1.76	0.11	OR76#	0.05	0.00	2.43	1.84
OR38	0.12	0.00	17.71	10.85	OR77*	31.97	13.03	1.08	1.84

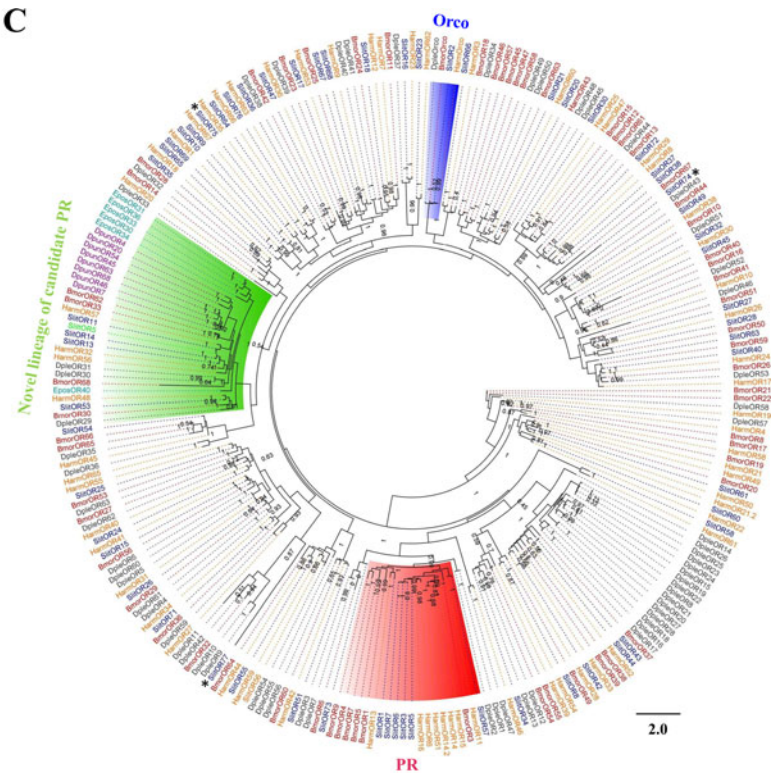
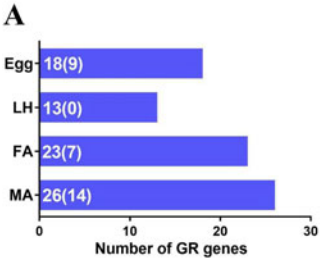


Fig. 4. The OR genes of *S. litura*. (a) The number of OR genes in SlitEgg, SlitLH, SlitFA, and SlitMA. The digits in the histogram represent the number of ORs that are highly expressed in different samples; (b) The FPKM values of ORs are in different samples. Orange represents a highly expressed gene and the red representing the FPKM values of these genes are in the top 2 of all LH expression genes. '#' representing the OR was not found in the previous genomic study, and '*' representing the OR is the newly discovered gene in this study; (c) Phylogenetic tree of ORs from *S. litura* and other moths. The *S. litura* ORs are shown in blue. Bmor, *Bombyx mori*; Harm, *Helicoverpa armigera*; Dpun, *Dendrolimus punctatus*; Epos, *Epiphyas postvittana*; Slitt, *Spodoptera littoralis*, and Dple, *Danaus plexippus*. '*' representing the OR is a newly discovered gene in this study.



B

Gene	Egg	LH	FA	MA
GR1	0.00	0.05	0.49	1.39
GR2	0.07	0.00	3.80	6.78
GR3	0.00	0.00	4.79	3.89
GR4	0.05	0.00	2.19	3.95
GR5	0.00	0.06	4.92	7.69
GR6	0.00	0.06	1.25	3.28
GR7	0.28	0.00	3.63	3.56
GR8	2.44	0.20	2.71	6.88
GR9	2.58	0.40	0.05	0.09
GR10	0.39	0.00	1.89	3.01
GR13	111.33	39.23	5.90	4.06
GR14	0.00	0.00	1.17	0.57
GR15	0.00	0.00	0.00	0.67
GR16	0.00	0.00	0.11	0.00
GR19	0.00	0.00	0.00	0.18
GR20	0.18	0.00	0.00	0.00
GR23	0.00	0.00	0.00	0.12
GR24	0.00	0.00	0.00	0.14
GR25	0.11	0.00	0.00	0.00
GR26	0.07	0.00	0.57	0.71
GR35	0.00	0.00	0.00	0.07
GR160	0.07	0.03	0.74	0.27
GR169	0.21	0.03	0.02	0.00
GR175	0.00	0.07	0.36	0.45
GR177	0.27	0.22	0.23	0.29
GR225	2.93	0.15	1.64	1.51
GR239	0.29	0.00	0.09	0.14
GR240*	45.96	323.39	645.79	202.70
GR241*	0.22	0.00	0.12	0.12
GR242*	28.51	3.40	1.37	2.95

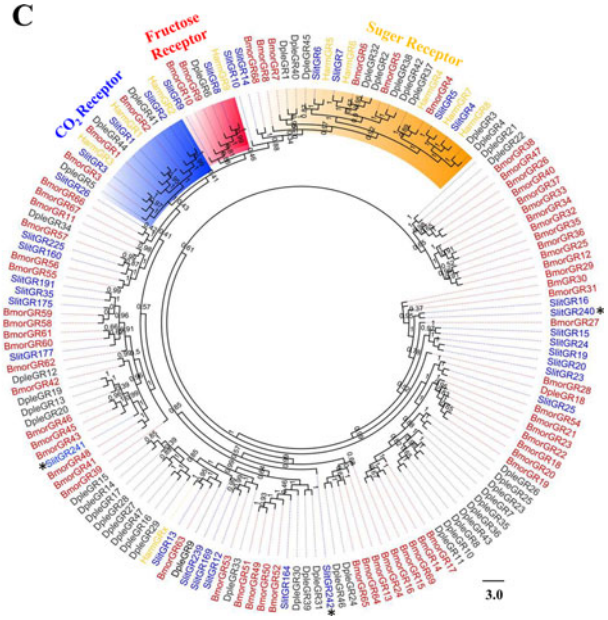


Fig. 5. The GR genes of *S. litura*. (a) The number of GR genes in SlitEgg, SlitLH, SlitFA, and SlitMA. The digits in the histogram represent the number of GRs that are highly expressed in different samples; (b) The FPKM values of GRs are in different samples. Orange represents a highly expressed gene, and the red representing the FPKM values of these genes are in the top 3 of all LH expression genes. '*' representing the GR is the newly discovered gene in this study; (C) Phylogenetic tree of GRs from *S. litura* and other moths. The *S. litura* GRs are shown in blue. Bmor, *Bombyx mori*; Harm, *Helicoverpa armigera*, and Dple, *Danaus plexippus*. '*' representing the GR is the newly discovered gene in this study.

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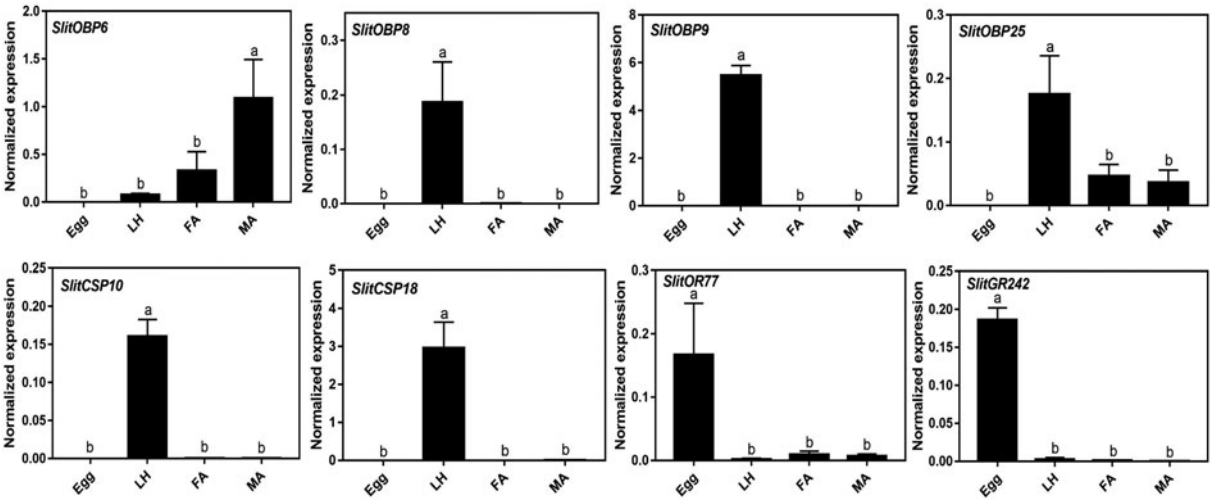

Fig. 6. qRT-PCR validation of the randomly selected chemosensory genes in different samples of *S. litura*. The different low case letters mean significance between tissues. LH, 5th larval head; MA, male antenna; FA, female antenna. Error bar represents mean ± SEM.

SlitOR2clustered with the Orco genes of other moths, indicating that the gene belong to the conserved Orco subfamily.

GRs

We obtained 30 different unigenes encoding putative SlitGRs in *S. litura*, three of which were previously unreported (Cheng *et al.*, 2017). However, this number is far less than that identified based on the *S. litura* genome (Cheng *et al.*, 2017). This may be explained by the presence of GRs in other chemosensory tissues of *S. litura,* such as the larval mouthparts or adult labial palps, which should be evaluated further. Sequence analysis showed that 27 sequences had full-length ORFs that encode 339–482 amino acids with 5–8 TMDs. Based on the FPKM results, 18, 13, 23, and 26 SlitGRs were found in the Egg, LH, FA, and MA tissues, respectively. Similar to SlitORs, SlitGRs were most frequent in MA (14 genes), followed by FA (seven genes, including the new gene SlitGR240), Egg (nine genes, including two new genes SlitGR241 and 242), and LH (0 genes) (fig. 5), indicating that these SlitGRs may play key roles in the chemosensory process of adult moths, based on the antennae.

A phylogenetic tree was constructed based on GRs of *S. litura*, *B. mori*, *H. armigera*, and *D. plexippus*. Three SlitGRs (SlitGR1, 2, and 3) belong to the carbon dioxide receptor subfamily, 2 SlitGRs (SlitGR8 and 9) belong to the fructose receptor subfamily, and 4 SlitGRs (SlitGR4, 5, 6, and 7) belong to the sugar receptor subfamily (fig. 5c), indicating that these SlitGRs participate in the recognition of carbon dioxide (Jones *et al.*, 2007; Kwon *et al.*, 2007), fructose (Jiang *et al.*, 2015), and sugar (Sato *et al.*, 2011).

Dynamic expression of chemosensory genes in the heads of *S. litura* larvae

We randomly selected eight chemosensory genes (*SlitOBP6*, *SlitOBP8*, *SlitOBP9*, *SlitOBP25*, *SlitCSP10*, *SlitCSP18*, *SlitOR77*, and *SlitGR22*) for validation using qRT-PCR. The qRT-PCR results were consistent with the FPKM data (fig. 6).

To estimate the function of chemosensory genes during larval feeding in *S. litura*, we measured the dynamic expression levels of genes with LH-biased expression using qRT-PCR. Prior to these experiments, it was necessary to establish the criteria for determining distinct stages of larval development. According to previously established criteria, the larvae were assigned to six instars according to the size of the larval head capsule (Sannino and Espinosa, 1999). In total, 2 (*SlitCSP1* and *SlitCSP7*) and 7 (*SlitOBP8*, *SlitOBP9*, *SlitOBP25*, *SlitCSP18*, *SlitOR34*, *SlitGR240*, and *SlitGR242*) genes had higher expression in the heads of 3- to 5-day-old larvae than at other stages ($P < 0.05$), respectively (fig. 7). Usually, 3- to 5-day-old larvae of *S. litura* and other noctuid moths are crucial for crop feeding (Jacquin-Joly *et al.*, 2001; Huang *et al.*, 2009; Zou *et al.*, 2016). SexiOBP13 of *S. exigua* is highly expressed in the heads of 3-day-old larvae and can bind to two kinds of host volatiles (nerolidol and farnesol) (Jin *et al.*, 2015). SexiOBP2 of *S. exigua* displays LH-biased expression and functional studies have indicated that it may participate in larval food searching (Liu *et al.*, 2015a). De Fouchier *et al.* (2018) found nine ORs in *S. littoralis* larvae that are involved in host searching. BmorGR66 of *B. mori* shows larval maxilla-biased expression and is a major factor affecting the larvae feeding preference (Zhang *et al.*, 2019). Based on these findings, we predicted that the nine chemosensory genes of *S. litura* may play key roles in larval feeding (Jacquin-Joly *et al.*, 2001). However, the other three genes (*SlitCSP6*, *SlitOR77*, and *SlitGR13*) of *S. litura* did not show similar expression characteristics, suggesting that they may be involved in olfactory and other physiological behaviors of larvae; however, experimental confirmation is needed.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485321000109

Acknowledgments. The authors thank Miss Yong-Jie Zhang for insect photography, Dr Tingcai Cheng for kindly proving the genomic data of *S. litura*, and Professor Kazuei Mita for kindly proving the sequences of some *S. litura* chemosensory genes.

Financial support. This work was supported by the National Natural Science Foundation of China (31970456), Anhui Provincial Natural Science

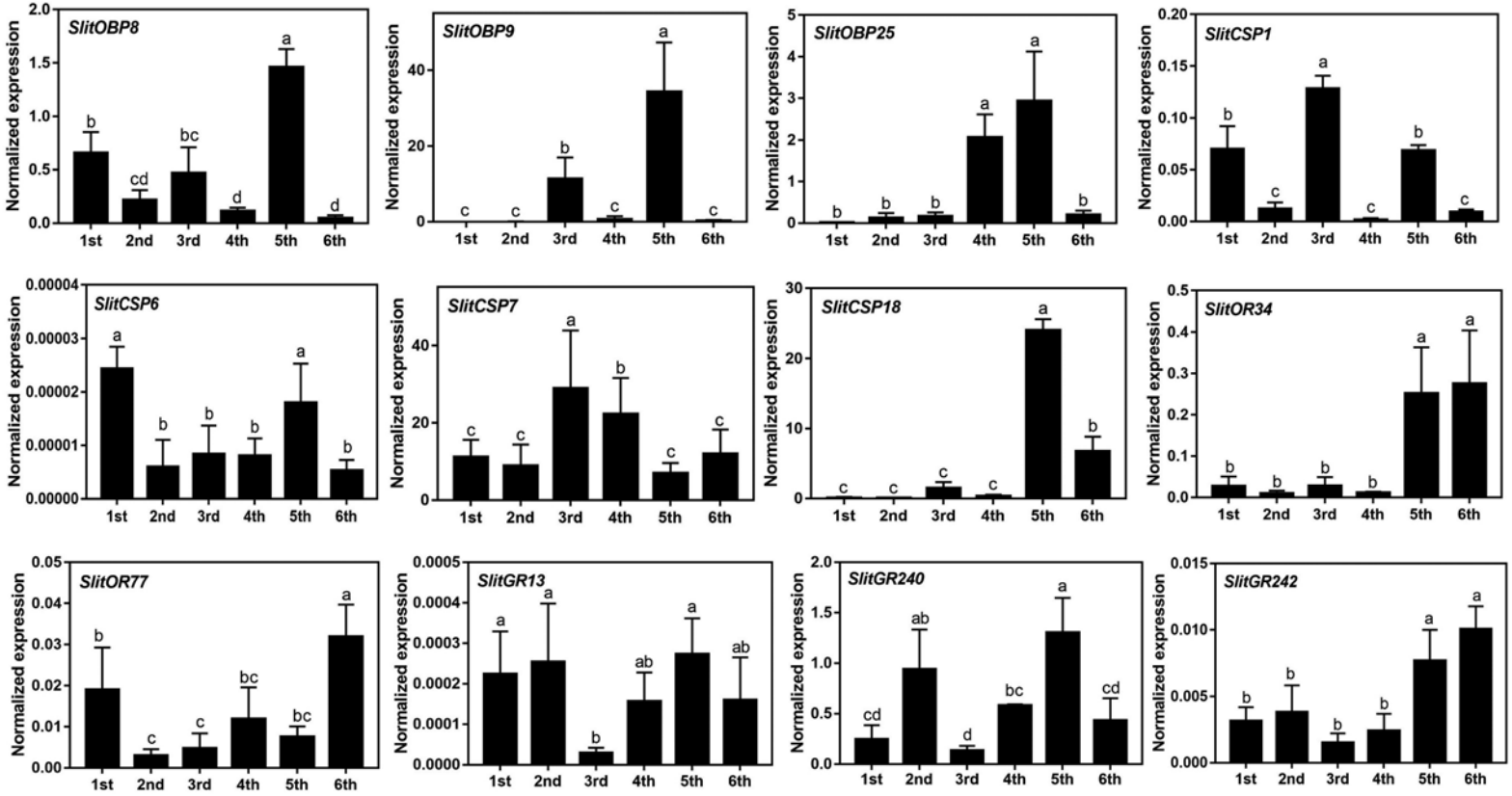

Fig. 7. The dynamic expression of chemosensory genes in the heads of *S. litura* larvae. The different low case letters mean significance between tissues. Error bar represents mean ± SEM.

Foundation (2008085MC63), and Natural Science Fund of Education Department of Anhui Province, China (KJ2019A0586 and KJ2019B09).

Conflict of interest. The authors declare that they have no conflict of interest.

Ethical standards. This article does not include any study on human participants or animals performed by any of the authors.

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