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Analysis of a cDNA library from the antenna of *Cnaphalocrocis medinalis* and the expression pattern of olfactory genes

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ABSTRACT

Chemoreception is a key feature in selection of host plants by insects. In this study, preliminary characterization and isolation of cDNA clones from *Cnaphalocrocis medinalis* antennal libraries identified eight olfactory genes, including two putative general odorant-binding proteins (GOBPs), three pheromone binding proteins (PBPs) and three chemosensory proteins (CSPs). The expression profiles of these eight genes in different tissues (antenna, head (without antennae), thorax, abdomen, leg and wing) were measured by real time qPCR. The results showed that GOBP and PBP genes in *C. medinalis* seemed to be antenna-specific, but differentially expressed in male and female antennae; while CSP genes were expressed ubiquitously during different developmental stages, but with an extremely elevated transcript level in antennae, legs and wings compared to head, thorax and abdomen. And also, the transcription levels of olfactory genes depended on the age, sex, and mating status of the adults. These findings support the hypothesis that OBPs and CSPs play dynamic roles during development of *C. medinalis* and are likely to be involved in broader physiological functions.

1. Introduction

A sensitive olfactory system seems to be particularly important in insects for reproduction (locating mates and oviposition sites) and nutrition (detecting food sources) [1]. Binding proteins provide the initial molecular interactions for chemical signals (semiochemicals) such as pheromones and host odors and are thought to ferry the semiochemical molecules across the antennal sensillum lymph to the olfactory receptors (ORs). To date, two classes of small, globular, water-soluble, extracellular proteins, odorant binding proteins (OBPs) and chemosensory proteins (CSPs), have been identified in the sensillar lymph of chemosensilla [2–4]. OBPs are members of a multi-gene family that includes the pheromone-binding proteins (PBPs), general odorant-binding proteins (GOBPs) and antennal binding proteins (ABPs) in Lepidoptera insects [5]; they all displayed limited sequence homology but have the same sequence motif of the six conserved cysteine residues. CSPs contain four cysteines, sharing neither sequence homology nor structural similarity with OBPs [3]. Although more than two decades of research have accumulated a very large amount of structural data on insect binding proteins [6], no convincing model for the role of OBPs and CSPs in chemoreception has yet been produced.

The leaf folder, *Cnaphalocrocis medinalis* (Guenée) is a migratory rice pest which is widely distributed in humid tropical and temperate regions of Asia, Oceania and Africa [7]. The larvae damage plants by folding the leaves and scraping the green leaf tissues within the fold, causing yield loss by reducing photosynthetic activity [8]. Chemical treatments are often impracticable due to the cryptic feeding habit. Consequently, it is an urgent need to develop a sustainable method of controlling the *C. medinalis*. Regulating olfactory chemoreception to control target insect pests is a potential pest management measure.

In order to study the function of the OBP and CSP genes, we established a cDNA library from antennae of *C. medinalis*, and identified eight olfactory-related genes. The temporal and spatial expression patterns of these eight genes in *C. medinalis* were further studied by real time qPCR.

2. Materials and methods

2.1. Insects and tissues

C. medinalis larvae or pupae were collected in Wuxue, China (115°45′E; 30°00′N). Sexed pupae were kept inside glass tubes until moths emerged. Immediately after emergence, female and male adults were provided with a 10% sucrose solution. To obtain mated females, newly emerged (0 day) male and female moths were paired in plastic-screen cages (20 × 20 × 10 cm).

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The antennae from male and female adults were mixed to construct a cDNA library. For tissue and spatial expression, antenna (At), heads (without antennae) (H), thoraces (T), abdomina (Ab), legs (L) and wings (W) were isolated from the adult virgin and mated males and females, and from mated males and females at different stages of development after eclosion. Tissues were collected from 0-day-old, 1-day-old and 3-day-old male and female moths, while for females, 5-day-virgin and 5-day-mated were also collected. Tissues were stored at −70 °C until used.

2.2. Construction and screening of antennal cDNA library

Total RNA was extracted from 200 mixed antennae from *C. medinalis* males and females (1:1) by using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and the concentration was assessed before reverse transcription. For cDNA synthesis and subsequent library construction, 1 μg of total RNA was used in accordance with the manufacturer's protocol of the SMART™ cDNA library construction kit (Clontech, Mountain View, CA). Inserts between 300 bp and 700 bp in length were selected and sequenced. Duplicate genes were identified by using the alignment module of DNAMAN software [9]. Homology analysis of the obtained sequences against some known protein sequences was performed using the BLASTP program and GenBank Database (http://www.ncbi.nlm.nih.gov/genbank/).

2.3. RACE and reverse transcription PCR

The preparation of 5′ and 3′ RACE-ready cDNA and PCR reactions were conducted following the instructions of the SMARTer™ RACE cDNA Amplification Kit (Clontech). Single strand cDNA was synthesized with Revert Aid™ First-Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). After obtaining the full length gene sequences, normal RT-PCR was performed with each specific primer pair (Supplementary Table 1) using rTaq DNA polymerase (Takara Bio Inc., Shiga, Japan). The resulting amplified products were subjected to electrophoresis on 1% (w/v) agarose gels in TBE and visualized with ethidium bromide.

2.4. Sequence analysis and phylogenetic tree analysis

By using the NCBI BLAST network server, protein sequences were identified and amino acid sequences of the same family in the same or different species were found and retrieved from GenBank. Data were aligned and compared using ClustalX (1.8), and then a phylogenetic tree was constructed by the Neighbor Joining (NJ) method using the MEGA5 program [10].

2.5. Analysis of tissue-specific expression

Total RNA was extracted from tissue samples according to the methods described and treated with DNase I (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using the One Step SYBR®PrimeScript® RT-PCR kit (Takara Bio Inc.) and then tested using the ABI 7500 Fast real time qPCR System (SYBR Green PCR Master Mix; Applied Biosystems). To check reproducibility, all test samples and the endogenous controls were amplified in triplicate. Cycling parameters were: 94 °C for 2 min, 40 cycles at 95 °C for 10 s and 60 °C for 40 s. Agarose gel electrophoresis, sequencing and melting curve analysis of products all indicated that none of the reactions produced non-specific amplification. CmedActin was used as an endogenous control to normalize the expression of target genes in olfactory tissues. Antennae of 0-day-old females were used as a calibrator to calculate ΔΔCt values between tissues (ΔCt male antennae or female tissues of any age − ΔCt female antennae of 0 day). Finally, the data were performed using the comparative $2^{-\Delta\Delta Ct}$ method [11] and represented as mean ± SE ($n = 3$) from three replicates, and then one-way ANOVA analysis was conducted to determine the difference.

3. Results

3.1. EST cloning and identification

The cDNA libraries of the antennae were constructed from a pool of total RNAs extracted from the antennae of male and female *C. medinalis*. The titer of the library was approximately 6×10^6 pfu/ml, and the quality of the library conformed to test requirements. A total of 398 clean clones were obtained, consisting of 111 contigs and 287 singlets. The EST length distributions are shown in Fig. 1. BLAST searches classified all the ESTs into clusters of orthologous groups (COGs), 55 of which were matched and classified into eight functional categories (Fig. 2). 154 high quality ESTs were acquired and 137 of them were unigenes after assembly. When blasted against the NCBI Nr Database, 56.2% were matched. Database searches were performed with the blast program (NCBI) and sequence alignment with DNAMAN, and then a final eight genes were scored at last, containing two full-length sequences and six segments.

3.2. Clone sequencing and bioinformatics analysis

Full length sequences were obtained from 3′ and 5′ RACE-PCR of segments. According to the BLAST analysis using GenBank, two GOBPs, three PBPs and three CSPs were identified (see Table 1).

GOBPs and PBPs were identified by their characteristic features, containing six conserved cysteines (Fig. 3). Performing an NCBI blast search on GOBPs showed that all of them belonged to the (pfam01395), the PBP/GOBP family, and that PBPs belonged to PhBP (smart00708), insect pheromone/odorant binding protein domains. They all showed high similarity to putative GOBPs and PBPs in Lepidoptera, with approximately 57–76% identity with other insects, respectively.

CSP genes identified from the cDNA library of antennae contained four conserved cysteines forming the classic CSP motif (except CmedCSP1): C1–X6–C2–X18–C3–X2–C4 (Fig. 3). All of the three CSPs were blasted in NCBI and the results showed that they belonged to the OS-D (pfam03392), insect pheromone-binding family, A10/OS-D. CmedCSP1 showed only 25–35% similarity to putative CSPs, while CmedCSP2 and CmedCSP3 showed greater similarity to Lepidoptera CSPs, displaying high similarity with DpleCSP at 74% and 71%, respectively.

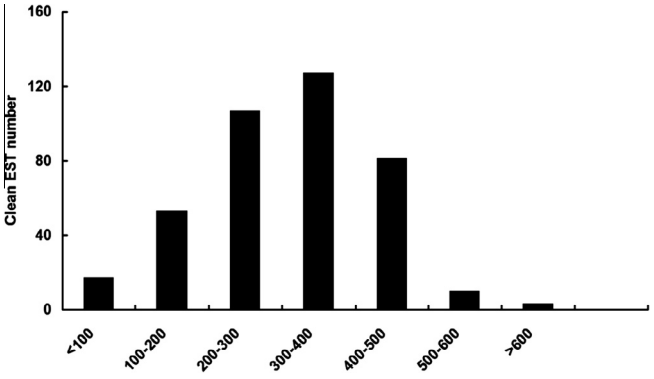

Fig. 1. Clean EST length distribution of the *C. medinalis* libraries. A total of 598 ESTs were identified after elimination of vector sequence and low quality sequences.

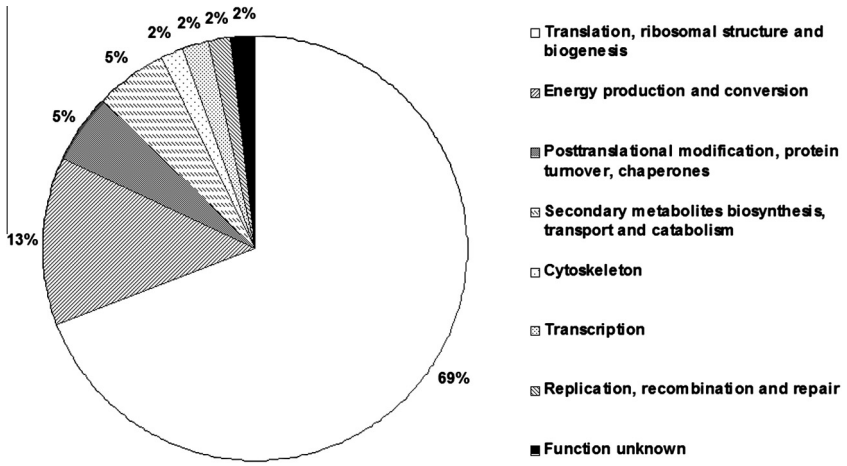

Fig. 2. Classification of ESTs from the cDNA library of *C. medinalis* based on their putative functions.

Table 1
List of OBPs and CSPs in *C. medinalis.*

Gene name	ORF	Length	Accession number	Homology search with known proteins			
				Score	E-value	Identify	Species
CmedGOBP2	363	120	KC507183	200	7.00E −64	76	*B. mori*
CmedGOBP3	423	140	KC507179	213	4.00E −68	80	*D. plexippus*
CmedPBP2	495	164	KC507181	242	7.00E −29	65	*D. indica*
CmedPBP3	549	182	KC507184	170	1.00E−50	60	*L. sticticalis*
CmedPBP4	447	148	KC507185	197	2.00E−61	57	*D. indica*
CmedCSP1	330	110	KC507178	84.3	2.00E−18	35	*H. armigera*
CmedCSP2	375	125	KC507180	186	3.00E−58	75	*D. plexippus*
CmedCSP3	372	124	KC507182	186	6.00E−58	71	*D. plexippus*

Abbreviations: Gene name, name of genes identified from *C. medinalis*; ORF, open reading frame; Length, number of amino acids including signal peptide region; E-value, the statistical significance of reported matches; Identities (%), percentage of amino acid identities between *C. medinalis* and homologs; Species, source species of homologous gene. *B. mori: Bombyx mori, D. plexippus: Danaus plexippus, D. indica: Diaphania indica, L. sticticalis: Loxostege sticticalis, H. armigera: Helicoverpa armigera.*

Fig. 3. Alignment of the OBPs and CSPs in C. *medinalis*. Their full-length amino acid sequences were aligned by DNAMAN. Black boxes show the conserved cysteine motif.

3.3. Phylogenetic analysis

The phylogenetic relationships of the predicted GOBPs, PBPs and CSPs in Lepidoptera and other insect species are shown in Fig. 4. From this phylogenetic tree, it could be seen that OBP and CSP genes first formed two distinct branches, then PBP and GOBPs separated. Database searches suggest that CmedPBP3 has the highest identity (60%) with LstiPBP-F1, and the neighbor-joining tree sequence analysis also shows that they are in the same branch. All olfactory genes in *C. medinalis* show high similarity in species of Lepidoptera, with the exception of CmedCSP1. CmedCSP1 is in the branches of previously CSPs, however, the identities are all quite low and the neighbor-joining tree analysis of sequences also shows low similarity with the others.

3.4. Expression patterns

To further study the function of olfactory genes identified in *C. medinalis*, we precisely compared the relative transcript levels of each gene in different tissues. All the genes were found to have distinct patterns of expression (Table 2). The transcript levels of them were all very low in the pupal stage. The expression levels of OBPs were high in the moth antenna but very low in the other tissues tested through all the days after eclosion (0, 1, 3, and 5 days). CSPs could be detected in all of the tissues by real time qPCR, although some of them had low transcript levels.

The PBPs and GOBPs were all expressed more highly in male antennae than in females. The expression level of CmedPBP2 in 0-day-old male antennae was about 95-fold higher than in females. Expression levels of OBPs in female antennae were also affected by the moths' age, for the highest expression of CmedPBP2, CmedPBP3, CmedPBP4 was observed in 0-day-old, 1-day-old and 5-day-old moths respectively, while GOBPs were in those of 5-d-old. The expression of OBPs had no significant difference between mated and unmated females ($P < 0.05$), although CmedGOBP2 and CmedGOBP3 in mated females were slightly higher than in virgins, indicating that the mated status of the moths did not affect expression of the OBPs in female antennae, neither PBPs nor GOBPs.

In contrast to OBPs, the expression levels of all three CSPs were higher in wings and legs, and there was no significant difference between males and females. In general, the expression levels of the three CSPs in wings increased with the moth's development, although that in 5-day-old females was lower than in those of 3-day-old, and there was no obvious expression difference in the legs at different ages. However, the transcript levels of the three CSPs in both legs and wings of mated females were significantly higher than unmated moths ($P < 0.05$). With the exception of CmedCSP1, which was expressed at levels in male antenna which were about 5-fold higher than in females at 0-day old, there was no significant difference between males and females with regard to expression of CmedCSP2 and CmedCSP3, or of CmedCSP1 at all other ages examined. In addition in other tissues, the transcript levels of all three CSPs showed no significant differences with age (Supplementary Table 2).

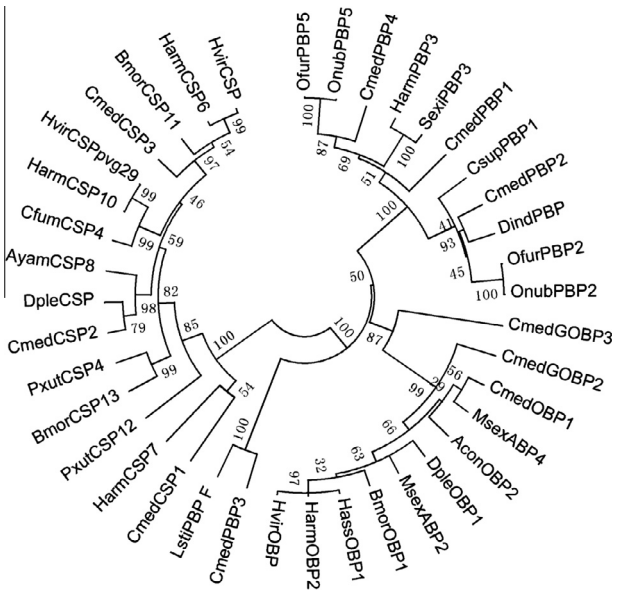

Fig. 4. Neighbor-joining tree of all known OBP sequences from other insects created using MEGA 5.0. Full-length amino acid sequences of CmedOBPs and CmedCSPs were used in this analysis. Numbers on the branches show the values from 1000 replicate bootstrap analyses. The accession numbers of the eight olfactory genes related are listed in Table 2. The other insect species listed are as follows: HvirCSP: ACX 53813, HarmCSP6: AEX 07267, BmorCSP11:NP_001091779, HvirCSPpvg29:EZ407157, HarmCSP10:AFR92094, CfumCSP4:AAW23971, AyamCSP8: ADV36661, DpleCSP: EHJ73337, PxutCSP4: BAF91714, BmorCSP13:NP_001037180, PxutCSP12:-BAG71920, HarmCSP7:HQ874663, LsitPBPF:ACF48467, HvirOBP: ACX53711, HarmOBP2:AEB54586, HassOBP1:AEX07275, BmorOBP1:NP_001140185, MsexABP2: AAL60416, DpleOBP1:EHJ65653, AconOBP2:AFD34178, MsexABP4:AF393490_1, OnubPBP2:ADT78496, OfurPBP2:ADT78501, DindPBP:BAG71419, CsupPB-P1:ADK66921, SexiPBP3:ACY78413, HarmPBP3:AAO16091, CmedOBP1:AFG72998, CmedPBP1:AFG72999, OnubPBP5:ADT78499, OfurPBP5:ADT78504.

4. Discussion

We constructed the first antennal cDNA library of *C. medinalis* and sequenced 398 positive clones. The COG classification shows that these clones have a variety of different functions, with roles in translation, ribosomal structure and biogenesis, energy production and conversion, posttranslational modification, protein turnover, chaperoning, secondary metabolite biosynthesis, transport and catabolism, the cytoskeleton, transcription, replication, recombination and repair, as well as some with unknown function. After discrimination and classification, we identified two GOBP, three PBP and three CSP genes from the library. These orthologs and numbers are consistent with CSPs and OBPs identified in transcriptomes of other Lepidoptera [12–17].

The OBPs of insects have been described as transport proteins that transfer hydrophobic semiochemicals in the sensillum cavity. However, the exact functions of GOBPs have not yet been clarified, except for some binding studies which have confirmed that OBPs have binding affinity to a broad range of odorants, suggesting that GOBPs might play a more important role in selectivity and specificity of odorant perception [18–23].

The expression patterns of OBPs in *C. medinalis* may help us to characterize the function of these proteins in future research. The results of real time qPCR showed that the expression levels of all OBPs in *C. medinalis* were very low in the pupal stage, and during the adult stage were mainly expressed in the antennae, particularly in the male antennae. Soluble PBPs in the sensillum lymph surrounding the dendrites are thought to transfer the usually hydrophobic pheromone molecules to the dendrite membrane of the sensory neurons [24], and are male biased in expression [25,26]. Several studies supported the role of PBP in pheromone detection, since female moths release a blend of sex pheromones to attract males over long distances, and males detect the released pheromones with extreme sensitivity and selectivity [27]. Because of the similar expression pattern of CmedGOBP2 and CmdPBP4, we believe that CmedGOBP2 may play the same role as a PBP, especially since BmorGOBP2 has already been reported to bind the *B. mori* sex pheromone component [21]. In females, the transcription levels of CmedOBPs also correlated with age, as in males, and did not depend on mated status. Females and males *C. medinalis* reach

Table 2
Relative expression of eight olfactory genes in *C. medinalis* (mean ± SE).

Tissues	Female						Males			
	P	0d	1d	3d	5dV	5dM	P	0d	1d	3d
CmedOBP2-At	0.027 ± 0.004b	0.679 ± 0.210ab	0.757 ± 0.441ab	0.260 ± 0.076b	1.877 ± 0.710a	2.781 ± 0.035ns	0.011 ± 0.004C	15.150 ± 1.573A	9.707 ± 0.084B	14.612 ± 1.735A
CmedOBP3-At	0.037 ± 0.012b	0.633 ± 0.184b	0.554 ± 0.252b	0.457 ± 0.113b	2.190 ± 0.609°	3.263 ± 0.826ns	0.048 ± 0.005C	2.197 ± 1.031AB	0.914 ± 0.373BC	3.789 ± 0.552A
CmedPBP2-At	1.80E−04 ± 5.65E−05b	0.334 ± 0.332b	1.122 ± 0.263a	0.655 ± 0.005ab	3.51E−03 ± 3.64E−05b	4.30E−03 ± 0.91E−05ns	4.03E−04 ± 1.27E−05C	31.722 ± 5.549A	17.769 ± 1.658B	36.675 ± 5.550A
CmedPBP3-At	0.015 ± 0.003c	1.121 ± 0.082a	0.870 ± 0.140ab	0.861 ± 0.297ab	0.382 ± 0.030bc	0.324 ± 0.083ns	0.010 ± 0.001C	3.458 ± 0.570A	1.399 ± 0.338B	3.638 ± 0.649A
CmedPBP4-At	2.80E−03 ± 2.28E−04b	0.356 ± 0.322b	0.513 ± 0.115b	0.192 ± 0.076b	1.504 ± 0.407°	1.374 ± 0.208ns	4.27E−03 ± 2.97E−04C	4.521 ± 0.684A	0.704 ± 0.049C	1.723 ± 0.163B
CmedCSP1-At	0.322 ± 0.079 b	0.757 ± 0.121 a	0.178 ± 0.058 b	0.108 ± 0.022 b	0.169 ± 0.004 b	0.151 ± 0.005ns	0.189 ± 0.032B	2.707 ± 0.262A	0.225 ± 0.043B	0.223 ± 0.034B
CmedCSP1-L	—	0.502 ± 0.151 a	0.171 ± 0.009 ab	0.086 ± 0.011 b	0.072 ± 0.019 bc	0.617 ± 0.247*	—	1.235 ± 0.199A	0.164 ± 0.009B	0.134 ± 0.004B
CmedCSP-W	—	0.452 ± 0.079 b	0.726 ± 0.185 b	1.454 ± 0.256 a	1.019 ± 0.123 ab	2.087 ± 0.211*	—	0.651 ± 0.197B	0.520 ± 0.106B	1.761 ± 0.336A
CmedCSP2-At	0.574 ± 0.162 d	0.997 ± 0.177 cd	1.181 ± 0.194 c	9.222 ± 0.096 a	5.176 ± 0.047 b	5.845 ± 0.261ns	0.617 ± 0.083D	2.779 ± 0.618C	4.252 ± 0.571B	9.102 ± 0.320A
CmedCSP2-L	—	0.750 ± 0.209 b	2.483 ± 0.603 b	4.464 ± 0.576 a	4.082 ± 0.834 ab	53.878 ± 18.128*	—	0.899 ± 0.448C	3.733 ± 0.045B	6.287 ± 0.514A
CmedCSP2-W	—	0.074 ± 0.024 c	9.607 ± 1.143 bc	27.355 ± 5.976 a	13.099 ± 2.167 b	28.406 ± 1.525*	—	0.023 ± 0.003C	8.370 ± 1.735B	21.698 ± 3.888A
CmedCSP3-At	0.045 ± 0.017 b	0.446 ± 0.033b	0.310 ± 0.172 b	0.679 ± 0.068 b	1.330 ± 0.343 a	1.675 ± 0.041ns	0.042 ± 0.005C	5.245 ± 0.776A	1.361 ± 0.005B	3.168 ± 0.149A

One of the 0 day antennae samples was used as the calibrator, P: pupae, 0d, 1d, and 3d refer to the adult at 0, 1 and 3 days after eclosion, 5dV and 5dM refer to the 5-day-old female adult, 5d virgin or mated, respectively. The script in lower case letters shows the result of one way ANOVA between different stages of females while the upper case letters shows the same in males. Comparing 5-d female virgin and mated moths by one-way ANOVA, ns indicates no significant difference while * indicates a significant difference ($P < 0.05$).

sexual maturity at 5 days and 1 day after emergence, respectively, and oviposition usually commences within 24 h of mating [28]. The high expression levels of CmedGOBPs in 5-day-old females may help them locate suitable hosts in preparation for ovipositioning. CmedPBPs also showed similar expression levels to GOBPs in females, with the exception of CmedPBP2; consequently we considered that CmedPBP3 and CmedPBP4 were likely to share functions not only with PBPs but also with GOBPs.

CmedCSP genes were expressed ubiquitously, while different CmedCSP genes displayed different expression patterns during different stages of development and in different tissues. In moths, CSPs or their transcripts have been identified generally in the thorax, abdomen, leg and head tissue [29]. CmedCSP1 and CmedCSP2 are highly expressed mainly in the antennae, but also in legs and wings. Labeling was observed in the sensilla chaetica of the antennae, but not in the olfactory sensilla or sensilla coeloconica, leading to the suggestion that in Orthoptera, CSPs are involved in contact chemoreception; this is also the case in Lepidoptera. Many insects show high expression levels in antennae, legs and wings but a lower level in the abdomen, thorax and head in both sexes [12]. Similar to OBPs, the expression levels of CmedCSPs differed with age; moreover the expression of CmedCSP1 and CmedCSP2 was also affected by the mated status of *C. medinalis,* with mated individuals showing significantly higher expression than unmated ones. It has been reported that mated *P. xylostella* individuals showed much higher expression levels of PxylCSPs at 24 h post-emergence compared to their unmated counterparts [30]. It has also been reported that many adult female insects do not automatically oviposit once they reached the spawning place, but some first judge its appearance through the tarsal sensilla [31]. Therefore, we suspect that adult female moths may be attracted to a potential host, however, before ovipositioning, they may make some judgment on the surface of the leaves using their legs. CmedCSP3 is only expressed in any great quantity in the antennae and has an extremely low level of expression in other tissues, similar to the expression pattern of CmedOBPs. All these results indicate that CSPs in *C. medinalis* may not only have an olfactory function in finding a host after eclosion or in seeking a place for ovipositioning, but might also play roles in gustation. In *Apolygus lucorum*, AlucCSP1 has proved to have a high affinity with most cotton secondary metabolites [32].

During different developmental stages, *C. medinalis* exhibits different behavior; therefore, it may be expected that it could be sensitive to different odors, which would result from changes in the expression of different OBPs and CSPs. Future work will focus on the binding characteristics of these proteins and their relationship with volatile compounds released from paddy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.038.

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