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Deep sequencing of antennal transcriptome from *Callosobruchus chinensis* to characterize odorant binding protein and chemosensory protein genes

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ABSTRACT

The sophisticated olfactory system of insects has a key role in their growth and reproduction, and elucidating the molecular mechanisms of this system in economically important insect species will be useful for development of new and effective insecticides. *Callosobruchus chinensis* (Linnaeus) (Coleoptera, Bruchidae) is a destructive pest of stored food, and is widely distributed around the world. However, our understanding at the molecular level of the olfactory mechanism in this beetle is limited. In the present study, antennal transcriptomic data was obtained using Illumina sequencing, and a total of 69,847 unigenes was assembled. Bioinformatics analyses identified a set of putative olfactory genes, including 21 odorant binding proteins (OBPs) and five chemosensory proteins (CSPs). We constructed phylogenetic trees of these OBPs and CSPs together with those from other insect species, and found high orthology with coleopteran species. The expression levels of OBPs and CSPs were estimated by the reads per kilobase per million mapped reads (RPKM) method. Our results provide the foundation to identify other chemoreception-related genes. It is to be hoped that some of these genes will be potential targets for developing novel behavioral blocker compounds and pesticides to interfere with the olfactory system of *C. chinensis* and help reduce population densities.

1. Introduction

Insects have a complex life cycle that makes use of a sophisticated olfactory system to control many aspects of growth, feeding, and reproduction, such as foraging, host location, mate choice, and identifying oviposition sites (Field et al., 2000; Leal, 2013). In this olfactory system, the antennae have crucial roles (Li et al., 2015b). Insects use their antennae to detect odors and to sense the external environment (Gu et al., 2015a). Specifically, various odorant molecules, including host plant volatiles and sex pheromones, pass through the polar holes of sensilla that are widely distributed on the surface of the antennae (Steinbrecht, 1997). A series of olfaction-related proteins are involved in this complex biochemical process, such as odorant binding proteins (OBPs), chemosensory proteins (CSPs), and other chemosensory receptors (Jacquin-Joly and Merlin, 2004; Leal, 2013; Zhang et al., 2013a, 2016; Zhou, 2010). In insect olfaction, external odorants are first recognized and captured by OBPs/CSPs, which are located in the antennal sensilla lymph, and then the OBPs/CSPs deliver the odorants through the lymph to chemosensory receptors (Pelosi et al., 2006). Thus, OBPs and CSPs act as transporters and regulate insect

Abbreviations: PCR, Polymerase chain reaction; cDNA, Complementary DNA; BLAST, Basic local alignment search tool; RPKM, Reads per kilobase per million mapped reads; Nr, Non redundant; COG, Clusters of orthologous groups of proteins; GO, Gene ontology; OBP, Odorant binding protein; CSP, Chemosensory protein.

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physiological behavior by interacting with target binding odorants.

Insect OBPs were first identified in the antennal sensilla lymph of males of the silk moth *Antheraea polyphemus* (Saturniidae) (Vogt and Riddiford, 1981). Since then, a large number of OBPs have been found in other insect species. The physico-chemical characteristics of OBPs have been investigated and it has been shown that OBPs are small, hydrosoluble proteins with three disulphide bridges that stabilize their three-dimensional structure (Sandler et al., 2000; Zhou et al., 2009). Notably, there is no homology between insect OBPs and vertebrate OBPs (Pelosi et al., 2014; Zhou et al., 2009). In insects, OBPs are generally divided into three subfamilies according to protein structure and amino acid sequence similarities: classic OBPs (with six conserved cysteines), minus-C OBPs (four conserved cysteines), and plus-C OBPs (more than six conserved cysteines) (Schultze et al., 2012; Spinelli et al., 2012; Zhou, 2010). Compared with OBPs, CSPs are smaller and show greater evolutionary conservation, even in phylogenetically distant species (Ban et al., 2003; Jacquinjoly et al., 2001). Following the identification of the first insect CSP-class gene (OS-D) in *Drosophila melanogaster* (Diptera, Drosophilidae) (McKenna et al., 1994; Pikielny et al., 1994), a number of CSPs have now been reported. In addition, CSP protein structures differ from those OBPs notably by containing two disulphide bridges with four conserved cysteines (Lartigue et al., 2002; Pelosi et al., 2005; Zhang et al., 2013b).

The adzuki bean beetle, *Callosobruchus chinensis* (Linnaeus) (Coleoptera, Bruchidae), is a destructive storage pest that is widely distributed around the world (Duan et al., 2016). The beetle has become a growing threat to harvested products of leguminous crops, such as mung bean (Shlnoda et al., 1991), adzuki bean (Tomooka et al., 2000), pigeon pea (Nahdy et al., 1998), and soybean (Duan et al., 2014). Adult *C. chinensis* oviposit eggs on the bean surface, and the larvae then penetrate and feed on the beans. As *C. chinensis* selects legumes to complete its life cycle, it is an egregious pest of leguminous crops (Tuda et al., 2004).

In the current study, we performed a sequence analysis of the transcriptome of *C. chinensis* antennae, and identified 21 OBP and 7 CSP genes after analyzing the transcriptomic data. We constructed phylogenetic trees of OBPs and CSPs to investigate orthologues between *C. chinensis* and other insect species. Gene expression levels were estimated by the reads per kilobase per million mapped reads (RPKM) method, which reflects the abundance of gene transcripts in the antennae. The results may provide a further understanding of the chemosensory mechanisms regulated by OBPs/CSPs in *C. chinensis* and provide new insights for the development of improved pest management strategies.

2. Materials & methods

2.1. Insect rearing and antennae collection

C. chinensis were fed on mung beans using the standard culture conditions, including a temperature of 25 ± 1 °C, relative humidity of 75 ± 10%, and 14 h light/10 h dark. In order to minimize inter-individual differences, 400 newly-emerged beetles (200 males and 200 females) were collected at the same time. The antennae of the collected beetles were dissected and flash frozen in liquid nitrogen, and stored at −80 °C until required for later analyses.

2.2. RNA extraction and cDNA library construction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA purity and degradation was tested by NanoPhotometer® spectrophotometry (IMPLEN, CA, USA) and 1% agarose gels. The cDNA library construction and Illumina sequencing of antennae samples were performed at Novogene Bioinformatics Technology Co., Ltd., Beijing, China. Sequencing libraries were generated using NEBNext® Ultra RNA Library Prep Kit for Illumina® (New England Biolabs, USA) following the manufacturer's recommendations. First strand cDNA was synthesized using random hexamer primers and M-MLV Reverse Transcriptase (RNaseH). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. After end-repair, adenylation of 3′ ends, and ligation of adaptors, the library fragments were purified with an AMPure XP system (Beckman Coulter, Beverly, USA) to select cDNA fragments of 150–200 bp. The size-selected and adaptor-ligated cDNAs were mixed with 3 μl USER Enzyme (NEB, USA) and incubated at 37 °C for 15 min followed by 5 min at 95 °C. PCR was then performed using Phusion High-Fidelity DNA polymerase, universal PCR primers, and index (x) primer. The PCR conditions were 98 °C for 1 min (1 cycle), 98 °C for 10 s, 50 °C for 30 s and 72 °C for 60 s (30 cycles), followed by 72 °C for 5 min. Finally, PCR products were purified (AMPure XP system) and library quality was assessed using an Agilent Bioanalyzer 2100 system.

2.3. Transcriptome sequencing and assembly

The libraries were sequenced on an Illumina Hiseq™ 4000 platform, and paired-end reads were generated. Clean reads were obtained by removing impurities of raw data such as adapters, oversized insertions, low quality reads, poly N reads, and small reads. The transcriptome assembly program Trinity (Grabherr et al., 2011; Li et al., 2010) was used to generate transcripts, based on clean and high quality data. The remaining longest transcript for each gene was selected as an unigene.

2.4. Gene functional annotation and sequence analysis

All unigenes obtained from the *C. chinensis* antennal transcriptome were aligned to protein databases, including the non-redundant (Nr) database, Swiss-Prot, Pfam, and COG, using the BLAST search tool to discover the highest similarity to the given unigene and their candidate functional annotations (E-value $<10^{-5}$). In addition, GO annotation was carried out with Blast2GO software (Gotz et al., 2008), and diverse GO terms were enriched to obtain functional annotations using GOseqR packages (Young et al., 2010). The open reading frame (ORF) was predicted with the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptides of chemosensory gene proteins were predicted with SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). Similarity searches for each gene were performed at the NCBI-Blast network platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Gene expression analysis of OBPs and CSPs

Gene expression levels of OBPs and CSPs were estimated by the RPKM method (Mortazavi et al., 2008), using the formula: RPKM (A) = (1,000,000 × C × 1000)/(N × L), where RPKM (A) is the abundance of gene A, C is the number of reads that uniquely align to gene A, N is the total number of reads that uniquely align to all genes, and L is the number of bases in gene A. The RPKM method minimizes the influence of sequencing depth and gene length when estimating gene expression levels.

2.6. Phylogenetic analysis

Phylogenetic trees were constructed using the amino acid sequences of OBPs and CSPs from *C. chinensis* and other insect species (Table S1). The OBP data set contained 203 sequences from seven