Gene 591 (2016) 425–432

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Identification of novel odorant binding protein genes and functional characterization of *OBP8* in *Chilo suppressalis* (Walker)

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ARTICLE INFO

Article history:
Received 20 February 2016
Received in revised form 15 June 2016
Accepted 24 June 2016
Available online 1 July 2016

Keywords:
Chilo suppressalis
Odorant binding protein
Expression profile
Binding experiment

ABSTRACT

At the peripheral level of the insect olfaction, odorant binding proteins (OBPs) are thought to bind and transport exogenous hydrophobic volatiles to the odorant receptors (ORs) located on the dendrite membrane of the olfactory neurons. In this study, cDNA sequences of 29 OBP genes from *Chilo suppressalis*, a notorious rice pest, were identified, with 15 of them being newly reported. The tissue and temporal expression patterns of these CsupOBPs were determined by RT-PCR, revealing that *CsupOBP8*, *10* and *24* were specifically expressed in the heads at larval stage and in antennae of both sexes at adult stage. In addition, *CsupOBP 6*, *9* and *18* were expressed in much higher levels in heads than abdomen at the larval stage, while *CsupPBP4* and *CsupOBP1*, *3*, *8*, *11* and *24* were specifically expressed in antennae of both sexes at the adult stage. Such expression profile strongly suggests olfactory roles of these genes. Furthermore, the function of CsupOBP8 was chosen to be investigated by the competitive fluorescence binding assay. Results showed that CsupOBP8 displayed high binding affinities (Ki = 4.9–15.0 μM) with plant volatile β-ionone, nerolidol, farnesol and 2-hexanone, suggesting that CsupOBP8 plays olfactory roles through binding and transporting the plant volatiles. The study provides an important base for understanding the olfactory mechanisms in *C. suppressalis*.

1. Introduction

Insects, or moths in particular, have evolved a complex and sensitive olfactory system to detect volatiles in the environments, which is crucial for food seeking, mating, and oviposition site selection. It is generally accepted that environmental hydrophobic volatile molecules are bound and transported across the hydrophilic antennal lymph to the olfactory receptor (OR) by odorant-binding proteins (OBPs) (Vogt et al., 2002; Pelosi et al., 2006; Garczynski et al., 2013), and thus OBPs play important roles in the olfaction. OBPs are small soluble proteins existing in high concentration in lymph of insect antennae and other sensory organs (Pelosi et al., 2014b; De Biasio et al., 2015). Classic OBPs are characterized by 6 conserved cysteines that form 3 disulphide bonds, which maintain the stabilization of OBP conformation. In addition, non-classic OBPs with various conserved cysteines have also been found, and designated as the Plus-C OBP (8 conserved cysteines), Minus-C OBP (4 conserved cysteines), Dimer OBP (12 conserved cysteines), and Atypical OBP (9–10 conserved cysteines) (Zhou, 2010).

Since the first OBP protein was identified from *Antheraea polyphemus* as a female sex pheromone carrier (Vogt and Riddiford, 1981), a huge number of OBPs have been molecularly identified in various species of different insect orders. The number of OBPs varies from 5 in *Pediculus humanus humanus* (Kirkness et al., 2010) to 81 in *Anopheles gambiae* (Holt et al., 2002), indicating that different species employ different arrays of OBPs to adapt volatiles in their specific environments. Most OBPs are specifically or mainly expressed in chemosensory organs, such as antennae and mouthpart appendages (Iovinella et al., 2011), whereas some OBPs are also and even only expressed in non-chemosensory organs, such as male reproductive organs (Ban et al., 2013; Sun et al., 2012). This expression pattern strongly suggests that OBPs play physiological roles in addition to chemosensation (olfaction and gustation). It is now widely accepted that OBPs act as carriers, bind hydrophobic semiochemicals and deliver them directly to ORs, or to the sensory neuron membrane proteins (SNMP), which then delivers to the ORs (Zhou, 2010). However, the functional complexity and diversity of OBPs remain largely unknown. The genome and transcriptome analyses provide important platforms to identify OBP genes in large

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; cDNA, complementary DNA; 1-NPN, 1-*N*-phenyl-naphthylamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Ki, dissociation constant; ANOVA, analysis of variance; SE, standard error.

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http://dx.doi.org/10.1016/j.gene.2016.06.052
0378-1119/

numbers, and consequently to explore their functional diversities in insects (Zhang et al., 2013).

Chilo suppressalis walker (Lepidoptera: Pyralidae) is a notorious pest of a variety of crops including rice, wheat, corn, and sugarcane. The larvae bore into and eat inside stems, causing yellowing, withering, rotten and eventually death of the plants (Ghareyazie et al., 1997). The control of *C. suppressalis* for a long time has been dependent on chemical insecticides, which result in various problems such as insecticide resistance by the pest and high level of insecticide residue in the rice (Qu et al., 2003). Therefore, alternatives are urgently anticipated to control this pest. Being important in insect behavior and physiology, olfactory system is considered as a new target for the pest management. Characterization of OBP genes in *C. suppressalis* not only is important for understanding insect olfactory mechanism, but also could provide potential target genes for novel pest management technology. Previous analyses of antennal transcriptome data, 27 putative OBP genes were identified (Cao et al., 2014; Gong et al., 2015). In this study, we reported the identification of 29 OBP genes (13 OBPs were previously unreported) based on analysis of a *C. suppressalis* genome data (Yin et al., 2014), which brings the total number of *CsupOBP* genes to 40. In addition, a comprehensive examination of tissue and developmental stage expression profiles of these genes was conducted by revers transcription-PCR (RT-PCR). Furthermore, gene *CsupOBP8*, characteristic by specific expression in heads at larval stage and in antennae at adult stage was functionally analyzed using the in vitro expression and ligand binding assay.

2. Materials and methods

2.1. Insects and sample collection

A *C. suppressalis* population was initially collected from paddy fields in Jiangsu Province, and was raised in the laboratory on rice seedlings in glass containers under 26 ± 1 °C, 80–90% relative humidity and 16 h light:8 h dark photoperiod. For developmental and tissue specific expression pattern studies, whole body of larvae of first and second instars, head or body of 3–6 instar larvae and 4 day-old pupae, and different tissues of adults including antenna, leg, wing, and abdomen were collected separately. The larval age was determined based on morphology standards (Song et al., 1958) examined under a stereoscopic microscope. After collection, the samples were immediately stored at −70 °C until use. All samples were collected with 2 biological replications.

2.2. Identification of OBPs from the genome data of C. suppressalis

To identify possible OBP exons, the 3rd assembly version of *C. suppressalis* genome (Yin et al., 2014) was queried by tblastn using Lepidopteran OBP sequences in the GenBank. Genomic scaffold sequences of exons found in the tblastn were used to construct putative OBP sequences manually using Sequencher v4.5 (Gene Codes, Inc., Ann Arbor, MI) and were refined using Splice Predictor (http://deepc2.psi.iastate.edu/cgi-bin/sp.cgi/). The correctness of the constructed sequences was verified by PCR experiments and sequencing of the PCR products (see RT-PCR analysis).

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted by SV 96 Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's protocol, including a step of DNase I treatment of the RNA extracts. RNA quality was checked with a spectrophotometer NanoDrop™ 1000 (NanoDrop™ 1000, Thermo Fisher Scientific, USA). The single-stranded cDNA template was synthesized using 1 μg total RNA with oligo (dT) 18 primer as the anchor primer, by M-MLV reverse transcriptase (TaKaRa, Dalian, Liaoning, China) at 42 °C for 1 h, according to the provided protocol. The synthesized cDNA template was stored at −20 °C for future use.

2.4. Phylogenetic analysis

Based on the OBP amino acid sequences with the signal peptide removed, a phylogenetic tree of OBP was reconstructed with other Lepidoptera insects for a phylogenetic evaluation. The tree contained 40 sequences from *C. suppressalis* (29 in full length), 19 from *Manduca sexta*, 43 from *Bombyx mori*, 15 from *Spodoptera littoralis*, 4 from *Cnaphalocrocis medinalis*, 6 from *Ostrinia furnacalis* and 5 from *Ostrinia nubilalis*. Sequences were aligned by ClustalX 5.0 and unrooted trees were constructed by MEGA6.0 using the neighbor-joining method, with Poisson correction of distances. Node support was assessed using a bootstrap procedure base on 1000 replicates.

2.5. RT-PCR analysis

Gene specific primers of OBP genes were designed using Primer Premier 5.0 (PREMIER Biosoft International, CA, USA) and listed in Table S1. PCR experiments including negative controls (no cDNA template) were carried out in a MyCycler™ Thermal cycler (Bio-Rad, USA) under the following conditions: 94 °C for 3 min, followed by 32 cycles at 94 °C for 30 s, 60 °C for 40 s, and 72 °C for 50 s, and final incubation for 10 min at 72 °C. The reaction volume was 25 μL with 1 μL of single-stranded cDNA (5 ng), 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.4 mM for each primer and 1.25 U r*Taq* DNA polymerase (TaKaRa, Dalian, Liaoning, China). PCR products were analyzed by electrophoresis on 1.5% w/v agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM $Na_2EDTA{\cdot}H_2O$) and the resulting bands were visualized with ethidium bromide and digitized using a GelDoc 2000 (Bio-Rad, USA). The *C. suppressalis β-actin* gene was used as a positive control.

To validate the predicted sequences of the 24 OBP genes identified, the PCR products obtained from cDNA samples of adult antennae were purified using the AxyPrep™ PCR Cleanup Kit (Axygen, USA), and then sub-cloned into the pEASY-T3 plasmid cloning vector system (TransGene, Beijing, China) following the manufacturer's instructions. The plasmid DNA was used to transform into Trans1-T1 competent cells. Positive clones were sequenced by GenScript (Nanjing, Jiangsu, China).

2.6. In vitro expression and purification of the recombinant CsupOBP8

Signal peptides were predicted and removed according to SignalP4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The CsupOBP8 sequence encoding mature protein was amplified by primers including *Bam*HI and *Xho*I restriction sites. The sequences of the 2 primers are listed in Table S1. The plasmids containing the inserts were digested by *Bam*HI and *Xho*I FastDigest® restriction enzymes (Fermentas, Thermo Fisher Scientific, USA). The expected bands were purified from the agarose gels and ligated into the expression vector pET-30a (+) (Novagen, USA) which was previously digested by the same enzymes. After ligation, expression of the recombinant protein was carried out in Luria–Bertani (LB) (100 μg/mL, kanamycin) with *Escherichia coli* BL21 (DE3) cells at 37 °C following the recommended protocols. The positive clones were validated by PCR and sequencing. Expression of the recombinant protein was induced by addition of IPTG to a final concentration of 1 mM when the LB medium culture reached an OD600 value of 0.6. The protein was purified by XK-16 Column with Ni Sepharose High performance (GE Healthcare Life Sciences, USA). The His-tag was cleavaged by enterokinase (GenScript Biology Company, Nanjing, Jiangsu, China) and separated from the protein by the column mentioned above. This purified protein was then desalinated by dialysis against 5 gradient buffers, in which NaCl concentration ranged from 0.4 M to zero. For each buffer, the protein was dialyzed for 5 h at 4 °C according to the previous study (Liu et al., 2012). The resulted protein was freeze-dried and kept at −70 °C.