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Function and Immunocytochemical Localization of Two Novel Odorant-Binding Proteins in Olfactory Sensilla of the Scarab Beetle *Holotrichia oblita* Faldermann (Coleoptera: Scarabaeidae)

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Abstract

Odorant-binding proteins (OBPs) are found in both insects and vertebrates, and it is believed that they are involved in chemical communication. In this study, we identify and express 2 OBPs from the scarab beetle, *Holotrichia oblita* Faldermann (Coleoptera: Scarabaeidae). HoblOBP1 shows more similarities with other scarab beetle OBPs, whereas HoblOBP2 is more diverse. *N*-phenyl-1-naphthylamine (1-NPN) is used as a fluorescent probe in ligand-binding experiment, and results indicate that both HoblOBPs prefer plant volatiles to putative *H. oblita* sex pheromones. HoblOBP1 shows binding affinity to a wider range of test compounds, but HoblOBP2 displays more specific binding affinity. Cinnamaldehyde and 2,4-di-*tert*-butylphenol bind to HoblOBP1 can elicit strong electrophysiological responses of the antennae from female *H. oblita* adults, respectively. Methyl salicylate also shows good affinity to HoblOBP2 and it can elicit moderate electrophysiological responses. Although, β-ionone is one of the ligands of the strongest binding, it elicits a weak electrophysiological response. In the immunocytochemical analysis, we observe that HoblOBP1 and HoblOBP2 are coexpressed in sensilla basiconica and placodea in both sexes.

Key words: electroantennogram, *Holotrichia oblita*, immunocytochemical localization, ligand-binding experiments, odorant-binding proteins

Introduction

The scarab beetle, *Holotrichia oblita* Faldermann (Coleoptera: Scarabaeidae), is a predominant underground pest in the northern parts of China and causes great economic loss (Wei et al. 1989). A sensitive olfactory system is vital to insects for finding food, mating, and choosing a site for oviposition (Field et al. 2000). An increasing number of studies have reported that at least 5 types of proteins are involved in olfactory coding in insects, and they include odorant-binding proteins (OBPs, Vogt and Riddiford 1981), olfactory receptors (ORs, Wetzel et al. 2001; Sakurai et al. 2004), odorant-degrading enzymes (ODEs, Vogt 2003), sensory neuron membrane proteins (SNMPs, Rogers et al. 1997; Vogt 2003; Jin et al. 2008), and G proteins (Laue et al. 1997). OBPs are believed to be responsible for the binding and transport of odors from the environment to ORs located on the membranes of olfactory neurons in the early stages of insect odorant reception (Pelosi and Maida 1990; Vogt et al. 1985). The features of an OBP include its small size (15–20 kDa), an α-helix pattern with 6 cysteines paired to make 3 disulphide bridges, its globular water-soluble nature and the possession of a signal peptide (Vogt and Riddiford 1981; Vogt et al. 1985; Krieger et al. 1993; Scaloni et al. 1999; Leal et al. 1999; Calvello et al. 2003; Liu et al. 2010).

The binding of OBPs to small organic compounds is an essential part of their physiological function. Scientists are interested in whether these proteins can selectively bind compounds representing different and numerous semiochemicals in the olfactory system. In an earlier study of the first identified insect OBP, the authors incubated tritium-labeled pheromone with *Antheraea polyphemus* antennal extract and detected its binding ability by native gel electrophoresis (Vogt and Riddiford 1981). Another method used to study

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ligand binding, called the cold binding assay, is based on separation of the complex from the free ligand using a centrifugal filter device and evaluation of the bound ligand after gas chromatography–mass spectrometry extraction (Leal et al. 2005). Fluorescent binding assays have been widely used to assess the binding of putative ligands. As a result of the competitive replacement of fluorescent probe by ligands, the fluorescent intensity of probe undergoes obvious changes, on which we are able to evaluate the binding character of OBPs (Campanacci et al. 2001; Bette et al. 2002; Ban et al. 2003; Calvello et al. 2003; Yu et al. 2009). Binding properties of several OBPs have been studied by using this method. For example, *Mamestra brassicae* PBP (pheromone-binding protein) 1 binds all 3 pheromone components, including *cis*-11-hexadecenol, *cis*-11-hexadecenal, and *cis*-11-hexadecenyl acetate, with small dissociation constants (Campanacci et al. 2001). The pea aphid OBP, ApisOBP3, specifically binds the alarm pheromone, (E)-β-farnesene (Qiao et al. 2009). The *Leucophaea maderae* cockroach PBP exhibits significant selectivity for 3-hydroxy-butan-2-one, which is a component of their pheromone blend (Riviere et al. 2003). It has been observed that the *A. polyphemus* moth PBP1 specifically binds to insect pheromones, but it also binds to some structurally related compounds (Bette et al. 2002). Although insects recognize and bind pheromones with high specificities, the OBP-binding data indicate that a rather broad pattern of binding specificity exists (Pelosi et al. 2006).

Since the identification of the first OBP, more than 400 OBP genes have been isolated and cloned. Among these genes, at least 150 are from lepidopteran species (Zhou 2010). In contrast to Lepidoptera, less work has focused on coleopteran OBPs. OBPs from some scarab beetles have been studied for 2 decades (Wojtasek et al. 1998). It is known that scarab beetles possess 2 OBP families, OBP1, which has 116 amino acids, and OBP2, which has 133 amino acids. OBP1 is well conserved among all the identified scarab beetle species, whereas OBP2 is more divergent and it is found in not all the species. There has been only one PBP detected in the antennal extracts of *Anomala osalkana* and *Popillia japonica*, respectively, and the sequences from these 2 species show a 96% similarity; both PBPs have been shown in a tritium-labeled binding experiment with equal affinity for (R)- and (S)-japonilure which are the sex pheromones of the 2 species, respectively (Leal 2001). However, our knowledge of OBPs as identified in scarab beetles is still insufficient, and more investigations are required.

In this paper, we identify 2 *H. oblita* OBPs and characterized their ligand-binding specificities using a fluorescence competitive binding assay with the *N*-phenyl-1-naphthylamine (1-NPN) fluorescent probe. Electroantennograms (EAGs) are used to record the reaction of the antennae with the different compounds that bind proteins. Using transmission electron microscopy combined with immunohistochemistry, we observe the coexpression of HoblOBP1 and HoblOBP2 in the sensilla of *H. oblita*.

Materials and methods

Insects

Adult insects were caught in the field of Cangzhou City, Hebei Province of China. The antennae of the females were excised, immediately frozen in liquid nitrogen, and stored at –70 °C until use.

Identification of the gene encoding OBP1 and OBP2

Total RNA was extracted from 50 antennae of *H. oblita* females using Trizol (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized using a 1st Strand cDNA Synthesis Kit (Takara). The degenerate polymerase chain reaction (PCR) primers, OBP1 (partial) and OBP2 (partial), were designed by aligning the OBP gene sequences from other scarab beetles. These primers amplified part of the coding sequence of *HoblOBP1* and *HoblOBP2* from cDNAs that were reverse transcribed from *H. oblita* messenger RNA. The complete cDNA sequence of the clones of interest were then obtained by 5′ and 3′ Rapid-amplification of cDNA ends (RACE) using the 5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0 (Takara) according to the manufacturer's protocol. The 5′ and 3′ RACE gene-specific primers (GSPs) were designed from the partial coding sequences of *HoblOBP1* and *HoblOBP2*. The sequences of oligonucleotide primers are listed in Table 1.

Cloning and sequencing

The crude PCR products were ligated into the pGEM (Promega) vector using a 1:5 (plasmid: insert) molar ratio. After transforming the ligation product into DH5-α cells and plating, positive colonies were identified by PCR and sequenced using an ABI3730 sequencer.

Sequence analysis

The putative signal peptides and the most likely cleavage sites were predicted using the SignalP 3.0 Server. The sequences used included several OBP sequences from coleopteran insects in the GenBank sequence database, and they were aligned using CLUSTAL 1.83. Phylogenetic analysis was conducted using MEGA 4.0 without the putative signal peptide sequence by the neighbor joining method, and the sample was bootstrapped 1000 times (Tamura 2007).

Bacterial expression and purification

The primer pairs, OBP1 and OBP2, were designed to amplify the coding sequences of the *HoblOBP1* and *HoblOBP2* genes (Table 1). pGEM plasmids that contained the nucleotide sequences encoding the mature proteins were digested with *Xho* I and *Eco*R I to produce fragments for ligation into the pET30a expression vector (Novagen). Protein expression was induced in BL-21(DE3) pLys cells upon addition of

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Table 1 Oligonucleotide primers used for clone and expression analysis of the OBPs in *Holotrichia oblita*

Primer name	Sequence	Position (bp)
OBP1(partial)-FP	5′-GAGGAAATGGAGGAATTGGCGAAAC-3′	81–105
OBP1(partial)-RP	5′-TATACGATCATATACGAATTAGGAT-3′	400–425
OBP2(partial)-FP	5′-ARATGCTGGTGCARAATG-3′	91–109
OBP2(partial)-RP	5′- RMARATTRAYGCCTGTRTTACATTC-3′	363–386
OBP1 5′RACE GSP1	5′-ACCGTCATCACCCACAATAGCC-3′	231–254
OBP1 5′RACE GSP2	5′-CCCACAATAGCCATCTCGGTCA-3′	223–244
OBP2 5′RACE GSP1	5′-CGATGTCCTTGACCTTCTCGATATTG-3′	266–291
OBP2 5′RACE GSP2	5′-CTACCTCTGTTGACTGTTCCATCGG-3′	232–256
OBP1 3′RACE GSP1	5′-AGCTGCACGATGATTGCGTTGCTC-3′	106–129
OBP1 3′RACE GSP2	5′-GTGAAGGATCAAAAGGGATTTCCGG-3′	162–186
OBP2 3′RACE GSP1	5′-AATGAAGGAACAGATACCCGACAG-3′	152–175
OBP2 3′RACE GSP2	5′-GATACCCGACAGTAGAGCAGGATT-3′	164–187
OBP1-FP	5′-GAATTCATGTCAGAAGAAATGGAAGC-3′	75–94
OBP1-RP	5′-CTCGAGCTATACGATCATATACGAC-3′	407–425
OBP2-FP	5′-GAATTCTGATACTCTACAAGAGC-3′	47–63
OBP2-RP	5′-CTCGAGCAGAAACCCATTGTTTCCT-3′	430–448

The restriction sites of *Xho* I and *Eco*R I are underlined.

0.7 mM Isopropyl β-D-1-Thiogalactopyranoside. After sonication of the bacterial pellet and centrifugation, all the protein was present in the supernatant. The proteins were purified by Ni ion affinity chromatography (GE-Healthcare). To prevent the His-tag from affecting the HoblOBP functional studies, the His-tag was removed by recombinant enterokinase (rEK) (Bio Basic Inc). Any remaining, undigested His-tag proteins were removed by a second round of Ni ion affinity chromatography.

Preparation of antisera

Antisera were obtained by injecting an adult rabbit subcutaneously and intramuscularly with 1 mg of recombinant protein, followed by 3 additional injections of 500 μg at the 21st, 35th, and 49th day. The protein was emulsified with an equal volume of Freund's complete adjuvant at the first time of injection and Freund's incomplete adjuvant at the second injection. The antiserum was tested by enzyme-linked immunosorbent assay. The rabbits were bled at the 7th day after the last injection and the serum was used without further purification.

Ligand-binding experiments

To measure the affinity of the 1-NPN fluorescent ligand to the recombinant proteins, a 2 μM solution of the protein in 50 mM Tris–HCl, pH 7.4, was titrated with aliquots of 1 mM ligand in methanol to final concentrations of 1–20 μM. The affinity of other ligands was evaluated in competitive binding assays using both the protein and the 1-NPN fluorescent probe at a 2 μM concentration while increasing the final concentration of each competitor from 4 to 24 μM. Scatchard plots were used to measure the 1-NPN dissociation constants with the proteins. The dissociation concentration of the competitors was calculated from the corresponding IC_{50} values using the equation, $K_i = [IC_{50}]/(1 + [1\text{-NPN}]/K_{1\text{-NPN}})$, where [1-NPN] was the free 1-NPN concentration and $K_{1\text{-NPN}}$ was the protein/1-NPN complex dissociation constant.

Electroantennogram

We tested the compounds for binding affinity to HoblOBP proteins. The basal and distal antennal segments were immobilized between 2 electrode holders with nondrying clay (Spectra 360 Electrode Gel) and placed on the EAG Micromanipulator MP-15 (Syntech) platform. The EAG signals were amplified, monitored, and analyzed with the EAG-Pro software (Syntech). The preparation was held in a humidified air stream delivered by the Syntech stimulus controller (CS-55 model; Syntech) at 500 mL/min to which a stimulus pulse of 40 mL/min was added for 0.5 min. Signals were recorded for 10 s beginning at 2 s before the onset of the stimulus pulse. An aliquot (10 μL) of a stimulus was loaded onto a filter paper strip. When a sample was finished, hexane was tested as a control. Throughout the study, the airflow was purified by activated charcoal. Following strong stimuli, a pause for at least 1 min was taken between stimulations.

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Statistical analysis was performed using SPSS Statistics V17.0. The differences between electrophysiological responses were analyzed by Duncan's multiple range test. The absolute EAG was equal to the EAG response of the test sample minus the value of the control.

Immunocytochemistry

Antennal lemalla were excised from adult beetles and chemically fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (2%) in 0.1 M phosphate-buffered saline, pH 7.4, and then embedded in LR White resin (Taab). Ultrathin sections (70–90 nm) were treated with primary antisera against HoblOBP1 and HoblOBP2 diluted at 1:5000–1:20 000, and 1:1000–1:6000, respectively. The secondary antibody was anti-rabbit IgG, which coupled to 10-nm colloidal gold. The secondary antibody was diluted at 1:20. Gold granules were size-increased by silver intensification, and 2% uranyl acetate was used to increase the contrast for analysis by transmission electron microscopy (HITACHIH-7500).

Results

The coding and amino acid sequences

By using RACE PCR, 2 full-length cDNAs encoding *HoblOBP1* and *HoblOBP2* were cloned from *H. oblita* and named *HoblOBP1* and *HoblOBP2* (GenBank IDs: GQ856258 and GQ856257; Figure 1). HoblOBP1 was a 141 amino acid protein with a predicted signal peptide within its initial 25 amino acids. HoblOBP2 was a 149 amino acid protein that also contained a predicted signal peptide within its initial 16 amino acids. The calculated molecular weight and isoelectric points of these mature proteins were 12.873 and 14.559 kDa and 4.49 and 4.85, respectively. Both sequences contained the typical 6-cysteine OBP signature and belonged to the classical group of OBPs. They both had a common pattern as follows:

HoblOBP1: X15-Cys-X27-Cys-X3-Cys-X37-Cys-X8-Cys-X8-Cys-X12 and

HoblOBP2: X20-Cys-X26-Cys-X3-Cys-X43-Cys-X10-Cys-X8-Cys-X17,where X was any amino acid. Between the second and third cysteines, there were 3 amino acids, and between the fifth and sixth cysteines, there were 8. Such an amino acid sequence pattern was in accord with the insect OBP "signature" (Zhou 2010). HoblOBP1 showed high amino acid identity with *Heptophylla picea* HpicOBP1 (93%), *Holotrichia parallela* HparOBP1 (93%), *Anomala schonfeldti* AschPBP (93%), *A. osakana* AosaPBP (93%), *Popillia japonica* PjapPBP (93%), *A. cuprea* AcupPBP1 (88%), and *Phyllopertha diversa* PdivOBP1 (87%). HoblOBP2 showed a lower amino acid identity with OBPs from other scarab beetles, including HpalOBP2 (85%), HpicOBP2 (67%), *Anomala rufocuprea* ArufPBP2 (54%), AschPBP2 (51%), and PdivOBP2 (52%) and thus demonstrated more divergent. Phylogenetic tree analysis indicated that 27 Coleoptera insect OBPs could be divided into 3 subgroups (Figure 1C). The 2 HoblOBPs reported here were in different branches, and the distance between them was quite far. HoblOBP3 and HoblOBP4, which we recently identified as new *H. oblita* members (Guan unpublished data), were in a different branch and were separated from HoblOBP1 and HoblOBP2, thereby demonstrating the diversity of the HoblOBP family.

Bacterial expression and purification

The recombinant HoblOBP1 and HoblOBP2 proteins were expressed in bacteria. Both proteins were obtained at a high yield (more than 20 mg/L of culture) and were completely soluble. To express the recombinant protein so that it most resembled the native protein, we used rEK to remove the His-tag. The protein was purified by 2 rounds of Ni ion affinity chromatography; the first round was intended to purify the recombinant protein from total proteins, and the second round was intended to remove the His-tag and undigested His-tagged proteins (Figure 2). The purified recombinant proteins were then used for investigation of their binding properties and for the production of polyclonal antibodies.

Binding specificity of HoblOBP1 and HoblOBP2

We used 1-NPN as a fluorescent probe to perform ligand-binding experiments with the 2 OBPs. We also tested the fluorescent probes, 1-aminoanthracene (1-AMA) and 1-anilinonaphthalene-8-sulphonic acid (1,8-ANS) but neither worked as well as 1-NPN. When excited at a wavelength of 337 nm, 1-NPN displayed a weak emission peak at 480 nm. A strong blue shift was observed in the presence of HoblOBP1 and HoblOBP2 with a fluorescence peak at 420 nm. 1-NPN bound recombinant HoblOBP1 and HoblOBP2 with a dissociation constant of approximately 2.42 and 8.0 μM, respectively (Figure 3).

We performed competitive binding assays with 73 organic compounds, including compounds from the leaves of green plants, plant volatiles, compounds known to attract scarab beetles, a sex pheromone component of another beetle species (Leal et al. 1992), and 2 putative *H. oblita* pheromones (Wang 2002). Table 2 listed the dissociation constants for each of these ligands.

The results showed that α-ionone and β-ionone had high binding affinities to both proteins. Some differences were demonstrated between HoblOBP1 and HoblOBP2. HoblOBP1 demonstrated affinity to 2,4-di-*tert*-butylphenol, cinnamaldehyde, and myrcene. HoblOBP2 demonstrated affinity to methyl salicylate. Interestingly, C_6–C_{16} alkanes could not bind these 2 proteins, although hexane could bind HoblOBP1 weakly. Most aromatic compounds in these experiments bound to both proteins. The 2 putative sex pheromones had limited binding ability to both HoblOBP1 and HoblOBP2.

We also tested whether linear aliphatic aldehydes and alcohols could bind the 2 proteins. Interestingly, we observed that their binding capacity was completely lost when the

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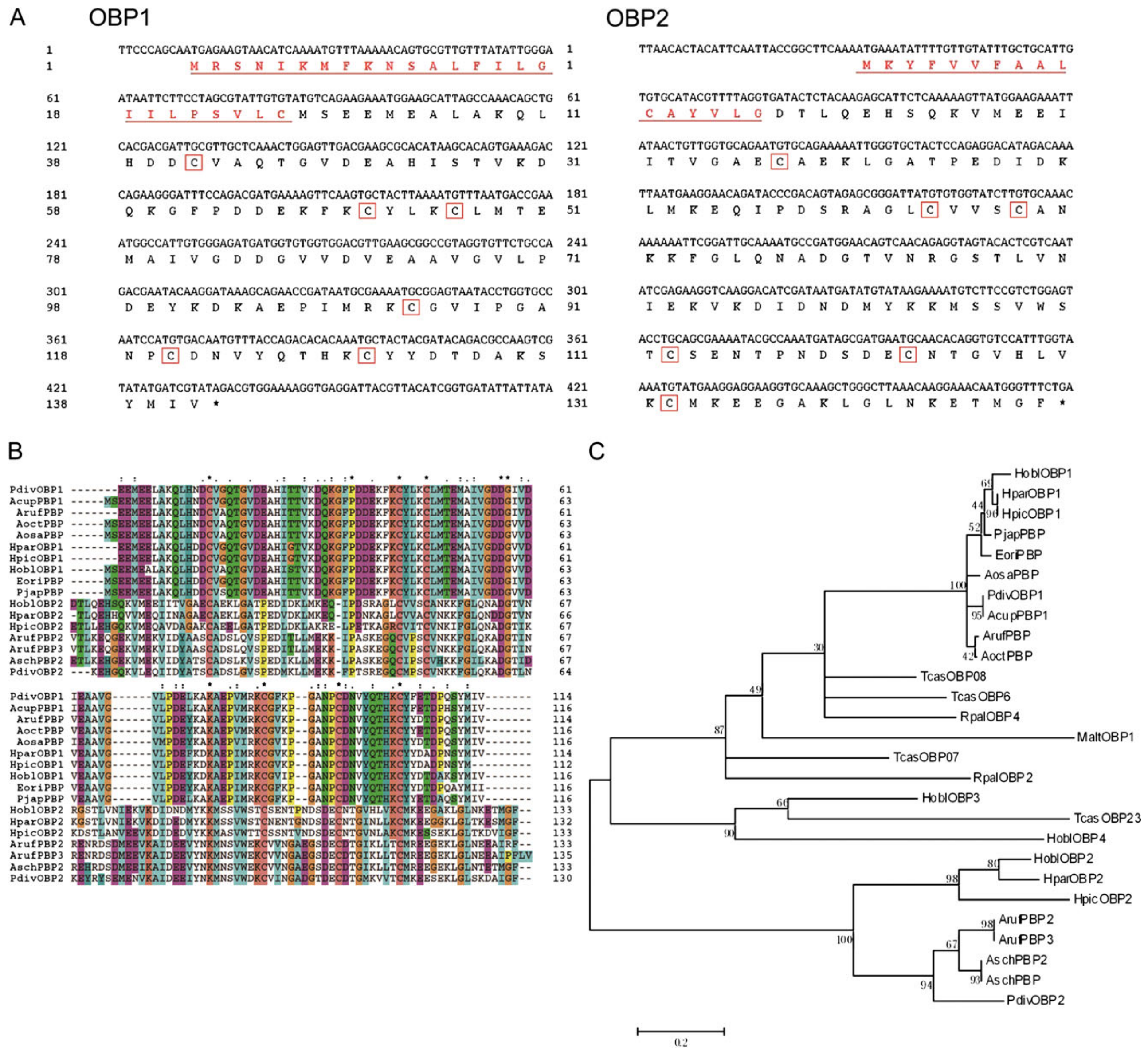

Figure 1 Sequence analysis of HoblOBP1 and HoblOBP2. **(A)** Nucleotide and deduced amino sequences of OBP1 and OBP2 from *Holotrichia oblita*. The 6 cysteines are marked in boxes, and the predicted signal peptide was underlined. **(B)** Amino acid sequences of HoblOBP1 and HoblOBP2 aligned with those of other scarab beetles. Fully conserved residues in all sequences are marked by "*". ":" indicates that one of the "strong" groups was fully conserved; "." indicates that one of the "weak" groups was fully conserved. **(C)** Phylogenetic tree of the *H. oblita* OBPs and OBPs from other coleoptera insects, including *Holotrichia parallela* (Hpar)*, Heptophylla picea* (Hpic), *Popillia japonica* (Pjap), *Exomala orientalis* (Eori), *Anomala osakana* (Aosa), *Phyllopertha diversa* (Pdiv), *Anomala cuprea* (Acup), *Anomala rufocuprea* (Hpar), *Anomala octiesostata* (Aoct), *Anomala schonfeldti* (Asch)*, Rhynchophorus palmarm* (Rpal), *Monochamus alternates* (Malt), and *Tribolium castaneum* (Tcas). This figure appears in color in the online version of *Chemical Senses*.

number of carbon atoms in the carbon chain exceeded 10. In contrast, hexyl benzoate, with a long side chain, demonstrated high affinity to HoblOBP1 and HoblOBP2. The affinity of HoblOBPs to benzoate was going down with decreasing carbon number in the benzoate side chain.

Electroantennogram

Based on the results of the competitive binding assays, we selected β-ionone, myrcene, cinnamaldehyde, 2,4-di-*tert*-butylphenol, and methyl salicylate, which was a plant volatile, to perform EAGs due to their high affinities to one or both of the OBPs (Figure 4). These components represent aliphatic terpenoids, aromatic compounds and aliphatic aldehydes. The EAG recordings demonstrated that 2,4-di-*tert*-butylphenol and cinnamaldehyde elicited strong electrophysiological responses of the antennae from female *H. oblita* adults at 10 μg/μL. β-ionone, which was the best competitive binding assay ligand, had a weak effect on female antennae. Methyl salicylate and myrcene showed an intermediate response in the EAG recordings.

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Immunocytochemical localization

In immunocytochemical localization experiments, anti-HoblOBP1 and antiHoblOBP2 were used to stain adult antennal sections. The outer lymph surrounding the dendrites showed positive staining; however, the dendrites themselves and the inner lymph were not stained. The sensilla placodea (Figure 5A,B,E, and G) and basiconica (Figure 5C,D,F, and H) of both sexes were strongly labeled with anti-HoblOBP1 and antiHoblOBP2. Moreover, the results of labeling 2 continuous sections by antiOBP1 and antiOBP2 antiserum, respectively, showed that the 2 HoblOBPs are coexpressed in sensilla placodea and basiconica. Sensilla coeloconica were not labeled (not shown).

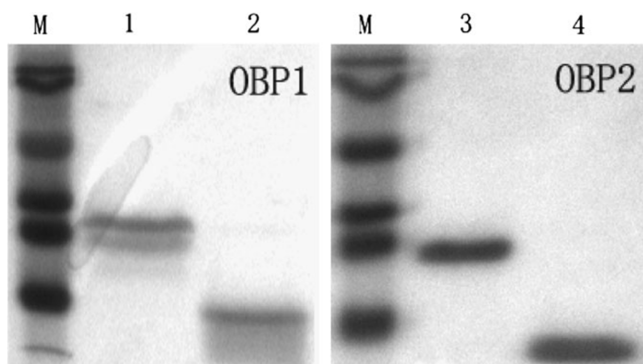

Figure 2 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the expressed products of pET/HoblOBP1 and pET/HoblOBP2. M: protein molecular weight marker (from the top: 94, 62, 40, 30, 24, and 16 kDa). 1, the purified fusion protein, pET/HoblOBP1; 2, a second round purification of HoblOBP1 protein after rEK digesting. 3, the purified fusion protein, pET/HoblOBP2; 4, a second round purification of HoblOBP2 protein after rEK digesting.

Discussion

We have identified 2 OBPs in *H. oblita*, and they are similar to others, previously reported in scarab beetles (Leal 2001). Both HoblOBPs sequences share all the common features such as a conserved 6-cysteine pattern, and they are 2 new members of OBP family. We have known that OBPs are grouped into 3 clusters in lepidopteran cladograms, called PBPs, general odorant-binding proteins (GOBPs), and antennal-binding protein X (ABPx), with GOBP further subdivided into 2 clusters, GOBP1 and GOBP2 (Zhou 2010). However, more OBP data from Coleoptera species are needed to better describe their evolutionary relationships.

Geraniol, eugenol, and *cis*-3-Hexen-1-ol have been reported to attract both sexes of the garden chafer, *Phyllopertha horticola* (Ruther 2004). In ligand-binding experiments, geraniol and eugenol demonstrate affinity for HoblOBP1 and HoblOBP2, respectively. *Cis*-3-Hexen-1-ol exhibit moderate binding affinity to both HoblOBPs. 2,4-di-*tert*-butylphenol, cinnamaldehyde, linalool, and *cis*-3-hexen-1-ol are major volatile components of castor beans, which have the ability to attract *H. oblita* (Song et al. 1996; Zhang 2005), and they

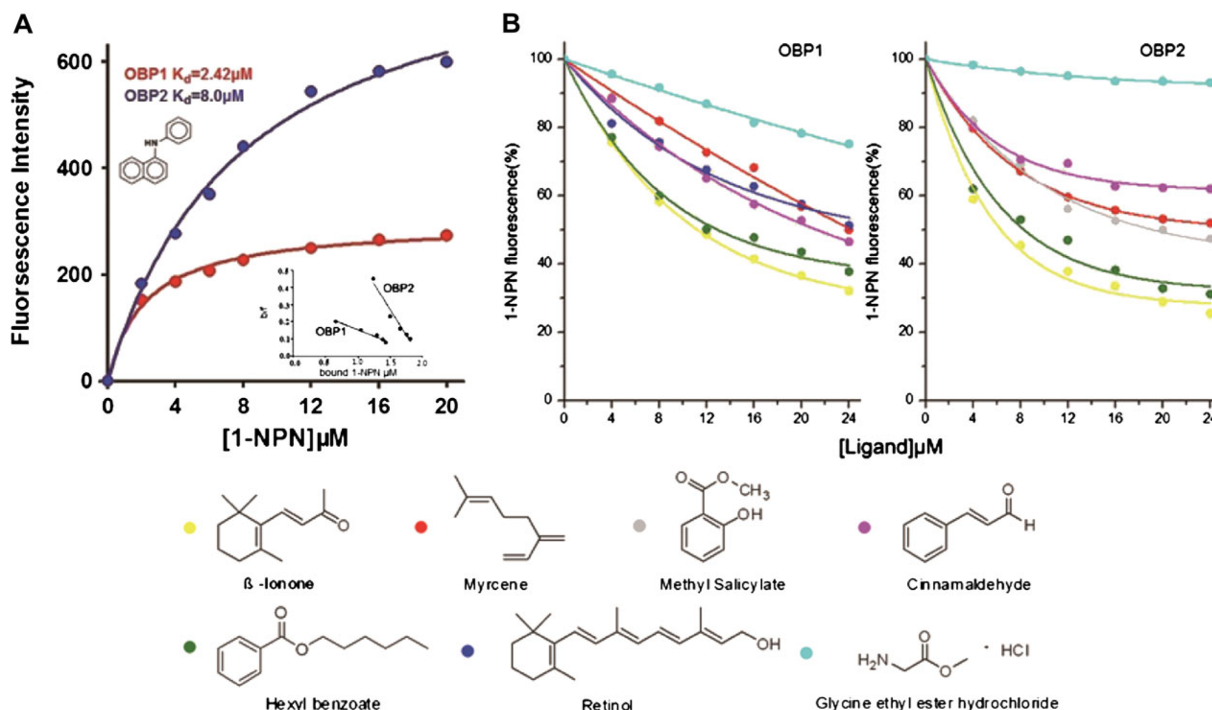

Figure 3 Ligand-binding experiments. **(A)** Binding curve and relative Scatchard plot. **(B)** Competitive binding curves of selected ligands to the 2 beetle OBPs. The chemical structures of the ligands are shown at the bottom of the panel. Mixtures of proteins and 1-NPN both at a 2 μM concentration were titrated with a 1 mM ligand solution in methanol.

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Table 2 Binding affinities of *Holotrichia oblita* odorant-binding proteins for experimental ligands in a binding assay with the fluorescent probe, 1-NPN

Ligands	OBP1 K_i	OBP2 K_i	Ligands	OBP2 K_i	OBP2 K_i	Ligands	OBP1 K_i	OBP2 K_i
Aliphatic alcohols			Aliphatic terpenoids			Aromatic compounds		
trans-2-hexenol	—	68.66	α-Terpinene	52.85	64.59	Dimerhyl phthalate	31.42	48.33
Hexanol	—	64.59	R-(−)-linalool	43.52	—	Eugenol	—	32.15
1-heptanol	—	—	β-Ionone	6.35	5.36	2,4-di-*tert*-butylphenol	8.31	80.92
1-octanol	49.09	N	α-Ionone	8.31	10.41	Methyl salicylate	28.45	16.04
Nonanol	28.45	—	myrcene	13.38	24.08	Benzaldehyde	40.46	32.14
1-octen-3-ol	—	49.95	Geraniol	22.58	—	Phenethyl alcohol	52.85	44.27
cis-2-hexenol	—	—	Terpinen-4-ol	34.41	—	Anisole	28.45	N
cis-3-hexen-1-ol	33.21	40.23	Aliphatic ester derivatives			Aliphatic aldehydes		
4-*tert*-butylcyclohexanol	19.68	N	Glycine ethyl ester hydrochloride	52.85	—	*tran*-2-Hexenal	22.58	48.3
Retinol	13.38	N	L-isoleucine methyl ester hydrochloride	52.85	76.83	Valeraldehyde	—	41.84
Aliphatic alkanes			L-proline methyl ester hydrochloride	—	N	Hexaldehyde	46.61	N
hexane	40.46	—	Propyl benzoate	17.96	60	Heptanal	N	71.93
Aliphatic ketones			Butyl benzoate	17.96	15.98	Nonanal	N	—
6-methyl-5-hepten-2-one	—	68.67	Hexyl benzoate	6.63	8.0	Cinnamaldehyde	12.25	48.33
2-cyclohexen-1-one	27.27	N	*cis*-3-hexenyl acetate	—	72.49			

Dissociation constants of ligands whose IC_{50} exceeded 100 mM are represented as "—". Compounds that showed no binging affinity are represented as "N". Other potential ligands were tested, but the remaining 33 potential ligands did not bind either OBP. These compounds included methyl anthranilate, β-caryophyllene, 2-tridecanone, nerolidol, phellandrene, limonene, α-pinene, β-pinene, octanal, decanal, undecylic aldehyde, dodecanal, tridecanal, tetradecanal, pentadecanal, palmitic aldehyde, 1-decanol, undecanol, dodecanol, tridecanol, tetradecanol, pentadecanol, hexadecanol, heptane, octane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, and hexadecan.

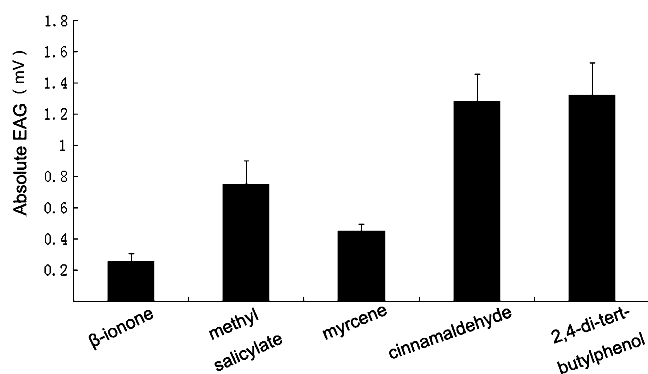

Figure 4 The EAG response of female *Holotrichia oblita* antennae to 5 plant volatiles that demonstrated affinity to HoblOBPs. The displayed responses were recorded with a 10 μg/μL source dose. Bars represent the mean ± SE ($N = 6$).

have been found to elicit a strong EAG response of their antennae (Deng et al. 2011). These compounds show good affinity to HoblOBP1 or HoblOBP2. As both OBPs bind compounds with different structures and properties, the broad OBP ligand specificity may be related to the wide variety of *H. oblita* hosts (Wei et al. 1989). R-(–)-Linalool, a trace component of the *H. parallela* sex pheromone (Leal et al. 1993), shows a moderate affinity for HoblOBP1. It appears that HoblOBP1 and HoblOBP2 do not function as PBPs, although they show high similarities to PBPs from other beetle species.

In our study, β-ionone is one of the best ligands for HoblOBP1 and HoblOBP2, and it shows a higher affinity for the latter. α-ionone also shows affinity for both proteins, but it is lower than that of β-ionone. These data indicate that isomers can influence affinity in fluorescence binding experiments. However, although β-ionone shows the highest affinity for HoblOBP1 and HoblOBP2, it fails to elicit a strong electrophysiological response in the beetle antennae. It is well known that the EAG only represents an overall activity of all the sensilla on the antenna, and therefore, even highly sensitive specialist OR neurons may not show up, if their total number is low. We have also observed that phenethyl alcohol and *trans*-2-hexenol can elicit high electrophysiological responses on *H. oblita* antenna but do not show affinity for either HoblOBP in this study (Deng et al. 2011). Similar results have been observed in *Microplitis mediator* OBPs and the *Drosophila melanogaster* OBP LUSH (Zhou, Huang, et al. 2004; Zhou, Zhang, et al. 2004; Zhang et al. 2010). In *D. melanogaster* and *Anopheles gambiae*, genome annotation has identified 61 and 72 OBPs, respectively (Hekmat-Scafe

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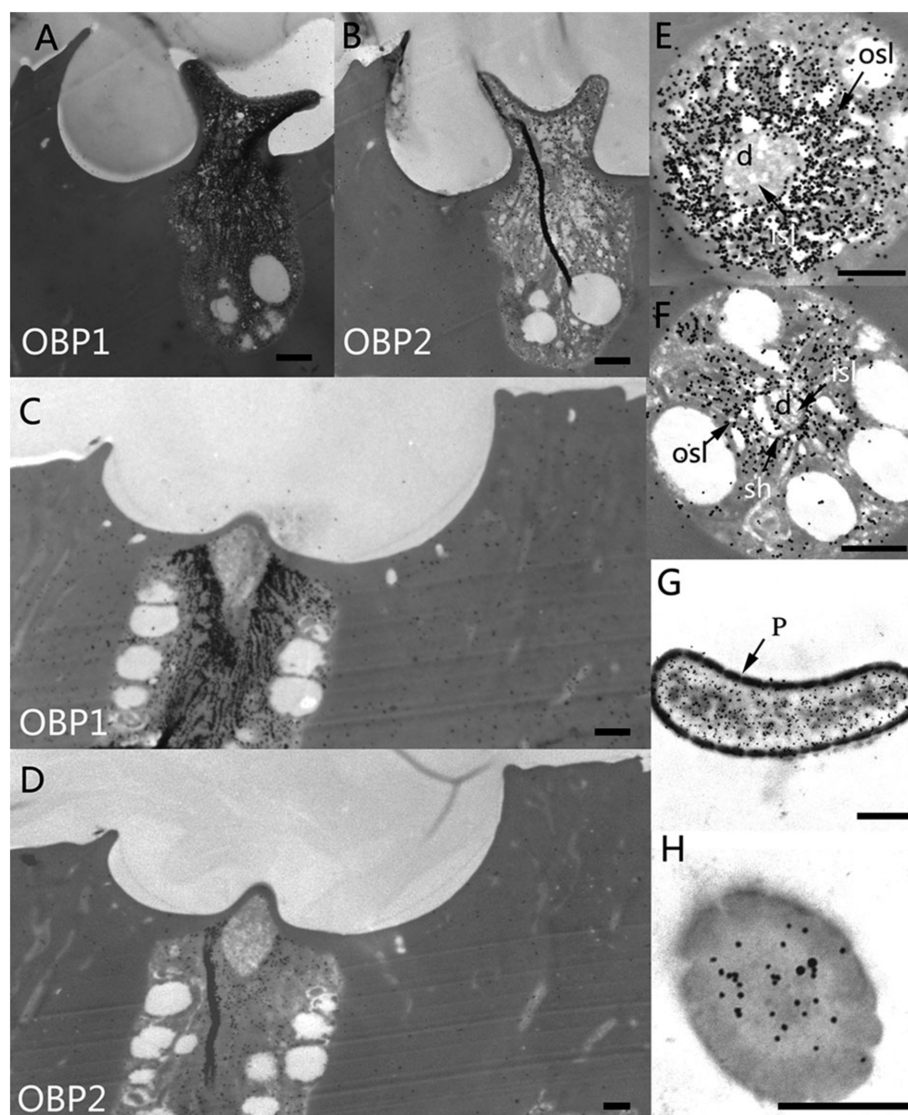

Figure 5 Immunocytochemical localization of OBPs in the olfactory sensilla of adult *Holotrichia oblita*. Sensilla placodeum **(A**, **B**, **E**, and **G)** and basiconica **(C**, **D**, **F**, and **H)** expressed OBP1 and OBP2. From A to D, 2 continuous sections labeled by different antiserum are shown. The sections below the hair base of sensilla placodeum (E) and basiconica (F) are shown. Inner (isl) and outer (osl) sensillum lymph cavities were separated by the dendrite sheaths (sh) that enveloped the dendrites. Bars, 1 μm in A–G; 0.5 μm in H.

et al. 2002; Biessmann et al. 2002; Vogt et al. 1985; Xu et al. 2003). Therefore, we infer that more than 4 OBPs might exist in *H. oblita*, of which diversified functions might be discovered later.

In fluorescence assays, some benzoates are used to define the size requirement for a tight binding. Benzoates have shown affinities for *Aedes aegypti* OBP22, *Locusta migratoria* OBP1, particularly the *n*-hexyl and *n*-butyl derivatives, and the binding affinity of these ligands decreases when the size of the carbon chain is reduced (Li et al. 2008; Yu et al. 2009). We find the carbon chain indeed important to the affinities between proteins and ligands. Interestingly, compounds composed of aldehydes or alcohols with long carbon chains cannot fit well inside the proteins tested in our experiments, but we observe the opposite situation in benzoates. Therefore, we suspect that a suitable group, such as a benzene ring, double bonds or ester carbonyl combined with a long carbon chain, forming a single compound, will facilitate protein affinity. The structure of retinol is close to that of benzoates in some way, they both have a ring and a long chain. Especially the existence of a conjugated series of double bonds on the carbon chain maintains the alkyl chain in a stretched position, which might be very important for a tight binding (Li et al. 2008). We presume that this special group combination may be a reason why retinol shows affinity to HoblOBP1.

The *H. oblita* antennae are sexually dimorphic (Kim and Leal 2000); there is a recognizable difference in the size of

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the antennal lamellae and the type of sensilla. Sensilla placodea and basiconica are the most common types in *H.oblita*, whereas sensilla coeloconica are fairly rare. The number of sensilla placodea on the antennal lamellae in females is approximately the same, while basiconica are significantly high in males. Therefore, it has been suggested that the basiconic sensilla are pheromone-sensitive sensilla, whereas sensilla placodea might be used in the detection of *H. oblita* plant volatiles (Wang 2006). The immunocytochemical localization results demonstrate that both HoblOBP1 and HoblOBP2 are expressed in the sensilla basiconica and placodea of both sexes. The 2 sensilla belong to wall-pore olfactory sensilla (Altner 1977), suggesting that the 2 OBPs are involved in chemoreception. Much of the cellular localization work have been done in Lepidoptera insects, the fly and locust (Steinbrecht et al. 1992; Laue et al. 1994; Steinbrecht et al. 1995; Zhang et al. 2001; Shanbhag et al. 2005; Jin et al. 2005; Yu et al. 2009). In *A. polyphemus*, *A. penyi*, *Bombyx mori*, and *D. melanogaster*, immunocytochemical studies have demonstrated that the PBP and GOBP are present in different sensillum types. Generally, PBPs are expressed in the pheromone-sensitive sensilla trichodea, whereas some sensilla basicona that responded to general odorants mainly express GOBPs (Steinbrecht et al. 1992, 1995; Shanbhag et al. 2005; Zhou 2010). Our experiments are the first to show that the sensilla placodea also expressed OBPs in Coleoptera. The 2 OBPs of *H. oblita* are widely expressed in the most common sensilla, and no significant sexual difference is observed. It has been suggested that these OBPs are important for sensing odorants, particularly from plant volatiles. There are 3 OBPs found coexpressed in sensilla trichodea of *D. melanogaster* (Shanbhag et al. 2005), similarly, 2 *A. gambiae* OBPs are also found in coexpression (Qiao et al. 2011). The coexpression of different OBPs within the same sensillum potentially could broaden the range of odorants to which the olfactory receptor neurons can respond (Hekmat-Scafe et al. 1997). Considering the extremely high concentration, these OBPs are likely to shift toward heterodimer in insect's sensillum lymph for combinatorial coding (Vogt and Riddiford 1981; Hekmat-Scafe et al. 1997; Qiao et al. 2011). We suppose that HoblOBP1 and HoblOBP2 might also have the possibility of this kind of cooperative function.

Investigating the binding affinity and distribution of expression of HoblOBPs is useful for understanding the physiological function and mechanism of olfactory recognition and for determining how *H. oblita* locates its mates and hosts. Such studies may assist in devising strategies to disrupt the aggregation behavior of this species.

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