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Functional characterization of odorant-binding proteins from the scarab beetle *Holotrichia oblita* based on semiochemical-induced expression alteration and gene silencing

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ABSTRACT

With the advent of next-generation sequencing, it is now possible to rapidly identify the entire repertoire of olfactory genes likely to be involved in chemical communication of an insect species. It remains, however, a challenge to identify olfactory proteins, such as odorant receptors and odorant-binding proteins (OBPs), vis-à-vis the odorants they detect. It has been reported that exposing the olfactory system to a physiologically relevant odorant alters the transcript levels of odorant receptor(s) involved in the detection of the tested odorant. We applied this paradigm in an attempt to identify putative OBPs from the scarab beetle *Holotrichia oblita* involved in the reception of plant-derived kairomones. Twenty-nine OBP genes were identified in the *H. oblita* transcriptome, 20 of which were enriched in antennae compared with nonolfactory tissues. Of these, 2 OBP genes, *HoblOBP13* and *HoblOBP9*, were upregulated upon exposure to one of the female attractants (*E*)-2-hexenol and phenethyl alcohol; none of the OBP transcripts changed upon exposure to methyl anthranilate, which does not attract *H. oblita* females. Binding assays showed that HoblOBP13 and HoblOBP9 have high affinity for (*E*)-2-hexenol and phenethyl alcohol, respectively. RNAi treatment showed that transcripts of both *HoblOBP13* and *HoblOBP9* declined in a time-course manner 24–72 h postinjection. OBP-dsRNA-treated female beetles showed significantly lower attraction to (*E*)-2-hexenol and phenethyl alcohol than did water-injected beetles and those treated with GFP-dsRNA. We, therefore, concluded that HoblOBP13 and HoblOBP9 are essential for *H. oblita* reception of the plant-derived kairomones (*E*)-2-hexenol and phenethyl alcohol.

1. Introduction

With the advent of next-generation sequencing, it is now possible to rapidly identify the entire repertoire of olfactory genes likely to be involved in chemical communication of an insect species (eg, [Jia et al., 2018]). Genome sequencing coupled with transcriptome analysis allows identification of putative odorant receptors (ORs), ionotropic receptors (IRs), odorant-binding proteins (OBPs), chemosensory proteins (CSPs), and odorant degrading enzymes (ODEs) (Leal, 2013). It remains a major challenge, however, to unambiguously identify the olfactory genes that detect odorants, including pheromones, plant attractants, repellents, and other semiochemicals, that have crucial roles in the chemical ecology of a species of interest. Typically, receptors are deorphanized using heterologous systems, such as the *Xenopus* oocyte recording system (Wetzel et al., 2001) and the empty neuron of the fruit fly (Dobritsa et al., 2003), leading to identification of specific, narrowly tuned, broadly responding and "silent" receptors (Wang et al., 2016). On the other hand, dozens of OBP genes are further characterized by tissue expression analysis and binding assays considering that genes expressed exclusively in olfactory tissues and proteins that bind certain semiochemicals are functional OBPs (Pelosi et al., 2018). Recently, it has been demonstrated that exposure of an odorant leads to alteration in the transcripts of olfactory receptor genes involved in the reception of that odorant (von der Weid et al., 2015). This technique called deorphanization of receptors based on expression alteration of mRNA levels (DREAM) (von der Weid et al., 2015) opened the door for the identification of ORs vis-à-vis odorants they detect. We then surmised that this approach may be applicable for the identification of functional

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OBPs. With transcriptome, we have identified dozens of putative OBP genes in the antennae of the scarab beetle, *Holotrichia oblita* Faldermann (Coleoptera: Scarabaeidae; Melolonthinae), which cause severe loses to soybeans, peanuts, corn, potatoes, and turf grass in China (Luo et al., 2008, 2009). First, we identified OBP genes that are predominantly expressed in antennae compared with nonolfactory tissues (legs). Then, we exposed female beetles to (*E*)-2-hexenol, a plant kairomone attractive to *H. oblita* (Deng et al., 2011) and compared transcript levels of antennae-specific OBP in treated and control beetles. Similar treatments were performed by applying phenethyl alcohol, a "food type lure" for scarab beetles (Leal et al., 1994), and methyl anthranilate, which is attractive to rutelines (Imai et al., 1997), but not melolonthine beetles (Toshova et al., 2016). Two OBPs (HoblOBP9 and HoblOBP13) whose transcript levels increased significantly when treated with (*E*)-2-hexenol or phenethyl alcohol, but not with methyl anthranilate, were expressed and demonstrated to have high affinity for these ligands. Phenotypes with transcript levels of OBP9 and OBP13 reduced by RNAi treatment had significantly less attraction to (*E*)-2-hexenol and phenethyl alchohol than water-injected and GFP-dsRNA-injected beetles had. Taken together these experiments led to the deorphanization of HoblOBP13 and HoblOBP9 as olfactory proteins involved in the reception of (*E*)-2-hexenol and phenethyl alchohol, respectively.

2. Materials and methods

2.1. Insect rearing

H. oblita adults of both sexes were obtained from Cangzhou City, China and were maintained in plastic containers (100 × 50 × 50 cm) with fresh elm leaves as the food source. Beetles were reared under controlled conditions at 25 ± 1 °C with 70% R.H. and a photoperiod of 12:12 h L:D. Antennae and legs (nonolfactory tissues) (Leal, 2013) were removed and flash-frozen in liquid nitrogen in 1.5-mL microcentrifuge tubes and stored at −80 °C until used to isolate RNA.

2.2. Identification of putative OBP genes from H. oblita transcriptome

We have previously performed a comparative transcriptome analysis of olfactory and nonolfactory tissues in *H. oblita* (Li et al., 2017). The dataset has been uploaded to SRA with the accession number SRR5461278. To identify candidate unigenes that encoded putative OBPs in *H. oblita*, the tBLASTn program was run with the available sequences of OBPs from other species (*Tribolium castaneum, Ips typographus, and Dendroctonus ponderosae*) as a "query." All candidate OBPs (Supplementary Table S1) were manually checked with the BLASTx program at the National Center for Biotechnology Information (NCBI).

2.3. Phylogenetic analyses

The phylogenetic reconstruction of *H. oblita* chemosensory genes was performed as previously reported (Li et al., 2017). Amino acid sequences were aligned using Clustal Omega (Sievers et al., 2011). Phylogenetic trees were constructed by the neighbour-joining method with the Jones-Taylor-Thornton (JTT) amino acid substitution model as implemented in MEGA 5.10 software. Node support was assessed using a bootstrap procedure with 1000 replicates.

2.4. Quantitative real-time PCR analysis

Total RNA was isolated from different tissues (100 male antennae, 100 female antennae, 12 legs [6 males and 6 females mixed]) of 2-3-day-old beetles using Trizol reagent (Invitrogen, USA). The expression of putative chemosensory genes in different tissues was investigated using quantitative real-time PCR (qRT-PCR). qRT-PCR was conducted on an ABI Prism 7500 Fast Detection System using a GO Taq®qPCR kit (Invitrogen, USA). An aliquot of 10 micrograms of total RNA sample was used for the synthesis of first-strand cDNA with reverse transcriptase (Takara, Japan), and the cDNA was then used as a template for qRT-PCR. Following a previously reported protocol (Li et al., 2017), β-actin was used to normalize the target gene expression and to correct for sample-to-sample variation. TaqMan primers and probes were designed using Primer Express 3.0 (Applied Biosystems, USA) (Supplementary Table S2). The specificity of each primer set was validated by melting curve analysis, and the efficiency was calculated by analyzing the standard curves with a fivefold cDNA dilution series. Each amplification reaction was performed using a 20-μL reaction mixture under the following conditions: denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and at 60 °C for 30 s. Relative quantification was performed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All data were normalized to endogenous β-actin rRNA levels from identical tissue samples, and the relative fold change in the different tissues was calculated with the transcript level of the legs as the calibrator. The relative fold change in different tissues was assessed by comparing the expression level of chemosensory genes in antennae with that in the legs. Each reaction was performed with 3 biological replicates, and each biological replicate was assessed 3 times (3 technical replicates). Data of relative expression levels in various tissues were subjected to one-way analysis of variance (ANOVA) followed by least significant difference test (Tukey's) for means comparison. The data were analysed directly by using the SAS 9.20 statistical software package (SAS Institute, Cary, North Carolina, USA). Different small letters indicate significant differences in transcript abundances ($P < 0.05$).

2.5. Probing HoblOBP expression alteration by exposure to semiochemicals

To examine the expression profile of the HoblOBPs after an environmental stimulus, a bioassay was conducted using host plant volatile (*E*)-2-hexenol, which elicited a strong response in *H. oblita* female antennae (Deng et al., 2011), phenethyl alcohol, and methyl anthranilate. Phenethyl alcohol is a food-type lure (Leal et al., 1994), whereas methyl anthranilate is a negative control given that it is an attractant for some beetles, but not for melolonthine beetles (Imai et al., 1997; Toshova et al., 2016). The bioassays were run in the laboratory at 25 °C using a Plexiglas cage (80 × 80 × 60 cm). Three-day-old adults (brown elytra) were obtained that had been starved for 24 h to control for differences among food sources. For each experiment, 1 rubber septum loaded with 100 μM of 1 of the semiochemicals (eg, (*E*)-2-hexenol) was placed in the center of the cage, and 150 starved females were released in the corners of the cage and kept in this environment for 24 h. Then, 100 female antennae were removed and flash-frozen in liquid nitrogen in 1.5 mL microcentrifuge tubes and stored at −80 °C until use. The expression levels of HoblOBPs were obtained by qRT-PCR. The protocols for total RNA, cDNA, and qRT-PCR were the same as above.

2.6. Purification of HoblOBPs and fluorescence assays

Ten *H. oblita* OBP proteins (HoblOBP1, 2, 3, 4, 5, 7, 8, 9, and 13) were expressed and purified for binding studies. The appropriate insertion plasmid of different pET/HoblOBPs was obtained from the Soil Insect Pests Laboratory of IPP, CAAS (Beijing, China) and was verified using DNA sequencing. BL21(DE3)plysS *Escherichia coli* competent cells were transformed with the plasmids pET/HoblOBPs, and protein synthesis was induced at an optical density of 600 nm (OD_{600}) of 0.8 with isopropyl β-D-1-thiogalactopyranoside (0.7 mM) for 3 h (Deng et al., 2012). All proteins were found to be expressed in the supernatant, and the recombinant proteins were purified by Ni ion affinity chromatography (GE-Healthcare, Sangon Biotech Co., Ltd, Shanghai, China). SDS-PAGE was used to monitor protein expression and purification. The purified proteins were stored at −20 °C in 50 mM Tris-HCl pH 7.4 buffer. Protein concentration was determined by the standard BCA method (Sangon Biotech Co., Ltd). The His-tag from each protein was removed by recombinant enterokinase (rEK) (Sangon Biotech Co., Ltd) so as to avoid possible interference with protein function. Remaining uncleaved His-tag proteins were removed by a second round of Ni ion affinity chromatography. Fluorescence spectra were recorded in a right-angle configuration on a Lengguang 970CRT (Shanghai Jingmi, Shanghai, China) at room temperature using a 1-cm light path fluoremeter quartz cuvette. Slit widths of 10 nm were selected for both excitation (set at 337 nm) and emission (recorded from 350 to 600 nm). The spectral data were obtained with high-speed scan and processed with the 970CRT 2.0 l software. The fluorescence reporter N-phenyl-1-naphthylamine (1-NPN) was dissolved in methanol to yield a 1.0 mM stock solution. Binding affinity for 1-NPN was titrated by adding aliquots into a 2-μM protein sample to final concentrations of 2–40 μM. Competitive binding of ligands was measured using 1-NPN (2 μM) and the final concentration of each ligand dissolved in HPLC purity grade methanol ranged from 2 to 40 μM.

To determine the binding ability of HoblOBPs, (*E*)-2-hexenol (or phenethyl alcohol) was added to displace 1-NPN from the HoblOBP/1-NPN complex. Binding data were collected for 3 independent measurements. The concentrations of competitors that caused a reduction in fluorescence to half-maximal intensity (IC50 values) were used as a measure of the binding dissociation constants and were calculated from the corresponding IC50 values by using the formula: $K_d = [IC50]/(1 + [1\text{-NPN}]/K_{1\text{-NPN}})$. In the equation, [1-NPN] is the free concentration of 1-NPN, and $K_{1\text{-NPN}}$ is the dissociation constant of the complex HoblOBP/1-NPN.

2.7. RNAi-mediated gene silencing

dsRNAs of HoblOBP9 and HoblOBP13 (for primers, see Supplementary Table S3) were synthesized *in vitro* using the T7 RiboMAX™ Express RNAi System (Promega, WI, USA) following the manufacturer's instructions. Double-stranded RNAs were dissolved in DEPC-treated water. Before injection, dsRNA concentration was determined by a Nanodrop™ 1000 (Thermoscientific, Lithuania), and its integrity was analyzed on 1.2% agarose gels. Then, 2 μg dsRNA was injected into the head of each 3-5 day-old female beetle with a microINJECTOR™ System MINJ-1 (Tritech Research, Los Angeles, CA, USA). As controls, GFP-dsRNA injected and water-injected beetles were also prepared. The GFP gene (GenBank number: ACY56286) was used to design dsGFP (414bp; from 79 to 492bp). The following primers were used: dsGFP-Forward: AAGTTCAGCGTGTCCG, dsGFP-Reverse: CACCTTGATGCCGTTC. After injection, beetles were housed in a cage at 25 ± 1 °C with 70% RH and a photoperiod of 12:12 h L:D. Individual female antennae were dissected in liquid nitrogen 1 d, 2 d, and 3 d postinjection, and RNA from each treatment was extracted with Trizol reagent (Invitrogen) to measure the transcript levels by qRT-PCR as described above.

The responses of female beetles to different treatments were assessed in a Y-tube olfactometer with a diameter of 3 cm, and 25-cm long stem and 25-cm long arms, 72 h postinjection. Compounds were tested at a concentration of 10 μg/μL in paraffin oil. In these assays, 10 μL of the odorant was applied to a rubber septum (Pherobio Technology Co., Ltd., China), which was then placed in one of the arms of the olfactometer. In the other arm, 10 μL of paraffin oil was used as a negative control. A group of 30–40 female beetles were individually tested for each replicate. Each group of 30 responding females was considered as 1 replicate. Tests were performed with 15 replicates for each semiochemical and each treatment. Insects were inspected with a red-light source to avoid light interference. The source of the air that passed through the Y-tube was a pressurized tank of pure air. The air was first filtered through active carbon and then humidified by bubbling it through a bottle filled with double-distilled (dd) water before entering the Y-tube. The airflow travelled through both arms of the Y-tube olfactometer at a flux of 300 mL/min. Each individual beetle was placed at the entrance of the olfactometer, and their choice was recorded when the beetle entered an arm and stayed for 30 s (Supplementary Video V1). Beetles that made no choice within 3 min, moved out of the arena, and those that moved upwind but changed course (Supplementary Video V2) were all considered nonresponders, which accounted for 10–15% of the beetles tested. The Y-tube olfactometer was cleaned with dehydrated alcohol and allowed to air-dry between trials with different treatments or odorant dilutions. The positions of the odour sources were exchanged after every 10 beetles. An attraction index was calculated from each replicate using the formula: (no. of beetles in the treatment arm – no. of beetles in the control side of the arena)/total number of responding beetles, which was set at 30 for all tests. Differences between treatments were compared with Prism 7 (GraphPad, La Jola, CA, USA) using the unpaired, Mann-Whitney test.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ibmb.2018.11.002.

3. Results and discussion

3.1. Putative OBP genes in H. oblita transcriptome

We searched our transcriptome dataset (Li et al., 2017) to identify by blastx *H. oblita* putative OBP genes with the “OBP sequence motif of Coleoptera” C_1- X_{26}- C_2- X_3- C_3- X_{42}- C_4- X_{11}- C_5 -X_8- C_6 (where X is any amino acid) (Xu et al., 2009). Twenty-nine separate unigenes were identified as putative OBP genes (Supplementary Table S1). The number of OBP genes identified for *H. oblita* was low compared with 49 OBP genes in the genome of *Tribolium castaneum* (Tribolium Genome Sequencing et al., 2008), but relatively high compared with other species of Coleoptera for which transcriptomic data are available: 25 OBPs in *Holotrichia parallela* (Ju et al., 2014), 15 OBPs in *Anomala corpulenta* (Li et al., 2015b), 19 OBPs in *Tenebrio molitor* (Liu et al., 2015), 26 OBPs in *Colaphellus bowringi* (Li et al., 2015c), 15 OBPs in *Ips typographus* (Andersson et al., 2013) and 31 OBPs in *Dendroctonus ponderosae* (Andersson et al., 2013). Of notice, the number of OBP in adult beetles may be an underestimation of the total OBP genes in a species given that some of the genes might be expressed only in immature stages (Dippel et al., 2014; Poivet et al., 2013).

Of the 29 putative HoblOBP genes, 25 were full-length OBPs whose signal peptides were identified by SignalP. The length of the 25 translated OBP protein sequences varied from 105 to 206 amino acids, and the sequences shared only 10.2–23.3% amino acid similarity with one another (Supplementary Table S4). Insect OBPs are further grouped into the following categories: classic OBPs (with a typical 6-cysteine signature), dimer OBPs (with two 6-cysteine signatures), plus-C OBPs (with 2 additional conserved cysteines plus 1 proline), minus-C OBPs (with the 2 conserved cysteines lost) and atypical OBPs (with 9–10 conserved cysteines and a long C-terminus) (Hekmat-Scafe et al., 2002; Pelosi et al., 2006). From the 29 genes in this study, we identified 19 classic OBPs (*HoblOBP1*, *2*, *3*, *4*, *5*, *6*, *7*, *8*, *9*, *10*, *11*, *12*, *14*, *16*, *17*, *18*, *19*, *20*, and *29*), 3 plus-C OBPs (*HoblOBP15*, *27*, and *28*) with 2 additional conserved cysteines and a conserved proline located downstream of C6, and 7 minus-C OBPs (*HoblOBP13*, *21*, *22*, *23*, *24*, *25*, and *26*) that were missing C2 and C5.

A phylogenetic tree based on neighbour-joining (NJ) for the *H. oblita* OBPs is shown in Fig. 1. In the phylogenetic tree, 29 HoblOBPs were scattered on different branches, and each HoblOBP was on the same branch as the homologous protein in *A. corpulenta* (Li et al., 2015a), *H. parallela* (Ju et al., 2014), *T. molitor* (Liu et al., 2015), *C. bowringi* (Li et al., 2015c), *I. typographus* (Andersson et al., 2013), *D. ponderosae* (Andersson et al., 2013), and *T. castaneum* (Andersson et al., 2013) (Fig. 1). Members of the minus-C class formed a large clade in the tree, which included the HoblOBP13, 21, 22, 24, 25 (Fig. 1). HoblOBP15 also clustered within the plus-C OBP clade containing the previously reported plus-C OBPs AcroOBP13, TcasOBP47, ItypOBP2/13, DponOBP2 and CbowOBP25.

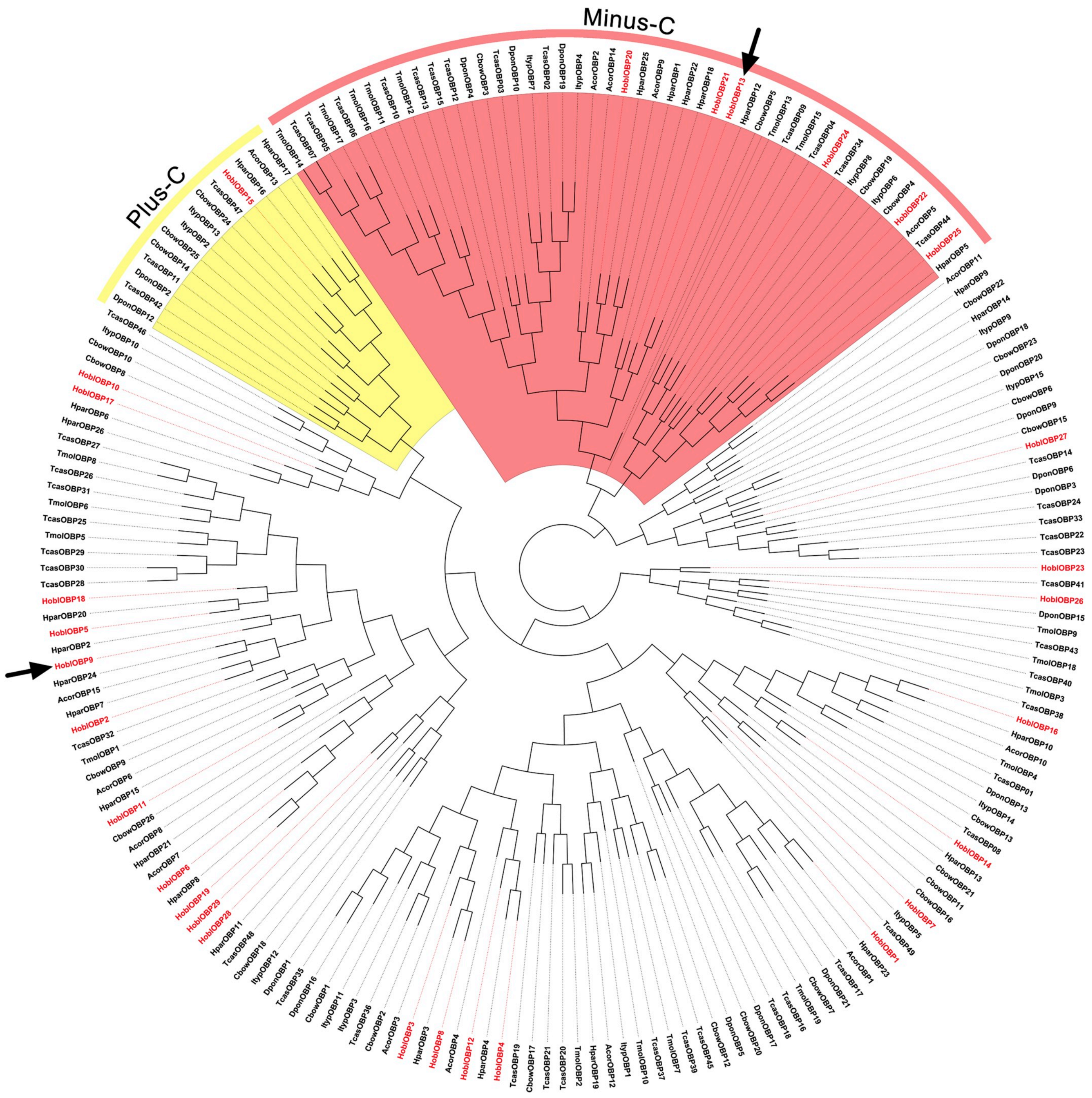

Fig. 1. Neighbor-joining phylogram of coleopteran OBPs constructed with MEGA 5.0 software. *Holotrichia oblita OBPs* (HoblOBPs) are displayed in red. Other OBPs included are from *Holotrichia parallela* (HparOBPs), *Anomala corpulenta* (AcorOBPs), *Calaphellus bowringi* (CbowOBPs), *Tenebrio molitor* (TmolOBPs), *Tribolium castaneum* (TcasOBPs), *Ips typographus* (ItypOBPs), and *Dendroctonus ponderosae* (DponOBPs). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Expression of OBP genes in olfactory and nonolfactory organs

The expression patterns of all 29 *OBP* genes were analysed by qRT-PCR with RNA extracted from male and female antennae and legs. Transcripts for 20 *OBP* genes were predominantly expressed in antennae (Fig. 2), whereas 9 *OBP* genes were enriched in legs (Fig. S1) and were unlikely to be involved in the chemosensation (Leal , 2013; Wang et al., 2016). Interestingly, we found only 3 *OBPs* that were slightly more enriched in male than in female antennae, namely *HoblOBP5, 14* and *19*. By contrast, at least 10 *OBPs* (*HoblOBP1, 3, 7, 10, 11, 12, 13, 15, 16* and *20*) were highly enriched in female antennae; the other OBPs being more expressed in female than in male antennae (Fig. 2). Of note, females of the *Holotrichia* species tend to feed on host plants while calling males by extruding an abdominal pheromone glad (Leal, 1998). It is, therefore, conceivable that some of these female-rich OBPs are involved in the reception of plant kairomones.

3.3. Semiochemical-induced alteration of OBP transcripts

It has been recently reported that exposure to volatile chemicals alters the number of transcripts corresponding to the odorant receptors involved in sensing the tested odorants (von der Weid et al., 2015). We,

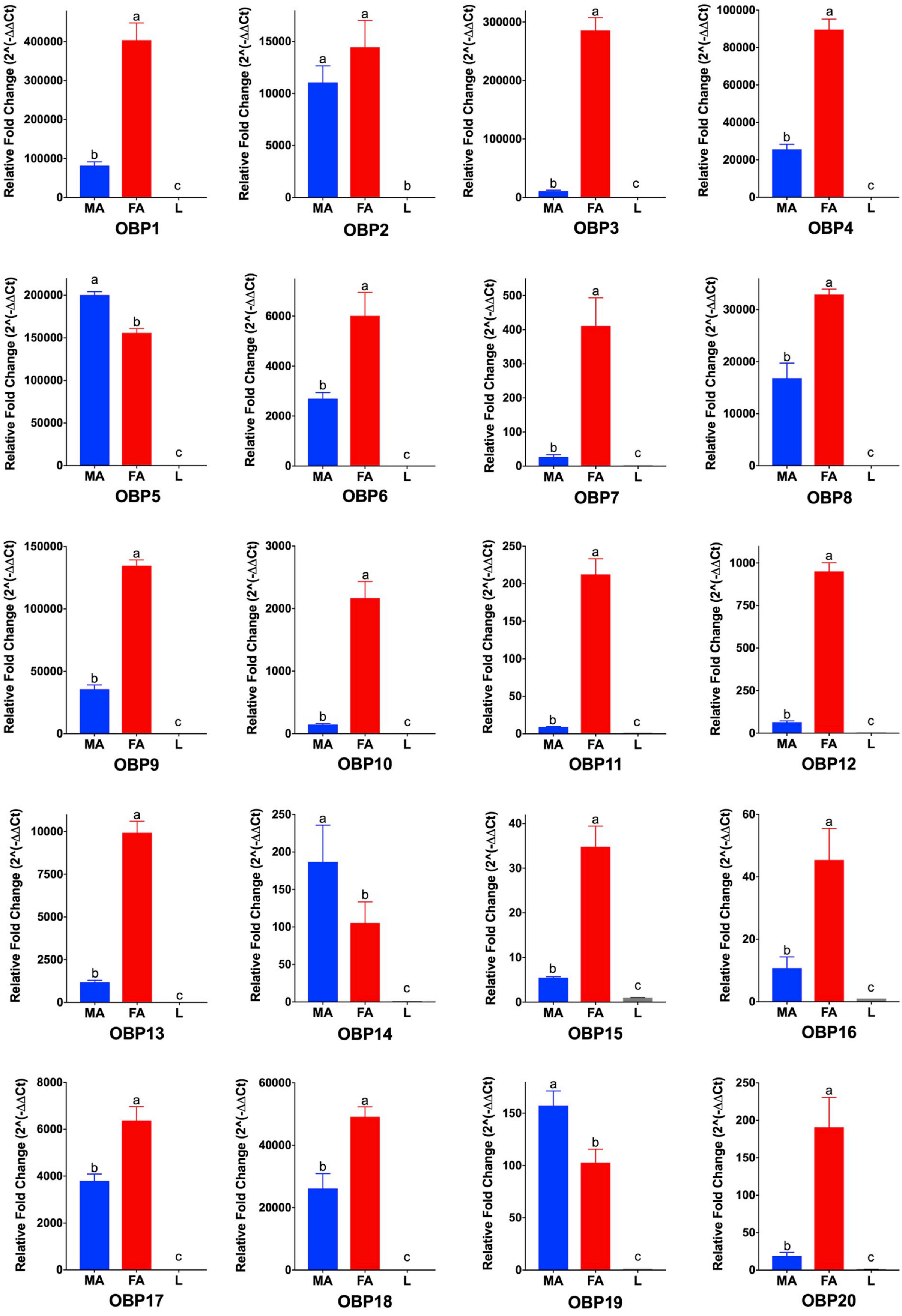

Fig. 2. qRT-PCR analysis of *H. oblita OBP* gene expression levels in male antennae (MA), female antennae (FA), and legs (L). For each graphic, columns with the same label (a, b, or c) are not significantly different (ANOVA, Turkey's multiple comparisons test, $P < 0.05$).

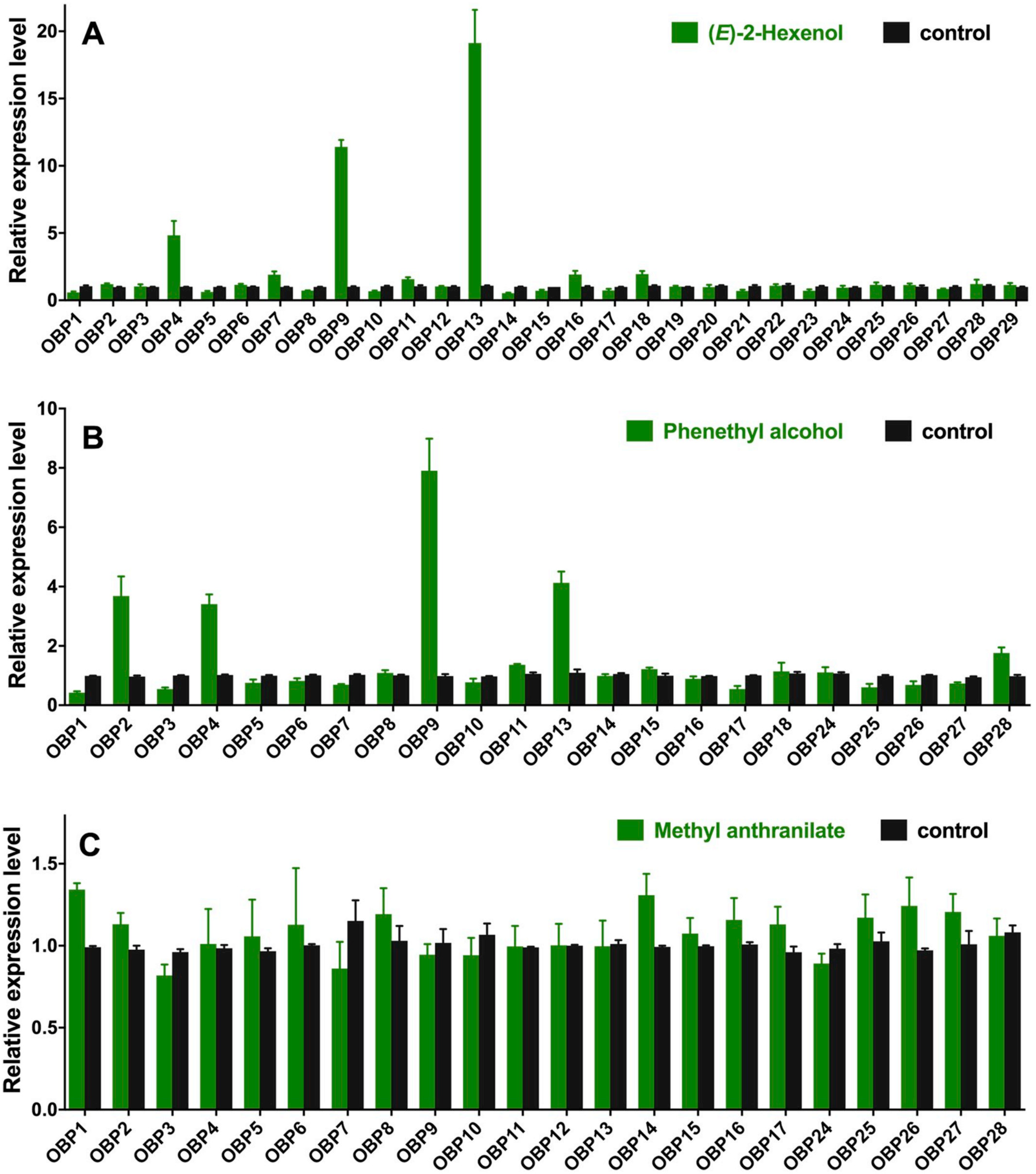

Fig. 3. Effect of exposure to plant-derived, volatile semiochemicals on transcript levels of *OBP* genes in antennae of *H. oblita* females. Templates from beetles treated (green bars) with (**A**) (*E*)-2-hexenol and (**B**) phenethyl alcohol, or (**C**) methyl anthranilate were compared with those from untreated beetles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

therefore, surmised that exposing female beetles to plant kairomones may lead us to OBPs involved in the reception of these odorants. We then exposed female beetles to (*E*)-2-hexenol, phenethyl alcohol, or methyl anthranilate and then compared the transcript levels of OBPs in treated and control beetles. Previously, it has been demonstrated that (*E*)-2-hexenol is an attractant for female *H. oblita* (Deng et al., 2011), whereas phenethyl alcohol is a known food-type lure for scarab beetles (Leal et al., 1994) and methyl anthranilate was discovered to be an attractant for *Anomala rufocuprea* (Rutelinae) (Imai et al., 1997) and other beetles (Toshova et al., 2016), but it is not an attractant for melolonthine beetles. The expression levels of *HoblOBP13, HoblOBP9, and HoblOBP4* increased dramatically in female beetles exposed to (E)-2-hexenol compared with control beetles. Likewise, the transcript levels of *HoblOBP9*, and to a lower extent the transcript of *HoblOBP2, HoblOBP3*, and *HoblOBP13*, increased in beetles exposed to phenethyl alcohol. By contrast, no significant change occurred in the transcript levels of *OBP* genes in female beetles exposed to methyl anthranilate compared with control beetles. Previous attempts to expose insects to semiochemicals led to upregulation of some *OBP* genes and downregulation of others (Paula et al., 2016, 2018), but in our case only upregulation of a few *OBP* genes took place. Although it remains to be determined whether this "DEARM-like approach" can be used to "deorphanize" OBPs, further functional analysis of the upregulated HoblOBPs suggests that this approach may allow the screening of a large repertoire of olfactory genes.

3.4. Binding of HoblOBPs to (E)-2-hexenol

To determine whether OBPs upregulated by exposure to these plant kairomones bind these semiochemicals, we evaluated by using a fluorescence displacement assays their binding activity and compared it with those of OBP not upregulated. For example, we tested binding of

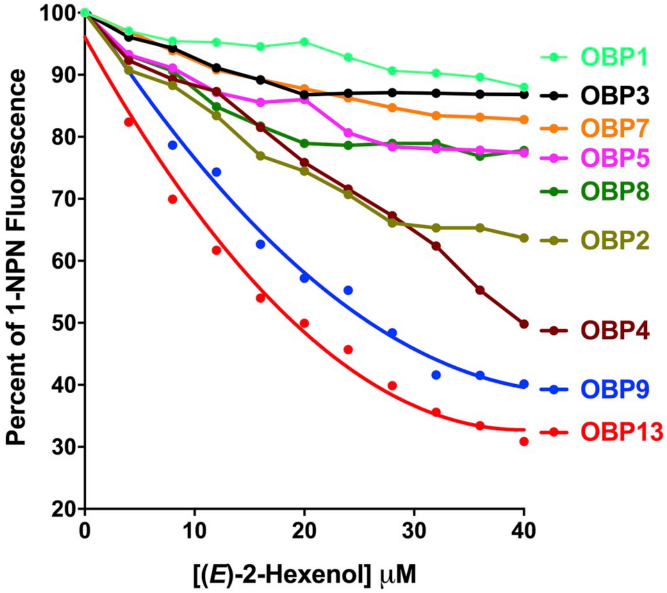

Fig. 4. Competitive binding curves of upregulated and control HoblOBPs to the plant-derived volatile (*E*)-2-hexenol. A sample of each protein (2 μM) was first bound to the fluorescence reporter 1-NPN (2 μM) and subsequently titrated with (*E*)-2-hexenol. Error bars were omitted for clarity. For OBP13, they were 1.18, 2.16, 4.05, 1.87, 3.67, 3.46, 4.73, 4.98, 4.60, and 4.79 for 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 μM, respectively.

(*E*)-2-hexenol to HoblOBP13, 9 and 4, compared it with the bindings of control OBPs: 1, 2, 3, 5, 7 and 8. Recombinant OBPs were expressed in *E. coli* at high yields (more than 2 mg/L), and purified using Ni ion affinity chromatography and anion exchange chromatography (Fig. S2). After their histidine-tags were removed by rEK, recombinant OBPs were tested for their binding properties using 1-NPN as a reporter. The dissociation constants were calculated for each HoblOBP (Supplementary Table S5). The results showed that only 3 upregulated HoblOBPs showed affinity for (*E*)-2-hexenol (Fig. 4A; see also Supplementary Table S5), with HoblOBP13 showing the highest affinity. It has been previously shown that one odorant might be detected by multiple OBPs (Deng et al., 2012; Wang et al., 2014; Yin et al., 2012). We, therefore, concluded that HoblOBP13 and HoblOBP9, and perhaps HolbOBP4, are involved in the reception of (*E*)-2-hexenol. Likewise, out of the upregulated OBPs upon exposure to phenethyl alcohol, HoblOBP9 showed the highest affinity for this ligand. The other OBPs whose genes were upregulated, HoblOBP2, 13, and 4, showed moderate binding affinity, whereas a negative control, HoblOBP7 (somewhat downregulated, Fig. 3) showed no affinity for phenethyl alcohol (Fig. S3). Next, we tested whether silencing these genes would have an effect on the phenotype.

3.5. *Effect of dsRNA treatment on HoblOBP9 and HoblOBP13*

To test the effect of reducing transcript levels of *HoblOBP9* and *HoblOBP13* genes on beetle's behavior, we injected female beetles with dsRNAs, determined by qRT-PCR the best timing for behavioral measurements, and compared their attractiveness to (*E*)-2-hexenol or phenethyl alcohol with that of control beetles. First, we compared transcript levels of control (noninjected), water-injected, and OBP-dsRNA-injected beetles at 24, 48, and 72 h postinjection. Throughout the studied time course, no difference occurred in transcript levels of *HoblOBP13* and *HoblOBP9* in control beetles and those injected with water (Fig. 5). The transcript levels of both *HoblOBP13* and *HoblOBP9* decreased over time, with 40% or lower transcript levels after 72 h (Fig. 5). Similar results were obtained when water-injected and GFP-dsRNA-injected beetles were used as negative controls (Fig. S4). In short, water injection or GFP-dsRNA treatment did not affect the transcript levels of *HoblOBP13* and *HoblOBP9*, but treatment with OBP-dsRNA did (Fig. 5 and S4). The reduction in transcript levels implies that protein levels may have decreased, which would ultimately affect behavior.

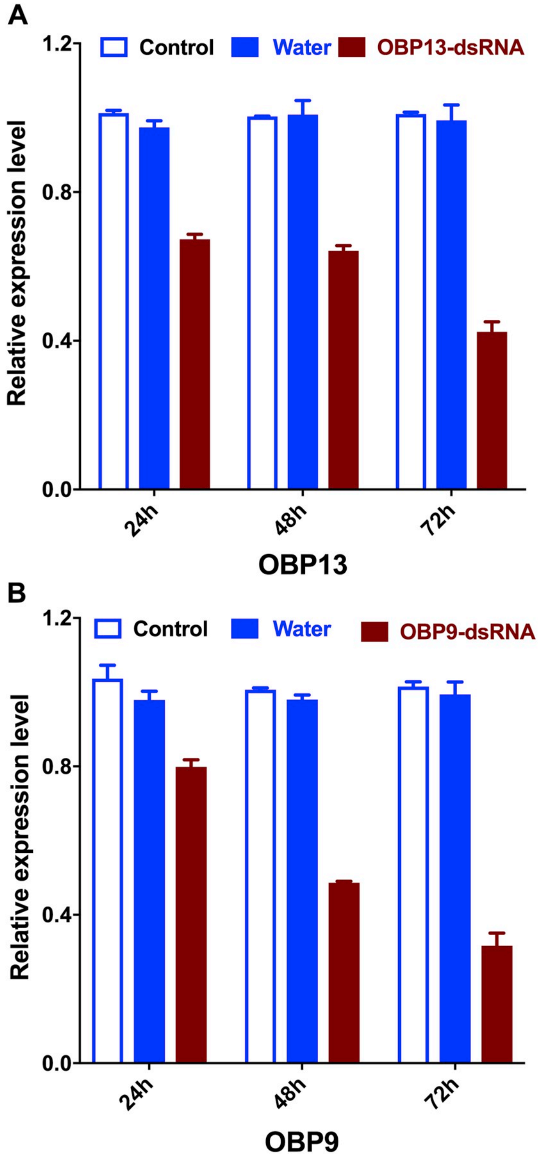

Fig. 5. Effect of RNAi treatment on the transcript levels of (**A**) *HoblOBP13* and (**B**) HoblOBP9 over time. Transcript levels in the antennae of untreated (control) female beetles were compared with those obtained from beetles injected either with water or OBP-dsRNA, 24, 48, and 72 h postinjection.

Lastly, we compared the attractiveness of female beetles treated with OBP-dsRNA and negative controls to (*E*)-2-hexenol (Fig. 6) or phenethyl alcohol (Fig. S5). Although the attraction indexes of beetles treated with OBP13-dsRNA were lower than those injected with OBP9-dsRNA, no significant difference existed between these treatments ($P < 0.0001$) (Fig. 6). Similar results were obtained when attraction to phenethyl alcohol was measured (Fig. S5). We, therefore, concluded that both HoblOBP13 and HoblOBP9 are essential for the reception of (*E*)-2-hexenol and phenethyl alcohol, 2 plant-derived attractants for *H. oblita.*

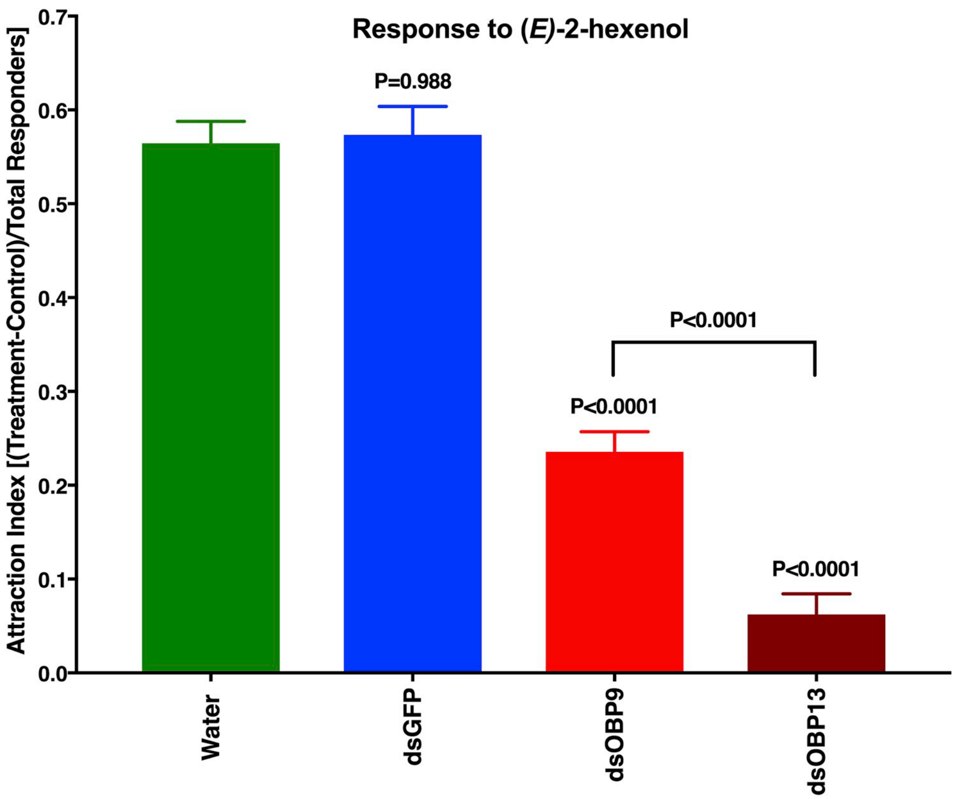

Fig. 6. Behavioral responses of *H. oblita* phenotypes to (*E*)-2-hexenol in a Y-olfactometer. In each experiment, the responses of 30 responding beetles of each type were recorded with 15 replicates. Water, beetles injected with water; dsGFP and dsOBPs are abbreviations for GFP-dsRNA-, and OBP-dsRNA-injected beetles. For each experiment (response of 30 beetles) a data point was generated by calculating an attraction index, ie, (no. of beetles in the treatment arm – no. of beetles in the control side of the arena) divided by total (30) number of responding beetles. N = 15, number of responding beetles in each bar: 450.

CRediT authorship contribution statement

Jiao Yin: Formal analysis, Formal analysis, Formal analysis, Writing - original draft, Writing - review & editing. **Chaoqun Wang:** Formal analysis, Writing - review & editing. **Chiqin Fang:** Formal analysis, Writing - review & editing. **Shuai Zhang:** Writing - review & editing. **Yazhong Cao:** Writing - review & editing. **Kebin Li:** Formal analysis, Writing - review & editing. **Water S. Leal:** Formal analysis, Formal analysis, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

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