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ORIGINAL PAPER

Identification of odorant-binding proteins and functional analysis of antenna-specific AplaOBP1 in the emerald ash borer, *Agrilus planipennis*

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Abstract

Olfaction-based strategies have been successfully applied to monitor the emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae); however, roles of chemosensory-associated proteins in olfactory perception of *A. planipennis* are rarely reported. Here, we identified 11 odorant-binding proteins (OBPs) from the *A. planipennis* transcriptome and genome. Sequence and phylogenetic analyses revealed that *A. planipennis* OBPs (AplaOBPs) could be classified into four subfamilies. The reverse transcription PCR (RT-PCR) analysis showed that all three members of the ABPII, one plus-C OBP, and one minus-C OBP were specifically or predominantly expressed in the antennae of both sexes, indicating their possible involvement in chemoreception. Subsequently, an antenna-specific OBP, AplaOBP1, was investigated through in situ hybridization, immunocytochemistry, and fluorescent competitive binding assays. It was found that the AplaOBP1 gene was detected under the base of the antennal sensilla, and the AplaOBP1 protein was expressed in the lymph of multiparous sensilla basiconica types I and III. In vitro, recombinant AplaOBP1 exhibited high binding affinities (K_i = 3.38–9.25 μM) with nine volatiles including five host-tree terpenes (e.g., myrcene, limonene, nerolidol, α-farnesene, and ocimene), which indicated that AplaOBP1 might be involved in the detection of host volatiles. The electrophysiological activities of all AplaOBP1 ligands were further confirmed using electroantennography, whose results imply that these volatiles may act as putative semiochemicals for *A. planipennis*. In conclusion, our results provide valuable insights into the molecular basis of olfaction in *A. planipennis* and help us use OBPs as targets to design novel olfactory regulators for management of *A. planipennis*.

Keywords *Agrilus planipennis* · Odorant-binding proteins · Sensilla basiconica · Fluorescence binding assay · Electroantennography

Key message

- *Agrilus planipennis* is a destructive invasive pest of ash trees, but little is known about the molecular basis of olfaction in this insect.

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- Eleven AplaOBPs were identified, and five OBPs were mainly expressed in the antennae.
- AplaOBP1 expressed in sensilla basiconica and showed high binding affinities with host volatiles. All ligands of AplaOBP1 could trigger antennal responses of both males and females.
- AplaOBPs are potential targets to discover novel olfactory regulators for management of *A. planipennis*.

Introduction

In insects, olfaction plays a crucial role in locating food sources, searching for sexual partners, selecting oviposition sites, and avoiding predators. Such behaviors are primarily dependent on the perception of variety of semiochemicals, such as sex pheromones, host plant volatiles, and animal odorants (Bruce et al. 2005; Ebrahim et al. 2015; Mattiacci and Dicke 1995; Metcalf and Kogan 1987; Nunez 1982; Takken 1991; Takken and Knols 1999). The olfactory signals are typically detected by neurons housed within different morphology olfactory sensilla present on the insect antennae; for example, neurons in trichoid sensilla sense sex pheromones and neurons in basiconica sensilla help to detect most of the host plant volatiles (Bruyne et al. 2001; Dweck et al. 2015; Kaissling et al. 1989). Olfactory sensilla are perforated by numerous pores, forming a hollow structure filled with aqueous lymph that harbors the dendritic branches of the olfactory receptor neurons (ORNs). Odorants enter into the olfactory sensillum cavity through pore canals, across the aqueous lymph, activate the ORNs, and ultimately lead to multi behavioral responses (Hansson and Stensmyr 2011; Leal 2013; Steinbrecht 1997; Zacharuk 1980). Among the several proteins involved in olfactory perception, the odorant-binding proteins (OBPs) are major peripheral olfactory proteins and play essential functions in insect olfactory systems (Larter et al. 2016; Pelletier et al. 2010; Pelosi et al. 2014; Vogt et al. 1989; Vogt and Riddiford 1981; Xu et al. 2005).

Inside olfactory sensilla, OBPs are synthesized in auxiliary cells and secreted in the sensillum lymph at extraordinarily high concentrations (Laue et al. 1994; Maida et al. 1993; Vogt et al. 1989). Within a species, various OBPs are expressed in different antennal sensilla; some are co-expressed in the same sensilla (Jiang et al. 2018; Larter et al. 2016; Pikielny et al. 1994; Schultze et al. 2012; Shanbhag et al. 2005; Wang et al. 2018). Although the specific physiological roles of OBPs in olfactory perception remain poorly understood, it is widely believed that they play essential roles in binding, solubilizing, and transferring hydrophobic odorants across the aqueous sensillum lymph to olfactory receptors in the dendrites of the ORNs (Gomez-Diaz et al. 2013; Leal 2013; Leal et al. 2005; Xu et al. 2005). Various studies have evidenced that OBPs have the ability to bind odorants, with different degrees of affinity and selectivity for different OBPs (Gong et al. 2010; Huang et al. 2018; Leal et al. 2005; Li et al. 2014; Ye et al. 2017; Yin et al. 2013; Zhang et al. 2011). But the model of odorant transport via OBPs may not apply to all olfactory sensilla. A recent study found that some basiconic sensilla do not require an OBP for the transport of odorants to the olfactory receptor (Larter et al. 2016).

Since the first insect OBPs were reported in Lepidoptera (Vogt and Riddiford 1981), many OBP genes have been identified in different insect species (Dippel et al. 2014; Forêt and Maleszka 2006; Gong et al. 2009). Based on their structural features and phylogenetic relationships, OBPs are divided into different subfamilies: classic, minus-C, plus-C, dimer, PBP/GOBP, ABPI and ABPII, CRLBP, and D7 (Hekmat-Scafe et al. 2002; Vieira and Rozas 2011). These subfamilies are unequally distributed across insect species, and some of them are absent in certain species (Vieira and Rozas 2011). In *Tribolium castaneum*, 50 OBPs form a group subdivided into four subfamilies: classic, minus-c, plus-C, and ABPII (Dippel et al. 2014). As the list of identified OBP members has increased recently, the analysis of their expression in different insects has revealed that only a part of them are restricted to the antennae (Dippel et al. 2014; Forêt and Maleszka 2006; Pelosi et al. 2018; Wang et al. 2018). In insect antennae, OBPs are expressed not only in chemosensory sensilla but also in humidity-detecting sensilla. In *Drosophila*, one OBP was found to be expressed in hygrosensory sensilla and contributes to the humidity detection (Larter et al. 2016; Sun et al. 2018), indicating the diverse roles of antennal OBPs.

The emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), native to Asia, is a destructive and invasive forest pest of ash trees (Herms and McCullough 2014). Since the first detection of this pest in North America in 2002, it has killed hundreds of millions of ash trees (Morin et al. 2017). In *A. planipennis*, antennal transcriptomic approaches have already led to the identification of various olfactory genes, such as nine OBPs and two odorant receptors (Mamidala et al. 2013), which now await functional characterization. In the present study, we identified 11 putative OBP sequences from the genomic and transcriptomic data of this species. Moreover, the expression patterns of these OBPs in different tissues were evaluated using reverse transcription PCR (RT-PCR). Subsequently, we focused on the AplaOBP1, one of the antennae-specific OBPs in *A. planipennis*, to determine its potential roles in odorant detection. The in situ hybridization, immunocytochemistry, and fluorescent competitive assays were performed to investigate the expression feature and ligand-binding characteristics of AplaOBP1. Finally, electrophysiological activities of AplaOBP1 ligands were confirmed using electroantennography (EAG) recordings.

Materials and methods

Insect culture, tissue collection, and RNA extraction

In April 2018, infested ash trees with overwintering larvae of *A. planipennis* were felled in the suburbs of Beijing, China and cut into 50-cm-long logs. The logs were placed in a rearing cage at room temperature of 20–23 °C and relative humidity (RH) > 60% to allow the beetles to emerge. The emerging beetles were then collected daily and fed on ash leaves.

Different tissues (100 antennae, 6 heads without antenna, 4 bodies [mixture of thoraxes, abdomens, legs, and wings]) were dissected separately from 1- to 3-day-old female and male beetles, immediately frozen in liquid N_2, and stored at − 80 °C until RNA isolation. Total RNA was extracted from different tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The integrity and quantity of RNA samples were checked by using 1.2% agarose gel electrophoresis and NanoPhotometer N60 (Implen, München, Germany), respectively.

Identification of odorant-binding proteins in *A. planipennis*

The FASTA files of the predicted genome transcript of *A. planipennis* (https://www.ncbi.nlm.nih.gov/genome/?term=Agrilus+planipennis) and the transcriptome unigene sequences (Jun et al. 2015) were entered into BioEdit v7.1.3.0 (Hall 1999) to create local nucleotide database files. The local tBLASTn program was performed, using available sequences of OBP proteins from *Dendroctonus valens* (Gu et al. 2015), *Monochamus alternatus* (Wang et al. 2014), *Rhynchophorus ferrugineus* (Antony et al. 2016), and *T. castaneum* (Dippel et al. 2014) as "queries" to identify candidate putative OBP sequences in *A. planipennis*.

Verification of OBP sequences by cloning and sequencing

Putative ORFs and associated amino acid sequences were determined using ORF FINDER (https://www.ncbi.nlm.nih.gov/orffinder/). Gene-specific primers were designed manually to clone the ORF of the *A. planipennis* OBP genes (Table S1). The template cDNA was synthesized from 2 µg of female antennal RNA using the Fast Quant RT kit (Tiangen, Beijing, China) following the manufacturer's protocol. Next, PCRs were conducted using one unit of KOD DNA polymerase (Taihe, Beijing, China) and 200 ng cDNA under the following conditions: denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min. The final extension step was at 68 °C for 10 min. The PCR products were cloned into the Blunt vector (Taihe, Beijing, China) and sequenced.

Sequence and phylogenetic analysis

Amino acid sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). N-terminal signal peptide sequences were predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). A Coleoptera-based phylogenetic analysis was performed, including 11 OBPs from *A. planipennis*, 11 OBPs from *Agrilus mali* (Cui 2018), and 50 OBPs from *T. castaneum* (Dippel et al. 2014). A neighbor-joining tree was constructed using the p-distance model and pairwise deletion gaps in MEGA 6.0 program. Bootstrapping was performed by resampling the amino acid positions of 1000 replicas.

RT-PCR measurement

The expression of OBP genes in antennae, heads, and bodies of both sexes were analyzed by semiquantitative RT-PCR using Taq DNA polymerase (Biomed, Beijing, China). cDNAs synthesis from different tissues was performed as described above. Each PCR volume (25 µL) contained 200 ng of cDNA from different tissues and was used as a template. The following cycling conditions were applied: 94 °C for 4 min and, for the subsequent 30 cycles: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The final extension step was at 72 °C for 5 min. The elongation factor 1-α (AplaEF-1α, XM_018476784.2) was employed to assess the cDNA integrity for all of the samples. For each amplification, negative controls using water template were performed. The amplification products were checked on 1.2% agarose gels. For each gene, one amplification product was sequenced to confirm its identity. The gene-specific primers were designed with the Primer 3 program (http://primer3.ut.ee/) and are listed in Table S1.

Expression and purification of recombinant AplaOBP1

The DNA sequences that encode the mature AplaOBP1 protein were chemically synthesized and cloned into pET30a (+) between the NdeI and XhoI sites by GenScript (Nanjing, China). The plasmid containing the correct insert was transformed into BL21 (DE3) competent cells. The protein was expressed in LB at 15 °C for 16 h through induction with 1 mM of isopropyl-*β*-D-thiogalactopyranoside (IPTG). The cultures were harvested by centrifugation and resuspended in 50 mM Tris buffer (pH 7.4). After sonication and centrifugation, the recombinant proteins, which were mainly present in the supernatant, were purified by a standard Ni column (GE Healthcare, Waukesha, WI, USA). His-tag was removed using recombinant enterokinase (Novagen, Madison, WI, USA) following the manufacturer's protocol. Purified AplaOBP1 was dialyzed in Tris buffer, and its concentration was determined by the Bradford method (Bradford 1976).

AplaOBP1 antisera production and Western blot assay

The antibody against AplaOBP1 was generated in rabbits and produced by GenScript (Nanjing, China). The purified AplaOBP1 protein was denatured and separated on 10% SDS–PAGE, and the gel was electrotransferred onto NC membranes. After the protein was transferred to the membranes, GenScript's ONE-HOUR Western TM Kit was used for Western blot analysis. The results showed that the anti-AplaOBP1 antiserum reacted specifically with the purified recombinant AplaOBP1 protein (Fig. S1).

In situ hybridization

Plasmids containing the coding regions of AplaOBP1 (Table S2) were linearized with Sal I (Takara, Dalian, China). Digoxigenin (DIG)-labeled RNA probes was synthesized using the DIG RNA Labeling Kit (T7) (Roche, Mannheim, Germany) following the protocol.

RNA in situ hybridization was performed as previously described (Wang et al. 2017). In brief, antennae were dissected and embedded in the Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA). Twelve-micrometer antennal cryosections were collected and placed on SuperFrost Plus microscope slides (Fisher Scientific, USA). Sections were fixed at 4 °C and pretreated at room temperature. After being prehybridized, the sections were incubated with the hybridization solution containing RNA probes at 60 °C overnight. DIG-labeled probe detection was performed using an anti-DIG AP-conjugated antibody (Roche, Mannheim, Germany) in combination with HNPP/Fast Red (Roche, Mannheim, Germany). The sections were analyzed using a Zeiss LSM 880 laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), and the images were processed with ZEN 2 software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Fluorescence competitive binding assays

The binding abilities of AplaOBP1 to 58 volatiles were measured on an F-380 fluorescence spectrophotometer (Tianjin, China) using 10-nm slits and a 1-cm light path. As the fluorescent probe, the 1-NPN was excited at the wavelength of 337 nm and emission spectra were recorded between 390 nm and 530 nm. To measure the affinity of 1-NPN to the AplaOBP1 protein, a 2-μM solution of purified protein in 50-mM Tris–HCl at pH 7.4 was titrated with aliquots of 1-mM 1-NPN dissolved in methanol to final concentrations ranging from 2 to 16 μM.

Competitive binding was measured by titration of the solution of both AplaOBP1 protein and 1-NPN at a concentration of 2 μM by adding aliquots of 1-mM methanol solution of ligand to final concentrations of 2–16 μM. Dissociation constants of the competitors were calculated by the equation $K_i = IC_{50}/(1 + [1-NPN]/K_{1\text{-}NPN})$, where IC_{50} is the concentration of ligands halving the initial fluorescence value of 1-NPN, [1-NPN] is the free concentration of 1-NPN, and $K_{1\text{-}NPN}$ is the dissociation constant of the AplaOBP1/1-NPN complex. The experiments were performed in triplicate, except for the ligands that did not show significant binding; these were analyzed in single experiments.

Electrophysiological recordings

The antennal responses of *A. planipennis* to the nine candidate ligands of AplaOBP1 were evaluated by the EAG technique. Antennae of the 1–3-day-old beetles were dissected, and their last segment was cutoff. A glass capillary filled with 3 M KCl, which served as the reference electrode, was connected to the base of each antenna. The antennal tip was connected a similar glass capillary, which provided the recording electrode. Twenty microliters of tested chemicals (10 mg/mL, diluted in paraffin oil) was applied to filter paper strips (1.0 cm × 5.0 cm) and inserted into a glass Pasteur pipette (Camlab, Cambridge, UK) as a cartridge. The test cartridge was connected to a stimulus controller (CS-55; Syntech, Kirchzarten, Germany) that generated a 0.5-s stimulus at 30-s intervals, with a constant flow of 10 mL/s. The signals generated by the antennae were recorded with EAG Pro software (Syntech). Preliminary experiments had shown that (*Z*)-3-hexen-1-yl acetate had the capacity to elicit stable EAG responses, and thus, it was used as the standard stimulus. The solvent control and standard stimulus were applied at the beginning and end of the stimulation of each antenna. For each compound, EAG responses were recorded from eight antennae of different insects of each sex. Responses to the solvent control were subtracted from all EAG responses elicited by the test odor stimuli. The normalized EAG values were presented as percentages of the standard stimulus responses.

Results

Odorant-binding proteins in *A. planipennis*

Based on the sequence similarity to insect OBPs, 11 full-length candidate OBP genes (ORF range, 396–591 bp) were identified from the *A. planipennis* genome and transcriptome (Table 1). The nucleotide sequences of *A. planipennis* OBP genes were verified by molecular cloning and sequencing (Table S3). All *A. planipennis* OBP proteins had a predicted N-terminal signal peptide with a length from 16 to 25 aa (Table 1). The comparison with previously identified OBPs revealed that four candidates were new OBPs. In addition, we found that the previously identified ApOBP2 and ApOBP4 were actually identical to ApOBP8 and ApOBP6 (Mamidala et al. 2013). We renamed the *A. planipennis* OBP genes AplaOBP1–11, following the conventions for

Table 1 List of OBPs identified in *A. planipennis*

Gene name	Previous name	Genome databases Gene ID	Transcriptome databases Gene ID	ORF (bp)	Signal peptide	Subfamily	*Agrilus mali* homolog	
							Protein ID	% identity
AplaOBP1	ApOBP1	XM_025981437.1	ND	396	1–18aa	ABPII	AVU05010.1	81.7
AplaOBP2	ApOBP3	XM_025974145.1	EABT21324	426	1–22aa	ABPII	AVU05011.1	78.8
AplaOBP3	ApOBP4/6	XM_025977151.1	EABT24710	423	1–19aa	ABPII	AVU05012.1	88.6
AplaOBP4		XM_018478260.2	EABT5835	447	1–16aa	Classic	AVU05013.1	73.7
AplaOBP5		XM_025978523.1	EABT666	468	1–25aa	Classic	AVU05014.1	78.5
AplaOBP6	ApOBP2/8	XM_018477087.2	EABT22002	591	1–17aa	Plus-C	AVU05015.1	56.2
AplaOBP7	ApOBP9	XM_018473296.1	EABT8276	402	1–19aa	Minus-C	AVU05016.1	55.8
AplaOBP8		XM_018475634.1	EABT36384	405	1–18aa	Minus-C	AVU05017.1	85.8
AplaOBP9		XM_018473294.2	ND	408	1–20aa	Minus-C	AVU05018.1	84.4
AplaOBP10	ApOBP7	XM_018473295.2	EABT16054	402	1–18aa	Minus-C	AVU05019.1	81.2
AplaOBP11	ApOBP5	XM_018478572.2	EABT6497	450	1–18aa	Minus-C	AVU05020.1	90.6

Previous names are from Mamidala et al. 2013. Subfamilies are derived from Fig. 1. Amino acids identity percentages were calculated using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). ND, not detected

the homologous genes in *A. mali* (Cui 2018). The AplaOBP family members shared relatively low amino acid identities (11.3–36.8%) (Table S4). However, the amino acid identities of orthologous OBPs in *A. planipennis* and *A. mali* are much higher (e.g., 90.6% among the OBP11 orthologs) (Table 1).

The phylogenetic relationships of the 11 AplaOBPs, as well as the 11 OBPs from *A. mali*, were used to construct a phylogenetic tree clustering with 50 OBPs from *T. castaneum* (Fig. 1). The 11 AplaOBPs were significantly divergent from each other and clustered together with

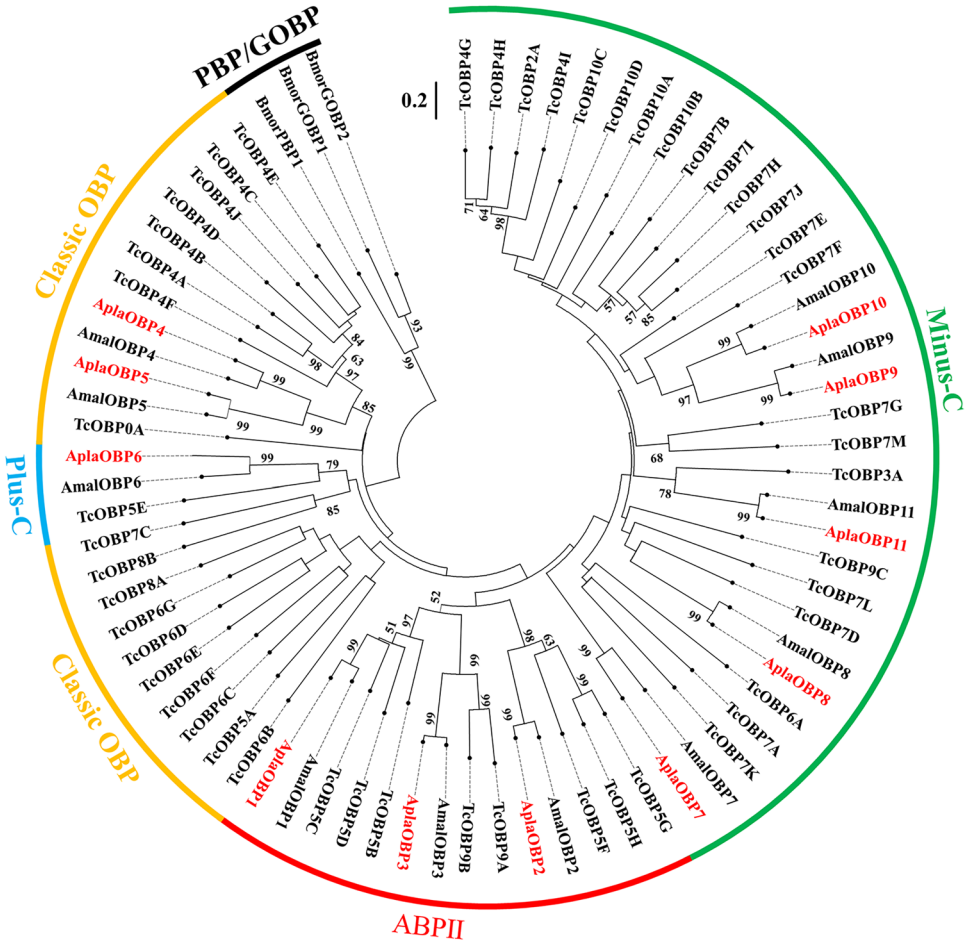

Fig. 1 Phylogenetic relationship of candidate OBPs from *A. planipennis* and other Coleoptera insects: Apla, *A. planipennis*; Amal, *A. mali*; and Tcas, *T. castaneum*. The PBP/GOBP subfamily members in *Bombyx mori* (BmorGOBP1, BmorGOBP2, and BmorPBP1, Krieger et al. 1996) were used as out-group. Genes from *A. planipennis* are labeled with red. The outer rings indicate the phylogenetic subfamily (ABPII in red, plus-C in blue, classic in orange, minus-C in green, and PBP/GOBP in black)

their orthologs in *A. mali* and *T. castaneum* (Fig. 1). In *A. planipennis*, three OBPs were grouped in the ABPII subfamily; two were clustered in the Classic OBP subfamily; one was identified in the plus-C OBP subfamily; and the remaining five proteins were assigned to the minus-C OBP subfamily (Fig. 1). Within each subfamily, most of the AplaOBP proteins had a conserved cysteine pattern. The only exception was the AplaOBP7, a minus-C subfamily member, which, unusually for the family, had six cysteine residues. Conversely, its ortholog in *A. mali* (AmalOBP7) had four cysteine residues. The amino acid alignments of *A. planipennis* and *A. mali* OBPs from these groups are depicted in Fig. 2.

Expression profiles of AplaOBPs

The 11 OBP expression patterns in different tissues of both males and females were investigated by semiquantitative RT-PCR. The elongation factor 1-α (AplaEF-1α) gene was constitutively expressed in all tissues, providing a stable

Fig. 2 Amino acid alignments of four subfamilies of OBPs between *A. planipennis* and *A. mali*. **a** Classic OBPs and ABPII; **b** plus-C OBPs; **c** minus-C OBPs. The signal peptides are in blue boxes. The conserved cysteine residues are indicated by yellow shading

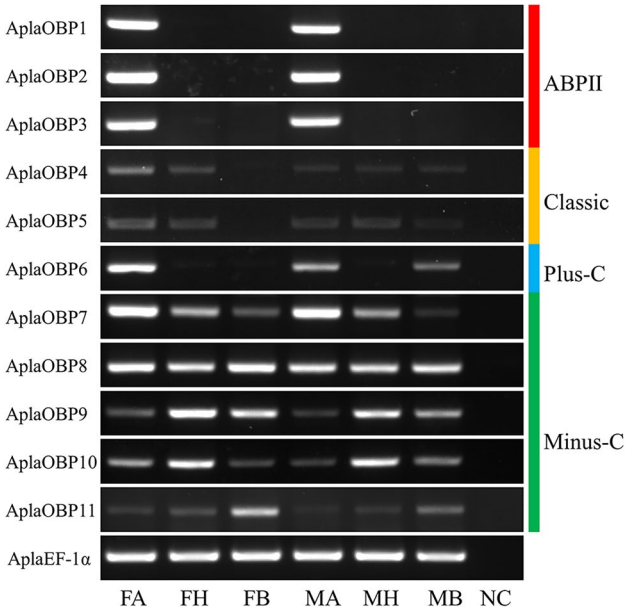

Fig. 3 Tissue- and sex-specific expression of the 11 OBP genes in *A. planipennis*. Abbreviations: *FA* female antennae, *FH* female heads, *FB* female bodies, *MA* male antennae, *MH* male heads, *MB* male bodies, *NC* no template control. Right bars indicate the different subfamilies (ABPII in red, classic in orange, plus-C in blue, and minus-C in green)

control for the integrity of the cDNA templates (Fig. 3). All three ABPIIs (OBP1, OBP2, and OBP3) were specifically expressed in the antennae of both sexes. The two classic OBPs, OBP4 and OBP5, were present at low expression levels in all tissues. The plus-C OBP (OBP6) was predominant in the antennae of both sexes but was also present in the body of the male individuals. Of the five minus-C OBPs, only OBP7 was predominantly expressed in the antennae of both sexes. The remaining four minus-C OBPs (OBP8, OBP9, OBP10, and OBP11) were expressed in all tissues and had low expression levels in the antennae.

Location of AplaOBP1 in *A. planipennis* antennae

The precise expression pattern of antenna-specific AplaOBP1 was further determined by in situ hybridization and immunocytochemistry localization. AplaOBP1 hybridization signals were detected at the bases of the sensilla basiconica by in situ hybridization (Fig. 4). The *A. planipennis* antenna is covered with three types of s. basiconica (I, II, and III) (Crook et al. 2008a). The immunocytochemistry results indicated that s. basiconica types I and III were strongly labeled by anti-AplaOBP1 antisera; the gold granules were concentrated at the sensillum lymph in the sensillum hair lumen (Fig. 5). However, s. basiconica type II was not labeled by the anti-AplaOBP1 antisera.

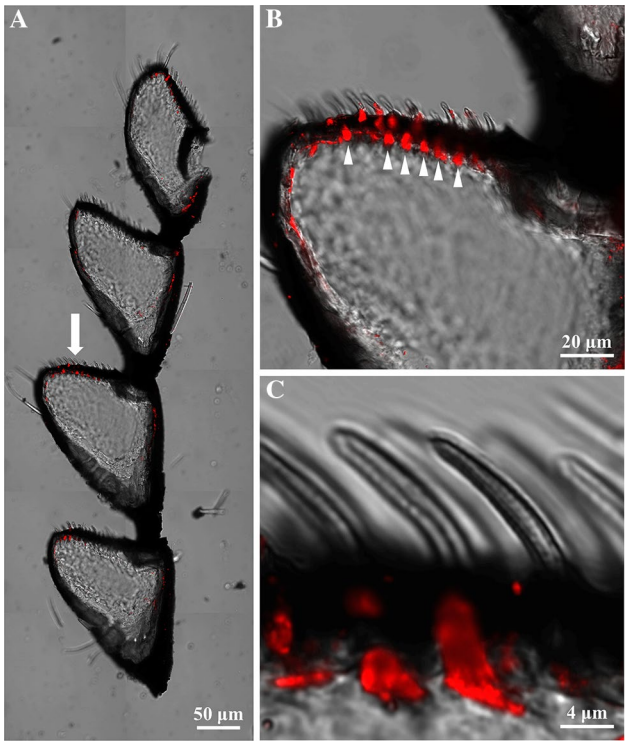

Fig. 4 In situ hybridization of AplaOBP1 in female antennae of *A. planipennis*. DIG-labeled antisense RNA probes for AplaOBP1 were hybridized to longitudinal sections through the female antennae and visualized using red fluorescence. **a** Gene expression of AplaOBP1 in four segments of the female antenna. The white arrow indicates the AplaOBP1 probe-labeled region; **b** One segment showing the distribution of AplaOBP1 probe-labeled cells (triangles) in the female antennae; **c** Higher magnification of the AplaOBP1-labeled cells under the sensilla basiconica. In situ hybridization data of AplaOBP1 in male antennae were consistent with in female antennae (data not shown here). The negative controls are provided in Fig. S2

The antennae of males and females showed consistent in situ hybridization and immunocytochemistry data for AplaOBP1.

Binding characteristic of recombinant AplaOBP1

The specific expression of AplaOBP1 in multiporous s. basiconica suggests that AplaOBP1 is involved in olfactory recognition processes. To screen the putative ligands for AplaOBP1, we first expressed AplaOBP1 in a bacterial system. The protein was purified by affinity chromatography on Ni-columns and used for polyclonal antisera production, as well as for ligand-binding experiments. The size and purity of the recombinant protein were examined by SDS–PAGE (Fig. S1) and mass spectrometric analysis (data not shown here). We measured the protein affinity toward 58 volatile compounds in competitive binding experiments by using *N*-phenyl-1-naphthylamine (1-NPN) as a fluorescent probe. The

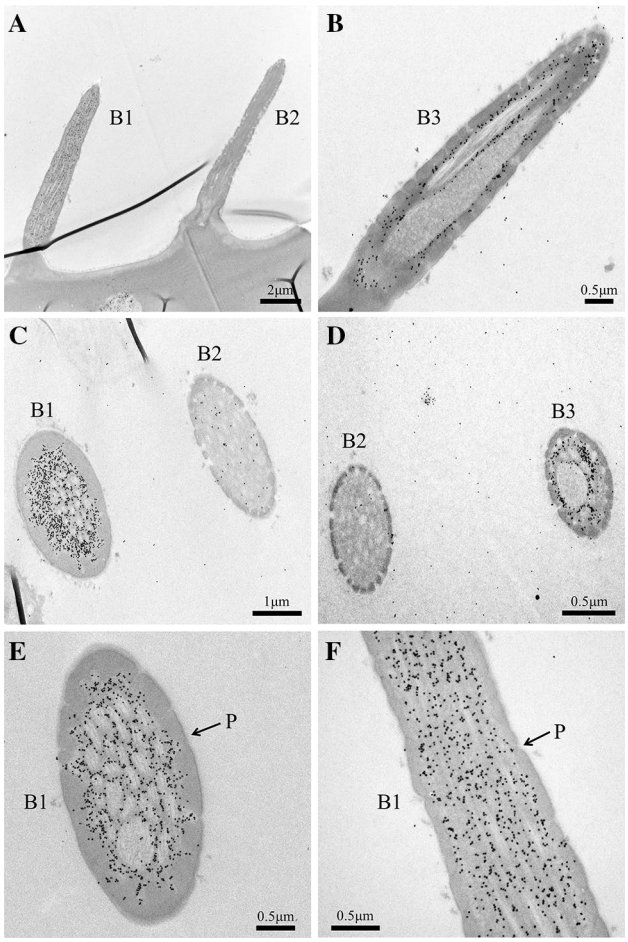

Fig. 5 Immunocytochemical localization of AplaOBP1 proteins in the olfactory sensilla of female *A. planipennis* antennae. Black spots in the sensillum lymph of hair lumen represent immunostained AplaOBP1. The longitudinal sections (**a**, **b**, **f**) and the cross sections (**c**, **d**, **e**) showing the sensillum lymph of hair lumen of the sensilla basiconica type I and type III were heavily labeled by anti-AplaOBP1 antisera, while the hair lumen of s. basiconica type II was never labeled. The few grains found in s. basiconica type II represent a non-specific background. The dilution of the primary antibody was 1:3000, and the secondary antibody was anti-rabbit IgG conjugated with 10-nm colloidal gold granules at a dilution of 1:20. Abbreviations: B1, sensilla basiconica type I; B2, s. basiconica type II; B3, s. basiconica type III; and P, pore. The immunocytochemical data of AplaOBP1 in antennal sensilla of males were consistent with that in females (data not shown here). The negative controls are provided in Fig. S3

candidate compounds were selected from known plant secondary metabolites including 27 ash plant volatiles (Table 2).

First, the affinity constant was measured for AplaOBP1 to 1-NPN. AplaOBP1 binds reversibly with 1-NPN with a dissociation constant of 1.95 ± 0.10 μM (Fig. 6a, b), indicating that 1-NPN is a suitable fluorescent reporter. Of the 58 tested odorants, only nine compounds showed binding affinities for AplaOBP1, including octane, hexyl butyrate, (−)-citronellal, citral, myrcene, (+)-limonene, nerolidol, α-farnesene, and ocimene. The binding affinities of these ligands ranged from 3.38 ± 0.39 to 9.25 ± 0.28 μM (Table 2, Fig. 6c, d).

Electrophysiological activities of putative ligands of AplaOBP1

To determine whether the AplaOBP1 ligands have biological activity, we measured the electrophysiological responses of male and female *A. planipennis* to these volatiles using EAG recordings. The results clearly indicated that all volatiles could elicit electrophysiological responses in the antennae of both male and female *A. planipennis* (Fig. 7). Of note, (+)-limonene elicited the highest EAG response among all ligands in both males and females. Nerolidol elicited only weak responses in the male and female antennae. All volatiles, except for nerolidol, elicited significantly higher EAG responses from the female antennae than from the male ones.

Discussion

We identified 11 OBP genes by analyzing genomic and transcriptomic data of *A. planipennis*, four of which are newly reported. We detected all previously described *A. planipennis* OBPs (Mamidala et al. 2013) and confirmed the predicted open reading frames (ORFs) of all 11 OBPs by molecular cloning and sequencing. The number of *A. planipennis* OBPs identified here is less than the 50 OBPs identified in *T. castaneum* (Dippel et al. 2014), but the same as in *A. mali* (Cui 2018). Despite the fact that *A. planipennis* OBP members are highly divergent, all of which have a clear ortholog in *A. mali*. The high conservation in OBP ortholog sequence identities between the two *Agrilus* species indicates that each OBP has a conserved function and evolved from a common ancestor without subsequent duplication. This finding may offer important insights for future comparative studies of OBP functions between these two species.

The AplaOBPs can be grouped into four subfamilies. Our expression data showed that all of the ABPII are specifically expressed in the antennae, indicating that this subfamily is likely involved in olfactory perception. The antennal expression of *A. planipennis* ABPII is consistent in the other species. All ABPII members of *T. castaneum*, *Anopheles gambiae*, and *Drosophila melanogaster* are highly expressed in the antennae (Dippel et al. 2014; Hekmat-Scafe et al. 2002; Pitts 2011). Genes in this subfamily have been shown to have a specific role in olfaction. For example, *Acyrthosiphon pisum* ApisOBP7 is involved in alarm pheromone perception (Feng et al. 2012) and *D. melanogaster* LUSH (DmelOBP76a) participates in sex pheromone detection (Xu et al. 2005). In contrast to ABPII, most of the minus-C OBPs of *A. planipennis* had significantly diversified expression profiles, indicating that these OBPs might exert broader

Table 2 Dissociation constants (K_i) of the complexes between the ligands and OBP1 of *A. planipennis*

Ligand	CAS number	K_i (μM)	Ligand	CAS number	K_i (μM)
1-Hexanol*	111-27-3	–	Hexyl acetate*	142-92-7	–
trans-2-Hexen-1-ol	928-95-0	–	*cis*-3-Hexenyl acetate*	3681-71-8	–
cis-2-Hexen-1-ol	928-94-9	–	2-Heptanone	110-43-0	–
trans-3-Hexen-1-ol	928-97-2	–	3′, 4′-Dimethoxyacetophenone	1131-62-0	–
cis-3-Hexen-1-ol*	928-96-1	–	4′-Ethylacetophenone*	937-30-4	–
trans-4-Hexen-1-ol	928-92-7	–	2-Octanone	111-13-7	–
1-Octanol	111-87-5	–	(−)-Citronellal	5949-05-3	6.26 ± 0.10
Nonanal*	124-19-6	–	Citral	5392-40-5	5.68 ± 0.06
Valeraldehyde	110-62-3	–	1,8-Cineole*	470-82-6	–
Octanal	124-13-0	–	Linalool*	78-70-6	–
Benzaldehyde	100-52-7	–	*β*-Pinene*	127-91-3	–
Butyraldehyde	123-72-8	–	Myrcene*	123-35-3	8.19 ± 0.41
trans-2-Heptenal	18829-55-5	–	(+)-3-Carene*	13466-78-9	–
Hexanal*	66-25-1	–	(+)-Limonene*	5989-27-5	9.25 ± 0.28
trans-2-Hexenal*	6728-26-3	–	(1*R*)-(+)-*α*-Pinene*	7785-70-8	–
Tridecane	629-50-5	–	Terpineol	8000-41-7	–
Decane*	124-18-5	–	*α*-Terpinene	99-86-5	–
Undecane	1120-21-4	–	Nerolidol*	7212-44-4	5.09 ± 0.06
Octane	111-65-9	6.84 ± 0.50	*β*-Ionone	14901-07-6	–
Dodecane*	112-40-3	–	*α*-Humulene*	6753-98-6	–
Indole*	120-72-9	–	*α*-Copaene*	3856-25-5	–
Methyl phenylacetate	101-41-7	–	Geraniol	106-24-1	–
Methyl salicylate*	119-36-8	–	*α*-Farnesene*	502-61-4	3.38 ± 0.39
cis-3-Hexenyl butyrate*	16491-36-4	–	Ocimene*	13877-91-3	7.42 ± 0.42
Methyl benzoate	93-58-3	–	*β*-Caryophyllene*	87-44-5	–
Isobutyl acrylate	106-63-8	–	(−)-Caryophyllene oxide*	1139-30-6	–
Hexyl Butyrate	2639-63-6	5.78 ± 0.22	Pyrrolidine	123-75-1	–
trans-2-Hexenyl acetate	2497-18-9	–	Isobutyl tiglate	61692-84-0	–
trans-2-Hexenyl butyrate	53398-83-7	–	2-Phenylethanol	60-12-8	–

Dissociation constants are reported only where IC_{50} values could be measured. "–" indicates data not available; "*" denotes the volatiles found in the ash tree (Crook et al. 2008b; Rigsby et al. 2017; Rodriguez-Saona et al. 2006)

physiological functions. The general characteristics of the expression patterns of the minus-C OBP subfamily in *A. planipennis* are similar to those in other species (Dippel et al. 2014; Forêt and Maleszka 2006).

The OBPs expressed in olfactory sensilla are most likely involved in olfactory perception. Focusing on the ABPII, we examined the expression of the AplaOBP1 at the sensillum morphology level. In accordance with previous studies (Huang et al. 2018; Laue et al. 1994; Maida et al. 2005; Steinbrecht et al. 1995), the present study found that the OBP gene is detected in cells at the base of the antennal sensilla, and the expressed protein fills the olfactory sensilla lymph. The *A. planipennis* antenna is covered with three types of olfactory sensilla (s. basiconica I, II, and III) (Crook et al. 2008a), each of which is probably involved in the detection of different types of volatiles. AplaOBP1, which is selectively expressed in two of the three olfactory sensilla, might transport different types of odorants to meet the requirements of each olfactory sensilla type. In fact, our fluorescent binding results strongly support a selective binding of AplaOBP1 to different odorants with quite high affinities. Of the nine AplaOBP1 ligands, five volatiles were detected in ash trees (e.g., myrcene, limonene, nerolidol, *α*-farnesene, and ocimene) (Crook et al. 2008b; Rigsby et al. 2017; Rodriguez-Saona et al. 2006), indicating that AplaOBP1 is likely involved in host volatile recognition. In the present study, none of the candidate green leaf volatiles (GLVs) [e.g., (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, hexanol, and

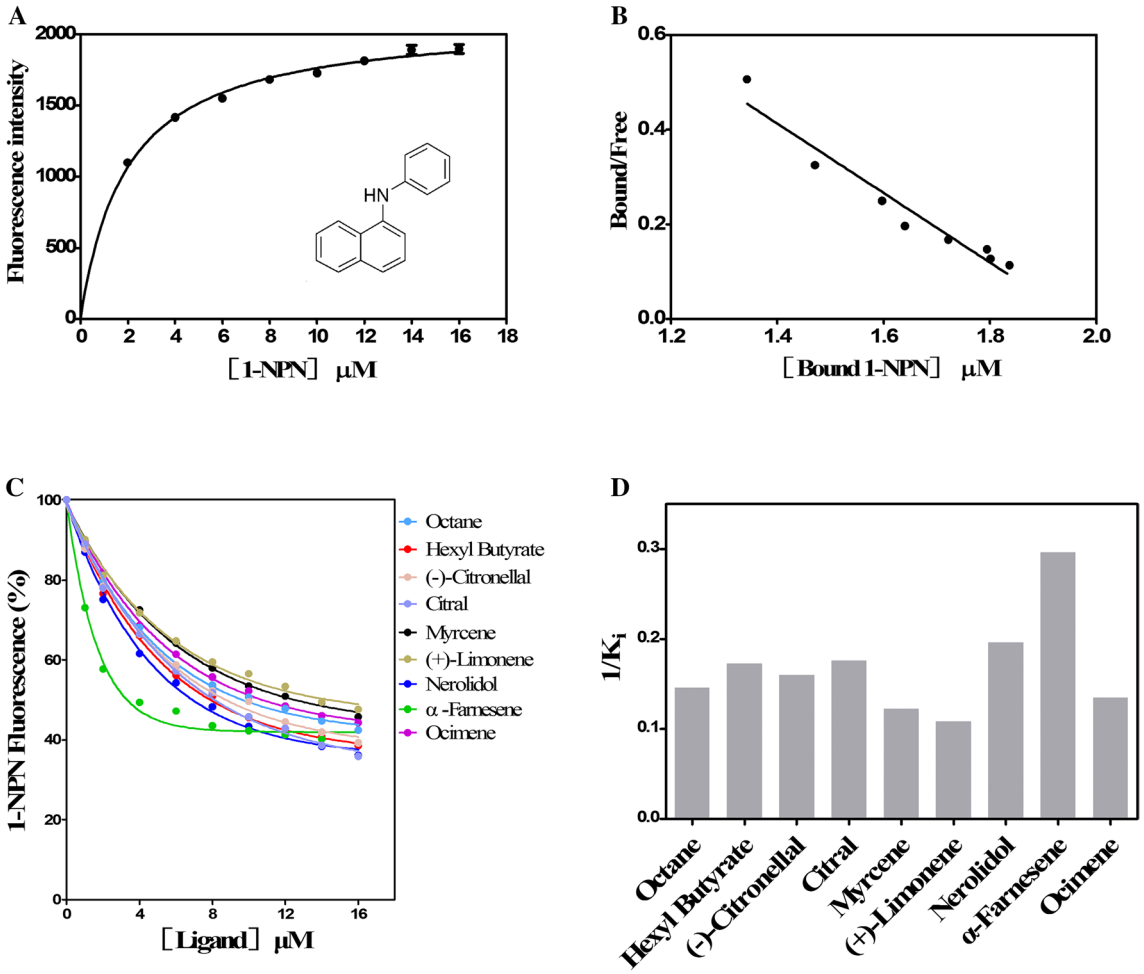

Fig. 6 Fluorescence competitive binding assay of *A. planipennis* AplaOBP1. Binding curve (**a**) and Scatchard plot (**b**) of the fluorescence probe 1-NPN to AplaOBP1. The binding curve and the relative Scatchard plot indicate the binding constants of AplaOBP1/1-NPN complex are 1.95 ± 0.10 μM for AplaOBP1; **c** competitive binding curves of tested volatiles to AplaOBP1; **d** reverse values of dissociation constants (K_i) were measured with the putative ligands of AplaOBP1

(*Z*)-3-hexenyl acetate] could bind to AplaOBP1, although some of them have been shown to elicit behavioral responses in *A. planipennis* (Crook et al. 2008b; Grant et al. 2010, 2011). It is probable that other AplaOBPs could participate in GLVs transportation.

Electrophysiological studies have shown that *A. planipennis* exhibit antennal responses to a wide range of host volatiles (Crook et al. 2008b; Rodriguez-Saona et al. 2006), and lures based on host volatiles have been used to catch adult males and females in the field [e.g., (3*Z*)-hexenol] (Crook et al. 2008b). The nine ligands of AplaOBP1 can elicit antennal responses from both males and females, indicating that they may act as putative semiochemicals for *A. planipennis*. Among these volatiles, two monoterpene compounds, limonene and myrcene, evoked the largest EAG responses from female antennae. Although the behavioral activities of limonene and myrcene were not confirmed in the present investigation, these two monoterpene compounds might play a key role in the host location or the oviposition preference of female *A. planipennis*, considering that they have been shown to elicit behavioral responses in some insects (Gitau et al. 2013; Miller 2007).

In conclusion, we identified 11 OBP genes from *A. planipennis*, which can be divided into four subfamilies. The ABPII subfamily may have a specific role in *A. planipennis* olfactory perception, since all members are specifically expressed in the antennae of both sexes. The olfactory sensilla expression and the ability to bind host volatiles demonstrates that one of the ABPII members (AplaOBP1) plays olfactory roles through binding and transporting odorants.

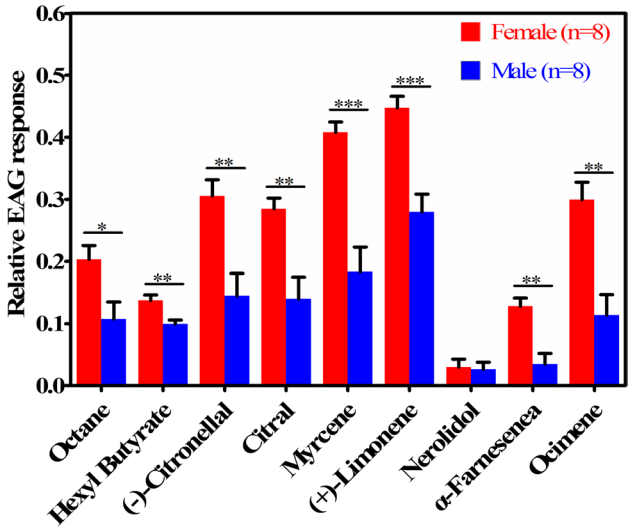

Fig. 7 EAG activity of male and female *A. planipennis* antennae to different ligands of AplaOBP1 (10 mg/mL). EAG amplitudes were adjusted to the control stimulus [(Z)-3-hexen-1-yl acetate], and are given as the mean ± SE. $*P<0.05$; $**P<0.01$; $***P<0.001$, significant differences for male versus female comparisons (one-way ANOVA test)

AplaOBP1 ligand activity was confirmed at the antennal level by EAG, indicating that these volatiles may act as potential semiochemicals for *A. planipennis*. In future, further studies are necessary to confirm the behavioral activity of these semiochemicals as well as their possible applications for the monitoring and control of *A. planipennis* populations.

Authors' contribution

SNW, FZ, and YJZ conceived and designed research. GYY and HW supplied insects. SNW conducted the experiments. SNW and SS analyzed the data and wrote the manuscript. KHD and AK revised the manuscript.

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