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AlepPBP2, but not AlepPBP3, may involve in the recognition of sex pheromones and maize volatiles in *Athetis lepigone*

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Abstract

Athetis lepigone Möschler (Lepidoptera, Noctuidae) is a common maize pest in Europe and Asia. However, there is no long-term effective management strategy is available yet to suppress its population. Adults rely heavily on olfactory cues to locate their optimal host plants and oviposition sites. Pheromone-binding proteins (PBPs) are believed to be responsible for recognizing and transporting different odorant molecules to interact with receptor membrane proteins. In this study, the ligand-binding specificities of two AlepPBPs (AlepPBP2 and AlepPBP3) for sex pheromone components and host plant (maize) volatiles were measured by fluorescence ligand-binding assay. The results demonstrated that AlepPBP2 had a high affinity with two pheromones [(Z)-7-dodecenyl acetate, Ki = 1.11 ± 0.1 μM, (Z)-9-tetradecenyl acetate, Ki = 1.32 ± 0.15 μM] and ten plant volatiles, including (-)-limonene, α-pinene, myrcene, linalool, benzaldehyde, nonanal, 2-hexanone, 3-hexanone, 2-heptanone and 6-methyl-5-hepten-2-one. In contrast, we found that none of these chemicals could bind to AlepPBP3. Our results clearly show no significant differences in the functional characterization of the binding properties between AlepPBP2 and AlepPBP3 to sex pheromones and host plant volatiles. Furthermore, molecular docking was employed for further detail on some crucial amino acid residues involved in the ligand-binding of AlepPBP2. These findings will provide valuable information about the potential protein binding sites necessary for protein-ligand interactions which appear as attractive targets for the development of novel technologies and management strategies for insect pests.

Introduction

In most insects, the chemosensory system plays an extremely important role in detecting food sources, mating, and searching for suitable oviposition sites (Hansson and Stensmyr, 2011; Lassance and Löfstedt, 2013; Renou and Anton, 2020). The main function of odorant-binding proteins (OBPs) located in the lymphatic fluid of the chemosensory organs is to detect, discriminate and transport the odor molecules from the environment to the dendritic membrane of the olfactory receptor neurons (ORNs), where the odor molecules interact with odorant receptors (ORs) (Pelosi *et al*., 2018), which could affect the behavior of insects concurrently.

OBPs are presented in high concentrations in the lymphatic fluid of the main olfactory organ (antennae). They are small water-soluble proteins characterized by six highly conserved cysteine residues and six α- helices that appear to provide a pocket for binding hydrophobic ligands (Song *et al*., 2018). A large number of different OBP subtypes were found to be highly expressed in insects and vary greatly between species using distinct functions. Pheromone-binding proteins (PBPs) are the common and special proteins among those classic proteins. It has long been thought that PBP, as the name implies, was a kind of protein to specifically bind sex pheromones secreted by female glands in corresponding insect populations (Li *et al*., 2018b). PBPs expression have mostly been identified in male antennae; however, lower levels of expression have been found in female antennae and mainly divided into three types in most moths, namely PBP1, PBP2 and PBP3, such as *Antheraea polyphemus* (Maida *et al*., 2000), *Plutella xyllotella* (Sun *et al*., 2013), *Helicoverpa armigera* (Zhang *et al*., 2012), *Tryporyza intacta* (Fang *et al*., 2018), *Spodoptera litura* (Liu *et al*., 2013), *A. ipsilon*

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(Gu *et al*., 2013), *M. brassicae* (Maïbèche-Coisné *et al*., 1998), *Heliothis virescens* (Campanacci *et al*., 2001; Abraham *et al*., 2005), *Chilo suppressalis* (Chang *et al*., 2015; Dong *et al*., 2019).

Several types of research have shown that host plant volatiles, produced by both anabolic and catabolic processes, played an important role in the cooperative evolution of plants and insects and had an important influence on insect behavior (Harrewijn *et al*., 1994). Generally, many insect species used a chemosensory system to detect and discriminate the chemicals released by host plants, such as allyl isothiocyanate, dipropyl thiosulfinate, and other plant-specific odor components (Lecomte *et al*., 1998). Different types of insect behaviors e.g. finding the suitable host plants through volatiles and oviposition sites using plant secondary metabolites have been previously studied in many species (Dicke *et al*., 1990; Binder *et al*., 1995; Lucas-Barbosa *et al*., 2015), which revealed that host plant volatiles extremely affected insect behavior. Additionally, many plant species can emit volatile organic compounds (VOCs), such as terpenoids, nitrogen and sulfur compounds, upon insect pests attack to reduce damage caused and/or induced resistance in plants against them (Maffei, 2010). It was found that some allelochemicals released by plants, mainly secondary metabolites, participate in the defense mechanisms of plants against herbivore attack which in turn affect the reproductive activities of insects (Harrewijn *et al*., 1994). This study indicated that plant volatiles are particularly abundant and play an important role in mediating the interaction between insects and their host plants. Therefore, in-depth studies investigating the mechanisms involved in insect chemoreception systems will help to decode this sophisticated communication language to make better decisions about pest control and crop protection.

It has been found that organisms maintain inter-group or intra-group communication through corresponding ways of information transmission, to coordinate biological behaviors. Sex pheromones, a kind of tiny special chemical substance, were secreted *in vitro* by the homogenous sex individual to induce courtship and mating behavior. In insects, many volatiles sex pheromones have been detected or identified, such as cis-8-dodecenyl acetate of *Grapholita molesta* (Roelofs *et al*., 1969), (Z, Z)-11,13-Hexadecadienal of *Amyelois transitella* (Liu *et al*., 2010), Z-11-hexadecenyl acetate of *M. brassicae* (Veire and Dirinck, 1986), (Z,E)-9,12-tetradecadien-l-ol acetate and (Z)-9-tetradecen-l-ol of *Spodoptera exigua* (Tumlinson *et al*., 1981), (Z)-11-hexadecenal, (Z)-11-hexadecenyl acetate and (Z)-11-hexadecenyl alcohol of *P. xylostella* (Chisholm *et al*., 1979; Lee *et al*., 2005), and Bombykol and Bombykal of *B. mori* (Odinokov *et al*., 1993). Related behavioral experiments have shown that sex pheromones were usually released at certain times during the life cycles of insects that influence their activities of mating recognition and altering predator-prey interactions (Mondor *et al*., 2004; Curkovic and Ferrera, 2012; Lo Pinto *et al*., 2013), revealing that the effects of sex pheromones on insect behaviors were very complex which need further investigation.

Athetis lepigone Möschler (Lepidoptera, Noctuidae) is one of the most important polyphagous destructive insect pests of economic crops worldwide (Jiang *et al*., 2011) It caused a great loss to the production of summer maize after the first report in China in 2011 (Fu *et al*., 2014). Synthetic insecticides are now the primary agents for their control, but the excessive use of insecticides may lead to the damage of ecosystems and increase the resistance levels of the target pest. Therefore, it is necessary to strengthen dynamic monitoring, timely make pest predictions to reduce the damage or develop novel green pest control strategies in modern pest management (Sarfraz *et al*., 2005; Choo *et al*., 2018; Hackett and Bonsall, 2019). The development of novel green behavioral inhibitors based on olfactory genes has become a hot spot research field for pest control (Pelosi *et al*., 2018; Caballero-Vidal *et al*., 2021). In order to fully understand the concept of insect chemical communication, we have recently identified two female sex pheromone of *A. lepigone* as (Z)-7-dodecenyl acetate (Z7-12:Ac) and (Z)-9-tetradecenyl acetate (Z9-14:Ac) with a ratio of 1:5 by analyzing the extracts of the female sex pheromone gland (Yan *et al*., 2018), and obtained three antennae-enriched PBP genes (AlepPBP1-3) in *A. lepigone* by antennal transcriptome analysis (Zhang *et al*., 2017). Afterward, we expressed and purified the recombinant AlepPBP1 protein in a prokaryotic expression system. Moreover, the ligand-binding assay showed that AlepPBP1 had a higher binding affinity to two sex pheromones of *A. lepigone* (Zhang *et al*., 2020*a*, 2020*b*, 2020*c*). In this study, we further analyzed and explored the potential functions of the other two AlepPBP candidates (AlepPBP2 and AlepPBP3) according to their different binding affinities to sex pheromones and host plant volatiles. Furthermore, some key amino acid residues involved in the process of AlepPBP2 recognition and their binding properties to different ligands were identified based on their molecular docking approach.

Materials and methods

Total RNA extraction, cDNA synthesis

The total RNAs were extracted from 100 adult male antennae using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After checking the RNA quality, the first cDNA strand was synthesized using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China).

Molecular cloning and preparation of expression vectors

We amplified the full-length open reading frame (ORFs) of AlepPBP2 and AlepPBP3 using the PCR approach and following the protocol recently described in Zhang *et al*. (2017). Then, the amplified products were directly cloned into the expression vector pET-30a(+) (Novagen, Darmstadt, Germany) for expression of recombinant proteins, following the manufacturer's instructions. The details of the cloning and construction of the expression vectors are displayed in the supplementary information.

Expression and purification of the recombinant AlepPBPs

Plasmids were transformed into *E. coli* BL21 (DE3) to express these proteins according to the method recently described by us and other researchers (Damberger *et al*., 2013; Katti *et al*., 2013; Zhu *et al*., 2016; Wang *et al*., 2020; Zhang *et al*., 2020*b*, 2020*b*). After the *E. coli* cells were crushed by ultrasound, the recombinant AlepPBP2 and AlepPBP3 were detected to be insoluble inclusion body by SDS-PAGE analysis. Similar to the previous experiments, the recombinant AlepPBPs were dialyzed and purified followed our previous studies methods (Zhang *et al*., 2020*a*, 2020*b*). After removing His-tag, further purification and dialysis were needed. The active AlepPBPs without His-tag were used for *in vitro* binding assays.

Molecular docking

Amyelois transitella (Walker) (Lepidoptera: Pyralidae) AtraPBP1 (PDB ID: 4INW) was employed as an appropriate template.

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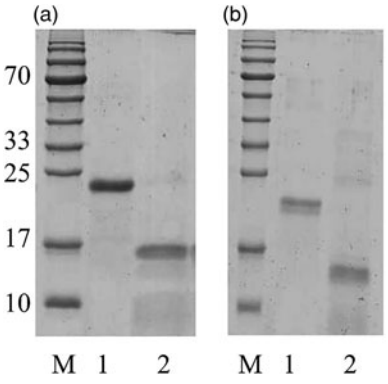

Figure 1. Expression and purification of AlepPBP2 (A) and AlepPBP3 (B) by SDS-PAGE analysis. The purified fractions (lane 1) of recombinant proteins pET/AlepPBPs with His-tag. Re-purification of AlepPBPs after His-tag removal by recombinant enterokinase (lane 2). M: Protein molecular mass marker of 70.0, 33.0, 25.0, 17.0 and 10.0 KDa.

The homology proteins modeling and molecular docking were performed by using MODELER version 9.19 (http://salilab.org/modeller/), Autodock Vina version 1.1.2 (Trott and Olson, 2009), AutoDock Tools version 1.5.6 (Morris *et al.*, 2009), and PyMOL version 1.9.0 (http://www.pymol.org/), according to our recent studies (Zhang *et al.*, 2020*b*).

In vitro binding assays of AlepPBPs and data analysis

Different binding affinities of 1 mM N-phenyl-1-naphthylamine (1-NPN) as the fluorescent reporter to AlepPBP2 and AlepPBP3 were measured by a fluorescent competitive combination experiment. Then, sex pheromones or host plant volatiles were used to titrate the AlepPBPs, according to our described methods (Zhang *et al.*, 2014; Zhang *et al.*, 2020*a*, 2020*b*). The details of binding assays are displayed in the supplementary data.

Results

Cloning, expression and purification of AlepPBP2 and AlepPBP3

In an initial step, the genes encoding AlepPBP2 and AlepPBP3 were amplified from cDNA using the primers containing restriction sites. After agarose gel electrophoresis of PCR products and recycle, these genes were cloned into cloning vector pEASY-T3 or expression vectors pET-30a(+). The recombinant AlepPBPs expressed existed in the *E. coli* as insoluble inclusion bodies, and the proteins were purified using affinity chromatography and 6 × His-tags were removed using enterokinase (fig. 1). According to previous experiments, the proteins denatured in urea and reconstituted in a series of dialysis (Liu *et al.*, 2015). The SDS-PAGE results indicated that the molecular mass of AlepPBP2 and AlepPBP3 was approximately 16.6 KDa and 16.37 KDa, respectively. These results are consistent with our recent findings (Zhang *et al.*, 2020*b*).

Ligand-binding assay of two AlepPBPs

Generally speaking, ligand-binding assay is the most reliable analytical method for screening which ligand can bind to PBP, which has been confirmed by *in vivo* experiments in different insects (Dong *et al.*, 2017*a*; Zhu *et al.*, 2019). First, the binding affinities of 1-NPN to AlepPBP2 and AlepPBP3 were measured, with the Kd = 5.47 ± 0.34 μM and Kd = 13.76 ± 0.75 μM, respectively, and the saturation and linear Scatchard plots were observed (fig. S1). The results showed that the two AlepPBPs had activity and there is a single binding site between AlepPBPs and 1-NPN, which are similar to our recent research findings (Zhang *et al.*, 2020*b*). We detected the binding affinities of two AlepPBPs to two sex pheromone components (Z7-12: Ac and Z9-14: Ac), using fluorescence competitive binding assays with 1-NPN as a fluorescent probe. By comparison, it was found that the binding affinities of AlepPBP2 to both sex pheromones (Z7-12: Ac and Z9-14: Ac) were much high (Z7–12:Ac, Ki = 1.11 ± 0.1 μM; Z9–14:Ac, Ki = 1.32 ± 0.15 μM] (table 1 and fig. 2a). However, AlepPBP3 gave the exact opposite trend. In addition, the binding affinities of AlepPBPs to ten different host plant volatiles were also examined. Among the different alkenes (limonene, α-pinene and myrcene), the binding affinities to AlepPBP2 were observed similarly (fig. 2b). Several ketones have been found to exhibit a strong affinity for AlepPBP2 (Ki < 9 μM), such as 2-hexanone, 3-hexanone, 2-heptanone, and 6-methyl-5-hepten-2-one (fig. 2d). Furthermore, aldehydes (nonanal and benzaldehyde) and alcohol (linalool) have also been found to bind AlepPBP2 (fig. 2c). However, we observed that all of the above volatile ligands of maize displayed no binding affinities to AlepPBP3 (fig. 3).

Protein modeling, molecular docking of ligands and AlepPBP2

According to homology protein modeling and conformation of small molecules, the protein structure of AlepPBP2 was obtained (Zhang *et al.*, 2020*b*) and used to identify the key amino acid residues that strongly interact with the ligands by molecular docking. Previous studies reported that the hydrophobic cavity of PBPs was detected which bind a broad array of hydrophobic ligands (Gong *et al.*, 2010). We also found in the present study that the same amino acid residues were involved in the binding of AlepPBP2 to both sex pheromone components by analyzing the binding models (fig. 4). There were two hydrophobic residues (Phe-15 and Phe-39 underlined by the solid line) in AlepPBP2 that bind to all examined ligands (including sex pheromones) (fig. 5), suggesting that these residues may play important roles in the interaction between AlepPBPs and ligands. Additionally, some other residues have been found to play key roles in binding to plant volatiles (fig. 5) and were listed in table 2. Moreover, there was a significant difference in the number of key amino acid residues (6–11), among which the residues (polar Ser-12 and Lys-123, and Trp-40, Phe-122, and Ile-138) played major roles in most of the host plant volatiles (more than 70%). Interestingly, only Val residue (Val-118) is presumed to play a special role in the interaction with linalool.

Discussion

Many studies have found that PBPs not only have a high binding affinity with sex pheromones, but also can bind several host plant volatiles (Robertson *et al.*, 1999; Picimbon and Gadenne, 2002). Although, sex pheromones and host plant volatiles were abundant in the surrounding environment (Linn *et al.*, 1987; Kehat and Dunkelblum, 1990). Since the first identification of sex pheromones in insects (Karlson and Butenandt, 1959), the importance of insect sex pheromones involved in insect life activities has been demonstrated in a growing body of literature. This indicated that

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Table 1. Binding data of different ligands to AlepPBP2 and AlepPBP3.

		PBP2		PBP3	
Ligand name[a]	CAS number	IC_{50} (μM)	Ki (μM)	IC_{50} (μM)	Ki (μM)
Sex pheromones					
Z7-12:Ac	14959-86-5	1.28 ± 0.12	1.11 ± 0.1	>4	–
Z9-14:Ac	61319-25-3	1.52 ± 0.17	1.32 ± 0.15	>4	–
Alkenes					
(-)-limonene	5989-54-8	8.69 ± 1.49	7.56 ± 1.32	>20	–
α-pinene	7785-26-4	7.17 ± 0.79	6.23 ± 0.7	>20	–
myrcene	123-35-3	8.25 ± 0.4	7.15 ± 0.34	>20	–
Alcohols					
linalool	78-70-6	10.1 ± 2.25	8.76 ± 1.96	>20	–
Aldehydes					
benzaldehyde	100-52-7	11.46 ± 2.06	9.94 ± 1.81	>20	–
nonanal	124-19-6	9.34 ± 0.68	8.1 ± 0.58	>20	–
Ketones					
2-hexanone	591-78-6	6.55 ± 0.25	5.67 ± 0.23	>20	–
3-hexanone	589-38-8	8.98 ± 1.63	7.79 ± 1.43	>20	–
2-heptanone	110-43-0	6.07 ± 0.32	5.26 ± 0.28	>20	–
6-methyl-5-hepten-2-one	110-93-0	9.91 ± 2.36	8.54 ± 2.04	>20	–

[a]The ligands were identified as sex pheromones or host plant volatiles according to literatures; Mixtures of protein and 1-NPN, both at the concentration of 2 μM, were titrated with 0.1 mM solutions of sex pheromone to final concentrations of 0.2–4 μM and were titrated with 1 mM solutions of each ligand (non sex pheromone) to final concentrations of 1–20 μM. '>20' means that IC_{50} could not be calculated directly with the tested ligand concentrations, and subsequently the Ki of the ligand is designated as '–'.

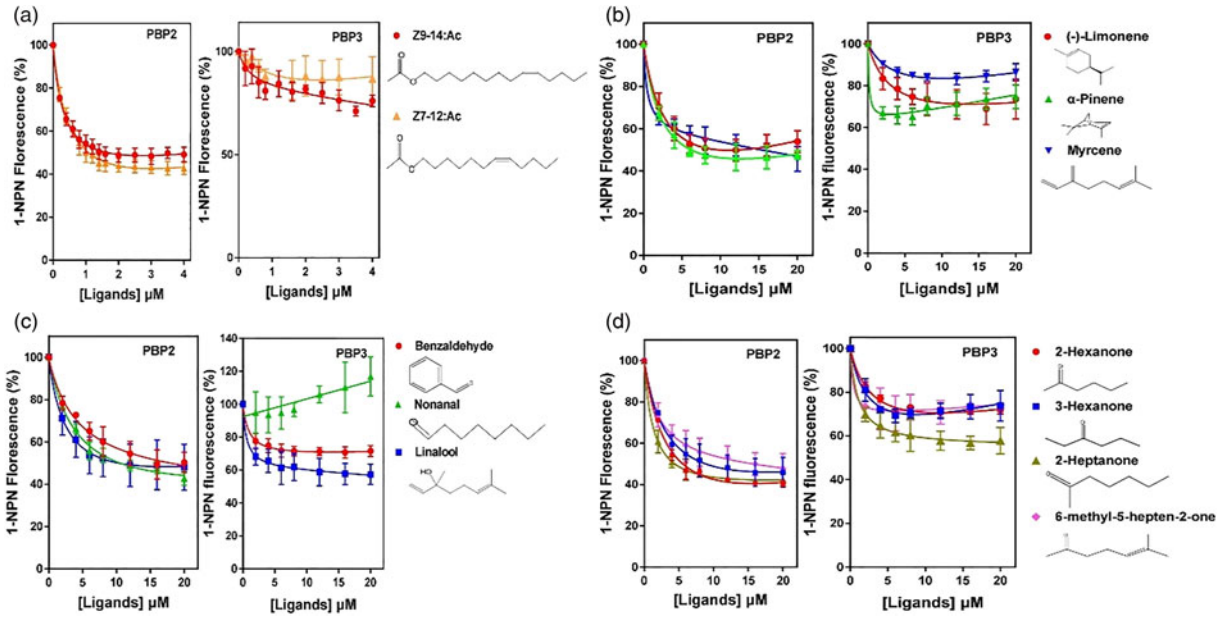

Figure 2. Ligand-binding assay for AlepPBPs. Binding curves of selected ligands to two AlepPBPs, including *A. lepigone* sex pheromones (a), Alkenes (b), Aldehydes and Alcohol (c), and Ketones (d). The ligand names are shown on the right of the curves.

insect sex pheromones participate in the regulation of various behavioral responses of insects, for instance, insect population assembly (Soroka *et al*., 2005), the release of warning and defensive signals (Purnamadjaja and Russell, 2005), and attraction of male insects to mate which affects female laying-eggs (Lo Pinto *et al*., 2013). Correspondingly, plant volatiles have been found to influence the life activities of insects with varying degrees of success (Sweeney *et al*., 2004; Xu and Turlings, 2018). For

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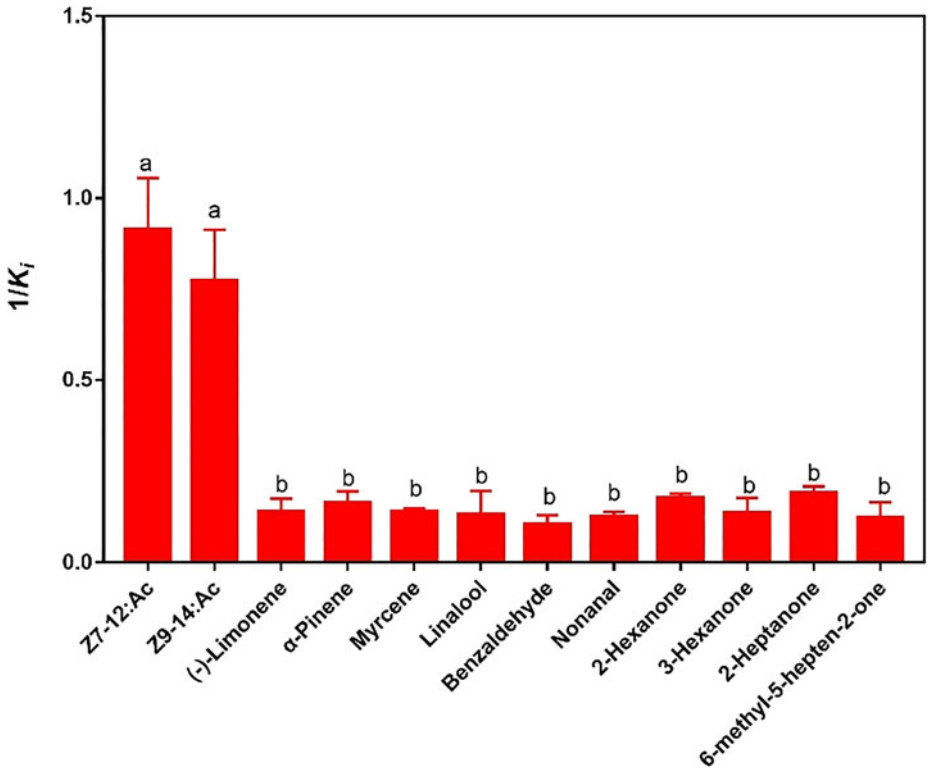

Figure 3. Comparison of the binding affinities (indicated by 1/Ki) for AleppBP2 to different components. Between different compounds were analyzed with one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$ and significant differences are marked with different letters: a, b for AlepPBP2.

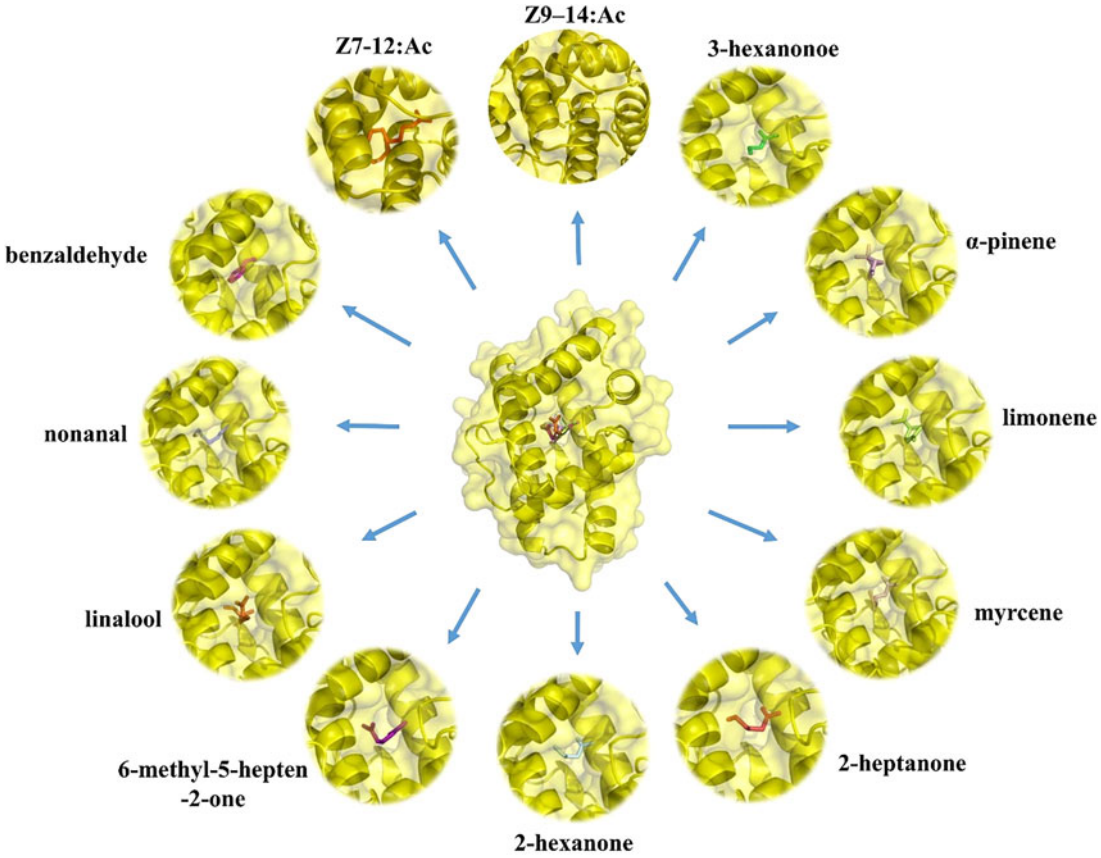

Figure 4. Binding modes of AlepPBP2 to different ligands. Two sex pheromones (Z7-12:Ac and Z9-14:Ac), (-)-limonene, α-pinene, myrcene, linalool, benzaldehyde, nonanal, 2-hexanone, 3-hexanone, 2-heptanone, and 6-methyl-5-hepten-2-one, in the putative binding pocket of AlepPBP2.

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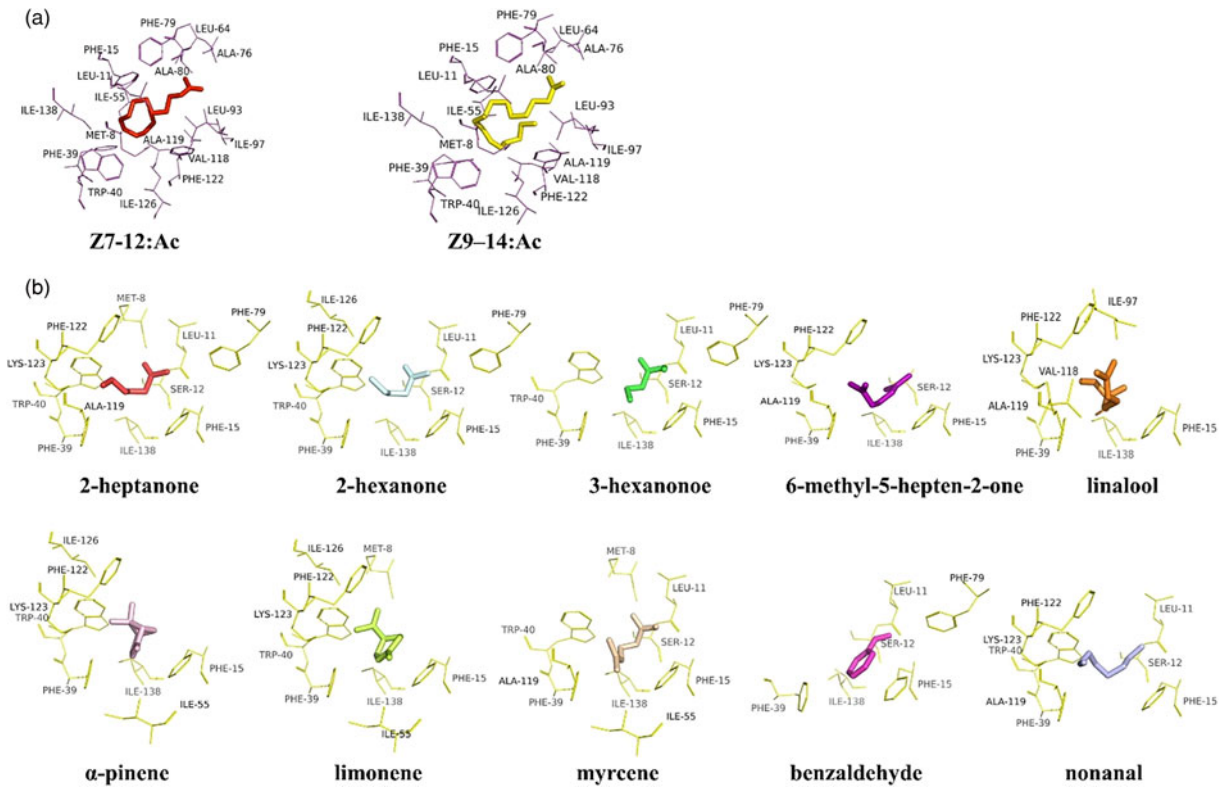

Figure 5. The key residues of the different ligands. Two sex pheromones (Z7-12:Ac and Z9-14:Ac), (-)-limonene, α-pinene, myrcene, linalool, benzaldehyde, nonanal, 2-hexanone, 3-hexanone, 2-heptanone, and 6-methyl-5-hepten-2-one, that interact with AlepPBP2. The residues of the ligands are highlighted in light purple or yellow.

Table 2. Prediction of key amino acid residues during the docking of AlepPBP2 to different ligands.

	Key amino acid residues	
Ligands	Nonpolar	Polar
Z9-14:Ac	M8, L11, F15, F39, W40, I55, L64, A76, F79, A80, L93, I97, V118, A119, F122, I126, I138	–
Z7-12:Ac	M8, L11, F15, F39, W40, I55, L64, A76, F79, A80, L93, I97, V118, A119, F122, I126, I138	–
(-)-Limonene	M8, F15, F39, W40, I55, F122, I126, I138	K123
α-Pinene	F15, F39, W40, I55, F122, I126, I138	K123
Myrcene	M8, L11, F15, F39, W40, I55, A119, I138	S12
Linalool	F15, F39, I97, V118, A119, F122, I138	K123
Benzaldehyde	L11, F15, F39, I97, I138	S12
Nonanal	L11, F15, F39, W40, A119, F122,	S12, K123
2-Hexanone	L11, F15, F39, W40, F79, I F122, I126, I138	S12, K123
3-Hexanonoe	L11, F15, F39, W40, F79, 138	S12,
2-Heptanone	M8, L11, F15, F39, W40, F79, A119, F122, I138	S12, K123
6-methyl-5-hepten-2-one	F15, F39, A119, F122, I138	S12, K123

Note: Key amino acid residues common to all ligands are underlined by the solid line.

example, (1) (Z)-3-hexenol and (E)-β-farnesene could influence the flight time and host plant acceptance of *Episyrphus balteatus* (Verheggen *et al.*, 2008), (2) the mixtures of (E)-2-hexenyl acetate and the sex pheromone of *Holotrichia parallela* resulted in significantly higher male catches than the sex pheromone alone (Ju *et al.*, 2017), (3) host plant volatiles could help egg parasitoids distinguish host habitats with parasitized hosts from those without (Li *et al.*, 2020). PBPs usually played crucial roles in the process of sexual communication of moths. Some studies found that PBPs are involved in the recognition of several key ligands, such as sex pheromones and host plant volatiles (Picimbon *et al.*, 1997; Grater *et al.*, 2006; Guo *et al.*, 2012; Yang *et al.*, 2017; Fu *et al.*,

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2018). Additionally, it was worth noting that insect PBPs were generally divided into three sub-groups in the phylogenetic tree (Picimbon and Gadenne, 2002), indicating that they may diverge in the direction of evolution. Therefore, in order to better explain the evolution mechanism of PBPs functional differentiation, we need to carry out this research using different insect models.

In this study, we found that AlepPBP2 exhibited higher binding affinities for both sex pheromone components (Z7-12:Ac and Z9-14:Ac), reflecting the vital function of AlepPBP2 in sexual communication in accordance with the result of previous tissue expression analysis (Zhang *et al.*, 2017). However, the AlepPBP3 highly expressed in both male and female antennae (Zhang *et al.*, 2017), had almost no binding affinity to sex pheromones, suggesting that AlepPBP3 had produced functional differentiation in evolution and portended that it may have other unknown functions (Xiu *et al.*, 2008; Liu *et al.*, 2013). Moreover, these results bore strong resemblances to those of the noctuid moth, *S. litura* (Liu *et al.*, 2013). Other studies have found that all identified PBPs of *C. suppressalis* (Dong *et al.*, 2019), *A. polyphemus* and *Antheraea pernyi* (Maida *et al.*, 2003), had the ability to bind to their sex pheromones. However, AlepPBP3 had not binding affinities to test ligands, which is similar to SinfPBP3 of *Sesamia inferens* (Noctuidae) (Jin *et al.*, 2014), indicating that AlepPBP3 and SinfPBP3 may play the least role (if any). In addition, proteins expressed by olfactory related genes of insects have been proved to bind to insect sex pheromones, such as GOBP2 (Ziegelberger, 1995; Zhou *et al.*, 2009; Zhang *et al.*, 2020*a*), OBP-LUSH (Laughlin *et al.*, 2008), and SinfCSP19 (Zhang *et al.*, 2014). It was also observed in other studies that PBPs enhanced the sensitivity of olfactory receptors to sex pheromones (Syed *et al.*, 2006; Chang *et al.*, 2015). All these findings indicated that sex pheromones are essential for insect life activities and complete their vital roles in a variety of ways. It also pointed out to us that the roles of sex pheromones in male recognition and sexual behavior in female are also very complex.

From previous experiments (Chang *et al.*, 2015; Khuhro *et al.*, 2017; Pelosi *et al.*, 2018), we found that the relationship between the olfactory proteins and host plant volatiles is not one to one, and this may present a considerable challenge in further studying the functions of certain proteins. Therefore, the binding affinity between the host plants volatiles and the PBPs in the olfactory system of insects can be also lucubrated. In this study, we found that AlepPBP2 had high binding affinities to certain alkenes and ketones released by the host plant-maize, suggesting that AlepPBP2 also had the partial function of OBPs, like locating the host and food sources (Matsuo *et al.*, 2007). Furthermore, we also found that there was a lower binding affinity of AlepGOBPs to the examined plant volatiles than sex pheromones used in our recent study (Zhang *et al.*, 2020*a*). This information indicated that there might be compensation and interactions between PBPs/GOBPs and OBPs in the insect olfactory system (Xu *et al.*, 2005), and also showed that AlepPBP2 mainly functions by binding to sex pheromones, while the reorganization of host plant volatiles was a secondary function. Other experiments have also proved that the recognition of plant volatiles might modulate the sensitivity of insects to sex pheromones. For example, linalool and phenylacetaldehyde enhanced the sensitivity of *H. armigera* and *S. litura* to sex pheromones, respectively, while high concentration reduced the sensitivity (Ochieng *et al.*, 2002; Kaissling, 2013), and α-pinene, as an attractant and synergistic agent, had been found to play a role in the insect-host relationship (Sweeney *et al.*, 2004). In addition, in the study of the interaction between insect sex pheromones and plant volatiles, it was easy to observe that the combination greatly improved the attraction to insects (Varela *et al.*, 2011), and the plant volatiles were used to compete with sex pheromones to inhibit neuronal excitation and could be as synergistic agents to increase the control effect of pests (Hanks *et al.*, 2012). These data suggested that there is a possible synergy between sex pheromones and host plant volatiles (Harrewijn *et al.*, 1994; Yang *et al.*, 2004; Varela *et al.*, 2011; Hanks *et al.*, 2012; Collignon *et al.*, 2016), and showed that the binding of AlepPBP2 to maize volatiles in *A. lepigone* serves as a secondary auxiliary function to regulate insect life activities. Our findings will lay the foundation for revealing the mutual recognition mechanisms between proteins and ligands.

Furthermore, we also revealed the key amino acid residues involved in the recognition process through molecular docking of AlepPBP2 and different ligands. We found that residues (Phe-15 and Phe-39) involved in all ligand binding which might play important roles in binding to ligands, and similar results were found in other studies (Dong *et al.*, 2017*b*). In addition, all residues predicted were found to be consistent in the binding to both sex pheromones, indicating that these residues may be involved in the specific recognition of sex pheromones. It has been found that some residues may affect the structure of proteins (Dong *et al.*, 2017*b*; Mazumder *et al.*, 2018). Therefore, X-ray diffraction of protein–ligand complexes (Mazumder *et al.*, 2018) and the mutant binding assay (Laughlin *et al.*, 2008; Zhang *et al.*, 2020*b*) could be used to further analyze the molecular mechanisms of the interaction between insect PBPs and ligands.

Reverse chemical ecology has emerged as a method of screening for behaviorally active odorants (such as attractants and repellents) based on their molecular interactions with olfactory proteins (OBPs or ORs) (Leal *et al.*, 2008; Zhu *et al.*, 2017; Venthur and Zhou, 2018). Usually, the interaction between olfactory proteins and different odorants are studied *in vitro* to screen out the odorants that can highly bind to olfactory proteins, and then conduct behavioral experiments to further determine which highly bound odorants have behavioral attraction or repellent activity. This has been successfully applied in different insects, such as *Culex quinquefasciatus* (Choo *et al.*, 2018), *Rhodnius prolixus* (Franco *et al.*, 2018), *Aenasius bambawalei* (Li *et al.*, 2018a), and *Spodoptera littoralis* (Caballero-Vidal *et al.*, 2021). Therefore, the assays on the binding affinity of AlepPBPs and volatiles in our study belongs to a part of reverse chemical ecology, the results will provide important help for us to develop attractants or repellents in the future.

It could be concluded that AlepPBP2 displayed a higher binding affinity with both sex pheromone components and some host plant volatiles than AlepPBP3. This suggests that AlepPBPs might produce differences in evolution and have different roles in the recognition process of distinct ligands. Some key residues involved in the ligand-binding of AlepPBP2 were revealed by the molecular docking and we found the hydrophobic residues (Phe-15 and Phe-39) in AlepPBP2 bind to all ligands, which could achieve the target for further study of ligand-binding mechanism. These findings will not only lay a foundation to understand the molecular mechanisms by which PBP bind to ligands, but also help to use sex pheromones and host plant volatiles for target insect pest control.

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Ethical standards. This article does not include any study on human participants or animals performed by any of the authors.

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