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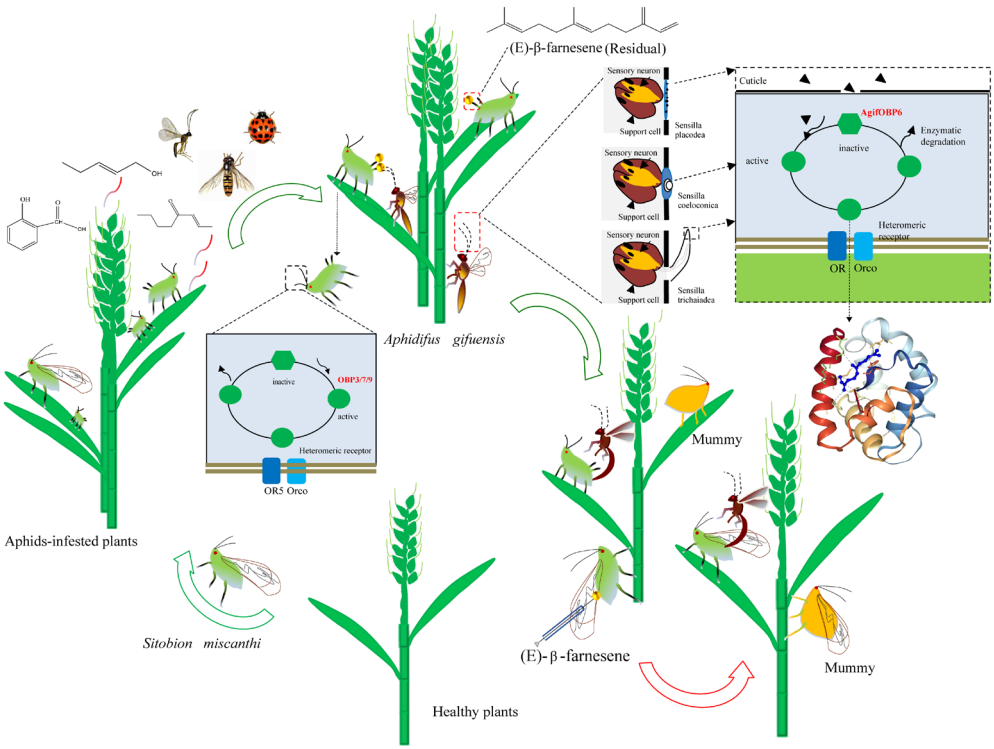
Functional investigation of odorant-binding proteins in *Aphidius gifuensis* and its host *Sitobion miscanthi* revealed convergent evolution



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Promotors: Profs Frédéric Francis & Julian Chen

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COMMUNAUTÉ FRANÇAISE DE BELGIQUE

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Functional investigation of odorant-binding proteins in *Aphidius gifuensis* and its host *Sitobion miscanthi* revealed convergent evolution

Xin Jiang

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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Abstract

Xin Jiang (2023). "Functional investigation of odorant-binding proteins in *Aphidius gifuensis* and its host *Sitobion miscanthi* revealed convergent evolution" (Ph.D Dissertation in English).

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187 pages, 32 figures, 12 tables.

Aphids are the most devastating agricultural pests, either directly damaging agricultural crops or serving as vectors of plant viral diseases, which cause significant yield loss each year. Tritrophic interactions among host plants, aphids and natural enemies have been the highlights in agricultural research for a long time. As the natural enemies of aphids, parasitoid wasps have evolved the sophisticated olfactory system to perceive infochemicals, some of which attract wasps to locate the preys or hosts, some repel for long or short-range. In a review, we summarized the interactions of infochemicals, aphids and their parasitoid wasps from the perspective of behavior traits to olfactory perception in Chapter I.

The grain aphid *S. miscanthi* is a severe sap-sucking insect pest, specialist for cereal plants. *S. miscanthi* is a dominant cereal species, and widely distributed in wheat-growing regions of China, but has been mis-reported as *Sitobion avenae* in China before. *S. miscanthi* is significant for both basic and applied research. However, the genome of information is not available. Therefore, we sought to publish the genome information for *S. miscanthi* here. We first present the chromosome-level genome sequence of the *S. miscanthi* strain Langfang-1in Chapter II, which displays higher-quality assembly data indexes than prior scaffold-level aphid genomes. A 2n=18 karyotype for *S. miscanthi* was supported by the majority of the sequences assembling into 9 scaffolds. There were 8 different aphid species represented in the genome annotation data, including the pea aphid *Acyrthosiphon pisum*, peach aphid *M. persicae*, soybean aphid *Aphis glycines*, Russian wheat aphid *Diuraphis noxia*, cherry-oat aphid *Rhopalosiphum padi*, and black cherry aphid *Myzus cerasi*, the cotton aphid *Aphis gossypii*, and the corn leaf aphid *Rhopalosiphum maidis*. Repeat sequences and phylogenetic analysis show that *S. miscanthi* is closely linked to *A. pisum*, with a time interval between their divergence and that of *S. miscanthi* being roughly 25.0-44.9 million years. The detail of the results is presented in Chapter II.

Aphidius gifuensis is one of dominatant endoparasitoids of the green peach aphid *Myzus persicae* and grain aphid *S. miscanthi* in the agroecosystem in China. Insect odorant-binding proteins (OBPs) play the vital roles in odor perception during feeding, host searching, mating and oviposition. In Chapter III, a comparative antennal transcriptomic analysis was applied between male and female adult of *A. gifuensis*. The spatial expression patterns among antennae, heads, thoraxes, abdomens and legs of OBPs in both sexes were further profiled. Results showed that fifteen *AgifOBP*s were predicted, and 14 of them were identified by gene cloning, including 12 classic OBPs and 2 minus-C OBPs. As expected, all OBPs were mainly expressed at high levels in antennae, heads or legs which are sensory organs and tissues. Finally, ligand

binding properties of 2 OBPs (AgifOBP7 and AgifOBP9) were further evaluated. Female leg specifically expressed AgifOBP9 displays a broad and high binding property to aphid alarm pheromones, plant green volatiles and aphid sex pheromones (Ki <10 μM). However, female leg specifically expressed AgifOBP7 displays poor affinity for all tested ligands except CAU-II-11 ((E)-3, 7-dimethylocta-2,6-dien-1-yl-2-hydroxy-3-methoxybenzoate), a reported (E)-β-farnesene (EBF) analog with an exceptionally high binding affinity (Ki = 1.07 ± 0.08 μM). In summary, the spatial expression pattern of the OBP repertoire in *A. gifuensis*, and further studied the binding properties of OBP7 and OBP9, which are mainly expressed in female legs, laying the foundation for the dissection of the contribution of OBPs to chemosensation in *A. gifuensis*. The detail of the results is presented in Chapter III.

(E)-β-farnesene (EBF) is one typical and ecologically important info-chemicals in tri-trophic level interactions among plant-aphid-natural enemies. While the molecular mechanism of parasitoid recognizing and utilizing EBF is unclear. In Chapter VI, we functionally characterized eight AgifOBPs from *A. gifuensis*, among which, AgifOBP6 was the only OBP up-regulated by various doses of EBF, it showed a strong binding affinity to EBF in vitro as well. The lack of homology between AgifOBP6 and the EBF binding proteins from aphids or from other aphid natural enemies, supported that this is a convergent evolution among insects from different orders driven by EBF. Molecular docking of AgifOBP6 with EBF revealed interactive key residues and hydrophobic forces as the main forces. And whole-mount immunolocalization showed that this is a widely expressed OBP among various antennal sensilla. Furthermore, two bioassays using grain aphids *S. miscanthi* indicated that trace amount of EBF may promote the biological control efficiency of *A. gifuensis* to aphid, especially to the winged aphid.

Our present work indicates that OBPs between *A. gifuensis* and its host aphid were separately evolved but eventually driven to a common biological function by convergent evolution in aphid's alarm hormones identification. And also offers a novel perspective on the biological control of aphid by reducing the initial population of migrant biotype in source area with a low concentration of EBF application, to promote biological control efficiency by the parasitoid to the winged aphid before immigration. The detail of the results is presented in Chapter VI.

Keywords: odorant-binding protein (OBPs), (E)-β-farnesene (EBF), convergent evolution, genome, spatial expression pattern, olfactory plasticity.

Résumé

Xin Jiang (2023). "L'étude fonctionnelle des protéines de liaison aux odeurs chez *Aphidius gifuensis* et son hôte *Sitobion miscanthi* a révélé une évolution convergente" (thèse de doctorat en anglais).

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Résumé:

Les pucerons sont des ravageurs importants endommageant directement les cultures agricoles ou servant de vecteurs de maladies virales des plantes, ce qui entraîne des pertes de rendement importantes chaque année. Les interactions tritrophiques entre les plantes hôtes, les pucerons et leurs ennemis naturels sont depuis longtemps les points forts de la recherche agronomique. En tant qu'ennemi naturel des pucerons, les guêpes parasitoïdes ont développé un système olfactif sophistiqué pour percevoir les sémiochimiques, dont certains sont attractifs et nécessaires à la localisation de leurss proies ou de leurss hôtes, et d'autres sont répulsifs à longue ou courte distance. Dans le chapitre 1, une revue de la littérature présente les interactions basées sur les sémiochimiques entre les pucerons et leurs guêpes parasitoïdes ,et ce du point de vue des traits de comportement à la perception olfactive.

Le puceron des céréales *S. miscanthi* est un ravageur de type suceur de sève spécialiste des plantes céréalières. Il s'agit d'une espèce céréalière dominante et largement distribuée dans les régions productrices de blé de Chine, qui y a déjà été signalée à tort comme *Sitobion avenae*. *S. miscanthi* est également employé pour la recherche fondamentale et appliquée. Cependant, le génome de cette espèce n'est pas disponible. Par conséquent, nous avons d'abord cherché à publier ici les informations sur le génome de *S. miscanthi*. Dans le chapitre 2, nous présentons les séquence du génome des chromosomes de la souche Langfang-1, qui affiche des indices de données d'assemblage de meilleure qualité que les génomes de pucerons au niveau de l'échafaudage antérieurs. Un caryotype 2n = 18 pour *S. miscanthi* a été pris en charge par la majorité des séquences s'assemblant en 9 échafaudages. Il y avait 8 espèces de pucerons différentes représentées dans les données d'annotation du génome, y compris le puceron du pois *Acyrthosiphon pisum*, le puceron du pêcher *M. persicae*, le puceron du soja *Aphis glycines*, le puceron russe du blé *Diuraphis noxia*, le puceron de l'avoine du cerisier *Rhopalosiphum padi* et le puceron noir du cerisier *Myzus cerasi*, le puceron du cotonnier *Aphis gossypii* et le puceron du maïs *Rhopalosiphum maidis*. Les séquences répétées et l'analyse phylogénétique montrent que *S. miscanthi* est étroitement lié à *A. pisum*, avec un intervalle de temps entre leur divergence et celle de *S. miscanthi* étant d'environ 25,0 à 44,9 millions d'années.

En Chine, *A. gifuensis* est l'un des endoparasitoïdes les plus courants du puceron vert du pêcher *Myzus persicae* et du puceron des céréales *Sitobion miscanthi*. Les protéines de liaison aux odeurs (*Odor binding proteins* - OBPs) jouent un rôle essentiel dans la perception des odeurs pendant l'alimentation, la recherche d'hôtes, l'accouplement et la ponte. Dans le chapitre III, une analyse transcriptomique

antennaire comparative a été appliquée sur les males et les femelles d'*A. gifuensis*. Les modèles d'expression des OBPau sein des antennes, des têtes, des thorax, des abdomens et des pattes ont été profilés plus en détail pour chaque sexe. Les résultats ont prédits quinze OBP chez *A. gifuensis* (AgifOBPs). Parmi celles-ci, 14 AgifOBPs ont été identifiés par clonage de gènes, dont 12 OBP classiques et 2 OBP moins-C. Comme prévu, tous les OBPs étaient principalement exprimés à des niveaux élevés dans les parties présentant des organes sensoriels tels que les antennes, la tête ou les jambes. Enfin, les propriétés de liaison des ligands de 2 OBP (AgifOBP7 et AgifOBP9) ont été évaluées plus en détail. L'AgifOBP9 est exprimé spécifiquement dans les jambes des femelle et affiche une propriété de liaison large et élevée aux phéromones d'alarme de puceron, aux odeurs vertes des plantes et aux phéromones sexuelles de puceron (Ki <10 μM). Cependant, AgifOBP7 spécifiquement exprimé dans les jambes féminines AgifOBP7 spécifiquement exprimé affiche une faible affinité pour tous les ligands testés à l'exception de CAU-II-11 ((E)-3,7-diméthylocta-2,6-dien-1-yl-2-hydroxy-3 -méthoxybenzoate), un analogue (E)-β-farnésène (EBF) rapporté avec une affinité de liaison exceptionnellement élevée (Ki = 1,07 ± 0,08 μM). En résumé, lesd'expression d'OBP chez *A. gifuensis* ont été étrudiés selon les différentes parties morphologiques, plus les propriétés de liaison des OBP7 et des OBP9, qui sont principalement exprimés dans les jambes des femelles, jetant les bases de la dissection de la contribution des OBP à la chimiosensation chez *A. gifuensis*. Le détail des résultats est présenté au chapitre III.

Le (E)-β-farnésène (EBF) est un produit chimique d'information typique et écologiquement important dans les interactions de niveau tritrophique plantes-pucerons-enemis naturels. Les mécanismes moléculaires régulant la reconnaissance au niveau des parasitoïdes percevant l'EBF demeurent peu connus. Dans le chapitre VI, nous avons caractérisé fonctionnellement huit AgifOBP d'*Aphidifus gifuensis*, parmi lesquels AgifOBP6 était le seul OBP régulé à la hausse par diverses doses d'EBF. Une forte affinité de AgifOBP6 pour la liaison à l'EBF a également été mise en évidence in vitro.. L'absence d'homologie entre AgifOBP6 et les protéines de liaison à l'EBF des pucerons ou d'autres ennemis naturels des pucerons a confirmé qu'il s'agit d'une évolution convergente parmi des insectes de différents ordres. L'ancrage moléculaire d'AgifOBP6 avec EBF a révélé des résidus clés interactifs et des forces hydrophobes comme forces principales. L'immunolocalisation complète a montré qu'il s'agit d'un OBP largement exprimé dans diverses sensilles antennaires. De plus, deux essais biologiques menés sur *S. miscanthi* ont indiqué que des traces d'EBF favorisent la lutte biologique contre *A. gifuensis*, en particulier sur les pucerons ailés.

Notre travail actuel indique que les OBP entre *A. gifuensis* et son puceron hôte ont évolué séparément mais ont finalement mené à une fonction biologique commune par convergence évolutive. Notre travail And offre également une nouvelle perspective sur la lutte biologique contre les pucerons en allant de la réduction de la population initiale de pucerons biotypes migrateurs dans les zones d'origine : par une faible concentration d'application d'EBF et en favorisant l'efficacité de la lutte biologique via des parasitoïdes sur les pucerons ailés avant l'immigration. Le détail des résultats

est présenté au chapitre VI

Mots clés: protéine de liaison aux odeurs (OBP), (E)-β-farnésène (EBF), évolution convergente, génome, patron d'expression spatiale, plasticité olfactive.

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Xin Jiang
05/12/2022 in Gembloux, Belgium

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List of Abbreviations

ACN	Acetonitrile
A. confusum	*Aulacocentrum confusum*
***A. gifuensis*/Agif**	*Aphidifus gifuensis*
Agly	*Aphis glycines*
***A. gossypii*/Agos**	*Aphis gossypii*
alate-BC	Alate blank control
alate-NC	Alate negative control
alate-T	Alate treatment
A. mellifera	Apis mellifera
***A. pisum*/Apis**	*Acyrthosiphon pisum*
apterous-BC	Apterous blank control
apterous-NC	Apterous negative control
apterous-T	Apterous treatment
CAU-II-11	(E)-3,7-dimethylocta-2,6-dien-1-yl-2-hydroxy-3-methoxybenzoate
CHC	Cuticular hydrocarbon
COI	Mitochondrial cytochrome oxidase I
***C. pallens*/Cpal**	*Chrysopa pallens*
CSP	Chemosensory protein
DMNT	(E)-4,8-dimethyl-1,3,7-nonatriene
D. noxia	*Diuraphis noxia*
EAG	Electroantennogram
***E. balteatus*/Ebal**	*Episyrphus balteatus*
EBF	(E)-β-farnesene
***E. corollae*/Ecor**	*Eupeodes corollae*
FA	Female antennae
GLVs	Green leaf volatiles
GO	Gene Ontology
GOBP	General odorant-binding protein
***H. axyridis*/Haxy**	*Harmonia axyridis*
HIPVs	Herbivore-induced plant volatiles
HSD	Honestly significant difference
IgG	Immunoglobulin G
IPTG	Isopropyl-1-thio-b-D-galactopyranoside
IRs	Ionotropic receptors
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOG	EuKaryotic Orthologous Groups
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
***L. erysimi*/Lery**	*Lipaphis.erysimi*
LTQ	Linear trap quadrupole

MA	Male antennae
M. dirhodum	*Metopolophium dirhodum*
Mb	Megabyte
MeSA	Methyl salicylate
MHO	6-methyl-5-hepten-2-one
M. mediator	*Microplitis mediator*
***M. persicae*/Mper**	*Myzus persicae*
M. pulchricornis	*Meteorus pulchricornis*
MS	Mass spectrometry
***M. viciae*/Mvic**	*Megoura viciae*
nLC	Nano liquid chromatography
NR	Nonredundant protein
nymph-NC	Nymph negative control
nymph-T	Nymph treatment
OBP	Odorant-binding protein
OR	Olfactory receptor
ORNs	Olfactory receptor neurons
PBP	Pheromone-binding protein
PBST	PBS containing 0.05% Tween-20
PDB	Protein Data Bank
PVDF	Polyvinylidene fluoride membrane
***R. padi*/Rpad**	*Rhopalosiphum padi*
S. avenae/Save	*Sitobion avenae*
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S. miscanthi	*Sitobion miscanthi*
SNMPs	Sensory neuron membrane proteins
***T. aestivum* L.**	*Triticum aestivum L.*
TAG	Trimyristoyl triglyceride
TIC	Triglycerides
TrEMBL	Translation of European Molecular Biology Laboratory
TRITC	Tetramethylrhodamine
VOCs	Volatile organic compounds
1-NPN	N-phenyl-1-naphthylamine
2-DE	two-dimensional gel electrophoresis
4-DMP	4-allyl-2,6-dimethoxyphenol

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Chapter I General introduction

I.1. Context and objectives

I.1.1. Context

Aphids are the most devastating agricultural pests, either directly damaging agricultural crops or serving as vectors of plant viral diseases, which causes significant yield losses each year. *S. miscanthi* is a dominant cereal species, and widely distributed in wheat-growing regions of China. At present, overuse of insecticide result in serious '3R' problem, with the society development, people's willing of getting more healthy food become more and more urgent. In the tritrophic interactions among host plants, aphids and natural enemies have been the highlights in agricultural research for a long time. Ultilize nature enemies is a vital part to control aphids. Among them, *Aphidius gifuensis* Ashmead belongs to Hymenoptera, and is a dominate parasitoid of aphids such as *Sitobion miscanthi* Fabricus and *Myzus persicae* Sulzer (Bi and Ji, 1993; Ohta and Honda, 2010). Previous studies have revealed that the main component of aphid alarm pheromone is trans-bete-farnesene(Bowers et al., 1972; Francis et al., 2005), and three types of EBF-binding proteins were identified within 9 aphid species (Wang et al., 2021). For the *A.gifuensis*, which respound to a variety of odor compounds at behavioral and electrophysiological levels (Liu, et al 2001; Dong et al., 2008; Fan et al., 2018; Song et al., 2021), For example, *A. gifuensis* can distinguish between healthy, mechanically damaged, and aphid-infested plants (Dong et al., 2008). Additionally, both female and male *A. gifuensis* were reported to present a positive electroantennogram (EAG) response to EBF and many tobacco volatiles, including trans-2-hexenal, methyl salicylate, benzaldehyde, cis-3-hexen-1-ol, and 1-hexanal (Song et al., 2021). The volatile sex pheromone has also been shown to be released by female Aphidius, causing intense sexual orientation in males (Fan et al., 2018). Besides, a lot of natural enemies such as *Aphidius ervi*, *Aphidius uzbekistanicus*, and *Adalia bipunctata* show attractant behavior to EBF (Buitenhuis et al., 2004). It remains unknown how such interactions are established within infochemical between aphids and parasitoids at the molecular level.

In this study, we wanted to unravel the genome of host aphid *S. miscanthi* for the understanding of holograms of aphid olfactory genes. And further obtain the candidate OBPs in the parasitoid *A. gifuensis* through antennae transcriptome and their expression characteristics. Finally, the molecular mechanisms used by the aphid parasitoid *A. gifuensis* to detect EBF. We adopted phylogenetic relationship analysis between aphid alarm pheromone binding proteins (OBP3, OBP7, OBP9) and the OBPs of its 5 natural enemies to illustrate their convergent evolution; prokaryotic expression and ligand binding assays, homology modeling, molecular docking, induction assay with EBF, immunolocalization also conducted to unravel the molecular base of perceiving EBF. Additionally, two bioassays were designed to test the functional of infochemical between the two species. Results indicated that OBPs between *A. gifuensis* and its host aphid were separately evolved but eventually driven to a common biological function by convergent evolution. The results also suggest that *A.gifuensis* may involve a more sensitive olfactory ability to detect the trace of EBF produced by aphids over a short distance.

I.1.2. Objectives

Three specific objectives are as follows:

(1) The genome sequencing of host aphid *S. miscanthi* for the understanding of holograms of aphid olfactory genes.
(2) The candidate OBPs in the parasitoid *A. gifuensis* through antennae transcriptome and their expression characteristics.
(3) The functional investigation of OBPs in identification of aphid alarm hormone (EBF) by *A. gifuensis* and its host *S. miscanthi.*

I.2. Research roadmap and outline

I.2.1. Research roadmap

The research roadmap is presented in Figure 1-1.

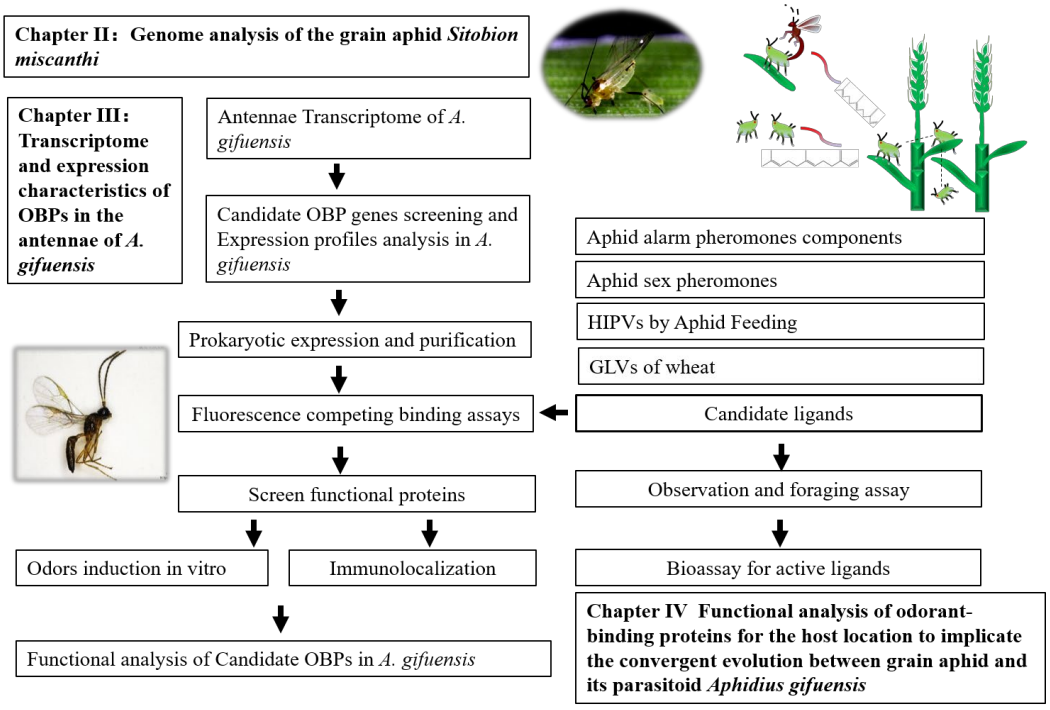

Figure 1-2 The overview contents and research roadmap of thesis

I.2.2. Outline

Chapter III A chromosome-level draft genome of the grain aphid *Sitobion miscanthi*.

Reference: Xin Jiang, Qian Zhang, Yaoguo Qin, Hang Yin, Siyu Zhang, Qian Li, Yong Zhang, Jia Fan, Julian Chen. (2019). A chromosome-level draft genome of the

grain aphid *Sitobion miscanthi*. *Gigascience*, 8(8), giz101.

Chapter IV Spatial Expression Analysis of Odorant Binding Proteins in Both Sexes of The Aphid Parasitoid *Aphidius gifuensis* and Their Ligand Binding Properties.

Reference: Xin Jiang, Yaoguo Qin, Jun Jiang, Yun Xu, Frédéric Francis, Jia Fan, Julian Chen. (2022). Spatial Expression Analysis of Odorant Binding Proteins in Both Sexes of The Aphid Parasitoid *Aphidius gifuensis* and Their Ligand Binding Properties. *Frontiers in physiology*, 13:877133.

Chapter V Functional analysis of odorant-binding proteins for the parasitic host location to implicate convergent evolution between the grain aphid and its parasitoid Aphidius gifuensis.

Reference: Xin Jiang, Jun Jiang, Miaomiao Yu, Siyu Zhang, Yaoguo Qin, Yun Xu, Frédéric Francis, Jia Fan, Julian Chen. (2023). Functional analysis of odorant-binding proteins for the parasitic host location to implicate convergent evolution between the grain aphid and its parasitoid *Aphidius gifuensis*. *International journal of Biological and Macromolecules*, 226, 510-524.

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Chapter II Literature review in parasitoid wasp chemical ecology based on chemical communication at tritrophic level among plant-herbivore -parasitoid

II.1 Introduction

Insects are capable of responding to certain info-chemical signal associated with cues like odors, color, sound, shape, or size. These cues may be helpful for at long or short-range to habitat selection, risk reversion, foraging, mating, reproducing, in short, to survive. The chemical ecology of insects, specially semiochemistry, has been thoroughly studied, due to their role in the spread of diseases to animals including human, as well as their direct predator attacks on crops, livestock, and other living things, but also, in some circumstances, due to their beneficial output. Semiochemicals are chemical compounds that communicate information between two animals in an ecological setting, eliciting a behavioral or physiological reaction that is adapted to either one of the creatures or to both of them (Vet & Dick, 1992). Aphids are crucial pests for widely food production through directly damaging agricultural crops or acting as plant virus carriers. The aphids and their natural enemies, such as predators and parasitic insects, have developed subtle peripheral nervous systems that alter them to sight a variety of info-chemicals, whereas it's tough to seek out an acceptable place to prey in exceedingly sophisticated surroundings with several distinct plants and animal species. Species with overlapping habitats like species among multi-trophic levels have typical interaction characteristics, creating them wonderful subjects for exploring co-adaptive evolution, this suggests that it even shows there are additional obvious clues of adaptative evolution. Rather, the main content of this chapter is to review olfaction-mediated host location from the perspective of behavior traits to olfactory perception focusing on the info-chemicals utilized by aphids and their parasitoid wasps. Right-smart knowledge about how info-chemicals encode identity and suitability for host-seeking aphids and their parasitoids as well as why only highly-specific blends of volatiles could trigger corresponding behavioral responses has been gathered in recent years. In the initial part of this chapter, a number of key info-chemicals utilized by aphids and their parasitoid wasps will be reviewed. Then, the host selection process for parasitoids represented by volatiles driven behavioral responses is discussed. Finally, recent advances in molecular mechanism connected within the behavior response of host location between aphids and their parasitoid wasps are also summerized and present.

II.1.1 The main crop of aphid species and damages

Aphids, often known as greenflies and blackflies, are tiny sap-sucking insects that belong to the superfamily Aphidoidea. These sap-feeding hemipterans have developed parthenogenesis which leads to an incredibly rapid reproductive rate and winged dimorphism which supports passive wind dispersal. All above may render individuals disposable, and yet guarantees survival and flourishment of whole population. As humans began the agricultural period, aphids were among our most worthy foes due to their incredible evolutionary adaptations (Sorensen, 2009). Of the 5000 species that live on a variety of host plants, more than 5% of aphids cause significant crop damage (Van Emden & Harrington, 2017; Miller & Foottit, 2009). Due to their ability to directly or indirectly harm its host in horticulture and agriculture by feeding on them, spreading plant viruses, and producing honeydew.

A few commercially significant species of these aphids have drawn a lot of interest

(Baranyovits, 1973; Morrison & Peairs 1998; Oerke, 1994; Calevro et al., 2019). For instance, the devastating green peach aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae) has a host range that includes more than 400 species in 40 different plant families, many of which are significant crop plants. It is a serious dangerous, global polyphagous pest, *M. persicae* can spread over 100 plant viruses, both persistent and non-persistent, including the persistent potato leaf roll virus and the non-persistent cucumber mosaic virus, in addition to directly harming plants (Eskandari et al., 1979; Bwye et al., 1997). The *Acyrthosiphon pisum* is another noxious sap-feeding aphid in the Aphididae family (Harris). It is one of the most important aphid species for agronomy since it consumes numerous varieties of legumes (plant family Fabaceae), including feed crops like pea, clover, alfalfa, and broad beans (Van Emden, 2017) and as a model insect. *Sitobion miscanthi* (Takahashi) and *S. avenae* (Fabricius) are often mistaken for one another in China. The combined genomic and morphological investigations revealed that *S. avenae* is only found in the Yili region of Xinjiang, China, and that the aphids previously known as *S. avenae* but discovered elsewhere in China are actually *S. miscanthi* (Zhang, 1983; Liu, 2009; Jiang et al., 2019). While *S. miscanthi* predominates in China's majority of wheat-growing regions, it also poses a threat to sorghum, rice, corn, barley, oats, and sugarcane (Wu, 2002). Apart from the *S. miscanthi*, the bird cherry-oat aphid *Rhopalosiphum padi*, the greenbug aphid *Schizaphis graminum*, and *Metopolophium dirhodum* are also the important cereal aphid pests of gramineous crops in China (Homoptera: Aphididae) (Blackman et al., 2006; Chen JL, 2013; Zhang et al., 2019). And yet, the *Aphis gossypii* Glover (Lagos-Kutz et al., 2014), *Rhopalosiphum maidis* (Fitch) (El-Ibrashy et al., 1972), *Aphis glycines* (Liu et al., 2014) also attracted the attention of scientists.

II.1.2 general introduction of parasitoid wasps

Following a number of findings made during an *A. gossypii* outbreak early in the summer of 1920, biological study of the aphid parasites was initiated. The parasitoid wasp genus known as Braconidae is the second most species-rich family in the animal kingdom. Jones et al (Jones et al., 2009) and Jones & Quicke (Dolphin and Quicke, 2001) estimated that there were 50,886 and 42,653 species, respectively. There are roughly 70,000 species worldwide when the ratio of Braconidae to vascular plants in Europe (0.3) is utilized (Chen & van Achterberg, 2019). This family contains almost all primary parasitoids, which primarily prey on the larvae or adults of insects from more than 120 different families. Numerous of these species aid in the biological and natural control of insect pests in horticulture, forestry, and agriculture (Ribeiro et al., 2013; Wharton, 1973). Aphidius is a worldwide-ranging insect genus in the Braconidae family, which are endoparasites of aphids that lay their eggs inside of them. The parasitoid turns to face the aphid, tucks its abdomen under its head and thorax to strike the aphid with the ovipositor at the tip of its abdomen, usually laying an egg at that very moment. The aphidius larvae eat the inside of the aphid as they grow, which eventually causes the hosts to enlarge or become mummified, turning grey or yellow. When the adult parasite bites a hole in the mummies by removing the sugar, the aphidius goes through a complete metamorphosis. Adult aphidius often have a size of less than 1/8 inch (3 mm). The genus aphidius contains a large number of species that

function as biological pest controllers for aphids on agricultural crops, in greenhouses, in urban environments, and in backyard gardens.

II.1.3 The host selection process for the parasitoids

The host selection process for parasitoids was first descripted into three distinct processes by Virson (1976). The first step is determining the location of the habitat, then the host, and the last step has been separated into host recognition and host acceptance for parasitoids (Michaud & Mackauer, 1994; Muratori et al., 2006). The host recognition process involves both physical and chemical factors (Godfray, 1994). Host body size (Shirota et al., 1983; Kouame´ & Mackauer, 1991), host cuticle texture (Arthur,1981), shape (Vinson, 1985), and color (Ankersmit et al., 1981, 1986; Michaud & Mackauer, 1994, 1995; Battaglia et al., 2000), as well as host movements (Arthur, 1981; Mackauer et al., 1996; Dippel & Hilker, 1998) act as cues triggering the attack behavior of female parasitoids. Finding a plant first, then looking for aphids, may not be the most effective tactic because aphids only occupy a small portion of all host plants. Although it is more likely for natural enemies to start looking for hosts before landing on the plant, it is more important for aphid natural enemies to distinguish between finding a plant and recognizing an aphid on the plant. According to Hatano et al. (2008a), host location refers to finding the aphid when its natural adversary is already on the host plant and habitat location refers to finding the aphid itself. As a result, aphid natural enemies look for "habitat with host" rather than "host habitat". And the habitat's status in this terminology is equivalent to that of a food plant. The starting point of the aphid natural enemies' quest is an aphid habitat, or a host plant where aphids may be present. It has been discovered that host plants are attracted to aphid parasitoids (Hymenoptera: Braconidae), including *Diaretiella rapae* (M' Intosh) (Read et al., 1970), *Lysiphlebus testaceipe* (Cresson) (Schuster & Starks,1974), *Trioxys indicus* Subba Rao and Sharma (Singh & Sinha, 1982), *Aphidius uzbekistanicus* Luzhetski, *Aphidius ervi*, Haliday (Powell & Zhang, 1983), *Aphidius rhopalosiphi* De Stephani-Perez, *Lysiphlebus fabarum* (Marshall), *Praon sp*. (Van Emden,1988), and *Aphidius funebris* Mackauer (Pareja et al., 2007), making it seem as though aphid parasitoids are generally affected by this phenomena. According to Vinson's nomenclature, host acceptance, also referred to as the proper act of oviposition or host/prayer eating, is the last step in the host selection process (Steidle & van Loon, 2002; Virson (1976)). In the searching behavior for herbivores, natural enemies face the dilemma of reliability versus detectability (Vet & Dick, 1992). Due to the low biomass of aphids, volatiles produced by herbivores directly provide reliable information about their presence, but they are present in low concentrations in the environment. Plant volatiles are less reliable due to the possibility of herbivores present, despite their high biomass and ease of detection. Natural adversaries commonly use the following strategies to circumvent the reliability-detectability issue: (1) utilize more obtrusive semiochemicals from herbivore stages besides those that are vulnerable to assault, (2) reaction that focuses on cues brought on by certain herbivore-plant interactions, (3) Learn to connect dependable but challenging to detect inputs with simple to detect stimuli (Vet & Dick,1992).

II.1.4 The terminology of Semiochemicals

Chemicals that carry information (infochemicals) were discovered by Dick & Sabelis (1988) to be substances that transmit information during a natural interaction between two individuals, eliciting in the recipient a behavioral or physiological response that is adaptive to either one of the interactants or to both. Semiochemical terms mainly refer to pheromone, allelochemical, allomone, kairomone, and synomone (Nordlund & Lewis, 1976). An infochemical called a pheromone facilitates interactions between creatures of the same species, with benefits to the origin-related organism ([+, -] pheromone), the recipient ([-, +] pheromone), or both ([+, +1 pheromone). Allelochemicals are infochemicals that facilitate communication between members of different species. Allomone is an allelochemical important to the biology of an organism (organism 1) that, upon coming into contact with a member of a different species (organism 2), causes the recipient to exhibit behavioral or physiological responses that are adaptively advantageous to organism 2 but unfavorable to organism 1. When it comes into contact with a member of a different species (organism 2), a kairomone causes the recipient to exhibit behavioral or physiological responses that are adaptively beneficial to organism 1 but unfavorable to organism 2. A synomone is an allelochemical relevant to the biology of an organism (organism 1) that, upon contact with a member of a different species (organism 2), causes the recipient to exhibit behavioral or physiological characteristics that are advantageous to both organisms 1 and 2 in terms of adaptation (Nordlund & Lewis, 1976).

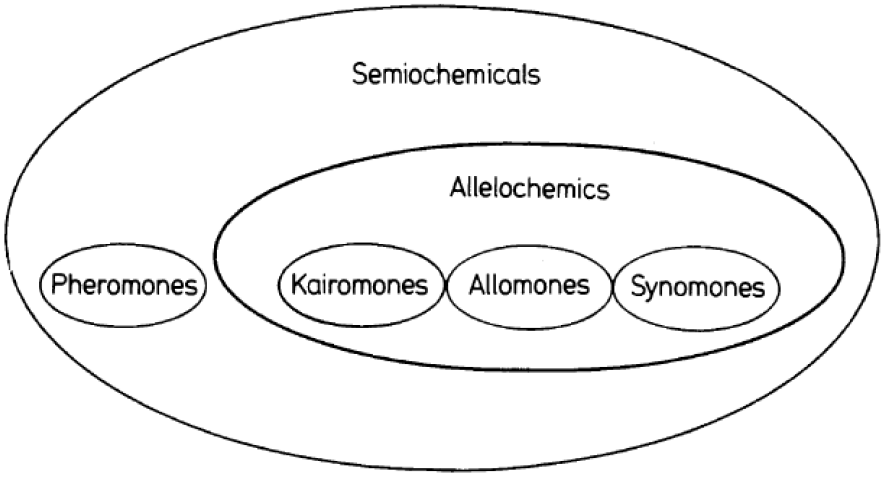

Figure 1-1. Structure of current semiochemical terminology according to Nordlund & Lewis (1976).

II.1.5 Volatile organic compounds

Plants produce and emit a wide range of volatile organic compounds (VOCs) as defenses against biotic and abiotic threats, interacting with their environment (Dudareva et al., 2013). More than that VOCs released by plants as well as herbivores act as infochemicals in the tritrophic relationship. Healthy plants normally release relatively little amounts of volatiles. Reduced pressure steam distillation-extraction, gas chromatography-mass spectrometry, and co-chromatography were used to identify nonanal and related unsaturated C9 aldehydes and alcohols, as well as some other aldehydes, alcohols, and a ketone, from the aerial parts (foliage and culms) of wheat plants (Hamilton-Kemp & Andersen, 1984). Gas chromatography-mass spectrometry (GC-MS) and headspace solid microextraction (HS-SPME) were the two techniques most frequently used to examine organic compounds (GC-MS). A fused silica fiber coated with polymeric organic liquid is inserted into the headspace above the sample as part of the HS-SPME method, which is a solvent-free sample preparation approach. The coating concentrates and extracts the volatile organic analytes, which are then transferred to the analysis device for desorption and analysis (Zhang, Z., & Pawliszyn, 1993). To identify different substances in a test sample, an analytical method known as GC-MS combines the benefits of gas chromatography and mass spectrometry (Sparkman et al., 2011). There were 25 different volatile substances discovered connected to the wheat plant's leaves (and stems) first using Tenax adsorbent trapping and examined by capillary GC-MS (Butter, 1985). The main volatile substances include caryophyllene, (Z)-3-hexenyl acetate, (Z)-3-hexanol, (E)-β-ocimene, and (E)-2-hexena. A-copaene, a-farnesene, and linalool oxides are unusual substances.

II.1.6 The infochemical elicits behavior and EAG responses within aphids.

The use of olfaction by host-seeking aphids is widely documented, and in recent decades, a wealth of knowledge has been gathered about how volatiles may encode host identity and appropriateness as well as the specific behaviors they activate in aphids. These olfactory signals may be used to identify a host using species-specific molecules or specific ratios of common components. Aphids must be able to find and identify host plants in order to survive, which is made possible, among other things, by the utilization of olfactory cues (Pickett et al., 1992; Pickett & Glinwood, 2008). Electroantennogram (EAG) technology allows researchers to examine the usual reactions of these receptors to plant scents. This can be used to demonstrate the existence of specific plant volatiles that are probably essential for separating the aroma blends of host and non-host plant species during orientation and landing. Several aphid species, such as the English grain aphid *Sitobion avenae* (F.), the rose-grain aphid *Metopolophium dirhodum* (Walk.) (Yan & Visser, 1982; Visser & Yan, 1995), the black bean aphid *Aphis fabae* Scop. (Hardie et al., 1995), the vetch aphid *Megoura viciae* Buckton, the peach-potato aphid *Myzus persicae* (Sulz.) and the cabbage aphid *Brevicoryne* have been observed to exhibit EAGs in response to plant volatiles (Visser & Piron, 1994, Visser et al., 1996). In general, (a) the general green leaf volatiles, such as (E)-2-hexenal, (E)-2-hexenol-1, (Z)-3-hexenyl acetate, hexanol-1, hexanal, (E)-2-

heptenal, and 2-heptanone, (b) the benzaldehydes, e.g., 4-methoxybenzaldehyde, (c) the carvones, (-)-(R)-carvone, (d) the monoterpene aldehyde citronetlal, (e) the nitriles, i.e., hexanonitrile and heptanonitrile, and (f) some isothiocyanates, e.g., butyl and 4-pentenyl isothiocyanate show distinct EAG response profiles. the reactivity of two cereal aphids, *Macrosiphum avenae* and *R. padi*, to volatiles produced by aphid feeding on wheat plants was assessed by Guo and Liu (2005). The two aphid species were considerably attracted to E-2-hexenal, E-2-hexenol, and E-3-hexenyl acetate while the alatae and apterae of *M. avenae* and apterae of *R. padi* were most attracted to 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, and methyl salicylate.

During the fall, many species of aphids reproduce sexually on their main hosts, and the sexual females generate a pheromone that attracts males. Its main constituents are (1R, 4aS, 7S, 7aR)-nepetalactol and (4aS, 7S, 7aR)-nepetalactone (Dawson et al., 1987). To protect the plants they have colonized from predators and other hazards, aphids have created cutting-edge defense mechanisms. They depart from the host plant and release repulsive droplets that warn nearby conspecifics to run away (Kislow & Edwards, 1972; Wientjens et al., 1973; Nault & Bowers, 1974; Montgomery & Nault, 1977; Clegg & Barlow, 1982). These secretions contain alarm pheromone, and the main ingredient in many aphid species is the sesquiterpene EBF, including *A. pisum*, *M. persicae*, *S. miscanthi*, *R. padi* and *A. gossypii* (Bowers et al., 1972; Kislow & Edwards, 1972; Wientjens et al., 1973; Nault & Bowers, 1974; Edwards et al., 1973; Pickett & Griffiths, 1980; Mostafavi et al., 1996; Francis et al., 2005; Zhong et al., 2012; Zhang et al., 2017; Qin et al., 2019; Wang et al., 2021). Aphids are repelled by EBF, and the amount of EBF secreted varies among aphid species. Take *S. avenae* (Fabricius) for instance, which emits only quite low amounts of EBF (0.7 ng on average) per cornicle droplet (Micha and Wyss, 1996). The nature of an aphid's response to EBF differs depending on the context of predation and the costs of escaping (Montgomery and Nault, 1978; Montgomery and Nault, 1977; Arakaki, 1989; Roitberg and Myers, 1978). While aphids normally only release cornicle droplets after being physically assaulted by a predator, not when they first come into contact with one (Nault and Phelan, 1984), Only 10% of attacks result in the emitter escaping (Dixon, 1958; Edwards,1966). Only one or a few aphids at a time are targeted simultaneously within an aphid colony, and the signal is not magnified by the emission of nearby aphids (Hatano et al., 2008b). Natural enemies may use plant-derived EBF as a synomone to detect aphid-infested plants through a changed plant volatile bouquet because it emits in very low concentrations and is unstable; it is only present after an attack (Vosteen et al., 2016).

II.1.7 Infochemical mediate behavior response within the parasitoid of aphids

Previous research has suggested that the healthy plant's volatiles may serve as a trigger for parasitoid species. Semiochemicals are usually cited as mediating host recognition (Strand and Vinson, 1982; Grasswitz and Paine, 1992; Battaglia et al., 1993). This has also been proven by many parasitoid species (Takabayashi et al., 1991; Whitman and Eller, 1992; Röse et al., 1998; Hoballah and Turlings, 2005). For instance, the common green leaf volatile (Z)-3-hexenyl acetate was discovered to react

with *Aphidius funebris* (Gouinguené et al., 2005). Both male and female *A. ervi* responded to wheat and bean leaves. Male *A. uzbekistanicus* responded to a variety of plant materials, whereas females solely responded to wheat leaves (Powell & Zhang, 1983).

Several investigations have shown that plant-aphid combinations are attractive to aphid parasitoids. It can often be challenging to determine whether this attraction is caused by plant volatiles, aphid volatiles, or aphid leftovers on the plants. HIPVs are also essential markers for parasitoids to find hosts (Vet & Dicke, 1992; Hare, 2011) and these HIPVs are capable of being exploited by parasitoids as a reliable and simple indicator to find plants with herbivore infestations (Dicke & Baldwin, 2010). The first proof that plants can alter their volatile emissions in response to aphid assault was provided by Guerrieri et al. (1993) using the parasitoid A. ervi's attraction to aphid-damaged plants. In fact, Read et al. (1970) showed that the aphid Brassica olerassicae (L), which causes damage to the plant, attracted Diaretiella rapae (M' Intosh) to mustard oil, also known as ally isothiocyanate, which was emitted by collard, *Brassica olerassicae* (Read et al., 1970). It is evident that the damaged plant produced the principal stimuli that parasitoids find attractive, and plants also create semiochemicals at levels that are detectable by parasitoids. This has been found in *Aphidus colemani* and *Aphelinus abdominalis,* which preferred the infested plants with *M. persicae* indicating that parasitoids could discriminate the infested cabbage and both the two parasitoids significantly responded to the plant odor (Ahmed et al., 2022).

Pareja et al. (2007) used a compositional approach for quantitative analysis, which treats each compound produced as an integral component of a blend rather than as a separate substance released into the environment. This approach enables analysis of the relative contributions of each compound to the blend as a whole. One example is *A. funebris* appears to be using a combination of chemical cues to locate host-infested plants (Pareja et al., 2007). And one and 10 ng of (Z)-3-hexenyl acetate, and 10 or 100 ng of 6-methyl-5-hepten-2-one were attractive to the parasitoid. Female parasitoids (*Cotesia chilonis*) were attracted to linalool and DMNT at low, medium, and high concentrations, while MeSA was appealing at low concentrations but repulsive at high concentrations, which are the main component of *Chilo suppressalis*-induced volatiles (Yao et al., 2022).

When aphids are attacked by predators or parasitoids, they emit the aphid alarm pheromone (E)-farnesene from their cornicles. Electrophysiological studies have shown that the seven-spot ladybird, *Coccinella septempunctata*, possesses specific olfactory receptors for (E)-β-farnesenc and β-caryophyllene. Laboratory studies show these compounds to have behavioural activity with *C. septempunctata*, suggesting that they may be involved in prey location. Even though many studies claim that EBF is the essential volatile to draw aphids' natural predators, some research also casts doubt on this claim (Joachim, C., and Weisser, W. W. 2015a; Joachim, et al., 2015b). Besides, the sesquiterpene hydrocarbon β-caryophyllene, which is reported to attract the lacewing *Chrysoperfa carnea*. There are founding related to the symbiont-mediated mechanism, the most intriguing finding is that EBF levels are typically lower in symbiont-defended compared to uninfected, indicating that *A. ervi* can distinguish between symbiont-infected and uninfected aphids and alter its oviposition activity to

maximize the likelihood of defeating symbiont-based defense *A. pisum* (Oliver et al., 2012).

This noncrop system also exhibits semiochemical-mediated parasitoid attraction, which has been shown to help explain patterns of parasitism in the field (Pareja, 2006). Sensilla on the ovipositor are most likely involved in the detection of contact or perhaps internal substances (Larocca et al., 2007) presumably following the detection and assessment of contact chemicals. The role that epicuticular characteristics play in aphidiine wasps' identification of hosts (Pennacchio et al., 1994). For instance, females need to contact the host species with their antennae in order to distinguish it from nonhost species when they are close together (Le Ralec et al., 2005). On rare occasions, aphid parasitoids have been recorded attempting to oviposit on host exuviae (Michaud, personal communication; Outreman, personal communication). The parasitoids of the hymenopteran cereal aphid *A. ervi* and *Aphidius uzbekistanicus* Luzhetski are another illustration, which sensitive to the scents of aphids. The generalist parasitoid *A. ervi* responded to *M. dirhodum* and *A. pisum*, *A. uzbekistanicus* responded to the cereal aphids *S. avenae* (F.) and *M. dirhodum* (Walker) (Glinwood, 1998). When aphid remains were removed from plants, the plant's volatile profile changed, according to research by Guerrieri et al., (1993). A higher proportion of native female parasitoids fall on broad beans, *Vicia faba* L., injured by *A. pisum* than on unharmed plants or aphids alone, demonstrating that parasitoid behavior is influenced by volatiles from both exuviae and feces as well as herbivore-induced plant volatiles, which can attract aphid parasitoids (Du et al., 1996).

Nonvolatile chemical substances are likely involved when antennal contact is necessary for host recognition, and a role for the cuticle texture must be taken into consideration (Godfray, 1994). From the perspective of chemical analysis, exuviae from *A. pisum* elicit attacks by *A. ervi* even when coarsely crushed (Battaglia et al., 2000). However, if the parasitoid is prevented from touching the exuviae with its antennae, the assault behavior is not seen. The notion that a cuticular molecule, acting as a recognition kairomone, is present in cornicle secretion and on the cuticle of exuviae was suggested by Battaglia et al. (2000).

Table 1-1. List of chemical compounds from different aphid-plant complexes and their effect on aphid parasitoid wasp responses

infochemical cue	aphid	Parasitoids	Effect	Reference
(E)-β-farnesene	*M. persicae*	*A. bipunctata*	Attract	Franics et al., 2004
	A. pisum			
	S. avenae	*A. Uzbekistanicus*	Attract	Micha &
		P. volucre	Attract	Wyss,1996
	S. miscanthi	*A. gifuensis*		Fan et al., 2018
6-methyl-5-hepten-2-	*A. pisum*	*A. ervi*	Attract	Du et al., 1998;
one (MHO)	*U. jacea*	*A. funebris*	Attract	Powel et al., 1998
	U. jacea	*A. uzbekistanicus*	Repel	Pareja et al., 2007
	R. padi	*A. rhopalosiphi*	None	Holler et al., 1994
				Gonzales et al., 1999
(Z)-3-hexenyl acetate	*U. jacea*	*A. funebris*	Attract	Pareja et al., 2007
Methyl salicylate	*A. glycines*			
(Z)-jasmone		*A. ervi*	Attract	Birkett et al., 2000
(4aS,7S,7aR)-		*A. ervi*	Attract	Glinwood et al.,
nepetalactone		*A. eadyi*	Attract	1999
(1R,4aS,7S,7aR)-		*Diaeretiella rape*		Gabrys et al., 1997
nepetalactol				
Cuticular hydrocarbon	*S. avenae*	*A. rhopalosiphi*	Accept	Muratori et al.,
(CHC)				2006

II.1.8 The molecular mechanism within the peripheral olfactory system

The behavioral response of insects to olfactory cues is essentially driven by feeding, reproduction and habitat selection (Pelosi et al., 2014). Several multi-gene families encode proteins with crucial roles in chemoreception systems. Molecular odorants enter the sensilla through pores and spread inside the hemolymph on the antennae due to odorant-binding proteins (OBPs) and/or chemosensory proteins (CSPs) (Pelosi et al., 2006; Leal, 2013). These odorants are then transported to olfactory receptors (ORs), ionotropic receptors (IRs), or sensory neuron membrane proteins (SNMPs), from which the chemical signals will be transmitted into electrophysiological signals for the brain (Leal, 2013; Pelosi et al., 2018).

II.1.8.1 Odorant binding proteins (OBPs)

Insect OBPs were initially discovered in antennae of the moth *Antheraea polyphemus* (Vogt & Riddiford, 1981). Their wide distributions in antennal sensilla indicated the first link of OBPs in the signal chain of odorant perception (Xu et al., 2009). OBPs are tiny, globular, water-soluble proteins with a molecular weight of 10-30 kDa (Pelosi et al., 2005). The presence of six highly conserved cysteine residues, which are paired in three interlocking disulfide bridges to maintain the protein's tertiary structure, is a common feature of classical OBPs (Pelosi et al., 2014). OBPs act as shuttles for hydrophobic odor molecules, transporting them through the sensillum lymph to odorant receptors (Zhou, 2010). After initiating receptors, OBPs may also concentrate odorants in the sensillum lymph and swiftly destroy odorant molecules (Vogt & Riddiford, 1981; Leal, 2013). Insect OBPs not only are expressed in the chemosensory system, but also occur in nonsensory tissues and organs, such as the cornicles (Wang et al., 2021), thoraxes (Xue et al., 2016; Gao et al., 2018; Wang et al., 2019), reproductive organs (Li et al., 2008; Sun et al., 2012), mandibular glands (Iovinella et al., 2011), salivary glands (Zhang et al., 2017), and wings (Calvello et al., 2003; Pelosi et al., 2005; Wang et al., 2021). Some insect OBPs have physiological functions other than binding odorants. For example, the sperm carrier function of OBPs has been reported in the male reproductive apparatus of mosquitoes (Li et al., 2008). Moreover, one OBP expressed by male moths is found on the surface of fertilized eggs, which functions to avoid cannibalistic behaviors among larvae (Sun et al., 2012). The prediction of the whole OBP family in species became quite simple due to the availability of more insect genomes and transcriptomes using next-generation sequencing techniques. For instance, the number of OBPs in Hymenoptera varies, *Apis mellifera* has 21 OBPs, *Microplitis mediator* has 18 OBPs, *Pieris rapae* has 14 OBPs, *Spodoptera exigua* has 34 OBPs, *Cotesia vestalis* has 20 OBPs, and 90 OBPs were predicted in *Nasonia vitripennis* (Foret & Maleszka, 2006; Peng et al., 2017; Li et al., 2020; Liu et al., 2015; Liu et al., 2020; Vieira et al., 2012).

Recently, the aphid EBF recognition mechanism has received extensive attention and progress has been well made. The first EBF-binding protein and the first OBP discovered in aphids, respectively, were both found in OBP3 from the pea aphid *A. pisum* (Qiao et al., 2009). Since then, other investigations have revealed that OBP7

also has a specific preference for EBF in a variety of aphid species, including the pea aphid *A. pisum* (Zhang et al., 2017), the peach aphid *M. persicae* (Sun et al., 2012), the grain aphid *S. avenae* (Zhong et al., 2012), and the bird cherry-oat aphid *Rhopalosiphum padi* (Fan et al., 2017) and *Megoura viciae* (Bruno et al., 2018). More recently, it was revealed that OBP9 has a wide range of affinities, including EBF (Qin et al., 2020; Wang et al., 2021). Since at least three EBF-binding proteins have been discovered thus far, multiple OBP contacts may be required for peripheral EBF transmission. There are 3 EBF binding proteins in aphid species, and they are all orthologs with strong sequence consensus (Qin et al., 2020; Wang et al., 2021). Additionally, the substantial upregulation of OBP7 and OBP9 expression in response to EBF induction demonstrates the remarkable olfactory flexibility of aphids' olfaction (Zhang et al., 2021). Additionally, OR5, an olfactory receptor from aphids, has been shown to be in charge of EBF signal transduction (Zhang et al., 2017). For the natural enemies, progress has also been made in recent years, for example, CpalOBP10 showed its affinities to green leaf volatiles as well as EBF. HaxyOBP15 displayed a broad binding profile with (E)-β-farnesene as well as multiple other odor ligands in ladybeetle *Harmonia axyridis,* an important natural enemy that consumes aphids (Qu et al., 2022). Further, one EBF olfactory receptor, EcorOR3, as well as EcorOBP15 have been identified to involve in the EBF perception in hoverfly *Eupeodes corollae* (Wang et al., 2022).

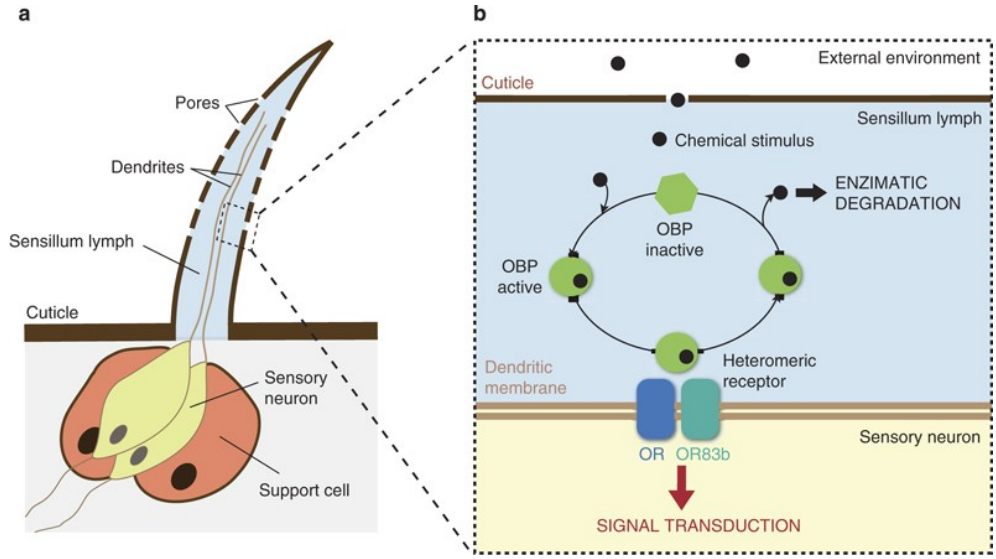

Figure 1-2 Schematic representation of the general structure of an insect olfactory hair and chemosensory signaling transduction pathway (Sánchez-Gracia et al., 2009).

II.1.8.2 chemosensory proteins (CSPs)

Insect CSPs, which are also called OS-D like proteins (McKenna et al., 1994) or sensory appendage proteins (SAPs) (Pikielny et al., 1994), represent a novel group of olfactory proteins involved in insect olfaction. CSPs have shown broad expression profiles in chemosensory tissues, including antennae (Robertson et al., 1999; Angeli et al., 1999; Liu et al., 2012; Jacquin-Joly et al., 2001; González et al., 2009; Gu et al., 2012), maxillary palps (Maleszka and Stange, 1997), labial palps (Maleszka et al., 1997; Jin et al., 2005), and the proboscis (Nagnan-Le et al., 2000). However, these proteins are also found in non-chemosensory organs, such as legs (Nomura et al., 1992; Kitabayashi et al., 1998), wings (Ban et al., 2003; Zhou et al., 2008), and pheromone glands (Jacquin-Joly et al., 2001). Insect CSPs have multiple functions in insect chemoreception, growth and development. In different species, variable expression of the genes occurs depending on, for instance, sex, tissue, or life stage (Liu et al., 2012; Guo et al., 2011).

II.1.8.3 olfactory receptors

At the molecular level, Odor decoding by insects is performed by arrays of olfactory receptor neurons (ORNs) located in different types of chemosensory hairs (sensilla) on the antennae (Benton, 2009; Rutzler & Zwiebel, 2005). Diverse proteins are involved in odor decoding (Abuin et al., 2011; Martin & Alcorta, 2011; Stengl & Funk, 2013; Leal, 2013). For most insects, ORNs responses rely on members of two large and divergent families of olfactory receptor proteins, which include odorant receptors (ORs) (Benton, 2009; Bohbot et al., 2007; Dahanukar et al., 2005; Ha and Smith, 2008; Neuhaus et al., 2005) and ionotropic glutamate-like receptors (IRs) (Rytz et al., 2013; Touhara, 2009; Wicher et al., 2008).

The ORs and GRs belong to the same receptor superfamily (Clyne et al., 1999; Robertson et al., 2003). The ORs detect volatile chemicals (odors) while the GRs are responsible for contact chemoreception and detection of carbon dioxide (Vosshall & Stocker, 2007; Robertson & Kent, 2009). Insect ORs were the first chemoreceptor family to be discovered in *Drosophila melanogaster* genome (Gao & Chess, 1999). Insect ORs belong to seven-transmembrane proteins, they are distinct from GPCRs and possess an inverse heptahelical topology, with the N-terminus being located in the intracellular section of the transmembrane protein and the C-terminus found extracellularly (Butterwick et al., 2018; Del et al., 2021; Benton, 2006). Insect ORs, with a high degree of divergence both within and across species, have been identified in many species, including *Apis mellifera* (Robertson & Wanner, 2006), *Macrocentrus cingulum* (Ahmed et al., 2016), *A. gifuensis* (Kang et al., 2017). The function of an insect OR depends on the presence of a non-ligand binding odorant receptor co-receptor (Orco), which functions as a ligand-gated ion channel (Stengl & Funk, 2013; Sato et al., 2008; Liu et al., 2016). In contrast to ORs, Orco is highly conserved across insect species sharing approximately 60% identity (Butterwick et al., 2018). Insect ORs can be broadly tuned or highly specific. For example, ApisOR4, from the pea aphid *A. pisum*, was screened against a panel of 57 odorants, it responded to a range of aromatic compounds, such as 4-ethylacetophenone and salicylaldehyde (Zhang et al., 2019).

IRs belong to an ancient family of chemosensory receptors that are relatives of ionotropic glutamate receptors (iGluR), and are divided into two subfamilies, the conserved "antennal" IRs and the species-specific "divergent" IRs (Abuin et al., 2011; Croset et al., 2010). The IRs have been identified in several insect species from different orders (Bengtsson et al., 2012; Li et al., 2015; Wu et al., 2015; Olivier et al., 2011; Gu et al., 2015). ionotropic glutamate receptor (iGluR)-related genes, named Ionotropic Receptors (IRs), were discovered in *D. melanogaster*, and are also ligand-gated ion channels, but with three transmembrane domains (Benton et al., 2009; Croset et al., 2010). The main functions of IRs are reported within IR8a and IR25a in *Drosophila*, which appear to operate as co-receptors, turning IRs sensory cilia targeting and IR-based sensory channels (Ai et al., 2013; Guo et al., 2014). While the latest research found that AsegIR75q.1 and AsegIR75p.1, which responded primarily to C_6-C_{10} medium-chain fatty acids, and their co-receptor AsegIR8a are not located in coeloconic sensilla as found in *Drosophila*, but in basiconic or trichoid sensilla (Hou et al., 2022).

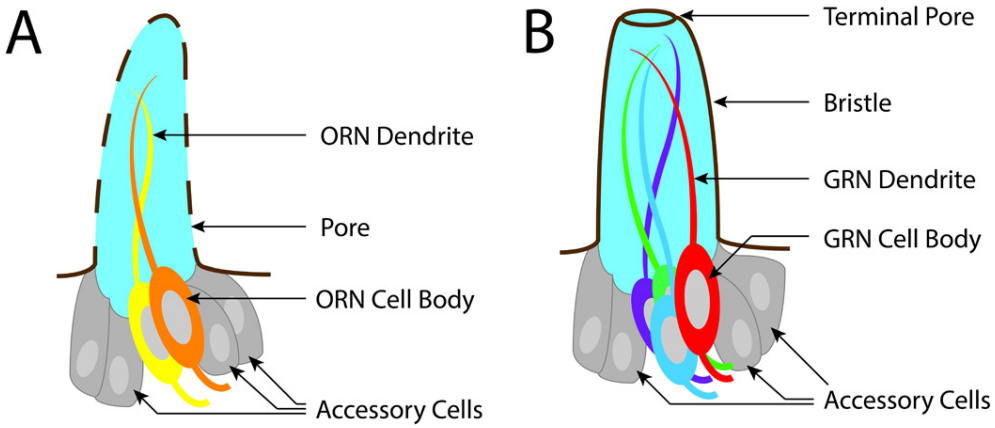

Figure 1-3 Chemosensory sensilla. (A) Olfactory and (B) gustatory sensilla in adult flies (Joseph & Carlson, 2015).

II.2. Conclusion and further trends

Info-chemicals mediated the behavior response is an old and yet hot topic in the tritrophic interaction system from plant-aphid-parasitoid wasps. Signal associated with cues like color, sound, shape, or size helpful for long or short-range attraction to prey or host. Both aphids and parasitoid wasps utilize inforchemicals in the same habitat and have developed subtle peripheral nervous systems that alter them to sight a variety of info-chemicals, whereas it's tough to seek out an acceptable place to pray in exceedingly sophisticated surroundings with several distinct plants and animal species.

Therefore, to promote the utilization of parasitoid wasp as agent to control aphids in the field, inforchemicals and molecular mechanism characteristics are necessary review between aphids and it's parasitoid wasps.

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3

Chapter III A chromosome-level draft genome of the grain aphid *Sitonion miscanthi*

This work is an original contribution, adapted from:
Xin Jiang[*], Qian Zhang[*], Yaoguo Qin[*], Hang Yin, Siyu Zhang, Qian Li, Yong Zhang, Jia Fan[†], Julian Chen. A chromosome-level draft genome of the grain aphid *Sitobion miscanthi.* Submitted in *GigaScience*, 8, 2019, 1-8.

Forward

Aphids are tiny sap-sucking insects that damage in yield and quality of wheat by plundering wheat nutrition, transmitting plant virus and excreting honeydew. Currently, the majority of measures rely heavily on chemical insecticide, which has led to an increase in the "3R" problem. This has been an urgent to develop green prevention and control technologies, for example, push-pull technology that uses semiochemicals to regulate the behavior of aphids. To gain a better understanding of that technology, a thorough understanding of the relationship between semiochemicals and the peripheral olfactory system of cereal aphids is needed. Among these cereal aphids, *S. miscanthi* is the dominant species and also an ideal model for studying three trophic levels interactions between insects and natural enemy. However, the genome information for this species has not been published yet. Therefore, from the perspective of green control of wheat pests and the basic frontier research on insects, the study of the genome of the *S. miscanthi* is of great importance.

Abstract

Background: *Sitobion miscanthi* is an ideal model for studying host plant specificity, parthenogenesis-based phenotypic plasticity, and interactions between insects and other species of various trophic levels, such as viruses, bacteria, plants and natural enemies. However, the genome information for this species has not been published yet. Here, we analyzed the entire genome of a female aphid colony using long-read sequencing and Hi-C data to generate chromosome-length scaffolds and a highly contiguous genome assembly.

Results: 1. The final draft genome assembly from 33.88 Gb of raw data was approximately 397.90 Mb with a 2.05 Mb contig N50. Nine chromosomes were further assembled based on Hi-C data to a 377.19 Mb final size with a 36.26 Mb scaffold N50. 2. The identified repeat sequences accounted for 26.41% of the genome, and 16,006 protein-coding genes were annotated. According to the phylogenetic analysis, *S. miscanthi* is closely related to *Acyrthosiphon pisum*, with *S. miscanthi* diverging from their common ancestor approximately 25.0-44.9 million years ago.

Conclusions: We generated a high-quality draft of the *Sitobion miscanthi* genome. This genome assembly promotes research on the lifestyle and feeding specificity of aphids and their interactions with each other and species at other trophic levels. It can serve as a resource for accelerating genome-assisted improvements in insecticide resistant management and environmentally safe aphid management.

Keywords: aphid, *Sitobion miscanthi*, *Sitobion avenae*, annotation, genome, long-read sequencing, Hi-C asse

III.1. Introduction

The grain aphid *Sitobion miscanthi* (NCBI: txid44668, Fig. 2-1) widely misreported as *Sitobion avenae* in China (Zhang, 1999), is a globally distributed sap-sucking specialist of cereal and a dominant species in wheat-growing regions across China. It threatens wheat production in various ways such as pillaging nutrition from the host, transmitting pathogenic plant viruses, and defecating sticky honeydew that further obstructs photosynthesis and reduces wheat quality. Together with its highly specialized host range, its simple parasitic life cycle, pleomorphism, and alternation of complete and incomplete life cycles make *S. miscanthi* significant for both basic and applied research. Therefore, we sought to publish the genome information for *S. miscanthi* here. Genomes with annotation information from a total of 8 aphid species, namely the pea aphid *Acyrthosiphon pisum* (International Aphid Genomics Consortium) peach aphid *Myzus persicae* (Mathers et al., 2017), soybean aphid *Aphis glycines* (Wenger et al., 2017), Russian wheat aphid *Diuraphis noxia* (Burger & Botha, 2017), cherry-oat aphid *Rhopalosiphum padi* (Thorpe et al., 2018), and black cherry aphid *Myzus cerasi* (Thorpe et al., 2018), the cotton aphid *Aphis gossypii* (Quan et al., 2019), and the corn leaf aphid *Rhopalosiphum maidis* (Chen et al., 2019) are available. However, no genome information for *S. miscanthi* has been published. Here, we report the chromosome-level genome sequence of the *S. miscanthi* isolate Langfang-1, which exhibits higher-quality assembly data indexes than other scaffold-level aphid genomes. Most of the sequences assembled into 9 scaffolds, which supported a 2n=18 karyotype for *S. miscanthi* (Kuznesova & Shaposhnikoy, 1973; Chen & Zhang, 1985). The repeat sequences and phylogenetic relationship of *S. miscanthi* with other insects were further analyzed.

Figure. 3-1 Winged and wingless *S. miscanthi*. Top, Winged adult; bottom, Wingless adult.

III.2. Methods and Results

III.2.1. Insects

Langfang-1, a grain aphid (*S. miscanthi*) isolate that was originally collected from wheat in Hebei province, was kept in our laboratory for genome sequencing. An isogenic colony was started from a single parthenogenetic female of *S. miscanthi* and was maintained on wheat (*Triticum aestivum*). Mother aphids were placed into culture dishes (dimeter of 9 cm) with moist absorbent paper on the bottom for 12 h. No newborn nymphs were fed during this period. Newborn nymphs within 12 h without feeding were collected for genome sequencing. In addition, 100 aphids of 1st and 2nd instars and 50 winged and wingless aphids at the 3rd instar, 4th instar and adult stages were collected for transcriptome sequencing.

III.2.2. Genome size estimation

High-quality genomic DNA for sequencing using the Illumina platform (Illumina Inc., San Diego, CA, USA) and PacBio Sequel sequencing (Pacific Biosciences of California, Menlo Park, CA, USA) was extracted from the newborn nymphs mentioned above. Data supporting the results of this article have been deposited at DDBJ/ENA/GenBank under Bioproject PRJNA532495 and the accession SSSL00000000. The version described in this article is version SSSL01000000. Other supporting data and materials including annotations and phylogenetic trees are available in the GigaScience GigaDB database (Jiang et al., 2019). The whole-genome size of *S. miscanthi* was estimated by *k*-mer analysis (*k*=19) based on Illumina DNA sequencing technology (Altschul ett al., 1990; Li et al., 2008). A short-insert library (270 bp) was constructed, and a total of ~42 Gb of clean reads was obtained for de novo assembly to estimate the whole-genome size using the standard protocol provided by the Illumina HiSeq X Ten platform. All clean reads were subjected to 19-mer frequency distribution analysis. The peak of 19-mer peak was at a depth of 89, and the genome size of *S. miscanthi* was calculated to be 393.1 Mb (Fig. 2-2, Table 1-1).

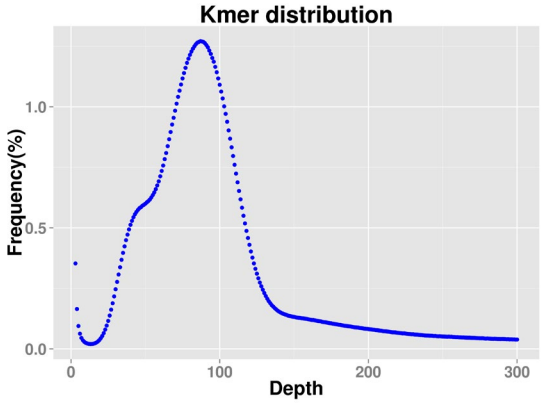

Figure. 3-2 19-mer distribution for the genome size prediction of *S. miscanthi*.

Table 3-1 Assessment results based on two strategies.

Genome feature/assessment strategy	19-mer analysis	PacBio
Genome size (Mb)	393.12	397.90
GC content (%)	31.70	30.25
Repeat sequence content (%)	35.07	24.14
Heterozygosity (%)	0.98	0.57

III.2.3. Genome assembly using PacBio long reads

The genomic DNA libraries were constructed and sequenced using the PacBio Sequel platform. Additionally, 4.35 million subreads (33.88 Gb in total) with an N50 read length of 12,697 bp were obtained after removing the adaptor (Fig. 3).

De novo genome assembly with long reads was performed using two pipelines, Canu (Canu, RRID:SCR 015880) and wtdbg (WTDBG, RRID:SCR 017225). Because of the high heterozygosity of *Sitobion miscanthi*, in the correction step, Canu first selects longer seed reads with the settings 'genomeSize=400000000' and 'corOutCoverage=50', then detects overlapping raw reads through the highly sensitive overlapper MHAP (mhap-2.1.2, option 'corMhapSensitivity=low/normal/high'), and finally performs an error correction with the falcon_sense method (option 'correctedErrorRate=0.025'). In the next step, with the default parameters, error-corrected reads are trimmed to remove unsupported bases and hairpin adapters to obtain the longest supported range. In the last step, Canu generates the draft assembly using the longest 80 coverage-trimmed reads with Canu v1.5 (Koren et al., 2017) to output more corrected reads and be more conservative at picking the error rate for the assembly to try to maintain haplotype separation.

Wtdbg is an SMS data assembler that constructs fuzzy Brujin graph (available at https://github.com/ruanjue/wtdbg). Wtdbg first generates a draft assembly with the command 'wtdbg -i pbreads.fasta -t 64 -H -k 21 -S 1.02 -e 3 -o wtdbg'. The use of error-corrected reads from Canu results in better assembly performance. Then, a consensus assembly is obtained with the command 'wtdbg-cns -t 64 -i wtdbg.ctg.lay -o wtdbg.ctg.lay.fa -k 15'.

To improve genome contiguity, two assemblies generated from the Canu and wtdbg pipelines were merged with three rounds of quickmerge (Chakraborty et al., 2016). Quickmerge uses contigs from wtdbg as query input and contigs from Canu as ref input. The two contigs are aligned through mummer (v4.0.0, available at https://github.com/mummer4/mummer) with the nucmer parameters '-b 500 -c 100 -l 200 -t 12' and delta-filter parameters '-i 90 -r -q', and then merged through quickmerge with the parameters '-hco 5.0 -c 1.5 -l 100000 -ml 5000'. The result was error corrected using Pilon (Chin et al., 2016). After all of the processing described above, the resulting genome assembly was further cleaned using Illumina NGS data, which were used in the 19-mer analysis above. The final draft genome assembly was 397.90 Mb, which reached a high level of continuity with a contig N50 length of 2.05 Mb (Table 2-2). The contig N50 of *S. miscanthi* was much higher than that of previous aphid genome assemblies constructed using DNA NGS sequencing technologies.

Table 3-2 Assembly statistics of the *S. miscanthi* genome and 7 other aphid genomes based mainly on NGS.

Genome assembly/species	*S. miscanthi*	*R. padi*	*D. noxia*	*Ac. pisum*	*Ap. glycines*	*M. persicae*	*M. cerasi*	*Ap. gossypii*
Assembly size (Mb)	397.9	319.4	393.0	541.6	302.9	347.3	405.7	294.0
Contig count	1,148	16,689	49,357	60,623	66,000	8,249	56,508	22,569
Contig N50 (bp)	1,638,329	96,831	12,578	28,192	15,844	71,400	17,908	45,572
Scaffold count	656	15,587	5,641	23,924	8,397	4,018	49,286	4,724
Scaffold N50 (bp)	36,263,045	116,185	397,774	518,546	174,505	435,781	23,273	437,960
Genome annotation								
Gene count	16,006	26,286	19,097	36,195	17,558	18,529	28,688	14,694
Mean gene length (kb)	7.805	1,543	1.316	1.964	1.520	1.839	1,222	1.964
Mean exon count per gene	6.7	5.20	3.0	5.0	6.2	6.1	3.7	10.1
Mean exon length (bp)	288	162	249.0	394.7/429	246	299	178	218

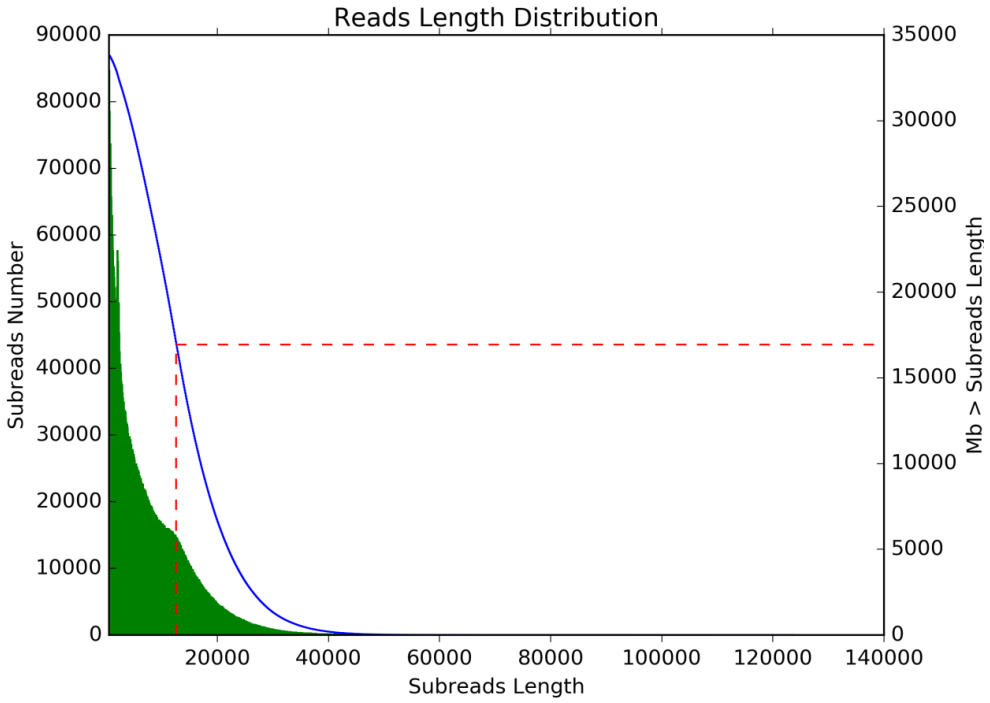

Figure. 3-3 Filtered subread length distribution

III.2.4. Genome quality evaluation

To assess the completeness of the assembled *S. miscanthi* genome, we subjected the assembled sequences to Benchmarking Universal Single-Copy Orthologs (BUSCO) version 2 (BUSCO, RRID:SCR 015008) (Simão et al., 2015). Overall, 1496 and 19 of the 1658 expected Insecta genes (insect_odb9) were identified in the assembled genome as having complete and partial BUSCO profiles, respectively. Approximately 143 genes were considered missing in our assembly. Among the expected complete Insecta genes, 1401 and 95 were identified as single-copy and duplicated BUSCOs, respectively (Fig. 2-4).

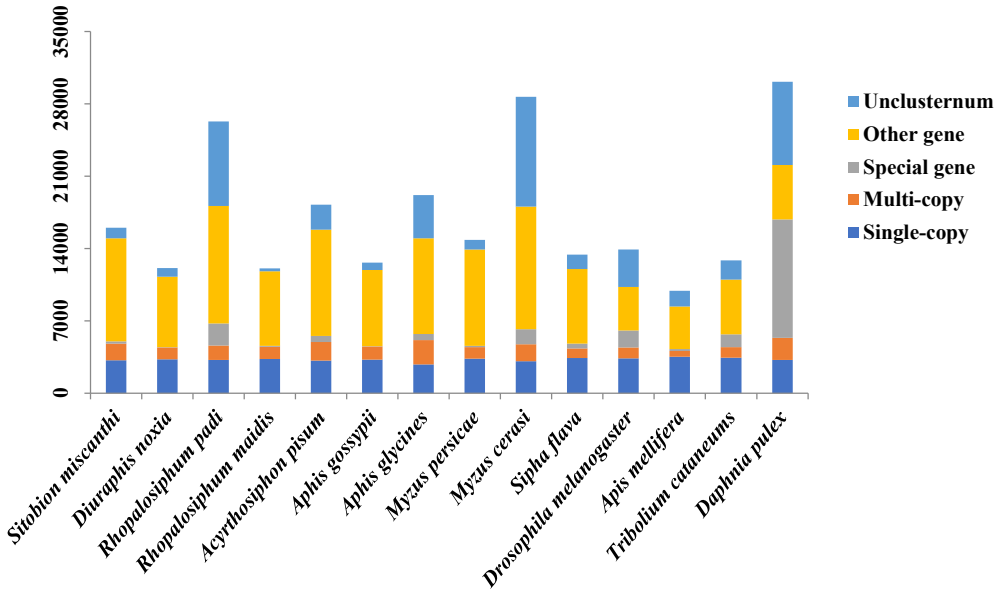

Figure. 3-4 Statistics of gene family clusters

III.2.5. Hi-C library construction and chromosome assembly

In this work, we used Hi-C to further assemble the genome of *S. miscanthi* at the chromosome level. Genomic DNA was extracted for the Hi-C library from the whole aphids of *S. miscanthi* mentioned above. Samples were extracted and sequenced following a standard procedure. Hi-C fragment libraries were constructed with insert sizes of 300-700bp and sequenced on the Illumina platform. Adapter sequences of raw reads were trimmed, and low-quality PE reads were removed for clean data. The clean Hi-C reads were first truncated at the putative Hi-C junctions, and then the resulting trimmed reads were aligned to the assembly results with BWA software (BWA, RRID:SCR 010910) (Li et al., 2009). Only uniquely alignable pairs reads whose mapping quality was more than 20 remained for further analysis. Invalid read pairs, including Dangling-End and Self-cycle, Re-ligation and Dumped products, were filtered by HiC-Pro (v2.8.1) (Servant et al., 2015).

In total, 38.44% of unique mapped read pairs were valid interaction pairs for scaffold correction and were used to cluster, order and orient scaffolds onto chromosomes by LACHESIS.

Before chromosome assembly, we first performed a preassembly for the error correction of scaffolds, which required the splitting of scaffolds into segments of 50 kb on average. The Hi-C data were mapped to these segments using BWA (version 0.7.10-r789) software. The uniquely mapped data were retained to perform assembly by using LACHESIS software. Any two segments that showed inconsistent connection with information from the raw scaffold were checked manually. These

corrected scaffolds were then assembled with LACHESIS. Parameters for running LACHESIS included CLUSTER_MIN_RE_SITES, 70; CLUSTER_MAX_LINK_DENSITY, 1; ORDER_MIN_N_RES_IN_TRUN, 19; ORDER_MIN_N_RES_IN_SHREDS, 19. After this step, placement and orientation errors exhibiting obvious discrete chromatin interaction patterns were manually adjusted. Finally, 774 scaffolds (representing 97.48% of the total length) were anchored to 9 chromosomes (Figure. 2-5, Table 2-3). A genome with a final size of 377.19 Mb and a scaffold N50 of 36.26 Mb was assembled, which showed a high level of continuity with a contig N50 of 2.05 Mb using 1,167 contigs. The contig N50 of the genome assembled using PacBio long reads and Hi-C assembly was much higher than that of the 7 previously published aphid genome assemblies constructed using DNA NGS technologies (Table 2-4).

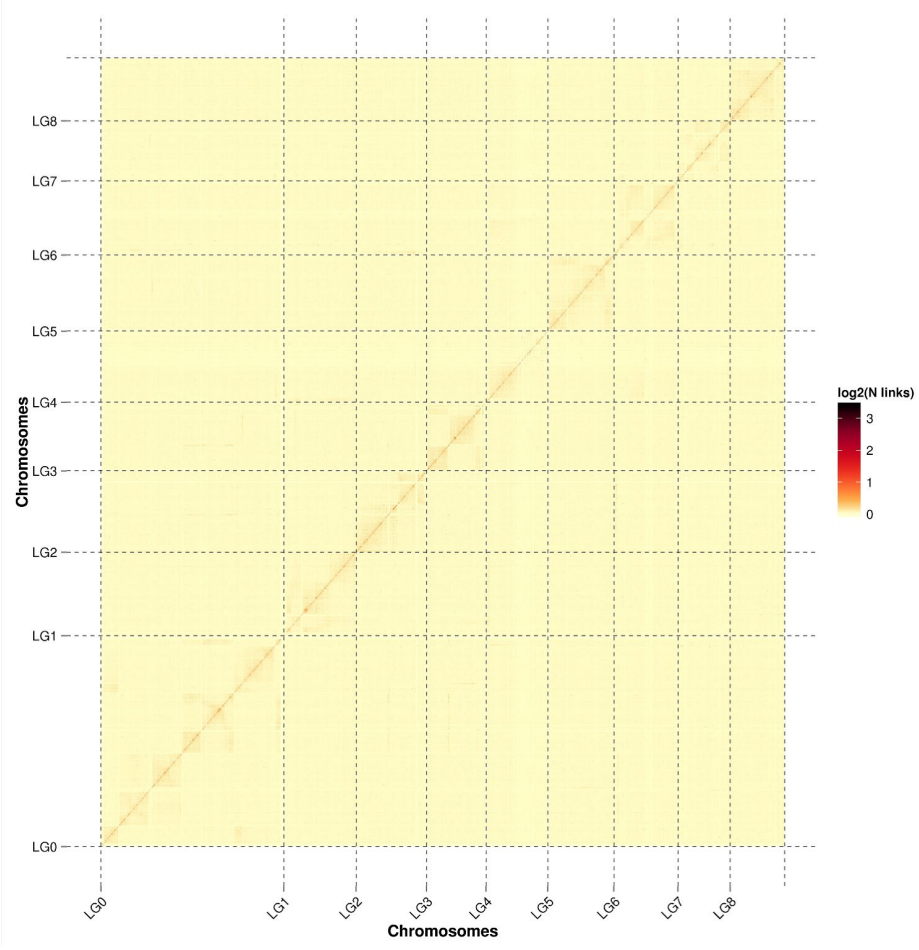

Figure. 3-5 Hi-C contact heatmap of the *S. miscanthi* genome.

Table 3-3 Summary of genome constructed to chromosome level of *S.avenae*

Group	Sequence Number	Sequence Length (bp)
Lachesis Group0	152	102338138
Lachesis Group1	79	41151279
Lachesis Group2	68	39866443
Lachesis Group3	57	33593668
Lachesis Group4	148	36747454
Lachesis Group5	58	36785619
Lachesis Group6	70	36121151
Lachesis Group7	79	30575005
Lachesis Group8	63	30712089
Total Sequences Clustered (Ratio %)	774 (67.48)	387890846 (97.48)
Total Sequences Ordered and Oriented (Ratio %)	501 (64.73)	377194755 (97.24)

Table 3-4 Summary of *S. miscanthi* genome assembly.

Statistics	Draft scaffolds	Corrected by HI-C
Contig number	1,039	1,167
Contig length	397,907,165	397,907,165
Contig N50 (bp)	2,049,770	1,565,814
Contig N90 (bp)	256,083	185,510
Contig max (bp)	11,219,273	10,100,000
Gap number/gap total length (bp)	0	0

III.2.6. Repeat sequences within the S. miscanthi genome assembly

To identify tandem repeats, we utilized 4 software, namely LTR_FINDER (v1.0.5; LTR Finder, RRID:SCR 015247)) (Xu et al., 2007), MITE-Hunter (v1.0.0) (Han & Wessler, 2010), RepeatScout (v1.0.5; RepeatScout, RRID:SCR 014653) (Price et al., 2005), and PILER-DF (v1.0) (Edgar & Myers, 2005) to build a de novo repeat library based on our assembly with the default settings. Subsequently, the predicted repeats were classified using PASTEClassifier (v1.0) (Hoede et al., 2014) and merged with Repbase (19.06) (Bao et al., 2015). Finally, using the resulting repeat database as the final repeat library, RepeatMasker v4.0.5 (RepeatMasker, RRID: SCR 012954) (Tarailo-Graovac & Chen, 2005) was used to identify repetitive sequences in the *A. nanus* genome with the following parameters: "-nolow -no is -norna -engine wublast." The repeat sequences accounted for 31.15% of the *S. miscanthi* genome, including

identified repeat sequences (26.42% of the genome), based on the de novo repeat library (Table 2-5).

Table 3-5 Detailed classification of repeats in the *S. miscanthi* genome assembly.

Type	Number	Length (bp)	Rate (%)
Class I	194093	51169345	12.86
DIRS	1,289	695,762	0.17
LINE	40,230	10,832,765	2.72
LTR/Copia	2,438	742,051	0.19
LTR/Gypsy	18,807	6,949,790	1.75
LTR/Unknown	7,534	3,195,404	0.8
PLE|LARD	115,765	28,920,417	7.27
SINE	6,665	1,075,456	0.27
SINE|TRIM	15	5,478	0
TRIM	1,116	1,281,655	0.32
Class I Unknown	234	26,384	0.01
Class II	188,820	44,184,063	11.1
Crypton	299	20,282	0.01
Helitron	5,688	1,871,785	0.47
MITE	7,972	1,434,924	0.36
Maverick	7,888	3,289,168	0.83
TIR	89,268	22,913,523	5.76
Class II Unknown	77,705	15,793,696	3.97
Potential Host Gene	926	251,812	0.06
SSR	2,611	381,142	0.1
Unknown	74,204	18,832,522	4.73
Identified	386,450	105,110,753	26.42
Total	460,654	123,943,275	31.15

III.2.7. Transcriptome sequencing to aid in gene prediction

Transcriptome sequencing (Illumina RNA-Seq and PacBio Iso-Seq) of cDNA libraries prepared from the whole newborn nymphs of *S. miscanthi* was conducted to aid in gene prediction. High-quality RNA was extracted using an SV Total RNA isolation kit (Promega, Madison, WI, USA). Reverse transcription was completed using a Clontech SMARTer cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA). A paired-end library was then prepared following the Paired-End Sample Preparation Kit manual (Illumina Inc., San Diego, CA, USA). Finally, a library with

an insert length of 300 bp was sequenced by an Illumina HiSeq X Ten in 150PE mode (Illumina Inc., San Diego, CA, USA). As a result, we obtained ~8.707 Gb of transcriptome data from RNA-seq. The quality of the transcripts was assessed by the proportion of gene regions covered by these transcripts, the higher being better. In this case, the proportion was 85.66%. The assembled transcripts were used to improve predictions of protein-coding genes in the *S. miscanthi* genome.

III.2.8. Gene annotation

Gene prediction of the *S. miscanthi* genome was performed using de novo, homology-based and transcriptome sequencing-based predictions. For de novo prediction, we employed Augustus v2.4 (Augustus, RRID:SCR 008417) (Stanke & Waack, 2003), GlimmerHMM v3.0.4 ((GlimmerHMM, RRID:SCR 002654) (Majoros et al., 2004), SNAP (version 2006–07-28; SNAP, RRID:SCR 007936) (Korf, 2004), GeneID v1.4 (Blanco et al., 2007) and GENSCAN (GENSCAN, RRID:SCR 012902) (Burge & Karlin, 1997) software to predict protein-coding genes in the *S. miscanthi* genome assembly. For homology-based prediction, protein sequences of closely related aphid species, namely, *Sipha flava*, *D. noxia*, *Ac. pisum* and *M. persicae,* were aligned against the *S. miscanthi* genome to predict potential gene structures using GeMoMa v1.3.1 (Keilwagen et al., 2016). For transcriptome sequencing-based prediction, we assembled the NGS transcriptome short reads into unigenes without a reference genome and then predicted genes based on unigenes using PASA v2.0.2 (PASA, RRID: SCR 014656) (Campbell et al., 2006). All of the above gene models were then integrated using EVM v1.1.1 (Haas et al., 2008) to obtain a consensus gene set. The final total gene set for the *S. miscanthi* genome was composed of 16,006 genes with an average of 6.74 exons per gene. The gene number, gene length distribution, and exon length distribution were all comparable to those of other aphid species (Table 2-2). Moreover, the indexes such as contig count and scaffold count were much improved.

To obtain further functional annotation of the protein-coding genes in the *S. miscanthi* genome, we employed the BLAST v2.2.31 (Altschul et al., 1990) program to align the predicted genes with functional databases such as the nonredundant protein (NR) (Marchler-Bauer et al., 2011), EuKaryotic Orthologous Groups (KOG) (Koonin et al., 2004), Gene Ontology (GO) (Dimmer et al., 2012), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000), and Translation of European Molecular Biology Laboratory (TrEMBL) (Boeckmann et al., 2003) databases (e-value $\leq 1e^{-5}$) (Figure. 2-6 and 2-7). Ultimately, 99.35% (15,902 genes) of the 16,006 genes were annotated based on at least one database (Table 2-6).

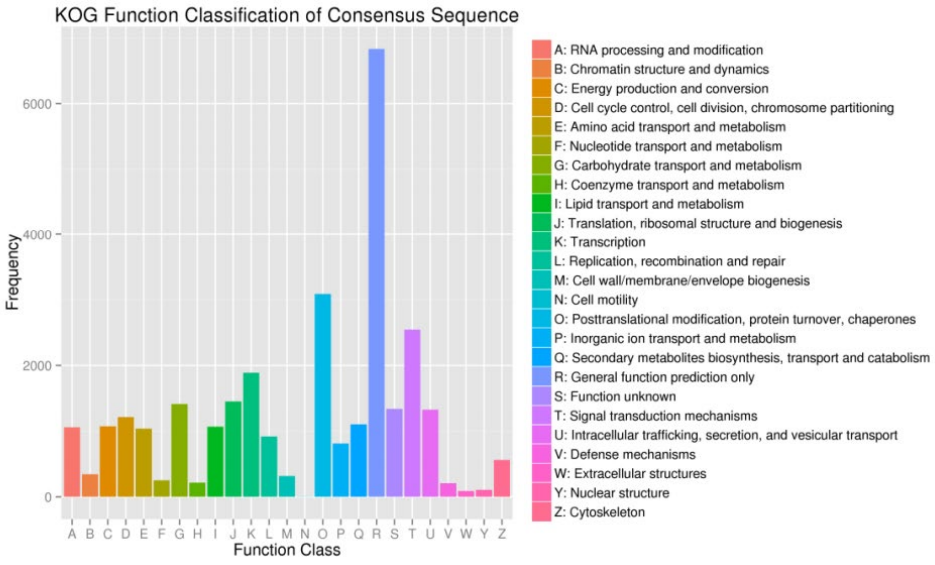

Figure. 3-6 KOG annotation result

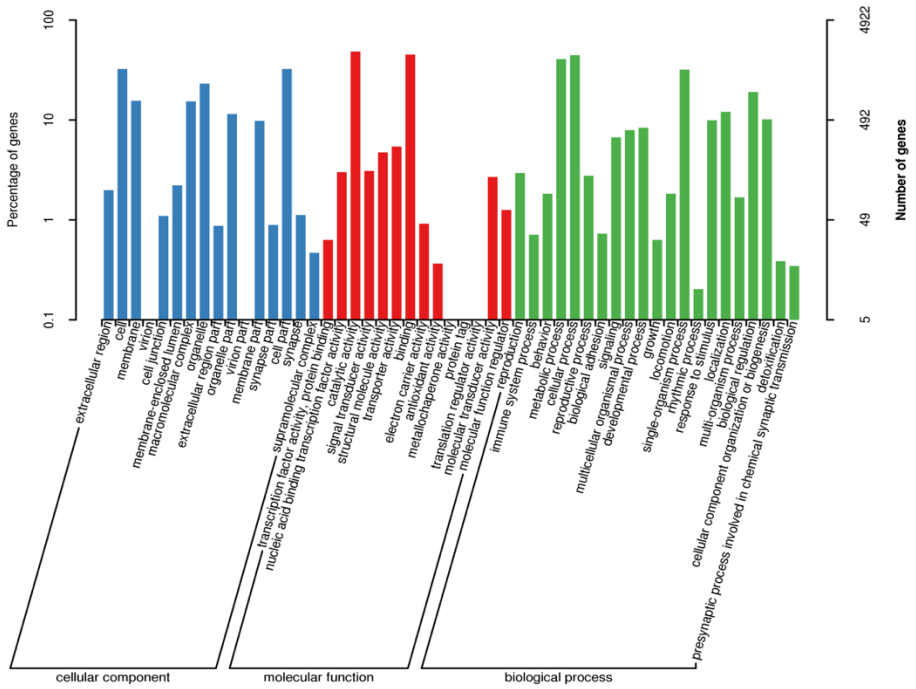

Figure. 3-7 KEGG annotation result

Table 3-6 genome annotation

Annotation Database	Annotated_Number	Percentage
GO	4922	30.75
KEGG	5970	37.30
KOG	9292	58.05
TrEMBL	15786	98.63
nr	15405	96.25
All_Annotated	15902	99.35

III.2.9. Gene family identification and phylogenetic tree construction

We used the OrthoMCL program (Li et al., 2003) with an e-value threshold of 1e-5 to identify gene families based on the protein alignments of each gene from *S. miscanthi* and those of other insect species, which included *R. padi*, *D. noxia*, *Ac. pisum*, *M. persicae*, *Ap. glycines, M. cerasi*, *Rhopalosiphum maidis*, *Ap. gossypii*, *S.*

flava (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/003/268/045/GCF_003268045.1_YSA_version1/GCF_003268045.1_YSA_version1_genomic.fna.gz), *Apis mellifera* (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/003/254/395/GCF_003254395.2_Amel_HAv3.1/GCF_003254395.2_Amel_HAv3.1_genomic.fna.gz), *D. pulex* (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/187/875/GCA_000187875.1_V1.0/GCA_000187875.1_V1.0_genomic.fna.gz), *Drosophila melanogaster* (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/001/215/GCF_000001215.4_Release_6_plus_ISO1_MT/GCF_000001215.4_Release_6_plus_ISO1_MT_genomic.fna.gz) and *Tribolium castaneum* (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/335/GCF_000002335.3_Tcas5.2/GCF_000002335.3_Tcas5.2_genomic.fna.gz). A total of 14,722 genes were identified by clustering the homologous gene sequences from 10,918 gene families (Figure. 4). One hundred thirty-eight gene families were specific to *S. miscanthi*. Subsequently, we selected 2,605 single-copy orthogroups from the abovementioned species to reconstruct the phylogenetic relationships between *S. miscanthi* and other arthropod species. A phylogenetic tree was constructed with the maximum-likelihood method implemented in the PhyML package (Guindon et al., 2010). We used the MCMCTree program to estimate divergence times among species based on the approximate likelihood method (Yang & Rannala, 2006) and with molecular clock data for the divergence time of medaka from the TimeTree database (Hedges et al., 2015). According to the phylogenetic analysis, *S. miscanthi* clustered with *Ac. pisum*. The divergence time between *S. miscanthi* and its common ancestor shared with *Ac. pisum* was approximately 76.8-88.4 million years (Fig. 2-8).

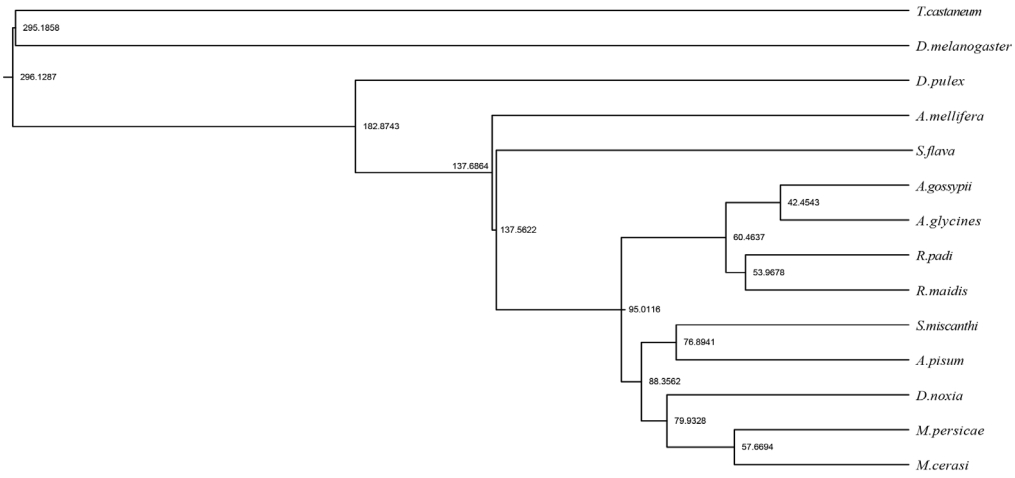

Figure. 3-8 The phylogenetic relationships of *S. miscanthi* with other arthropods.

III.3. Conclusions

1. We successfully assembled the chromosome-level genome of *S. miscanthi* based on long reads from the third-generation PacBio Sequel sequencing platform.
2. The size of the final draft genome assembly was ~397.90 Mb, which was slightly larger than the estimated genome size (393.12 Mb) based on k-mer analysis. The contigs were scaffolded
onto chromosomes using Hi-C data with a contig N50 of 2.05 Mb and a scaffold N50 of 36.26 Mb. We also predicted 16,006 protein-coding genes from the generated assembly, and 99.35% (15,902 genes) of all protein-coding genes were annotated.
3. We found that the divergence time between *S. miscanthi* and its common ancestor shared with *A. pisum* was ~76.8–88.4 million years.
4. The assembly of this genome will help promote research on the lifestyle and feeding specificity of aphids as well as their interactions with each other and other trophic levels and can serve as a resource for accelerating genome-assisted improvements in insecticide-resistant management as well as environmentally safe aphid management.

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4

Chapter IV Spatial expression analysis of odorant binding proteins in both sexes of the aphid parasitoid *Aphidius gifuensis* and their ligand binding properties

This work is an original contribution, adapted from:

Xin Jiang, Yaoguo Qin, Jun Jiang, Yun Xu, Frédéric Francis, Jia Fan, Julian Chen. Spatial Expression Analysis of Odorant Binding Proteins in Both Sexes of The Aphid Parasitoid *Aphidius gifuensis* and Their Ligand Binding Properties. Submitted in *Frontiers in physiology, 13:877133.*

Forward

Utilizing biocontrol agent to control aphides is an important strategy. *A. gifuensis* is one of the most common endoparasitoids of the green peach aphid *Myzus persicae* and grain aphid *S. miscanthi* in the field of China. Insect odorant-binding proteins (OBPs) play vital roles in odor perception during feeding, host searching, mating and oviposition. In addition, some OBPs are involved in other physiological processes such as gustation and reproduction. In the present study, a comparative antennal transcriptomic analysis was applied between male and female *A. gifuensis*. This part will lay solid foundation to clarify the potential odor binding protein genes and their expression characteristics.

Abstract

In China, *Aphidius gifuensis* is one of the most common endoparasitoids of the green peach aphid *Myzus persicae* and grain aphid *Sitobion miscanthi* in the field. Insect odorant-binding proteins (OBPs) play vital roles in odor perception during feeding, host searching, mating and oviposition. In addition, some OBPs are involved in other physiological processes such as gustation and reproduction. In the present study, a comparative antennal transcriptomic analysis was applied between male and female *A. gifuensis*. The spatial expression patterns among antennae, heads, thoraxes, abdomens and legs of OBPs in both sexes were further profiled. Fifteen *AgifOBP*s were predicted, and 14 of them were identified by gene cloning, including 12 classic OBPs and 2 minus-C OBPs. As expected, all OBPs were mainly expressed at high levels in antennae, heads or legs which are sensory organs and tissues. Finally, ligand binding properties of 2 OBPs (AgifOBP7 and AgifOBP9) were further evaluated. Female leg specifically expressed AgifOBP9 displays a broad and high binding property to aphid alarm pheromones, plant green volatiles and aphid sex pheromones (Ki <10 μM). However, female leg specifically expressed AgifOBP7 displays poor affinity for all tested ligands except CAU-II-11 ((E)-3,7-dimethylocta-2,6-dien-1-yl-2-hydroxy-3-methoxybenzoate), a reported (E)-β-farnesene (EBF) analog with an exceptionally high binding affinity (Ki = 1.07 ± 0.08 μM). In summary, we reported the spatial expression pattern of the OBP repertoire in *A. gifuensis*, and further studied the binding properties of OBP7 and OBP9, which are mainly expressed in female legs, laying the foundation for the dissection of the contribution of OBPs to chemosensation in *A. gifuensis*.

Key words: *Aphidifus gifuensis*, transcriptome, odorant-binding protein, spatial expression pattern, fluorescence binding assay.

IV.1. Introduction

Aphidius gifuensis is one of the most common endoparasitoids of green peach aphid *Myzus persicae* and grain aphid *Sitobion miscanthi* in China. *S. miscanthi* is also habitually called *Sitobion avenae* in China (Zhang et al., 1999; Jiang et al., 2019), and is the undisputed dominant Chinese dominant pest of wheat. Aphids has long been the

most damaging pest of crops and vegetables, causing yield and quality losses by stealing nutrients, transferring plant viruses, and excreting honeydew to block plant photosynthesis (Wu, 2002). In Yunnan and many other areas of China, *M. persicae* on tobacco has been successfully controlled by artificially released *A. gifuensis* as a powerful biocontrol tool (Ohta & Honda, 2010; Song et al. 2010).

The behavioral response of insects to olfactory cues is essentially driven by feeding, reproduction and habitat selection (Pelosi et al., 2014). Molecular odorants enter the sensilla through pores and spread inside the hemolymph on the antennae due to odorant-binding proteins (OBPs) and/or chemosensory proteins (CSPs) (Pelosi et al., 2006; Leal, 2013). These odorants are then transported to olfactory receptors (ORs), ionotropic receptors (IRs), or sensory neuron membrane proteins (SNMPs), from which the chemical signals will be transmitted into electrophysiological signals for the brain (Leal, 2013; Pelosi et al., 2018). Insect OBPs were initially discovered in antennae of the moth *Antheraea polyphemus* (Vogt & Riddiford, 1981). Their wide distributions in antennal sensilla indicated the first link of OBPs in the signal chain of odorant perception (Xu et al., 2009). OBPs are tiny, globular, water-soluble proteins with a molecular weight of 10-30 kDa (Pelosi et al., 2005). The presence of six highly conserved cysteine residues, which are paired in three interlocking disulfide bridges to maintain the protein's tertiary structure, is a common feature of classical OBPs (Pelosi et al., 2014). OBPs act as shuttles for hydrophobic odor molecules, transporting them through the sensillum lymph to odorant receptors (Zhou, 2010). After initiating receptors, OBPs may also concentrate odorants in the sensillum lymph and swiftly destroy odorant molecules (Vogt & Riddiford, 1981; Leal, 2013). The prediction of the whole OBP family in species became quite simple due to the availability of more insect genomes and transcriptomes using next-generation sequencing techniques. However, the number of OBPs in Hymenoptera varies; for example, *Apis mellifera* has 21 OBPs, *Microplitis mediator* has 18 OBPs, *Pieris rapae* has 14 OBPs, *Spodoptera exigua* has 34 OBPs, *Cotesia vestalis* has 20 OBPs, and 90 OBPs were predicted in *Nasonia vitripennis* (Foret & Maleszka, 2006; Peng et al., 2017; Li et al., 2020; Liu et al., 2015; Liu et al., 2020; Vieira et al., 2012). Insect OBPs not only are expressed in the chemosensory system, but also occur in nonsensory tissues and organs, such as the cornicles (Wang et al., 2021), thoraxes (Xue et al., 2016; Gao et al., 2018; Wang et al., 2019), reproductive organs (Li et al., 2008; Sun et al., 2012), mandibular glands (Iovinella et al., 2011), salivary glands (Zhang et al., 2017), and wings (Calvello et al., 2003; Pelosi et al., 2005; Wang et al., 2021). Some insect OBPs have physiological functions other than binding odorants. For example, the sperm carrier function of OBPs has been reported in the male reproductive apparatus of mosquitoes (Li et al., 2008). Moreover, one OBP expressed by male moths is found on the surface of fertilized eggs, which functions to avoid cannibalistic behaviors among larvae (Sun et al., 2012). Therefore, spatial expression patterns would be helpful to classify and analyze the possible functions of OBPs.

Herbivore-induced plant volatiles (HIPVs), green leaf volatiles (GLVs), and pheromones such as the aphid alarm pheromone E-beta-farnesene (EBF) are used by

natural enemies to find their prey during predation and parasitism (Song et al., 2010; Dong et al., 2008; CMD Moraes et al., 1998; Buitenhuis et al., 2004). *A. gifuensis* evolved a comprehensive chemosensory system to effectively detect the semiochemical cues of its host and plants (Song et al., 2010). For example, *A. gifuensis* can distinguish healthy, mechanically damaged, and aphid-infested plants (Dong et al., 2008). Additionally, both female and male *A. gifuensis* were reported to present a positive electroantennogram (EAG) response to EBF and many tobacco volatiles, including trans-2-hexenal, methyl salicylate, benzaldehyde, cis-3-hexen-1-ol, and 1-hexanal (Song et al., 2021). The volatile sex pheromone has also been shown to be released by female *Aphidius*, causing intense sexual orientation in males (Fan et al., 2018). OBPs, CSPs and chemosensory receptors in *A. gifuensis* have been widely predicted based ontranscriptome data (Kang et al., 2017; Fan et al., 2018). However, there is still a paucity of information on the expression profiles of odorant binding proteins in various sensory organs of *A. gifuensis.* Sequence identification is critical for further functional studies, not to mention the mechanisms of host foraging and mating behavior which are completely unknown.

In the present study, we performed gene prediction, identification, expression profiling of *AgifOBPs* and further performed a ligand competitive binding test on their recombinant proteins expressed in a prokaryotic expression system to discover two leg-specifically expressed OBPs (AgifOBP7 and AgifOBP9) in *A. gifuensis* as follows: 1) used *A. gifuensis* antennal transcriptome to predict *AgifOBPs*; 2) we identified *AgifOBPs* and profiled their spatial expression patterns among tissues and organs of both sexes; and 3) we revealed a partial mechanism of olfactory perception based on the ligand competitive binding test.

IV.2. Materials and methods

IV.2.1. Insect rearing and tissue collection

The laboratory population of *Aphidius gifuensis* was the same as that previously described by Fan (Fan et al., 2018). The mummies were collected and placed separately in petri dishes (3.5 cm in diameter). Newly emerged (within 0-12 h) *Aphidius* were transferred to larger petri dishes (9 cm in diameter and 2 cm in height) for another 24 h, and the two groups were divided by sex. Cotton balls dipped in a 25% aqueous solution of sucrose were constantly supplied as the diet for adult wasps. Approximately 500 pairs of antennae from each sex were collected for RNA sequencing. In total, for each replication of qRT-PCR, 100 antennae, 50 heads, 50 thorax, 50 abdomens, and 300 legs were collected. Three replicates were conducted for sampling. The dissected tissues were immediately frozen in liquid nitrogen and stored at -80 ℃.

IV.2.2. Total RNA extraction and synthesis of the first chain of cDNA

Total RNA was extracted using TRIzol reagent and combined with micro total RNA extraction kit (Tianmo, Beijing, China) following the manufacturer's instructions. The frozen tissues were homogenized with a liquid nitrogen cooled mortar and ground with a pestle into very fine dust. Homogenized tissues were treated with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA degradation and contamination were monitored on 2% agarose gels. RNA purity was checked using a Nanodrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). The RNA concentration was measured using a spectrophotometer RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Individual total RNA was isolated and cDNA was synthesized using the TRUEscript RT kit (LanY Science & Technology, Beijing, China) following the manufacturer's protocol.

IV.2.3. Transcriptome sequencing, assembly and functional annotation

A total of 3 μg of RNA sample with standard quality ratios ($1.8 < OD260/280 < 2.1$) was purified using poly-T oligo-attached magnetic beads. Divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×) were used for fragmentation. Single-stranded (ss) cDNA was synthesized using a random hexamer primer, M-MuLV Reverse Transcriptase and DNA Polymerase I and RNase H (NEB, USA). The 3' ends of the DNA fragments were adenylated and the NEBNext Adaptor was ligated to the fragments for hybridization. The library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, MA, USA) to size select cDNA fragments ~ 150-200 bp in length. Then, 3 μL of USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C prior to PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. The products were purified (AMPure XP system), and library quality was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, China) according to the manufacturer's instructions. The library preparations were sequenced on an Illumina HiSeq 2500 platform and paired-end reads (the sequencing strategy was PE125) were generated after cluster generation. After sequencing, the raw reads were processed to remove low quality and adaptor sequences by ng_qc, and then assembled into unigenes using Trinity r20140413p1 min_kmer_cov:2 and other default parameters (Grabherr et al., 2011). Then the unigenes were annotated using seven databases, including the nonredundant protein sequence (Nr, e-value = $1e^{-5}$), nonredundant nucleotide (Nt, e-value = $1e^{-5}$), Pfam (e-value = 0.01), Clusters of Orthologous Groups (KOG/COG, e-value = $1e^{-3}$), Swiss-Prot (e-value = $1e^{-5}$), Kyoto Encyclopedia of Genes and Genomes (KEGG, e-value = $1e^{-10}$) and Gene Ontology (GO, e-value = $1e^{-6}$) databases.

IV.2.4. OBP gene prediction and identification

The available sequences of *OBPs* from Hymenoptera species were used as "query" sequences to identify candidate unigenes that code OBPs in the *A. gifuensis* antennal transcriptome with the TBLASTn program with an e-value threshold of 10^{-5}. The sequences that fit the criteria were considered candidate OBPs. The open reading frames were searched by ORF finder (http://www.ncbi.nlm.nih.gov.orffinder/). The putative N-terminal signal peptides were predicted using the SignalP V4.1 program (http://www.cbs.dtu.dk/services/SignalP-4.1/) following the default parameters. Alignments of amino acid sequences were performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and edited using DNAMAN (Lynnon Biosoft San Ramon, CA, USA) software. According to the DEG results, the mean FPKM values for each gene in the antennae of males and females were then log-transformed ["$\log_2$ (FPKM + 1)"]. A heat map was generated using TBtools (Chen et al., 2020). A phylogenetic tree was constructed by MEGA11 using the maximum likelihood method with a LG+ mode to analyze the relationship of OBPs among species and reveal clues of their function (Tamura et al., 2021). Values indicated at the nodes are bootstrap values based on 1000 replicates presented with 95% cutoff. The orthologous protein sequences from the genomes and transcriptomes of the following Hymenoptera species were used in the analysis: *Apis mellifera* (Foret, S., & Maleszka, 2006); *M. mediator* (Zhang et al., 2009; Peng et al., 2017); *M. pulchricornis* (Sheng, et al., 2017) and *Aulacocentrum confusum* (Li et al., 2021). The amino acids of the sequences used are listed in Supplementary file 1. A circular phylogenetic tree was then generated and taxonomically color-coded using the online tool iTOL (https://itol.embl.de/itol.cgi). To identify the sequences of all candidate *AgifOBPs*, gene-specific primers (Table S1) were designed with Primer 5.0 (http://frodo.wi.mit.edu/primer5/). Polymerase chain reactions were conducted on an Eppendorf Mastercycler® gradient PCR machine using 2×TransStart FastPfu PCR SuperMix (Trans, Beijing, China) and antennal cDNA as a template. An initial denaturation step at 95 °C for 5 min followed by 35 cycles of 95 °C for 35 s, 58 °C as a melting temperature for 35 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide to ensure that the correct products were amplified. All targeted PCR products were purified using the AxyPrep PCR clean up Kit (CORING, Jiangshu, China), and then cloned into the pEASY Blunt clone vector (Trans, Beijing, China). After transformation of Escherichia coli DH5α competent cells with the ligation products, positive colonies were selected by PCR using the plasmid primers M13 F and M13 R and sequenced at San bo Biotech (Beijing, China). Individual clones confirmed to contain the desired sequence were incubated in LB/ampicillin medium.

IV.2.5. Spatial expression pattern of AgifOBPs

To explore the expression characteristics of the *AgifOBPs*, RT-qPCR with an ABI 7500 real-time PCR system (Applied Biosystems Fosters City CA, USA) was

conducted with cDNAs prepared from each tissue of male and female *Aphidius*. Briefly, 0.6 μL of both forward primer (10 μmol/L) and reverse primer (10 μmol/L) (Table S2) were used in a 20-μL reaction containing 10 μL of 2x SuperReal PreMix Plus, 2 μL of cDNA (from 250 ng of total RNA), 0.4 μL of 50x ROX reference dye, and 6.4 μL of ribonuclease-free ddH_2O following the instructions provided with the SuperReal PreMix Plus (SYBR Green) kit (FP205) (Tiangen, Beijing, China). The PCR program was as follows: initial 15-min step at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 32 s and elongation at 72 °C for 1 min and finally a 10-min step at 72 °C. For melting curve analysis, a dissociation step cycle was added automatically. The amplification efficiency was calculated using the equation: E= [10^(-1/slope)-1] ×100%, in which the slope was derived by plotting the cycle threshold (Ct) value against five 2-fold serial dilutions. Only primers with 95-105% amplification efficiencies were used for subsequent data analysis. Relative quantification was performed according to the $2^{-\Delta\Delta Ct}$ method **(**Livak K J, Schmittgen T D, 2001). *β-Actin* and (nicotinamide adenine dinucleotide) *NADH* were used as reference genes to normalize the data. All qRT-PCR analyses were performed in three technical and biological replications.

IV.2.6. Prokaryotic expression and purification of AgifOBP7 and AgifOBP9

The prokaryotic expression and purification procedures were consistent with previous studies (Prestwich, 1993; Wang et al., 2021). Gene-specific primers were designed to clone the full-length cDNAs encoding mature AgifOBP proteins. The PCR products were first cloned into the pEASY-T1 clone vector (TransGen Biotech, Beijing, China), and then excised and subcloned into the bacterial expression vector pET28a (+) (Novagen, Madison, WI) between the Nde I and EcoR I restriction sites, and reconstructed plasmids were verified by sequencing. The recombinant AgifOBP7 and AgifOBP9 in the present study contain no histidine-tagged peptide at the N-terminus.

Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM when the culture reached an OD_{600} value of 0.6. Cells were incubated for an additional 12 h at 28 °C and then harvested by centrifugation and sonicated at a low temperature (ice-water mixture). After centrifugation, the bands obtained were checked by 15% SDS-PAGE for their correspondence to the predicted molecular masses of the proteins. They were solubilized according to protocols for the effective rebuilding of the recombinant OBPs in their active forms (Prestwich, 1993). The soluble proteins were then purified by anion-exchange chromatography with RESOURCE Q15 HP column (GE HEALTH CARE, USA) and gel filtration (Superdex 75 10/300 GL column (GE HEALTH CARE, USA)), The crude extracts were passed over a pre-equilibrated RESOURCE Q15 HP column (20 mM Tris-HCl, pH 8.5), and then washed and eluted with Buffer B (20 mM Tris-HCL, 1 M NaCl, pH 8.5). And finally with two rounds of gel filtration through a Superdex 75 10/300 GL column, those eluted proteins were

collected and analyzed by 15% SDS-PAGE, and then, several successive dialyses were performed: (i) at 4 °C for 3 h, against 2 L of storage buffer (20 mM Tris-HCl, pH 8.5), (ii) at 4 °C for 3 h, against 2 L storage buffer (20 mM Tris-HCl, pH 8.5), and (iii) at 4 °C against 2 L of storage buffer (20 mM Tris-HCl, pH 8.5) overnight. Finally, the desalted protein samples were ultracentrifuged for 30 min using 3-kDa ultrafiltration at 4 °C, and 5000 rpm. Protein samples were analyzed by SDS-PAGE after every purification step, the concentration of purified protein was determined by a Protein Assay kit (Qubit™ Protein Assay kit, Q33211, Invitrogen), and the purified AgifOBPs were analyzed by mass spectrometry (LC-MS). The purity and concentration of the soluble proteins were evaluated using SDS–PAGE. Finally, stock solutions of AgifOBP7 and AgifOBP9 were collected and kept at -20 ℃ in Tris-HCl (50 mM, pH 7.4).

IV.2.7. Fluorescence competitive binding assays

To investigate the ligand-binding property of two AgifOBPs, five groups of competitive ligands were used: (i) aphid alarm pheromone components, including EBF, (-)-α-pinene, (-)-β-pinene and (+)-limonene which are released by other aphids following natural enemy predation or physical damage (Francis et al., 2005; Song et al., 2021), (ii) main components of the aphid sex pheromone: (4aSR,7SR,7aRS)-Nepetalactone; (iii) green leaf volatiles of wheat: (Z)-3-hexen-1-ol; (iv) aphid-induced plant volatiles(methyl salicylate, and 6-methyl-5-hepten-2-one); and (V) an EBF derivative artificial chemical, namely CAU-II-11, ((E)-3,7-dimethylocta-2,6-dien-1-yl-2-hydroxy-3-methoxybenzoate), which showed a high affinity for aphid EBF-binding proteins (OBP3/7/9, Qin et al., 2020), and was used to investigate the binding properties of purified AgifOBP7 and AgifOBP9. The classes, CAS numbers and purity of the chemicals used in this study are listed in Table 1.

Fluorescence intensity was recorded in a right angle configuration on a Lengguang 970CRT spectrofluorimeter (Shanghai Jingmi, China) at room temperature using a 1 cm light path fluorimeter quartz cuvette. A slit width of 10 nm was selected for both excitation and emission. The measured fluorescence intensities were corrected for both blank signals due to protein emission and scattered excitation light. The spectral data were processed using the software 970CRT 2.0l. Fluorescence binding experiments were conducted in 50 μM Tris-HCl buffer, pH 7.4, at room temperature. The binding affinity for N-phenyl-1-naphthylamine (1-NPN) was determined by adding aliquots of a 1 mM stock solution of 1-NPN dissolved in spectrophotometric grade methanol into a 2 μM protein sample. The fluorescence of 1-NPN was excited at 337 nm, and emission was recorded between 350 and 500 nm. Spectra were recorded with a high-speed scan. All ligands used in competitive experiments were dissolved in spectrophotometric grade methanol. In competition assays, aliquots of the competing ligands were added into a 2 μM protein solution in the presence of a given concentration of 1-NPN. To estimate the binding affinities of each AgifOBP for a variety of different ligands, we monitored the decrease in 1-NPN fluorescence due

to the ability of different odorants to displace 1-NPN and determined the Ki value for each compound. To determine the dissociation constants, the intensity values corresponding to the maximum fluorescence emissions were plotted against the cumulative 1-NPN concentration. The amount of bound ligand was calculated from the fluorescence intensity values by assuming that the protein was 100% active, with a stoichiometry of 1:1 protein: ligand at saturation. The curves were linearized using Scatchard plots. The value of $K_{1\text{-NPN}}$ was estimated on a direct plot by nonlinear regression with an equation corresponding to a single binding site using Prism 7 (GraphPad Software, Inc., USA), and the IC_{50} was defined as the concentration of a competitor that caused a 50% reduction in fluorescence intensity. The dissociation constants of the inhibitors (Ki) were calculated according to the formula $Ki = [IC50]/(1+[1\text{-NPN}]/K_{1\text{-NPN}})$, in which [1-NPN] represents the free 1-NPN concentration and $K_{1\text{-NPN}}$ represents the dissociation constant for AgifOBPs/1-NPN (Ban et al., 2003; Zhong et al., 2012; Sun et al., 2016; Fan et al., 2017; Qin et al., 2020; Wang et al., 2021). All fluorescence competitive binding assays were performed in three independent replicates, and Ki dates are present as means ± SD.

IV.2.8. Statistical analyses

For qRT-PCR analyses, the differences between means of biological replicates were tested using two-way ANOVA followed by multiple comparisons tests regardless of rows and columns using GraphPad Prism version 7.0.0 for Windows (GraphPad, Software, San Diego, California USA, www.graphpad.com). Differences between means for experiments with more than two treatments were distinguished using Tukey's honestly significant difference (HSD) test at the $P < 0.05$ significance level.

IV.3. Results

IV.3.1. Overview of transcriptomes

A total of 2.22 and 2.30 million raw reads were obtained from *A. gifuensis* antennae libraries from females and males, respectively. After removal of low-quality, adaptor, and contaminating sequences, 3.31 and 3.03 million clean reads were retained (Table 3-1) and assembled into 81235 distinct transcripts (mean length = 661 bp) and 65854 unigenes (mean length = 568 bp). The length distribution presented in (Figure 3-1). In total, 18,408 (27.95% of all 65854 unigenes), 5,625 (8.54%), 7,551(40.92%), 12,484(18.95%), 15,070 (22.88%), 15,951 (24.22%) and 9,462 (14.36%) transcripts from *A. gifuensis* antennae were annotated using the Nr, Nt, KO, Swiss-Prot, Pfam, GO and KOG databases respectively (Table 3-2). The most abundant GO terms were biological process terms, with *AgifOBP3* corresponding to the cellular process and *AgifOBP15* grouped with the membrane. The cluster for cellular process was the second largest group. Most transcripts that corresponded to molecular function were related to binding and catalytic activity (Figure 3-2). In the KOG classification, unigenes clustered into 26 categories (Figure 3-3). Among these categories, general function prediction was the dominant category, followed by signal transduction and

posttranslational modification, protein turnover and chaperon. All the unigenes annotated in the KO database were assigned to the 5 biological pathways described in the KEGG database: cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems (Figure 3-4). The most common pathway was metabolism followed by genetic information processing, organismal systems and cellular processes. Signal transduction was involved in 940 genes in the environmental information processing group.

Table 4-1 List of RNA-seq sequencing output data quality

Sample	Raw Reads	Clean reads	Clean bases	Error (%)	Q20(%)	Q30(%)	GC (%)
AgifantF_1	27586216	26482992	3.31G	0.03	96.17	92.90	34.19
AgifantF_2	27586216	26482992	3.31G	0.03	94.85	90.83	34.23
AgifantM_1	25116646	24271556	3.03G	0.03	95.27	91.73	31.02
AgifantM_2	25116646	24271556	3.03G	0.04	93.33	88.64	31.04

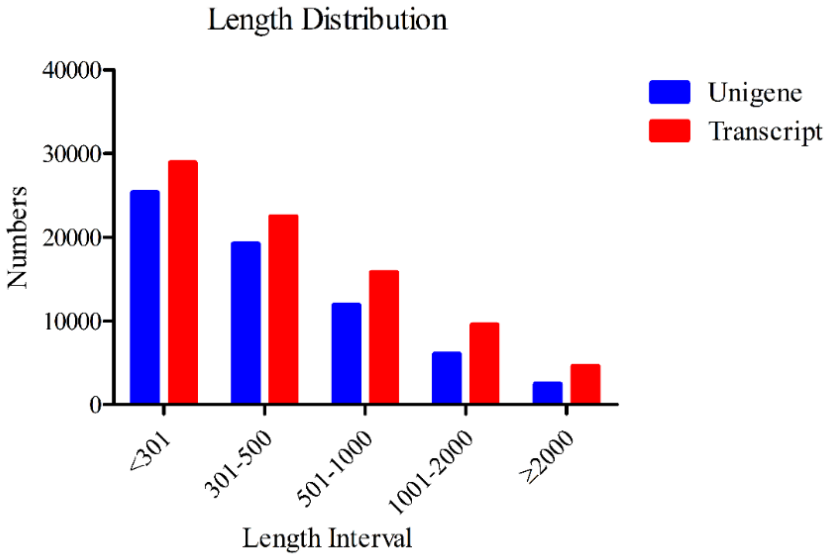

Figure. 4-1 Splice length distribution of unigene and transcript.

The abscissa is the length interval of the spliced transcript/unigene, and the ordinate is the number of times the spliced transcript/unigene of each length appears.

Table 4-2 Gene annotation success rate statistics

	Number of Unigenes	Percentage (%)
Annotated in NR	18408	27.95
Annotated in NT	5625	8.54
Annotated in KO	7551	11.46
Annotated in SwissProt	12484	18.95
Annotated in PFAM	15070	22.88
Annotated in GO	15951	24.22
Annotated in KOG	9462	14.36
Annotated in all Databases	2752	4.17
Annotated in at least one Database	22311	33.87
Total Unigenes	65854	100

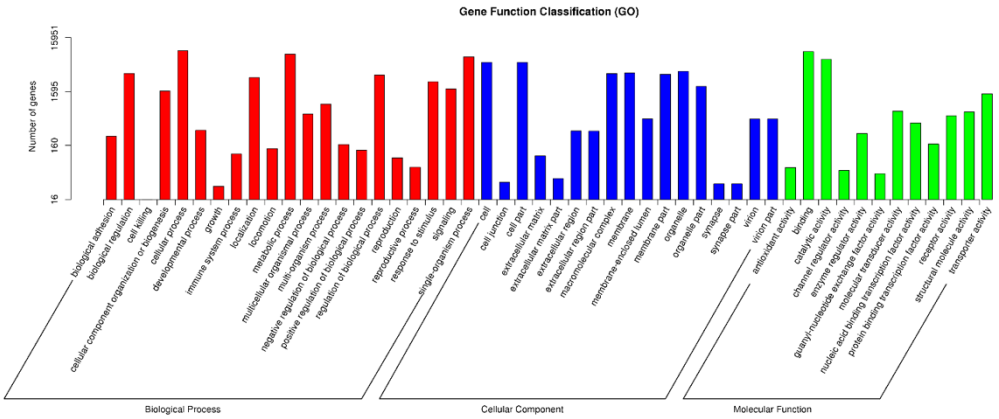

Figure. 4-2 gene function classification.

The abscissa is the next GO term of the three major categories of GO, and the ordinate is the number of genes annotated under the term (including subterms of the term). Three different classifications represent the three basic classifications of GO term (from left to right are biological processes, cellular components, and molecular functions)

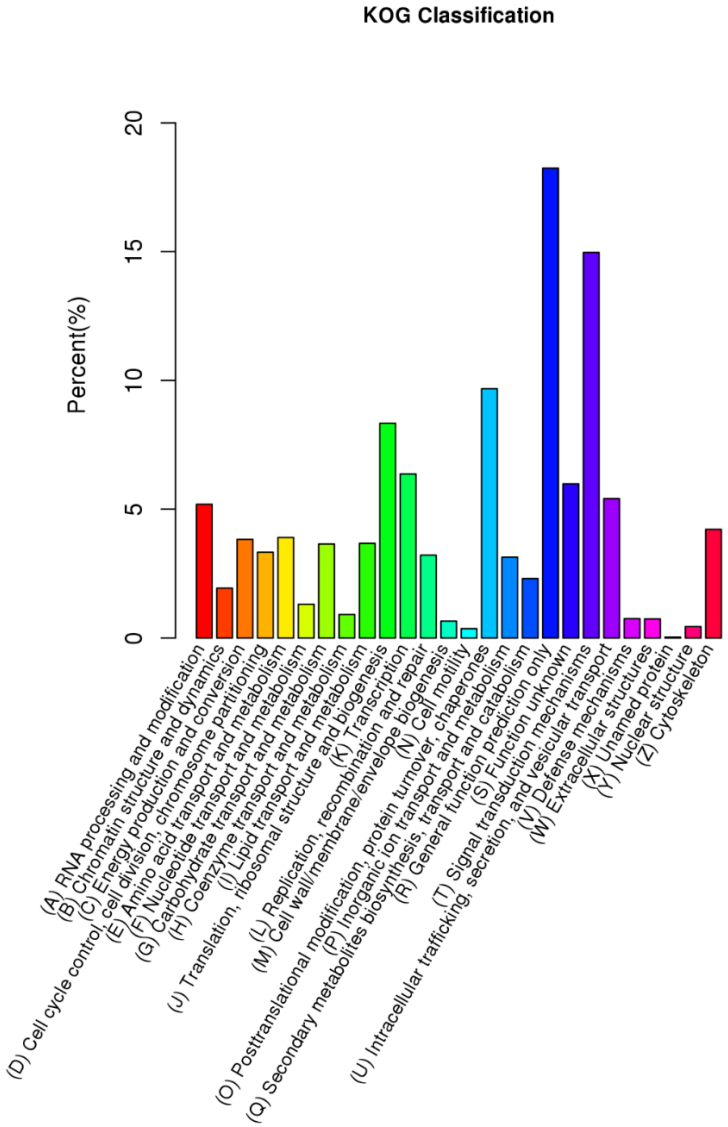

Figure. 4-3 KOG classification.

The abscissa is the names of 26 groups of KOG, and the ordinate is the ratio of the number of genes annotated to this group to the total number of genes annotated.

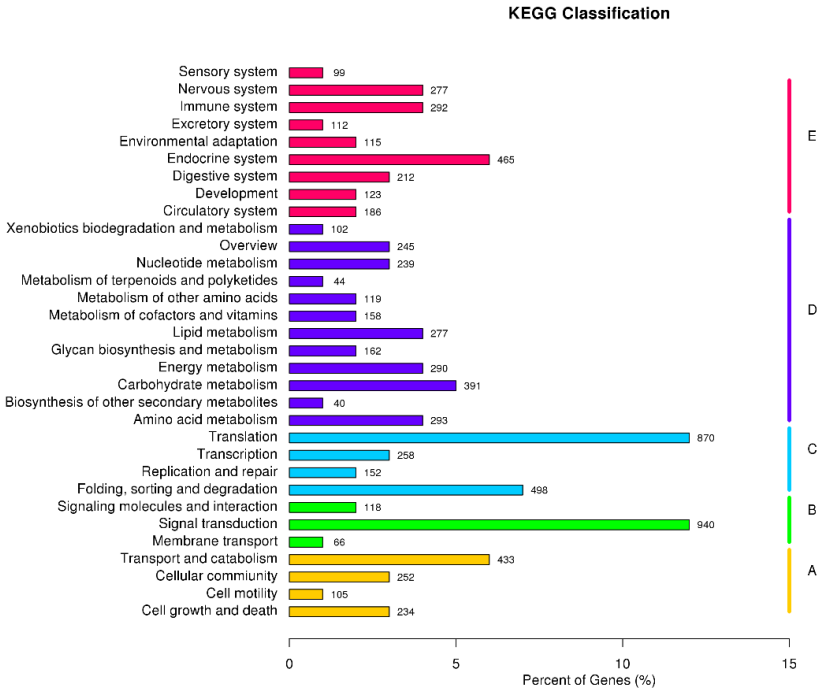

Figure. 4-4 KEGG classification.

The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the number of genes annotated under the pathway and their proportion to the total number of genes annotated. Divide genes into five branches according to the KEGG metabolic pathway involved: cellular process (A, Cellular Processes), Environmental Information Processing (B, Environmental Information Processing), Genetic Information Processing (C, Genetic Information Processing), metabolism (D, Metabolism), organic systems (E, Organicismal Systems).

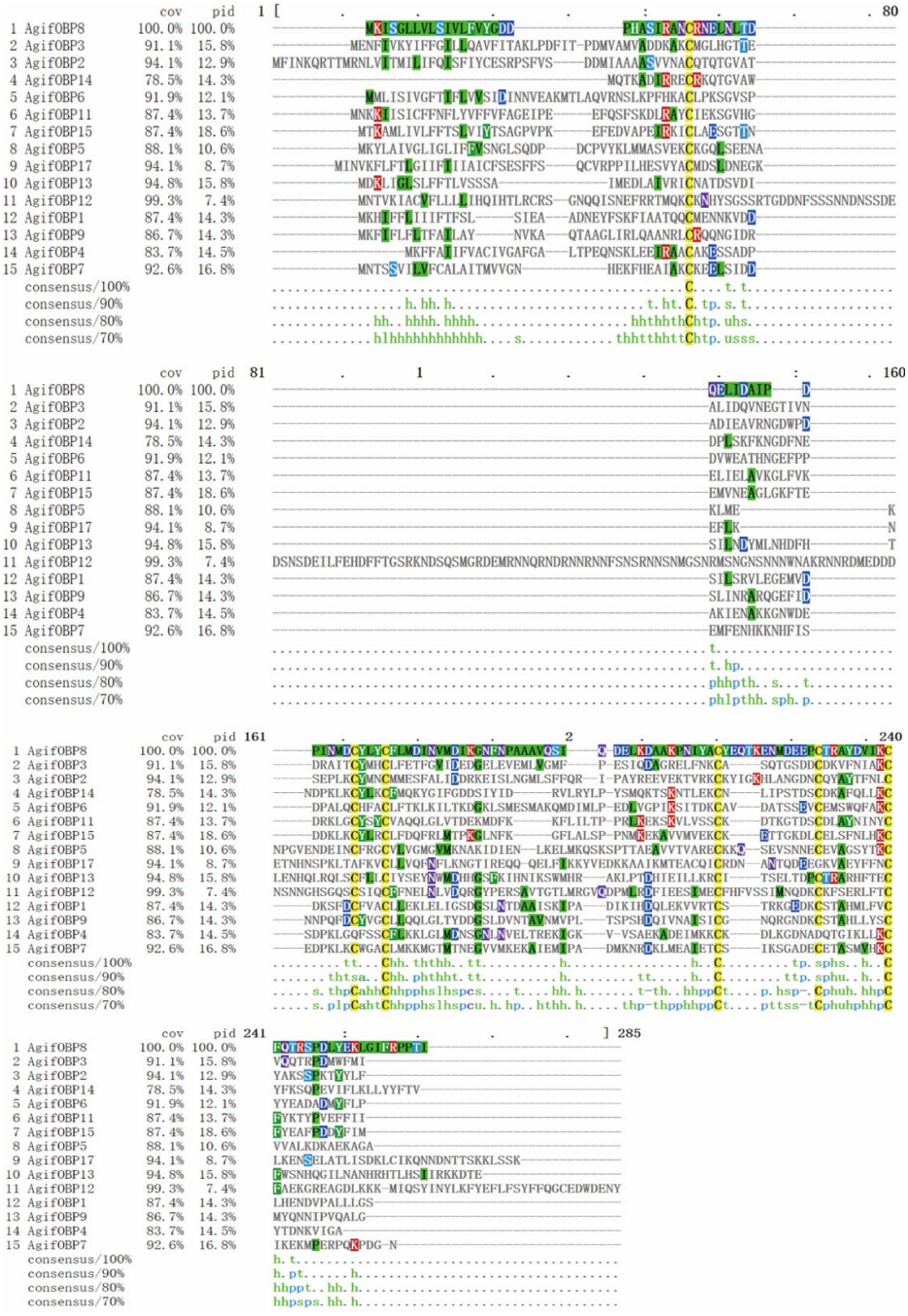

Figure. 4-5 Multiple sequence alignment of 15 odorant-binding protein (OBP) genes in *Aphidifus gifuensis*.

The conserved cysteine residues are highlighted by red boxes. The conserved Cys residues are indicated, representing conserved sequence identity >75%.

IV.3.2. OBP prediction and phylogenetic analysis

Fifteen putative OBPs with complete open reading frames were predicted from the antennal transcriptome data. We mainly named them following Fan's work (Fan et al., 2018). *AgifOBP10* with a partial ORF reported by Fan is missing here. All OBP transcripts were confirmed by molecular cloning, followed by sequencing, except for *AgifOBP14.* All 15 OBPs have the characteristic of insect OBP sequence motif (Yuan et al., 2015), and 13 *AgifO*BPs (*AgifOBP1-3,5-9,11,13,14,15*) of them have the classic OBP Cys motif (C_1-X_{22-32}-C_2-X_3-C_3-X_{36-46}-C_4-X_{8-14}-C_5-X_8-C_6) (Xu et al., 2009), while 2 *AgifOB*Ps (*AgifOBP4/17*) belong to the minus-C OBP Cys motif with four or five conserved cysteines (Figure 3-5). The heatmap in Figure.3-6 illustrates that OBP5, OBP6, OBP11, and OBP15 were highly expressed genes in in both sex antennae but OBP3/14/17 showed relatively low expression levels. The phylogenetic tree of Hymenoptera OBPs was built using MEGA11 (maximum likelihood method with an LG model) OBP sequences from 5 different species (*A. gifuensis*, *A. mellifera*, *M. mediator*, *M. pulchricornis* and *A. confusum*). *A. gifuensis* OBPs are clustered together to form three homologous subgroups (lineages). Among them, OBP1, OBP5, OBP7, OBP9 andOBP17 were in one subgroup, OBP2, OBP3, OBP6, OBP8 and OBP11-OBP15 were in the other subgroup, and OBP4 fell into the third subgroup. The results showed that AgifOBP*s* almost spread across in clades without species specificity (Figure 3-7). Among these AgifOBPs, AgifOBP4 was found in the MpulOBP4 clade. AgifOBP6 exhibited a rather high similarity to other orthologs such as AmelOBP6, MmedOBP6 and MpulOBP6. AgifOBP8 also showed a high similarity to MmedOBP8 and MpulOBP8 (Figure 3-7).

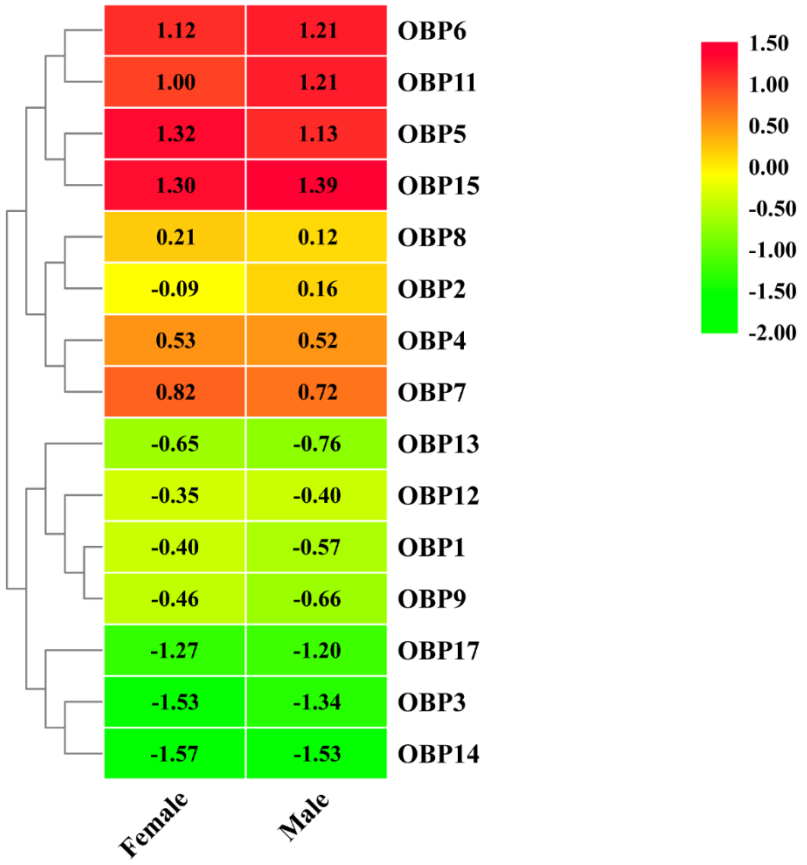

Figure. 4-6 Heatmap of differentially expressed OBP genes between females and males based on FPKM values of antennae transcriptomes.

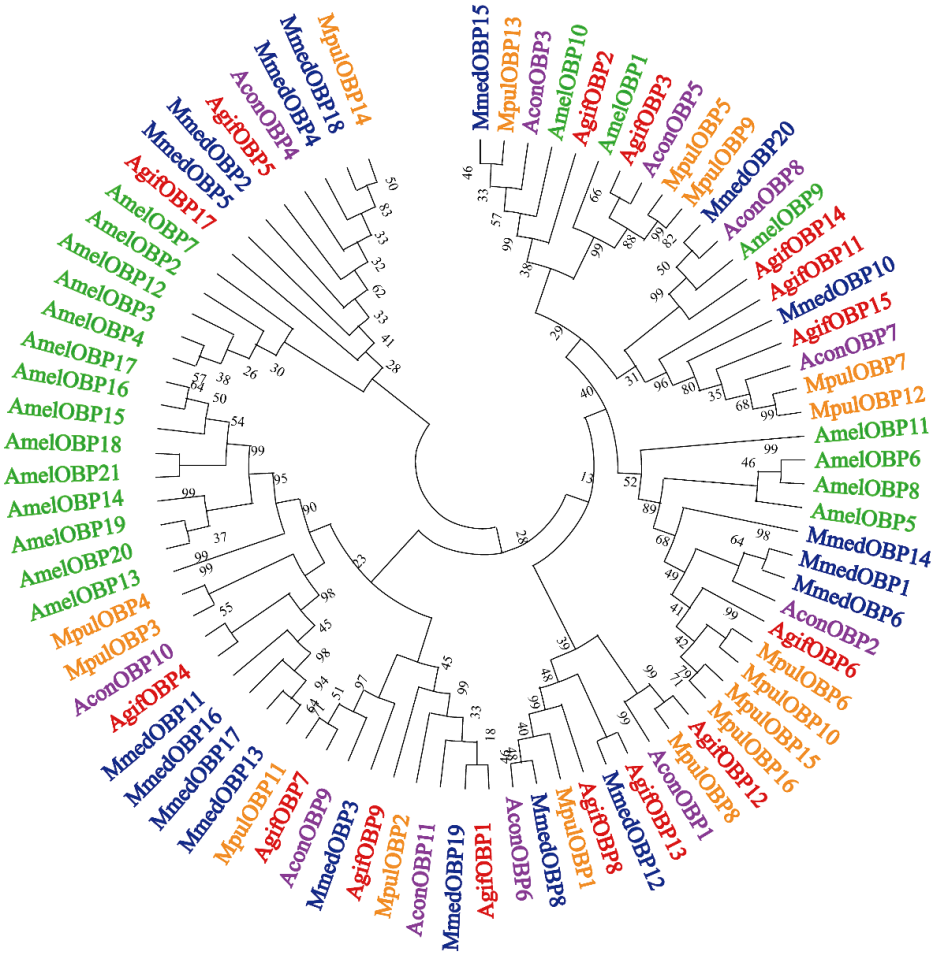

Figure. 4-7 Phylogenetic relationships of target parasitoid putative OBPs and 66 putative other hymenopteran OBPs

Detailed relationships of the putative *AgifOBPs* (in red), *MmedOBPs* (in blue), *AmelOBPs* (in green), *MpulOBPs* (in orange), and *AconOBPs* (in purple). The trees were constructed with MEGA 11 using an LG+ model and bootstrap support was calculated with 1000 rapid bootstrap replicates with a 95% cutoff.

IV.3.3. Spatial expression pattern of AgifOBPs

Compared to other tissues or organs, 8 of the 14 OBPs, namely, *AgifOBP3, AgifOBP5, AgifOBP6, AgifOBP7, AgifOBP8, AgifOBP11, AgifOBP12* and *AgifOBP15,* maintained higher expression in antennae (Figure 3-8, Figure 3-9; $p<0.05$). *AgifOBP17* was highly expressed in the head. *AgifOBP1/2/7/9* were expressed in legs with significantly higher expression levels (Figure 3-8, Figure 3-9, $p<0.05$). The other two *OBPs*, *AgifOBP4* and *AgifOBP13* were widely expressed among tissues and organs.

Specifically, *AgifOBP3/5/6/11/12/15* were specifically expressed in antennae. Among them, the expression levels of AgifOBP3/11 were even higher in male antennae. However, *AgifOBP12/15* were even higher in female antennae, and *AgifOBP6* showed no difference in antennae of both sexes. Moreover, *AgifOBP1/2/4/5/7/9/15* showed relatively higher expression levels in legs (Figure 3-8/9). Among them, *AgifOBP2* was specifically expressed in female legs. And *AgifOBP7/9/15* were expressed at comparatively higher levels in female legs. In contrast, *AgifOBP1/4/5* were expressed at higher levels in male legs. Notably *AgifOBP7* was female specific and was expressed directly in female antennae and legs. In males, *AigfOBP8* was specifically expressed in antennae. In females, it was relatively highly expressed in both the antennae and abdomen. We also found that the highest level of *AgifOBP17* was in the heads of both sexes. Although both *AgifOBP4* and *AgifOBP13* were widely expressed, *AgifOBP4* showed an even higher expression level in thoraxes of both females and males. *AgifOBP13* expression was significantly higher in the male abdomen. In addition, *AgifOBP1* and *AgifOBP9* were specifically or highly expressed in the legs of both male and female *A. gifuensis*. *AgifOBP2* was significantly expressed in the legs of females (Figure 3-9, $p<0.05$).

In summary, *AgifOBP3/5/6/11/12/15* were antennal specifically expressed OBPs. *AgifOBP2/9* were specifically expressed OBPs in legs. *AgifOBP17* is an OBP specifically in the head (Figure 3-8, $p<0.05$). In addition, *AgifOBP5/7* were female specific OBPs. *AgifOBP8* expression was significantly higher in the antennae of males and in both the antennae and abdomen of females (Figure 3-9, $p<0.05$).

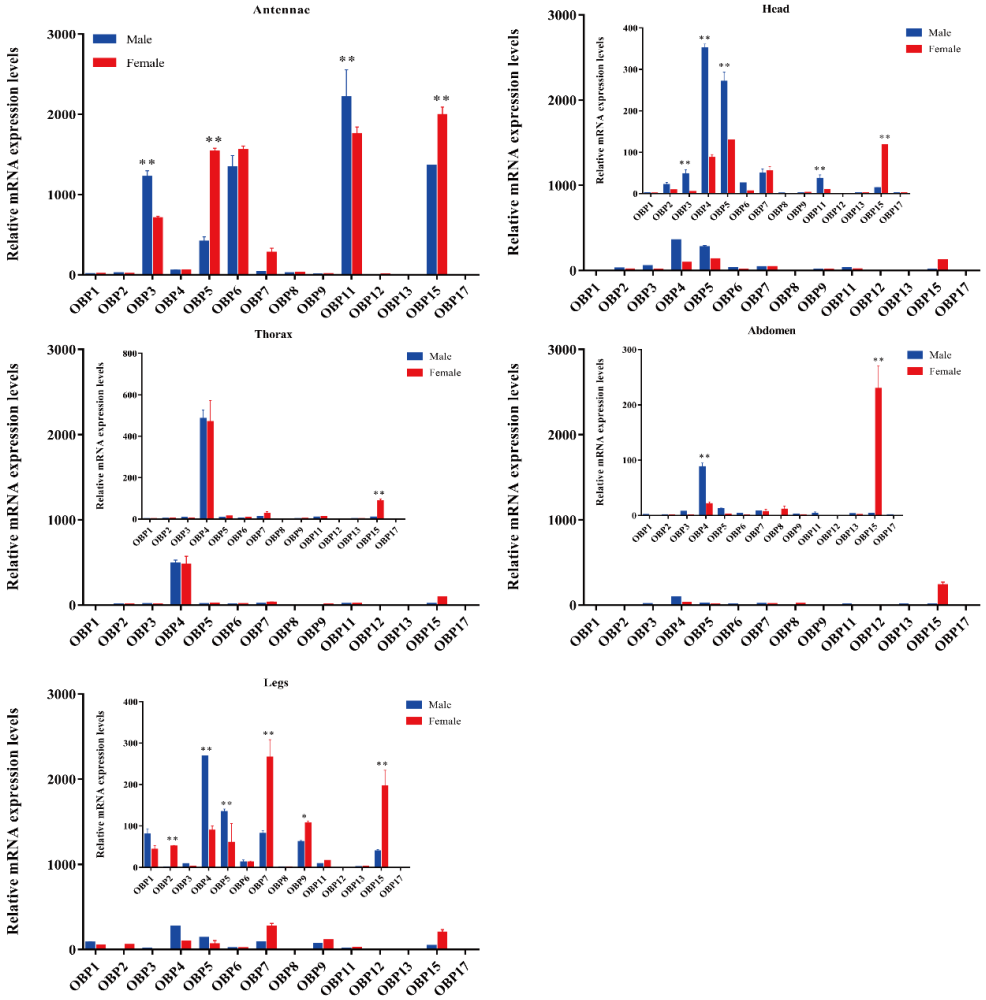

Figure. 4-8 The relative expression patterns of different AgifOBP genes in different tissues of males and females as measured by quantitative real-time polymerase chain reaction.

The fold changes are relative to the transcript levels of OBP13 in the male thorax. The NADH and ACTIN genes were used as references to normalize the expression of each tested gene. The data are presented as the mean ± SD. The asterisk * and ** above the bars indicate significant differences at $P<0.05$; and $P<0.01$, respectively, according to two-way ANOVA.

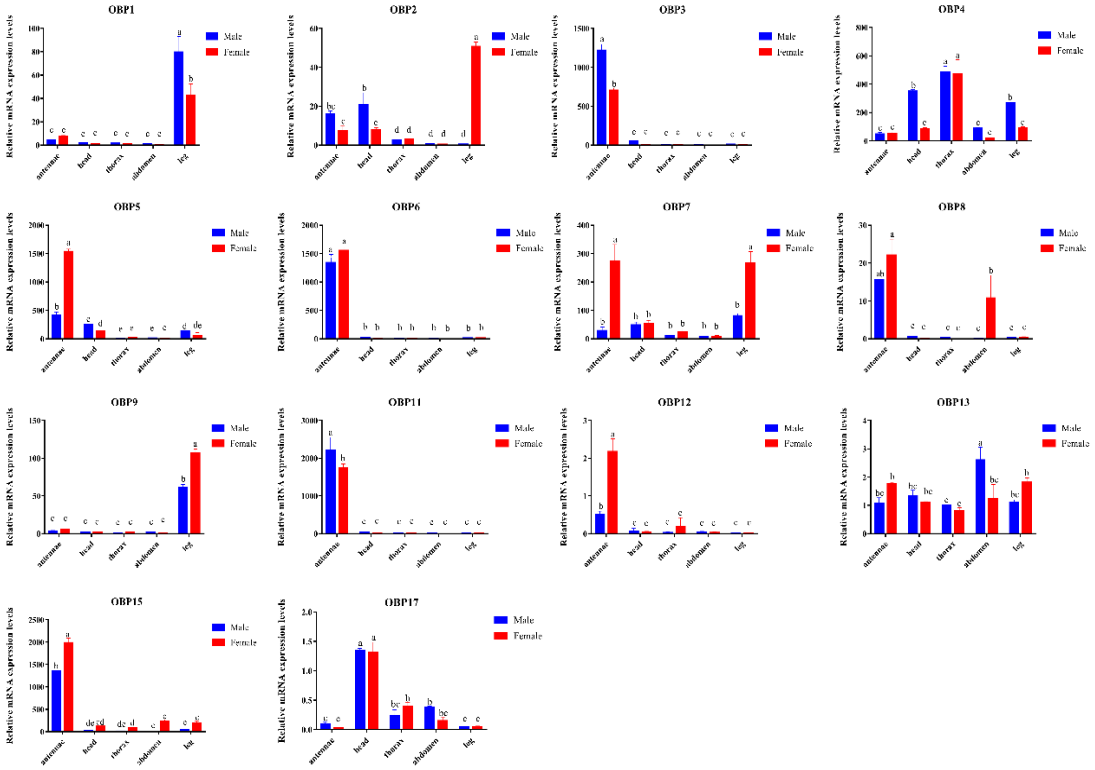

Figure. 4-9 Relative expression of AgifOBP genes in the different tissues of male and female *A. gifuensis* as measured by quantitative real-time polymerase chain reaction.

Relative fold changes were normalized to the transcript levels in the male thorax. The NADH and ACTIN genes were used as references to normalize the expression of each tested gene. The standard error is represented by the error bar (n=3) and the different lowercase letters (a, b, c, d, e) indicate significant differences in transcript abundances (two-way ANOVA followed by Tukey's HSD multiple comparison test, $P < 0.05$).

IV.3.4. Expression and purification of AgifOBP7 and AgifOBP9

AgifOBP7 and AgifOBP9 were successfully expressed in the inclusion bodies using a bacterial system. After a dissolving and refolding treatment, the refolded AgifOBP7 and AgifOBP9 were purified with yields of 0.25 mg/mL as soluble proteins (Figure 3-10A, Figure 3-10B). More than 15 mg of purified AgifOBP7 and AgifOBP9 was obtained using RESOURCE Q15 affinity columns, with the His-tag removed. The theoretical molecular weight values for AgifOBP7 and AgifOBP9 were very close to the measured values (AgifOBP7, 13.401 kDa; AgifOBP9, 12.498 kDa). The purified protein samples were further identified by LC-MS/MS (data not shown).

IV.3.5. Fluorescence competitive binding assays

To investigate the role of two OBPs in the odor perception of aphids, we chose alarm pheromones (EBF, (-)-α-pinene, (-)-β-pinene, (+)-limonene, EBF derivative (CAU-II-11), aphid sexual pheromones (4aSR 7SR 7aRS)-nepetalactone as well as volatiles of wheat green leaf (cis-3-hexen-1-ol) and aphid induced plant main volatiles (methyl salicylate, 6-methyl-5-hepten-2-one) as the candidate ligands for fluorescence competitive binding assays (Table 2-3). We first tested the binding affinities of both OBPs to the fluorescent probe N-phenyl-1-naphthylamine (1-NPN) as previously reported (Qiao et al., 2009; Fan et al., 2017). The dissociation constants of AgifOBP7/1-NPN and the AgifOBP9/1-NPN complex were 1.69±0.27 μM and 0.69±0.24 μM respectively (Figure 3-10C, Figure 3-10D).

In a subsequent experiment, we used a fluorescence competitive binding assay to determine the binding affinities of AgifOBP7 and AgifOBP9 to different odorants. Based on the binding curves, we calculated the median inhibitory concentration (IC50) and dissociation constant (Ki) values (Table 2-3). Among the tested odorants, EBF, (-)-α-pinene, (-)-β-pinene, (+)-limonene, (4aSR 7SR 7aRS)-nepetalactone; cis-3-hexen-1-ol, methyl salicylate and 6-methyl-5-hepten-2-one displayed relatively high binding affinities (Ki < 10 μM) to AgifOBP9 (Figure 3-10F). Interestingly, among all the tested odorants, CAU-II-11 bound most strongly (Ki = 1.07±0.08 μM) to AgifOBP9 (Table 2-3), which is the derivative of EBF (Ki = 4.60±0.43 μM). However, this was not the case with AgifOBP7, which only displayed weak binding with EBF (Ki = 20.30 ± 1.99) and (4aSR 7SR 7aRS)-nepetalactone (Ki = 16.12 ± 3.49), not much with (-)-α-pinene, (-)-β-pinene, (+)-limonene, cis-3-hexen-1-ol, methyl salicylate and 6-methyl-5-hepten-2-one. CAU-II-11, like AgifOBP9, showed the strongest binding affinity (Ki = 1.07±0.08 μM) to AgifOBP7 among the examined odorants (Figure 3-10E, Table 2-3).

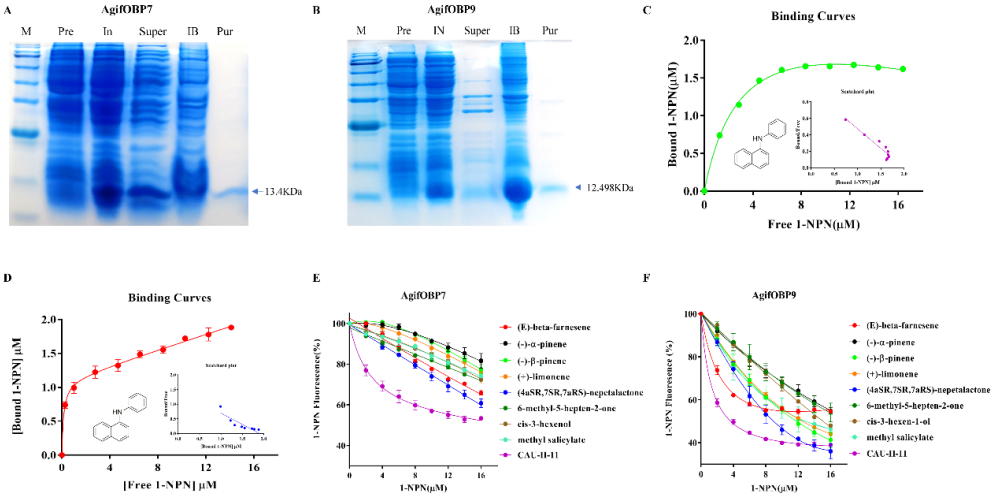

Figure. 4-10 Expression and binding properties of AgifOBP7 and AgifOBP9 with candidate ligands.

A: Expression and purification of AgifOBP7, B: Expression and purification of AgifOBP9. Line M: molecular weight PR1910 (11-180 KDa) Marker, 11, 17, 25, 35, 48, 63, 75, 100, 135, 180 KDa; IN: Induced pET-28a (+) / AgifOBP7/9; Super: pET-28a (+) / AgifOBP7/9 Supernatant; IB: pET-28a (+) / AgifOBP7/9 Inclusion body; Pur: Purified Purified pET-28a (+) / AgifOBP7/9 without His-tag. C, D: Binding curves of AgifOBP7 and AgifOBP9 with N-phenyl-1-naphthylamine (1-NPN) in 50 mM Tris-HCl buffer (pH 7.4). E, F: Competitive binding curves of AgifOBP7 and AgifOBP9 to components of aphid alarm pheromones ((E)-beta-farnesene, (-)-α-pinene, (-)-β-pinene, (+)-limonene, (E)-beta-farnesene derivative (CAU-II-11); aphid sexual pheromone (4aSR 7SR 7aRS)-nepetalactone, the volatiles of wheat green leaf (cis-3-hexen-1-ol) and aphid induced plant main volatiles (methyl salicylate, 6-methyl-5-hepten-2-one). A mixture of the recombinant protein and N-phenyl-1-naphthylamine (1-NPN) in 50 mM Tris-HCl buffer (pH 7.4) at the concentration of 2 μM was titrated with 1 mM solutions of each competing ligand to a final concentration range of 2 μM to 16 μM. Fluorescence values are presented as percent of the values in the absence of competitor. Date are the means ± SD of three independent experiments.

Table 4-3 Binding affinities of AgifOBP9 for candidate ligands, evaluated in displacement binding assays using the fluorescent probe, 1-NPN.

No.	Code	CAS	Purity	OBP7		OBP9	
				IC50	Ki(μM)	IC50	Ki(μM)
1	(E)-β-Farnesene	18794-84-8	≥85%	44.35±4.34	20.30±1.99	17.83±1.69	4.60±0.43
2	(-)-α-Pinene	80-56-8	≥95%	> 30	> 30	24.10±3.42	6.22±0.88
3	(-)-β-Pinene	19902-08-0	≥99%	> 30	> 30	11.86±1.27	3.06±0.33
4	(+)-Limonene	138-86-3	≥95%	> 30	> 30	12.85±0.25	3.32±0.06
5	Nepetalactone	21651-62-7	≥80%	> 30	16.12±3.49	9.03±0.31	2.33±0.08
6	6-Methyl-5-hepten-2-one	110-93-0	≥99%	> 30	> 30	22.32±3.38	5.76±0.87
7	cis-3-Hexenol	928-96-1	≥97%	> 30	> 30	17.01±0.33	4.39±0.09
8	Methyl salicylate	119-36-8	≥99%	> 30	> 30	13.31±2.99	3.43±0.77
9	CAU-II-11	-	≥98%	18.56±1.73	8.50±0.73	4.15±0.33	1.07±0.08

IV.4. Discussion

Odorant-binding proteins are classically defined as olfactory soluble proteins (Vogt, R. G., & Riddiford, L. M, 1981; Pelosi, 2006) and play an essential role in habitat searching and finding suitable mates. With the increase in insect genome projects and transcriptome sequencing projects, large numbers of OBPs have recently been identified in different insect species. In the present study, we constructed a cDNA library from the antennae of the endoparasitoid *A. gifuensis* for transcriptome sequencing and categorized the potential function of the odorant binding protein genes by bioinformatics approaches.

IV.4.1. OBP prediction, cloning and phylogenetic analysis

Fourteen OBPs in the *A. gifuensis* antennae transcriptome were identified in the present study. This number is similar to those in *A. mellifera* (Foret, S., & Maleszka, 2006), *M. mediator* (Zhang et al., 2009; Peng et al., 2017), *Meteorus pulchricornis* (Sheng et al., 2017), *Cotesia vestalis* (Zhou et al., 2021) and *Aulacocentrum confusum* (Li et al., 2021), therefore indicating that there are similar OBP numbers in

Hymenoptera insects.

The phylogenetic tree of these *AgifOBPs*, together with OBPs from 4 hymenopteran species, showed that the *AgifOBPs* segregate into the orthologous clades of the other species, rather than into *A.gifuensis* paralogous clades. *AgifOBP4* was found in the *MpulOBP4* clade, whereas *AconOBP4*, *MmedOBP4*, and *AmelOBP4* were clustered in the other one clade. *AgifOBP6* and *AgifOBP8* were present in the three wasps of *A. gifuensis*, *M. mediator* and *M. pulchricornis,* but their orthologs were rarely found in *Apis mellifera* (Figure 3-7). This also suggests that these *AgifOBPs* might play different roles in odor recognition or have roles other than olfaction. The comparatively conserved OBPs in hymenoptera wasps, particularly in parasitoid wasps implied that their function could be limited to the common olfactory physiology of these insects. Some study results on natural enemies of aphids support this hypothesis. For example, aphid OBP7 orthologs have been widely reported to have their affinities with the alarm pheromone EBF (Sun et al., 2012; Zhong et al., 2012; Fan et al., 2017; Qin et al., 2020). *CpalOBP10* in lacewing *Chrysopa pallens*, an aphid predator, belongs to the same lineage as aphid OBP7 such as in *S. avenae* and in *A. pisum,* and its affinity for EBF was also consistent with that of aphid OBP7 orthologs (Li et al., 2017; Li et al., 2019).

IV.4.2. Spatial expression pattern

The spatial expression profile of *AgifOBPs* was verified using qPCR. Our data revealed that five *OBPs*, namely *AgifOBP3*, *AgifOBP5*, *AgifOBP6*, *AgifOBP11*, and AgifOBP15, were expressed at a high level in the antennae (Figure 3-8/9), while four *OBPs*, *AgifOBP2*, *AgifOBP4*, *AgifOBP7* and *AgifOBP8*, were expressed at a medium level, and seven *OBPs*, *AgifOBP1*, *AgifOBP9*, *AgifOBP12/13*, and *AgifOBP17*, were expressed at alow level in the antennae (Figure 3-8/9). The antennal specific *OBPs* (Figure 3-9) suggest their function of recognizing and binding odorants from the environment. Six *OBPs*, *AgifOBP2, AgifOBP4, AgifOBP5, AgifOBP7, AgifOBP13* and *AgifOBP17,* showed expression patterns among sensory and nonsensory organs, indicating their possible multiple functions in olfactory perception as well as other physiological processes such as development and reproduction. Both *AgifOBP1* and *AgifOBP9* showed higher expression levels in the legs than the other four tissues (antennae, heads, thorax and abdomen), which could be related to the adaptation of *A. gifuensis* during migration as we have discussed in our previous study (Xue et al., 2016), and might be involved in the procedure of taste or volatile perception or be related to olfactory sensilla on the legs (Yasukawa et al., 2010; Harada et al., 2012). A similar condition was also found for *AgifOBP5*, which is expressed in small amounts in the head and leg, in addition to being expressed abundantly in antennae.

Apart from antennae, alternatively, these OBPs expressed in other tissues may be responsible for corresponding functions. For example, *NlugOBP3* is highly expressed in the abdomen of *Nilaparvata lugens* and may be involved in juvenile hormone transport and play an important role in metamorphosis (He et al., 2011). Insect OBPs

have been reported to act as carrier proteins in the male reproductive apparatus of mosquitoes (Li et al., 2008). After mating, the OBPs expressed by male moths are found on the surface of fertilized eggs, which helps the larvae to avoid cannibalistic behaviors (Sun et al., 2012). For parasitic wasps, *AconOBP8* was reported to be expressed predominantly in the abdomen (Li et al., 2021). Similar expression patterns of OBPs in the nonolfactory tissues were observed in *Sclerodermus sp*. (Zhou et al., 2015) and *M. pulchricornis* (Sheng et al., 2017). In our present study, qPCR analysis revealed that *AgifOBP8* was also expressed in the female abdomen, and it can be speculated that *OBP8* may potentially function as a pheromone-binding protein for identifying a particular signal such as the sex pheromone component in mating or oviposition behaviors, although the active component of sex pheromone in this species is still unclear.

The results obtained by qPCR are consistent with antennal transcriptome based differential expression analysis (heatmap, Figure 3-6). Nonetheless, any discrepancy between qPCR and differential expression analysis results illustrates the poor performance of showing local details by omics big data analysis.

IV4.3. Ligand-binding properties

Table 6 indicates that the proteins AgifOBP7 and AgifOBP9 have broad binding activities across the aphid alarm pheromone components, aphid sex pheromone, green leaf volatiles, aphid-induced plant volatiles and EBF derivatives. AgifOBP9 showed higher binding activities than AgifOBP7 with all the five types of compounds. Similar results have been found in its prey aphid *A. pisum*, in which ApisOBP9 also exhibited higher affinities with all the compounds than ApisOBP7 (Qin et al., 2020), although there is no evolutionary homology between the two species. For AgifOBP7 and AgifOBP9, EBF derivatives had higher binding properties than the lead EBF and other compounds. These results are consistent with studies on the characterized OBPs of ApisOBP1, ApisOBP3, and ApisOBP6-OBP10 in *A. pisum* (Sun et al., 2012; Qin et al., 2020). Both proteins show preferential binding to several related compounds. AgifOBP7 bound the above five types of compounds from strong to weak: EBF derivative, aphid sex pheromone main component, aphid alarm pheromone component EBF, and other alarm pheromone components, green leaf volatile and induced plant volatiles. AgifOBP9, bound the above five types of compounds from strong to weak: EBF derivative, aphid sex pheromone main component, aphid alarm pheromone component (-)-β-pinene and (+)-limonene, induced plant volatile methyl salicylate, green leaf volatile (Z)-3-hexen-1-ol, alarm pheromone component EBF, induced plant volatile 6-methyl-5-hepten-2-one and alarm pheromone component (-)-α-pinene. Our results suggest that there are substantial differences in their interactions such that AgifOBP7binds strongly to aphid pheromone components and derivatives and binds weakly to the others, which is similar to OBP7 in *S. avenea* (Zhong et al., 2012); and AgifOBP9 broadly binds to all kinds of compounds, which is likely OBP9 in *M. persicae* (Wang et al., 2021).

As the natural enemy of aphids, *A. gifuensis* locates aphids using the cues of aphid pheromones and plant volatiles (Powell et al., 1998; Kang et al., 2017). Our results indicate that AgifOBP7 is specific to aphid pheromone components and EBF derivatives, and that AgifOBP9 has a broad spectrum of binding to compounds. Other OBPs in aphid natural enemies also bound to aphid pheromone components and plant volatiles. For example, OBP3, OBP4, OBP6, OBP7, OBP9, and OBP10 in *Chrysopa pallens* bind plant volatiles and aphid alarm pheromone EBF, and, OBP10 specifically binds EBF (Li, et al., 2017; Li et al., 2019). The new OBPs from the aphid natural enemy *Eupeodes corollae*, OBP12, OBP15 and OBP16 also bound with EBF and plant volatiles, among which OBP12 and OBP15 strongly bound EBF (Wang et al., 2022).

Many natural enemies such as *Aphidius ervi*, *Aphidius uzbekistanicus*, and *Adalia bipunctata* are attracted to EBF **(**Buitenhuis et al., 2004**)**. To confirm the functions suggested by the phylogenetic tree and tissue expression profiles, AgifOBP7 and AgifOBP9 were selected to perform a potential functional study. Overall, the odorants exhibited relatively high binding affinities (Ki < 10 μM) to *AgifOBP9* (Figure 3-10F, Table 2-3). Interestingly, among all the tested odorants, CAU-II-11 had the strongest binding affinity (Ki = 1.07±0.08 μM) to AgifOBP9 (Table 2-3), which is the derivative of EBF (Ki =4.60±0.43 μM). This finding is in line with prior research on ApisOBP3/7/9 using CAU-II-11 (Qin et al., 2020). This result further supports that both aphid-induced volatiles as well as EBF are used by *A. gifuensis* in aphid location and that AgifOBP9 may be involved in this process.

In summary, we first predicted 15 *OBPs* based on the antennal transcriptome of both male and female *A. gifuensis*. Fourteen of these OBPs were verified by gene cloning. Furthermore, their detailed spatial expression pattern showed that most *OBPs* are mainly expressed in the sensory organs, but some are widely expressed in various tissues or organs such as the thorax and abdomen. Finally, at least one female particularly expressing OBP (AgifOBP9) showed affinity to EBF in a fluorescence competition experiment, which further indicated the likely molecular basement of sensing the aphid alarm pheromone at the molecular level in *A.gifuensis*. In addition, what cannot be ignored is the presence of *OBPs* expressed in other nonsensory organs such as the abdomen, which supports the existence of carrier transport functions other than for foreign chemicals and therefore broader ligand ranges of wasp *OBPs*. Our findings may shed insight into parasitic wasps' olfactory sensitivity to host hints, as olfactory organs recognize pheromones and odorant substances that influence both host hunting and oviposition activities and will help us better understand parasitic wasp host forging and mating behaviors, which will aid in the strengthening and better utilization of *A. gifuensis* as a powerful and natural biocontrol strategy. As a result, we anticipate that additional research into the aforementioned topics will improve the efficacy of parasitoid-based biological control approaches against aphid pests.

IV.5. Reference

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IV.6. Supplementary files

List of the protein names and amino acid sequences of OBPs between *A. gifuensis*, *Apis mellifera, Microplitis mediator, Meteorus pulchricornis, Aulacocentrum confusum* that used in the phylogenetic analysis.

>AgifOBP1
MKHIFFLIIIFTFSLSIEAADNEYFSKFIAATQQCMENNKVDDSILSRVLEGEM
VDDKSFDCFVACLLEKLELIGSDGSLNTDAAISKIPADIKIHDQLEKVVRTCST
RKGEDKCSTAHMLFVCLHENDVPALLLGS-
>AgifOBP2
MFINKQRTTMRNLVITMILIFQISFIYCESRPSFVSDDMIAAAAASVVNACQTQT
GVATADIEAVRNGDWPDSEPLKCYMNCMMESFALIDDRKEISLNGMLSFFQR
IPAYREEVEKTVRKCKYIGKHLANGDNCQYAYTFNLCYAKSSPKTYYLF-
>AgifOBP3
MENFIVKYIFFGILLQAVFITAKLPDFITPDMVAMVADDKAKCMGLHGTTEA
LIDQVNEGTIVNDRAITCYMHCLFETFGVIDEDGELEVEMLVGMFPESIQDA
GRELFNKCASQTGSDDCDKVFNIAKCVQQTRPDMWFMI-
>AgifOBP4
MKFFAIIFVACIVGAFGALTPEQNSKLEEIRAACAKESSADPAKIENAKKGNW
DESDPKLGQFSSCFLKKLGLMDNSGNLNVELTREKIGKVVSAEKADEIMKK
CKDLKGDNADQTGIKLLKCYTDNKVIGA-
>AgifOBP5

MKYLAIVGLIGLIFFVSNGLSQDPDCPVYKLMMASVEKCKGQLSEENAKLM
EKNPGVENDEINCFRGCVLVGMGVMKNAKIDIENLKELMKQSKSPTTAEAV
VTVARECKKQSEVSNNECEVAGSYTKCVVALKDKAEKAGA-
>AgifOBP6
MMLISIVGFTIFLVVSIDINNVEAKMTLAQVRNSLKPFHKACLPKSGVSPDV
WEATHNGEFPPDPALQCHFACLFTKLKILTKDGKLSMESMAKQMDIMLPED
LVGPIKSITDKCAVDATSSEVCEMSWQFAKCYYEADADMYFLP-
>AgifOBP7
MNTSSVILVFCALAITMVVGNHEKFHEAIAKCKEELSIDDEMFENHKKNHFI
SEDPKLKCWGACLMKKMGTMTNEGVVMKEKAIEMIPADMKNRDKLMEAI
ETCSIKSGADECETASMVHKCIKEKMPERPQKPDGN-
>AgifOBP8
MKISGLLVLSIVLFVYGDDPHASIRANCRNELNLTDQELIDAIPDPINMDCYL
YCFLMDINVMDIKGNFNPAAAVQSIQDELKDAAKPNIYACYEQTKENMDEE
PCTRAYDVIKCFQTRSPDLYEKLGIFRPPTI-
>AgifOBP9
MKFIFLFLTFAILAYNVKAQTAAGLIRLQAANRLCRQQNGIDRSLINRARQGE
FIDNNPQFDCYVGCLLQQLGLTYDDGSLDVNTAVNMVPLTSPSHDQIVNAISI
CGNQRGNDKCSTAHSLYSCMYQNNIPVQALG-
>AgifOBP11
MNKKIISICFFNFLYVFFVFAGEIPEEFQSFSKDLRAYCIEKSGVNGELIELAVK
GLFVKDRKLGCYSYCVAQQLGLVTDEKMDFKKFLILTPPRLKEKSKVLVSSC
KDTKGTDSCDLAYNINYCFYKTYPVEFFII-
>AgifOBP12
HMLRCRSGNQQISNEFRRTMQKCKNHYSGSSRTGDDNFSSSNNDNSSDEDS
NSDEILFEHDFFTGSRKNNSQSMGRDEMRNNQRNDRNNRNNFSNSRNNSN
MGSNRMSNGNSNNNWNAKRNNRDMEDDDNSNNGHSGQSCSIQCFFNELN
LVDQRGYPERSAVTGTLMRGVQDPMLRDFIEESIMECFHFVSSIMNQDKCKF
SERLFTCFAEKGREGCEDWDENY-
>AgifOBP13
MDKLIGLSLFFTLVSSSAIMEDLAIVRICNATDSVDISILNDYMLNHDFHTLEN
HQLRQLSCFLLCIYSEYNWMDHHGSFKIHNIKSWMHRAKLPTDHIEILLKRC
ITSELTDPCTRARHFTECFWSNHQGILNANHRHTLHSIIRKKDTE-
>AgifOBP14
MQTKADIRRECRKQTGVAWDPLSKFKNGDFNENDPKLKCYLKCFMQKYGI
FGDDSIYIDRVLRYLPYSMQKTSKNTLEKCNLIPSTDSCDKAFQLLKCYFKSQ
PEVIFLKLLYYFTV-
>AgifOBP15
MTKAMLIVLFFTSLVIYTSAGPVPKEFEDVAPEIRKICLAESGTTNEMVNEVG
LGKFTEDDKLKCYLRCLFDQFRLMTPKGLNFKGFLALSPPNMKEKAVIMVE
KCKETTGKDLCELSFNLHKCFYEAFPDDYFIM-
>AgifOBP17
MINVKFLFTLGIIFIIIAICFSESFFSQCVRPPILHESVYACMDSLDNEGKEFLKN

ETNHNSPKLTAFKVCLLVQFNFLKNGTIREQQQELFIKKYVEDKKAAIKMTE
ACQICRDNANAQNEEGKVAEYFFNCLKENSELATLISDKLCIKQNNDNTTSK
KLSSK-
>MmedOBP1
MKNIIIFTTAITIFTFINFSQTEARMTMTQIRNAMKPLGKTCLGKTGLSKEVQA
GQHNGEFPEDEALMCYHSCLLKLAKISDKSGNINLDTVHKQIDLMMPEDLI
ARAKAVTTDCFGEIKSTEICRMSFEFVKCYFIKGPEIVFFP-
>MmedOBP2
MKSIIFLGVLLTVLISNKAEAKSVQKRECPFKKPFEANAPKCMDKISEENMG
RMMQGNMDNDEIRCFVGCVFENAGFVKDNKVQMDKVREAVDNFVDDYK
YSKEVGDQVYGVVSDCAPQAEKGANNCEVSSNLLICFKTNNKFT-
>MmedOBP3
MRGSVLAIVACALVVGVLGDDDMKEKHKEIFKKCAEETGVTKEDLHNHKR
GEEPETKIKCFHACIAKADGAMVDGKLNKDKVIEKIPADLPDRERIIEAVTKC
SEQTAADECETAHLVFKCLRENKALPHPPHHHHHHHDE-
>MmedOBP4
MKCFTLTAAGILFTVLITVNNASSNSNMEELVKKSMEETFKACKDKLTPENF
ALLNKDPHADNQEIKCFKACGMNHAGIMADGKIQIEKMEEKLNSLLGEDK
KDFSKIIIGRAKPCVEEANKGENECDVAAGFEACVQKTINTKSDN-
>MmedOBP5
MKNFVVIVILALYFTATTESLQEIMNTFQKARLEVRAPCLHLLSNETLTTLKT
RRHLDNPEIRCFKACLMERQGYLKDNKIFIDEYEKLIDVNLKRIKELNMKFA
RACVNEAEKSENKCELAHNYNRCILHQTRKHYNQTAEENDENQNQHL-
>MmedOBP6
MKNTLFFTLAAAFLLGYNIPHVESRMSMAQTINTMKPLGKTCAAKTGLSKE
MQDGQHEGQFPEEEALMCYHTCLLKMAKVADKTGKLNIDAMVKQIDMLM
PEDLVDKAKTACSGCADEVTATEGCRPSWEFMKCWYGRAPELYFFP-
>MmedOBP8
MDSNIKYMCFLYIFIVVFMFFSEAIDQSDPHASTRKKCSGEFKLTDEILKLGE
QDPSDFSCYLFCLFKDINIMNQKGEFDPNLAAQEVQDNLREAARKYIFMCY
DLVKPNMTSDGCKNALEMVQCFKEKAPEMYEMLGLFHPPSNEPLKMTQ-
>MmedOBP10
MAKFLLSSVGVLVLIAYVQSGPVPEEFKDVQPTIRAACVKESGLTNEELVNK
AALGEFTDDPQLKCYLKCIFDQFRLVSKRGINFDAMLALSPPSMKENAIKMV
KECRDTKGKEGDLCDLSFEVTKCLYNSNPETYFIL-
>MmedOBP11
MKIFAVIFAICIVYAVAIGNLTEEERVELDRLANICVNETGFYEGHNSDDPAKN
WISYGFKLQCYFSCMLKKMNIMNEDGTLNEEMIRKKIGDEVPADKIDAVITK
CKDLKGANKCETATMIMKCYSDERLSLDPAEKSV-
>MmedOBP12
MAIVRICNTTDPVDLRVLNDYLMNHNLNRLHIKSHHPLACFLLCVYSEFNW
MDRHGGFKVHNIKAWMLRAELSENDTDILLRKCISLELTDPCTRAQYFTECF

WTNHQDVTVDHRHSLHSIMHKDVHQDKIYN-
>MmedOBP13
MKIIAVIFAVCFAGALAELTVEQLAKLREHSTACITETLVDDANVDAAMHHNI
WRMDDLKLRCYFFCLLKKLKVMNEDGKLNEEITRQRLANLFPADRIDGVIM
KCKEMKGADACETAILMAKCHADERGLLGPSPRSA-
>MmedOBP14
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HLVEGSVKAYTVCSRAAISEDPCEKAFQYAKCYYETDAPSYFYP-
>MmedOBP15
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>MmedOBP16
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CKDLSGGDVCETAMLMMKCYADEKALTKIITEKSS-
>MmedOBP17
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MMKCKDMKGADMCETAMMMMKCYADEKAFTKIITEKSS-
>MmedOBP18
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KAIGESMINGAKNCAGPAEEGENECEVAHRFITCLMEHAAEEKKKHNE-
>MmedOBP19
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>MmedOBP20
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FKAQPEVLKSVSFV-
>MpulOBP1
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DHLGCYLFCFLKDLEVMDDKGSFDPAAATDAVEEELREASRPEIYSCYESYS
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>MpulOBP2
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QRGDDKCETAHMLYQCMQEKNIPTTTLLG-
>MpulOBP3
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QWQEGDEKLQCFAACLLKKLGMMAEDGKLNEEVSLAKMTLDVGAEKARE
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>MpulOBP4
MKTIAVVFAVCIVGALGNLTEEHKAKLREHRDKCIRETKVDRTLVDKAHGG
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IWDNCKDKTGANTCAKGFELMKCYTSKKTLLLA-
>MpulOBP5
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QSTATNILGACAAQPGADNCEKMYNIGVCVQAKDPSMFFML-
>MpulOBP6
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>MpulOBP8
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DGDPRQSCIVQCIFEELNSVDQRGFPERASVTRMLLRGIQDPMVHDFIGESIL
QCFQFLSSEMNYDKCTFSQNLLNCLADKGKEQCEDWND-
>MpulOBP9
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STATNILGACAAQPGADNCEKMYNIGVCVQAKDPSMFFML-
>MpulOBP10
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DKIDAMLPEDLAQRVKENSKICFSKITSNDLCVMSWEFTKCFYELDSSLFFFP
-
>MpulOBP11
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>MpulOBP12
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CRDTTAKPDDMCDLSFNLHKCFYNSSPDQYFIM-

>MpulOBP13
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VSIALRECNGIGKYLAHGDNCTYAYTFNMCYAMLSPKTYYLF-
>MpulOBP14
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DETADNEEIRAFKACVMIHGGILEDQKIKIDKIQEMLKNHVEEEEMENVMTV
MKICQGEAEIGADDGEVATLFINCFKKATADSNAAK-
>MpulOBP15
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VEKTHEGEFPTDPALQCYFACILKLMKVVSKDEHIDLDMMHKQADLLMVQ
NLANQVKQLTQTCYENITSSEVCEMSWELVKCYHELDSSMYFFP-
>MpulOBP16
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GVSKDLIDKTHEGEFPPDPQLQCYYACIFKMMKVVTKDEQVDLNLILKQIN
MLALEELGKQITPIVQDCDAKITATEVCEVSWAFAKCLWETDQSMYFFP-
>AmelOBP2
MNTLVTVTCLLAALTVVRGIDQDTVVAKYMEYLMPDIMPCADELHISEDIAT
NIQAAKNGADMSQLGCLKACVMKRIEMLKGTELYVEPVYKMIEVVHAGNA
DDIQLVKGIANECIENAKGETDECNIGNKYTDCYIEKLFS
>AmelOBP1
MASNTKQAFIYSLALLCLHAIFVNAAPDWVPPEVFDLVAEDKARCMSEHGT
TQAQIDDVDKGNLVNEPSITCYMYCLLEAFSLVDDEANVDEDIMLGLLPDQ
LQERAQSVMGKCLPTSGSDNCNKIYNLAKCVQESAPDVWFVI
>AmelOBP6
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LLDGQFRGEFPQDERLMCYMKCIMIATKAMKNDVILWDFFVKNARMILLEE
YIPRVESVVETCKKEVTSTEGCEVAWQFGKCIYENDKELYLAP
>AmelOBP5
MHVKSVLLLITIVTFVALKPVKSMSADQVEKLAKNMRKSCLQKIAITEELVD
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>AmelOBP14
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VEDKKVQLYCECILKNFNILDKNNVFKPQGIKAVMELLIDENSVKQLVSDCS
TISEENPHLKASKLVQCVSKYKTMKSVDFL
>AmelOBP4
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GCFEACVFQKLHFMDGNTLNVEKLESGTRELTPDDFTEDVHEIIEQCVSKAA
DEDECMVARKYIDCALEKMKFLDDELEKIAGN
>AmelOBP11
MKAAEIWLVSLYWYLILQIALVYGEISDIDEFREMTSKYRKKCIGETKTTIED
VEATEYGEFPEDEKLKCYFNCVLEKFNVMDKKNGKIRYNLLKKVIPEAFKEI

GVEMIDSCSNVDSSDKCEKSFMFMKCMYEVNPIAFIAP
>AmelOBP9
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DQNLKCYLKCFMTKHGILDKNAEVDVQKALRHLPRSMQDSTKKLFNKCKS
IQNEDPCEKAYQLVKCYVEFHPEVLQTVPFL
>AmelOBP12
MLYNNLTIVIILIMCGVQNLRARSVNIFQDIADCVDRSNMTFHELKKLRDSSE
ARIKLINEEENFRNYGCFLACIWQQTGVMNGSELSTYNIAGIIEGQYHDDED
LKTFFHKIALTCEDDVHRKFLHVNDECDVALSFKLCMLKAMRNYP
>AmelOBP13
MKTIIFIFAFCLVGILAVSEESINKLRKIESVCAEENGIDLKKADDVKKGIFDK
NDEKLACYVDCMLKKVGFVNADTTFNEEKFRERTTKLDSEQVNRLVNNCK
DITESNSCKKSSKLLQCFIDNNLMKIFE
>AmelOBP7
MKKFLVIFVYILSVAVIIRANGINEILKIMAVSMKDIRYCIIHMGLTFKDFIKMQ
ELLQEEDISEGNIKKYLTNYSCFITCALEKSHIIQNDEIQLDKLVEMANRKNISI
DVKMLSECINANKSTDKCENGLNFIICFSKLLSDMYEDTFEDTLKHKSYV
>AmelOBP21
MKTIVIISAICVCVGALTLEELQIGLRAVIPVCRIDSGIDEKKEDDFRNGIIDVE
NEKVQLFSECLIKKFNAYDDGGNFNEVVVREIAEIYLDENEVNKLITECSAIS
DADIHLKSSKLIKCFAKYKTLKEIMNE
>AmelOBP20
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VEDEKPQRYNECILKQFNIVDESGNFKENIVQELTSIYLDENVIKKLVAECSVI
SDANIYIRFNKLVKCFGKYKTMKEVLNL
>AmelOBP19
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VEDEKPQRYNECILKQFNIVDESGNFKENIVQELTSIYLDENVIKKLVAECSVI
SDANIYIRFNKLVKCFGKYKTMKEVLNL
>AmelOBP18
MKTFVIISAICVCVGALTLEEFQIGLRAVVPICRIETSIDQQKEDDFRDGNIDVE
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ADLAVKSAKLLKCIGKYKTLKEMLSG
>AmelOBP16
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CSPISDANVHIKISKIFQCFMKYKTITDILNS
>AmelOBP17
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MDDENVLLFIECTMKKFNVVDENANFNEKISSDIVRAVLNDNEADQLLAEC
SPISDPNALIKISKILECFFKYKTINQILNS
>AmelOBP15

MKTILIISAICICVGALSIKDFQNAIRMGQSICMAKTGINKQIINDVNDGKINIE
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TNVHLKITKIFQCITKFKTINDILNS
>AmelOBP3
MKTIVILLFTLCIVSYMMVRCDDITLCLKQENLNLDDIDSLLEDESERMLRK
RGCIEACLFHRLALMNDNVFDVSKFDVYLNDTDMDMDLKDSIRKIIRQCVD
NAKNEDKCLTAQKFSRCVIDYVKFHITQYMISNANSNTTSEEESSDNST
>AmelOBP8
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ATKAMKNDVILWDFFVKNARMILLEEYIPRVESVVETCKKEVTSTEGCEVA
WQFGKCIYENDKELYLAP
>AmelOBP10
MKYSILLSLLITCLICSPSVHCGTRPSFVSDEMIATAASVVNACQTQTGVATV
DIEAVRNGQWPETRQLKCYMYCLWEQFGLVDDKRELSLNGMLTFFQRIPAY
RAEVQKAISECKGIAKGDNCEYAYRFNKCYAELSPRTYYLF
>AconOBP1
MNSINVFCLFVLLFRQIDALQCRSGNEQTSDELRKIMEKCNHRQTDGKHDD
NSSVDYSSDNSSEEMMFSKDFFTNNKKKTENVTKNSGSTSGIDSSDHYKRQF
SNRPYDINYSNYSGLQQTTNTKNSNDDTAKISCNIHCFFDELNLVDQRGFPER
ISVTKSMIKNIHYSELRDFIEESILECFQFLSNDPNQDKCEYSQNLVNCFADKG
KEGCEDWDE
>AconOBP2
MNHHKSINISSIFIAIVIIAAVNIFNIEARMTMAQIQNAMKPAGKTCAGKTGVA
KEVLAQTREGIFPEDRDLMCYHACLLKMMKMMTKDNKIAIETMMKQIDLM
MPEDLIQRTKDVSQKCYDELTTDEPCEMSWQFVKCYSDTDRSLYFFP
>AconOBP3
MQNILRFLCLVQVVLVACGSRPSFVSDEMIAGAASVVNACQTQTGVATTDIE
AVRNGEWPDSRSLKCYMYCLWAQFGLVDNNRELSLNGMLTFFQRMPAYRA
EVDRAIRECKGLSKYYANGDDCQYAYTFNQCYAEHSPQTYYLF
>AconOBP4
MNSFTVASLIVLVTLCAGFLPTHAEEEDDDNPLKALAKESIEHCKDKLSDEN
KELLKKNRKADNREIRCFQACVLNHVGALKDTKFDMDKIKQVVNDNVDEV
ERDDWIAAINVCREDGEKETDECDVAGAFVQCFNNYEESDEE
>AconOBP5
MSRIIVNCVLLGIILQAGLVPAKRPDFVTDEMVAMIKDDKDRCMQEHGTTEA
LIERVNDGDIPNDKAITCYMYCLFESFSVIDEDGVLEADMLTGFFPEDIQAKG
GPILSACASQDGADNCEKVYNIAKCVHSKMPEMWFMV
>AconOBP6
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FGCYLSCFLQNLNIMDDKGVFDPAVATQSVAADLREESKNDIYACYEMRKDE
PTDDLCKTAYGMINCFRERSPKLYEMMGIFRAPGK
>AconOBP7
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DFADDETLKCYLKCVFDQFRLISTKGFNYKAFLTLAPPDLKDKAAKLIELCG
ETTGKPGDMCDLSWNINKCMYNAYPDVYFIF
>AconOBP8
MVILNRTIRMNFLQILIVSLVLIQTNADIRRDCREQTGVSWDALKRLKAADF
NQTDHKLKCYLKCFMTMNGIFNEGDVDVERVLRHLPRSLQESSRTTLEYCK
KFPSKDACDKAFQLAKCYFKFQPEVLRSVSFV
>AconOBP9
MKSFIAVLLCTFVVGVLSGGPMHEKIVKCKEELGVEEDAIRNMMKNNDYN
DPTVRCFNACLMKSFGKMAEDGTVNKDAVSEHVPPHVDREQFIEAATVCM
EEKGTDECDTANLIHKCLKDKKVIPSGLPPPPPQ
>AconOBP10
MKTLVVILVVCIVGVFGGLSDEQKEKLRIHRKTCETETGVEKTLVDNAHRGN
WAESDPKLRCFAACMLKRMAMMDDSGNFNEAETRKKISSDIPADKVDEVIN
KCKDMKGADSCETGLKLMKCYNDQRAVIMA
>AconOBP11
MACLLQGLKLVNPDGSLNSQVAIDKIPDTIDSRDIIVNAINVCSQRKGSEPCST
AHELFNCIHENKIPELLLG

Supplementary Table 1. Primer lists for RT-PCR

Gene names	direction	Primer Sequences（5'→3'）
OBP1	F	CGGGATCCAATGAATATTTTTCAAAATTCATTG
	R	CCGCTCGAG TTATGAACCAAGAAGTAGTGCAGGA
OBP2	F	CCCATATGCGTCCCAGTTTTGTATCAGATGA
	R	CGGAATTCTTAAAACAGGTAATATGTTTTTGGT
OBP3	F	CCCATATGAAATTGCCGGATTTTATAACAC
	R	CGGAATTCTCATATCATAAACCACATATCAGGT
OBP4	F	CCCATATGGCATTGACTCCTGAGCAAAATTCTA
	R	CGGAATTCTTAAGCTCCAATGACTTTGTTGTCT
OBP5	F	CCCATATGCAGGATCCAGATTGTCCAGTTTAT
	R	CGGAATTCTTAGGCTCCAGCTTTTTCTGCTTTG
OBP6	F	CCCATATGAAAATGACTTTGGCCCAAGTTA
	R	CGGGATCCTTAAGGCAAAAAATACATATCTGCA
OBP7	F	CCCATATGAATCATGAAAAATTTCATGAAGC
	R	GCGAATTCTTAGTTACCATCTGGTTTTTGGG
OBP8	F	CCCATATGGATGATCCTCATGCATCAATCAG
	R	CGGAATTCTCATATAGTTGGTGGACGAAAAATT
OBP9	F	CCCATATGCTTATTAGGCTTCAAGCTGCTAA
	R	GCGAATTCTCAGCCTAAAGCTTGTACTGGAA
OBP11	F	CCCATATGTTTTTTGTTTTTGCTGGTGAAATTC
	R	GCGAATTCTTATATGATGAAAAATTCTACTGGA
OBP12	F	CCCATATGCTGAGATGTCGTTCAGGTAATCA
	R	CGGGATCCTTAGTAGTTTTCGTCCCAATCTTCA
OBP13	F	CCCATATGATCATGGAAGATCTTGCAATC
	R	CGGGATCCCTATTCGGTATCTTTTTTTCTAATT
OBP15	F	CCCATATGTCTGCTGGTCCAGTACCAAAAG
	R	GCGAATTCTTACATGATGAAATAATCATCAGG
OBP17	F	GTTCGACCTCCAATTCTT
	R	TCAGCAACTTTCCCTTC

Supplementary Table 2. Primer lists for qRT-PCR.

Gene names	direction	Primer Sequences（5'→3'）
OBP1	F	TACAGATGCTGCTATTTC
	R	CACTTATCTTCTCCCTTT
OBP2	F	TTGCACTTATTGATGACAGA
	R	TTTTGGTGATGATTTTGC
OBP3	F	GTTGGAATGTTCCCAGAA
	R	CGCAGTCATCAGAGCC
OBP4	F	GCTTGTATTGTTGGTGCTTT
	R	GACTCATCCCAATTTCCTTT
OBP5	F	AAATCGCCAACAACAG
	R	AGCCACTTCGCACTC
OBP6	F	AAAGCATAACCGACAA
	R	TCGGCTTCATAATAACAC
OBP7	F	AAAGTGTTGGGGAGC
	R	GATCATTTCGATAGCCT
OBP8	F	GTGGCTTACTTGTCTTATC
	R	AATTCAGCTCATTTCG
OBP9	F	AAATCGTGCTCGTCA
	R	AAGTCAGTCCCAGTTGT
OBP11	F	AGGATTAGTAACCGATGAA
	R	GCTATCAGTTCCCTTTGT
OBP12	F	GAATGCGAAACGAAA
	R	GTGCCTGTTACTGCTGA
OBP13	F	GATCATCATGGCAGTTT
	R	CGAGTACAAGGGTCTG
OBP15	F	GAAGCTGGACTTGGA
	R	TTTGGTGGAGATAACG
OBP17	F	AAAATGACTGAGGCTTGT
	R	TTAATGTCGCTAATTCTGA
Actin	F	ACAGCAGCATCATCATCAA
	R	TCTGGACAACGGAATCTTT
NADH	F	CTGGCACTGGGATAAAAC
	R	TCAGGAATTGGTGAAAGC

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Chapter V Functional analysis of odorant-binding proteins for the parasitic host location to implicate the convergent evolution between grain aphid and its parasitoid *Aphidius gifuensis*

This work is an original contribution, adapted from:

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Forward

In the old and vibrant tritrophoic interaction system of plant-aphid-nature enemy, (E)-β-farnesene (EBF) is the well-studied infochemical among those semiochemicals. Which can repel aphid and attract the natural enemy. Until now, at least 3 types of EBF binding proteins have been found within 9 aphid species. Previous works also have illustrated the behavior traits within aphids and the predators, although several works question the function of EBF. The potential molecular mechanisms have been made within the predator and the main points were support the idea that predator mainly utilize the HIPV as a reliable cue to locate their host. In this part, we furtherly discovered the potential convergent evolution mechanisms between host aphids and it's parasitoid wasp.

Abstract

(E)-β-farnesene (EBF) is one typical and ecologically important info-chemicals in tri-trophic level interactions among plant-aphid-natural enemies. However, the molecular mechanisms of parasitoids recognizing and utilizing EBF are unclear. In this study, we functionally characterized eight AgifOBPs from *A. gifuensis*, among which, AgifOBP6 was the only OBP up-regulated by various doses of EBF, it showed a strong binding affinity to EBF in vitro as well. Lack of homology between AgifOBP6 and EBF binding proteins from aphids or from other aphid natural enemies supported that this is a convengent evolution among insects from different orders driven by EBF. Molecular docking of AgifOBP6 with EBF revealed ineractive key residues and hydrophobic forces as the main forces. And whole-mount immunolocalization showed that this is a widely expressed OBP among various antannal sensilla. Furthermore, two bioassays using grain aphids *S. miscanthi* indicated that trace EBF may promote the biological control efficiency of *A. gifuensis,* especially on winged aphids. Our present work offers a novel perspective on the biological control for aphids from reducing the initial population of migrant biotype aphids from source areas: promoting the biological control efficiency of parasitoids to winged aphids before the immigration, by low concentration of EBF application.

Keywords*: A. gifuensis,* odorant-binding protein (OBPs), (E)-β-farnesene (EBF), parasitoid, convergent evolution, olfactory plasticity.

V.1. Introduction

Evolutionary adaptation fuels the genetic diversification of living organisms, driving speciation and emergent biodiversity (Lamichhaney et al., 2015, Simões et al., 2016). Species with overlapping habitats have typical interaction characteristics, making them excellent subjects for exploring co-adaptive evolution. Which means that there are more obvious clues of adaptive evolution among multi-trophic levels. Aphid alarm pheromone, for example, is one of the most typical and ecologically important info-chemicals in three-trophic level interactions among plant-aphid-natural enemies. It was contained in the aphid cornicle droplets emitted when aphids are physically attacked (Dixon, 1958; Nault and Phelan, 1984). And induces behavioral responses in receiving conspecifics (Kislow and Edwards, 1972; Müller, 1983). Aphids get the warning signal typically cease feeding, move away from the signal, and drop off sometime (Pickett et al., 1992, Zhang et al., 2017). (E)-β-farnesene (EBF) is the primary active component of alarm pheromone in most aphid species (Bowers et al., 1972; Francis et al., 2005).

Interestingly, it also attracts aphid natural enemies such as *Aphidius uzbekistanicus, Coleomegilla maculata*, *Chrysoperla carnea,* Aphidius nigripes*, Adalia bipunctata*, *Episyrphus balteatus*, *Harmonia axyridis*, *Aphidius ervi*, *A. gifuensis* (Micha et al., 1996, Zhu et al., 1999, Buitenhuis et al., 2004, Francis et al., 2004, 2005b, Verheggen et al., 2007, 2008, Heuskin et al., 2012, Fan et al., 2018). Besides, apparently, plants are also involved in this complicated tri-trophic level interaction, EBF has been reported to be released by many plants such as maize (Schnee et al., 2002), and Mentha x piperita, L. (Crock et al., 1997). Despite a large number of works reporting that EBF is the key volatile to attract natural enemies of aphids, some studies also question such a hypothesis (Joachim, C., and Weisser, W. W. 2015a; Joachim, et al., 2015b). As EBF emitted by aphids in very low amounts and unstable, it was, therefore, suggested that natural enemies might use plant-derived EBF as a synomone to identify aphid-infested plants via an altered plant volatile bouquet (Vosteen et al., 2016).

Insect odorant-binding proteins (OBPs) play important roles in peripheral olfactory signal transduction, which connects info-chemicals in habitat with olfactory receptors (ORs) located on the olfactory nerve (Vogt, R. G., & Riddiford, L. M, 1981; Pelosi, 2006; Leal, 2013). Recently, aphid EBF recognition mechanism has received extensive attention and progress has been well made. Three EBF binding proteins, namely OBP3, OBP7 and OBP9 have been identified in succession so far in aphids such as *M. persicae* (Wang et al., 2021), *Sitobion avenae* (Zhong et al., 2012), *A. pisum* (Qiao et al., 2009, Zhang et al., 2017, Qin et al., 2021), *Aphis glycines* (Wang et al., 2020), *Megoura viciae* (Bruno et al., 2018) and *Rhopalosiphum padi* (Fan et al., 2017). There has always been confusion between *Sitobion avenae* (Fabricius) and *S. miscanthi* (Takahashi) in China. Thanks to a systematic study of both aphid species, it was found that *S. avenae* is only distributed in the Yili region of Xinjiang, China, the aphids distributed in other parts of China that were originally named after *S. avenae* were *S. miscanthi* (zhang,1999; Liu et al., 2009; Jiang et al., 2019). Each of 3

EBF binding proteins among aphid species are orthologs with highly sequence consensus. And the positive response to EBF induction by strongly up-regulated expressions of OBP7 and OBP9 indicates the strong olfactory plasticity of aphids' olfaction (Zhang et al., 2021). More than that, OR5, an aphid olfactory receptor, has been demonstrated to be responsible for EBF signal transduction (Zhang et al., 2017).

During predation and parasitism, natural enemies utilize herbivore-induced plant volatiles (HIPVs), green leaf volatiles (GLVs), or volatiles from aphids to locate their hosts (Dong et al., 2008, CMD Moraes et al., 1998, Buitenhuis et al., 2004, Wang et al., 2022). Lacewing *Chrysopa pallens* was reported to utilize plant-derived compounds, pest-induced volatiles, and EBF as foraging cues (Li et al., 2017). And CpalOBP10 showed its affinities to green leaf volatiles as well as EBF. HaxyOBP15 displayed a broad binding profile with (E)-β-farnesene as well as multiple other odor ligands in ladybeetle *Harmonia axyridis,* an important natural enemy that consumes aphids (Qu et al., 2022). Further, one EBF olfactory receptor, EcorOR3, as well as EcorOBP15 have been identified to involve in the EBF perception in hoverfly *Eupeodes corollae* (Wang et al., 2022).

Aphid parasitoids as natural enemies could also detect plants that previously infested by host aphids (Du et al., 1997, 1998; Guerrieri et al., 1999; Powell et al., 2006). And it is believed that parasitoid tracks EBF as it indicates the existence of aphids (Battaglia et al., 1993, Micha et al., 1996, Buitenhuis et al., 2004, Heuskin et al., 2012, Fan et al., 2018, Jiang et al., 2022). *A. gifuensis* is one of the most common endoparasitoids of tobacco aphid *Myzus persicae* as well as wheat aphid *Sitobion miscanthi* in China (Ohta & Honda, 2010; Pan et al., 2018). And has evolved a powerful peripheral chemosensory system. It distinguishes among healthy, mechanically damaged, and aphid-infested plants and chooses the last as the one that is most likely to harbor their potential attack targets (Dong et al., 2008; Yang et al., 2009). Both female and male *A. gifuensis* were attracted by EBF as well as many plant volatiles, including trans-2-hexenal, methyl salicylate, benzaldehyde, cis-3-hexen-1-ol, 1-hexanal and so on (Buitenhuis et al., 2004; Zhang et al., 2020; Fan et al., 2018, Song et al., 2021a). In addition, the intense sexual orientation of males to females in distance indicated the existence of sex pheromone (Fan et al., 2018). However, the molecular mechanism underlying the attraction of parasitoids to either EBF or any other olfactory cues stays completely unknown to date.

Female parasitoids utilize electromagnetic radiation, sounds, visuals (such as the color and shape of the aphids, (Harmon et al., 1998) and odors to locate food and oviposition sources (Vinson, 1976). Although visual cues, such as the color and shape of the aphids, may contribute to some extent, olfactory stimuli are predominant. This notion has been demonstrated in some species (Read et al., 1970; Singh and Sinha, 1982; Wickremasinghe and van Emden, 1992; Powell et al., 1998; Storeck et al., 2000; Hatano et al., 2008). Several investigations also reported on the involvement of multiple sensory signals as well as environmental conditions, and the influence of EBF on predators affects parasitism. For example, parasitized aphid colonies are less

attractive than healthy ones (Pineda et al., 2007). When the species richness of parasitoid wasps increases, the parasitism ratio decreases (Yang et al., 2021). Furthermore, both host density and natal host significantly affected self superparasitism of *A. gifuensis* on *S. avenae* (Pan et al., 2018). When two or three host species were offered in the same quantity to pairs of parasitoids, the level of parasitism was lower than when only one host species was offered (Saeed et al., 2018). The presence of aphid predator chemical cues, such as *H. axyridis* related cues, influenced the foraging behavior and activity of *A. gifuensi* (Xia et al., 2021).

In the present study, we hypothesized that the EBF recognition of parasitoids could also be strengthened through olfactory plasticity, thus help on host (aphid) detection and parasitism. EBF induction was carried out on *A. gifuensis* to explore the OBPs up-regulated in response (plasticity), and their binding affinities to EBF were verified *in vitro*. Homology modeling, molecular docking and immunolocalization of AgifOBP6 unraveled the molecular mechanisms of perceiving EBF in *A. gifuensis*. Meanwhile, data *in vivo* of EBF application test was obtained for evaluating the promotion of EBF on parasitoid behavior.

V.2. Materials and methods

V.2.1. Plants and Insects

Parasitoid wasps (*A. gifuensis*) were originally collected from *M. persicae* mummies in August 2019 in Kunming, Yunnan province, China, and they had been cultured on *S. miscanthi* which are parthenogenic clones (Langfang-1) reared on wheat (*T. aestivum* L.) 'AK58' at our laboratory in the Institute of Plant Protection of Chinese Academy of Agricultural Sciences (Beijing, China) for 2 years with an air-conditioned insectary: 23 ± 2 °C with 55 ± 10% relative humidity and a photoperiod of 16:8 (L: D) h. The rearing methods in details were referred to Fan et al., (2018) and Jiang et al., (2022).

V.2.2. EBF induction assay

Olfactory stimulation is an ideal tool for evaluating insect responses at molecular level to odors such as in the blowfly *protophormia terraenovae* (Barbarossa et al., 2007) and aphid *S. miscanthi* (Zhang et al., 2021). Here, EBF induction were performed to screen out the OBP proteins that respond with up-regulated expressions in *A. gifuenss.* The protocol mainly followed previous studies (Pan et al., 2019; Hu et al., 2021; Zhang et al., 2021; Ma et al., 2022). The mummies were separated individually and waits for emergence. And 10 female adults one day after emergence were collected into one *Petri* dish (13 cm in diameter and 2 cm in height) at 10:00-11:00 am. Then, each Petri dish was treated with EBF of a certain concentration (a total of 4 concentrations, namely, 0.4 ng/μL, 4 ng/μL, 40 ng/μL, and 400 ng/μL). And triplicates in total. Their corresponding negative control was set up. A total volume of 10 μL EBF (Wako, Japan) that dissolved in hexene was loaded on a filter paper at the

bottom of the Petri dish to induce for 0.5 h. Antennae, the olfactory organ, were collected immediately in RNase-free tubes with bottom immersed in liquid nitrogen, ultimately stored at −80 °C until total RNA extraction.

V.2.3. Total RNA extraction and cDNA preparation

Antennae were dissected from female adult wasps, which were merged within 24-36h. In total, there were three replicates for the sampling. For each group, 100 female antennae, control and EBF-treated were collected, respectively. Total RNA was extracted using Trizol reagent and combined with a micro total RNA extraction kit (Tianmo, Beijing, China) following the manufacturer's instructions. RNA degradation and contamination were monitored on 2% agarose gels. RNA purity was checked using a Nanodrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). RNA concentration was measured using a spectrophotometer RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). cDNAs were synthesized using the TRUEscript RT kit (LanY Science & Technology, Beijing, China) following the manufacturer's protocol.

V.2.4. Expression investigation of AgifOBPs

Real Time-quantitative PCR (RT-qPCR) with an ABI 7500 real-time PCR system (Applied Biosystems Fosters City CA, USA) was conducted to explore the responses of *AgifOBPs* at mRNA level after being induced with EBF. Primers were described in our previous work (Jiang et al., 2022) and used a 20-μL reaction containing 10 μL of 2 x SuperReal PreMix Plus, 0.6μL of forwarding primer (10 μmol/L), 0.6μL of reverse primer (10 μmol/L), 2 μL of cDNA (50ng/μL), 0.4μL of 50 x ROX reference dye, and 6.4 μL of nuclease-free ddH_2O followed the instructions provided with the SuperReal PreMix Plus (SYBR Green) kit (FP205) (Tiangen, Beijing, China). The PCR program was as follows: an initial 15-min step at 95°C, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 32s, and elongation at 72°C for 45s, and finally 10-min step at 72°C. For melting curve analysis, a dissociation step cycle was added automatically. Relative quantification was performed according to the $2^{-\Delta\Delta Ct}$ method **(**Livak K J & Schmittgen T D, 2001). β-actin and NADH were used as reference genes to normalize the data. All qPCR analyses were performed in three technical and biological replicates.

V.2.5. Heterologous expression and purification of AgifOBPs

Given that AgifOBP (7/9) was obtained in our previous work (Jiang et al., 2022). In this part, Eight OBPs (AgifOBP1-6, AgifOBP8 and AgifOBP11), including those in response to EBF induction, were expressed in prokaryotes. The expression and purification methods are consistent with the previous study (Prestwich, 1993; Wang et al., 2021; Jiang et al., 2022). The PCR products with were first cloned into a pEASY-T1 clone vector (Trans, Beijing, China), which was assembled each AgifOBP to form a fusion protein without histidine-tagged peptide, and then subcloned into the

bacterial expression vector pET28a (+) (Novagen, Madison, WI) between Nde I and either EcoR I or Bam H I restriction sites, and reconstructed plasmid were verified by sequencing. This plasmid protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM when the culture had reached an OD_{600} value of 0.6. Cells were incubated for an additional 12 h at 28 ℃ or 37 ℃ and then harvested by centrifugation and sonicated in 5 seconds with a purse at 3 seconds for 15 minutes at a low temperature (ice-water mixture). After centrifugation, the bands obtained were checked by 15% SDS-PAGE for their correspondence to the predicted molecular masses of the proteins. They were solubilized according to protocols for the effective rebuilding of the recombinant OBPs in their active forms (Prestwich, 1993, Qin et al., 2020, Wang et al., 2021). The soluble proteins in the refolded proteins were then purified by anion-exchange chromatography with RESOUCE Q15 HP column (GE HEALTH CARE, USA) and finally with two rounds of gel filtration through a Superdex 75 10/300 GL column (GE HEALTH CARE, USA). The concentration of purified protein was determined by a Protein Assay kit (Qubit™ Protein Assay kit, Q33211, Invitrogen), and the purified AgifOBP(1-6/8/11) was analyzed by mass spectrometry (LC-MS). The purity and concentration of the soluble proteins were evaluated using SDS-PAGE. Finally, stock solutions of AgifOBP (1-6/8/11) were collected and kept at -20 ℃ in Tris-HCl (50 mM, pH 7.4).

V.2.6. Fluorescence competitive binding assays

To investigate the ligand-binding property of AgifOBPs, five groups of competitive ligands were used: (i) aphid alarm pheromone components, including E-β-farnesene (EBF), (-)-α-pinene, (-)-β-pinene and (+)-limonene, which is released by other aphids following natural enemy predation or physical damage (Francis et al., 2005a; Song et al., 2021b); (ii) main components of the aphid sex pheromone (4aSR,7SR,7aRS)-Nepetalactone); (iii) green leaf volatiles of wheat (Z)-3-hexen-1-ol); (iv) aphid-induced plant volatiles (methyl salicylate and 6-methyl-5-hepten-2-one); and (V) an EBF derivative artificial chemical, namely, CAU-II-11 ((E)-3,7-dimethylocta-2,6-dien-1-yl-2-hydroxy-3-methoxybenzoate), which showed a high affinity for aphid EBF-binding proteins (OBP3/7/9, Qin et al., 2020). The classes, CAS numbers, and purity of the chemicals used in this study are listed in Table 4-2. Fluorescence competition assays were conducted following previous work (Ban et al., 2003; Zhong et al., 2012; Fan et al., 2017; Qin et al., 2020; Wang et al., 2021). The decrease in 1-NPN fluorescence due to the ability of different odorants to displace 1-NPN from the binding cavity of AgifOBPs were observed and recorded, and then, Ki value for each compound was determined. The intensity values corresponding to the maximum fluorescence emissions were plotted against the cumulative 1-NPN concentration for calculating dissociation constants. The amount of bound ligand was calculated from the fluorescence intensity values by assuming that the protein was 100% active, with a stoichiometry of 1:1 protein: ligand at saturation. The curves were linearized using

Scatchard plots. The value of $K_{1\text{-NPN}}$ was estimated on a direct plot by non-linear regression with an equation corresponding to a single binding site using Prism 9.0 (GraphPad Software, Inc., USA), and the IC_{50} was defined as the concentration of a competitor that caused a 50% reduction in fluorescence intensity. The dissociation constants of the inhibitors (Ki) were calculated according to the formula Ki = [IC50]/(1+[1-NPN]/$K_{1\text{-NPN}}$), in which [1-NPN] represents the free 1-NPN concentration and $K_{1\text{-NPN}}$ represents the dissociation constant for AgifOBPs/1-NPN. Referring to previous work (Wang et al., 2021), we consider the ligand-binding affinity with AgifOBPs as high when Ki < 2 μM, medium when 2 μM < Ki < 10 μM, and weak when Ki > 10 μM.

V.2.7. Sequence analysis and structure prediction

AgifOBP6 was chosen for further homology modeling and molecular docking. First of all, amino acid sequences of AgifOBP6 were aligned with other EBF binding proteins, and the sequence consistency was analyzed to determine whether there was homology. Alarm pheromone binding proteins in aphids (OBP3, OBP7, and OBP9) and in natural enemies were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/) or UniProt database (https://www.uniprot.org/). The amino acid identity of AgifOBP6 with EBF binding OBPs was analyzed in MEGA11 using the maximum likelihood method with an LG+ mode (Tamura et al., 2021). Values indicated at the nodes are bootstrap values based on 1000 replicates presented with a 95% cutoff. The orthologous protein sequences from the genomes and transcriptomes of the following species were used in the analysis: *Myzus persicae* (Wang et al., 2019), *Sitobion miscanthi* (Xue et al., 2016), *Aphis gossypii* (Gu et al., 2013), *Acyrthosiphon pisum* (Zhou et al., 2010), *Aphis glycines* (Wang et al.,2020), *Megoura viciae* (Bruno et al., 2018), *Rhopalosiphum padi* (Kang et a., 2018), LeryOBP3 (AJO61166), LeryOBP7 (AJO61167)) and the OBP protein sequences from parasitoid *A. gifuensis* (Fan et al., 2018; Jiang et al.,2022); the predators *Harmonia axyridis* (Qu et al., 2021), *Chrysopa pallens* (Li et al., 2013), *Episyrphus balteatus* (Wang et al., 2017) and *Eupeodes corollae* (Wang et al., 2017; Jia et al., 2019). The amino acids of the sequences used are listed in Supplementary file 1. Then, homology modeling was carried out using SWISS-MODEL (https://swissmodel.expasy.org/). The amino acid sequence of AgifOBP6 was submitted to the NCBI BLASTp server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for searching a proper template in the PDB database. Identity between template and target protein was above 30% was taken into consideration. Three methods, namely Verify_3D, Procheck, and ERRAT were used at UCLA-DOE LAB-SAVES v6.0 (https://saves.mbi.ucla.edu/) to assess the final 3D model of AgifOBP6 protein (Laskowski et al., 1996). At last, molecular docking was conducted to investigate the mode of ligand binding. Docking calculations for AgifOBP6 with EBF and its analog CAU-II-11 were performed using the UCSF Dock6.9 protocol in the Yinfo Cloud Computing Platform (https://cloud.yinfotek.com/). The chemical structure of the

small molecule EBF and its analog CAU-II-11 were drawn by JSME and converted to a 3D structure with energy minimization in the MMFF94 force field. The crystal/NMR structure of CpalOBP4 was automatically downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/). All redundant atoms except chain A were deleted and then the protein structure was carefully treated in several steps including residue repairing, protonation, and partial charges assignment in the AMBER ff14SB force field. The DMS tool was employed to build the molecular surface of the receptor using a probe atom with a 1.4 Å radius. The binding pocket was defined by the crystal ligand and spheres were generated to fill the site by employing the Sphgen module in UCSF Chimera (Pettersen et al., 2004). A box enclosing the spheres was set with a center of (-29.58, -2.026, -13.65) and sizes of (31.592, 31.441, 35.657), within which grids necessary for rapid score evaluation were created by the Grid module. Finally, DOCK 6.9 (Lang et al., 2009; Mukherjee et al., 2010) program was utilized to execute semi-flexible docking where 10000 different orientations were produced. Clustering analysis was performed (RMSD threshold was set 2.0 Å) for candidate poses and the best-scored ones were output. The top-ranked pose, as judged by the Vina docking score, was subjected to visual analysis using PyMOL v.1.9.0 (http://www.pymol.org/).

V.2.8. Western blot assay

The rabbit antiserum against a recombinant protein of AgifOBP6 was produced by Xinnuojingke Biotech (Beijing, China). Crude antennal proteins were extracted using RIPA buffer (Solarbio, Beijing). Protein samples were separated by 15% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (PVDF) (Millipore, Carrigtwohill, Ireland). The membrane was blocked using 5% fat-free milk (BD Biosciences, San Jose, CA, USA) in PBS containing 0.05% Tween-20 (PBST) at 4°C overnight. After washing three times with PBST (10 min each), the blocked membrane was incubated with rabbit anti-AgifOB6 antiserum (1:5000) at room temperature for 1 h. After three additional washes with PBST, the membrane was incubated with Goat Anti-Rabbit immunoglobulin G (IgG), and HRP-conjugated antibody (CWBIO, Jiangsu, China) (1: 5000) at room temperature for 2 h. Finally, the membrane was developed using Immobilon Western Chemiluminescent HRP Substrate (Merck, Beijing, China), then exposed and imaged on an ImageQuant LAS 4000 mini (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

V.2.9. Whole-Mount Immunolocalization of AgifOBP6

Given that AgifOBP6 strongly bunded with EBF, they were chosen to further study the expression characters at the subcellular localization. Fluorescence in whole-mount was performed to identify the location of AgifOBP6 in antennae according to a previous study (Daniele B et al., 2018; Li et al., 2022). Antennae from virgin female specimens were dissected under the microscope after 24h to 36h of the adult stage and washed twice with PBS, pH=7.4. After the washing step, samples were fixed in 4% paraformaldehyde in PBS for 2 h and then washed twice with the same buffer. Samples

were then incubated for 30 min with PBS containing 2% BSA (to reduce non-specific binding) and 0.1% of the detergent Tween 20 (Sigma) to permeabilize tissues favoring the entrance of antibodies. Samples were then incubated for 1 h at room temperature with antisera raised in rabbit. The recombinant protein was used to produce antibodies against the entire amino acid sequences of AgifOBP6 except for signal peptides. Antibodies of AgifOBP6 were diluted at 1:15000 in tris buffered saline with 2% bovine serum albumin (BSA). Samples were washed with PBS and incubated for 1h in a dark chamber with the secondary goat anti-rabbit tetramethylrhodamine (TRITC)-conjugated antibody (Jackson, Immuno Research Laboratories Inc., West Grove, PA, United States) diluted 1:200 in blocking solution containing 0.1% Tween 20. This (TRITC)-conjugated antibody has been previously used in experiments on the aphid *M. viciae* OBPs (Daniele B et al., 2018). Primary polyclonal anti-AgifOBP6 antibody was omitted or substituted with rabbit pre-immune serum (1:200), and sections were treated with a blocking solution containing 0.1% Tween 20 (Sigma) and incubated only with the secondary antibody in all controls. Coverslips were mounted with City fluor (City fluor Ltd., London, United Kingdom), and immunofluorescence was analyzed using an inverted laser-scanning confocal microscope (LSM880, Carl Zeiss, Germany.) equipped with a Plan APO 40 x 0.95 NA objective. Images were acquired using Zeiss ZEN 2.1 software (emission windows fixed in the 561 nm) without saturating any pixel. Fluorescence and bright-field images were combined with Adobe Illustrator 2020 (Adobe Systems Incorporated, San Jose, CA, United States).

V.2.10. Investigation for the phenotypic host preference

In this section, experiments were carried out mainly followed previously published work (Xia et al., 2012; Rezaei et al., 2019). Two phenotypes of aphid adults (winged and wingless adults) as well as 2nd stars nymph were employed as three groups to be treated with EBF (4 ng/μL diluted into trimyristoyl triglyceride (TAG, APPLYGEN)). Fifteen aphids were introduced to three wheat seedlings (AK 58) with a density of five per wheat seedlings kept in a peri dish. The roots of wheat seedlings were placed in a 1.5 ml tube and sealed with scraped cotton to moisturize. Let the introduced aphids colonize and start the experiment after 24 hours. EBF (4ng/ul, 0.05 ul using a flat-mouth microsyringe with 5 μL range) was coated on the dorsal abdomen of *S. miscanthi* quickly, and then one female aphidius was put in each petri dish. Probing and tapping by antennae of aphidius were defined as probing. And the action of attacking aphids for oviposition were defined as parasitism. Parameters mentioned above as well as for aphids, their defense behaviors against aphidius' attack present the behavioral responses within 10 minutes of either aphidius or aphid were all recorded (Song et al., 2021a). Negative control and blank control were also set up. The aphids of the negative control were treated with trimyristoyl triglyceride and the aphids of the blank control were not treated without anything. Place a 6W incandescent lamp 20 cm above the light source to eliminate any light source interference. Fisher LSD one-way ANOVA was used to calculate significant

differences ($p < 0.05$).

V.2.11. Investigation for the foraging behavior of A. gifuensis

We further compared behaviors to aphids of the aphidius with and without EBF treatment, to evaluate the effects of nonogram EBF on both host (aphid) preference and parasitism ratio (0.2 ng/ aphids). Both winged and wingless adults and 2nd instar nymphs were separately tested. This work was conducted in a wheat seedlings system (Figure. 4-10F), for the details, 10 wheat seedlings (AK58) at their two leaves period in a pot were placed in a climate chamber (16: 8 h L: D; 22 ± 1 ℃). Twenty aphids were introduced to the 10 wheat seedlings and covered by a plastic insect cage (13 cm in diameter and 30 cm in height) with screen mesh caps. Remove the newborn aphids after 24 hours of colonization of adults. Either 20 adults or 20 nymphs were applied evenly with 1μL in total of EBF solution (4 ng/μL of EBF + trimyristoyl triglyceride (TAG)) to the 20 aphids. The same amount of TAG was applied as negative control group. And the blank control group was not applied with any chemical. Single female aphidius merged between 24-36 h was then introduced to EBF treated aphids as well as their control aphids and allowed them to forage and parasite for 24 h. Experiments were performed in ten replications. The number of mummies were recorded after 12 days, parasitism was defined as the proportion of mummies to original 20 aphids. And significant differences were analyzed using one-way ANOVA followed by Fisher LSD ($p < 0.05$).

V.2.12. Statistical analyses

For qRT-PCR and bioassays, the differences between control and treatments of biological replicates were tested using one-way ANOVA followed by multiple comparisons tests regardless of rows and columns using GraphPad Prism version 9.0.0 for Windows (GraphPad, San Diego, California USA, www.graphpad.com). Differences among treatments were distinguished using Tukey's honestly significant difference (HSD) test at the $P < 0.05$ significance level.

V.3. Results

V.3.1. Response of AgifOBPs expression to EBF induction

EBF induced extensive changes in the expression of OBPs. Among them, AgifOBP6/9/12 were up regulated to 164%, 191% and 152%, while, OBP7/15 were down regulated to 80.8% and 32.6%, and the expression of remaining OBPs were generally stable (Figure. 4-1). In the present study, the EBF application was set on a 10-fold gradient (0.4/4/40/400ng), most OBPs except OBP6 responded only to a certain concentration of EBF. For example, only 4ng dose of EBF caused the up-regulation of OBP9 expression, while the other doses, 40 or above, did not induced any responses of OBP9 at mRNA level. And for AgifOBP12, 400 ng EBF significantly up-regulated its expression. However, the expressions of AgifOBP7 and

AgifOBP15 were decreased by 40 ng and 4000 ng EBF respectively. AgifOBP6, one of the OBP with the highest expression abundances, was significantly up-regulated at various amounts of EBF amount (from 4 to 4000 ng, Figure. 4-1).

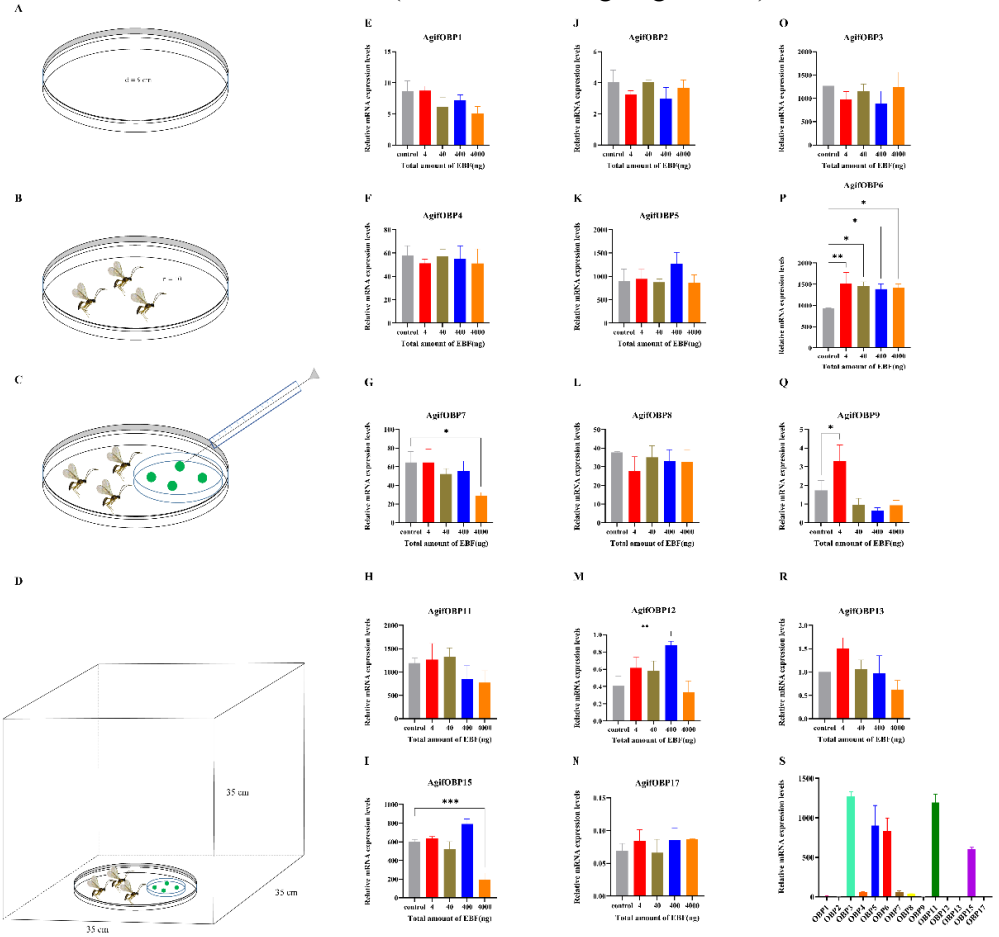

Figure. 5-1. The induction diagram shows the relative expression levels of *AgifOBPs* after being treated with different dosages of EBF (4-4000 ng) in the antennae.

(A)-(D): The diagram of inducing assay with different dosages of EBF that dissolved in hexane; (E)-(R): The relative expression levels of *AgifOBPs* in antennae after being induced with EBF; (D): The relative expression levels of *AgifOBPs* in the control group. Data are means of three independent experiments and presented as mean ± SD. The standard error is represented by the error bar and the star above each bar denotes significant differences (one-way ANOVA, $P < 0.05$).

V.3.2. Expression and purification of AgifOBP(1-6/8/11)

SDS-PAGE showed that AgifOBPs were present as inclusion bodies using a bacterial system (AgifOBPs/pET-28a, (BL21 DE3)), and mature AgifOBPs with no fusion tags were purified accomplished with serial chromatographic steps on anion-exchange resins RESOUCE15 Q/SP HP columns (GE Healthcare Biosciences, Uppsala, Sweden). After a dissolving and refolding treatment, the refolded AgifOBP(1-6/8/11) were purified as soluble proteins, with yields of 0.21 mg/mL to 0.63 mg/mL (Figure. 4-2, Figure. 4-6A) and more than 15 mg of recombinant AgifOBP(1-6/8/11) were obtained. Whereas, the remaining OBPs were not successfully expressed and purified. The theoretical molecular weight values for AgifOBP(1-6/8/11) were close to the measured values (AgifOBP1, 13.01 kDa; AgifOBP2, 14.15 kDa; AgifOBP3, 13.41 kDa; AgifOBP4, 12.53 kDa; AgifOBP5, 13.18 kDa; AgifOBP6, 13.65 kDa; AgifOBP8, 13.53 kDa; AgifOBP11, 13.90 kDa). The purified protein samples were further identified by LC-MS/MS.

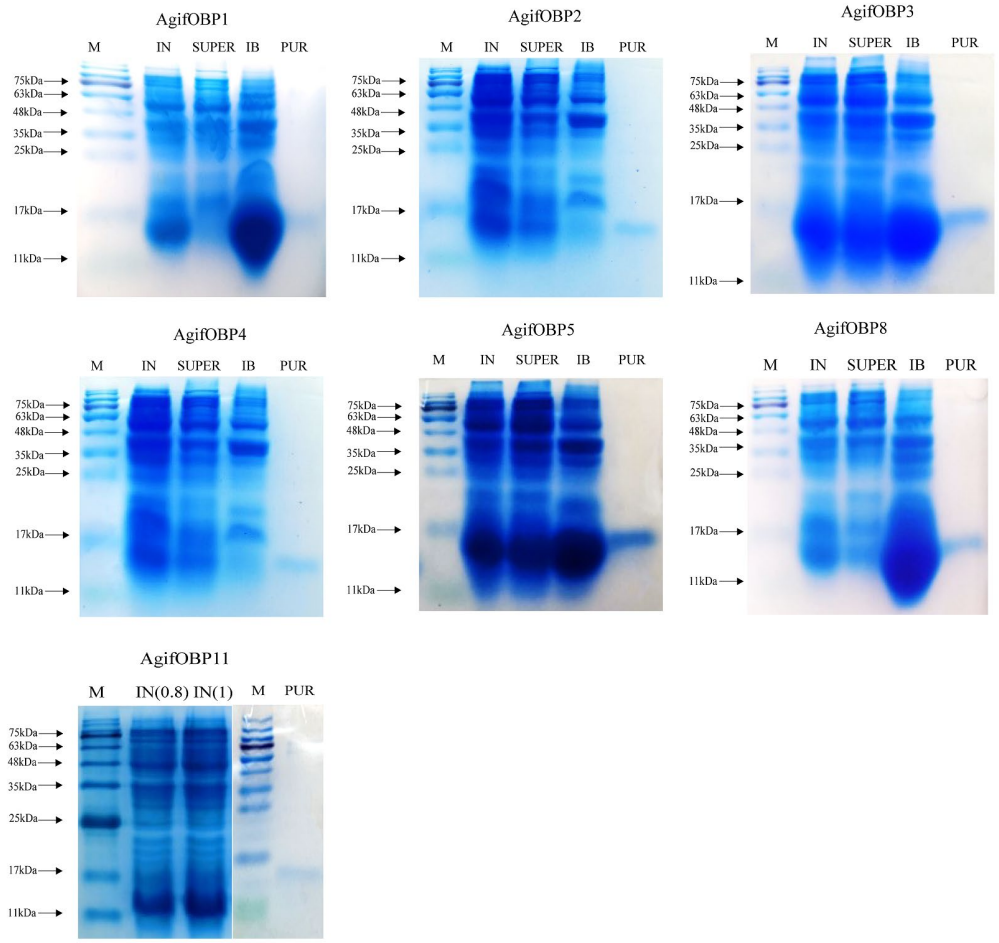

Figure. 5-2 Prokaryotic Expression and purification of AgifOBP(1-5/8/11).

Line M: molecular weight PR1910 (11-180KDa) Marker, 11, 17, 25, 35, 48, 63, 75, 100, 135, 180 KDa; IN: Induced pET-28a (+) / AgifOBP(1-5/8/11); Super: pET-28a (+) / AgifOBP(1-5/8/11) Supernatant; IB: pET-28a (+) / AgifOBP(1-5/8/11) Inclusion body; Pur: Purified Purified pET-28a (+) / AgifOBP(1-5/8/11).

V.3.3. Fluorescence competitive binding assays

We first tested the binding affinities of both AgifOBPs to the fluorescent probe N-phenyl-1-naphthylamine (1-NPN). Every single OBP exhibited a regular saturation binding curve to 1-NPN and a linear Scatchard plot (Figure. 4-3A, Figure. 4-3D). The dissociation constants of AgifOBPs were listed in table 1. Interestingly, in the following fluorescence competitive binding assays, all purified proteins AgifOBPs (AgifOBP1-6/8/11) showed strong binding affinities with CAU-II-11, a previously published EBF analog (Figure. 4-3, Table 4-2). However, among the 8 AgifOBPs, AgifOBP6 and AgifOBP8 displayed a strong binding affinity for EBF with Ki values of $6.26E^{-7} \pm 3.82E^{-6}$ μM and 0.51 ± 0.02 μM, respectively (Figure. 4-3J, Figure. 4-3K, Table 4-2). The binding property of AgifOBP6 to diluted EBF (c = 20 nM/L) was still quite strong with a Ki 0.48 ± 0.01 μM (Figure. 4-3J). (-)-α-pinene, (-)-β-pinene and (+)-limonene are 3 active components of aphid alarm pheromone reported in few aphids, such as *Megoura viciae* (Song et al., 2021), AgifOBP8 also showed a strong binding property to (-)-α-pinene, with Ki values of 1.94 ± 0.02 mM (Figure. 4-3K, Table 4-2). And AgifOBP1 and AgifOBP4 had a medium binding affinity to (-)-α-pinene with Ki values of 8.18 ± 1.37 μM and 8.33 ± 0.39 μM, respectively (Figure. 4-3E, Figure. 4-3H, Table 4-2), while the affinities of remaining OBPs including AgifOBP6 were weak for (-)-α-pinene. Both AgifOBP6 and AgifOBP8 showed a high affinity for (-)-β-pinene, with Ki values of 1.38 ± 0.35 μM and 1.26 ± 0.02 μM, respectively. For (+)-limonene, the AgifOBP6 and AgifOBP8 also showed a strong affinity with Ki values of 0.93 ± 0.02 μM and 0.39 ± 0.05μM, respectively (Figure. 4-3J, Figure. 4-3K, Table 4-2). While AgifOBP2 and AgifOBP4 displayed a medium binding affinity for both (-)-β-pinene and (+)-limonene (Figure. 4-3F, Figure. 4-3H, Table 4-2), AgifOBP1, AgifOBP3, AgifOBP5, and AgifOBP11 showed poor binding or no binding property for the above three chemicals (Table 4-2).

For the main component of aphid sex pheromone nepetalactone, we found that AgifOBP6 displayed a high affinity to nepetalactone with a Ki value of 0.72 ± 0.01μM, while AgifOBP1, AgifOBP2, AgifOBP3, AgifOBP8, and AgifOBP11 displayed a medium binding affinity for nepetalactone (Figure. 4-3J, Table 4-2), AgifOBP4 and AgifOBP5 showed weak binding or no binding to the tested ligands.

Besides, both AgifOBP6 and AgifOBP8 exhibited better binding abilities with some wheat volatiles such as 6-Methyl-5-hepten-2-one (MHO) compared with other AgifOBPs (Figure. 4-3J, Figure. 4-3K, Table 4-2). Yet AgifOBP1, AgifOBP2, AgifOBP3, AgifOBP4, and AgifOBP11 showed medium binding properties (Figure.

4-3E- Figure. 4-3H, Figure. 4-3L, Table 4-2), while, AgifOBP5 showed weak binding abilities to candidate ligands with Ki values more than 10 μM (Figure. 4-3I, Table 4-2). Only AgifOBP6 had a high affinity with methyl salicylatec (MeSA), while AgifOBP1, AgifOBP2, AgifOBP8, and AgifOBP11 displayed a medium binding affinity (Figure. 4-3J, Table 4-2). For the plant volatile cis-3-hexen-ol, we found that AgifOBP6 had a high affinity with Ki values of 0.86 ± 0.01μM, and AgifOBP8 displayed a medium binding affinity (Table 4-2, Figure. 4-3)

Table 5-1. Calculated association constants of AgifOBPs/1-NPN probe complexes.

Protein name	AgifOBP1	AgifOBP2	AgifOBP3	AgifOBP4	AgifOBP5	AgifOBP6	AgifOBP8	AgifOBP11
Kd(μM)	0.89±0.09	0.58±0.06	2.11±0.41	2.21±0.56	4.77±0.46	0.21±0.09	0.86±0.06	1.94±0.12

Table 5-2. Binding affinities of AgifOBPs for candidate ligands, evaluated in displacement binding assays using the fluorescent probe, 1-NPN.

No.	Code	CAS	Purity	OBP1	OBP2	OBP3	OBP4	OBP5	OBP6	OBP8	OBP11
				Ki (μM)							
1	(E)-β-farnesene	18794-84-8	≥85%	5.53±0.54	4.08±0.22	2.78±0.07	6.16±0.35	19.48±0.40	6.26E-07±3.82E-06	0.51±0.02	6.62±2.23
2	(-)-α-pinene	80-56-8	≥95%	8.18±1.37	19.20±3.02	-	8.33±0.39	-	16.20±0.07	1.94±0.02	-
3	(-)-β-pinene	19902-08-0	≥99%	16.06±5.56	4.07±0.09	24.98±2.83	6.99±0.70	-	1.38±0.35	1.26±0.02	-
4	(+)-limonene	138-86-3	≥95%	20.24±7.01	3.13±0.17	-	7.39±0.42	-	0.93±0.02	0.39±0.05	-
5	Nepetalactone	21651-62-7	≥80%	4.03±0.34	3.66±0.62	8.95±0.69	13.38±0.21	28.47±3.61	0.72±0.01	2.08±0.09	5.21±0.58
6	6-methyl-5-hepten-2-one	110-93-0	≥99%	9.42±1.50	19.83±2.48	23.04±1.93	-	23.04±2.18	1.00±0.07	1.88±0.10	5.38±0.85
7	cis-3-hexenol	928-96-1	≥97%	11.44±2.09	10.95±1.06	26.21±1.74	-	20.13±3.28	0.86±0.01	2.18±0.01	10.56±1.11
8	methyl salicylate	119-36-8	≥99%	6.95±1.69	5.23±0.51	-	27.90±.3.31	-	0.84±0.19	2.69±.026	6.69±0.36
9	CU-II-11	-	≥98%	9.44±0.61	0.65±0.02	1.50±0.20	7.82±1.37	3.35±0.27	1.95±0.01	1.76±0.07	-

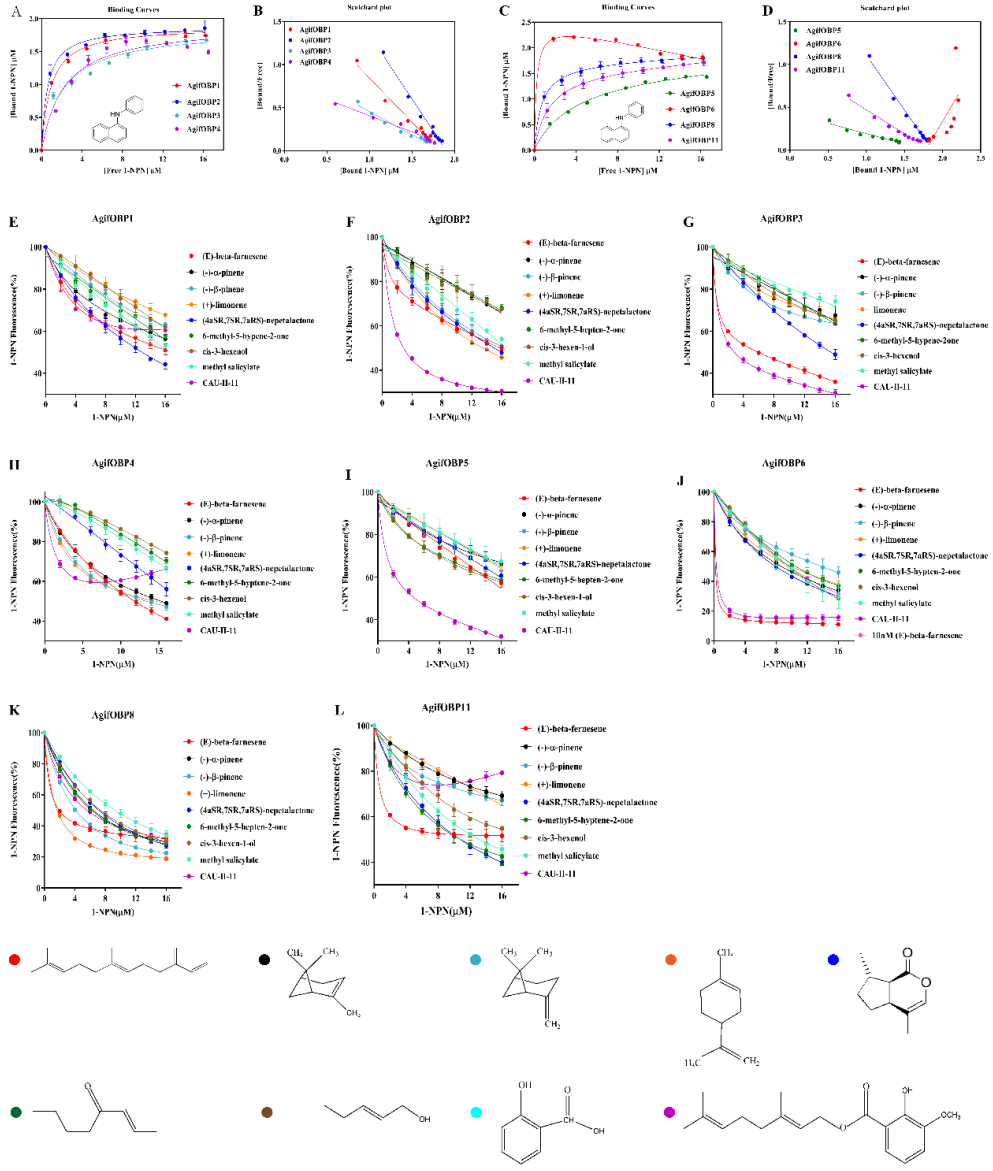

Figure. 5-3 Competitive binding assays of AgifOBP1-6/8/11 with candidate ligands.

AgifOBP1-6/8/11 was 2 μM in tris buffer (50 mM/L, pH 7.4). Aliquots of a 1 mM 1-NPN in methanol solution were added to the protein to final concentrations of 2-16 μM, and the emission spectra were recorded between 350 and 500nm. A mixture of the recombinant protein and N-phenyl-1-naphthylamine (1-NPN) in 50 mM Tris-HCl buffer (pH 7.4) both at the concentration of 2 μM was titrated with 1 mM solutions of

each competing ligand to the final concentration range of 2 - 16 μM. Fluorescence values were presented as percent of the values in the absence of a competitor. Dates are the means ± SD of three independent experiments.

V.3.4. Homology Modeling and Molecular Docking

Based on the results of EBF induction bioassy and fluorescence competitive ligand binding test, AgifOBP6 is the only OBP that was up regulated by EBF and showed a strong affinity for EBF. Therefore, it was chosen to further explore the mechanism of EBF perception in *A. gifuensis*. No homology was found between AgifOBP6 with amino acid of either aphids' OBPs or natural enemies' OBPs (Figure. 4-4). AgifOBP6 shared less than 30% amino acid sequence identity with reported EBF binding proteins in aphids (OBP3, OBP7, OBP9) as well as in natural enemies such as EcorOBP15 in *E. corollae* (Wang et al., 2022) and CpalOBP10 in *C. pallens* (Li et al., 2017) (Figure. 4-4). Anyhow, the crucial binding residue prediction of AgifOBP6 that interacts with EBF is critical for OBPs functional mechanism analysis. Considering that no crystal structure of any orthologue of AgifOBP6 is available, we employed a computational procedure to firstly choose a template for AgifOBP6 and then model the three-dimensional (3D) structure. The sequence alignment analysis using the Protein Data Bank (PDB) database of a web server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that AgifOBP6 shares 33.9% amino acid identity with CpalOBP4 (PDB ID: 6JPM, has a resolved protein crystal structure) of *C. pallens*, which is greater than the minimum requirement (30%) for protein modeling(reference). The 3D structure of the AgifOBP6 protein with CpalOBP4 as the template was predicted using the program SWISS-MODELel. As shown in Figure. 4-4, the structural comparison revealed that the amino acids of AgifOBP6 form seven α-helical structures (α1–α6) (Figure. 4-4B, Figure. 4-4C) as CpalOBP4 does, which is similar to those OBPs of LUSH (Laughlin et al., 2008), *Anopheles gambiae* (Ziemba et al., 2013)*, H. axyridis* (Qu et al., 2022). Moreover, AgifOBP6 has a similar internal cavity structure as CpalOBP4's (Figure. 4-4A). Which suggests that CpalOBP4 could be a reliable model in subsequent analyses. The models predicted by Homology modeling were named as Mod-AgifOBP6. For the predicted protein model, VERIFY3D, ERRAT, and Procheck were used to analyze the accuracy and reliability. The VERIFY3D (Figure. 4-5), ERRAT (Figure. 4-6), and Procheck (Figure. 4-7) showed that the models of Mod-AgifOBP6 were reasonable. The protein structure of AgifOBP6 was composed of six typical α-helices, forming a hydrophobic binding cavity, which are the important features of insect OBPs (Figure. 4-4C). According to the affinities between recombinant proteins and chemicals, we selected (E)-β-farnesene and its analog CAU-II-11 as the target ligand to study the docking conformation and binding energy with AgifOBP6 protein. The binding energy between AgifOBP6 and EBF and CAU-II-11 was then calculated, and the results showed that the docking binding energies were negative and the total value are -35.116863, -48.893936, respectively. And the distances of all potential interaction residues were less than 4 Å (Figure. 4-4F, Figure. 4-4I), indicating that

there is a strong interaction of AgifOBP6 with EBF and CAU-II-11. For AgifOBP6, seven residues, including ARG32, LEU35, LYS36, HIS39, TRP53, PHE144, and PRO146, were critical for binding affinity to EBF based on hydrophobic interactions. And LEU28, VAL31, ARG32, TRP5339, HIS57, and PHE144, were critical for binding affinity to CAU-II-11. Hydrophobic interactions were the important linkages of AgifOBP6 with EBF and its analog CAU-II-11.

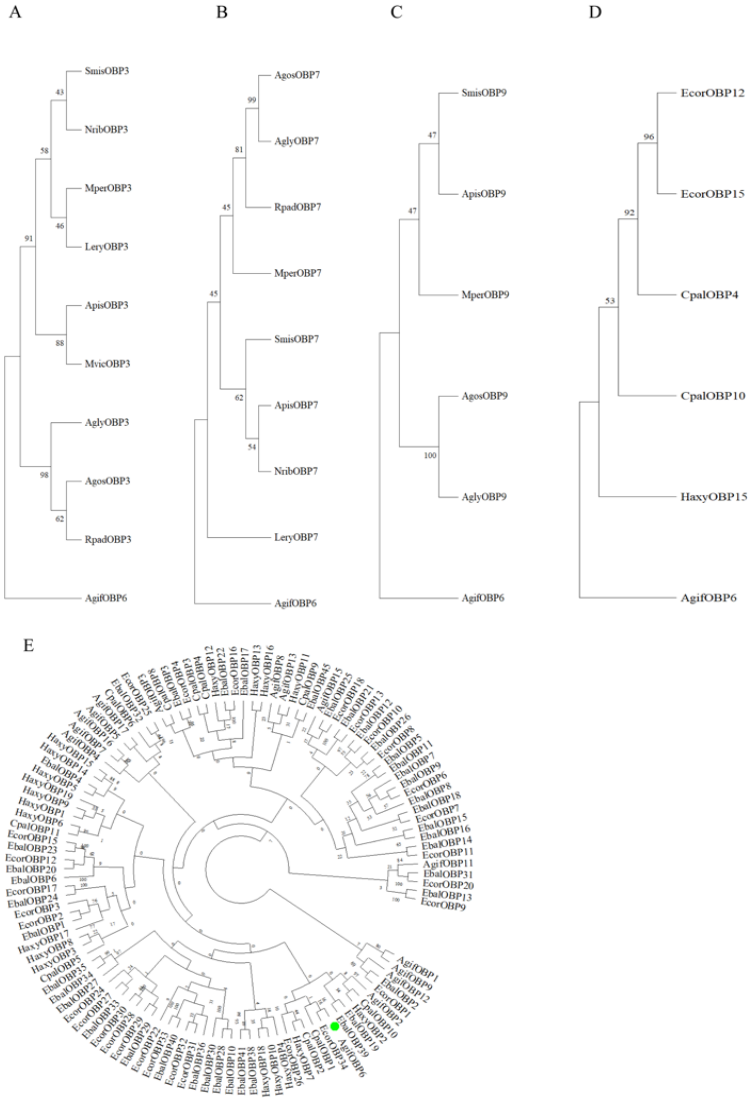

Figure. 5-4 The phylogenetic tree of odorant-binding proteins (OBPs) between aphids and their natural enemies.

A: The phylogenetic tree of odorant-binding proteins (OBPs) between parasitoids and predators. B: The phylogenetic tree of AgifOBP6 and OBP3 in aphids. C: The phylogenetic tree of AgifOBP6 and OBP7 in aphids. D: The phylogenetic tree of AgifOBP6 and OBP9in aphids. E: The phylogenetic tree of AgifOBP6 and alarm pheromone binding OBPs in natural enemies of aphids. These phylogenetic trees were listed with 22 putative OBP3/7/9 from 9 aphid species and 114 OBPs from 5 natural enemy species of aphids. Details and abbreviations of the 9 aphid species are *Myzus persicae* (Mper), *Sitobion avenae* (Save), *Aphis gossypii* (Agos), *Acyrthosiphon pisum* (Apis), *Aphis glycines* (Agly), *Megoura viciae* (Mvic), *Rhopalosiphum padi* (Rpad) and *Lipaphis.erysimi* (Lery); the natural enemy species are *Aphidifus gifuensis* (Agif), *Harmonia axyridis* (Haxy), *Chrysopa pallens* (Cpal), *Episyrphus balteatus* (Ebal) and *Eupeodes corollae* (Ecor).

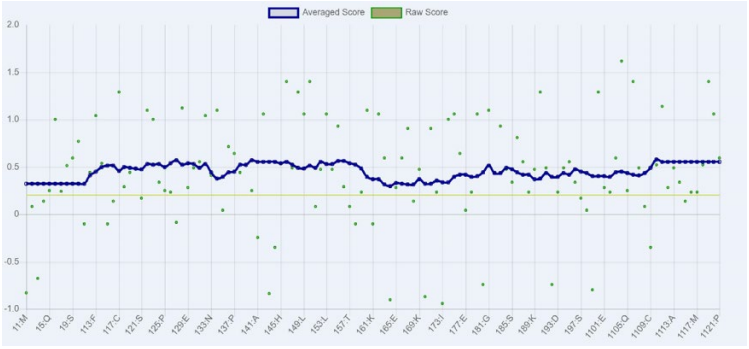

Figure. 5-5 The VERIFY3D of models Mod-AgifOBP6

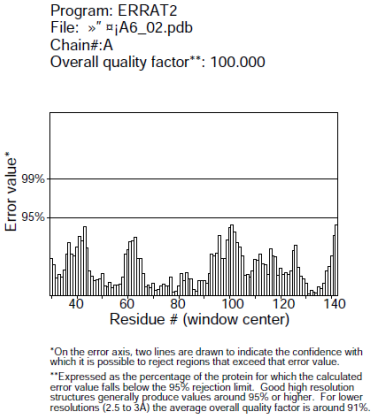

Figure. 5-6 The ERRAT result of models Mod-AgifOBP6

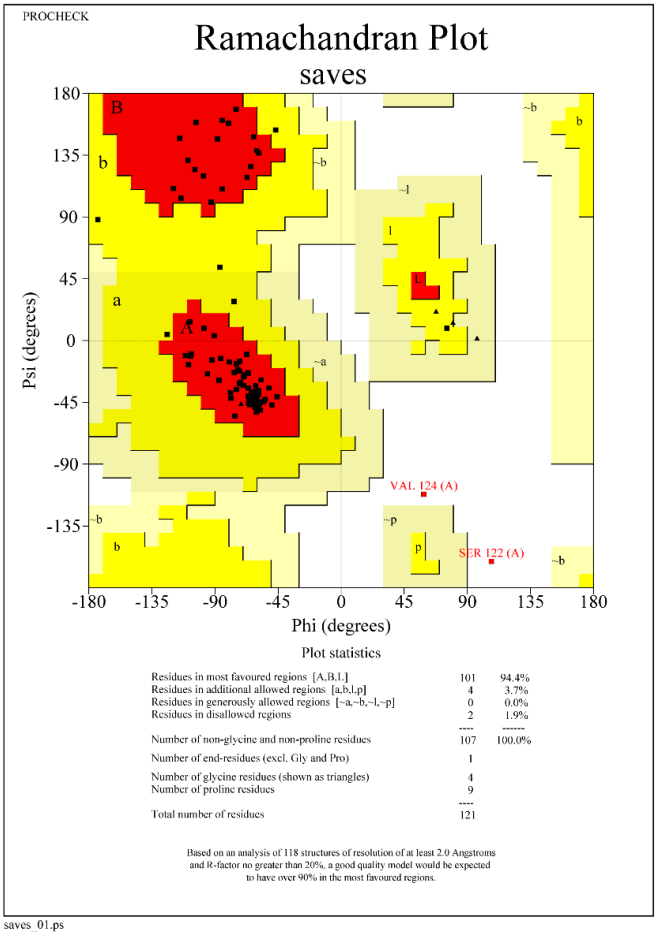

Figure. 5-7 The Procheck result of the models Mod-AgifOBP6

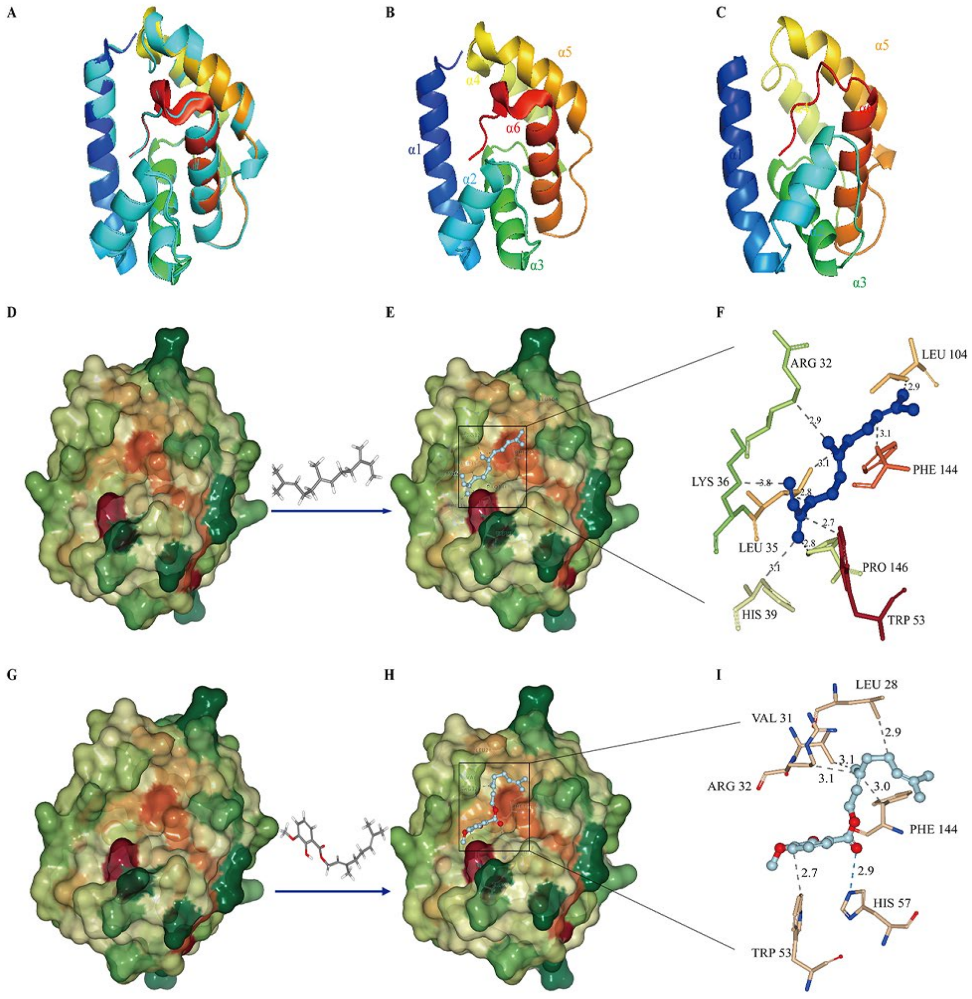

Figure. 5-8 Structural modeling and patterns analysis of AgifOBP6 for the molecular interactions with E-β-farnesene and its analog CAU-II-11.

(A): Superposition of the two odorant-binding proteins (OBPs) from the matching panels B and C in the same orientation. (B): CpalOBP4 (PDB ID: 6JPM). (C): AgifOBP6. (D),(G): Surface hydrophobicity of AgifOBP6; (E),(H): Surface hydrophobicity of AgifOBP6 binding with E-β-farnesene and its analog CAU-II-11 (dodger green, most hydrophilic; orange, intermediately hydrophobic; red, most hydrophobic). Key Residues within 4 Å of the E-β-farnesene and its analog CAU-II-11 are presented in F and I, respectively.

V.3.5. Western blot and Immunocytochemical localization of AgifOBP6

Previous works had revealed that AgifOBP6 was mainly expressed in the antennae of both sexes of *A. gifuensis* at the transcript level (Jiang et al., 2022). In the Western blot examination, staining of recombinant OBP6 and antennal extract with anti-AgifOBP6 antiserum revealed a prominent band at 15 kDa, which was comparable in size to the expected AgifOBP6 (approximately 13.65 kDa) (Figure. 4-9B). It was further complemented by the whole-mount immunolocalization investigations (Figure. 4-9A). Results showed sensillum distribution in details of AgifOBP6. It is commonly abundant in the lymph surrounding the sensory dendrites of the olfactory sensilla. In particular, sensilla placodea which located equally distributed around all flagellomeres except the first one (Figure. 4-9C, Figure. 4-9D); and also on the antennal tip (Figure. 4-9E); besides, AgifOBP6 is expressed in Sensilla coeloconica presented on each antenna segment (Figure. 4-9F, Figure. 4-9G) as well as in the sensilla trichaidea which are found on all segments except the radicula (Figure. 4-9H, Figure. 4-9I).

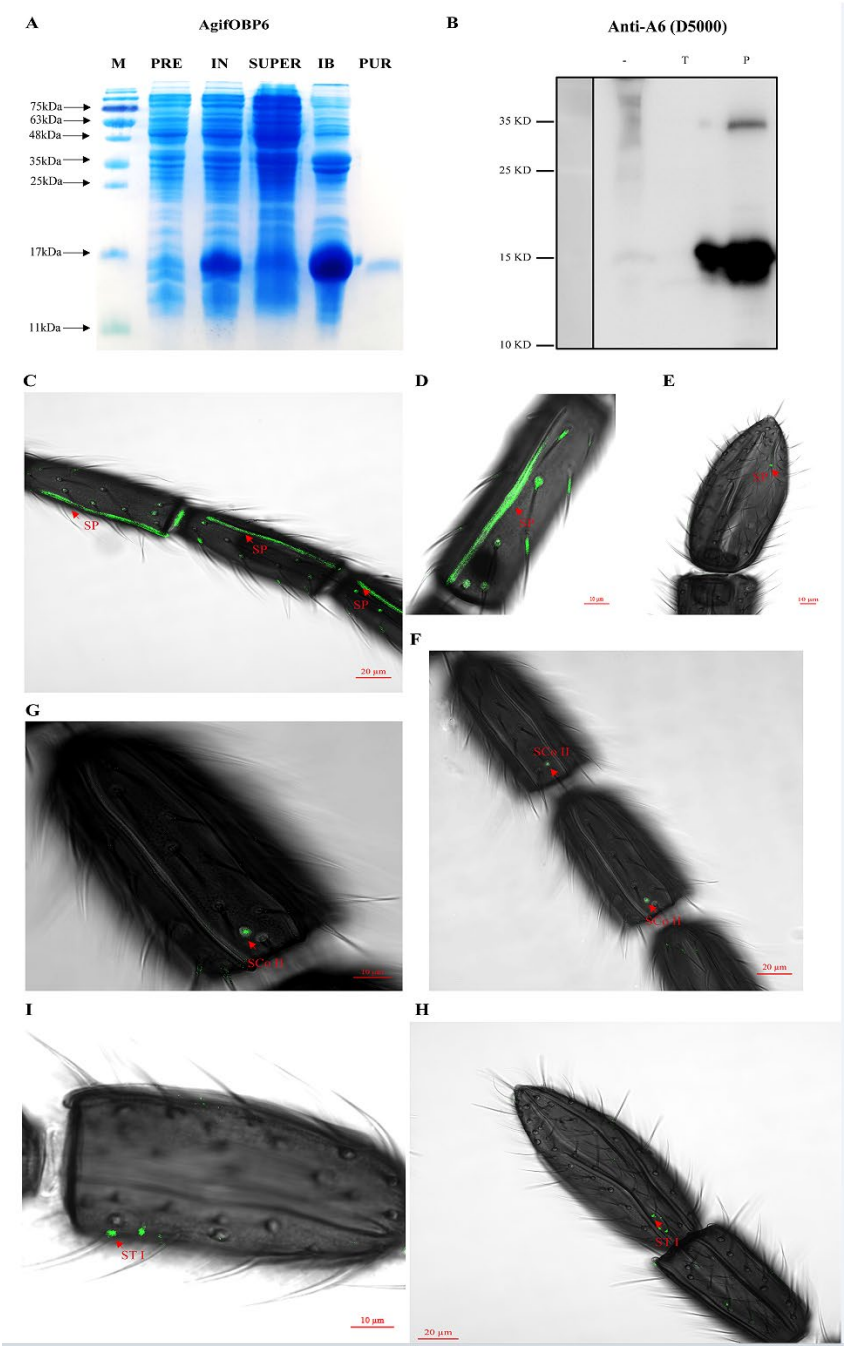

Figure. 5-9 Heterologous expression, western blot and immunolocalization analysis of AgifOBP6.

(A): the mature AgifOBP6 protein was heterologous expressed and purified. Lane M: molecular weight PR1910 (11-180 kDa); M: Marker,11, 17, 25, 35, 48, 63, 75, 100, 135, 180 kDa; PRE: None induced pET-28a (+)/AgifOBP6; IN: Induced pET-28a (+)/AgifOBP6; Super: pET-28a (+)/AgifOBP6 Supernatant; IB: pET-28a (+)/AgifOBP6 inclusion body; Pur: Purified pET-28a (+)/AgifOBP6 without His-tag. (B): western blot analysis of AgifOBP6. The line "-": Negative control; Line "T": the antennae crude of female *A. gifuensis*; Line "P": the recombinant AgifOBP6. (C), (D); immunolocalization of AgifOBP6 expressed in Sensilla placodea located equally distributed around all flagellomeres of *A. gifuensis* female, except the first flagellomere and on the antennal tip (E); (F), (G): antiserum of anti-AgifOB6 was detected on Sensilla coeloconica presented on each antenna segment; (H), (I) antiserum of anti-AgifOB6 was detected on Sensilla trichaidea which are found on all segments except the radicula of *A. gifuensis*. Bars in (C), (F), (H) 20 μm; bars in (D), (E), (G), (I): 10 μm.

V.3.6. EBF induced interactions between aphid S. miscanthi and aphidius A. gifuensis

The behavior traits were grouped and separately collected from aphidius. Data were listed in terms of probe times and attacking times. The probing results showed less interest of aphidius in winged aphids of aphidius than nymphs as well as wingless adults. Whereas, there were not any significant differences between the nymph and wingless adults (Figure. 4-10D). Further, EBF did not significantly stimulated the already high level of probing times of both nymph and wingless aphids to a higher level (Figure. 4-10D). Aphidius showed no more differences on probes between EBF treated winged aphids and nymphs as well as wingless aphids. Hence, EBF treatment significantly increased the probe times on winged aphids. It, therefore, reached the level of no statistical difference with EBF treated wingless adults and nymphs as well as their controls.

Interestingly, the following attacks on aphids (parasitism) were corresponding to the probes. Aphidius were more excited (more probes) to EBF treated aphids than trimyristoyl triglyceride (solvent) and blank control

In the aphid group, after 24h to 36h of free walking, the final parasitism rate was calculated using data collected on the 12th day and all offsprings were removed during entire investigation period. The percentage of mummies in winged adult was the lowest as 35%, and mummies of both nymphs and wingless aphids were 78.5% and 90% respectively. They were all significantly higher than winged adults but not significantly different from each other (Figure. 4-10G). The mummy rate of EBF treated winged adults significantly increased than blank control which is consistent with results of investigation on probe and attacking (Figure. 4-10G). While, another investigation after 12 d of free walking with all 2nd generation offspring retained showed that, 1. a significantly higher survival number of winged adults than both nymph and wingless adults (Figure. 4-10H). 2. for EBF treated winged adults, aphidius finally reduced their populations to a level that was the same as wingless adults, although their parasitoid rate was lower as 53.5% (Figure. 4-10H).

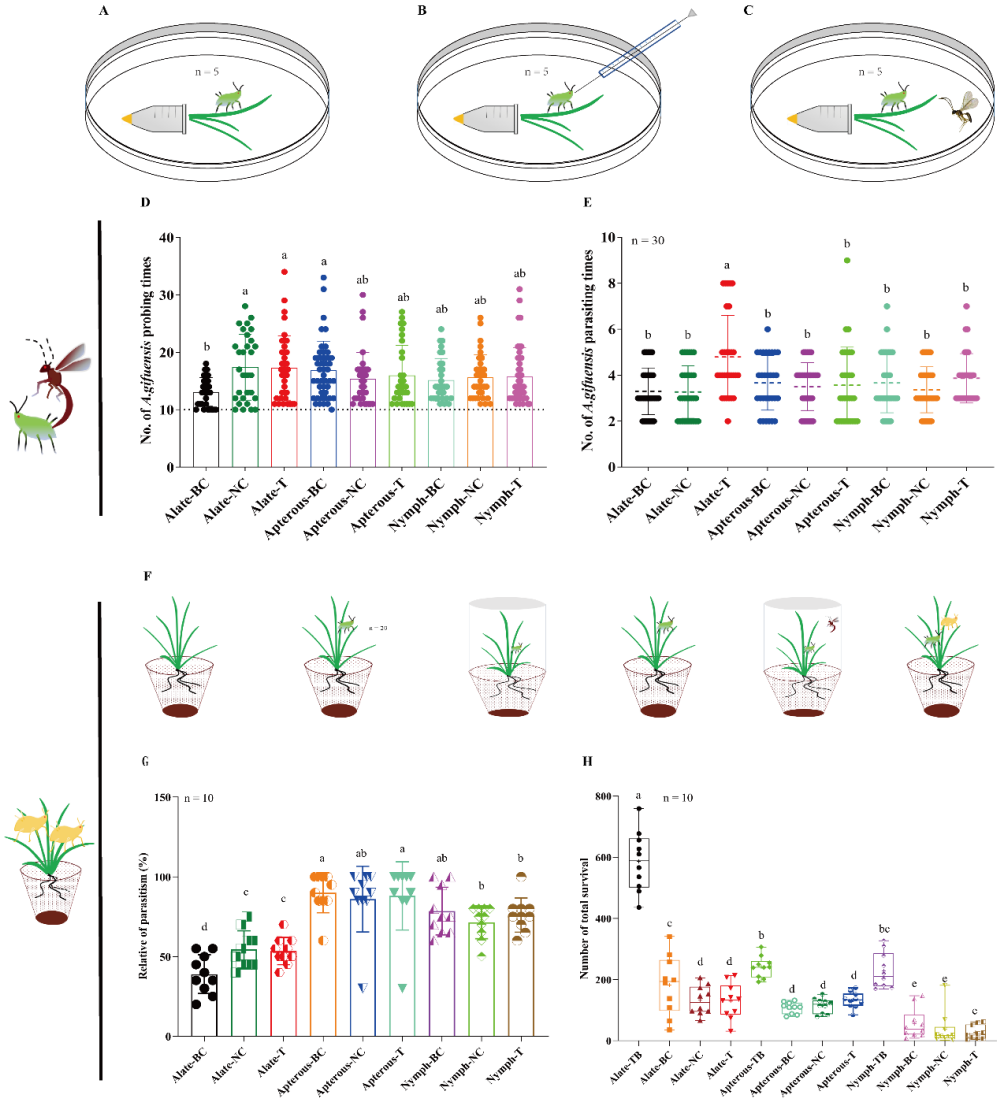

Figure. 5-10 The schematic of coating low concentration of E-β-farnesene on the pronotum of *S. miscanthi* and the foraging behavior of *A. gifuensis* responses.

(A)-(C): The diagram of coating low concentration of E-β-farnesene on the pronotum of *S. miscanthi*; (D): Number of *A. gifuensis* probing times, n ≥ 30; (E): Number of *A. gifuensis* parasitizing times, n ≥ 30; (F): The diagram of *A. gifuensis* foraging behavior based on coating EBF on *S. miscanthi*. (G): Relative of *S. miscanthi* parasitism. (H): Total number of surviving aphids. nymph-NC: nymph negative control, nymph-T:

nymph treatment; apterous-BC: apterous blank control, apterous-NC: apterous negative control, apterous-T: apterous treatment; alate-BC: alate blank control, alate-NC: alate negative control, alate-T: alate treatment. The different lowercase letters (a, b, c) indicate significant differences (one-way ANOVA followed by Tukey's HSD multiple comparison test, $P < 0.05$).

V.4. Discussion

V.4.1. Screening for EBF binding protein

Aphid OBPs extensively respond to EBF with complex patterns, which shows strong olfactory plasticity among the aphid species. However, related researches on their parasitoids are scarce. In our present work, there were up to 5 OBPs in total responded to EBF induction. Interestingly, AgifOBP6 was the only OBP responded to EBF in a wide range of doses from low to high (4ng to 4000ng) by up regulation. The affinity of AgifOBP6 to EBF was further confirmed by a subsequent competitive ligand binding test, which indicated that AgifOBP6 may play in the molecular basis of EBF recognition in *A. gifuensis*. While, the up-regulation of AgifOBP9 and AgifOBP12 were limited to a specific dose of 4 and 400 ng EBF respectively, and the down-regulation of AgifOBP7 and AgifOBP15 was limited to a high dosage of 4000ng. Like AgifOBP6, AgifOBP7 and AgifOBP9 were identified as having affinity for EBF as well, although the affinities of them were much weaker as weak and mediate, respectively. However, the affinity data of AgifOBP12 and AgifOBP15 with EBF could not be obtained as they were not able to be purified successfully. The olfactory perception in insects as well as other animals is generally dose-dependent. Dose beyond the threshold range, either too high or too low will lead to a decrease in response. Thus, the up- regulation induced by low dose EBF i.e 40ng suggested that AgifOBP9 was associated with the high sensitivity of aphidius to EBF. Therefore, AgifOBP9 could collaborate with AgifOBP6 to ensure the sensitivity of *A. gifuensis* in low doses of EBF. And once EBF dose is above the threshold, AgifOBP7/15, the two down-regulated OBPs by EBF induction may neutralize AgifOBP6 by down-regulating their expressions, and the response to excessively high doses of EBF was achieved through the cooperation of three OBPs (AgifOBP6, AgifOBP7 and AgifOBP15 at 4000ng).

In summary, a basic pattern was figured out for EBF induced OBPs. AgifOBP6 is always up-regulated by EBF induction, and there are two more up-regulated EBF-binding proteins interacting with AgifOBP6 at lower concentrations. While, at higher doses of 4000ng, down-regulated EBF-binding proteins may interact with AgifOBP6 were found.

Besides, we noticed that AgifOBP7 and AgifOBP9, the other two EBF binding OBPs reported in our previous works are mainly expressed in leg of female, instead of antennae (Jiang et al., 2022), which suggests the possibility of other physiological functions than olfaction. Although tried numerous experimental settings reported earlier (Prestwich, 1993; Wang et al., 2021; Jiang et al., 2022), we failed obtaining AgifOBP12, AgifOBP13, AgifOBP15, and AgifOBP17.

The most notable binding affinity was between AgifOBP6 and EBF, which had demonstrated the best binding property with Ki values of 6.26E-7 ± 3.82E-6 μM (Figure. 4-3J, Table 4-2). And even diluting 100 times (c = 20 nM/L), AgifOBP6 still had showed a strong binding property with EBF (Ki = 0.48 ± 0.01 μM) (Figure. 4-3J). Western blotting and immunocytochemical localization of AgifOBP6 further validated the expression in antenna. AgifOBP6 was found in sensilla placodea (Figure. 4-9C, 4-9D). There are many multiple pores on the surface of SP, which is consistent with the putative theory that sensilla placodea likely has an olfactory function (Ochieng et al., 2000); AgifOBP6 is also labeled in sensilla coeloconica presented on each antenna segment (Figure. 4-9F, 4-9G), which is consistent with the discovery that SCo I and SCo II are thought to have olfactory or thermos functions (Bourdais et al., 2006; Xi et al., 2010). Furthermore, the antiserum of AgifOBP6 was detected on the sensilla trichaidea (Figure. 4-9H, 4-9I), which is prevalent on all segments except the radicula of *A. gifuensis*. We hypothesize that sensilla trichaidea might have more function in female antennae beyond the putative mechano-receptive function in male and female *A. gifuensis* (Das et al., 2011).

The binding mechanisms of AgifOBP6 with two ligands (EBF and its analogue CAU-II-11) that displayed highly binding affinities were explored. As the three-dimensional (3D) structure of AgifOBP6 (Figure. 4-9C) that employed by a computational procedure, the docking results revealed negative docking binding energies were the main force to maintain such binding properties. Aphid EBF binding OBPs shared their orthologous genes among species. However, there obviously was no homology of EBF binding OBPs between aphids and aphidius, nor between aphidius and other aphid natural enemies such as *E. balteatus* and *Chrysopa pallens* (Wang et al., 2022; Li et al., 2017). It is clear that when diverged into different species, aphids acquired homologous EBF-binding proteins from their common ancestor. Since then, natural enemies co-evolved with aphids and gradually acquired the ability to detect EBF. Thus, their EBF binding proteins were independently evolved from each other and driven by convergent evolution.

V.4.2. EBF effects on parasitism of A. gifuensis

First of all, the probing times of aphidius wasps to aphids with different phenotypes or at different developmental stages were different. Once aphidius arrive near their prey, they generally begin frequent, excited probing. And residual EBF on the surface of wingless adults as well as nymph abdomen could stimulate probing even more. But for winged aphids, the wing tissue covers abdomen, preventing antennae from detecting residual EBF. The wing tissue interferes with the contact between antennae and the residual EBF, which leads to the lack of interest in winged aphid, so the probing times is less than that of wingless adult and nymph. However, we found that no significant difference in the number of subsequent attacks on winged adults, wingless adults as well as nymph. Interestingly, the differences in the number of mummies between each group were not consistent with the results of parasitism times. The winged aphid did not thus obtain a comparable number of mummies to the wingless aphid and the nymph, but significantly fewer of them. Which suggested that

the wing tissue prevented aphid from being parasitized to the correct site, thus reducing the success parasitism rate.

EBF promoted the efficiency of biological control, specially on winged aphids. Our results showed that the application of EBF on the dorsal abdomen of winged aphid caused more frequent probing by aphidius wasp, which was as frequent as that of the wingless adult and nymph aphid. This supported the idea that there were remains of EBF on the surface of aphid that attracts the aphidius' attention and gets it excited and probe frequently. For example, EBF was detected from the fresh and dried cornicle of *S. avenae* (Micha & Wyss, 1996) and recent work also found that the extraction from the surface of *A. pisum* contains EBF which serves as a short-range guide for the larve of *E. corollae* (Wang et al., 2022). The length of the wing tissue covers the entire abdomen and extends beyond the end of the abdomen. Which may physically interfere with the detection of antennae to abdominal EBF, thus reducing the interest of aphidius to winged adults. But when apply EBF to winged aphid, it caused the part of wing tissue that covered the abdomen to be coated by EBF. This explained why trace EBF significantly increase the frequency of winged aphid probe and attacks, further increased the rate of mummies. Although EBF significantly increased the parasitism rate of winged aphid, it was still significantly lower than that of wingless aphid and nymph which may also due to the obstruction of wing tissue. For nymph and wingelss aphids, compared with body size, it is more difficult for the aphidius accurately attack the smaller aphid. Which may result in a difference in parasitism rate between them. The total survival of the winged aphid was significantly higher than that of the wingless and nymph in TB groups (Figure. 4-10H), a reasonable explanation being that the winged aphid is also divided into colonized aphids and migratory aphids, and when the winged aphid is colonized, the flight muscle is decomposed in its body, and it has the ability to transform into a reproductive aphid (Bai et al., 2022). The wingless and nymphs were parasitized by a large number of initial aphids, whereas the winged aphids were parasitized by a small number. As a result, the winged aphids that survived produced a considerably higher number of offspring nymphs than the wingless and nymphs. The number of surviving aphids in the nymphal aphid groups (BC, NC, T) was much lower than in the wingless adult aphid groups, owing to the less productive aphids in the nymphal aphid had 2-3 days less developmental length than the wingless adult aphid, but the nymph likewise grew into an adult after 2-3 days. Adult aphid numbers have also grown.

EBF as a critical infochemical in the tri-trophic level interactions among plant-aphid-aphid natural enemy, its biological significance to aphid seems to be more likely to ensure the survival of the aphid population through a few escapes than to ensure the overall survival of the current generation. The majority of aphid species respond to EBF at a pretty low dosage of 1ng to 10 ng in the field (Jing-Gong et al., 2002). Whereas, aphids typically emit cornicle droplets only after being physically attacked (Nault & Phelan, 1984), resulting in the emitter's escape in about 10% of attacks (Dixon, 1958; Edwards, 1966). Further, within an aphid colony, generally a single or a few aphids are attacked at the same time, and the signal is not amplified by the emission of neighboring aphids (Hatano et al., 2008). It is important to note that EBF applied in this study was dissolved in triglycerides (TIC). And the reason we chose

TIC is that the major component of aphid droplet secreted from cornicle are TIC (Callow et al., 1973). Although the solubility of EBF in different solvents is different, the amount of EBF volatilized out is definitely not the same as the amount we applied to insects, which was not identified in our present study.

Wasp parasitism rates are relatively low when evaluated in the field, for example, the highest parasitic rate of aphids in maize fields was only 1.79% in Jilin Province, China (Zhang et al., 2020), the calculation of the parasitism ratio of S. miscanthi is estimated to be between 10% to 15% (Yang et al., 2017; Xu et al., 2018; Liu et al., 2021). *A. gossypii* showed a similar phenomenon (Yang et al., 2021). While the biological significance to natural enemies seems to be taking advantage of EBF to ensure every full meal. EBF triggers attack behavior in predators (Kindlmann et al., 2010) and parasitoids, obviously serving as a stimulant for host/prey finding and attacking. For instance, the release of alarm pheromone by *S. avenae* is attractive for *A. rhopalosiphi* parasitoids (Micha & Wyss, 1996), while the emission of cornicle secretions by *A. pisum* stimulates a strong oviposition attack response from *A. ervi* females (Battaglia et al., 1993). Our work confirmed that EBF could promote the biological control efficiency of *A. gifuensis*, especially for winged aphids, by coating trace levels of EBF on the dorsal abdomen of *S. miscanthi*.

V.5. Conclusion

In the present study, our results demonstrated a high plasticity of OBPs on EBF recognition in *A. gifuensis*. AgifOBP6 was up-regulated by EBF in a wide dose range and showed a strong affinity for EBF. Low consistency between AgifOBP6 and EBF binding OBPs from aphids as well as other natural enemies, indicated that the EBF recognition OBPs evolved separately in aphidius *A. gifuensis* and its host aphid, but were eventually driven to a common biological function by convergent evolution.

Homology modeling and molecular docking confirmed that hydrophobic interactions were the main forces between AgifOBP6 with EBF and its analog. And AgifOBP6 is expressed in the peripheral sensilla organs of antennae by whole-mount immunolocalization, such as the sensilla placodea, sensilla coeloconica, and sensilla trichaidea. Then, by observing the behavioral interaction manipulated by EBF between aphid and aphidius and investigating the mummies and survival individuals, it was confirmed that EBF could promote the biological control efficiency of *A. gifuensis*, especially on winged aphids. Our findings may shed insight into parasitic wasps' olfactory sensitivity to host hints, as olfactory organs recognize pheromones and odorant substances that influence both host hunting and oviposition activities and will help us better understand parasitic wasp host forging behaviors, this will aid in the strengthening and better utilization of *A. gifuensis* as a powerful and natural biocontrol strategy.

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V.7. Supplementary file

List of the protein names and amino acid sequences of OBPs between aphid and its natural enemies used in the phylogenetic analysis.

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> EbalOBP2
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> EbalOBP3
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> EbalOBP4
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> EbalOBP5
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ENCADDNSEGSSPLDWGYRYYKCFKDNEVLYETMRKARFIQPDVVGKQ-
> EbalOBP6
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-
> EbalOBP7
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> EbalOBP8
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> EbalOBP9
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E-
> EbalOBP10
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> EbalOBP11
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> EbalOBP12
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> EbalOBP17
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> EbalOBP18
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> EbalOBP24
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> EbalOBP26
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> EbalOBP27
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AFRLSNCFSPALIELLQKKV-
> EbalOBP28
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> EbalOBP29
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REMFDFCKDESGGEDECDKPFKIYLCLKKLSDIFKY-
>EbalOBP30
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EFGFHKCETAIKLHRCFLGVRNYAITQIYSS
> EbalOBP31
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> EbalOBP32
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> EbalOBP34
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RTRRRAALLEVLSRKV-
> EbalOBP35
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> EbalOBP36
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> EbalOBP38
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> EbalOBP39
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> EbalOBP40
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> EbalOBP41
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> EbalOBP45
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> EcorOBP1
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> EcorOBP2

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> EcorOBP3

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> EcorOBP4

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> EcorOBP6

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>EcorOBP7

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>EcorOBP8

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>EcorOBP9

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>EcorOBP10

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>EcorOBP11

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>EcorOBP12
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>EcorOBP13
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>EcorOBP17
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>EcorOBP18
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>EcorOBP20
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>EcorOBP24
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>EcorOBP25
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>EcorOBP27
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>EcorOBP28
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>EcorOBP29
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KKLSDIFKY
>EcorOBP30
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>EcorOBP32
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CKNEPGSGCEVAMNMHLCFLKNKAY
>EcorOBP34
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PCDCGYNVFKCVLDGMMAMEEQ

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Chapter VI General discussion, conclusion and perspectives

VI.1. General Conclusion

Aphids are tiny sap-sucking insects that damage in yield and quality of agro products by plundering wheat nutrition, transmitting plant virus and excreting honeydew. Among these cereal aphids, *S. miscanthi* is the dominant species and also an ideal model for studying three trophic levels interactions between insects and natural enemy. However, the genome information for this species has not been published yet. Therefore, from the perspective of green control of wheat pests and the basic frontier research on insects, the study of the genome of the *S. miscanthi* is of great importance. Here, we analyzed the entire genome of a female aphid colony using long-read sequencing and Hi-C data to generate chromosome-length scaffolds and a highly contiguous genome assembly. The final draft genome assembly from 33.88 Gb of raw data was approximately 397.90 Mb with a 2.05 Mb contig N50. Nine chromosomes were further assembled based on Hi-C data to a 377.19 Mb final size with a 36.26 Mb scaffold N50. 2. The identified repeat sequences accounted for 26.41% of the genome, and 16,006 protein-coding genes were annotated. According to the phylogenetic analysis, *S. miscanthi* is closely related to *Acyrthosiphon pisum*, with *S. miscanthi* diverging from their common ancestor approximately 25.0-44.9 million years ago.

Utilizing biocontrol agent to control aphides is an important strategy. *A. gifuensis* is one of the most common endoparasitoids of the green peach aphid *M. persicae* and grain aphid *S. miscanthi* in the field of China. Insect odorant-binding proteins (OBPs) play vital roles in odor perception during feeding, host searching, mating and oviposition. In our following works, a comparative antennal transcriptomic analysis was applied between male and female *A. gifuensis* to lay the solid foundation of covering the potential odor binding protein genes and their expression characteristics. In this part, fifteen *AgifOBP*s were predicted, and 14 of them were identified by gene cloning, including 12 classic OBPs and 2 minus-C OBPs. As expected, all OBPs were mainly expressed at high levels in antennae, heads or legs which are sensory organs and tissues.

In the old and yet vibrant tritrophoic interaction system of plant-aphid-nature enemy, (E)-β-farnesene (EBF) is the well-studied infochemical among those semiochemicals. EBF can repel aphid and attract the natural enemy. Until now, at least 3 types of EBF binding proteins have been found within 9 aphid species. Previous works also have illustrated the behavior traits within aphids and the predators, although several works question the function of EBF. The potential molecular mechanisms have been made within the predator and the main points were support the idea that predator mainly utilize the HIPV as a reliable clue to locate their host. In this part, we furtherly discovered the molecular mechanisms of parasitoids recognizing and utilizing EBF and found a potential convergent evolution mechanism between host aphids and it's parasitoid wasp. Functionally work revealed that ten AgifOBPs from *A. gifuensis*, among which, AgifOBP6 was the only OBP up-regulated by various doses of EBF, it showed a strong binding affinity to EBF in vitro as well. Lack of homology between AgifOBP6 and EBF binding proteins from aphids or from other aphid natural enemies

supported that this is a convengent evolution among insects from different orders driven by EBF. Molecular docking of AgifOBP6 with EBF revealed ineractive key residues and hydrophobic forces as the main forces. And whole-mount immunolocalization showed that this is a widely expressed OBP among various antannal sensilla. Furthermore, two bioassays using grain aphids *S. miscanthi* indicated that trace EBF may promote the biological control efficiency of *A. gifuensis,* especially on winged aphids.

In total, we generated a high-quality draft of the *Sitobion miscanthi* genome. This genome assembly promotes research on the lifestyle and feeding specificity of aphids and their interactions with each other and species at other trophic levels. It can serve as a resource for accelerating genome-assisted improvements in insecticide resistant management and environmentally safe aphid management. Further, the prediction of OBPs in *A. gifuensis* and their spatial expression patterns, which are mainly expressed in female legs, laying the foundation for the dissection of the contribution of OBPs to chemosensation in *A. gifuensis*. Additionally, our work found that OBPs between *A. gifuensis* and its host aphid were separately evolved but eventually driven to a common biological function by convergent evolution. And also offers a novel perspective on the biological control for aphids from reducing the initial population of migrant biotype aphids from source areas: promoting the biological control efficiency of parasitoids to winged aphids before the immigration, by low concentration of EBF application.

VI.2. General discussion and perspectives

Aphids (Hemiptera: Aphididae) are a group of phloem feeding insects numbering more than 5 000 extant species (Favret, 2014), and most are common crop pests. *S. miscanthi*, a grain aphid, is frequently mistaken for *Sitobion avenae* (Fabricius 1775) in China (Zhang, 1999). It is one dominant species in China's wheat-growing regions and is a widely dispersed specialists in sucking grain sap. We present for the first time a chromosome-level genome sequence of the *S. miscanthi* strain Langfang-1, which displays higher-quality assembly data indexes than prior scaffold-level aphid genomes. A 2n=18 karyotype for *S. miscanthi* was supported by the majority of the sequences assembling into 9 scaffolds (Chen et al., 1985; Chen et al., 2019). In total of 8 aphid species have been assembled and analyzed in the genome annotation data, including the pea aphid *A. pisum* (International Aphid Genomics Consortium), peach aphid *M. persicae* (Mathers et al., 2017), soybean aphid *A. glycines* (Wenger et al., 2017), Russian wheat aphid *Diuraphis noxia* (Burger & Botha, 2017), cherry-oat aphid *R. padi* (Thorpe et al., 2018), black cherry aphid *Myzus cerasi* (Thorpe et al., 2018), the cotton aphid *A. gossypii* (Quan et al., 2019), and the corn leaf aphid *Rhopalosiphum maidis* (Chen et al., 2019). Phylogenetic analysis show that *S. miscanthi* is closely linked to *A. pisum*, with a time interval between their divergence and that of *S. miscanthi* being roughly 25.0–44.9 million years.

More recently, the English (*Sitobion avenae*) and Indian (*Sitobion miscanthi*) grain

aphids, have just been deciphered and compared based on a new generation of chromosome-scale genome assemblies. Low levels of genetic divergence exist between *S. avenae* and *S. miscanthi* and comparison of haplotype-resolved assemblies revealed that the *S. miscanthi* isolate used for genome sequencing is likely a hybrid, with one of its diploid genome copies being closely related to *S. avenae* (~0.5% divergence) and the second being substantially more divergent (> 1%) (Mathers et al., 2022). *S. avenae* and *S. miscanthi* are members of a species complex with numerous very distinct lineages that predate the beginnings of agriculture, according to analyses of genotyping-by-sequencing (GBS) data of grain aphids from the UK and China. Grain aphid diversity and, consequently, the evolutionary potential of this significant pest species have both been greatly aided by hybridization (Mathers et al., 2022). With the development of genomics, the genomics of more cereal aphids are uncovering, including *S. miscanthi, S. avenae* and *M. dirhodum* (Jiang et al., 2019; Mathers et al., 2022; Zhu et al., 2022). Those work will serve as a crucial source of information for comprehending the olfactory evolution, wing dimorphism, and detoxifying metabolism of aphid insects.

The behavioral response of insects to olfactory cues is essentially driven by feeding, reproduction and habitat selection (Pelosi et al., 2014). Insect odorant-binding proteins (OBPs) play important roles in peripheral olfactory signal transduction, which connects info-chemicals in habitat with olfactory receptors (ORs) located on the olfactory nerve (Vogt, R. G., & Riddiford, L. M, 1981; Pelosi, 2006; Leal, 2013).

Once getting get the warning signal from the alarm pheromone, aphids typically cease feeding, move away from the signal, drop off sometime (Pickett et al., 1992, Zhang et al., 2017). (E)-β-farnesene (EBF) was demonstrated as the primary active component of alarm pheromone in most aphid species (Bowers et al., 1972; Francis et al., 2005). EBF perception received extensive attention since then. And recently, the progress on aphid EBF recognition mechanism has been well made. The first EBF-binding protein and the first discovered OBP in aphids, respectively, were both OBP3 from the pea aphid *A. pisum* (Qiao et al., 2009). OBP7 was subsequently showed to have a specific affinity for EBF in a variety of aphid species, including the pea aphid *A. pisum* (Zhang et al., 2017), the peach aphid *M. persicae* (Sun et al., 2012), the grain aphid *S. avenae* (Zhong et al., 2012), and the bird cherry-oat aphid *Rhopalosiphum padi* (Fan et al., 2017) and *Megoura viciae* (Bruno et al., 2018). More recently, it was revealed that OBP9 has affinities for a wide range of ligands, including EBF (Qin et al., 2020; Wang et al., 2021). So far at least 3 EBF-binding proteins have been discovered, which suggest that multiple OBP contacts may be required for peripheral EBF transmission. All 3 EBF binding proteins are orthologs among aphid species (Qin et al., 2020; Wang et al., 2021). Additionally, the substantial upregulation of OBP7 and OBP9 expression in response to EBF induction demonstrates the remarkable olfactory flexibility of aphids' olfaction (Zhang et al., 2021). Additionally, OR5, an olfactory receptor from aphids, has been shown to be in charge of EBF signal transduction through working with ORco (Zhang et al., 2017).

Natural enemies of aphids employ chemicals such as EBF, herbivore-induced

volatiles (HIPVs), green leaf volatiles (GLVs), and others to locate their target hosts for parasitism or predation (Song et al., 2010; Dong et al., 2008; CMD Moraes et al., 1998; Buitenhuis et al., 2004; Wang et al., 2022). Natural enemies must choose between dependability and detectability while analyzing the behavior of herbivores (Vet & Dick, 1992). Due to the low biomass of aphids, volatiles produced directly by herbivores are present in low concentrations in the environment yet provide reliable information about their presence. Thus, contact kairomones in the host cuticle and cornicle secretion are crucial in host recognition, while plant volatiles, especially those generated by aphid feeding, are utilized as long-range cues (Powell et al. 1998). Additionally, significant advancements were made in the molecular basis of olfaction. For instance, it has been noted that the lacewing *Chrysopa pallens* uses plant-derived substances, volatiles produced by pests, and EBF as foraging cues (Li et al., 2017). And CpalOBP10 demonstrated its affinity for both EBF and the volatiles found in green leaves. In the ladybeetle *Harmonia axyridis*, an essential natural enemy that consumes aphids, HaxyOBP15 demonstrated a wide binding profile with (E)-β-farnesene as well as other odor chemicals (Qu et al., 2022). Additionally, EcorOR3 and EcorOBP15, two EBF olfactory receptors, have been discovered to have a role in the sensing of EBF in hoverflies (*Eupeodes corollae*) (Wang et al., 2022).

Aphid parasitoids respond to host and host plant volatiles, like most parasitoid species, in order to find potential hosts (Du et al. 1997, 1998; Royer & Boivin, 1999). Although parasite-induced behavioral manipulation is a common occurrence, our understanding of the underlying neural pathways is still in its infancy. We sought to understand the chemosensory foundation of host discrimination by *A. gifuensis*, which enables it to select the "best" host available, in light of the knowledge already available about how and when the parasitoid marks the host. In order to find sources of food and oviposition, female parasitoids use electromagnetic radiation, sounds, sights (such as the color and shape of the aphids, Harmon et al., 1998), and scents (Vinson, 1976). Olfactory stimuli predominate, even though visual cues like the aphids' color and shape may occasionally play a role.

The mechanism by which parasitoids choose their hosts was divided into three different phases by Virson in 1976. During the host acceptance phase, the female's preference is directly influenced by the host's appropriateness and quality, as seen in *A. colemani* (Benelli et al., 2014). Poor quality hosts can cause development to be delayed, mortality to rise, and longevity and fertility to decrease (Mackauer et al. 1996, Roitberg et al. 2001). Larger aphids are typically regarded as better hosts (Cloutier et al. 2000). It has been demonstrated that *A. colemani* grown on larger hosts (*M. persicae*) are bigger and more fertile than parasitoids grown on smaller aphids (number of eggs per ovariole) (*Ap. gossypii*). However, parasitization rates on *Ap. gossypii* were more than three times greater than on *M. persicae* (Sampaio et al. 2008), indicating that parasitoid preferences are not exactly matched with host quality features and cannot be anticipated by just taking host qualities into account (Sampaio et al. 2001, Ode et al. 2005).

Even though there is little information available for *A. colemani*, the honeydew produced by aphid hosts serves as a source of host searching kairomones used by Aphidius species (such as *A. nigripes* and *Ephedrus cerasicola* Stary'). Even in the absence of an infection, some indications from the plant itself can help *A. colemani* locate its hosts. For instance, Lo Pinto et al. (2004) showed that uninfested plants might generate volatile cues that aid parasitoids in finding the host's environment.

Aphidius gifuensis is one of the most common endoparasitoids of green peach aphid *M. persicae* and grain aphid *S. miscanthi* in China. To successfully perceive the semiochemical cues of its host and plants, *A. gifuensis* evolved a complex chemosensory system. *A. gifuensis* (Yang et al., 2009), for instance, can tell the difference between healthy, mechanically injured, and aphid-infested plants (Dong et al., 2008). Additionally, it was discovered that both male and female *A. gifuensis* responded favorably to EBF and a variety of tobacco volatiles, including trans-2-hexenal, methyl salicylate, benzaldehyde, cis-3-hexen-1-ol, and 1-hexanal (Song et al., 2021). It has also been demonstrated that female Aphidius release the potent sex pheromone, which causes acute sexual orientation in males (Fan et al., 2018). It has also been demonstrated that female Aphidius release the potent sex pheromone, which causes acute sexual orientation in males (Fan et al., 2018).

Based on transcriptome data, many OBPs, CSPs, and chemosensory receptors in *A. gifuensis* have been predicted (Kang et al., 2017; Fan et al., 2018; Li et al., 2021). However, there is still a lack of information regarding the expression patterns of odorant-binding proteins in the different sensory organs of *A. gifuensis*. In our chapter III, we performed a comparative antennal transcriptomic analysis between male and female *A. gifuensis* and screened candidate odorant binding protein genes in this aphid parasitoid. Fifteen AgifOBPs were predicted, and 14 of them were identified by gene cloning, including 12 classic OBPs and 2 minus-C OBPs. The phylogenetic tree of these *AgifOBPs*, together with OBPs from 4 hymenopteran species, showed that the *AgifOBPs* segregate into the orthologous clades of the other species, rather than into *A.gifuensis* paralogous clades, this also suggests that these *AgifOBPs* might play different roles in odor recognition or have roles other than olfaction. Previous work found that OBPs are relatively conserved, which suggests that their function may be restricted to the common olfactory physiology of these insects. This theory is supported by several research findings about aphids' natural enemies. For instance, it has been extensively documented that the alarm pheromone EBF has affinities with the aphid OBP7 orthologs (Sun et al., 2012; Zhong et al., 2012; Fan et al., 2017; Qin et al., 2020). CpalOBP10, an aphid predator found in the lacewing *Chrysopa pallens*, shares a lineage with aphid OBP7 found in *S. avenae* and *A. pisum*, the affinity for EBF was likewise in line with that of aphid OBP7 orthologs (Li et al., 2017; Li et al., 2019). However, in our work, we found that no homology was found between AgifOBP6 with amino acid of either aphids' OBPs or natural enemies' OBPs, based on the results of EBF induction bioassay and fluorescence competitive ligand binding test, AgifOBP6 is the only OBP that was up regulated by EBF and showed a strong affinity for EBF. And AgifOBP6 shared less than 30% amino acid sequence identity

with reported EBF binding proteins in aphids (OBP3, OBP7, OBP9) as well as in natural enemies such as EcorOBP15 in *E. corollae* (Wang et al., 2022) and CpalOBP10 in *C. pallens* (Li et al., 2017). It is clear that when diverged into different species, aphids acquired homologous EBF-binding proteins from their common ancestor. Since then, natural enemies co-evolved with aphids and gradually acquired the ability to detect EBF. Thus, their EBF binding proteins were independently evolved from each other and driven by convergent evolution.

The spatial expression patterns among antennae, heads, thoraxes, abdomens and legs of AgifOBPs in both sexes revealed that five *OBPs*, namely *AgifOBP3*, *AgifOBP5*, *AgifOBP6*, *AgifOBP11*, and AgifOBP15, were expressed at a high level in the antennae, while four *OBPs*, *AgifOBP2*, *AgifOBP4*, *AgifOBP7* and *AgifOBP8*, were expressed at a medium level, and seven *OBPs*, *AgifOBP1*, *AgifOBP9*, *AgifOBP12/13*, and *AgifOBP17*, were expressed at a low level in the antennae. The antennal specific *OBPs* suggest their function of recognizing and binding odorants from the environment. Six *OBPs*, *AgifOBP2, AgifOBP4, AgifOBP5, AgifOBP7, AgifOBP13* and *AgifOBP17,* showed expression patterns among sensory and nonsensory organs, indicating their possible multiple functions in olfactory perception as well as other physiological processes such as development and reproduction. Both *AgifOBP1* and *AgifOBP9* showed higher expression levels in the legs than the other four tissues, which could be related to the adaptation of *A. gifuensis* during migration as we have discussed in previous study (Xue et al., 2016), and might be involved in the procedure of taste or volatile perception or be related to olfactory sensilla on the legs (Yasukawa et al., 2010; Harada et al., 2012). A similar condition was also found for *AgifOBP5*, which is expressed in small amounts in the head and leg, in addition to being expressed abundantly in antennae. Apart from antennae, alternatively, these OBPs expressed in other tissues may be responsible for corresponding functions. For example, *NlugOBP3* is highly expressed in the abdomen of *Nilaparvata lugens* and may be involved in juvenile hormone transport and play an important role in metamorphosis (He et al., 2011). Insect OBPs have been reported to act as carrier proteins in the male reproductive apparatus of mosquitoes (Li et al., 2008). After mating, the OBPs expressed by male moths are found on the surface of fertilized eggs, which helps the larvae to avoid cannibalistic behaviors (Sun et al., 2012). For parasitic wasps, *AconOBP8* was reported to be expressed predominantly in the abdomen (Li et al., 2021). Similar expression patterns of OBPs in the nonolfactory tissues were observed in *Sclerodermus sp*. (Zhou et al., 2015) and *M. pulchricornis* (Sheng et al., 2017). In this part, qPCR analysis revealed that *AgifOBP8* was also expressed in the female abdomen, and it can be speculated that *OBP8* may potentially function as a pheromone-binding protein for identifying a particular signal such as the sex pheromone component in mating or oviposition behaviors, although the active component of sex pheromone in this species is still unclear.

Ligand binding properties of AgifOBPs were further evaluated. We first analyzed two female leg specifically expressed AgifOBPs. AgifOBP9 displays a broad and high

binding property to aphid alarm pheromones, plant green volatiles and aphid sex pheromones. However, female leg specifically expressed AgifOBP7 displays poor affinity for all tested ligands except CAU-II-11 ((E)-3, 7-dimethylocta-2,6-dien-1-yl-2-hydroxy-3-methoxybenzoate), a reported (E)-β-farnesene analog with an exceptionally high binding affinity (Qin et al., 2020). In the chapter IV, we tested the binding characteristics of another 8 AgifOBPs *in vitro.* The most notable binding affinity was between AgifOBP6 and EBF, which had demonstrated the best binding property. And even diluting 100 times, AgifOBP6 still had showed a strong binding property with EBF. Western blotting and immunocytochemical localization of AgifOBP6 further validated the expression in the antenna. AgifOBP6 was found in sensilla placodea. There are many multiple pores on the surface of SP, which is consistent with the putative theory that sensilla placodea likely has an olfactory function (Ochieng et al., 2000); AgifOBP6 is also labeled in sensilla coeloconica presented on each antenna segment, which is consistent with the discovery that SCo I and SCo II are thought to have olfactory or thermos functions (Bourdais et al., 2006; Xi et al., 2010). Furthermore, the antiserum of AgifOBP6 was detected on the sensilla trichaidea, which is prevalent on all segments except the radicula of *A. gifuensis*. We hypothesize that sensilla trichaidea might have more function in female antennae beyond the putative mechano-receptive function in male and female *A. gifuensis* (Das et al., 2011). The binding mechanism of AgifOBP6 with EBF and it's analoge was illustrated by a computational procedure, and the docking results revealed negative docking binding energies were the main force to maintain such binding properties.

Olfactory stimulation is an excellent method for assessing insect reactions to scents at the molecular level. In recent years, odors have gained popularity as a stimulus to examine neuronal electrophysiological signals and olfactory system protein expression. Females of the Rockefeller strain of Aedes aegypti (Diptera: Culicidae) were subjected to a variety of doses of insect repellents, including geranyl acetate, bisabolol, nerolidol, and DEET (Portilla et al., 2022). After stimulation with 4-allyl-2,6-dimethoxyphenol (4-DMP) and methyl eugenol (ME), five OBPs, including BdorOBP5, BdorOBP19d, BdorOBP22, BdorOBP50e, and BdorOBP56a, were likewise considerably elevated in the midlegs of the male *Bactrocera dorsalis* (Hu, et al., 2021). The stimulation of -caryophyllene dramatically changed the expression levels of AnilOBP9 and AnilCSP6 in *Anagrus nilaparvatae*, a significant egg parasitoid wasp of the rice planthopper, indicating that these two genes may be connected to host detection (Ma et al., 2022). The complex patterns of aphid OBPs' extended responses to EBF demonstrate the olfactory flexibility of the different aphid species (Zhang et al., 2021). There aren't many related studies on their parasitoids, though. In our present work, there were up to 5 OBPs in total responded to EBF induction. Interestingly, AgifOBP6 was the only OBP that responded to EBF in a wide range of doses from low to high (4ng to 4000ng) by upregulation. This indicated that AgifOBP6 may play in the molecular basis of EBF recognition in *A. gifuensis*. While, the up-regulation of AgifOBP9 and AgifOBP12 were limited to a specific dose of 4 and 400 ng EBF respectively, and the down-regulation of AgifOBP7 and AgifOBP15

was limited to a high dosage of 4000ng. Like AgifOBP6, AgifOBP7 and AgifOBP9 were identified as having an affinity for EBF as well (Jiang et al., 2022). However, the affinity data of AgifOBP12 and AgifOBP15 with EBF could not be obtained as they were not able to be purified successfully. The olfactory perception in insects is generally dose-dependent. Dose beyond the threshold range, either too high or too low will lead to a decrease in response. One such example is the attraction of the parasitoid *Cotesia chilonis* wasps to linalool and DMNT at low, medium, and high concentrations. Furthermore, MeSA was repellent at high concentrations but appealing at low ones. Hexadecane showed no effect on the attraction of parasitoids at any dose (Yao et al., 2022). Higher doses of synthetic EβF (1.4 μg to 5.7 μg) attracted the specialized parasitoid *Aphidius uzbekistanicus* (female). The generalist parasitoids *Aphidius ervi* and *Praon volucre* responded to high dosage (0.3-30.0 μg) (Micha & Wyss, 1996; Du et al., 1998; Heuskin et al., 2012). Thus, the up-regulation induced by low dose EBF i.e 40ng suggested that AgifOBP9 was associated with the high sensitivity of aphidius to EBF. Therefore, AgifOBP9 could collaborate with AgifOBP6 to ensure the sensitivity of *A. gifuensis* in low doses of EBF. And once EBF dose is above the threshold, AgifOBP7/15, the two down-regulated OBPs by EBF induction may neutralize AgifOBP6 by down-regulating their expressions, and the response to excessively high doses of EBF was achieved through the cooperation of three OBPs (AgifOBP6, AgifOBP7 and AgifOBP15 at 4000ng).

Finally, we test the EBF effects on foraging behavior of *A. gifuensis* by two bioassays using grain aphids *S. miscanthi.* Results indicated that trace EBF may promote the biological control efficiency of *A. gifuensis,* especially on winged aphids. It is important to note that EBF applied in this part was dissolved in triglycerides (TIC). And the reason we chose TIC is that the major component of aphid droplet secreted from cornicle are TIC (Callow et al., 1973). Although the solubility of EBF in different solvents is different, the amount of EBF volatilized out is definitely not the same as the amount we applied to insects, which needs to further identify in the future.

The female aphid parasitoids change the extent of their exploitation once they have discovered an aphid colony based on a variety of indications. EBF is a critical infochemical in the tri-trophic level interactions among plant-aphid-aphid natural enemies, it's biological significance to aphids seems to be more likely to ensure the survival of the aphid population through a few escapes than to ensure the overall survival of the current generation. The majority of aphid species respond to EBF at a pretty low dosage of 1ng to 10 ng in the field (Jing-Gong et al., 2002). Whereas, aphids typically emit cornicle droplets only after being physically attacked (Nault & Phelan, 1984), resulting in the emitter's escape in about 10% of attacks (Dixon, 1958; Edwards, 1966). Further, within an aphid colony, generally a single or a few aphids are attacked at the same time, and the signal is not amplified by the emission of neighboring aphids (Hatano et al., 2008).

Aphid parasitoids show different levels of host specialization. Some specialize on a few aphid species, whereas others specialize on habitat and attack most aphid species

within a given habitat (Moreno-Mari et al. 1999; Stilmant et al. 2008). Different cues are used by generalists and specialists to locate their environment and vital resources. Although generalists can utilize a greater variety of resources, specialists are anticipated to be more effective in utilising a single resource than generalists (Stilmant et al. 2008). It seems that generalist parasitoid wasps are less sensitive to EBF than specialist ones. For instance, *Aphidius colemani* Viereck (Hymenoptera: Braconidae: Aphidiinae), a pan-tropical, broadly oligophagous, solitary endoparasitoid of many aphids of economic importance, females are attracted by plant-extracted nepetalactone (a component of aphid sex pheromone), when it was in high concentration; while the wasps do not respond to (E)-beta-farnesene (Benelli et al., 2014). Another generalist parasitoid, *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae: Aphidiinae), parasitizes more than 60 aphid species worldwide, such as *R. padi*, *M. persicae*, *Brevicoryne brassicae*, and *Lipaphis erysimi*, is widely used parasitoid against aphids (Silva et al., 2011; Kant & Minor, 2017; Soni et al., 2022). Recent work found that *Diaeretiella rapae* females generally showed no significant behavioral response to these alarm pheromone components and analogs under low concentrations (0.1 μg/μL). While, their olfactory response to these compounds gradually enhanced with increasing concentrations in the olfactory bioassays (Qin et al., 2022).

Apart from EBF, Other chemicals also found effects on the improvement of parasitism rates. For example, by applying cis-jasmone (CJ) in three brassica cultivars (Pak choi, Samurai and Wesway), the largest increase in parasitism rates was observed on Samurai (121 % increase) (Ali et al., 2021). β-caryophyllene, a volatile organic compound chemical that can attract *A. nilaparvatae* (Lou et al., 2005), which also used as a stimulus to compare changes in the expression levels of eight soluble chemical communication proteins. As the expression levels of AnilOBP9 and AnilCSP6 indicated that they were significantly altered by the stimulation of β-caryophyllene, suggesting that these two genes may be related to host detection in the parasitoid wasp *Anagrus nilaparvatae* (Hymenoptera: Mymaridae) (Ma et al., 2022).

Another aspect is regarding the detection of sex pheromones in parasitoid wasps, which may improve the parasitism rate of the host. Mated females spend more time searching for hosts, lay more eggs, and have higher parasitism rates than virgin females because they are more effective at parasitizing (Tagawa et al., 1987; Michaud & Mackauer 1995; Kugimiya et al., 2010). This notion is verified by the insect parasite *Campoletis chlorideae* Uchida, which found the species-specified sex pheromones components, and has an increase in host larval parasitism when parasitoid wasp sex pheromones are prevalent (Guo, et al., 2022). The plants spiked with the sex pheromone and (Z)-jasmone operate as a rendezvous point for mating, which encourages mating (Guo, et al., 2022). This extrapolation is fair because it has been noted that plants serve as mate-hunting insects' rendezvous points and because plant volatiles can increase the creation of sex pheromones and make the partners more receptive (Xu & Turlings, 2018).

Specialization in resource usage can be used to explain the phenomena of species

coexistence, which occurs when multiple species use the same resource in the same habitat (Egas et al. 2004).

For parasitoids, switching costs may include the time spent evaluating, accepting, or responding to the host's defense, as well as costs connected to greater developmental mortality in a less-than-ideal host. In the wheat field, four main species are coexisted, including *S. avenae*, *R. padi*, *Schizaphis graminum*, and *M. dirhodum* (Blackman et al., 2006; Chen JL, 2013; Zhang et al., 2019). However, it is difficult to deduce actual parasitoid preference patterns in the field during the entire season. For example, the field-collected habitat specialist *Aphidius rhopalosiphi* did not exhibit any preference for *S. avenae* or *M. dirhodum* (Powell and Wright, 1988). The relative abundance of host species and parasitoid species that were observed during the entire season may have changed, which could have a biased effect on patterns that are observed in the field. The learning process and local strain adjustments may also have an impact on the results. Apart from the hosts, the hyperparasitoid of parasitoid wasps and predators of aphids also influence the foraging behavior (Buitenhuis et al., 2004; Song et al., 2021).

It is important to note, though, that improper application of the info-chemical could cause volatiles to confuse parasitoids searching for mates or hosts, particularly in the field. Odors that originate from plants, parasitoids, predators and their hosts are often intermingled and fluctuate, and parasitoids employ sophisticated olfactory coding mechanisms to detect the chemical space. It will take a far better foundational understanding of how parasitoids actually exploit scents in complicated and highly variable contexts to successfully use volatiles to affect parasitoid behaviors. Wasp parasitism rates are relatively low when evaluated in the field, for example, the highest parasitic rate of aphids in maize fields was only 1.79% in Jilin Province, China (Zhang et al., 2020), the calculation of the parasitism ratio of *S. miscanthi* is estimated to be between 10% to 15% (Yang et al., 2017; Xu et al., 2018; Liu et al., 2021). *A. gossypii* showed a similar phenomenon (Yang et al., 2021). While the biological significance to natural enemies seems to be taking advantage of EBF to ensure every full meal. EBF triggers attack behavior in predators (Kindlmann et al., 2010) and parasitoids, obviously serving as a stimulant for host/prey finding and attacking. For instance, the release of alarm pheromone by *S. avenae* is attractive for *A. rhopalosiphi* parasitoids (Micha & Wyss, 1996), while the emission of cornicle secretions by *A. pisum* stimulates a strong oviposition attack response from *A. ervi* females (Battaglia et al., 1993). Our work confirmed that EBF could promote the biological control efficiency of *A. gifuensis*, especially for winged aphids, by coating trace levels of EBF on the dorsal abdomen of *S. miscanthi*. Our results confirmed that EBF binding proteins between aphids and their natural enemies evolved separately and were directed to the same function by convergent evolution. This is consistent with the notion that the genetic diversification of living things is fueled by evolutionary adaptation, causing speciation and emerging biodiversity (Lamichhaney et al., 2015; Simes et al., 2016). And their inherent interactional traits, species with overlapping habitats provide great

research subjects for studying co-adaptive evolution. This implies that among multi-trophic levels, there are more overt signs of adaptive evolution.

Most of the studies focused on the attractant properties of alarm pheromones on natural enemies that have been conducted under controlled laboratory conditions. For example, expression of (E)-β-farnesene synthase gene in *Arabidopsis thaliana* increased aphid parasitoid attraction toward plants (Beale et al., 2006). Three kinds of cereal aphids were repelled and foraging was increased for a parasitic natural enemy by genetically modifying a hexaploid type of wheat to emit (E)-β-Farnesene in laboratory behavioral trials (Bruce et al., 2015). While there are different results regarding the defensive role of (E)-β-farnesene against aphids. Constitutive releases of (E)-β-farnesene in transgenic Arabidopsis had no impact on the behavior or settling of the green peach aphid (*M. persicae*) (Kunert et al., 2010). However, even if alarm cues are ineffective in directly affecting aphids, their emission may improve the attractiveness of aphid predators and boost biological control (Du et al., 1998; Verheggen et al., 2007, 2008; Zhu et al., 1999; Vosteen et al., 2016). Field trials are needed to further evaluate the application of EBF. As previous work has found that constitutive releases of EBF in field trials neither reduced the number of cereal aphids nor increase parasitism (Bruce et al., 2015). Our present work offers a novel perspective on the biological control for aphids from reducing the initial population of migrant biotype aphids from source areas: to promoting the biological control efficiency of parasitoids to winged aphids before the immigration, by low concentration of EBF application. To optimize the parasitic power of parasitoids in the field, Insect populations of parasitoid wasps are important for early determination of the community composition of Aphidiinae parasitoids, until recently, the COI and 16S primers in combination for monitoring the parasitism rates on *S. miscanthi* in wheat fields was developed (Hu et al., 2022). Thus will enhance the efficiency of parasitism. Together, the environmental factors call for additional tests or a more accurate impersonation of alarm pheromone emission in plants when contemplating this method. it is specifically promised to spray EBF, combine large-scale breeding of this parasitoid wasp, and deliberately release the wasps in accordance with the wasp-to-host population ratio.

VI.3. References

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Appendix-Publications

1**. Xin Jiang**, Jun Jiang, Miaomiao Yu, Siyu Zhang, Yaoguo Qin, Yun Xu, Frédéric Francis, Jia Fan, Julian Chen. (2023). Functional analysis of odorant-binding proteins for the parasitic host location to implicate convergent evolution between the grain aphid and its parasitoid *Aphidius gifuensis. International journal of Biological and Macromolecules*, 226, 510-524.

2. **Xin Jiang**, Yaoguo Qin, Jun Jiang, Yun Xu, Frédéric Francis, Jia Fan, Julian Chen. (2022). Spatial Expression Analysis of Odorant Binding Proteins in Both Sexes of The Aphid Parasitoid *Aphidius gifuensis* and Their Ligand Binding Properties. *Frontiers in physiology*, 13:877133.

3. **Xin Jiang**, Qian Zhang, Yaoguo Qin, Hang Yin, Siyu Zhang, Qian Li, Yong Zhang, Jia Fan, Julian Chen. (2019). A chromosome-level draft genome of the grain aphid *Sitobion miscanthi*. *Gigascience*, 8(8), giz101.

4. Zhang, S., Zhang, Q., **Jiang, X**., Li, Q., Qin, Y., Wang, W., Fan J, Chen, J. (2021). Novel temporal expression patterns of EBF-binding proteins in wing morphs of the grain aphid *Sitobion miscanthi*. *Frontiers in physiology*, 12:732578.