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Transcriptome analysis used to identify and characterize odorant binding proteins in *Agasicles hygrophila* (Coleoptera: Chrysomelidae)

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The transcriptomes of *Agasicles hygrophila* eggs and first instar larvae were analyzed to explore the olfactory mechanism of larval behavior. The analysis resulted in 135,359 unigenes and the identification of 38 odorant-binding proteins (OBPs), including 23 Minus-C OBPs, 8 Plus-C OBPs, and 7 Classic OBPs. Further analysis of differentially expressed genes (DEGs) revealed 10 DEG OBPs, with 5 (*AhygOBP5*, *AhygOBP9*, *AhygOBP12*, *AhygOBP15* and *AhygOBP36*) up-regulated in first instar larvae. Verification of expression patterns of these 5 *AhygOBPs* using qPCR showed that *AhygOBP9* and *AhygOBP36* were mainly expressed in the adult stage with gradually increasing expression in the larval stage. *AhygOBP5*, *AhygOBP12*, and *AhygOBP15* were not expressed in eggs and pupae, and their expression in larvae and adults showed no clear pattern. These 5 *AhygOBPs* may play an olfactory role in larval behavior, providing a basis for further investigation of their specific functions and clarifying the olfactory mechanism of *A. hygrophila*.

Key words: Agasicles hygrophila, odorant-binding protein, transcriptome analysis, egg, first instar larvae

Introduction

Insect odorant-binding proteins (OBPs) are a class of water-soluble globular proteins consisting of 100–150 amino acids and a molecular weight of approximately 15–20 kD (Field et al. 2000). They have hydrophobic cavities that can recognize and bind hydrophobic compounds. OBPs are generally divided into 3 different subgroups based on the number of conserved cysteine residues: the Classic subgroup, which contains 6 highly conserved cysteine residues; the Minus-C subgroup, which lacks 2 conserved cysteine residues; and the Plus-C subgroup, which has 2–4 additional cysteine residues compared to the Classic subgroup (Zhou et al. 2004, Li et al. 2013).

Agasicles hygrophila (Coleoptera: Chrysomelidae) is a leaf beetle native to South America that feeds exclusively on *Alternanthera philoxeroides* (Coulson 1977, Zhao et al. 2016). Herbivorous insects use plant volatile compounds to locate their host plants for feeding and egg deposition (Wang et al. 2020). Previous studies have shown that *A. hygrophila* female beetles use olfactory cues to select plants for feeding and oviposition (Li et al. 2017). The monophagous beetle *A. hygrophila* uses (E)-4,8-dimethyl-1,3,7 nonanetriene (DMNT) as a volatile compound to recognize its host plant *A. philoxeroides* (Wang et al. 2020). *A. philoxeroides* releases DMNT in response to attacks by *A. hygrophila*, which actively encourages *A. hygrophila* feeding and oviposition (Li et al. 2017, Wang et al. 2020). However, only the *A. hygrophila* odorant receptor coreceptor (AhygOrco) has been reported to be associated with foraging and mate locating, and inhibition of AhygOrco reduces host and partner recognition (Ning et al. 2023). Chemosensory mechanisms other than AhygOrco in *A. hygrophila* have not been well studied.

Based on experimental observations, we have discovered that *A. hygrophila* displays host specificity from its larval stage. To investigate the olfactory mechanism underlying this larval behavior, we sequenced and analyzed the transcriptome of eggs and 1st instar larvae. Through bioinformatic analysis, we identified a total of 38 *AhygOBPs*. To confirm our findings, we used quantitative real-time

PCR (qPCR) to verify the up-regulated expression of 5 OBP genes at various developmental stages. Our results shed light on the olfactory mechanism behind larval foraging.

Materials and Methods

Insect Rearing and Sample Collection

Insects were raised under controlled conditions at 25 ± 1 °C, with a 12-hour light:12-hour dark photoperiod and 75 ± 5% relative humidity. For transcriptome sequencing, eggs were collected 48 h after laying, while first instar larvae were collected 48 h after hatching. Expression profiling studies were conducted using samples from a total of 6 developmental stages, including eggs, first to third instar larvae, pupae, and female and male adults. All samples were represented by at least 3 biological replicates.

Table 1. Summary of BLASTP search results of unigenes against public protein databases

Databases	Numbers of unigene hits	Percentage
NCBI nr	31,327	23.1%
Swiss-prot	16,714	12.3%
GO	20,590	15.2%
KEGG	6,322	4.7%
Total unigenes	135,359	

RNA Extraction and cDNA Synthesis

TR150 Micro Total RNA Rapid Extraction Kit (with DNase I) (JIANSHI BIOTECH) was used to extract total RNA from each sample individually, and the concentration and purity of RNA were assessed using a NanoDrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). The first-strand cDNA was synthesized from 1 μg RNA usingHifair III 1st Strand cDNA Synthesis SuperMix for qPCR (Yisheng Bio), and cDNA templates were stored at −20 °C.

Sequencing was performed by UW Genetics Ltd (Shenzhen, China) using the Illumina HiSeq 4000 sequencing platform. Trimming of lowquality reads and adapter sequences was performed using Trimmomatic software. The get_longest_isoform_seq_per_trinity_gene.pl script in TRINITY was used to obtain the longest isoforms for each gene (Grabherr et al. 2011; Zhang et al. 2022); transcripts with 95% similarity were clustered using cd-hit and then annotated

Table 2. Summary of AhygOBP sequence information

Gene ID	Length of AA	Classification	Signal peptide	Mw(kD)	PI
AhygOBP1	102	Minus-C	–	11.2	9.6
AhygOBP2	104	Minus-C	–	11.8	4.9
AhygOBP3	117	Classic-C	16	12.4	6.1
AhygOBP4	123	Classic-C	17	13.6	5.0
AhygOBP5	126	Minus-C	16	14.1	6.8
AhygOBP6	127	Classic-C	20	14.8	4.6
AhygOBP7	128	Minus-C	17	14.4	5.5
AhygOBP8	128	Minus-C	19	14.5	6.1
AhygOBP9	128	Minus-C	19	14.5	5.4
AhygOBP10	129	Minus-C	16	14.7	4.7
AhygOBP11	129	Minus-C	20	14.3	4.7
AhygOBP12	129	Minus-C	–	15.0	5.4
AhygOBP13	129	Minus-C	19	14.8	5.8
AhygOBP14	129	Plus-C	22	15.1	5.1
AhygOBP15	131	Minus-C	17	14.6	6.2
AhygOBP16	131	Plus-C	16	14.3	4.2
AhygOBP17	132	Minus-C	17	14.5	5.9
AhygOBP18	133	Minus-C	17	15.3	9.0
AhygOBP19	133	Minus-C	18	14.6	5.1
AhygOBP20	134	Minus-C	16	14.9	7.7
AhygOBP21	134	Minus-C	16	15.0	7.7
AhygOBP22	134	Minus-C	19	15.2	5.4
AhygOBP23	135	Classic-C	19	15.8	7.6
AhygOBP24	136	Classic-C	19	15.4	5.1
AhygOBP25	136	Minus-C	16	15.4	9.0
AhygOBP26	137	Plus-C	16	15.4	8.1
AhygOBP27	138	Plus-C	19	15.7	9.3
AhygOBP28	139	Minus-C	19	16.0	8.3
AhygOBP29	141	Plus-C	22	16.4	4.5
AhygOBP30	143	Plus-C	19	16.1	8.4
AhygOBP31	146	Minus-C	25	16.4	7.1
AhygOBP32	147	Minus-C	–	16.6	6.2
AhygOBP33	147	Minus-C	17	16.7	5.9
AhygOBP34	148	Classic-C	16	16.6	4.9
AhygOBP35	152	Plus-C	32	16.9	5.6
AhygOBP36	155	Minus-C	33	17.3	8.3
AhygOBP37	165	Classic-C	21	19.2	8.5
AhygOBP38	173	Plus-C	–	20.1	8.6

by searching various databases, including the NCBI Non-Redundant Protein Sequence Database (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Pfam, Protein Orthologue Group (KOG), and Gene Ontology (GO) (Li et al. 2020).

Gene Identification and Bioinformatic Analysis

The OBP genes were identified based on the conserved structural domain of the OBP gene family (PBP_GOBP:PF01395). The PBP_GOBP HMM profile was downloaded from the Pfam database, and then the candidate genes were screened using the hmmsearch program of HMMER (version 3.1b2). The identified candidates were then subjected to further validation screening by submitting them to NCBI Batch CD-search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi).

The open reading frame (ORF) of each individual gene was determined using the ORF Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). To assess sequence conservation, multiple sequence alignment was conducted using DNAMAN. Signal peptides were predicted using SignalP 6.0 (Almagro Armenteros et al. 2019). Motif structure was predicted using MEME (https://meme-suite.org/meme/tools/meme).

Phylogenetic Analysis

To explore the evolutionary relationship between the *AhygOBP* genes and their orthologues, we utilized 162 OBP genes from 5 Coleopteran species, including *A. hygrophila*, *Dendroctonus ponderosae*, *Anoplophora glabripennis*, *Agrilus planipennis*, *Tribolium castaneum*, and *Rhynchophorus ferrugineus*, to construct a phylogenetic tree. The 4 other Coleopteran insects were selected due to their extensively studied olfactory genes, and the amino acid sequences of OBP genes were retrieved from the NCBI database or literature attachments. To align all amino acid sequences, the Clustalw method in MEGA (Yang et al. 2018) was used, and the neighbor-joining method with 1,000 bootstraps (Tamura et al. 2004) was employed to construct the phylogenetic tree. The phylogenetic tree was visualized and annotated using FigTree (Zhu et al. 2022).

Expression Profiling Analysis

The gene expression levels between eggs and first instar larvae were compared using fragments per kilobase of transcript per million fragments mapped (FPKM). DESeq2 software was used to analyze genes that were significantly differentially expressed, with absolute values of log2 (fold change) (>1) and *P*-values (<0.05) (Anders and Huber 2010).

To verify the 5 up-regulated OBP genes in first instar larvae, qPCR was performed with ribosomal protein S18 (RPS18) as the internal reference gene (Jia et al. 2020). The primers used for qPCR were designed and synthesized by Shanghai Biological Engineering Co., Ltd. (China). The qPCR reactions were performed using the Applied Biosystems Step One Plus Real-Time PCR System, with a reaction volume of 20 µl. The reactions were established with 10.0 µl Hieff qPCR SYBR Green Master Mix (High Rox Plus), 0.4 µl of each primer (10 µM), 1.0 µl of cDNA template, and 8.2 µl of sterilized ultrapure H_2O. The reaction conditions included predenaturation at 95 °C for 5 min, followed by 40 cycles of 10 s at 95 °C, and 30 s at 60 °C. Three biological replicates per tissue and 3 technical replicates per biological replicate were used. Data were processed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008), one-way analysis of variance (ANOVA) was performed using SPSS, and multiple comparisons were performed using the LSD (least significance difference) method. The results were visualized with GraphPad Prism software.

Results

Transcriptome Sequencing and Assembly

The Illumina HiSeq 4000 sequencing platform generated a total of 1,426,068,984 reads from 8 (egg: 3 samples; 1st instar larvae: 5

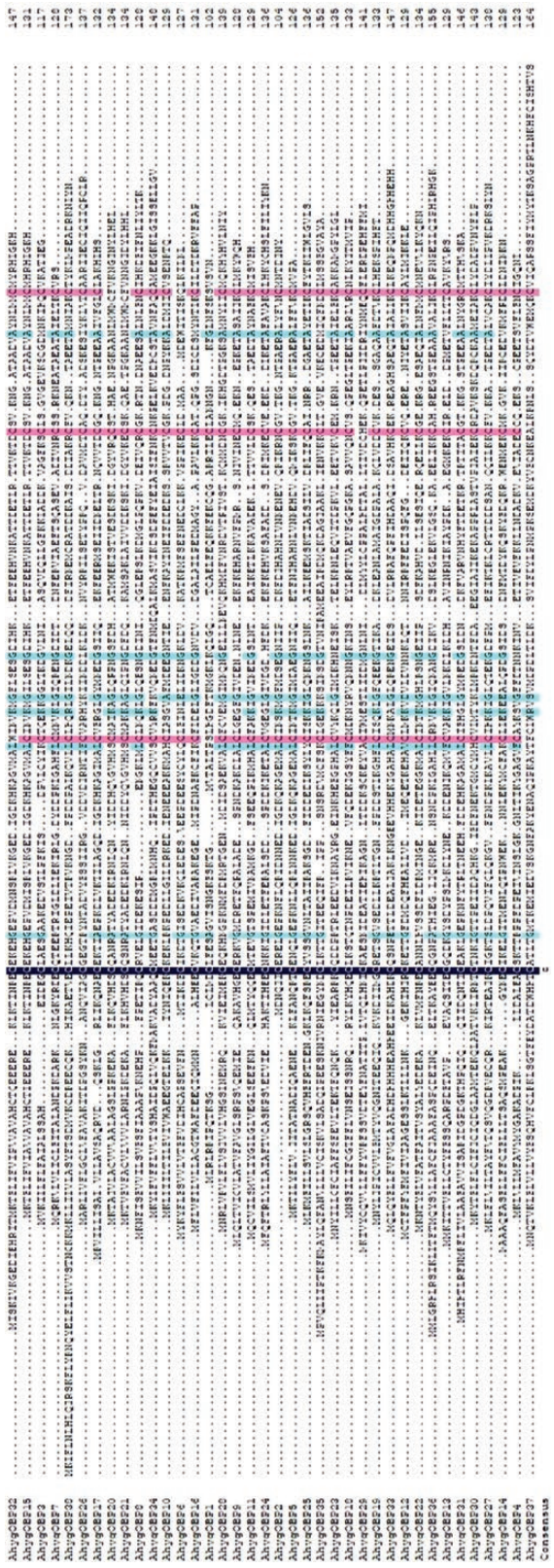

Fig. 1. Multiple sequence alignments of AhygOBPs.

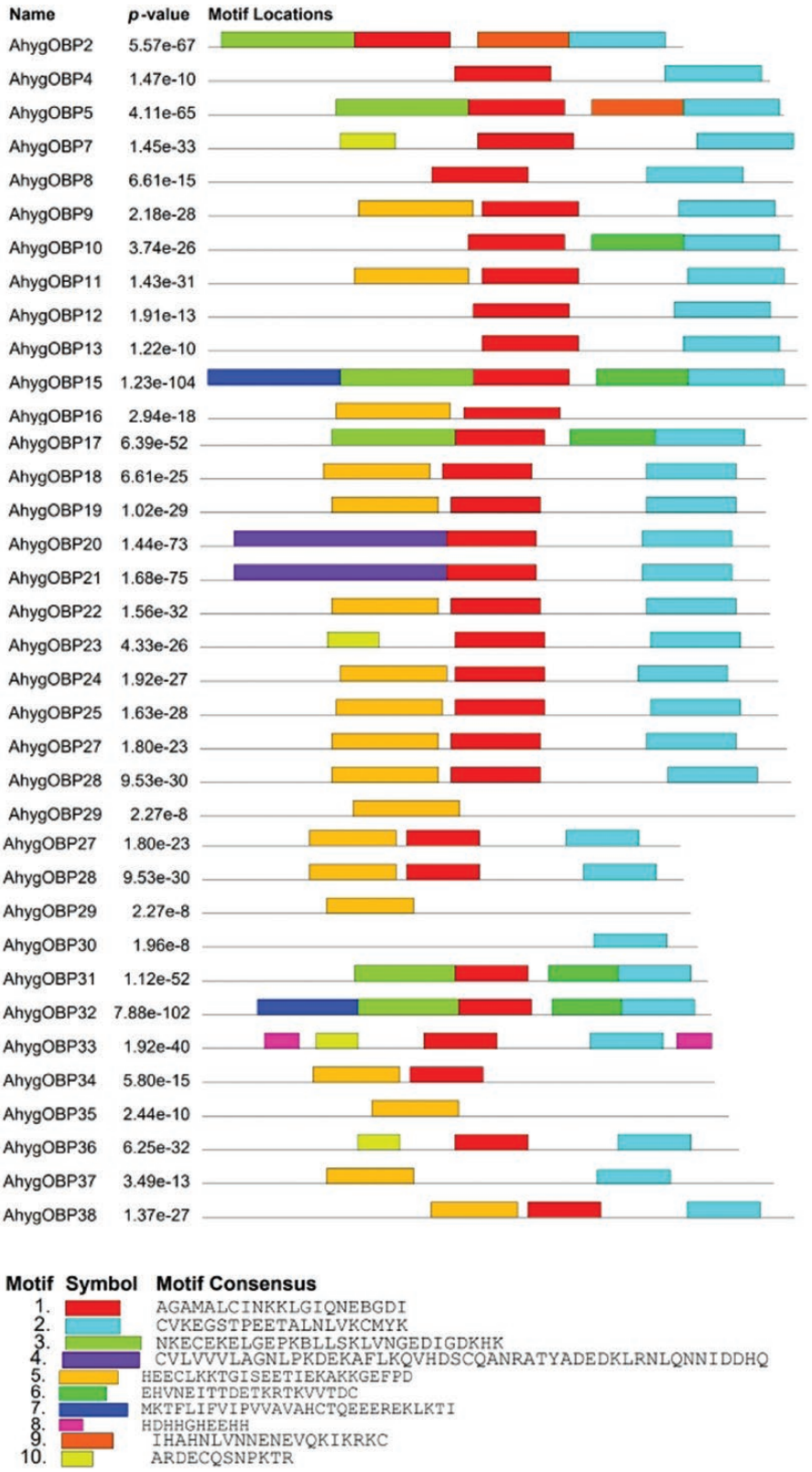

Fig. 2. Motif information of *AhygOBPs;* Rectangles in different colors represent different Motifs, and the sequences represented by different motifs are shown in the legend.

samples) RNA-Seq libraries with a total size of 142.5 Gb. A total of 272,894 transcripts were generated after pruning and recombination. Similarity clustering by searching for the longest isoforms and transcripts for each gene resulted in 135,359 nonredundant single genes. Further classification of the single genes by function (Table 1) showed that 31,327 single genes were annotated by the

Nr database (23.1%), 16,714 single genes by the Swiss-Prot database (12.3%), 20,590 single genes by the GO database (15.2%), and 6,322 single genes by the KEGG database (4.7%).

Identification, Characterization, and Phylogenetics of *AhygOBPs*

A total of 38 OBPs were identified from the transcriptome assembly of *A. hygrophila* and named as AhygOBP1-38 based on their sequence length (Table 2). These 38 *AhygOBPs* encode 102–173 amino acids, with 7 of them belonging to the Classic OBPs, 23 belonging to the Minus-C OBPs, and 8 belonging to the Plus-C OBPs. From the 38 *AhygOBPs*, 33 have a signal peptide at the N-terminus, while the remaining 5 have no signal peptide. The signal peptide is composed of 16–33 amino acids. The molecular weights of the 38 *AhygOBPs* range from 11.2 to 20.1 kD, and their isoelectric points range from 4.2 to 9.6.

The multiple sequence alignment of 38 *AhygOBP* genes showed that a total of 4 C on the alignments (Fig. 1), indicating a highly conserved OBP gene family structure. To identify conserved structural domains in the sequence, we used MEME and found that the sequences contained multiple identical motifs, with the red motif being the most conserved and the blue motif being the next most conserved (Fig. 2).

Phylogenetic analysis showed that the OBP genes are relatively conserved, with most of the OBP genes of the same species clustered in the same family and only a few clustered with other Hymenoptera (Fig. 3). For instance, *AhygOBP6* and *AglaOBP52*, *AhygOBP25*, and *DponOBP39*, *AhygOBP8*, and *AplaOBP2*, and *AhygOBP1* and *TcasOBP15* clustered in the same family.

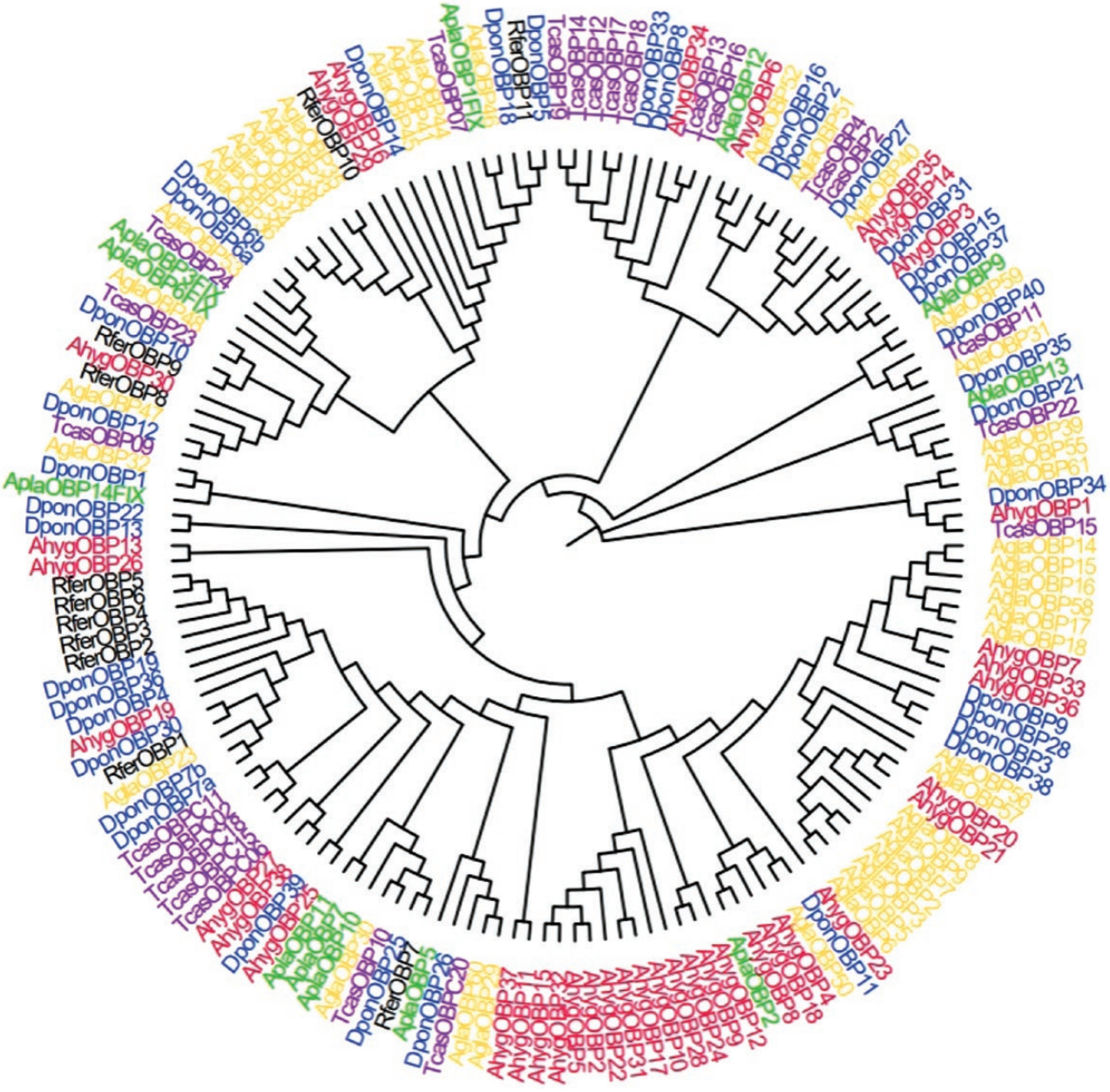

Fig. 3. Phylogenetic tree of OBP genes from *A. hygrophila* and other coleopteran species (neighbor-joining tree, NJ); Ahyg: *Agasicles hygrophila*, Agla: *Anoplophora glabripennis*, Apla: *Agrilus planipennis*, Dpon: *Dendroctonus ponderosae*, Tcas: *Tribolium castaneum*, Rfer: *Rhynchophorus ferrugineus*.

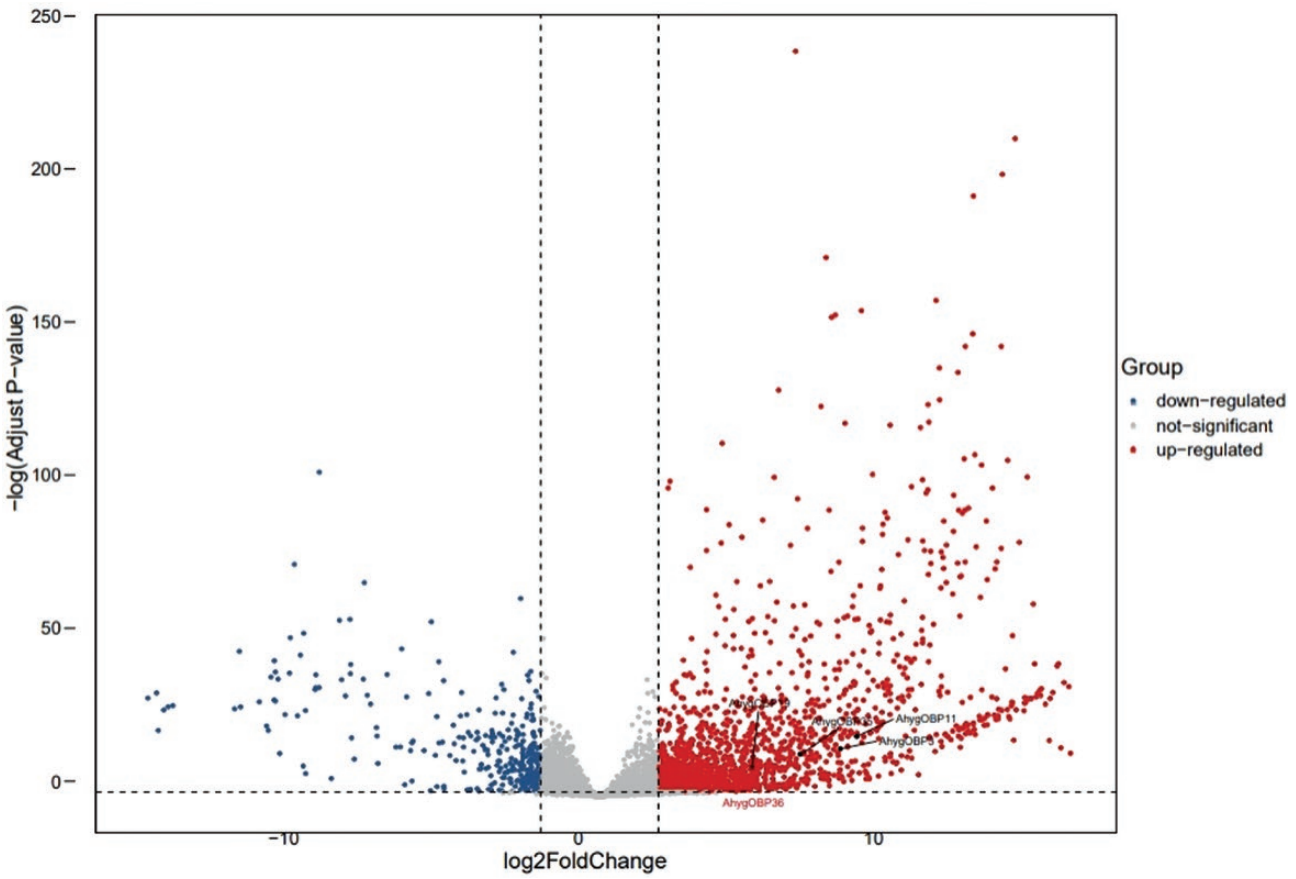

Fig. 4. Volcano plot of differentially expressed genes in the eggs and first instar larvae of *A. hygrophila*; The OBP genes up-regulated in first instar larvae were *AhygOBP5, AhygOBP9, AhygOBP12, AhygOBP15,* and *AhygOBP36*.

Transcriptome-based Identification of Differentially Expressed Genes

Based on transcriptome read counts in *A. hygrophila,* we determined the expression profiles of all unigenes using DESeq2. Among the 1,914 differentially expressed genes (DEGs) identified, 1,560 were highly expressed in the first instar larvae, while 354 were highly expressed in the egg stage. DEGs highly expressed in the first instar larvae, include 5 OBP genes, namely *AhygOBP3*, *AhygOBP11*, *AhygOBP19*, *AhygOBP35*, and *AhygOBP36*. The expression of these 5 OBP genes across both periods is shown in Fig. 4.

Expression Profiles during Different Developmental Stages

The expression of the 5 up-regulated *AhygOBP* genes in the first instar larvae was further validated by qPCR at different developmental stages. The results showed that all 5 OBP genes had higher expression levels in the first instar larvae compared to the egg stage. However, the expression levels of each OBP varied at different developmental stages (Fig. 5). *AhygOBP11* and *AhygOBP35* had similar expression patterns, with low expression in eggs and pupae, gradually increasing in the larval stage, and being highest in the adult stage (Fig. 5). The expression of *AhygOBP11* was higher in female adults, while *AhygOBP35* had higher expression in male adults (Fig. 5). *AhygOBP3* was most highly expressed in the first instar larvae, with low expression in eggs and pupae, and not significantly different in adult and second and third instar larval stages (Fig. 5). *AhygOBP36* was mainly expressed in the larval stages, with lower expression in eggs, pupae, and male adults, and not significantly different. *AhygOBP19* had low expression in eggs and pupae and showed no significant differences in the larval and adult stages (Fig. 5).

Discussion

The identification of olfactory genes is a crucial step towards understanding the molecular mechanisms of olfaction. Among these genes, OBPs are known to be important chemosensory proteins in insects, playing a key role in odorant perception. Although it has been shown that the function of OBPs is not limited to olfactory effects, and OBPs have been found to be present in organs not generally associated with chemoreception, their binding molecular signatures remain indisputable (Bruno et al. 2018).

In our previous experiments, we observed olfactory functions in *A. hygrophila* at the larval stage, and therefore analyzed the transcriptome of both eggs and larvae in order to elucidate the olfactory mechanisms at different developmental stages. In this study, we generated a total of 1,426,068,984 reads from eggs and first instar larvae, resulting in a total of 135,359 of the longest transcripts. Among these, we identified a total of 38 OBPs genes, with the Minus-C subclade having the most OBPs (23), followed by the Plus-C subclade (8), and the Classic-C subclade (7). Previous studies have suggested that Minus-C OBPs are more abundant in primitive insects, whereas Plus-C OBPs are more abundant in higher species (Vieira and Rozas 2011, Spinelli et al. 2012). This implies that Minus-C OBPs may be the ancestors of both classical OBPs

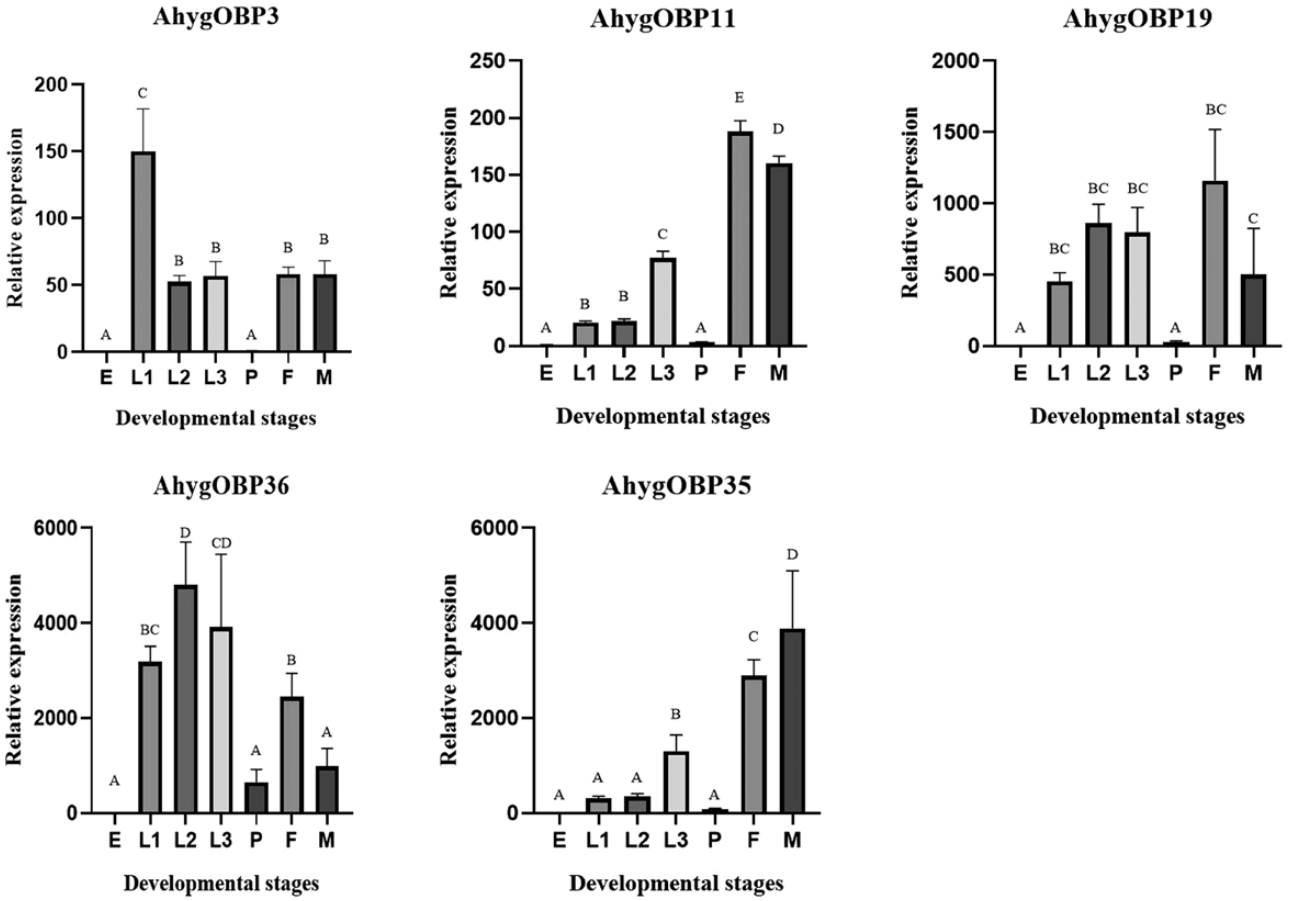

Fig. 5. Relative expression of the 5 *AhygOBP* genes throughout different developmental stages; E: egg; L1–L3: 1st-3rd instar larvae; P: pupa; F: female; M: male. The internal reference genes were RPS18, and the relative expression levels are expressed as the mean ± SEM of 3 biological replicates. Multiple comparisons were performed using the LSD method, and different letters on the bars indicate significant differences ($P < 0.05$).

and Plus-C OBPs, and that OBP evolution has resulted in more disulphide bonds and more complex structures over time.

However, it should be noted that the number of OBP genes identified in this study may not represent the complete set of *A. hygrophila* OBPs gene families. Some genes may have been missed during the RNA-seq process due to low expression levels or lack of expression in the tested transcriptome. Additionally, some olfactory genes with significant differences from known query gene sequences may not have been detected in the transcriptome analyses using homology search methods (Li et al. 2022).

Recent studies have shown that OBPs are not only expressed in chemoreceptive tissues such as antennae, but also in the nonchemoreceptive tissues such as pheromone glands, where they play a role in pheromone release (Jacquin-Joly et al. 2001, Li et al. 2008, Dani et al. 2011, Gu et al. 2013). For example, MvicOBP3 and MvicOBP8 are highly expressed in cornicles and cauda of the hemipteran insect *Megoura viciae* (Bruno et al., 2018). In the present study, OBP genes were identified during the larval stage, and they may have a putative role in development (Maleszka et al. 2007, Marinotti et al. 2014).

Based on the analysis of differential genes combined with FPKM values, we identified 5 OBPs that were up-regulated at the larval stage. It is hypothesized that these 5 OBPs may be involved in the olfactory recognition function of the larval stage. We further validated the spatio-temporal expression patterns of these genes using qPCR, which showed that they were not only expressed at low levels in the egg stage but also largely absent in the pupal stage. *AhygOBP11* and *AhygOBP35* were mainly expressed in the adult stage, with increasing expression in the larval stage, suggesting their role in olfactory function. However, the expression patterns of *AhygOBP3*, *AhygOBP19*, and *AhygOBP36* did not follow the above pattern, indicating that they may have functions other than olfaction.

Our findings provide a foundation for clarifying the roles of *A. hygrophila* OBP genes, and the highly expressed OBP genes in the larval stage should be further explored for their unique functions.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

Changhong Dong (Data curation [Lead], Formal analysis [Lead], Methodology [Equal], Visualization [Lead], Writing—original draft [Lead]), Huang Cong (Data curation [Equal], Methodology [Lead], Project administration [Equal], Writing—review & editing [Lead]), Xiaoyu Ning (Formal analysis [Equal], Methodology [Equal]), Bo Liu (Funding acquisition [Equal], Project administration [Equal], Writing—review & editing [Equal]), Xi Qiao (Funding acquisition [Equal], Project administration [Equal]), Wanqiang Qian (Funding acquisition [Equal], Project administration [Equal]), Daohong Zhu (Methodology [Equal], Project administration [Equal], Writing—review & editing [Equal]), and Fang-Hao Wan (Project administration [Equal], Writing—review & editing [Equal])

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