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ORIGINAL ARTICLE

Expression and function of two olfactory-related genes in response to methyl salicylate in the wolf spider *Pardosa pseudoannulata* (Araneae: Lycosidae)

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Abstract. The wolf spider (*Pardosa pseudoannulata*) is common in rice fields. Olfaction is crucial for perceiving chemical information, and our previous research indicated that methyl salicylate (MeSA), a common herbivore-induced plant volatile (HIPV), significantly attracts *P. pseudoannulata*. However, few studies have examined the functions of spiders' olfactory-related genes and their interactions with HIPVs. This study aimed to identify olfactory-related genes associated with MeSA in *P. pseudoannulata*. Transcriptome analysis revealed four differentially expressed olfactory-related genes in response to MeSA in *P. pseudoannulata*. Subsequent molecular docking analysis indicated that MeSA had a strong predicted binding affinity with proteins of two key olfactory-related genes (*PpseNPC2-7* and *PpseSNMP2*). These two genes were cloned, and their expression patterns were investigated in various body parts of male and female spiders. *PpseNPC2-7* and *PpseSNMP2* showed significantly higher expression in leg I, pedipalps, and cephalothorax compared to the abdomen in both males and females. Following RNA interference treatment targeting *PpseNPC2-7* in females and *PpseSNMP2* in males, Y-type olfactory choice experiments revealed a significantly reduced preference for MeSA in dsRNA-treated spiders. Additionally, notable declines in relative electrophysiological responses were observed in five body parts (pedipalps, cephalothorax, leg I, leg II, and leg IV) in dsRNA-treated male and female spiders. These findings demonstrate the significant roles of the *PpseNPC2-7* and *PpseSNMP2* genes in MeSA recognition by *P. pseudoannulata* and lay a strong foundation for further exploration into the olfactory molecular mechanisms underlying HIPV responses in spiders.

INTRODUCTION

The wolf spider *Pardosa pseudoannulata* (Bösenberg & Strand, 1906) (Araneae: Lycosidae) is a common predator in rice fields (Wang & Shi, 2002). This species is an important natural enemy of rice pests such as the brown planthopper *Nilaparvata lugens* Stål (Preap et al., 2001; Wang & Shi, 2002). Previous studies have demonstrated that *P. pseudoannulata*, which exhibits strong predatory ability, high fecundity, and strong resistance, plays a key ecological role in the control of rice pests (Wang, 2007; Xiao et al., 2015; Liu et al., 2016; Fu et al., 2022). However, research on its physiological and biochemical mechanisms is limited, as is research on its molecular biology, especially the function of olfactory genes. Further research in these areas could offer important clues about the mechanisms underlying this spider's role as a natural enemy of agricultural pests. In the present work, we examined olfactory-related genes in the common spider, *P. pseudoannulata*, one of the most extensively studied spiders to date.

Herbivore-induced rice volatiles (HIPVs) play a crucial role in the three-level trophic relationship between plants, herbivores, and natural enemies (Dicke, 2009; Allmann & Baldwin, 2010). When damaged by pests, rice plants release specific volatile substances, such as (*Z*)-3-hexene-1-ol, (*E*)-2-hexene-1-ol, 2-heptanone, octadecane, nerolidol, and methyl salicylate (MeSA) (Turlings et al., 1990; Xu et al., 2002). Research has shown that these rice volatiles attract the natural enemies *Anagrus nilaparvatae* and *Cyrtorhinus lividipennis* following damage by the brown planthopper *N. lugens* (Lou & Cheng, 1996; Rapusas et al., 1996). Parasitoids such as *Cotesia glomerata* can detect HIPVs released by damaged plants, demonstrating the value of HIPVs in pest control (Aartsma et al., 2019). Moreover, HIPVs are known to influence physiological

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activities in spiders. For example, volatiles such as (*E*)-β-caryophyllene, α-humulene, and 1,8-cineole can attract the East African jumping spider, *Evarcha culicivora* (Salticidae), without prior exposure to plants or compounds (Nelson et al., 2012). In addition, scholars have shown that the presence of 0.5 μg/μL linalool can significantly enhance the attraction of *Pirata subpiraticus* and *P. pseudoannulata*. Such exposure reduces predation latency in male *P. subpiraticus* and female *P. pseudoannulata*, and increases the daily predation capacity of female *P. pseudoannulata* (Liu et al., 2022). The inductive effects of 2-heptanone and linalool on male *P. pseudoannulata* have also been documented (Xiao et al., 2018).

Chemical communication is a common method of information exchange among organisms that significantly influences behaviors such as habitat selection, reproduction, foraging, and defense (Ren et al., 2017; Leung et al., 2022). The recognition of external odor molecules is a highly intricate process. Olfactory proteins, crucial to the initial processing of these odor molecules, can be classified into two categories: (1) carrier proteins, such as odorant-binding proteins (OBPs), chemosensory proteins (CSPs), and Niemann-Pick type C2 proteins (NPC2s); and (2) chemosensory membrane proteins, including olfactory receptors (ORs), ionotropic receptors (IRs), gustatory receptors (GRs), and sensory neuron membrane proteins (SNMPs) (Li et al., 2006; Renthal et al., 2017; Cassau & Krieger, 2021; Wicher & Miazzi, 2021).

Research on olfactory-related genes and their functions has predominantly focused on insect species, with fewer studies on arachnids and other arthropod classes. Recent genomic studies have identified multiple chemosensory gene families in spiders, including gustatory receptors (GRs), ionotropic receptors (IR/iGluRs), OBP-like proteins, NPC2 proteins, carrier proteins (CCPs), and CD36 SNMPs. For example, the annotated genome of the wandering spider *Dysdera silvatica* contains 127 GRs, 443 IR/iGluRs, 4 OBP-like genes, 7 NPC2 genes, 7 CCP genes, and 11 CD36-SNMP genes (Escuer et al., 2022). Comparative genomic analysis of multiple spider species, including *Tetragnatha kauaiensis*, further revealed that there was substantial interspecific variation in the copy numbers of these chemosensory gene families (e.g., GRs ranging from 84 to 1,436), and that transposable element expansion is a key driver of genome size variation in spiders (Cerca et al., 2021). Similarly, in the lone star tick (*Amblyomma americanum*), Renthal et al. (2017) identified two proteins structurally homologous to insect OBPs, with high expression in the pedipalps. Transcriptomic analysis of the storage mite (*Tyrophagus putrescentiae*) revealed two CSP genes, with three-dimensional (3D) modeling demonstrating a hydrophobic ligand-binding pocket formed by six α-helices. Molecular docking trials indicated strong predicted binding affinities of (-)-isolongifolene, 2-methylnaphthalene, and cyclopentadecane for TputCSP1 (Qu et al., 2016). Proteomic characterization of Haller's organ (the olfactory apparatus) and the pedipalps of the castor bean tick *Ixodes ricinus* identified 13 NPC2 proteins. Competitive binding assays revealed that IricNPC2-1 could bind α-amyl cinnamaldehyde and other organic compounds, suggesting a potential role in odorant transport (Iovinella et al., 2016). In the predatory mite *Neoseiulus barkeri* (Nb), NPC2 genes may play a role in chemical communication in adult males, with NbNPC2-1 exhibiting specific olfactory functions in females (Li et al., 2020). Transcriptome mining in *P. pseudoannulata* uncovered six NPC2 genes showing elevated expression in the adult chelicerae and pedipalps (Cao et al., 2018; Xiu et al., 2019).

Wandering spiders, characterized by their wide foraging range and high predation capacity, are important natural enemies in rice fields (Preap et al., 2001). As a wandering spider, *P. pseudoannulata* depends on visual, tactile, and auditory cues to capture prey, with olfaction playing a crucial role in long-distance prey localization and capture. MeSA is an important herbivore-induced plant volatile that participates in plant defense (Zhao et al., 2010). Not only does it serve as a defense mechanism but it also attracts insects and other invertebrates (Shimoda et al., 2002; De Boer & Dicke, 2004; Zhu & Park, 2005). In our previous work, olfactory choice experiments showed that 1.0 and 1.5 μg/μL MeSA had a significant attractant effect on male *P. pseudoannulata* (Xiao et al., 2018; Liu et al., 2022).

However, the molecular mechanisms underlying this attraction remain unclear. Previous studies have demonstrated that exposure to odorants can alter the mRNA levels of chemosensory genes in various animals (Wan et al., 2015; Paula et al., 2018; Mappin et al., 2023). For example, when *Spodoptera exigua* was exposed to plant volatiles, chemosensory genes such as *OBP1*, *OBP7*, *PBP1*, and *ABP2* were expressed at higher levels compared to controls exposed to ambient air (Wan et al., 2015); the same study further employed qRT-PCR to characterize the expression profiles of olfactory-related genes in *S. exigua* after pre-exposure to plant volatiles, and found that the transcript levels of *OR3*, *OR6*, *OR11*, *OR13*, *OR16*, *OR18*, *Orco*, *ABP2*, *OBP1*, *OBP7*, and *PBP1*, as well as the electroantennogram (EAG) responses, were significantly affected. In addition, transcriptome analysis of larval heads revealed that the odorant receptor *SfruOR51* was significantly upregulated in response to 2-hexynoic acid (Huang et al., 2025). Similarly, exposure to a single volatile organic compound led to the strong and non-specific upregulation of olfactory receptors (Llopis-Giménez et al., 2020). These findings confirm that both chemosensory gene expression and olfactory sensitivity can be affected by pre-exposure to plant volatiles. Accordingly, we adopted gene expression plasticity as a framework to explore olfactory responses to plant volatiles. In *Chrysoperla sinica*, an important predatory arthropod, 12 *CsinOBP* genes exhibit predominant expression in the antennae of both male and female individuals (Li et al., 2018), highlighting the conserved role of OBPs in chemosensory perception across predatory arthropods. Wang et al. (2018) identified 18 OBPs, 1 NPC2, and 3 SNMPs from the antennal transcriptomes of *Cyrtorhinus lividipennis* Reuter (Hemiptera: Miridae) and different olfactory-related genes showed differences in expression

profile between males and females. Based on these findings, we aimed to identify olfactory-related genes potentially responsive to MeSA in *P. pseudoannulata*. First, we identified olfactory-related genes upregulated in adult female and male *P. pseudoannulata* following treatment with 2.5 μg/μL MeSA. Molecular docking experiments were further performed to examine the interactions between olfactory proteins and MeSA. The functions of the above key genes were subsequently validated using RNA interference (RNAi) technology, behavioral choice experiments, and electrophysiological experiments. This study contributes valuable basic data essential for understanding the olfactory mechanism of *P. pseudoannulata*. Elucidating the olfactory mechanism of *P. pseudoannulata* in response to MeSA not only sheds light on its ecological adaptability but also provides a theoretical basis for biological control and ecological conservation.

MATERIAL AND METHODS

Spiders

The study primarily focused on *P. pseudoannulata* adults. Subadults and spiderlings of various ages were collected from rice fields in Bahuo Village, Huaxi District, Guiyang, Guizhou Province, China (26.41°N, 106.68°E). The spiders were individually placed in transparent polypropylene (PP) tubes (12 cm × 4 cm diameter) containing water-soaked cotton balls at the bottom for humidity control, and the tubes were sealed with sterilized cotton. The spiders were maintained in an artificial climate chamber at 25 ± 1°C, 65 ± 5% relative humidity, and a 14L : 10D photoperiod. Each spider was fed three to six housefly pupae twice per week, and molting status was observed daily until maturity. To ensure consistency across all experimental procedures, unmated adult spiders were used in all subsequent assays.

Transcriptome sequencing

Sample processing

Adult male and female *P. pseudoannulata* were exposed to 2.5 μg/μL MeSA, a concentration determined based on previous research (von der Weid et al., 2015; Xiao et al., 2018). In our previous work, olfactory choice experiments showed that 1.5 μg/μL MeSA had a significant attractive effect on male *P. pseudoannulata* (Xiao et al., 2018), and von der Weid et al. (2015) found that increasing the concentration of volatiles, together with large-scale transcriptional profiling of chemosensory neurons, could help identify receptor-ligand pairs in *Drosophila*. Accordingly, we used a concentration of 2.5 μg/μL prior to transcriptome sequencing to balance effective olfactory stimulation and transcriptional detectability. Experiments were conducted in an observation chamber (4.7 cm in diameter, 3.9 cm in height), where PCR (Polymerase Chain Reaction) tubes containing 2.5 μg/μL MeSA were attached to the walls, while the control group used 99% paraffin. Liquid paraffin, which was selected for its chemical stability, low volatility, and good solubility, served as both the solvent for MeSA and the control to avoid interference with the spiders' olfactory responses. After the observation chamber was saturated with volatiles, spiders that had been starved for 48 h were introduced for 1 min. This duration was based on preliminary qRT-PCR analyses, which indicated that the spiders showed no significant changes in olfaction-related gene expression after longer exposures (10, 15, or 30 min). Moreover, brief exposure can effectively avoid acute toxic reactions potentially induced by chemical compounds (Mappin et al., 2023) while better capturing the initial olfactory response before signal dampening by odorant-degrading enzymes (ODEs). The four groups comprised a female control group (CK-F), a male control group (CK-M), a female treatment group (MeSA-F), and a male treatment group (MeSA-M), with three spiders used in each group. The treated spiders were rapidly frozen in liquid nitrogen and stored at −80°C for subsequent experiments.

Transcriptome sequencing

Total RNA was extracted using the TRIzol method. RNA integrity and quality were assessed via 1% agarose gel electrophoresis, and RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Four groups' cDNA libraries were constructed and sequenced using the NovaSeq 6000 sequencing platform at Biomarker Technologies (Beijing, China) with paired-end 150-bp sequencing. Prior to data analysis, strict quality control was performed to ensure that the sequencing reads were of sufficient quality, including removing adapter-containing sequences and low-quality reads (reads with more than 10% ambiguous bases or with over 50% of bases having a quality score ≤ 10). The clean-data yields for CK-F, CK-M, MeSA-F, and MeSA-M were 6.01, 6.05, 6.06, and 5.70 Gb, respectively. High-quality reads were aligned to the *P. pseudoannulata* reference genome (Version GCA_032207245.1; source: https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_032207245.1/) using HISAT2 v2.2.1 (--dta -p 6 --max-intronlen 5000000) (Kim et al., 2015). According to the alignment statistics, mapping rates of reads to the reference genome ranged from 85.16% to 87.19%. Based on the reference genome sequence, mapped reads were assembled using StringTie v2.2.1 (--merge -F 0.1 -T 0.1) (Pertea et al., 2015), and the resulting transcripts were compared with existing genome annotations to supplement and improve the original genome annotation.

Analysis of Differential expression genes

Gene expression abundance was estimated using StringTie v2.2.1 and reported as FPKM values. Differential expression analysis was performed using EBSeq v1.30.0 (Leng et al., 2013), with the raw gene-level count matrix provided by Biomarker Technologies as input. Differentially expressed genes (DEGs) were identified using thresholds of $|\mathrm{Log_2(FC)}| \geq 1$ and FDR ≤ 0.01. Differential expression was assessed for CK-F versus MeSA-F and CK-M versus MeSA-M. DEG functional annotation was performed using multiple databases, including NR (NCBI non-redundant protein sequences; accessed in August 2023), GO (Gene Ontology), Swiss-Prot (a manually annotated and peer-reviewed protein sequence database; accessed in August 2023), eggNOG 4.5 (Huerta-Cepas et al., 2016), COG (Clusters of Orthologous Groups of proteins), and KEGG (Kyoto Encyclopedia of Genes and Genomes) using DIAMOND v2.0.15 (Buchfink et al., 2015). The predicted amino acid sequences were searched against the Pfam database (accessed in August 2023) using HMMER v3.4 (Finn et al., 2011), with hits considered significant at an E-value ≤ 1e-10. Olfactory-related genes, including OBP, CSP, NPC2, IR, and SNMP, were identified based on functional annotation results. Domain architectures of annotated proteins were further validated using the SMART online tool (http://smart.embl.de).

To confirm the accuracy of the transcriptome data, four upregulated and four downregulated genes were randomly selected from male and female groups for qRT-PCR validation. Reverse transcription was performed using the StarScript pro All-in-One RT Mix with the gDNA Remover kit (Genstar, Beijing, China), and qRT-PCR was conducted using the 2 × RealStar Fast SYBR qRT-PCR Mix kit (Genstar, Beijing, China) on the CFX96 Touch real-time PCR instrument (Bio-Rad, Hercules, CA, USA). Prim-

er sequences are listed in Table S1, and the reference gene for *β*-actin was used as described by Cao et al. (2018).

P. pseudoannulata OBPs phylogenetic analysis

For the *P. pseudoannulata* OBPs identified from transcriptome sequencing, we performed comparative sequence analysis that included both insect OBPs and spider OBP-like proteins. Representative insect OBP sequences were obtained from *Anopheles gambiae* (African malaria mosquito), *Apis mellifera* (western honeybee), and *Drosophila melanogaster* (fruit fly). For spiders, OBP-like sequences were retrieved from published genomic resources (Cerca et al., 2021), including *Araneus ventricosus* (orb-weaving spider), *Argiope bruennichi* (European wasp spider), *Dysdera silvatica* (nocturnal ground-dwelling spider), *Latrodectus hesperus* (western black widow spider), *Parasteatoda tepidariorum* (common house spider), *Stegodyphus mimosarum* (African social velvet spider), *Tetragnatha kauaiensis* (long-jawed spider), and *Trichonephila clavipes* (golden silk spider). Multiple sequence alignment was performed in MEGA v11.0.10 using ClustalW, and phylogenetic trees were constructed with the Neighbor-Joining (NJ) method based on the p-distance model. Bootstrap support was assessed with 1000 replicates, and gaps were treated by partial deletion with a site coverage cutoff of 50%. The resulting phylogeny was plotted, formatted, colored, and labeled using the iTOL web server (Letunic & Bork, 2024).

Molecular modeling and docking

The structures of proteins encoded by four differentially expressed olfactory-related genes were predicted using AlphaFold3 (https://alphafoldserver.com/) (Abramson et al., 2024). Each gene was modeled five times, and the structure with the highest predicted template modeling (pTM) score was selected for molecular docking with MeSA, whose 3D structure was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Two ionotropic receptors, PpseIR25a and PpseIR93a-3, which are highly expressed in the pedipalps (a major chemosensory organ in spiders; Tichy et al., 2001; Xiao, 2019), were selected as negative controls for molecular docking, as well as three rice volatiles known to be induced by herbivory: 2-heptanone, cis-3-hexen-1-ol, and linalool (Liu et al., 2022). Protein preprocessing in AutoDockTool 1.5.7 comprised water removal, hydrogen bond addition, the merging of non-polar hydrogens, receptor atom type identification, and the assignment of atomic charges and force fields, with the results saved in PDBQT format. For the ligand molecules, atomic charges and force fields were added, with the results also saved in PDBQT format. Molecular docking was performed using AutoDock Vina 1.2.7, with 50 docking results generated per group (Trott & Olson, 2010). Docking structures were visualized in PyMOL 2.5.7.

Cloning and expression patterns of olfactory-related genes in different body parts

Based on their relatively favorable predicted binding to MeSA, PpseNPC2-7 and PpseSNMP2 were selected for cloning, and their expression patterns in 7 different body parts (pedipalps, legs I–IV, cephalothorax, and abdomen) were investigated. After 1 min of MeSA exposure, male or female spiders were frozen in liquid nitrogen and then dissected on an ice-cold plate. Each body part from five different spiders was pooled as a single biological replicate, and three independent replicates were prepared per body part (42 samples in total, 7 body parts with 3 replicates per body part for each sex). RNA extraction, reverse transcription, and qRT-PCR experiments were conducted. Specific primers were designed in Primer 6.0 according to the transcriptome sequence data, and total RNA extraction and reverse transcription were completed following the protocol (primer information is provided in Table S2). PCR amplification was conducted using the 2 × Taq PCR StarMix (Dye) kit (Genstar, Beijing, China). PCR products were purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China) and ligated into the pGEM-T Easy vector (Promega, USA). Sequencing was performed at Shanghai Sangon Biotech Co., Ltd.

RNAi efficiency of two olfactory-related genes

RNAi was used to silence the expression of the two genes (*PpseNPC2-7* and *PpseSNMP2*) to verify their functions. Specific primers with T7 promoters (Table S3) were designed using the open reading frame (ORF) sequences. The PCR products were purified, and the fragments used for in vitro synthesis of double-stranded RNA (dsRNA) using the TranscriptAid T7 High Yield Transcription Kit according to the manufacturer's instructions. The dsRNA concentration and integrity were assessed using a Nanodrop 2000 spectrophotometer and 1% agarose gel electrophoresis, respectively. Healthy, active male and female *P. pseudoannulata* with minimal size variation were microinjected with 1 µL of dsRNA (1,000 ng/µL) or ds*GFP* as the control (Fu et al., 2022). Following injection, spiders were individually housed in an artificial climate chamber for later experiments. At 24, 48, and 72 h post-dsRNA injection, male and female spiders were snap-frozen in liquid nitrogen for 5 min and subsequently stored at −80°C. At each time point, three independent biological replicates were prepared separately for each sex. Each replicate consisted of three individuals of the same sex. Total RNA was then extracted and reverse-transcribed into cDNA, and RNAi efficiency was quantified via qRT-PCR.

Electrophysiological response and behavioral choice after RNAi

Based on the results of RNAi efficiency, female spiders at 48 h post-injection with ds*PpseNPC2-7* and male spiders at 24 h post-injection with ds*PpseSNMP2* were tested with 2.5 µg/µL MeSA as the stimulus and 99% paraffin as the control for Y-shaped olfactory choice experiments. Trials were conducted at 25°C with an odor flow rate of 400 mL/min in a dark environment and monitored using a head-mounted red flashlight to eliminate visual cues and ensure that the spiders' responses were primarily driven by olfactory stimuli. Behavioral responses were recorded when spiders entered an arm to a distance of > 10 cm for ≥ 30 s. Trials with no response within 15 min were excluded (Xiao et al., 2018). The Y-olfactometer was cleaned with 75% ethanol after every five tests, and odor directions were alternated every 20 min. Each treatment group comprised 30 individuals.

Electrophysiological experiments were performed to measure the responses of the pedipalps, legs I–IV, and the cephalothorax to 2.5 µg/µL MeSA. Following RNAi treatment (female spiders 48 h post-injection with ds*PpseNPC2-7* and male spiders 24 h post-injection with ds*PpseSNMP2*), the spider tissues were placed on the electrophysiological equipment's tuning fork electrodes and secured with conductive adhesive. Tests were initiated after baseline stabilization, with 10 µL MeSA applied to filter paper placed in a pipette tip connected to the gas stimulation device (400 mL/min for 0.5 s). Responses were averaged across five replicates per spider. Relative electrophysiological responses were calculated as follows: Relative electrophysiological response (mV) = electrophysiological response to MeSA (mV) − mean electrophysiological response to the control (liquid paraffin) (mV).

Data analysis

The data were organized using Microsoft Excel 2019. The verification of transcriptome sequencing data, the relative expression levels of olfactory-related genes in different body parts of male and female spiders, and the detection of interference efficiency

after olfactory gene interference were analyzed using the $2^{-\Delta\Delta Ct}$ method. Relative expression levels were expressed as mean ± standard error (SE), and the expression levels in the spider abdomen were used as controls. One-way ANOVA was used for the analysis of gene expression, followed by Tukey's procedure for multiple comparisons. Student's *t*-tests were used for differential comparison of olfactory-related genes between male and female spiders. Chi-squared tests were used to compare olfactory behavioral choice responses of *P. pseudoannulata*. In the electrophysiological experiments, Student's *t*-tests were used to compare results that conformed to a normal distribution. For results that did not conform to a normal distribution, the Mann-Whitney *U* test was used for differential comparison. Normally distributed data are presented as the mean ± SE, whereas non-normally distributed data are presented as the median (Q1–Q3). All figures were generated using Origin 2024.

RESULTS

Transcriptome analysis of *P. pseudoannulata*

Transcriptome sequencing yielded a total of 23.82 Gb of clean data, which has been uploaded to the National Center for Biotechnology Information (NCBI) database (BioProject: PRJNA1195908). Each sample's clean data reached at least 5.70 Gb, with a Q30 base percentage of 91.45% or higher (Table S4). The Venn diagram of expressed genes in the four samples is shown in Fig. S1. Compared with the control groups (CK-F: 11,227; CK-M: 8,172), the methyl salicylate treatments had 11,331 genes in MeSA-F and 7,507 in MeSA-M. To confirm the accuracy of the transcriptome data, qRT-PCR validation experiments demonstrated consistency between the transcriptome data and relative expression patterns, confirming the reliability of the transcriptome results (Figs S2 and S3).

DEG analysis

Comparative analysis identified 1,348 and 1,241 DEGs in the CK-F vs. MeSA-F and CK-M vs. MeSA-M groups, respectively (Fig. S4). The annotated DEGs for both the CK-F vs. MeSA-F and CK-M vs. MeSA-M groups are listed in Table S5 (Supplement S2). In the CK-F vs. MeSA-F comparison, we identified three DEGs: *PpseOBP1*, *PpseOBP2*, and *PpseNPC2-7*. In the CK-M vs. MeSA-M comparison, three olfactory-related DEGs were screened out: *PpseOBP1*, *PpseOBP2*, and *PpseSNMP2* (Table S6).

COG, GO, and KEGG functional analyses of DEGs

The DEG annotation results from COG classification are shown in Fig. S5. In the CK-F vs. MeSA-F group, 276 DEGs were annotated, while in the CK-M vs. MeSA-M group, 323 DEGs were annotated. The GO classification results of DEGs are presented in Fig. S6. There were 842 and 803 annotated genes in the CK-F vs. MeSA-F and CK-M vs. MeSA-M group comparisons, respectively. The KEGG classification enrichment analysis of DEGs is shown in Fig. S7. For the CK-F vs. MeSA-F and CK-M vs. MeSA-M comparisons, 285 and 307 genes were annotated in KEGG categories, respectively. COG enrichment analysis showed that the differentially expressed genes mediated by methyl salicylate were mainly enriched in categories R: General function prediction only and Q: Secondary metabolites biosynthesis, transport and catabolism (Fig. S5). GO enrichment analysis revealed that the differentially expressed genes were mainly enriched in biological processes such as cellular process, metabolic process, and biological regulation; in cellular components, they were mainly enriched in cellular anatomical entity, intracellular, and protein-containing complex; and in molecular functions, they were mainly enriched in binding, catalytic activity, and transporter activity (Fig. S6). KEGG results showed that the pathways with higher enrichment ratios were mainly concentrated in metabolism-related pathways. Among them, Tyrosine metabolism had the highest enrichment ratio (6.67%), suggesting that tyrosine metabolism may play an important role in the response to methyl salicylate stimulation. In addition, multiple lipid metabolism-related pathways were significantly enriched, including fatty acid metabolism, linoleic acid metabolism, and arachidonic acid metabolism, indicating that methyl salicylate treatment may induce lipid metabolism remodeling in *P. pseudoannulata*. Notably, signal transduction-related pathways such as neuroactive ligand-receptor interaction, ECM-receptor interaction, Hedgehog signaling pathway, and mTOR signaling pathway were also enriched, suggesting that plant volatile signals may mediate behavioral responses by regulating neural perception and cellular signaling networks. Moreover, similar enrichment patterns were observed in the differential gene analyses performed separately for males and females (Fig. S7).

Phylogenetic relationship of *P. pseudoannulata* OBPs

A phylogenetic tree constructed from the amino acid sequences of insect OBPs and spider OBP-like proteins revealed that both *P. pseudoannulata* OBPs clustered with spider OBP-like proteins, particularly those from *P. tepidariorum* (Ptep), *T. clavipes* (Tcla), and *S. mimosarum* (Smim). In contrast, they were clearly separated from insect OBPs (Fig. 1).

Molecular docking

AlphaFold3 predictions indicated that all olfactory-related proteins exhibited good structural confidence (Table S7). The 3D protein binding information of four differentially expressed olfactory-related proteins and two ionotropic receptors (PpseIR25a and PpseIR93a-3) with four HIPVs is shown in Table 1. The molecular docking results demonstrated that MeSA bound stably to PpseNPC2-7, PpseSNMP2, PpseOBP1, and PpseOBP2, with affinities of −5.8, −5.5, −5.3, and −5.1 kcal/mol, respectively. These values were typically lower than those of other tested compounds, indicating a stronger predicted binding potential. In addition, MeSA showed affinities of −4.7 and −4.8 kcal/mol for the ionotropic receptors PpseIR25a and PpseIR93a-3, respectively, indicating relatively weaker predicted interactions. The docking region showed that MeSA binds to PpseOBP1 in a deep cavity pocket, to PpseOBP2 in a channel-like binding mode, to PpseNPC2-7 in a shallow groove, and to PpseSNMP2 in a deep cavity pocket. Key amino acid residues that mediate MeSA binding in-

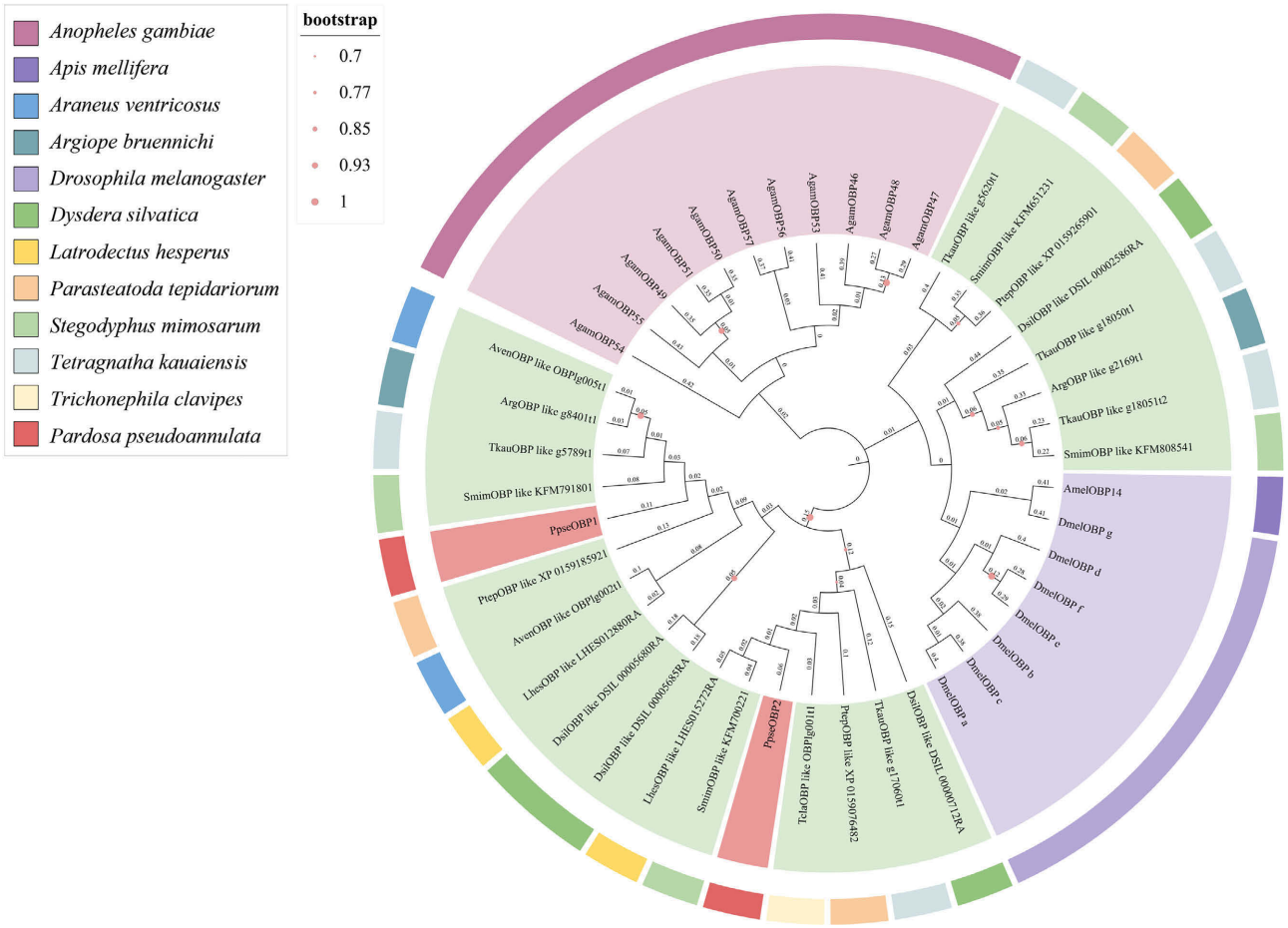

Fig. 1. Neighbor-Joining tree of PpseOBPs, insect OBPs, and spider OBP-likes. Included OBPs are from *Anopheles gambiae* (Agam), *Apis mellifera* (Amel), and *Drosophila melanogaster* (Dmel). Included OBP-likes are from *Araneus ventricosus* (Aven), *Argiope bruennichi* (Arg), *Dysdera silvatica* (Dsil), *Latrodectus hesperus* (Lhes), *Parasteatoda tepidariorum* (Ptep), *Stegodyphus mimosarum* (Smim), *Tetragnatha kauaiensis* (Tkau), and *Trichonephila clavipes* (Tcla).

Table 1. Molecular docking results of six receptor proteins and four HIPVs.

Receptor molecules	Ligand molecules	Affinity (kcal/mol)	Amino acid residues that form hydrogen bonds with the ligand molecule
PpseOBP1	2-Heptanone, HE	−4.4	LYS-67
	cis-3-hexen-1-ol, CH	−3.9	LEU-14, PHE-17
	Linalool, LI	−5.3	GLU-98, ILE-62
	Methyl salicylate, MS	−5.3	GLN-154, TYR-107
PpseOBP2	2-Heptanone, HE	−3.9	PHE-19, SER-20
	cis-3-hexen-1-ol, CH	−3.7	PHE-19, SER-20
	Linalool, LI	−4.8	SER-20
	Methyl salicylate, MS	−5.1	SER-20, LEU-18
PpseNPC2-7	2-Heptanone, HE	−4.9	THR-121
	cis-3-hexen-1-ol, CH	−4.4	PHE-21, THR-23
	Linalool, LI	−3.7	PHE-21, THR-23
	Methyl salicylate, MS	−5.8	GLY-81, ARG-83, GLN-106, LYS-90
PpseSNMP2	2-Heptanone, HE	−4.8	PHE-440
	cis-3-hexen-1-ol, CH	−4.1	MET-212
	Linalool, LI	−4.8	SER-237
	Methyl salicylate, MS	−5.5	LEU-293, LEU-222
PpseIR25a	2-Heptanone, HE	−3.7	LYS-668
	cis-3-hexen-1-ol, CH	−3.4	LYS-179, GLN-296
	Linalool, LI	−4.8	LYS-668, GLN-755
	Methyl salicylate, MS	−4.7	ASN-38, ASP-39, LYS-98
PpseIR93a-3	2-Heptanone, HE	−4.0	TRP-164
	cis-3-hexen-1-ol, CH	−3.6	—
	Linalool, LI	−4.3	ASP-157, ARG-159
	Methyl salicylate, MS	−4.8	ASN-257, LEU-753, GLN-754

clude GLY-81, ARG-83, GLN-106, and LYS-90 in PpseNPC2-7, as well as LEU-293 and LEU-222 in PpseSNMP2. The visualization of the docking interactions revealed hydrogen bonding and hydrophobic contacts between MeSA and both proteins. In addition, salt bridge formation was observed in the PpseNPC2-7-MeSA complex (Fig. 2).

Cloning and expression patterns of two olfactory-related genes in *P. pseudoannulata*

Based on the results of molecular docking, the binding stability of MeSA was greater toward PpseNPC2-7 and PpseSNMP2 than toward PpseOBP1 and PpseOBP2. Therefore, the genes *PpseNPC2-7* and *PpseSNMP2* were selected for subsequent functional experiments. The coding sequences of *PpseNPC2-7* and *PpseSNMP2* were validated through cloning and were assigned the GenBank accession numbers PQ855353.1 and PQ855354.1, respectively. The results indicate that *PpseNPC2-7* harbors an ML domain (PF02221), which is found in proteins rom plants, animals, and fungi, including epididymal secretory protein E1 (also known as NPC2) (Fig. S8). *PpseSNMP2* features a CD36 domain (PF01130) (Fig. S9).

The expression levels of *PpseNPC2-7* and *PpseSNMP2* were significantly different ($P < 0.05$) among the body parts of male and female *P. pseudoannulata* (Fig. 3). In females,

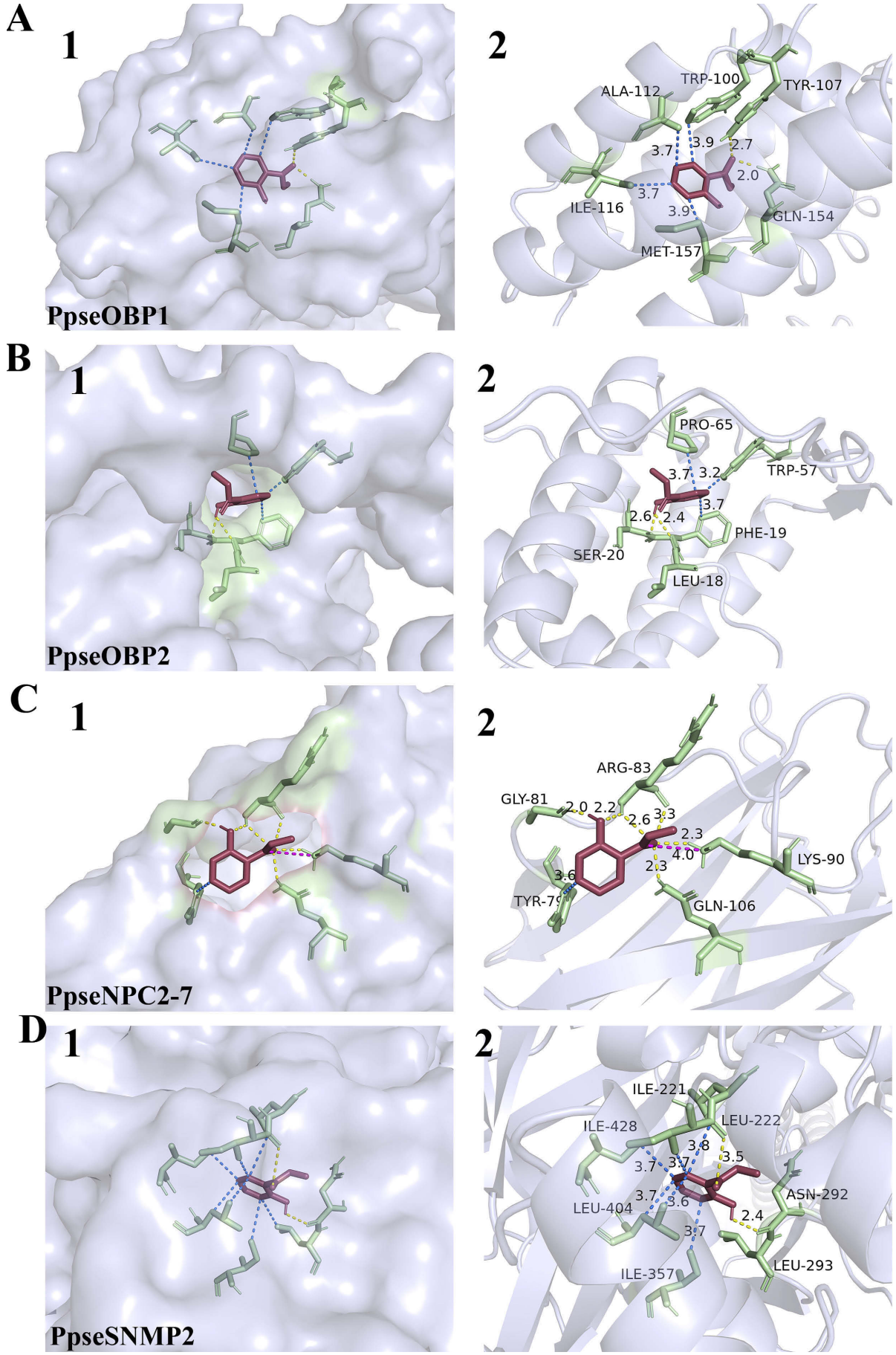

Fig. 2. Visualization results of molecular docking.

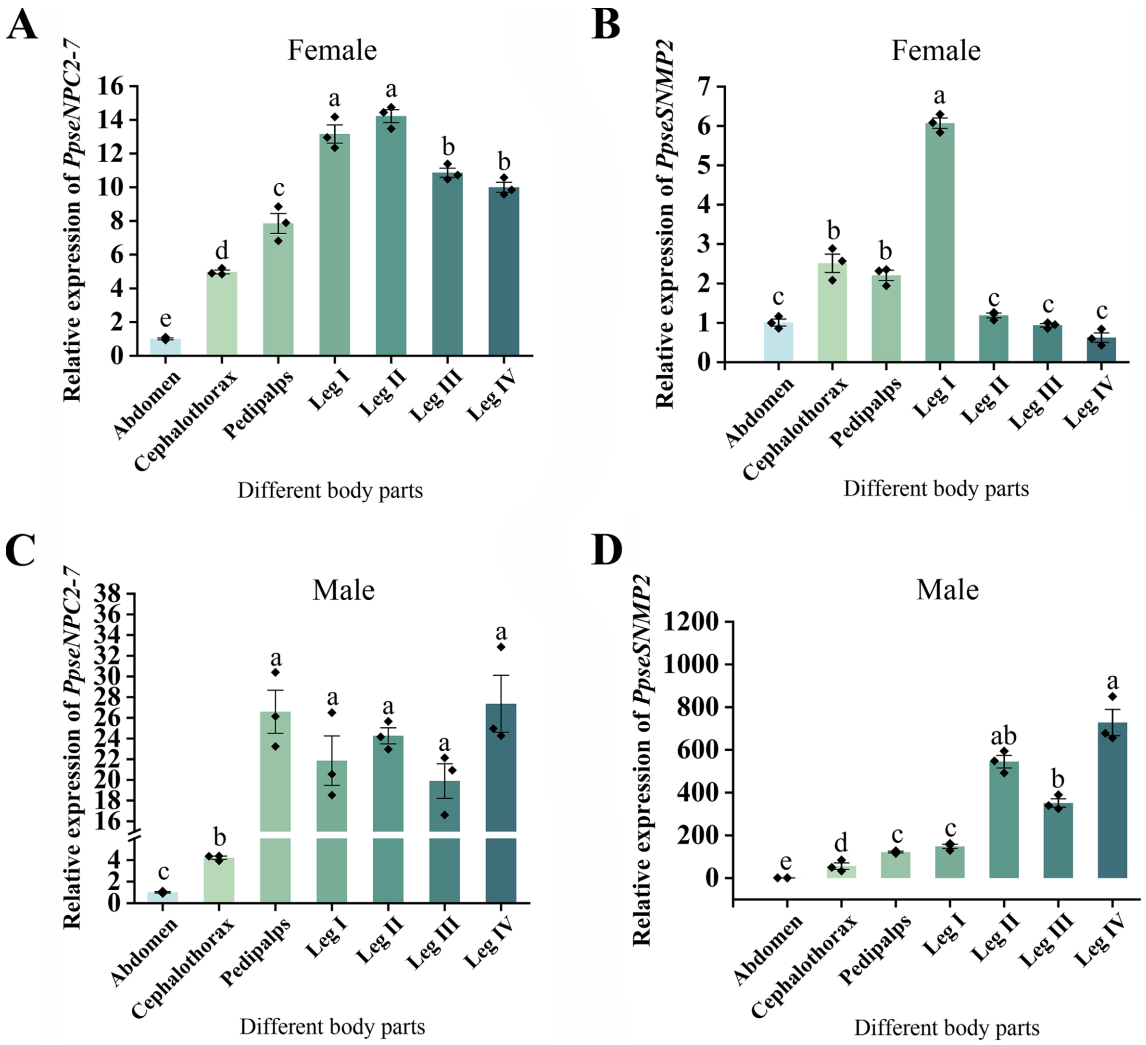

Fig. 3. Expression patterns of two genes in various body parts of spiders following treatment with 2.5 μg/μL methyl salicylate (MeSA). (A) Relative expression levels of *PpseNPC2-7* in female spiders; (B) relative expression levels of *PpseSNMP2* in female spiders; (C) relative expression levels of *PpseNPC2-7* in male spiders; and (D) relative expression levels of *PpseSNMP2* in male spiders. Different letters (a, b, c, d, e) above the bars indicate significant differences among body parts based on one-way ANOVA followed by Tukey's multiple comparisons ($P < 0.05$).

both *PpseNPC2-7* and *PpseSNMP2* displayed significantly higher expression in leg I, pedipalps, and cephalothorax tissues compared with the abdomen ($P<0.05$) (Figs 3A and 3B). In males, both *PpseNPC2-7* and *PpseSNMP2* exhibited significantly high expression across the cephalothorax, pedipalps, and legs I–IV, surpassing abdominal levels ($P<0.05$) (Figs 3C and 3D). Remarkably, the expression level of *PpseSNMP2* in leg IV was 703.34 times higher than that in the abdomen ($P<0.05$) (Fig. 3D).

RNAi efficiency detection of olfactory-related genes in *P. pseudoannulata*

We assessed RNAi efficiency ~~on~~for the olfactory-related genes *PpseNPC2-7* in females and *PpseSNMP2* in males at 24, 48, and 72 h post-dsRNA injection. The highest interference efficiency for ds*PpseNPC2-7* was observed 48 h after injection, with *PpseNPC2-7* expression being downregulated by 70.27% (Fig. 4A). The highest interference efficiency for ds*PpseSNMP2* was observed 24 h after injection, with *PpseSNMP2* expression being downregulated by 87.94% (Fig. 4B).

Behavioral choice results of *P. pseudoannulata* after RNAi

Based on the RNAi efficiency results, we conducted behavioral tests on female spiders at 48 h post-injection with ds*PpseNPC2-7* and on male spiders at 24 h post-injection with ds*PpseSNMP2*. For females in the control group (ds*GFP*), 30 replicate trials were conducted. Two spiders did not make a choice within 15 min, while the remaining 28 spiders selected between MeSA and paraffin oil (n = 28). Among them, 20 chose MeSA (71.43%), while 8 selected paraffin oil (28.57%) ($\chi^2 = 5.143$, $P = 0.023$). The difference was statistically significant ($P<0.05$), indicating that control females displayed a clear preference for MeSA. In the ds*PpseNPC2-7* group, 11 female spiders did not make a choice within 15 min. Among the 19 that did (n = 19), 8 selected MeSA (42.11%) and 11 chose paraffin oil (57.89%) ($\chi^2 = 0.474$, $P = 0.491$). No significant preference was observed ($P>0.05$). For male spiders, all 30 spiders in the control group (ds*GFP*) made choices (n = 30). Among them, 21 selected MeSA (70.00%), while 9 chose paraf-

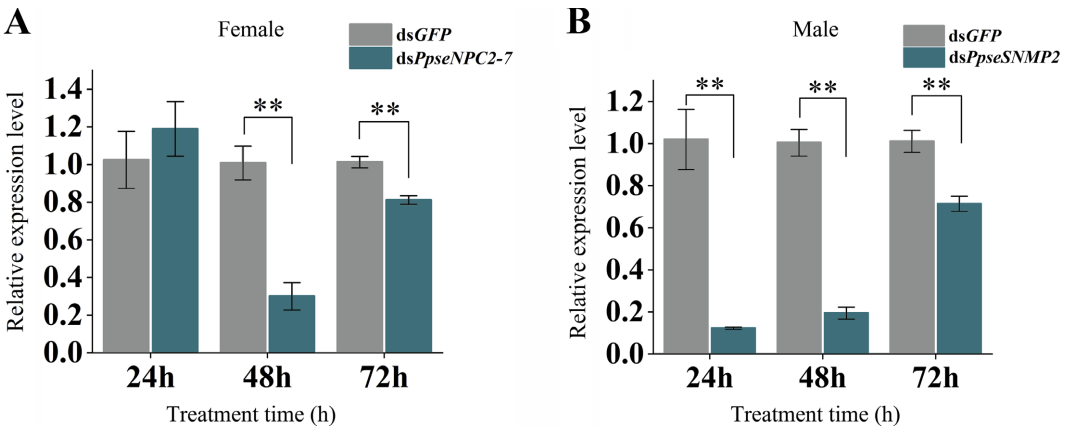

Fig. 4. Relative mRNA expression levels of two genes following dsRNA treatment in *Pardosa pseudoannulata*. (A) *PpseNPC2-7* gene in female spiders; (B) *PpseSNMP2* gene in male spiders. Asterisks (**) indicate a significant difference at the $P < 0.01$ level by Student's *t*-test.

fin oil (30.00%), demonstrating a significant preference for MeSA ($\chi^2 = 4.80$, $P = 0.028$). In the ds*PpseSNMP2* group, 8 spiders did not respond. Among the remaining 22, 6 chose MeSA (27.27%) and 16 opted for paraffin oil (72.73%) ($\chi^2 = 4.545$, $P = 0.033$), revealing a marked shift in choice behavior toward paraffin oil (Fig. 5).

Electrophysiological response results of *P. pseudoannulata* after RNAi

The relative electrophysiological responses of different body parts in female spiders after 48 h of ds*PpseNPC2-7* interference are shown in Fig. 6, while representative electrophysiological traces from key body parts are presented in Fig. S10. In one set of experiments, female spiders without *PpseNPC2-7* interference exhibited responses to MeSA stimulation ranging from 0.204–0.137 mV in the pedipalps (Fig. S10A), 0.220–0.175 mV in leg I (Fig. S10C), and 0.179–0.139 mV in leg II (Fig. S10E). Following ds*PpseNPC2-7* interference, the response ranges in these body parts decreased to 0.032–0.023 mV (Fig. S10B), 0.031–0.014 mV (Fig. S10D), and 0.021–0.016 mV (Fig. S10F), respectively. Overall, the responses of ds*PpseNPC2-7*-treated females to MeSA were significantly lower than those of ds*GFP*-treated controls ($P < 0.001$) across all body parts, except for leg IV ($P < 0.05$; Fig. 6E). In ds*GFP*-treated females, the highest responses were observed in leg I [0.178

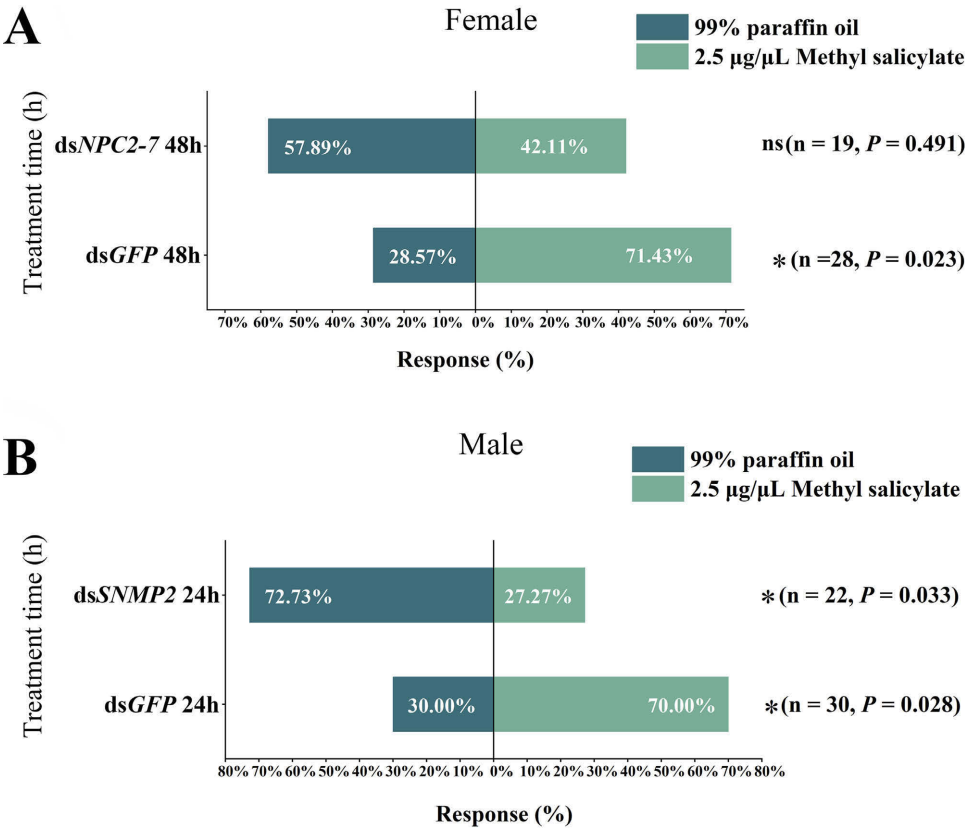

Fig. 5. Behavioral responses of *Pardosa pseudoannulata* after RNA interference. Asterisks (*) indicate significant differences at $P < 0.05$ based on the Chi-square test.

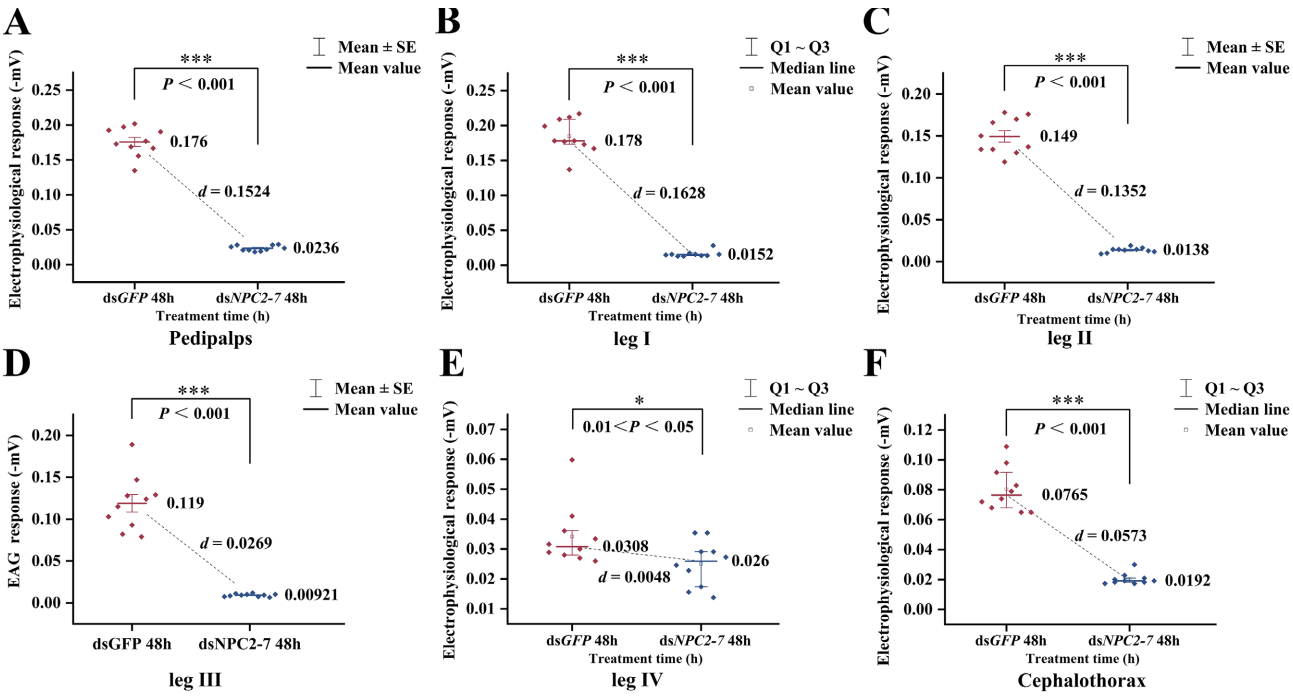

Fig. 6. Relative electrophysiological response values of each body part of female spiders after 48 h of ds*PpseNPC2-7* treatment. (A)–(F) Relative electrophysiological response values of spider pedipalps, leg I, leg II, leg III, leg IV, and the cephalothorax. Note: the Mann-Whitney *U* test was utilized for leg I (B), leg IV (E), and the cephalothorax (F) (***$P < 0.001$), while Student's *t*-test was employed for the other body parts (***$P < 0.001$).

(0.172–0.21) mV; Fig. 6B], pedipalps [0.176 ± 0.007 mV; Fig. 6A], and leg II [0.1494 ± 0.007 mV; Fig. 6C].

The relative electrophysiological responses of different body parts in male spiders after 24 h of ds*PpseSNMP2* interference are shown in Fig. 7, with representative traces presented in Fig. S11. In one set of experiments, male spiders without *SNMP2* interference exhibited responses to MeSA stimulation ranging from 0.117–0.073 mV in the pedipalps (Fig. S11A), 0.149–0.141 mV in leg I (Fig. S11C), and 0.145–0.140 mV in leg II (Fig. S11E). After *SNMP2* interference, the response ranges decreased to 0.059–0.034 mV (Fig. S11B), 0.048–0.032 mV (Fig. S11D), and 0.041–0.024 mV (Fig. S11F), respectively. Overall, except for leg III ($P > 0.05$), the responses of dsPpseSNMP2-treated

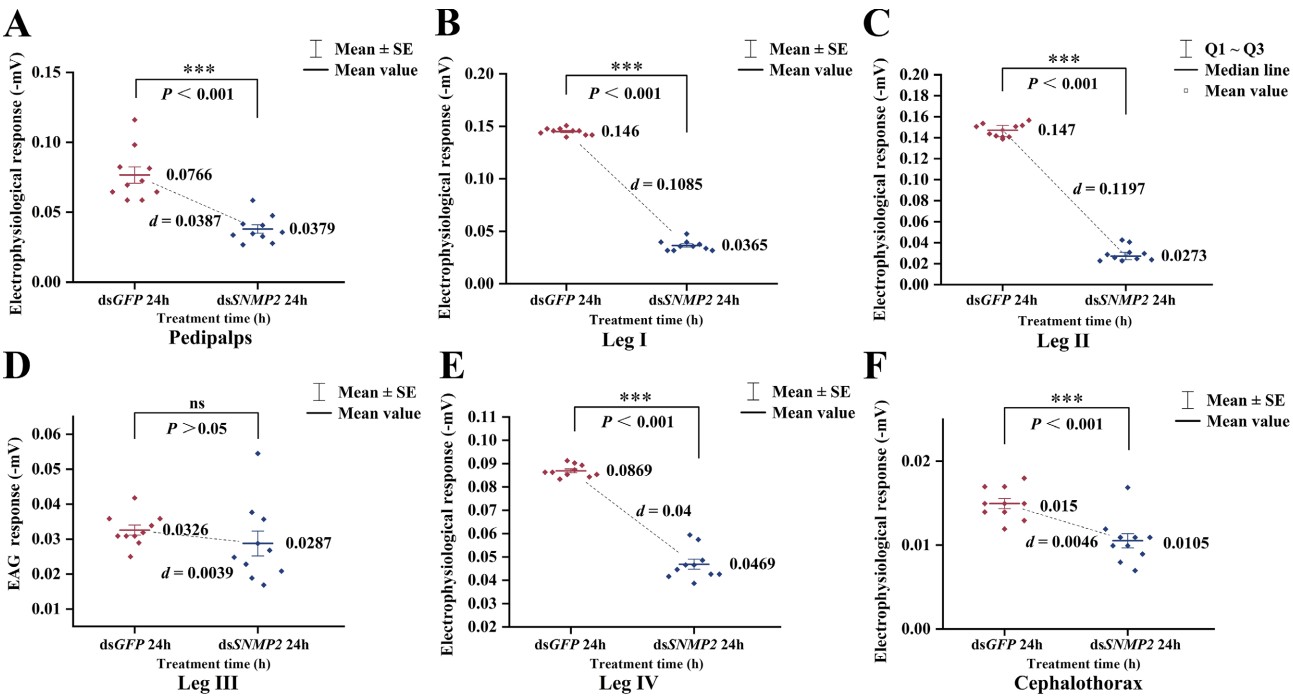

Fig. 7. Relative electrophysiological response values of each body part of male spiders after 24 h of ds*PpseSNMP2* treatment. (A)–(F) Relative electrophysiological response values of spider pedipalps, leg I, leg II, leg III, leg IV, and the cephalothorax. Note: the Mann–Whitney *U* test was used for leg II (C) (***$P < 0.001$), while Student's *t*-test was conducted for the other body parts (***$P < 0.001$).

males to MeSA were significantly lower than those of ds*GFP*-treated controls ($P < 0.001$). After ds*PpseSNMP2* treatment, the electrophysiological values for legs II, I, and IV were 0.0273 (0.0235–0.0332) mV (Fig. 7C), 0.0365 ± 0.002 mV (Fig. 7B), and 0.0469 ± 0.0021 mV (Fig. 7E), respectively.

DISCUSSION

Although olfaction plays a crucial role in spider behavior, the olfactory systems of spiders remain less studied compared to insects (Huber, 2005; Vizueta et al., 2017). Previous research has shown that spiders adopt different sensory strategies for prey localization depending on their habitat and behavioral traits. Unlike web-building spiders, wandering spiders can utilize olfactory cues for long-range prey detection, localization, and the identification of mates (Uhl, 2012). As an important HIPV, MeSA is widely present in the chemical composition of various plants and is involved in attracting natural enemies such as syrphid flies (Diptera: Syrphidae), green lacewings (Neuroptera: Chrysopidae) (Mallinger et al., 2011), ladybird beetles (Zhu & Park, 2005; Yang et al., 2023b), and predatory mites (Shimoda et al., 2002; Gadino et al., 2012), thus aiding in pest control. Specific concentrations of MeSA have been reported to attract male *P. pseudoannulata* (Xiao et al., 2018) and increase the predation rates of the wolf spiders *P. subpiraticus* and *P. pseudoannulata* (Liu et al., 2022).

In this study, adult *P. pseudoannulata* were exposed to MeSA, and transcriptome sequencing revealed changes in gene expression levels between treatment and control groups (CK-F vs. MeSA-F and CK-M vs. MeSA-M). The enrichment results of COG, GO, and KEGG showed that the differentially expressed genes of *P. pseudoannulata* after methyl salicylate treatment were mainly involved in pathways related to metabolic regulation, cellular degradation, and signal transduction. Notably, amino acid metabolism and lipid metabolic pathways were significantly enriched, suggesting that these physiological processes may participate in the adaptive molecular response of the spider to plant volatile stimulation (Mu et al., 2010).

Among olfactory-related genes, *PpseOBP1*, *PpseOBP2* and *PpseNPC2-7* were significantly differentially expressed in female spiders, while *PpseOBP1*, *PpseOBP2*, and *PpseSNMP2* displayed significantly differential expression in male spiders. The phylogenetic analysis showed that *P. pseudoannulata* OBPs are closer to the OBP-like proteins identified in spiders by Cerca et al. (2021), while remaining separated from insect OBPs. In addition, transcriptome-based identification of OBPs in *P. pseudoannulata* (Cao et al., 2018) showed that the two OBPs clustered together at the edge of the phylogenetic tree and exhibited a distant relationship with insect OBPs. Taken together, the findings indicate that although *P. pseudoannulata* OBPs differ structurally from insect OBPs, they may perform analogous roles in odorant binding and signal transmission. Moreover, structural divergence could result in distinct binding sites or affinities, leading to chemosensory specificities distinct from those observed in insects. Our results revealed distinct differences in gene expression patterns between male and female spiders, which may be attributed to multiple underlying factors. Extensive existing literature, particularly insect-focused studies, has thoroughly documented sexually dimorphic chemosensory gene expression patterns in response to volatile stimuli (Yang et al., 2016; Fan et al., 2018; Song et al., 2021; Ma et al., 2024; Yi et al., 2024). In this study, transcriptome sequencing identified 21 chemosensory-related genes (4 GR genes, 5 IR genes, 3 SNMP2 genes, 2 OBPs, and 7 NPC2 genes) between sexes and following methyl salicylate treatment. The sequences of all these genes are provided in Supplement S1. Differentially expressed genes were identified with criteria of $|\mathrm{Log}_2(\mathrm{FC})| \geq 1$ and FDR ≤ 0.01. Thus, in the CK-F vs. MeSA-F comparison, we identified three DEGs (*PpseOBP1*, *PpseOBP2*, and *PpseNPC2-7*). In the CK-M vs. MeSA-M comparison, three olfactory-related DEGs (*PpseOBP1*, *PpseOBP2*, and *PpseSNMP2*) were screened out. This divergence suggests that females and males may rely on distinct molecular pathways to perceive and respond to chemical cues. These findings support sexual dimorphism in chemosensory systems and extend this evidence to spiders, offering insights into arachnid chemical communication.

To address the core question of why such sex-biased olfactory gene expression occurs in spiders in response to plant volatiles, current research on spider chemosensory mechanisms remains extremely limited. Prior studies have only detected sex-specific behavioral responses to plant volatiles in spiders via Y-tube olfactometer assays (Xiao et al., 2018; Liu et al., 2022), with minimal mechanistic insights available. To date, only a few studies have explored the structural and physiological basis of spider olfaction, for instance, in the orb-weaving spider *Argiope bruennichi*, wall pore sensilla are abundant on all walking legs of mature males but absent in females and subadult males; these sensilla also display strong, concentration-dependent responses to sex pheromones (Talukder et al., 2025). Critically, no published empirical data can fully explain the molecular or physiological drivers underlying sex-specific responses to plant volatiles in spiders. Unraveling these elusive mechanisms will be a primary focus of our future research.

With the increasing application of computational technology in biology, molecular modeling and docking techniques have emerged as crucial tools for the study of arthropod sensory pathways (Peng et al., 2024). In this study, AlphaFold3 (Abramson et al., 2024; Wee & Wei, 2024) was employed to model the structures of *P. pseudoannulata* PpseNPC2-7 and PpseSNMP2, followed by docking analyses with MeSA in AutoDock Vina (Trott & Olson, 2010). All predicted protein structures achieved high levels (pTM > 0.5) (Abramson et al., 2024). Docking results indicated strong predicted binding affinities, with PpseNPC2-7 exhibiting the highest affinity (−5.8 kcal/mol). The intermolecular interactions between the two proteins and MeSA included hydrogen bonds and hydrophobic interactions.

SNMP2 belongs to the SNMP family. In insect olfaction, SNMP2 proteins exhibit broad expression in the support cells of various types of antennal sensilla and play a critical role in the detection of certain odorants, such as pheromones (Cassau & Krieger, 2021; Kohlmeier & Billeter, 2023). Kohlmeier & Billeter (2023) suggest that, in addition to pheromone detection, SNMP2 is also involved in general odorant detection. NPC2 proteins are present in all vertebrates and act as carriers for cholesterol and lipids (Zhu et al., 2018). Existing research indicates that NPC2 is an important carrier of chemical signals in arthropods, and it may provide similar functions to OBPs and CSPs (Xiu et al., 2019). In the parasitic wasp *Baryscapus dioryctriae*, research demonstrated that BdioNPC2b most strongly bound to 2-butyl-2-octenal, which could elicit an electrophysiological response and attract *B. dioryctriae* adults (Chen et al., 2024). In another parasitic wasp, *Macrocentrus cingulum* Brischke, McinNPC2 was reported to strongly bind with *β*-ionone (Maung et al., 2021). In *P. pseudoannulata*, the PpseNPC2-1 protein bound strongly to nerolidol, and molecular docking further validated the strong binding interaction between PpseNPC2-1 and nerolidol (Rao et al., 2025). PpseNPC2-7 belongs to the NPC2 protein family, and the molecular docking results suggest that PpseNPC2-7 can bind well with MeSA. In addition, salt bridges formed between PpseNPC2-7 and MeSA. These findings demonstrate that MeSA is an important natural enemy attractant (Li et al., 2022; Riahi et al., 2022; Qian et al., 2024). Identifying the optimal concentration of MeSA holds the potential to increase attraction and predation rates and reduce the attack latency period of *P. pseudoannulata*, ultimately contributing to pest control strategies.

In this study, we analyzed the expression patterns of *PpseNPC2-7* and *PpseSNMP2* across different body parts of male and female spiders exposed to 2.5 μg/μL MeSA. Both genes were predominantly expressed in the pedipalps and legs. *PpseNPC2-7* displayed high expression in the pedipalps and legs of both sexes, whereas *PpseSNMP2* was mainly expressed in the first pair of legs in females and in the pedipalps and all legs in males. This expression pattern corresponds with the previously reported distribution of chemosensory sensilla on spider pedipalps and legs (Foelix & Chu-Wang, 1973; Talukder et al., 2025), implying that spider legs and pedipalps may be important body parts for chemosensory function. Moreover, such sex-specific differences in gene expression may also be influenced by the pronounced sexual size dimorphism in *P. pseudoannulata* (Zhang et al., 2021). Previous studies have shown that olfactory-related genes are primarily expressed in spider pedipalps and the first pair of legs (Vizueta et al., 2017); this was also the case in *P. pseudoannulata*. Previous research found that *PpseNPC2-1*, *PpseNPC2-2*, *PpseNPC2-5*, and *PpseNPC2-6* were highly expressed in the pedipalps and chelicerae (Xiu et al., 2019), while two *SNMP* genes were identified in the transcriptomes of the pedipalps and the first pair of legs (Cao, 2018). In our study, these genes were also highly expressed in the pedipalps and legs of *P. pseudoannulata*, implying their involvement in olfactory detection. Similar expression patterns have been reported for chemosensory genes in the wandering spider *D. silvatica* (Vizueta et al., 2017). Taken together, these findings suggest that these genes may participate in the binding and recognition of various chemical stimuli, and we speculate that they may be key genes involved in chemical communication.

RNAi technology has shown tremendous potential in the management of agricultural pests (Aronstein et al., 2011; Nitnavare et al., 2021; De Schutter & Smagghe, 2022). It has also been widely applied in research on the growth, development, reproduction, and environmental adaptation of arthropods (Meng et al., 2015; Hou et al., 2021; Yang et al., 2023a). However, studies on olfactory-related genes in spiders remain scarce, as current research on olfactory-related genes and their functions primarily focuses on insect species (Gong et al., 2008). The potential functions of genes related to the olfactory process can be explored using RNAi technology in conjunction with behavioral (Lin et al., 2015; Liu et al., 2021; Guo et al., 2023) or electrophysiological experiments (Huang et al., 2008). In the present study, RNAi experiments were performed to silence *PpseNPC2-7* in female spiders and *PpseSNMP2* in males. Behavioral assays showed that gene silencing reduced the preference of both sexes for MeSA, and electrophysiological recordings revealed that the responses to MeSA were significantly attenuated in most body parts of the silenced individuals. These results suggest that *PpseNPC2-7* and *PpseSNMP2* may contribute to olfactory recognition and chemosensitivity in spiders. Similar phenomena have been reported in other arthropods: for example, silencing *DcitOBP7* in *Diaphorina citri* also reduced both preference and EAG sensitivity to MeSA (Liu et al., 2021); and knockdown of an OBP gene in *Hippodamia variegata* led to a significant decrease in preference for specific volatiles (Tang et al., 2023). This study provides the first functional validation of NPC2 and SNMP genes in spider olfaction via RNAi, filling a gap in the understanding of chemosensory mechanisms in non-insect arthropods.

In conclusion, we utilized MeSA to screen out two key olfactory-related genes from transcriptome data and molecular docking simulations in *P. pseudoannulata* and effectively silenced these genes using RNAi technology to examine their functions in olfaction. Our findings suggest that the *PpseNPC2-7* and *PpseSNMP2* genes may contribute to MeSA recognition in *P. pseudoannulata*. This study provides a preliminary understanding of the functions of these genes in olfactory behavior and a fresh perspective for comprehending the olfactory mechanisms and ecological adaptability of spiders. In the future, we will employ immunofluorescence localization and fluorescence competitive binding assays to further examine the distribution patterns of these olfactory-related genes in different spider tissues and explore their interactions with these HIPVs. Furthermore, we plan to conduct field and laboratory experiments to investigate the feasibility of utilizing MeSA or other HIPVs as behavioral regulators for spiders.

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AUTHOR CONTRIBUTIONS. Z.-L. Wang: Software, formal analysis, data curation, writing – original draft. C.-C. Li: Software, formal analysis, data curation, writing – original draft. F. Xiao & J.-Y. Zhu: Methodology, investigation. H.-R. Zhang: Software, resources. S.-S. Zhang: Validation. F.-F. Xia: Writing – review and editing. R. Xiao: Writing – review and editing, supervision, project administration. T.-X. Liu: Conceptualization, writing – review and editing, supervision.

DECLARATION OF COMPETING INTERESTS. The authors declare that they have no conflicts of interest.

DATA AVAILABILITY. The transcriptome data presented in this study are openly available in the NCBI SRA database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1195908) under accession number PRJNA1195908.

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Online supplementary files:

Supplement S1 (http://www.eje.cz/2026/023/S01.pdf). Figs S1–S11, Tables S1–S4, S6, S7. The sequences of chemosensory-related genes.

Supplement S2 (http://www.eje.cz/2026/023/S02.xls). Table S5. Annotation of DEGs in the CK-F vs. MeSA-F and CK-M vs. MeSA-M.