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Identification and Functional Characterization of an Odorant Receptor Expressed in the Genitalia of *Helicoverpa armigera*

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Abstract: Olfaction is critical for guiding the physiological activities of insects, with antennae being the primary olfactory organs. However, recent evidence suggests that other tissues may also participate in olfactory recognition. Among these, the genitalia of moths have received attention due to their roles in mating and oviposition. Sensilla and odorant receptors (ORs) in moth genitalia highlight the potential olfactory function of these structures. In this study, we examined the olfactory sensing capacity of the genitalia in *Helicoverpa armigera* by analyzing their structure in males and females and characterizing the expressed ORs. Scanning electron microscopy uncovered many sensilla distributed throughout the male and female genitalia. Transcriptome sequencing identified 20 ORs in the genitalia, with HarmOR68 exhibiting significant responses to methyl esters: methyl benzoate and salicylate. Our findings provide theoretical evidence that *H. armigera* genitalia may have significant olfactory perception functions.

Keywords: *Helicoverpa armigera*; genitalia; sensilla; olfactory receptor; scanning electron micrograph

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1. Introduction

Through evolution, insects have developed a highly sensitive olfactory system, which is important in the life of insects. Guided by the olfactory system, insects can locate food, avoid predators, and find mates by recognizing complex odor molecules in the surrounding environment [1–4]. Insects' life activities cannot be separated from olfactory guidance.

The antennae are the peripheral sensory organs that insects use to detect the external environment, and they are primarily tasked with olfactory detection [5,6]. Olfactory sensation in the antennae is facilitated by hairlike structures known as sensilla distributed on their surface, considered the smallest functional unit in olfactory sensation [7]. The number of sensilla on a single antenna can reach the thousands, exhibiting morphological diversity. According to their difference in external shape, antennal sensilla in moths can be sensilla trichoidea, sensilla basiconica, sensilla placodea, sensilla auricillica, or sensilla chaetica [8]. The functionality of sensilla is primarily mediated by olfactory genes expressed within this sensory organ [9,10]. These olfactory genes include two types of receptors, namely, odorant receptor (OR) and ionotropic receptor (IR), existing on the dendrite membrane of sensory neurons within the sensilla. There are other proteins involved in the receptor's ability to sense odorants, like odorant binding protein (OBP), chemosensory protein (CSP), and sensory neuron membrane protein (SNMP). When odor molecules enter the lymph fluid of the sensilla via pores on the sensilla's surface, OBPs or CSPs bind to odor molecules, and they are transmitted to the dendritic membrane of sensory neurons. The corresponding receptors on the dendritic membrane are activated, transmitting olfactory signals into nerve impulses [11–13]. These neural signals are received and processed by the brain, producing corresponding behaviors [14].

Given that ORs are predominantly expressed in the antennae of insects, these appendages have been the focal point of research into ORs. Insect ORs were first uncovered in *Drosophila melanogaster* through genome analysis and subsequently identified in other model insects [15,16]. Furthermore, the functions of these ORs have been systematically examined [17], elucidating olfactory recognition mechanisms. In recent years, numerous ORs have been identified through genomic and antennal transcriptome analysis in other insect species, especially in moths, including agriculture and forest pests [18–20]. Building upon this foundation, the functions of ORs have been examined extensively. Notably, pheromone receptors (PRs) were identified and functionally characterized in *Bombyx mori* and *Heliothis virescens*. BmorOR1 and BmorOR3 were found to recognize bombykol and bombykal, the sex pheromone components of *B. mori* [21,22]. In *H. virescens*, HvirOR13 and HvirOR6 are expressed on olfactory receptor neurons (ORNs) within specific trichoidea sensilla and elicit electrophysiological responses to the major pheromone components Z11-16:Ald and Z9-14:Ald, respectively [23,24]. Additionally, the functions of general ORs in many moths have been studied, and many ORs can recognize important plant volatiles and influence the feeding and oviposition behaviors of these moths. For instance, the ORs of *Spodoptera littoralis* are exceptionally efficient at recognizing short-chain aliphatic alcohols, aromatics, and terpenes, as they are the primary components of host plant volatiles [25]. The efficient recognition of these compounds by ORs may assist *S. littoralis* in better locating host plants. Isothiocyanates attract *P. xylostella* for oviposition, with OR35 and OR49 specifically responding to these compounds, thereby helping female *P. xylostella* select better host plants for laying eggs [26]. The above studies on moth ORs focus on those expressed in the antennae.

In addition to the antennae, other tissues may also participate in olfactory recognition, which drives their physiological activities. Several studies have identified that apart from the antennae, ORs are also present in nonolfactory organs like the proboscis, labial or maxillary palps, and legs. The maxillary palps of *Drosophila melanogaster*, covered with sensory hairs called sensilla, house odorant receptor neurons (ORNs) that express at least seven ORs [27]. RNA sequencing has identified 31 ORs expressed in the palps of *Schistocerca americana*, with further tissue-specific expression analysis revealing that SameOR63 only expressed palps, suggesting this receptor plays a palp-specific role [28]. Olfactory cells from the labial and maxillary palps of *Schistocerca gregaria* can detect pheromones, and SNMP and ORs were found to co-express in these organs [29]. ORs were found in the proboscis of *Eupeodes corollae* and varied depending on their feeding status [30]. RNA-seq identified a total of 95 ORs expressed in the leg RNA samples from 1-, 10-, and 20-day-old workers of *Apis cerana* [31]. Additionally, transcriptome sequencing results of the legs of *Apolygus lucorum* examined the expression of AlucOR109, potentially related to their olfaction and gustation [32].

Genitalia play an essential role in the mating behavior of insects, enabling them to reproduce and further their species. Genitalia comprise the external part of the reproductive system of insects, serving as a general term for the organs involved in mating, insemination, and oviposition. Genitalia are primarily composed of appendages in the genital segment of the abdomen [33]. The external genitalia of the female moth are called ovipositors [34], while those of the male moth are copulatory organs. The reproductive system includes gonads in the body [35,36], During the reproductive process in moths, the female releases a complex blend of pheromones from her genitalia to attract conspecific males and facilitate mating [37]. Female also use their genitalia to lay eggs in suitable host plants [38].

The expression of ORs has been found in the ovipositors of *Bactrocera dorsalis*, among which BdorOR43a-1 was identified to respond to benzothiazole and influence female egg-laying behavior [39]. The transcriptome sequencing of *Sesamia nonagrioides* and *Chilo suppressalis* demonstrated that several ORs are expressed in female ovipositors and may participate in olfactory functions [40,41]. Several studies have detected olfactory genes expressed in the *Manduca sexta* ovipositor and identified an olfactory function for neurons in ovipositor sensilla using single sensillum recordings (SSR) [42,43]. The expression of

HvirOR13 and PBP2 in the ovipositor of *H. virescens* suggests they may mediate abdominal responses to the primary pheromone component Z11-16:Ald [44]. Furthermore, transcriptome sequencing of the genitalia of *Spodoptera frugiperda* revealed the expression of 12 ORs throughout these structures. The expression level of SfruOR53 in the ovipositor was similar to that in antennae [45]. The transcriptome sequencing of the pheromone gland–ovipositor complex in *Helicoverpa assulta* revealed significantly higher expression of the odorant receptor gene HassOR31 in the ovipositor, which primarily responded to host plant volatiles like (Z)-3-hexene butyrate and which influences female egg-laying behavior by mediating host plant selection in gravid females [46].

H. armigera, a global agricultural pest, predominantly infests crops such as cotton and corn [47]. Our previous research analyzed the olfactory mechanism of this pest. Transcriptome analysis identified ORs expressed in the antennae, proboscis, and labial palps [48–50]. The ORs involved in sex pheromones, host plant volatiles, and oviposition pheromone perception in *H. armigera* were identified [51–53]. Furthermore, in a closely related species, *H. assulta*, several ORs were identified in the sex gland and oviposition complex, with HassOR31 mediating the host plant selection of gravid females [46]. In this study, we examined the olfactory sensing functionality of male and female genitalia in *H. armigera*. We identified a considerable number of sensilla distributed throughout the genitalia of both males and females. A total of 20 ORs were identified in the genitalia, and HarmOR68 exhibited significant responses to two methyl esters: methyl benzoate and methyl salicylate. These results suggest that genitalia may possess an olfactory function in *H. armigera*.

2. Materials and Methods

2.1. Insect Rearing and Tissue Collection

The *H. armigera* utilized in these experiments were obtained from a colony maintained at the Institute of Plant Protection at the Chinese Academy of Agricultural Sciences, Beijing, China. Larvae were grown on an artificial diet [54] at a controlled temperature of 26 ± 1 °C under a photoperiod of 14 h of light and 10 h of darkness. Male and female pupae were individually placed into separate glass tubes. Upon emergence, adult moths were fed a 10% honey solution. One thorax, one abdomen, three pairs of legs, 30 pairs of antennae, 30 proboscises, 60 labial palps, and 30 genitalia of female and male individuals were dissected two days after eclosion and flash-frozen in liquid nitrogen for storage at −70 °C until extraction.

2.2. Scanning Electron Micrographs (SEMs)

The genitalia of 2-day-old male and female adults were exposed by gently squeezing their abdomens with tweezers. The male and female genital structures were delicately excised using dissecting forceps and promptly submerged in a 2–4% glutaraldehyde solution for fixation, lasting between two and four hours at ambient temperature. Following fixation, the specimens underwent a graded dehydration process using ethanol solutions of increasing concentrations (70%, 80%, 90%, and 100%). After dehydration, the specimens were subjected to drying with a critical point dryer (LEICA CPD 030, Wetzlar, Germany). Gold coating of the specimen surfaces was performed using an ion sputtering machine (HITACHI MC 1000, Tokyo, Japan). Finally, the prepared specimens were imaged using a HITACHI SU8010 scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage ranging from 3 to 10 kV.

2.3. Total RNA Isolation

Total RNA was isolated from the harvested tissues using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), following the instructions precisely. The purity and concentration of the RNA were assessed utilizing a NanoDrop-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was validated via gel electrophoresis.

The RNA samples were then used for transcriptome sequencing, gene cloning, and quantitative real-time PCR (qPCR) analyses.

2.4. Transcriptome Sequencing and Gene Expression Analysis

Total RNA was extracted from the genitalia of five males and five females of *H. armigera* separately. Subsequently, 1 μg of RNA from each sample was utilized to construct cDNA libraries. The integrity and concentration of the constructed libraries were evaluated using the Qsep40 fragment inspection instrument and qubit concentration inspection instrument, respectively. Upon meeting the quality thresholds, the cDNA libraries underwent sequencing using the Illumina NovaSeq 6000 platform. Library construction and sequencing procedures were conducted at Biomarker Technologies (Beijing, China). Transcriptome sequencing was replicated in triplicate using three independent RNA samples. Following sequencing, low-quality reads (those with an N ratio exceeding 10% or over 50% of bases with a mass value of $Q \leq 10$) were discarded. The resulting high-quality clean data were retained in FASTQ format. Alignment of the clean data to the published *H. armigera* genome (genome version GCF_002156985.1_Harm_1.0) was conducted using HISAT2 (Kim et al., 2015). Subsequently, gene expression levels were quantified, and FPKM (fragments per kilobase of transcript per million mapped reads) values were acquired using StringTie [55].

A set of 67 ORs previously annotated in the genome was considered. Among these ORs, expression levels were estimated using the FPKM metric. Genes with FPKM values exceeding 0.1 were candidates for subsequent functional analysis. To visually represent the expression patterns of these ORs over the six samples, the pheatmap package in R was employed to generate a heatmap based on $\log_2$-transformed FPKM values, with a pseudocount of 1 added to accommodate 0 FPKM values.

2.5. cDNA Synthesis

A 2 μg sample of total extracted RNA was treated with DNaseI (TransGenBiotech, Beijing, China) to remove residual genomic DNA and then utilized for the synthesis of first-strand cDNA with an oligo-dT primer and a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The procedures followed the instructions provided with the RevertAid First Strand cDNA Synthesis Kit. The synthesized cDNA served as the template for gene cloning and qPCR analyses.

2.6. Gene Cloning

The specific primers for HarmOR68 and HarmOR47 cloning were designed based on the published sequence in GenBank (accession ID: XP_021193925.1) (Table 1). The entire open reading frame (ORF) was amplified using primeSTAR HS Mix (Takara, Tokyo, Japan). The PCR product was purified utilizing a PCR Purification Kit (TransGenBiotech, Beijing, China) and subsequently ligated into an pEASY-Blunt vector (TransGen Biotech, Beijing, China). The resulting vectors with the target fragment were transformed into Trans1-T1 competent cells (TransGenBiotech, China), and positive clones were sequenced by Sangon Biotech (Shanghai, China).

Table 1. The primers used in this study.

Usage	Primer Name	Primer Sequences
Gene cloning	HarmOR68-F	ATGGCAGAAAACTTATCGTATTTCGGC
	HarmOR68-R	TCAATTAGTGTTCTTCAGAAATG
	HarmOR47-F	ATGCCGTCCGATCAATCTAAAATGTTTG
	HarmOR47-R	CTATGTTTCTTGAGCATTTCTAATAAGTGTGAGAAAGA

Table 1. *Cont.*

Usage	Primer Name	Primer Sequences
Vector construction	HarmOR68E-F	TCAGGGCCCgccaccATGGCAGAAAACTTATCGTATTTCGGC (Apa I)
	HarmOR68E-R	TCAGCGGCCGCTCAATTAGTGTTCTTCAGAAATG (Not I)
	HarmOR47E-F	TCAGGGCCCgccaccATGCCGTCCGATCAATCTAAAATGTTTG (Apa. I)
	HarmOR47E-R	TCAGCGGCCGCCTATGTTTCTTGAGCATTTCTAATAAGTGTGAGAAAGA (Not. I)
qPCR	HarmOR68-F	AATCCTGCTGCGAGTATTTAACACC
	HarmOR68-R	AAGGTAGCCATATTGCGAACTCAAC
	HarmOR47-F	AAAATTATCAGCCGAAGAG
	HarmOR47-R	CTGGCATCAGCAAACTACAC

The restriction enzyme site of primer is underlined. Kozak sequence is lowercase.

2.7. Sequence and Phylogenetic Analysis

The transmembrane domain (TMD) prediction of HarmOR68 and HarmOR47 was performed using TOPCONS (12 February 2024, https://topcons.net/) and Protter (13 February 2024, http://wlab.ethz.ch/protter/start/). The dataset used for constructing the phylogenetic tree included the complete OR complement of four moth species within the Noctuidae family, encompassing 67 ORs from *H. armigera* [52], 71 ORs from *S. frugiperda* [56,57], 64 ORs from *H. assulta* [49], and 60 ORs from *S. littoralis* [58]. The amino acid sequences of these ORs were aligned using MAFFT (22 February 2024, https://www.ebi.ac.uk/Tools/msa/mafft). The maximum-likelihood phylogenetic tree of ORs was developed using FastTree with the Jones–Taylor–Thornton amino acid substitution model (JTT). The resulting phylogenetic tree was visualized and color-coded using FigTree 1.4.0 (25 February 2024, http://tree.bio.ed.ac.uk/software/figtree).

2.8. Real-Time Quantitative PCR (qPCR)

qPCR was conducted to evaluate the expression of HarmOR68 and HarmOR47 across various tissues (thoraxes, abdomens, legs, antennae, proboscis, labial palps, and genitalia) in both female and male *H. armigera* adults. The qPCR reactions were performed using synthetic cDNA templates and gene-specific primers (Table 1). Each reaction mixture consisted of a total volume of 20 µL, comprising 10 µL SYBR (TransGenBiotech, China), 8 µL RNase-free water, 1 µL cDNA, and 0.5 µL of each primer. The cycling conditions were established as follows: initial denaturation at 94 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 5 s, annealing at 60 °C for 15 s, extension at 72 °C for 10 s, and a final extension step at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Actin was utilized as the internal reference gene for sample normalization. The expression level of HarmOR68 was quantified using the $2^{-\Delta\Delta CT}$ approach [59]. Statistical analysis to evaluate the significance of differences in HarmOR68 and HarmOR47 expression between male and female tissues was conducted using *t*-tests in GraphPad Prism 8.0.1 software (Boston, MA, USA).

2.9. Gene Expression in Xenopus Oocytes and Electrophysiological Recordings

Mature and healthy *Xenopus* oocytes at stages V−VII were acquired following established protocols. The open reading frame (ORF) of the candidate gene was inserted into a pT7Ts expression vector. Subsequently, the cRNA of the candidate gene was synthesized using a mMESSAGE mMACHINE T7 kit (Thermo Fisher Scientific) and stored at −70 °C until further use. For microinjection, 27.6 ng of the cRNA mixture (candidate gene: HarmOrco 1:1) was injected into oocytes and cultured for 2−3 days at 18 °C. Stock solutions (1 mM) were prepared by dissolving the compound under examination (Table 2) in dimethyl sulfoxide (DMSO) and storing at −20 °C. Before the experiments, stock solutions were diluted in 1× Ringer's buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2 \cdot 2H_2O$, and 5 mM HEPES, pH 7.6) to a working concentration of 10^{-4} mM. Whole-cell currents were characterized using an OC-725C oocyte clamp (Warner Instruments, Hamden, CT, USA) with a holding potential of −80 mV. The oocytes were exposed to diverse odors for

20 s at a flow rate of 8 mL/minute. Data acquisition and analysis were conducted using Digidata1440A and PCLAMP 10.0 software (Axon Instruments Inc., Union City, CA, USA).

Table 2. Test compounds in this study.

No.	Odorant	CAS Number	No.	Odorant	CAS Number
1	2,6-di-tert-butylphenol	128-39-2	35	Heptanal	111-71-7
2	Acetophenone	98-86-2	36	Phenylacetaldehyde	122-78-1
3	Eugenol	97-53-0	37	4′-ethylacetophenone	937-30-4
4	2-phenylethanol	60-12-8	38	Jasmone	488-10-8
5	*trans*-3-hexen-1ol	928-97-2	39	(±)-camphor	76-22-2
6	3,7-dimethyl-3-octanol	78-69-3	40	2-pentadecanone	2345-28-0
7	(1r)-(-)-myrtenol	19894-97-4	41	(-)-piperitone	4573-50-6
8	(-)-*trans*-pinocarveol	547-61-5	42	Myrcene	123-35-3
9	(-)-linalool	126-91-0	43	(-)-*trans*-caryophyllene oxide	1139-30-6
10	Linalool	78-70-6	44	Farnesene, mixture of isom	502-61-4
11	(+)-cedrol	77-53-2	45	Ocimene	13877-91-3
12	*cis*-3-hexen-1-ol	928-96-1	46	(R)-(+)-limonene	5989-27-5
13	4-methoxybenzyl alcohol	105-13-5	47	A-pinene	80-56-8
14	1-octanol	111-87-5	48	(-)-β-pinene	18172-67-3
15	Benzyl alcohol	100-51-6	49	Camphene	79-92-5
16	4-hydroxy-4-methyl-2pentanone	123-42-2	50	A-Humulene	6753-98-6
17	1-octen-3-ol	3391-86-4	51	(S)-(-)-limonene	5989-54-8
18	Eucalyptol	470-82-6	52	A-terpinene	99-86-5
19	B-citronellol	106-22-9	53	(-)-*trans*-caryophyllene	87-44-5
20	Geraniol	106-24-1	54	Methyl benzoate	93-58-3
21	*cis*-2-hexen-1-ol	928-94-9	55	Decalactone	705-86-2
22	1-heptanol	111-70-6	56	Geranyl acetate	105-87-3
23	1s-(-)-verbenone	1196-01-6	57	(Z)-3-hexenyl acetate	3681-71-8
24	1-hexanol	111-27-3	58	Methyl 2-methoxy benzoate	606-45-1
25	(S)-cis-verbenol	18881-04-4	59	Butyl salicylate	2052-14-4
26	*trans*-2-hexen-1-al	6728-26-3	60	Methyl phenylacetate	101-41-7
27	Salicylaldehyde	90-02-8	61	*trans*-2-hexenyl acetate	2497-18-9
28	(±)-Citronellal	106-23-0	62	Benzyl acetate	140-11-4
29	Cinnamaldehyde	104-55-2	63	Methyl salicylate	119-36-8
30	4-ethylbenzaldehyde	4748-78-1	64	2-hexanol	626-93-7
31	3-vinylbenzaldehyde	19955-99-8	65	3-hexanol	623-37-0
32	Nonanal	124-19-6	66	*trans*-2-hexen-1-ol	928-95-0
33	(1r)-(-)-myrtenal	18486-69-6	67	Octanal	124-13-0
34	Benzaldehyde	100-52-7			

3. Results

3.1. The Ultrastructure of External Genitalia of Male and Female Helicoverpa armigera

SEM was employed to assess the presence of sensilla in the genitalia of male and female *H. armigera* and assess their ultrastructure. The abdomens of two-day-old female (Figure 1A) and male (Figure 1D) adult *H. armigera* individuals were gently squeezed to expose their external genitalia. Before SEM analysis, the hair pencils were removed from the male genitalia for improved observation (Figure 1D). Observation revealed defined structural differences between the female and male genitalia. The female genitalia appeared conical and were segmented into three sections, with sensilla located at the tip of the first segment and some sensory organs distributed along the third segment (Figure 1B,C). In contrast, the male genitalia were divided into two sections, with sensilla predominantly concentrated in the second section, while the first section lacked sensilla (Figure 1E,F).

In females, three distinct classes of sensilla were observed, namely, sensilla chaetica, sensilla trichoidea, and sensilla basiconica (Figure 2A). Among these, two subtypes of sensilla chaetica were found. Type I sensilla chaetica featured a surface covered with transverse threads and a visibly convex base, typically distributed at the tip of the genitalia, indicating a role in mechanical stimulation sensing. The presence of surface threads

suggests a potential chemical sensing role (Figure 2B). Type II sensilla chaetica exhibited a distinct longitudinal depression at their base and were shorter in length than type I sensilla chaetica. They were predominantly located in the third genitalia section (Figure 2C). Additionally, females possessed a single type of sensilla trichoidea, similar in shape to those on the antennae. These sensilla had a curved tip resembling hair and a surface covered with horizontal grains, indicating potential olfactory or gustatory functions (Figure 2D,E). Sensilla basiconica were also found in female genitalia, defined by their short length, smooth surface, and irregular holes at the tip. These sensilla may be involved in olfaction or taste perception, although they could also detect temperature or humidity (Figure 2F,G).

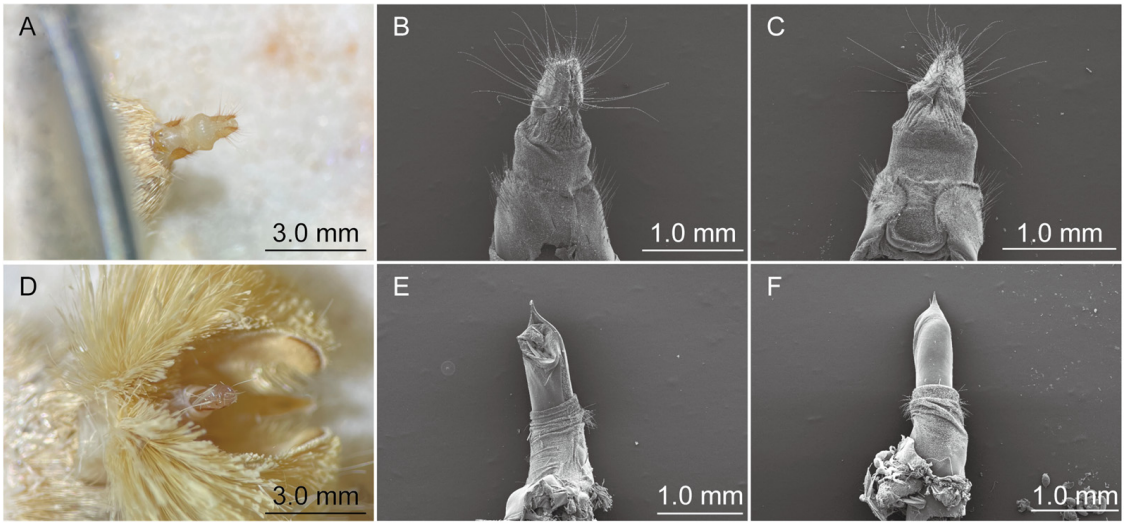

Figure 1. General morphology and ultrastructure of the genitalia of male and female adult *H. armigera*. (**A**) Female genitalia under an optical microscope. (**B**,**C**) Ventral and dorsal views of female genitalia under scanning electron microscope. (**D**) Male genitalia under an optical microscope (with hair pencils). (**E**,**F**) Ventral and dorsal views of male genitalia under scanning electron microscope.

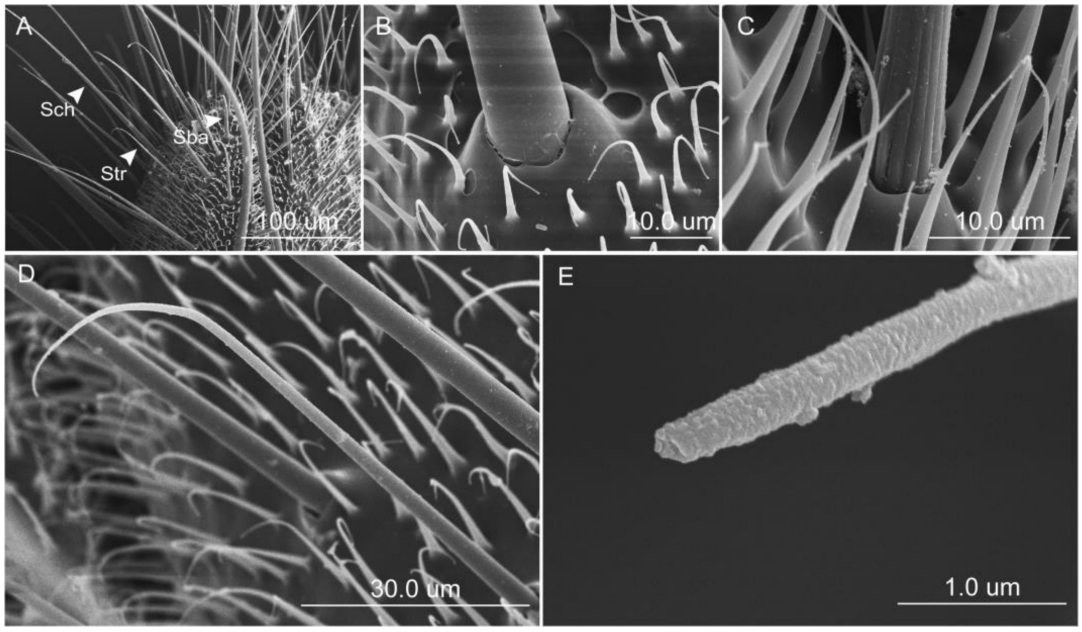

Figure 2. *Cont.*

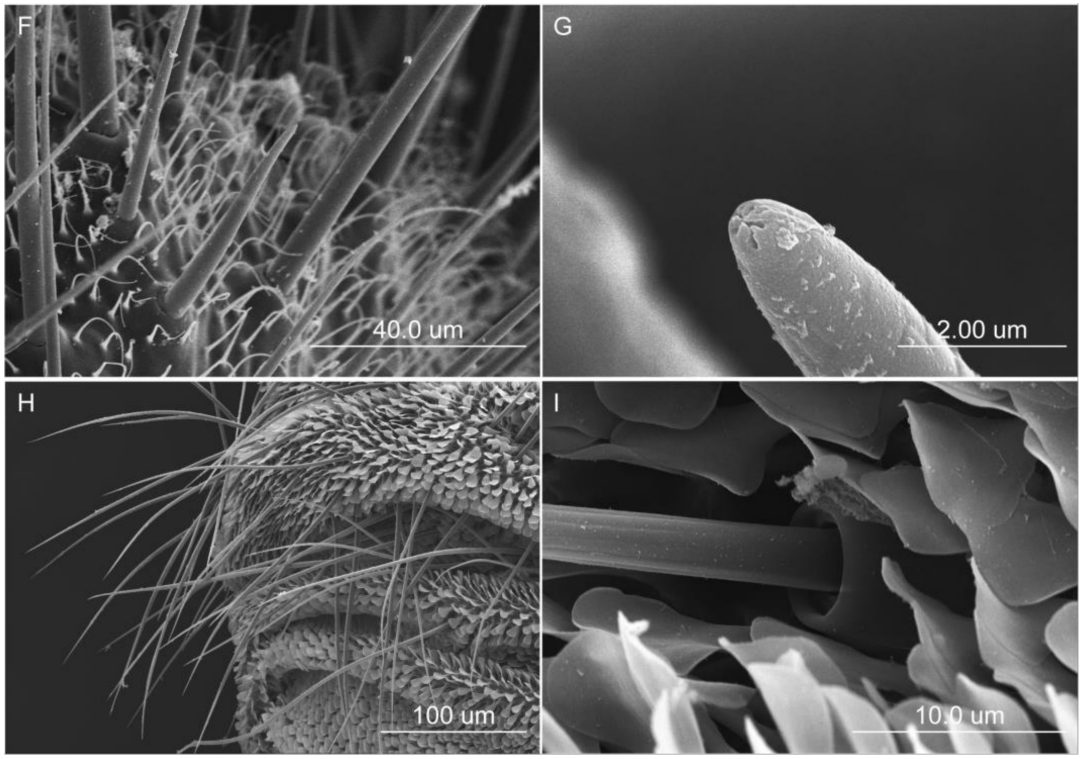

Figure 2. Scanning electron micrographs of sensilla on the genitalia of male and female adult *H. armigera*. (**A**) Distribution of sensilla on female genital tips. (**B**) Type I sensilla chaetica in females. (**C**) Type II sensilla chaetica in females. (**D**) Sensilla trichodea in females. (**E**) Apical orifice of female sensilla trichodea. (**F**) Sensilla basiconica in females. (**G**) Apical hole of female sensilla basiconica. (**H**) Distribution of sensilla on male genitalia. (**I**) Sensilla chaetica in males.

A single type of sensilla chaetica was found in males, typically within the second segment of the genitalia. Resembling the type II sensilla chaetica found in females, these structures exhibit a longitudinal depression at the base, suggesting a role in mechanical perception, alongside potential olfactory or gustatory sensing capabilities (Figure 2H,I).

3.2. Identification of Odorant Receptors in Genitalia in Helicoverpa armigera

Due to the abundant presence of sensilla in the genitalia, we aimed to characterize the potential expression of chemoreceptor genes throughout these structures. Transcriptome sequencing was employed to elucidate the presence of OR genes in both male and female genitalia. A total of 20 ORs were identified in the genitalia. Subsequently, their expression levels were quantified using the fragments per kilobase of transcript per million mapped reads (FPKM) method (Figure 3). The overall expression of these ORs in the genitalia was relatively modest. Notably, the OR exhibiting the highest expression level in the genitalia was HarmOR31, with FPKM values of 9.171 in females and 1.532 in males. Additionally, HarmOrco was detected with substantially lower FPKM values than HarmOR31. Among the ORs detected in the genitalia, only seven (HarmOR31, 26, 48, 67, 47, 44, and 68) exhibited relatively high expression levels (FPKM > 0.1). Notably, HarmOR31, OR26, OR48, OR67, and OR44 (unpublished data) have been previously investigated. Furthermore, we identified an OR annotated as OR1 in the genome assembly HaSCD2 (RefSeq: GCF_023701775.1), the functional properties of which remain uncharacterized. This OR was named HarmOR68. Given the established functions of the other genes, our future investigations will focus on elucidating the role of HarmOR68 and HarmOR47.

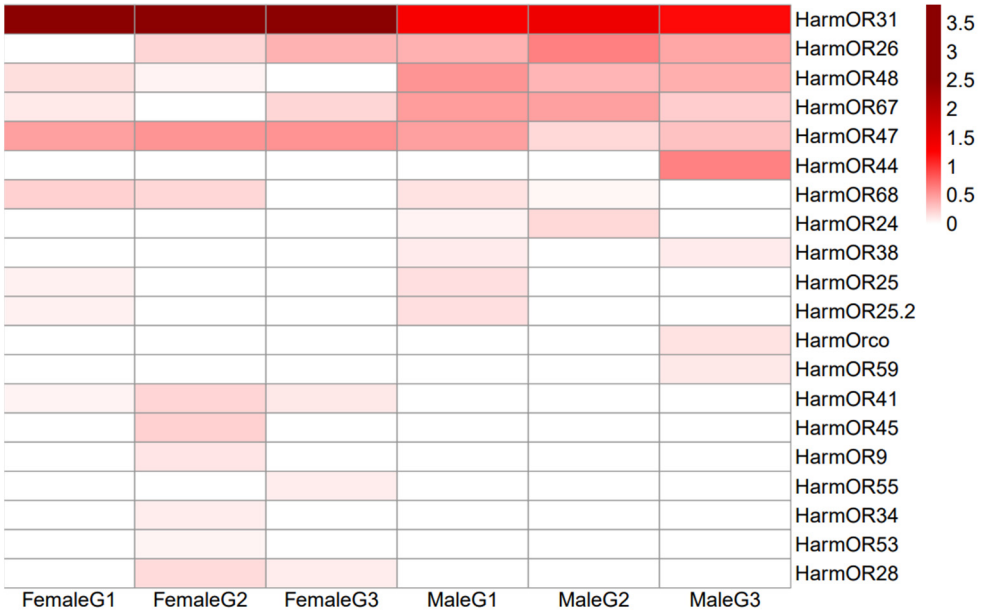

Figure 3. Heatmap of OR expression in male and female genitalia of *H. armigera*. The expression values are normalized as $\log_2$ (FPKM + 1).

3.3. Sequence and Phylogenetic Analysis

According to NCBI-published cotton bollworm odor receptor HarmOR68 (GenBank), The cDNA sequences of XP_021193925.1 and HarmOR47 (XP_021192462.2) were designed with gene-specific primers. We successfully cloned the complete open reading frame (ORF) sequences of HarmOR68 and HarmOR47. The ORF of HarmOR68 is 1179 bases (bp) long and encodes 392 amino acids. The ORF of HarmOR47 has a total length of 1173 bp, encoding 390 amino acids. Transmembrane domain prediction indicated that both HarmOR68 and HarmOR47 contained 7 TMDs, with the N-terminus located inside the cell and the C-terminal situated outside the cell, consistent with a characteristic arrangement typical of insect ORs [60] (Figure 4A,B).

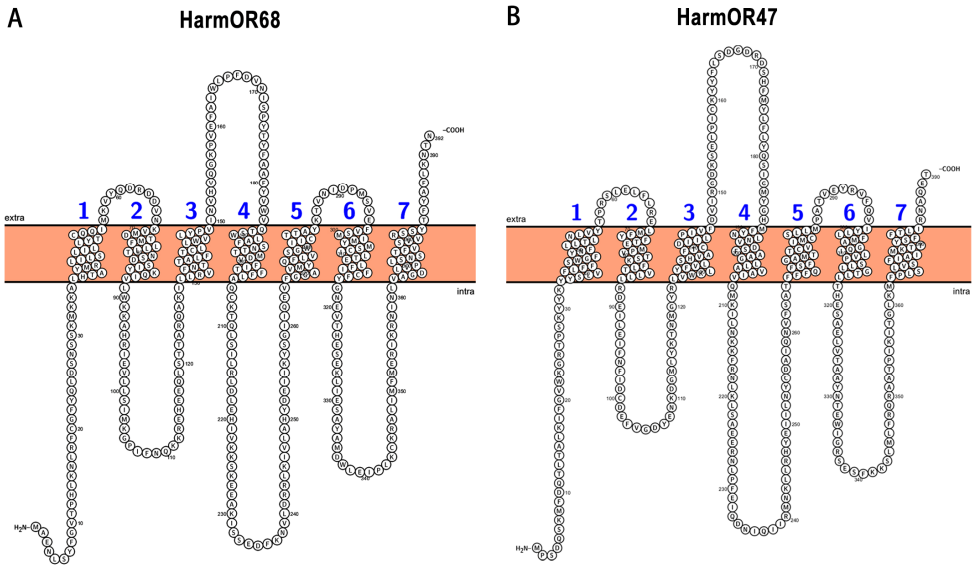

Figure 4. (**A**) The predicted transmembrane domain of HarmOR68. (**B**) The predicted transmembrane domain of HarmOR47.

To uncover the evolutionary relationships between HarmOR68 and ORs from other Lepidoptera species, we developed a phylogenetic tree (Figure 5) using sequences from various moth species, including *H. assulta*, *S. frugiperda*, *S. littoralis*, and *H. armigera*. Phylogenetic analysis revealed distinct clusters corresponding to Orco orthologs and PRs. Notably, HarmOR68 and HarmOR47 clustered with other general ORs and exhibited significant divergence from PRs and novel PRs, classifying HarmOR68 and HarmOR47 as members of the general OR repertoire in *H. armigera.*

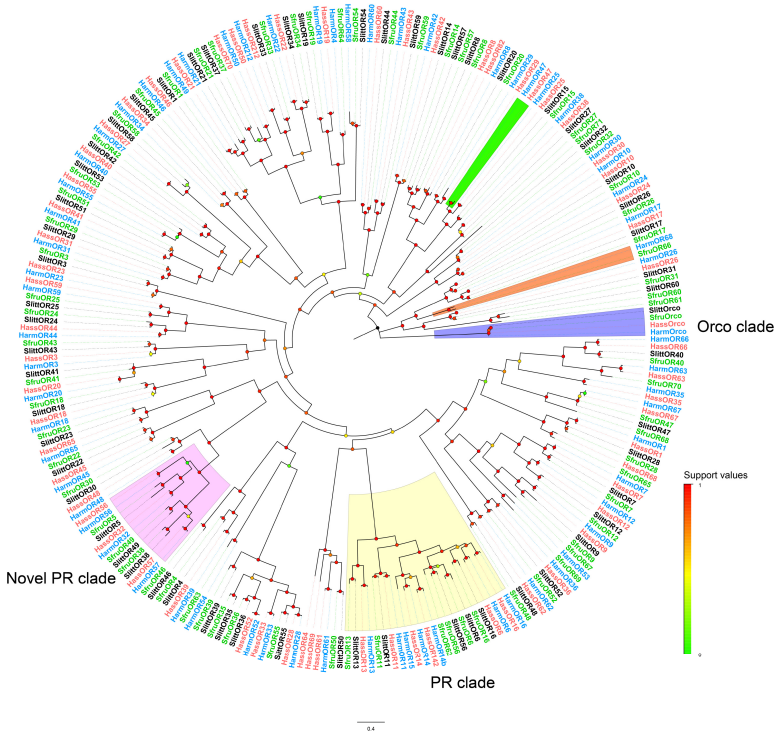

Figure 5. Phylogenetic tree of *H. armigera* ORs with known Lepidoptera ORs. Hass: *H. assulta* (red); Harm: *H. armigera* (blue); Sfru: *Spodoptera littoralis* (green); Slitt: *Spodoptera littoralis* (black). The clade in orange represents the HarmOR68 clade. The clade in green indicates the HarmOR47 clade. The clade in purple is the Orco clade. The clade in yellow indicates the PR clade. The clade in pink denotes the novel PR clade.

3.4. Tissue Expression Pattern of HarmOR68 and HarmOR47

To confirm the expression of HarmOR68 and HarmOR47 in the genitalia, we performed qPCR assays to assess the expression profiles across various tissues, including thoraxes, abdomens, legs, antennae, proboscis, labial palps, and genitalia in *H. armigera*. Our findings demonstrated that HarmOR68 transcripts were detected in all examined tissues. Specifically, in the antennae, the relative expression levels of HarmOR68 were markedly higher in males than females. However, no significant difference in gene expression was identified between males and females in the remaining six tissues. Notably, HarmOR68 exhibited substantial expression levels in the genitalia, mirroring its expression in other tissues. Furthermore, the expression pattern of HarmOR68 in the genitalia of both males and females was consistent with the findings obtained from transcriptome sequencing (Figure 6A). HarmOR47 was expressed in the antennae of males and females, the genitalia of males and females, and the lower lip of female moths. The expression level was highest in the antennae of males and females and was also expressed in the genitalia of male and female moths. The expression level in the lower lip of female moths was very weak (Figure 6B).

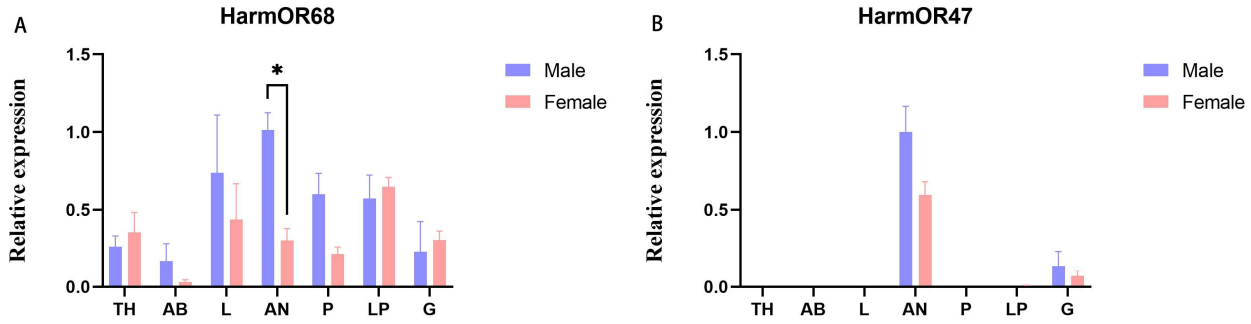

Figure 6. (**A**) Relative expression of HarmOR68 in different tissues of male and female *H. armigera*. (**B**) Relative expression of HarmOR47 in different tissues of male and female *H. armigera*. TH: thoraxes, AB: abdomens, L: legs, AN: antennae, P: proboscis, LP: labial palps, G: genitalia. The asterisks indicate differences between females and males (*t*-test, * $p < 0.001$). Error bars indicate the SEM ($n = 3$).

3.5. Functional Analysis of HarmOR68 and HarmOR47

To characterize the olfactory function of HarmOR68 and HarmOR47, electrophysiological recordings of *Xenopus* oocytes co-expressing HarmOR68/HarmOrco and HarmOR47/HarmOrco were conducted using a two-electrode voltage clamp system. A comprehensive panel of 67 chemicals with behavioral or electrophysiological activity in cotton bollworms was used to screen potential ligands for HarmOR68 (Table 2). Our findings demonstrate that HarmOR68 strongly responds to the stimulation of two specific compounds, methyl salicylate and methyl benzoate (Figure 7A). The average response of HarmOR68 to 10^{-4} M was evaluated at concentrations of methyl salicylate and methyl benzoate of 295.0 ± 13.02 nA and 182.6 ± 39.78 nA (Figure 7B). Notably, HarmOR68 exhibited high specificity, responding only to methyl salicylate and methyl benzoate in all 67 tested odors but not to the remaining 65 odors. Under stimulation by 67 different compounds, electrophysiological records were conducted on *Xenopus* oocytes co-expressing HarmOR47/HarmOrco with a dual-electrode voltage clamp system. The recording showed none of the 67 compounds caused electrophysiological reactions in *Xenopus* oocytes expressing HarmOR47/HarmOrco (Figure 7C).

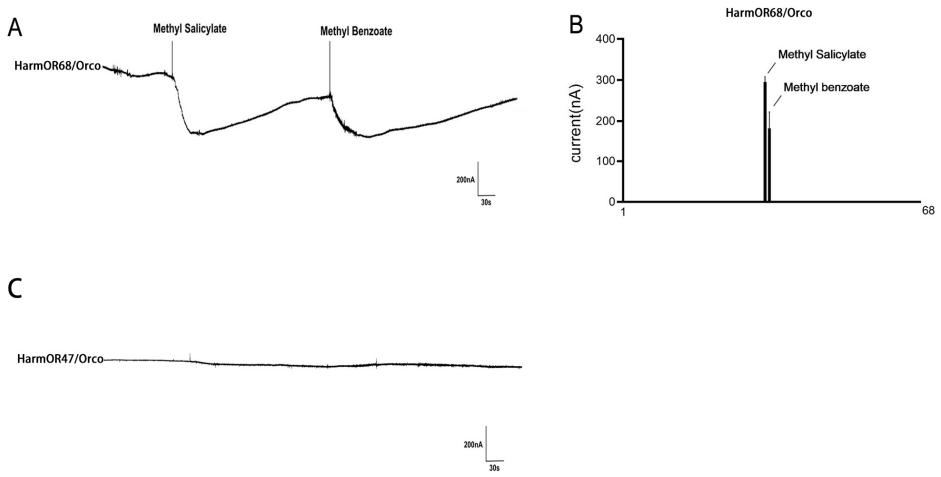

Figure 7. Functional analyses of HarmOR68 in *Xenopus* oocytes. (**A**) Inward current responses of *Xenopus* oocytes with expression of HarmOR68/HarmOrco. (**B**) Histograms of the tested compound response profile of *Xenopus* oocytes. Error bars indicate SEM ($n = 6$). (**C**) Inward current responses of *Xenopus* oocytes expressing HarmOR47/HarmOrco.

4. Discussion

To establish whether the genitalia of male and female *H. armigera* moths possess olfactory sensing functionalities, we examined their ultrastructure. Under electron microscopy,

sensilla were distributed on both male and female genitalia. Females have two types of sensilla chaetica, one type of sensilla trichoidea, and one type of sensilla basiconica. Males have only one type of sensilla chaetica, and the number of sensilla chaetica was significantly higher in females compared to males. This difference may arise from the distinct reproductive roles of females and males [33]. In moths, the female moth usually releases sex pheromones to attract males [37]. After mating, female moths typically find suitable host plants to ensure the survival of their offspring [61]. During egg laying, females extend their ovipositors into close contact with the host plant and deposit eggs on its surface. This behavior may have propelled the evolution of a more sensitive sense of touch or olfactory sense in female genitalia [62].

Prior studies have examined the genitalia of female *H. armigera* somewhat. In this study, transcriptome sequencing was performed in the pheromone gland–ovipositor complex. A total of 10 ORs expressed in the female gonadal ovipositor complex were identified [46], and 7 of them were also identified in our transcriptome results, with consistent results. The absence of three ORs in our findings may be attributed to differences in sampling approaches. In addition, we conducted transcriptome sequencing on male genitalia, identifying that while females had significantly more sensilla than males, the expression of ORs did not indicate a higher level in females, possibly because they do not serve olfactory functions [63], with neurons within them expressing chemoreceptors like IRs or GRs instead of ORs [64]. Behaviors like mating and oviposition influence the expression of ORs in insects [65]. In this study, we conducted transcriptome sequencing on the genitalia of female moths prior to mating. Specific ORs may be induced by mating or oviposition behaviors before exhibiting heightened expression in females [66]. Among the ORs investigated, HarmOR24, HarmOR59, HarmOrco, and HarmOR44 exhibited specific expression in males, while no significant difference was identified between males and females in the remaining highly expressed ORs. Given that our analysis was limited to the transcriptomes of three-day-old *H. armigera* genitalia in this study, some ORs expressed at other developmental stages may have been unidentified due to sampling limitations.

According to our prior studies, ORs expressed in the genitalia are also identified in antennae and other tissues, with no genital-specific ORs identified [48]. Many of the functions of highly expressed ORs in the genitalia are already well documented. For instance, HarmOR26 exhibits an electrophysiological response to the monoterpene (E)-nerolidol [52]. OR67 operates as a broad-spectrum receptor, exhibiting strong reactivity to 1-octanol, which impacts the communication between ovarian epithelial cells and oocytes in various insects [67]. The primary ligand for OR48 is (S)-(-)-verbenone, a plant volatile with specific repellent effects on Coleoptera [52,68]. The ligands for HarmOR44 include 2-phenylethanol and phenylacetaldehyde. 2-phenylethanol can induce more frequent lifting of the female cabbage looper moth abdomen and wing flapping, indicating its sexually stimulating effect on the female [69], and phenylacetaldehyde can also be used as a synergist for some compounds [70]. HarmOR31 is the most highly expressed OR throughout the genitalia; HarmOR31 and HassOR31 are homologous genes. According to the literature, HassOR31 has been implicated in mediating female oviposition, suggesting that HarmOR31 and HassOR31 may have a similar function. Myrcene is the optimal ligand for HarmOR31 [46]. We focused our exploration on HarmOR68 and HarmOR47, which have unknown functions. Typically, similar to the expression patterns observed for other ORs, HarmOR68 is expressed in the genitalia and other tissues, suggesting diverse functions for this receptor [49]. HarmOR47 was expressed in the antennae and genitalia of males and females. However, the function of OR47 remains unknown. According to the evolutionary tree, *H. assulta* is closely related to *H. armigera*. However, *H. assulta* has no homologous gene to HarmOR68 [71], while SfruOR66 in *S. frugiperda* shares a 81.63% similarity with HarmOR68. This may be due to the host range of *H. armigera* being very wide and overlapping with *S. frugiperda* [72]. Conversely, *H. assulta* exhibits a single host plant preference [73], which offers valuable insights into the species differentiation between *H. armigera* and *H. assulta*.

Our study revealed that HarmOR68 exhibits significant electrophysiological responses to methyl salicylate and methyl benzoate. During mating, male moths release sex pheromones by their hair pencils, enabling female receptivity to mating. Methyl salicylate, a crucial component of the male sex pheromone in *H. virescens* [37], closely related to *H. armigera* [74], may play a similar role. The expression of HarmOR68 in the genitalia may enhance female receptivity to mating with males. Methyl salicylate is a common plant volatile produced by plants when they are attacked by pests and pathogens [75] and can replace benzaldehyde as a synergist of the aggregation pheromone grandisoic acid in *Conotrachelus nenuphar* [76]. HarmOR68 can also sense methyl benzoate, documented to have insecticidal effects on *Drosophila suzukii*, *Hyalomorpha halys*, and *P. xylostella* of Lepidoptera [77], as well as being an effective control method for *Plodia interpunctella* [78]. HarmOR68 exhibits significant electrophysiological responses to methyl benzoate. It is also expressed in the genitalia, suggesting it may enhance female moth receptivity to mating with males or facilitate close-range selection of oviposition sites. Detection of the neurons in the female genitalia that respond to methyl benzoate would further demonstrate that female genitalia have olfactory functions. In future work, it is necessary to utilize SSR to detect the olfactory function of neurons present in *H. armigera* genitalia sensilla and design relevant behavioral experiments to assess the biological functions of the main ligands.

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