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Antennal ionotropic receptors IR64a1 and IR64a2 of the parasitoid wasp *Microplitis mediator* (Hymenoptera: Braconidate) collaboratively perceive habitat and host cues

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ABSTRACT

Ionotropic receptors (IRs), as a member of the conserved chemoreceptor families in the peripheral nervous system, play a critical role in the chemoreception of *Drosophila*. However, little is known about IRs in Hymenoptera insects. Here, we comprehensively characterized the gene structure, topological map and chemosensory roles of antennal IRs (MmedIRs) in the hymenopteran parasitoid wasp *Microplitis mediator*. We found that the IRs were conserved across various insect species. In the *in situ* hybridization assays, most IRs showed female antennae biased features, and there was no co-expression of the IRs and the olfactory receptor co-receptor (ORco). Moreover, three IR co-expressed complexes, IR75u-IR8a, IR64a1-IR8a and IR64a2-IR8a, were detected. Two genes with high similarity, IR64a1 and IR64a2, were located in distinct neurons but projected to the same sensillum. In two-electrode voltage-clamp recordings, IR64a1 was widely tuned to the chemicals from habitat cues released from host plants over long distances, whereas IR64a2 responded to a narrow range host cues and plant odors with low-volatility. Notably, IR64a2 was able to perceive *Z*9-14: Ald, a vital sex pheromone component that is released from *Helicoverpa armigera*, which is the preferred host of *M. mediator*. Furthermore, most ligands of IR64a1 and IR64a2 can trigger electrophysiological responses in female wasps. We propose that IR64a1 and IR64a2 collaboratively perceive habitat and host cues to assist parasitoids in efficiently seeking hosts.

1. Introduction

Chemoreception plays critical roles in insects survival and reproduction, and most insects rely on sensitive organs that express various chemosensation proteins to perceive chemical cues related to feeding, mating and oviposition in the external environment (Dahanukar et al., 2005; Kaupp, 2010; Leal, 2013; Sachse and Krieger, 2011; Vosshall and Stocker, 2007). The process of chemoreception in insects appears to be mediated by diverse chemoreceptor families, including odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), pickpocket (PPK) families and transient receptor potential (TRP) channel families. Among them, ORs, GRs and IRs have been extensively studied in different insects. These receptor families, which are housed in the specific dendrites of olfactory or gustatory sensory neurons (OSNs or GSNs), respond to various classes of semiochemicals (Ihara et al., 2013; Joseph and Carlson, 2015; Nei et al., 2008; Touhara and Vosshall, 2009). In contrast to ORs and GRs, IRs are highly conserved and have been recently discovered in insects (Benton et al., 2009; Croset et al., 2010).

As a large and highly divergent family of ionotropic glutamate receptors (iGluRs), IRs were initially found in the *Drosophila melanogaster* genome using bioinformatic and expression screening (Benton et al., 2009). According to the phylogeny and comprehensive expression analysis, *Drosophila* IR members are subdivided into two subfamilies:

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conserved "antennal IRs", which are specifically expressed in antennae and are mainly involved in olfaction; and species-specific "divergent IRs", which are found in various tissues and some of them can be responsible for the sensation of taste (Abuin et al., 2011; Benton et al., 2009; Croset et al., 2010; He et al., 2019; Jaeger et al., 2018; Joseph et al., 2017; Koh et al., 2014; Lee et al., 2018; Rimal and Lee, 2018; Rimal et al., 2019; Rytz et al., 2013; Tauber et al., 2017). Similar to ORs, the specific antennal IRs form heteromeric complexes with one or more co-receptors (IR8a, IR25a or IR76b) to perform their physiological functions (Abuin et al., 2011; Benton et al., 2009; Silbering et al., 2011).

Over the past decade, biological functions of 16 antennal IRs have been reported in *D. melanogaster*. Initially, antennal IRs were investigated as odorant-responding receptors. Specific IRs co-expressed with IR8a often responded to acids. For instance, the IR84a and IR8a (IR84a-IR8a) complex responds to phenylacetaldehyde and phenylacetic acid that promote male courtship (Grosjean et al., 2011). The IR31a-, IR64a-, and IR75-IR8a complexes mainly mediate acid detection (Ai et al., 2013; Rytz et al., 2013; Silbering et al., 2011). Specific IRs co-expressed with IR25a or IR76b generally perceive amines. For example, IR25a, IR76a, and IR76b work together to recognize phenylethyl amine (Abuin et al., 2011). IR41a-IR76b complex mediates the long-distance attraction to polyamine (Hussain et al., 2018). Recently, IRs were found to mediate modalities beyond olfaction including gustation, thermo-sensation, and humidity sensation (van Giesen and Garrity, 2017). For instance, IR25a and IR76b act as co-receptors and are co-expressed with divergent IRs together in non-antennae tissues responding to taste (Ganguly et al., 2017; Stewart et al., 2015). IR21a, IR93a, and IR25a are all required to mediate physiological and behavioral responses to cool temperatures (Knecht et al., 2016; Ni et al., 2016). In addition, IR25a functions as a thermosensor and mediates circadian rhythms (Chen et al., 2015). IR40a and IR68a are co-expressed with IR25a and IR93a, respectively, and they play roles in sensing humidity (Enjin et al., 2016; Knecht et al., 2016, 2017).

In addition to *D. melanogaster* IRs, limited studies on IR functions have been reported in *D. sechellia* and *Anopheles gambiae* (Liu et al., 2010; Pitts et al., 2017; Prieto-Godino et al., 2017). These works presented the conservative function of IRs in Dipterans. Recently, the IR repertoire of Lepidoptera was deeply investigated at the genome and molecular level (Liu et al., 2018; Tang et al., 2018; Zhu et al., 2018). However, as a conserved gene family across arthropods, functional studies of IRs in different species are limited.

Microplitis mediator (Haliday) (Hymenoptera: Braconidae) is a generalist endoparasitoid of a wide range of lepidopteran larvae including *Helicoverpa armigera*, *Mythimna separata*, *Mamestra brassicae* and other moth species (Khan, 1999; Lauro et al., 2005; Li et al., 2006). In China, *M. mediator* has been successfully mass-reared and used as a biological control agent in farmland (Li et al., 2006). Like most other parasitoid wasps, *M. mediator* utilizes chemosensory organs to detect host-related chemical cues for habitat searching, host location, and host assessment. In our previous study, 17 IRs (MmedIRs) were identified in *M. mediator*, among which ten were antennal IRs and most of them were highly expressed in female antennae (Wang et al., 2015, 2016). However, the physiological roles of these IRs have not been determined. In the current study, we investigated the gene structures and intron insertion sites of MmedIRs. In addition, we mapped the expression patterns and combinatorial modalities of the MmedIRs in antennae. Finally, we assessed the roles of functional IR complexes via *in vitro* expression combined with electrophysiology tests. These data will help explore the chemoreception mechanisms mediated by IRs, and further improve our understanding of IRs beyond model insects.

2. Materials and methods

2.1. Insect rearing and sample collection

Cocoons of *M. mediator* were incubated in a climate chamber under the following conditions: 28 ± 1 °C, 60 ± 10% R. H. (relative humidity) and 16L: 8D photoperiod. The emerged adults were fed 10% sucrose solution. Antennae of adult *M. mediator* were collected to perform the RNA extraction and cDNA synthesis. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. RNA extraction and cDNA synthesis

Total RNAs were extracted from homogenized antennae using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacture's protocol. The quantity of RNA was checked using 1.1% agarose gel electrophoresis and detected on a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, MA, USA). Total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to remove residual genomic DNA. For gene cloning and qPCR analysis, 1 μg of RNA was used to synthesize the first-strand cDNA using SuperScript™ III Reverse Transcriptase system (Invitrogen, Carlsbad, CA, USA).

2.3. Gene structure analysis and intron insertion site alignment

Genomic DNA of *M. mediator* was extracted using a TIANamp Genomic DNA kit (TianGen, Beijing, China) following the manufacturer's instructions. Genomic sequences of *MmedIRs* (Supplementary Data 1) were amplified using specific primers (Table S1). The exon/intron splice sites were analyzed by Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/). The graphics were generated with Exon-Intron Graphic Maker (http://www.wormweb.org/exonintron). Transmembrane domains (TMDs) were predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001). In addition to *M. mediator*, six insect species including *M. demolitor*, *Apis mellifera*, *Nasonia vitripennis*, *D. melanogaster*, *Bombyx mori* and *Tribolium castaneum* were chosen for comparison of the intron insertion sites of the IR genes. Blast searches were performed using candidate sequences as queries against the corresponding genome data in NCBI. Exon/intron splice sites were identified as described above (Supplementary Data 2). Alignment of intron insertion sites of IR genes was performed based on the result of amino acid sequences alignment using ClustalX 2.1 (Larkin et al., 2007) (Supplementary Data 3).

2.4. In situ hybridization

Digoxigenin (DIG)- and biotin-labeled antisense or sense RNA probes were transcribed from linearized recombinant plasmids containing the coding region of the target genes using the DIG RNA Labeling Kit (SP6/T7) and Biotin RNA Labeling Mix (Roche, Mannheim, Germany). Specific primers were designed to amplify the target gene sequences (Table S1). Labeled probes were fragmented to an average length of 400 bp by incubating in carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2) following Cox et al. (1984).

Antennae were embedded in Tissue-Tek optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA, USA) and cut into 12 μm slices at −26 °C by using a freezing microtome (Thermo scientific, Cryostar NX50, USA). Sections were collected on Superfrost plus microscope slides (Fisher Scientific, USA) and stored at −80 °C until use. Procedures of hybridization were conducted according to previous studies (Guo et al., 2014; Xu et al., 2013; Xu et al., 2017; Yang et al., 2012). Briefly, the cut sections of antennae were dried at room temperature for 30 min and fixed in 4% paraformaldehyde solution at 4 °C for 30 min, then incubated in 0.2 M HCl for 10 min and washed in PBS buffer. Slides were incubated for 1 h in 50% formamide with 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate) solution. Next, 100 μl

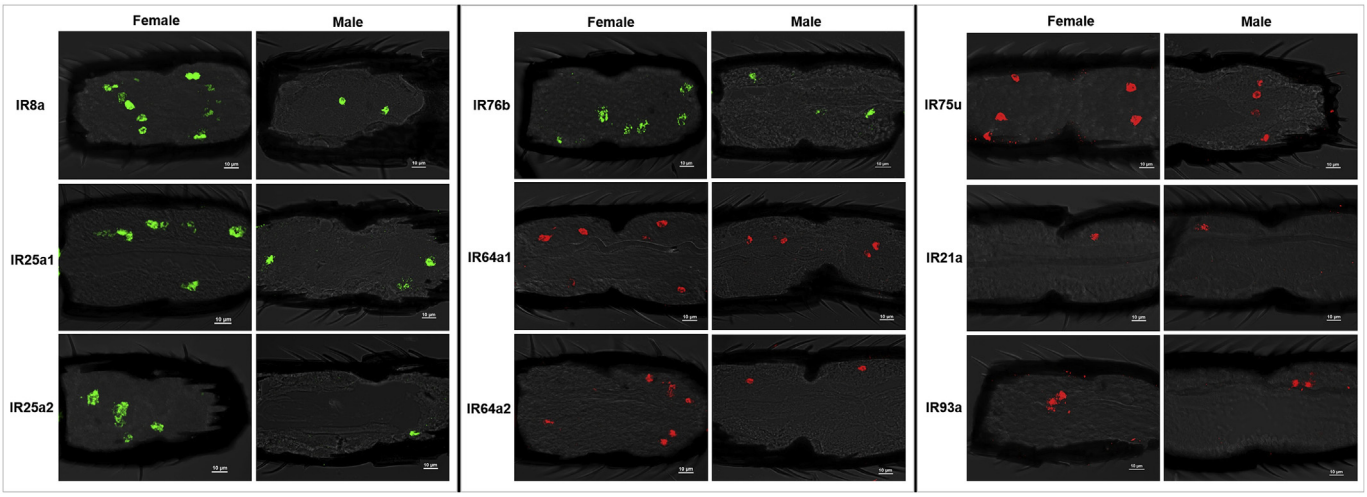

Fig. 1. MmedIRs expression in male and female antennae. Dig- or biotin-labeled antisense RNA probes of MmedIRs were labeled in OSNs of longitudinal sections of antennae segments from both sexes.

of hybridization solution containing RNA probes of the target gene was added to the corresponding slide, and the slides were incubated at 60 °C for at least 16 h. After hybridization, slides were washed three times in 0.1 × SSC at 60 °C for 20 min, and then incubated in 1% blocking reagent (Roche, Mannheim, Germany) diluted in TBS (100 mM Tris, 150 mM NaCl, pH 7.5) with 0.03% Triton X-100 at room temperature for 30 min. The detection of the DIG-labeled probe was conducted using anti-digoxigenin alkaline phosphatase conjugated antibody (Roche, Mannheim, Germany) combined with HNPP substrate (Roche, Mannheim, Germany). For the biotin-labeled probe, Strepavidin-HRP and the TSA Kit (Perkin Elmer, Waltham, USA) were employed to detect signals. Tissue sections were observed and imaged using a Zeiss LSM880 confocal microscope (Zeiss, Oberkochen, Germany).

2.5. In vitro expression and two-electrode voltage clamp recordings

Assays were conducted according to Wang et al. (2010). Briefly, the complete coding sequences of MmedIRs (IR8a, IR64a1, IR64a2, IR25a1, IR25a2 and IR76b) were cloned into the pT7TS vector. Next, recombinant plasmids were digested using a single restriction endonuclease. cRNAs of the target genes were synthesized from linearized vectors using a mMESSAGE mMACHINE® Kit (Invitrogen, Carlsbad, CA, USA). *Xenopus* oocytes (stage V-VI) were collected and treated with 1.3 mg/ml collagenase type I (Life Technologies, Grand Island, NY, USA) in 1 × Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl2, and 5 mM Hepes, pH 7.6) at room temperature for 1 h. Next, oocytes were injected with 27.6 ng of MmedIRs cRNA. After injection, oocytes were incubated for 3 day at 18 °C in 1 × Ringer's solution supplemented with 5% dialyzed horse serum, 50 μg/ml tetracycline, 100 μg/ml streptomycin, 550 μg/ml sodium pyruvate and 0.6 mM $CaCl_2$.

Ligand-induced whole-cell currents were recorded from injected *Xenopus* oocytes using a two-electrode voltage-clamp setup OC-725C oocyte clamp (Warner Instruments, Hamden, CT, USA) at a holding potential of −80 mV. Candidate ligands (Table S2) were dissolved in DMSO to create 1 M stock solution. Subsequently, stock solutions were diluted in 1 × Ringer's solution with 0.6 mM $CaCl_2$ to obtain working concentrations. Compounds used for stimulation were applied through a flowrate control system to the chamber holding oocytes for 15 s at a rate of 2 ml/min with extensive washing in Ringer's buffer at a rate of 4 ml/min between exposures that allowed the current to return to baseline. Data were collected by Digidata 1440A and analyzed using pClamp 10.0 software (Axon Instruments Inc., Union City, CA, USA).

2.6. Electroantennogram (EAG) tests

EAGs were performed to investigate the electrophysiological activities of the candidate compounds (Syed and Leal, 2011). The tested compounds were diluted in liquid paraffin at 10^{-7}-10^{2} μg/μl. Liquid paraffin was also used as a blank control. The antennae were carefully removed at the base, and a few terminal segments at the distal end were excised. The treated antennae were attached to electrode holders with electrode gel. A filter paper strip (4 mm × 30 mm) containing 10 μl test solution was inserted into a glass Pasteur pipette, and then the tip of the pipette was inserted into a hole in the wall of a metal tube directed at the antennal preparation. An air stimulus controller CS-55 (Syntech, Kirchzarten, Germany) was used for air flow. A continuous, carbon-filtered airflow was blown onto the antenna, and odor stimulation was delivered by a 0.2 s puff of air. Signals were recorded for 5 s with each substance at 30 s intervals. EAG recordings were made by an IDAC-2 recording unit with an amplifier and computer board (Syntech, Kirchzarten, Germany).

2.7. Data analysis

Statistical analyses were carried out using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD) and if needed, were transformed prior to analysis. In the EAG tests, significant differences were evaluated via multiple t-tests and Duncan's multiple range test.

3. Results

3.1. Gene structures of MmedIRs

The number of introns in the MmedIRs ranged from 4 to 14 and the intron sizes varied from 53 to 2022 bp. Two groups, IR25a1/IR25a2 and IR64a1/IR64a2/IR75u, shared the same number of introns (four and nine, respectively) (Fig. S1A). The alignment of the intron insertion sites of the MmedIRs with other homologous antennal IRs showed that there were multiple conserved sites and phases in each IR gene group throughout different orders. The homologous IR8a group almost shared seven complete conserved intron insertion sites. The IR64a and IR75 groups shared several conserved sites. Most of the sites and phases of each IR gene group from *M. mediator* and *M. demolitor* were completely conserved (Fig. S1B).

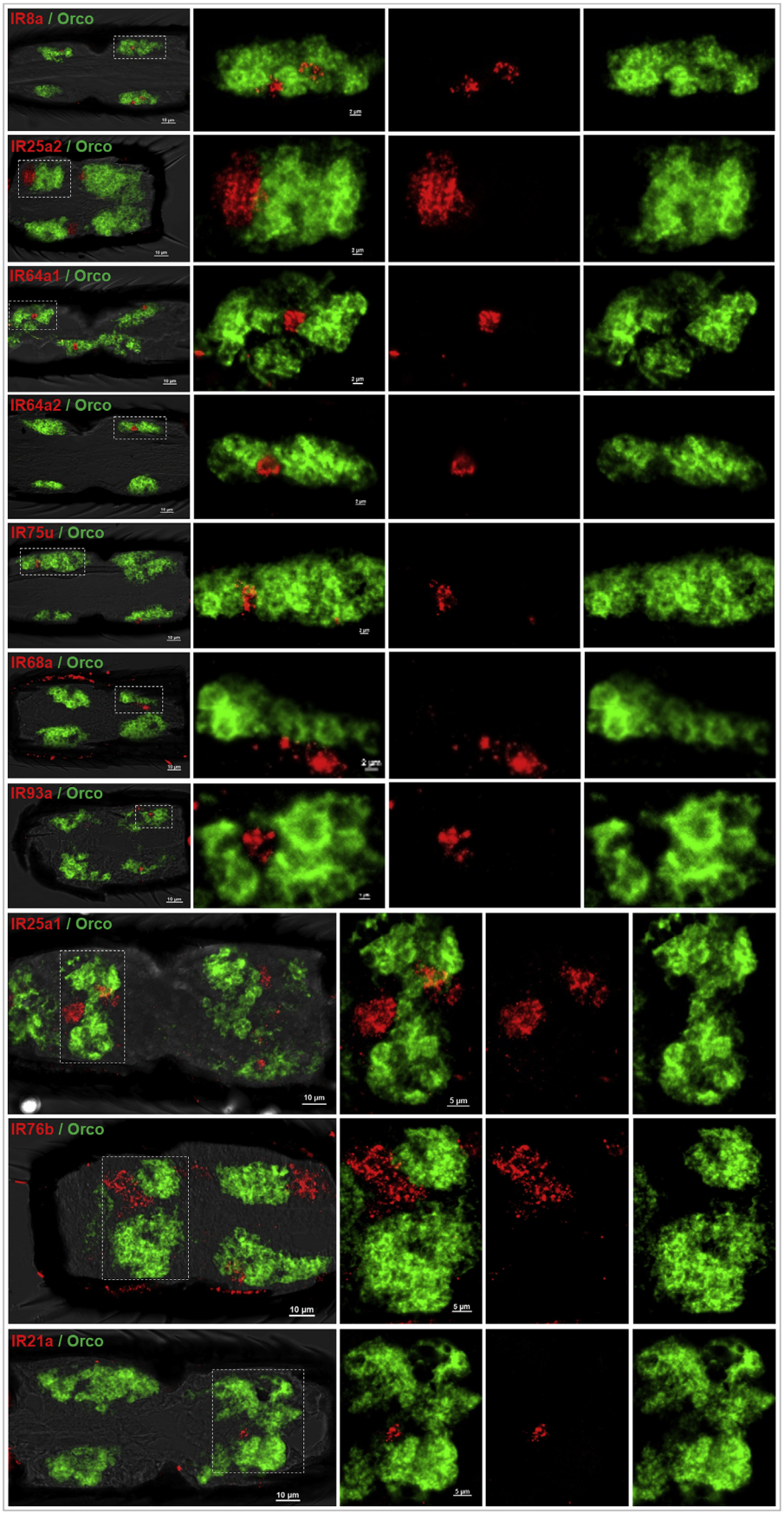

Fig. 2. Co-localization of MmedIRs and MmedOrco in female antennae. MmedIRs antisense RNA probes were Dig-labeled and visualized by red fluorescence. MmedOrco antisense RNA probe was biotin-labeled and visualized by green fluorescence. The dashed frame areas are enlarged and shown on the right. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

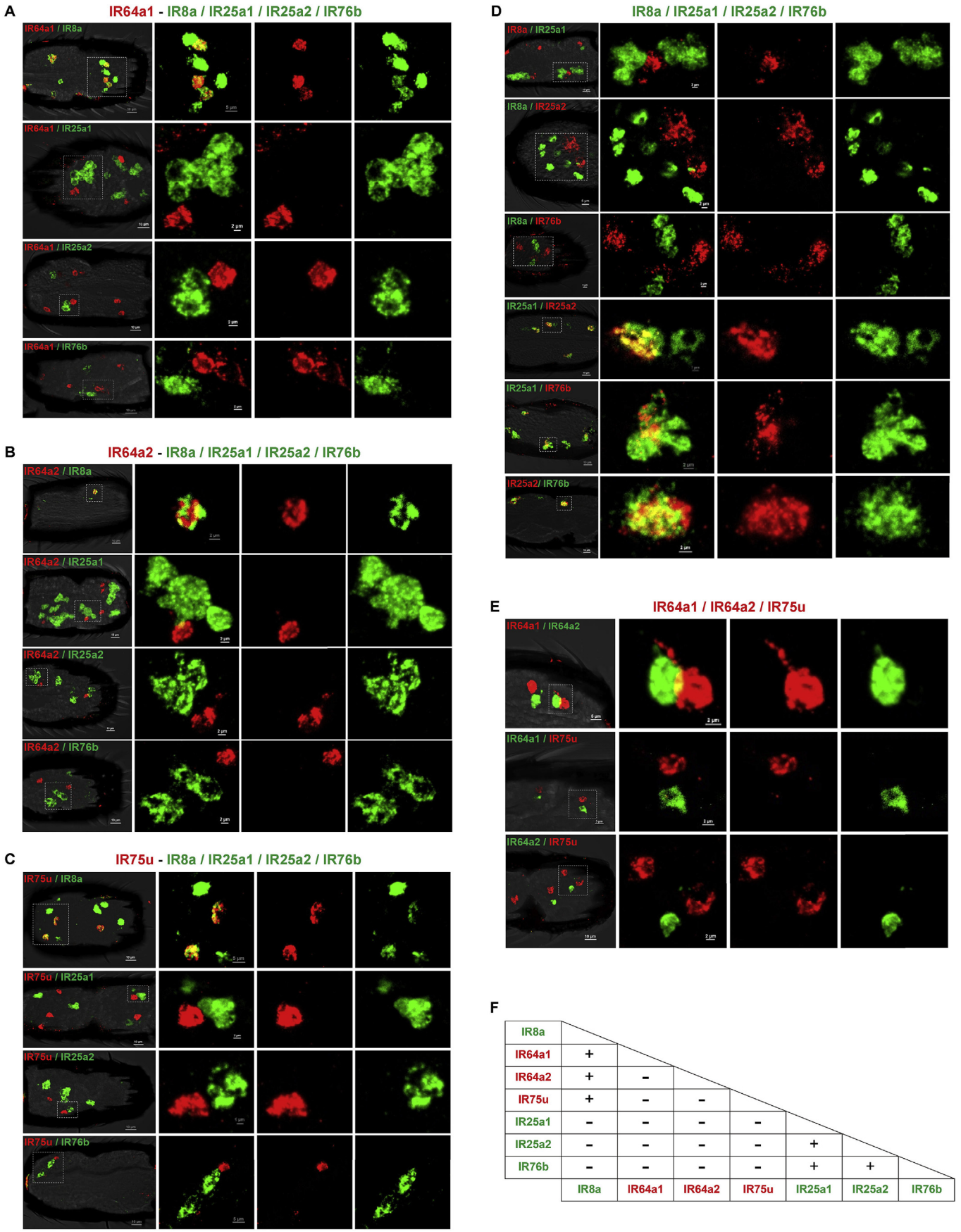

Fig. 3. Co-expressions of MmedIRs in female antennae. (A–C) Localization of specific MmedIRs (IR64a1, IR64a2 and IR75u) with MmedIRcos (IR8a, IR25a1, IR25a2 and IR76b). **(D)** Co-localization among MmedIRcos. **(E)** Co-expressions among specific MmedIRs. **(F)** Co-expression combinations between MmedIRs. "+" indicates that the corresponding two IR genes are co-expressed at the RNA level in the female antennae of *M. mediator*. "-" indicates that the two IR genes are not co-expressed.

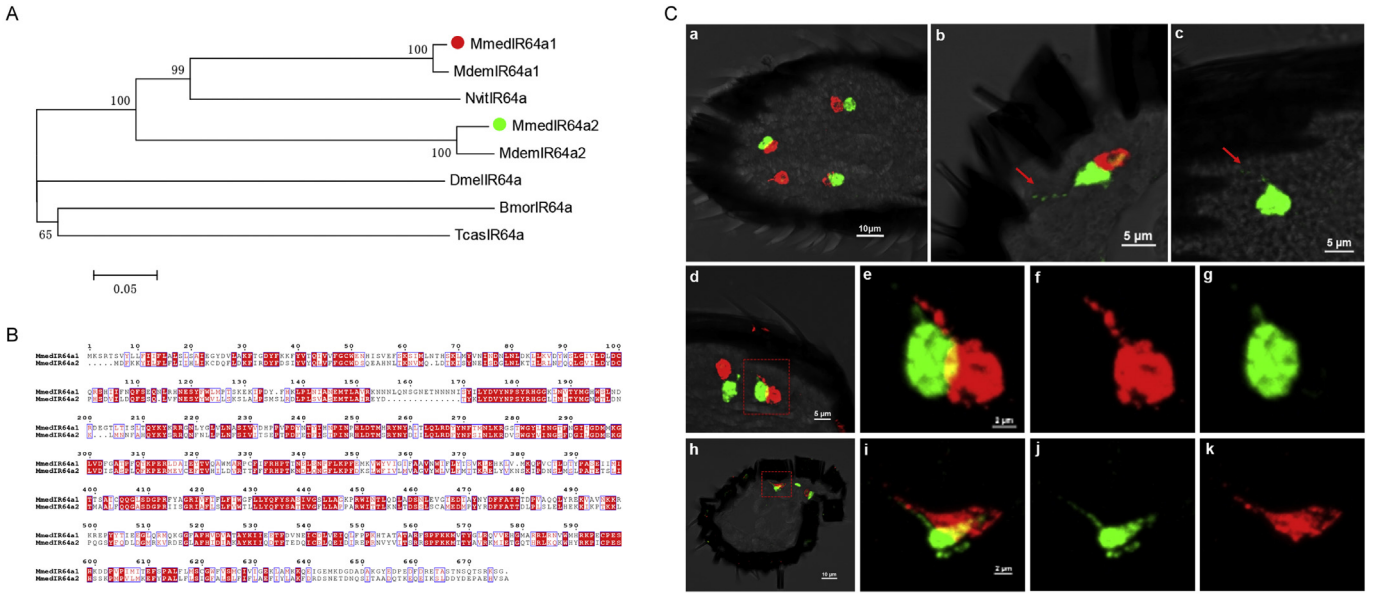

Fig. 4. Phylogenetic tree construction and location of MmedIR64a1 and MmedIR64a2. (A) Phylogenetic analysis of IR64a among hymenopteran insects and several model insects. The neighbor-joining tree was constructed using MEGA 5 with a *p*-distance model and pairwise by re-sampling the amino acid position 1000 times. **(B)** Sequence alignment of MmedIR64a1 and MmedIR64a2. Identical residues are shown in white letters with a red background. Amino acids with physical and chemical properties are shown in red letters. The similar and identical residues are framed in blue rectangles. **(C)** The location of MmedIR64a1 and MmedIR64a2 in the antennae. **(a)** MmedIR64a1 and MmedIR64a2 are generally expressed in adjacent cells; **(b, c)** The dendrites (indicated by red arrows) of OSNs expressing MmedIR64a2 are projected to the sensilla placodea; **(d–k)** Contiguous OSNs expressing MmedIR64a1 and MmedIR64a2 are projected to the same sensillum. The dashed frame areas are enlarged and shown in different fluorescence channel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Location characteristics of MmedIRs

In situ hybridization showed that labeled signals of all ten IR genes were detected in female antennae (Fig. S2). The labeled signals of the IRs in the female antennae were often significantly more than in those of the male antennae. Due to low expression, an expression difference in MmedIR68a between males and females was not observed (Fig. 1). Based on location characteristics, two types of IRs were defined: Type I including MmedIR8a, MmedIR64a1, MmedIR64a2 and MmedIR75u was mainly distributed in the antennal flagellums and expressed in one to three OSNs; and Type II including MmedIR25a1, MmedIR25a2 and MmedIR76b was located in the middle and distal segments of antennae and was often found at the apex of the last flagellum, and in most of cases, was expressed in clusters of OSNs (Fig. S3). However, the distribution features of MmedIR21a, MmedIR68a and MmedIR93a could not be defined due to low transcription levels.

3.3. Co-expression profile of MmedIRs

Although a large number of MmedOrco probe labeled cell clusters were detected in the antennal flagella, all MmedIRs and MmedOrco were localized within distinct cells in the female antennae (Fig. 2). Co-expression among the MmedIRs was also detected (Fig. 3). Among the IR co-receptors (IR8a, IR25a1, IR25a2 and IR76b), IR8a was not co-expressed with any other of the three genes (Fig. 3D), although the co-expression was found between IR25a1, IR25a2 and IR76b. Three specific IRs (IR64a1, IR64a2 and IR75u) were only co-expressed with IR8a, respectively (Fig. 3A–C). Moreover, these three IRs were not co-expressed with each other (Fig. 3E). Notably, two genes with high similarity, IR64a1 and IR64a2, were localized in distinct but adjacent cells, and the dendrites of these two cells projected together to the same sensillum. In some cases, we observed that the dendrite of the OSN-expressing IR64a2 gene projected to the sensilla placodea (Fig. 4).

3.4. Two-electrode voltage clamp recordings

To screen potential ligands, four co-expressed MmedIR complexes (IR64a1-IR8a, IR64a2-IR8a, IR75u-IR8a and IR25a1-IR25a2-IR76b) were expressed in *Xenopus* oocytes, respectively. Unfortunately, the two co-expressed complexes, IR75u-IR8a and IR25a1-IR25a2-IR76b, did not elicit oocytes responding against a panel of approximately 160 unitary candidate odorant chemicals. In contrast, the IR64a1-IR8a and IR64a2-IR8a complexes were sensitive to a range of tested compounds with different odor tuning properties, although oocytes expressing single IR alone did not respond to any candidate chemicals (Fig. 5A). The IR64a1-IR8a complex responded to a wide range of ligands including chemicals with 6–8 carbon atoms such as acids, aldehydes, esters and alcohols. However, the IR64a2-IR8a complex was active against a narrow range of compounds including acids and aldehydes with 8–14 carbon atoms (Fig. 5B–D, Fig. 6). Many of the ligands of these two complexes are components of habitat plant and host-derived cues (Fig. 7A and B; Table 1). The IR64a2-IR8a complex was exclusively sensitive to *Z*9-14: Ald (*cis*-9-tetradecenal), a sex pheromone component of *H. armigera*, but did not respond to other lepidopteran sex pheromone components (Fig. S4). In addition, the 5–7 carbons volatile carboxylic acids elicited much higher responses of IR64a1-IR8a complex, whereas the IR64a2-IR8a complex preferred the 10–12 carbons low volatile carboxylic acids (Fig. S5). In a dose-dependent manner, the IR64a1-IR8a complex responded with higher affinities to the tested ligands (EC_{50} values ranged from 5.81×10^{-7} to 3.21×10^{-5}) compared to that of the IR64a2-IR8a complex (EC_{50} values ranged from 1.37×10^{-4} to 2.84×10^{-3}) (Fig. 7C and D). Taken together, IR64a1 is widely tuned to high volatile habitat cues, whereas IR64a2 responds to a narrow range of host cues and low-volatility plant odors. Notably, IR64a2 can perceive sex pheromone components of *H. armigera*, indicating its crucial role in the accurate identification of hosts.

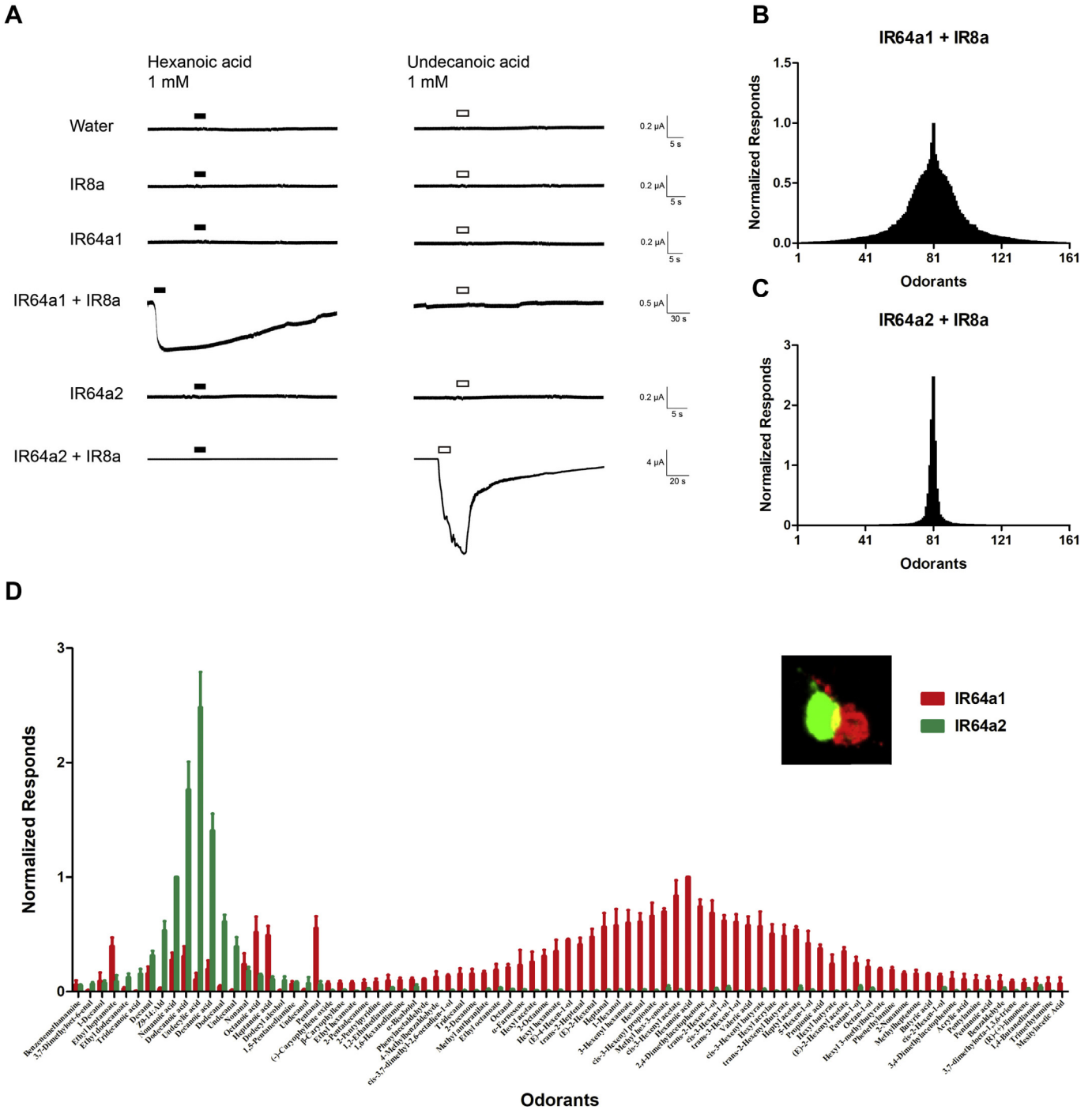

Fig. 5. Two-electrode voltage clamp recordings of the IR64a1 and IR64a2 complexes. (A) Whole cell current traces recorded at −80 mV. **(B)** Tuning curve of the MmedIR64a1 complex. **(C)** Tuning curve of the MmedIR64a2 complex. Odors with the strongest response values are in the center, and those with the weakest values are near the edges. **(D)** Functional characterization of the MmedIR64a1 and MmedIR64a2 complexes. The top 83 ligands of MmedIR64a1 (red) and MmedIR64a2 (green) complexes are listed. The response values were normalized to the positive control (hexanoic acid for the MmedIR64a1 complex and nonanoic acid for the MmedIR64a2 complex), n = 4–6. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. EAG assays

The electrophysiological responses of the female antennae to the main binding ligands (hexanoic acid, *cis*-3-hexenyl acetate, 2,4-dimethylacetophenone, methyl hex-3-enoate, *trans*-2-hexen-1-ol, *cis*-3-hexenyl propionate, *cis*-3-hexen-1-ol, 3-hexenyl hexanoate, valeric acid and 1-hexanol of the IR64a1-IR8a complex; undecylic acid, undecylic acid, decanoic acid, nonanoic acid, dodecanal, *Z*9-14: Ald, undecanal, nonanal, and tetradecanoic acid of the IR64a2-IR8a complex) were investigated by EAG recordings. Except for long carbon chain fatty acids, 2,4-dimethylacetophenone, methyl hex-3-enoate and *cis*-3-hexenyl propionate, most odorants at 10 μg/μl could elicit significant EAG responses compared with the control. Generally, the wasp antennae were sensitive to aldehydes, alcohols and esters, although their EAG values varied with the structure of tested chemicals. Dose-dependent curves indicated that the EAG values increased gradually with the

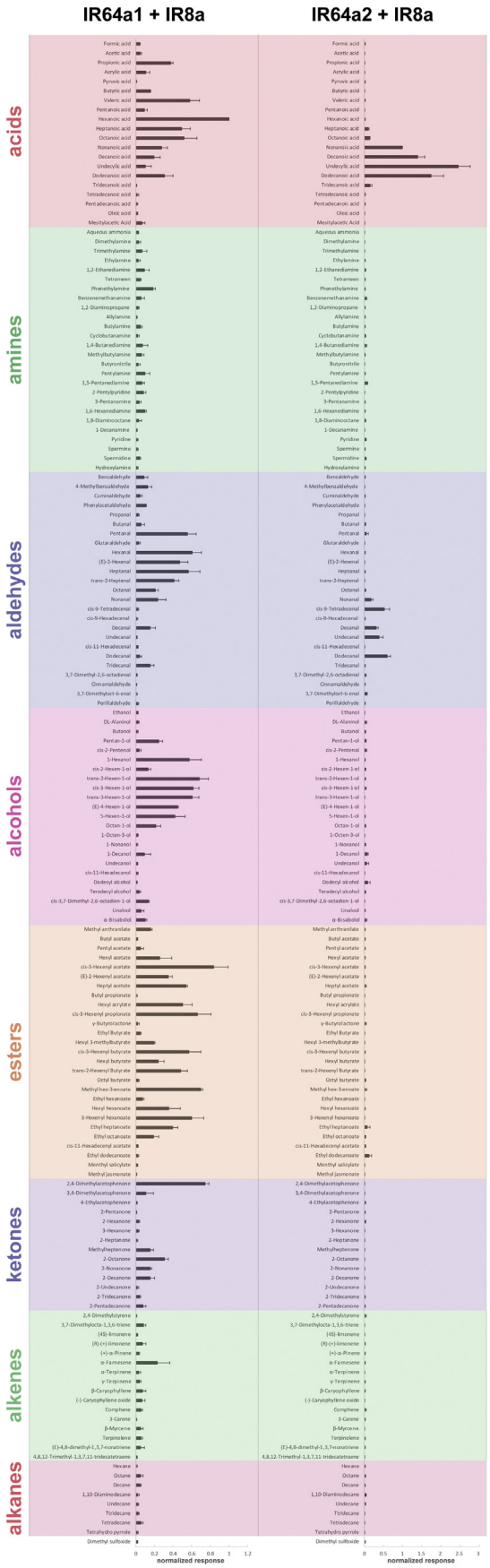

Fig. 6. Ligands binding profiles of the IR64a1 and IR64a2 complexes. Normalized inward current response values of the IR64a1 and IR64a2 complexes to the test chemicals, n = 4–6.

increase of the concentration of tested chemicals, and none of the compounds reached saturation at the stimulus load series (Table 2, Fig. S6A and B).

4. Discussion

Ionotropic receptors were first identified as a new class of olfactory receptors in *D. melanogaster*, and play broad roles in olfaction, gustation, thermo-sensation and humidity sensation (Croset et al., 2016; Enjin et al., 2016; Hussain et al., 2018; Knecht et al., 2016; Koh et al., 2014; Ni et al., 2016; Stewart et al., 2015; Zhang et al., 2013). Although ORs have been extensively investigated in various insect species, little is known about IRs function outside dipteran species (Liu et al., 2018; Zhang et al., 2017; Zhu et al., 2018). In the current study, we comprehensively characterized the function of IRs in the parasitoid wasp *M. mediator*, which is a prominent natural enemy of Noctuidae pests. Our work provides valuable data on the roles of chemoreceptor superfamilies in Hymenoptera species.

4.1. Conservative IRs

All antennal IRs in *M. mediator* have orthologs in *Drosophila* and other species including mosquitoes (Liu et al., 2010; Pitts et al., 2011), honeybees (Croset et al., 2010) and moths (Bengtsson et al., 2012; Große-Wilde et al., 2011; Legeai et al., 2011; Olivier et al., 2011). In contrast, insect ORs have only one co-receptor, Orco, which is conserved across different species (Jones et al., 2005; Rytz et al., 2013). As in *Drosophila*, the antennal IRs in *M. mediator* presented multiple introns (Croset et al., 2010). Specially, IR25a1/IR25a2 and IR64a1/IR64a2/IR75u shared the same numbers of intron, suggesting the functional conservation of these two orthologous groups in *M. mediator*. Moreover, most of the intron insertion sites and phases were clearly conserved within each individual IR group across insect orders. Notably, multiple conserved sites were also shared in distinct IR groups. Evolutionary relatedness and functional conservatism of antennal IRs may exist in all insects.

4.2. Distribution and co-expression patterns of MmedIRs

Most antennal IRs in the *M. mediator* were highly expressed in female antennae (Wang et al., 2015, 2016). *In situ* hybridization further verified that most of the MmedIRs were female antennae biased, suggesting their roles in female-specific physiological activities. Our FISH data did not support the IR/OR colocalization but rather suggested that there were cell-autonomous pathways of IR/OR in *M. mediator*, which was consistent with reports in *Drosophila* and mosquitoes (Benton et al., 2009; Pitts et al., 2017). Furthermore, we characterized two types of MmedIRs. Type I includes MmedIR8a, MmedIR64a1, MmedIR64a2 and MmedIR75u, which were evenly distributed in the antennal flagella and were expressed in one to three OSNs. IR64a1 and IR64a2 were expressed in adjacent cells of sensilla placodea on each antennal flagellum, and s. placodea were reported to respond to plant volatiles (Ochieng et al., 2000). Moreover, MmedIR8a that was detected in OSNs was also localized to s. placodea (Wang et al., 2016). Thus, we proposed that all Type I MmedIRs are likely expressed in s. placodea. Type II, including MmedIR25a1, MmedIR25a2 and MmedIR76b, were found in clusters of OSNs that were distributed in the middle and distal antennae flagella. Considering that the distribution of Type II IRs is coincident with s. basiconica type I (a type of gustatory sensilla) (Wang et al., 2018), we hypothesize that MmedIR25a1, MmedIR25a2 and MmedIR76b are expressed in this type of sensilla, but further verification is needed.

In *Drosophila*, broadly expressed "co-receptor" IRs (IR25a/IR8a/IR76b) form heteromeric partnerships with stimulus-specific IRs to generate an array of receptor complexes with diverse specificities (Ai et al., 2013; Rytz et al., 2013; van Giesen and Garrity, 2017). Three co-

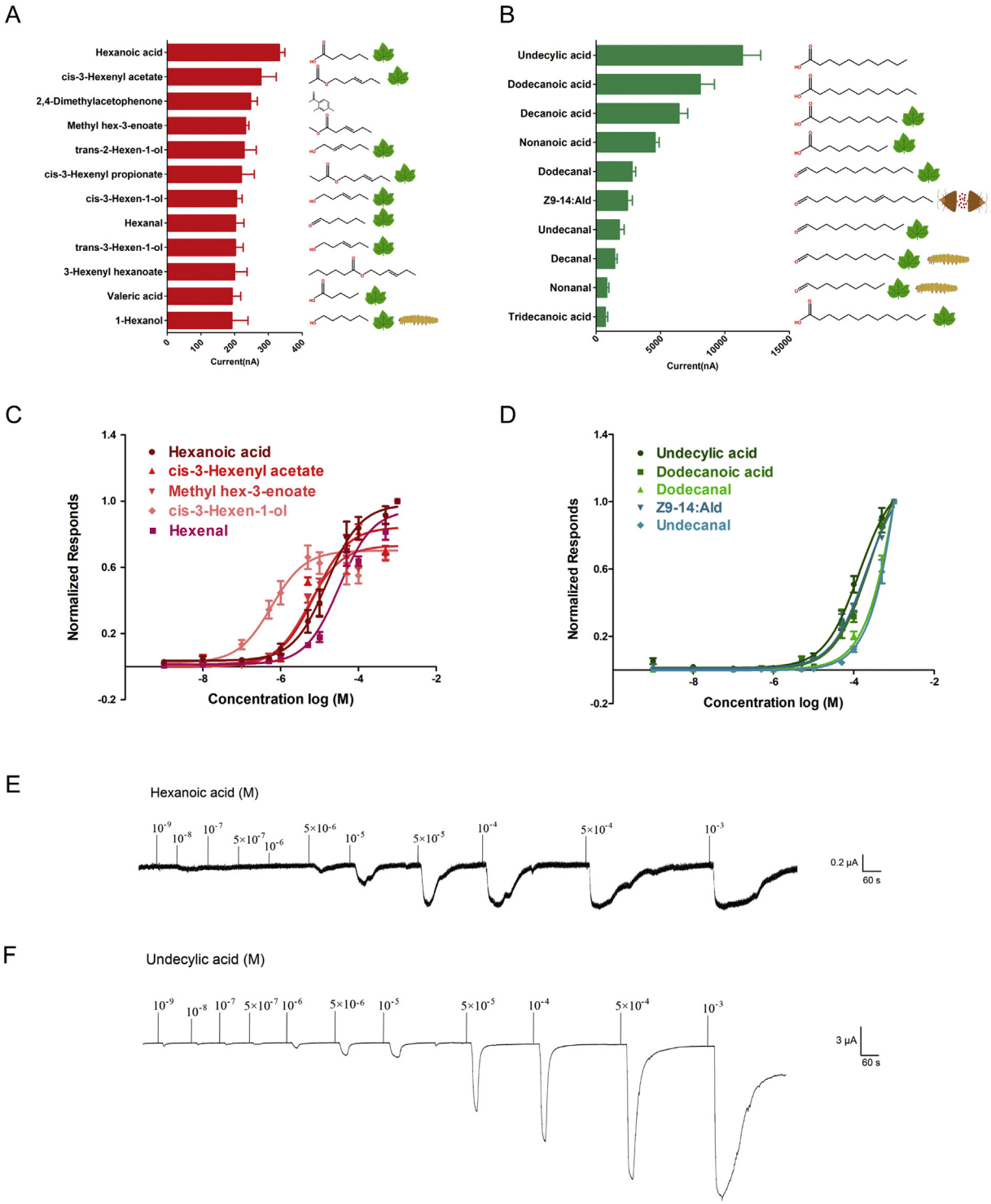

Fig. 7. Functional characteristics of the IR64a1 and IR64a2 complexes. (A, B) The IR64a1 and IR64a2 complexes respond to the main ligands. Green leaves: plant volatiles; Yellow larvae: volatiles from host bodies; Brown moths: sex pheromone components of lepidopteran insects. **(C, D)** Dose-dependent manner of the IR64a1 and IR64a2 complexes responding to the selected ligands. Half maximal effective concentration (EC_{50}): hexanoic acid (1.56×10^{-5} M), hexenal (3.21×10^{-5} M), *cis*-3-hexenyl acetate (7.37×10^{-6} M), methyl hex-3-enoate (5.03×10^{-6} M), *cis*-3-hexen-1-ol (5.81×10^{-7} M), undecylic acid (1.37×10^{-4} M), dodecanoic acid (2.48×10^{-4} M), dodecanal (1.62×10^{-3} M), *Z*9-14:Ald (2.06×10^{-4} M) and undecanal (2.84×10^{-3} M). Response values are normalized by defining the maximal response value as 1 in each group, n = 4–6. **(E, F)** Inward current responses of the IR64a1 and IR64a2 complexes to hexanoic acid and undecylic acid, respectively, with different dilutions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expressed complexes, IR64a1-IR8a, IR64a2-IR8a and IR75u-IR8a, are involved in acid-sensing in *Drosophila* (Abuin et al., 2011; Ai et al., 2013). In our study, the MmedIR64a1-IR8a, MmedIR64a2-IR8a and MmedIR75u-IR8a complexes that were mainly expressed in s. placodea of *M. mediator* may attribute to perceive plant volatiles.

The homologous IR25a and IR76b in *Drosophila* acted as co-

Table 1
List of the main ligands of the MmedIR64a1 and MmedIR64a2 complexes.

CAS No.	Odorants	Main carbon chain length	Target gene	Potential sources	EAG activity	Behavioral stimulation	References
142-62-1	Hexanoic acid	6	MmedIR64a1	Host plant	+[1]	+[1]	Shi et al. (2015)
3681-71-8	*cis*-3-Hexenyl acetate	6	MmedIR64a1	Host plant; HIPV	+[2]	+[2]	Dong et al. (2000a), b; Li et al. (1992); Whitman et al. (1992)
89-74-7	2,4-Dimethylacetophenone	6	MmedIR64a1				
2396-78-3	Methyl hex-3-enoate	6	MmedIR64a1				
928-95-0	*trans*-2-Hexen-1-ol	6	MmedIR64a1	HIPV		+[3]	Whitman et al. (1992)
33467-74-2	*cis*-3-Hexenyl propionate	6	MmedIR64a1	HIPV		+[3]	Whitman et al. (1992)
928-94-9	*cis*-3-Hexen-1-ol	6	MmedIR64a1	Host plant; HIPV	+[4]	+[3]	Dong et al. (2000a); Ochieng et al. (2000); Whitman et al. (1992)
66-25-1	Hexanal	6	MmedIR64a1	Host plant; HIPV	+[1]	+[2]	Dong et al. (2000a), b; Whitman et al. (1992)
928-97-2	*trans*-3-Hexen-1-ol	6	MmedIR64a1	Host plant			Unpublished data
31501-11-8	3-Hexenyl hexanoate	6	MmedIR64a1				
109-52-4	Valeric acid	5	MmedIR64a1	Host plant			Unpublished data
111-27-3	1-Hexanol	6	MmedIR64a1	Host plant; Host body surface	+[1]	+[1]	Shi et al. (2015)
112-37-8	Undecylic acid	11	MmedIR64a2				
143-07-7	Dodecanoic acid	12	MmedIR64a2				
334-48-5	Decanoic acid	10	MmedIR64a2	Host plant			Unpublished data
112-05-0	Nonanoic acid	9	MmedIR64a2	Host plant			Unpublished data
112-54-9	Dodecanal	12	MmedIR64a2	Host plant			Unpublished data
53939-27-8	*Z*9:14-Ald	14	MmedIR64a2	Host sex pheromone			Kehat et al. (1990)
112-44-7	Undecanal	11	MmedIR64a2	Host plant	+[1]		Zhang et al. (2011)
112-31-2	Decanal	10	MmedIR64a2	Host plant; Host body surface	+[1]	+[1]	Hedin (1975), 1976; Shi et al. (2015); Yu et al. (2007)
124-19-6	Nonanal	9	MmedIR64a2	Host plant; Host body surface	+[1]	+[1]	Hedin (1975), 1976; Shi et al. (2015); Yu et al. (2007)
638-53-9	Tridecanoic acid	13	MmedIR64a2	Host plant			Unpublished data

Note: CAS No.: Chemical abstract service number; EAG: Electroantennogram; HIPV: Herbivore-induced plant volatiles; +: The chemical could elicit the EAG or behavior responses of parasitoid wasps; Superscript 1: References on *M. mediator*; Superscript 2: References on *M. mediator* and *M. croceipes*; Superscript 3: References on *M. croceipes*; Superscript 4: The chemical could elicit the single sensillum recording (SSR) response of *M. croceipes*.

Table 2
EAG dose-dependent responsiveness of female wasps to the main ligands of the MmedIR64a1 and MmedIR64a2 complexes.

Chemical	Dosage (μg/μl)				
	0.01	0.1	1	10	100
Hexanoic acid	0.767 ± 0.111 d	1.080 ± 0.357 d	2.177 ± 0.376 c	3.361 ± 0.740 b	5.125 ± 0.559 a
cis-3-Hexenyl acetate	1.004 ± 0.252 c	1.205 ± 0.439 c	2.098 ± 0.679 c	3.827 ± 1.054 b	7.387 ± 2.019 a
2,4-Dimethylacetophenone	0.805 ± 0.173 b	1.054 ± 0.282 b	1.054 ± 0.282 b	2.245 ± 0.680 a	2.838 ± 0.967 a
Methyl hex-3-enoate	0.867 ± 0.211 c	1.659 ± 0.403 bc	1.189 ± 0.593 bc	2.601 ± 1.491 b	4.304 ± 1.840 a
trans-2-Hexen-1-ol	1.006 ± 0.169 c	1.028 ± 0.398 c	1.858 ± 1.084 c	4.329 ± 3.388 b	7.771 ± 3.455 a
cis-3-Hexenyl propionate	1.049 ± 0.259 b	1.630 ± 0.259 b	1.485 ± 0.878 b	2.390 ± 1.391 b	3.934 ± 1.831 a
cis-3-Hexen-1-ol	1.274 ± 0.542 c	1.569 ± 0.309 c	1.555 ± 0.308 c	3.209 ± 1.356 a	5.119 ± 1.345 b
Hexanal	0.787 ± 0.164 c	0.980 ± 0.249 c	1.410 ± 0.694 bc	2.561 ± 1.667 b	4.792 ± 2.077 a
trans-3-Hexen-1-ol	0.935 ± 0.144 c	1.114 ± 0.227 c	1.369 ± 0.209 c	3.127 ± 0.753 b	8.177 ± 2.575 a
3-Hexenyl hexanoate	1.084 ± 0.397 c	1.225 ± 0.281 bc	1.421 ± 0.353 bc	2.235 ± 0.599 b	3.725 ± 1.373 a
Valeric acid	1.299 ± 0.455 c	1.257 ± 0.449 c	1.178 ± 0.272 c	2.541 ± 0.709 b	4.305 ± 1.059 a
1-Hexanol	0.753 ± 0.547 c	0.997 ± 0.133 c	1.627 ± 0.584 c	3.903 ± 1.497 b	6.801 ± 2.793 a
Undecylic acid	0.831 ± 0.219 b	0.984 ± 0.372 b	1.039 ± 0.338 b	0.982 ± 0.222 b	1.559 ± 0.329 a
Dodecanoic acid	0.958 ± 0.340 a	1.034 ± 0.310 a	1.073 ± 0.226 a	0.980 ± 0.273 a	1.107 ± 0.187 a
Decanoic acid	0.821 ± 0.305 b	0.768 ± 0.146 b	0.822 ± 0.309 b	1.718 ± 0.655 a	1.990 ± 0.442 a
Nonanoic acid	0.807 ± 0.251 c	1.000 ± 0.546 c	1.638 ± 0.362 c	3.238 ± 0.712 b	5.931 ± 1.886 a
Dodecanal	0.798 ± 0.217 c	0.801 ± 0.119 c	1.183 ± 0.235 c	2.303 ± 0.559 b	4.505 ± 1.584 a
*Z*9:14-Ald	0.698 ± 0.203 c	0.842 ± 0.242 c	0.814 ± 0.226 c	2.142 ± 0.471 b	4.827 ± 0.714 a
Undecanal	0.915 ± 0.387 c	0.711 ± 0.186 c	1.390 ± 0.408 bc	2.225 ± 0.950 b	3.911 ± 1.566 a
Decanal	0.820 ± 0.343 c	1.208 ± 0.515 c	2.560 ± 0.992 bc	6.452 ± 3.256 b	12.108 ± 8.124 a
Nonanal	0.553 ± 0.228 d	0.738 ± 0.203 d	2.609 ± 0.741 c	5.338 ± 1.418 b	8.670 ± 2.108 a
Tridecanoic acid	0.664 ± 0.133 a	0.956 ± 0.526 a	1.216 ± 0.533 a	1.213 ± 0.430 a	1.116 ± 0.468 a

Note: Different letters following the data (mean ± SD) in a row indicate significant differences among five doses at $P < 0.05$ (Duncan's multiple range test).

receptors with specific IRs to mediate amine-sensing (Abuin et al., 2011; Hussain et al., 2018). Although our FISH results showed that MmedIR25a1, MmedIR25a2 and MmedIR76b co-expressed between two genes, we speculated that specific MmedIRs exist for these co-receptors but remain undiscovered. Wasps use antenna tips to touch and distinguish their hosts (Roux et al., 2005). MmedIR25a1, MmedIR25a2 and MmedIR76b were mainly distributed at the distal flagella of antennae, suggesting that specific IRs are co-expressed with the three co-receptors to recognize contact chemical cues.

Intriguingly, acid-sensing (IR8a-dependent) and amine-sensing (IR25a-dependent, except IR75d) IRs in *Drosophila* belong to two distinct clades, suggesting their derivation from ancestral IRs with this functional specialization (Silbering et al., 2011). Strikingly, regions of the target glomeruli receiving input from IR8a-dependent and IR25a-

dependent OSNs in the central nervous system, are distinct (Silbering et al., 2011). Acid-activated (IR8a-dependent) and amine-activated (IR25a-dependent) receptor complexes were also reported in mosquitoes. The co-expression of AgIR25a and AgIR76b with either AgIR41a or AgIR41c yield channels to respond against amines, whereas co-expression of AgIR8a and AgIR75k confers sensitivity to carboxylic acids (Kwon et al., 2006; Wang et al., 2010). Consequently, although the distribution of type I IRs (such as MmedIR8a) and type II IRs (such as MmedIR25a) in antennae of *M. mediator* is different, they may be related in evolution, function and nerve projection.

4.3. Functional characterization of the IR64a1 and IR64a2 complexes

In *Drosophila*, the sensory neurons expressing IR64a send axonal projections to either DC4 or DP1m glomeruli in the antennal lobe. DC4 neurons respond specifically to acids, whereas DP1m neurons respond to a broad spectrum of odorants. IR8a and IR64a formed a functional complex associated with acid-evoked physiological and behavioral responses (Ai et al., 2013). In this study, the primary ligands of the MmedIR64a1 and MmedIR64a2 complexes were acids, although these two complexes presented different odor tuning properties. The MmedIR64a1 complex responded to a relatively wide range of volatile short carbon chain compounds, and most of the ligands are components of plant volatiles released over a long distance. Conversely, the IR64a2 complex was tuned to narrow range of long carbon chain compounds, most of which are released from plants or host insects at close range. Notably, the IR64a2 complex was exclusively tuned to one sex pheromone component (*Z*9-14: Ald) of *H. armigera*. Ligands of both IR complexes are likely important chemical cues for accurate host-seeking in wasps.

4.4. Ecological significance of the IR64a1 and IR64a2 ligands

Most ligands of the MmedIR64a1 and MmedIR64a2 complexes can trigger electrophysiological responses in female wasps. Ligands of MmedIR64a1 are the important components of general plant volatiles or herbivore-induced plant volatiles (HIPVs) such as *cis*-3hexen-1-ol and *cis*-3-hexenyl acetate. These high-volatile six-carbon alcohols, aldehydes and derivative esters are largely released from plants or host caterpillar damaged plants, which act as crucial chemical cues to mediate host-seeking in parasitiod wasps (Eller et al., 1990; Röse et al., 1998; Tumlinson et al., 1993; Whitman and Eller, 1990). In the tritrophic chain, HIPVs serve as plant-to-parasitoid synomones that are released to benefit both the plant and the parasitoid but would be detrimental to caterpillar (Whitman and Eller, 1992). These compounds are named habitat cues (Webster and Cardé, 2017), which are easily detected over long distances and large area by parasitoids. Thus, wasps or other insects exploiting plant volatiles as a long-distance signal can be a cost-saving and efficient strategy to maximize their chances of subsequently encountering specific host cues.

In short distance host-seeking, host-related cues play more important roles in accurately locating hosts. Long carbon chain ligands of the MmedIR64a2 complex include the directed host cues, low volatile plant odors, and an important sex pheromone component (*Z*9-14: Ald) of *H. armigera* (Chang et al., 2017; Hillier and Thomas, 2016; Kehat et al., 1980; Kehat and Dunkelblum, 1990). These data indicate that, in the long-term coevolution process, parasitoid wasps could indirectly utilize host sex pheromones to accurately locate hosts in an intricate environment. Moreover, homologous MmedIR64a1 and MmedIR64a2 that are expressed in contiguous OSNs of the same sensilla may have evolved from the same ancestral IR to adapt to ecological contexts in collaborative and energy-saving ways.

5. Conclusion

In this study, the following speculative host-seeking strategy mediated by IRs in *M. mediator* was proposed: IR64a1 is widely tuned to habitat cues to guide the perceived behavior of wasps over long distances, whereas IR64a2 responds to narrow range host cues and low-volatility plant odors to benefit wasps to locate hosts precisely at short distances. IR64a1 and IR64a2 collaboratively perceive habitat and host cues to assist parasitoids to efficiently seek hosts in complex environments.

Our data indicate that antennal IRs are functionally conserved between dipteran and hymenopteran species across 350 million years of evolution. We boldly propose that the functional conservation of antennae IRs exists in all insects. Compared with insect ORs that may primarily detect species-specific odor cues, the IRs are promising molecular targets for the design of novel and broad-spectrum regulators to control insect olfactory behavior.

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Appendix A. Supplementary data

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