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# Activation of pheromone-sensitive olfactory neurons by plant volatiles in the moth *Agrotis ipsilon* does not occur at the level of the pheromone receptor protein

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In moths, mate finding relies on female-emitted sex pheromones that the males have to decipher within a complex environmental odorant background. Previous studies have shown that interactions of both sex pheromones and plant volatiles can occur in the peripheral olfactory system, and that some plant volatiles can activate the pheromone-specific detection pathway. In the noctuid moth *Agrotis ipsilon*, plant volatiles such as heptanal activate the receptor neurons tuned to the pheromone component (*Z*)7-12:OAc. However, the underlying mechanisms remain totally unknown. Following the general rule that states that one olfactory receptor neuron usually expresses only one type of receptor protein, a logic explanation would be that the receptor protein expressed in (*Z*)7-12:OAc-sensitive neurons recognizes both pheromone and plant volatiles. To test this hypothesis, we first annotated odorant receptor genes in the genome of *A. ipsilon* and we identified a candidate receptor putatively tuned to (*Z*)7-12:OAc, named AipsOR3. Then, we expressed it in *Drosophila* olfactory neurons and determined its response spectrum to a large panel of pheromone compounds and plant volatiles. Unexpectedly, the receptor protein AipsOR3 appeared to be very specific to (*Z*)7-12:OAc and was not activated by any of the plant volatiles tested, including heptanal. We also found that (*Z*)7-12:OAc responses of *Drosophila* neurons expressing AipsOR3 were not affected by a background of heptanal. As the *Drosophila* olfactory sensilla that house neurons in which AipsOR3 was expressed contain other olfactory proteins – such as odorant-binding proteins – that may influence its selectivity, we also expressed AipsOR3 in *Xenopus* oocytes and confirmed its specificity and the lack of activation by plant volatiles. Altogether, our results suggest that a still unknown second odorant receptor protein tuned to heptanal and other plant volatiles is expressed in the (*Z*)7-12:OAc-sensitive neurons of *A. ipsilon*.

KEYWORDS

**insect, olfaction, odorant receptor, sex pheromone, volatile organic compound**

## Introduction

In most moth species, females release a unique mixture of chemicals, called a sex pheromone, in an attempt to attract males, which specifically recognize it from far away (Kaissling, 2014). All the pheromonal signals released into the air are immersed in an olfactory environment: plants also emit their own volatile molecules, which have diverse functions including communication with other organisms. In addition, the trajectory of pheromones can be quite erratic because the air can have an unpredictable and turbulent movement. The pheromonal signal is therefore quite fragmented and drowned in a very rich odor background, which has been shown to modulate pheromone detection (Deisig et al., 2014; Renou et al., 2015).

Moth pheromone compounds, like any odorant molecule, are recognized by transmembrane receptors located in the dendrite membrane of olfactory receptor neurons (ORNs), located in several thousand sensilla on the antenna (Fleischer and Krieger, 2018). In insects, the major family of such receptors are the odorant receptors (ORs). Each ORN usually expresses only one type of OR, detecting a limited number of chemicals, in addition to the obligatory co-receptor named Orco (Larsson et al., 2004), but various lines or evidence indicate that each ORN can express more than just one OR and Orco (Couto et al., 2005; Fishilevich and Vosshall, 2005; Koutroumpa et al., 2014; Schultze et al., 2014; McLaughlin et al., 2021; Herre et al., 2022; Task et al., 2022). In moths, pheromone compounds are detected by receptors belonging to particular OR lineages (Zhang and Löfstedt, 2015; Bastin-Héline et al., 2019; Montagné et al., 2021). Whereas the majority of ORs have a broad activation spectrum, i.e., they can recognize several odorant molecules, pheromone receptors (PRs) are often very specific to their ligand or at least narrowly tuned to similar chemicals (Zhang and Löfstedt, 2015). Once interaction occurs between an odorant and the corresponding OR-Orco complex, the signal is transformed by the ORN into an electrical signal that is transmitted along the antennal nerve to the brain primary olfactory centers, the antennal lobes (ALs). There, peripheral inputs are processed, enabling the insect to extract relevant information and adopt the correct behavior (Masse et al., 2009; Touhara and Vosshall, 2009).

It has been demonstrated that volatile plant compounds (VPCs) can modulate the male pheromone response at ORNs, ALs, and behavioral level. At the behavioral level, both field and laboratory studies have shown a synergistic effect of VPC/pheromone mixtures on pheromone trapping (Light et al., 1993; Landolt and Phillips, 1997; Deng et al., 2004) and on male orientation in wind tunnel experiments (Schmidt-Busser et al., 2009; Varela et al., 2011). Such synergism has also been evidenced within the male macroglomerular complex (a set of enlarged glomeruli dedicated to the process of pheromones in the ALs) in *Cydia pomonella* (Trona et al., 2013). At the pheromone-responsive ORN level, studies are more contrasted. Addition of a VPC to the pheromone usually results in a suppressive effect on the pheromone response (Den Otter et al., 1978; Pophof and van der Goes van Naters, 2002). For instance, a background of linalool (one of the most common VPCs in nature) appears to halve the response of ORNs tuned to the major component of the sex pheromone in *Spodoptera littoralis* (Party et al., 2009). Similarly, a background of some VPCs – namely linalool, (*Z*)3-hexenyl acetate, hexanal and heptanal – reduces the firing rate of pheromone-responsive ORNs in the black cutworm moth *Agrotis ipsilon* (Renou et al., 2015). However, the effects have been shown to be VPC-dependent in *Heliothis virescens*. In this species, VPCs like linalool, geraniol or (*Z*)3-hexenyl acetate diminish the response of (*Z*)11-16:Ald-specific ORNs and this suppressive effect has been shown to occur at the level of the PR protein (Hillier and Vickers, 2010; Pregitzer et al., 2012). By contrast, a mixture of (*Z*)11-16:Ald and β-caryophyllene appears to enhance the activity of the (*Z*)11-16:Ald ORNs (Hillier and Vickers, 2010). In *Helicoverpa zea*, mixtures of linalool or (*Z*)3-hexenol and the major sex pheromone component also synergize the response of pheromone-responsive ORNs (Ochieng et al., 2002). Moreover, it has unexpectedly been shown in *A. ipsilon* that ORNs tuned to (*Z*)7-12:OAc – one of the three components of the sex pheromone – can respond to stimulation with VPCs alone, notably linalool, (*Z*)3-hexenyl acetate and heptanal (Rouyar et al., 2015; Conchou et al., 2021). Consequently, backgrounds of these volatiles mask pheromone responses and impact male behavior, notably by altering the perception of the ratio of pheromone constituents (Dupuy et al., 2017; Hoffmann et al., 2020). However, the mechanisms underlying this response of pheromone-sensitive ORNs to VPCs remain unknown.

Several hypotheses can be put forward. Although it is generally accepted that ORNs express only one type of OR, many exceptions have been documented (Couto et al., 2005; Fishilevich and Vosshall, 2005; Koutroumpa et al., 2014; Schultze et al., 2014; McLaughlin et al., 2021; Herre et al., 2022). First, *A. ipsilon* (*Z*)7-12:OAc-responsive ORNs could express several ORs, one specific for the pheromone and other(s) recognizing VPCs. Second, some moth PRs could be less specific than expected and able to bind certain VPCs in addition to pheromone compounds. In order to test these hypotheses and determine whether activation by VPCs occurs at the level of the PR or not, the aim of the present work was to identify the *A. ipsilon* receptor tuned to (*Z*)7-12:OAc, taking advantage of its recently published genome (Wang et al., 2021), and to study its functional properties in heterologous systems, isolated from its natural neuronal environment.

## Materials and methods

### Annotation of OR genes in the *Agrotis ipsilon* genome and identification of candidate pheromone receptors

*Agrotis ipsilon* OR (AipsOR) gene models were created by aligning a set of 485 full length amino acid OR sequences

previously identified in Noctuidae (including sequences from the *A. ipsilon* antennal transcriptome) on the genome assembly GCA_004193855.1 (BioProject PRJNA428387) using Exonerate v2.4.0 (Slater and Birney, 2005) with a score threshold set at 1,000, maximum intron length set at 10,000 and the three best results per query reported. Amino acid sequences translated from these gene models were extracted with the gffread utility from the Cufflinks package (Trapnell et al., 2010), then potential redundant sequences were clustered using CD-HIT protein (Fu et al., 2012).

To identify AipsORs belonging to the lineage of moth PRs, AipsOR sequences were aligned with PR sequences from *Bombyx mori* (Krieger et al., 2005), *Heliothis virescens* (Krieger et al., 2004) and *Spodoptera littoralis* (Meslin et al., 2022) using Clustal Omega v1.2.2 (Sievers and Higgins, 2018) and a phylogeny was built from this alignment using FastTree v2.1.11 (Price et al., 2009) as implemented in Geneious Prime® v2022.1 (Biomatters Ltd., Auckland, New Zealand). Then, a maximum-likelihood phylogeny of the PR lineage was built using PhyML 3.0 (Guindon et al., 2010) with default parameters.

## Heterologous expression of AipsOR3 in *Drosophila melanogaster* and single-sensillum recordings

The *AipsOr3* full-length open reading frame (ORF) was amplified by RT-PCR (forward primer 5′-ATGAAATTATTCTCT GATCTGTCTG-3′; reverse primer 5′-TTAATCTTCCTC CGCAACTG-3′) from cDNA prepared (Superscript II reverse transcriptase, Thermo Fisher Scientific, Carlsbad, CA, United States) from RNA extracted from male antennae (TRIzol® Reagent, Thermo Fisher Scientific), cloned into pCR™II-TOPO™ (Thermo Fisher Scientific) and then subcloned into the pUAST. attB vector. Plasmids were purified using the NucleoBond® Xtra Maxi Endotoxin Free kit (Macherey-Nagel, Düren, Germany). Plasmids were injected into fly embryos with the genotype *y1 M{vas-int.Dm}ZH-2A w**; *m{3xP3-RFP.attP}ZH-51C* to generate flies carrying the *UAS-AipsOr3* genetic construct on the region 51C of the second chromosome (BestGene Inc., Chino Hills, CA, United States). The *UAS-AipsOr3* line was then crossed to the $Or67d^{GAL4[2]}$ mutant knock-in line (Kurtovic et al., 2007) to generate flies with the genotype *w*; *UAS-AipsOr3*; $Or67d^{GAL4[2]}$ that express the AipsOR3 receptor instead of the endogenous receptor Or67d.

Single-sensillum recordings on at1 sensilla were performed as described in de Fouchier et al., 2015. For the screening experiment, stimulus cartridges consisted of Pasteur pipettes containing a filter paper loaded with either 10 µg/µl of an hexane solution of the pheromone compound (1 µg µl$^{-1}$) or 10 µl of a VPC diluted in mineral oil (1% v/v). Control cartridges contained 10 µl of hexane or mineral oil alone. Dose–response experiments with (*Z*)7-12:OAc were performed with decimal dilutions ranging from 1 µg µl$^{-1}$ down to 100 pg µl$^{-1}$, and those with heptanal were performed with 20, 10, 5 and 1% v/v dilutions (10 µl in the stimulus cartridge). Each of the recorded at1 ORNs was stimulated with all doses of the two compounds, as well as cartridges with solvent alone. For experiments with odorant backgrounds, stimuli were delivered using a custom-made device described in Rouyar et al., 2015. Backgrounds were generated using glass vials containing 1 ml of heptanal diluted in mineral oil (1% v/v) or mineral oil alone. Pheromone stimulations were made using a glass vial containing a filter paper loaded with 10 µl of (*Z*)7-12:OAc diluted in hexane (1 µg/µl). Source and purity of the chemicals used as stimuli can be found in Supplementary Table 1. Recordings were analyzed with the pCLAMP™ 11 software (Molecular Devices, San Jose, CA, United States) by subtracting the spontaneous firing rate before stimulation (in spikes·s$^{-1}$, measured during 500 ms before the onset of stimulation) from the firing rate during the stimulation (measured during a 500 ms time window which started 200 ms after the onset of stimulation). Statistical analyses were done with Prism 9.3.1 (GraphPad Software, San Diego, CA, United States) and RStudio (2022.02.3; RStudio Team, 2022).

## Heterologous expression of AipsOR3/ AipsOrco in *xenopus* oocytes and two-electrode voltage-clamp

The AipsOrco full-length ORF was synthesized *in vitro* by Synbio Technologies (Monmouth Junction, NJ, United States). AipsOr3 and AipsOrco ORFs were then sub-cloned into the pCS2+ vector (Synbio Technologies). Plasmids were linearized with *Not*I, then capped cRNAs were synthesized using SP6 RNA polymerase with the mMESSAGE mMACHINE™ SP6 transcription kit (Thermo Fisher Scientific). Purified cRNAs were resuspended in nuclease-free water at a 2 µg/µl concentration and stored at −80°C. Mature defolliculated oocytes were purchased from EcoCyte Bioscience (Dortmund, Germany) and microinjected upon delivery with the mixture of 27.6 ng of AipsOR3 cRNA and 27.6 ng of AipsOrco cRNA using a Nanoject III injector (Drummond Scientific, Broomall, PA, United States).

After 2–3 days of incubation at 18°C in Ringer's solution containing 5% dialyzed horse serum, 50 mg/L tetracycline, 100 mg/L streptomycin and 550 mg/L sodium pyruvate, two-electrode voltage-clamp recordings were performed at a holding potential of −80 mV. Intracellular glass electrodes were filled with 1:2 KCl (3 M): potassium acetate (3 M) and had resistances of 0.2–2.0 MΩ. Stock solutions of pheromone compounds and plant volatiles were prepared by diluting each compound to 1 M in dimethyl sulfoxide and were stored at −20°C until use. Before each experiment, the stock solution was diluted to the working concentration in Ringer's buffer ($10^{-5}$ M for pheromone compounds, $10^{-4}$ M for plant volatiles). Data acquisition and analysis were carried out with Digidata 1550A and pCLAMP™ 11 (Molecular Devices).

# Results

## Annotation of *Agrotis ipsilon* candidate pheromone receptors

Forty-one ORs were previously identified in the antennal transcriptome of *A. ipsilon*, including five ORs belonging to the PR clade: AipsOR1, 2, 3, 4, and 14 (Gu et al., 2014). We used the recently released genome of *A. ipsilon* (Wang et al., 2021) to annotate the complete repertoire of OR genes, and found a total of 76 genes (Supplementary Data Sheet 1). We notably identified five additional members of the PR clade, which were paralogues of AipsOR1 and AipsOR4 not found in the transcriptome (Figure 1). The phylogenetic analysis revealed that among those 10 candidate PRs, AipsOR3 was orthologous to the *A. segetum* receptor AsegOR4, which is narrowly tuned to (*Z*)7-12:OAc (Zhang and Löfstedt, 2013). Moreover, AipsOR3 was previously shown to be highly expressed in *A. ipsilon* male vs. female antennae (Gu et al., 2014). This receptor thus appeared as the best (*Z*)7-12:OAc candidate receptor.

## Functional characterization of AipsOR3 in *drosophila* at1 ORNs

To verify whether AipsOR3 was indeed the (*Z*)7-12:OAc receptor, we generated *D. melanogaster* lines expressing this receptor in at1 ORNs instead of the endogenous receptor DmelOR67d (Kurtovic et al., 2007). Antennae of transformed flies were stimulated with a range of pheromone compounds presented at high doses, including the three components of the *A. ipsilon* sex pheromone (*Z*)7-12:OAc, (*Z*)9-14:OAc and (*Z*)11-16:OAc (Gemeno and Haynes, 1998). Single-sensillum recordings showed that AipsOR3-expressing ORNs were significantly activated by (*Z*)7-12:OAc, with a mean response of ~200 spikes·s$^{-1}$ (Figure 2A). A very modest response was recorded for the *trans* isomer (*E*)7-12:OAc, but it was not statistically different from the control (solvent alone). We tested whether AipsOR3 could be activated by VPCs known to activate (*Z*)7-12:OAc ORNs in *A. ipsilon* male antennae, but found no response to any of the four compounds tested (Figure 2A). This was confirmed by dose–response analyses: heptanal, the most active VPC on (*Z*)7-12:OAc ORNs, was unable to activate AipsOR3-expressing ORNs even when stimulated with a 20% solution, equivalent to more than 1,500 µg loaded in the stimulus cartridge (Figures 2B,C). (*Z*)7-12:OAc significantly activated the same ORNs starting at a dose of 1 µg.

## Functional characterization of AipsOR3 in *xenopus* oocytes

To test whether the lack of activation by VPCs may be due to the neuronal environment, we also expressed AipsOR3 (together with its co-receptor AipsOrco) in *Xenopus* oocytes and conducted functional studies. Again, AipsOR3 was strongly and quite specifically activated by (*Z*)7-12:OAc, and a very low current was measured in response to stimulation with (*E*)7-12:OAc (Figures 3A,B). As in *Drosophila* ORNs, no response was found for any of the four VPCs. Overall, these results show that we probably identified the receptor expressed in (*Z*)7-12:OAc ORNs in *A. ipsilon* male antennae and that this receptor was not activated by plant volatiles, even at a high dose.

## Effect of a background of heptanal on AipsOR3 function

It has been shown previously in *A. ipsilon* that the response of (*Z*)7-12:OAc ORNs is diminished when pheromone stimulation is made in an odorant background of VPCs, notably heptanal (Rouyar et al., 2015; Conchou et al., 2021). We reproduced the same experiment with *Drosophila* at1 ORNs expressing AipsOR3 and found no difference between responses to (*Z*)7-12:OAc when presented in a heptanal background and a neutral background (Figure 4).

# Discussion

Although detection and central processing of sex pheromones and VPCs have been thought for long to be separated from each other, it is now clearly established that interactions between both classes of olfactory cues occur early in the peripheral olfactory system of male moths, usually resulting in a suppressive effect on the pheromone response (Den Otter et al., 1978; Pophof and van der Goes van Naters, 2002; Party et al., 2009). This is the case of pheromone-responsive ORNs of *A. ipsilon* males, for which a background of some VPCs – including heptanal – reduces the firing rate in response to a pulse of pheromone (Renou et al., 2015). The underlying mechanisms are not clearly understood, but another study conducted in *A. ipsilon* has demonstrated that some VPCs activate the pheromone ORNs by themselves (Rouyar et al., 2015), although it is not known if this phenomenon is direct or indirect. In this study, native *A. ipsilon* (*Z*)7-12:OAc-ORNs responded to heptanal starting at a 1% dilution with a spike frequency of around 60 spikes·s$^{-1}$, reaching 125 spikes·s$^{-1}$ at 10%. Interestingly, a previous report has already revealed that high doses of VPCs activate pheromone-responsive ORNs in a closely related species, *A. segetum* (Hansson et al., 1989). As it is generally accepted that ORNs express only one type of OR (but see exceptions to this, Couto et al., 2005; Fishilevich and Vosshall, 2005; Koutroumpa et al., 2014; Schultze et al., 2014; McLaughlin et al., 2021; Herre et al., 2022; Task et al., 2022), and considering that PRs are highly specific to pheromones, activation of pheromone ORNs by VPCs is puzzling. Would pheromone ORNs express more than one OR type? Would *A. ipsilon* PRs be less specific than previously thought?

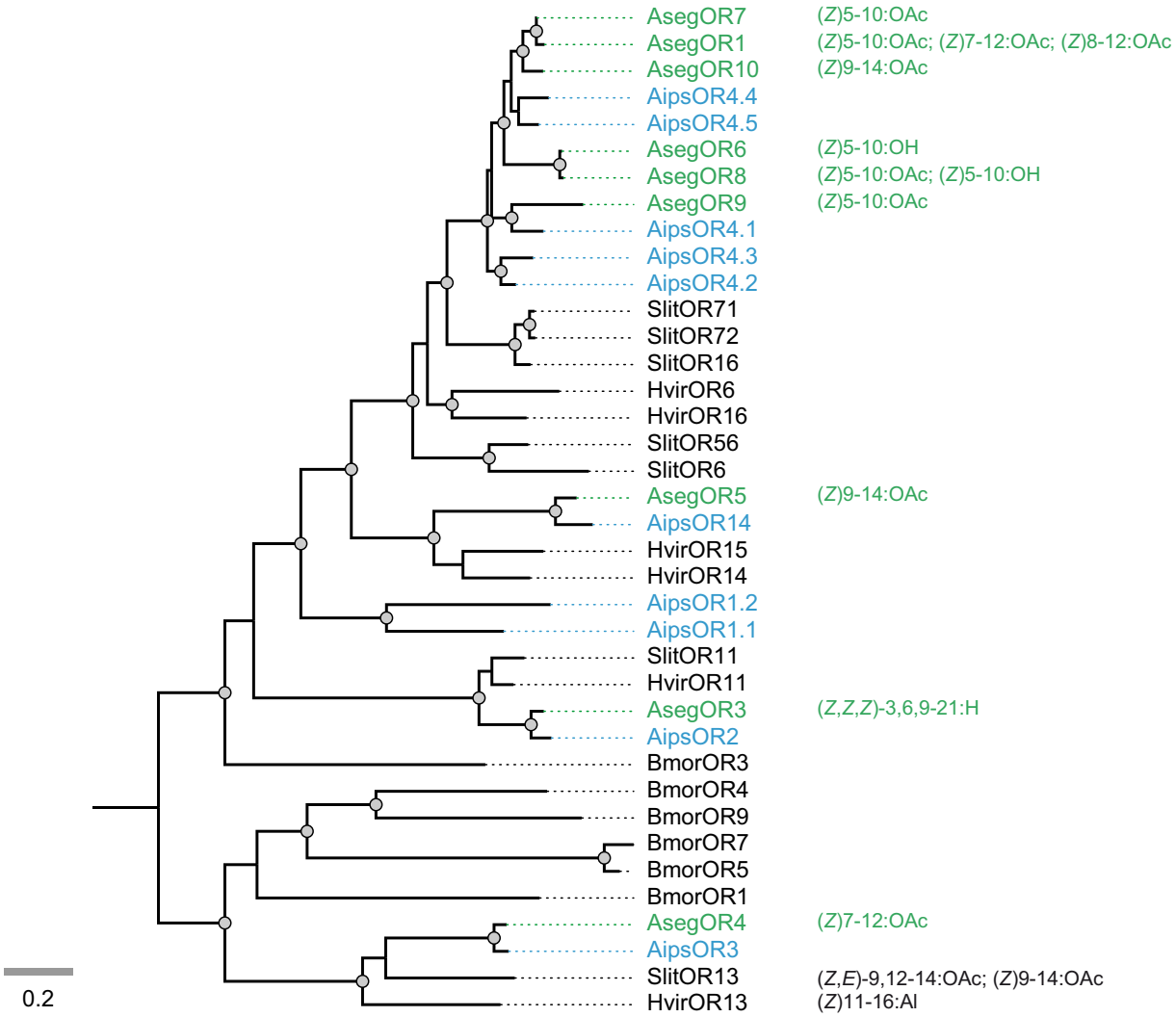


FIGURE 1
Phylogenetic position of *Agrotis ipsilon* ORs within the moth sex pheromone receptor clade. The maximum-likelihood tree was built from the alignment of amino acid sequences from *A. ipsilon* (Aips, in blue), *A. segetum* (Aseg, in green), *Bombyx mori* (Bmor), *Heliothis virescens* (Hvir) and *Spodoptera littoralis* (Slit). Nodes supported by the likelihood-ratio test (aLRT>0.9) are shown with gray dots. The best ligands of AsegORs identified previously (Zhang and Löfstedt, 2013; Zhang et al., 2016) are indicated, as well as those of the AipsOR3 orthologues HvirOR13 and SlitOR13 (Große-Wilde et al., 2007; de Fouchier et al., 2015).

To test these hypotheses, we worked at the level of the PR tuned to the pheromone compound (*Z*)7-12:OAc. First, we identified this receptor as AipsOR3. It belongs to the classical moth PR clade and is the orthologue of AsegOR4, also tuned to (*Z*)7-12:OAc in *A. segetum* (Zhang and Löfstedt, 2013). Interestingly, whereas some closely related PRs can respond to different pheromone compounds (Montagné et al., 2021), we have here a clear example of functional conservation. A large screening with 26 pheromone compounds revealed that AipsOR3, when expressed in *Drosophila* ORNs, was quite specific to (*Z*)7-12:OAc and did not respond to any of the four VPCs known to activate (*Z*)7-12:OAc ORNs in *A. ipsilon*. Moreover, a background of heptanal had no effect on the pheromone response of AipsOR3-expressing *Drosophila* ORNs, contrary to what has been observed in *A. ipsilon* (*Z*)7-12:OAc ORNs. Thus, heterologous expression of the receptor tuned to (*Z*)7-12:OAc does not recapitulate the functional properties of *A. ipsilon* (*Z*)7-12:OAc ORNs to VPCs.

The simpler interpretation of these results is that (*Z*)7-12:OAc ORNs express another OR responsible for the detection of VPCs. In fact, some cases of co-expression of several ORs within a single ORN have been revealed in different species. For instance, double *in situ* hybridization showed that at least two ORs are co-expressed in some *Anopheles gambiae* ORNs (Schultze et al., 2014). In *D. melanogaster*, some OR pairs are co-expressed in a single ORN and are both functional (Ebrahim et al., 2015; Lebreton et al., 2017). In the moth *Ostrinia nubilalis*, some pheromone-responsive ORNs co-express up to four different PR genes, and the broad tuning of these ORNs strongly suggest that at least several of these PRs are

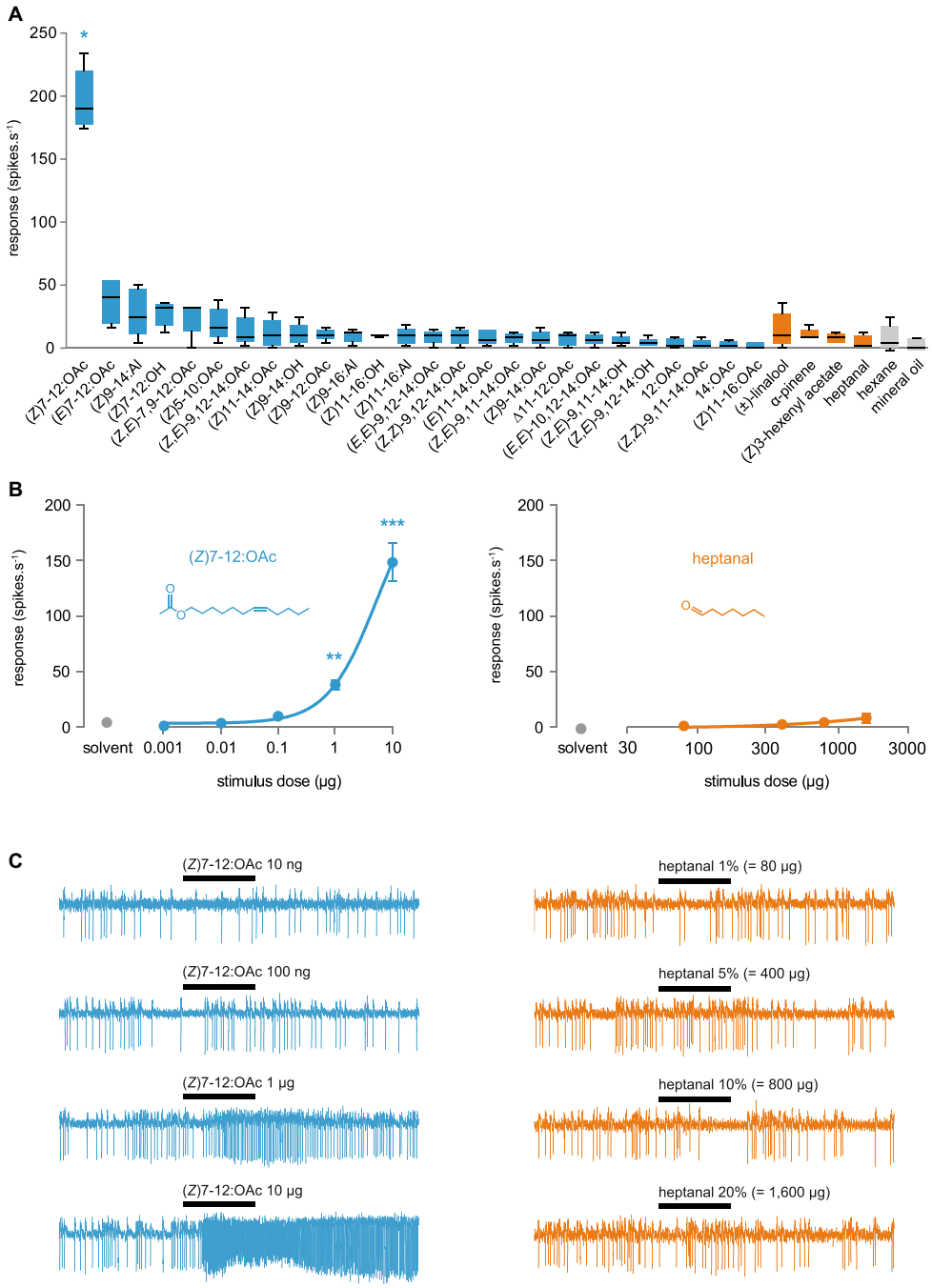


FIGURE 2
Functional analysis of AipsOR3 expressed in *Drosophila* at1 neurons. **(A)** Action potential frequency of at1 neurons expressing AipsOR3 in response to 26 moth pheromone compounds (10 µg in the stimulus cartridge – blue bars) and four volatile plant compounds known to be active on *A. ipsilon* (*Z*)7-12:OAc-responsive neurons (80 µg in the stimulus cartridge – orange bars). Boxes show the median and the first and third quartiles, and whiskers show the minimum and maximum values of the distribution ($n$=5). *$p$<0.05, significantly different from the response to solvent (Friedman ANOVA followed by a Dunn's *post-hoc* test). **(B)** Dose–response curves (mean response±SEM, $n$=11) of at1 neurons expressing AipsOR3 when stimulated with the *A. ipsilon* pheromone compound (*Z*)7-12:OAc or the volatile plant compound heptanal. ***$p$<0.001, **$p$<0.01, significantly different from the response to solvent (Friedman ANOVA followed by a Dunn's *post-hoc* test). **(C)** Example of a series of recordings obtained for an at1 neuron expressing AipsOR3. Black bars represent the stimulus (500 ms).

functional (Koutroumpa et al., 2014). Alternatively, non-OR membrane receptors could be responsible for VPC responses. In insects, olfactory detection is indeed performed not only *via* ORs but also *via* ionotropic receptors (IRs; Wicher and Miazzi, 2021). Whereas ORs and IRs are generally expressed in different ORN populations, a recent study in the mosquito *Aedes aegypti* has unexpectedly shown that IRs could be co-expressed with ORs in the same ORNs, and that both receptor types were functional

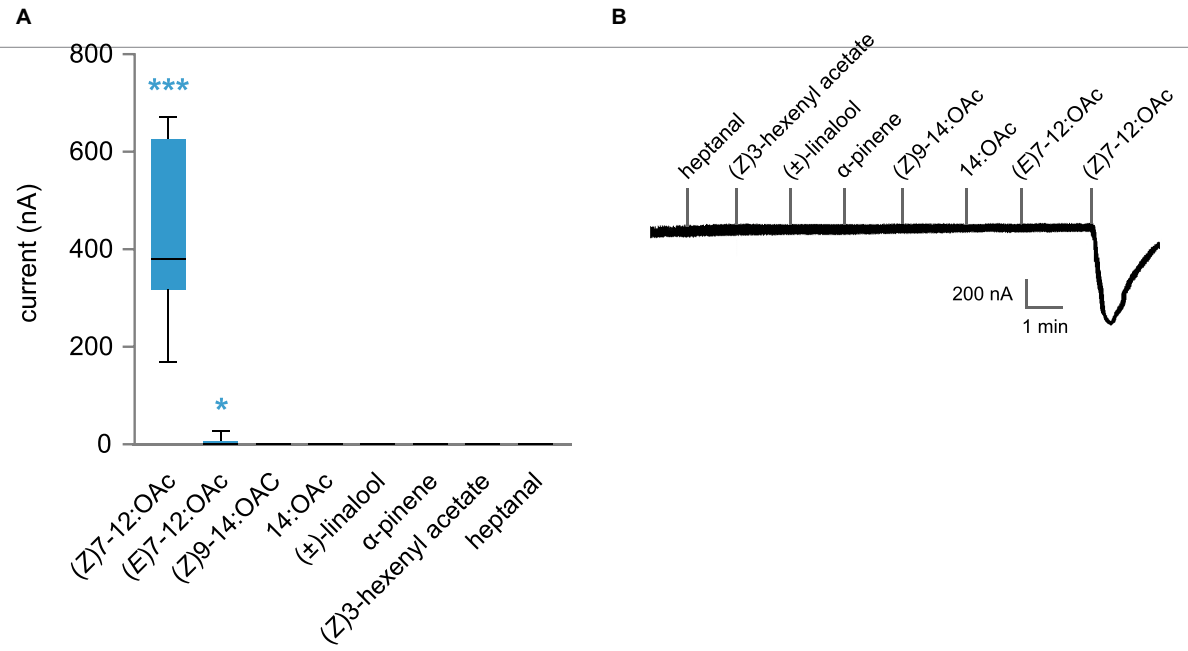


FIGURE 3
Functional analysis of AipsOR3 expressed in *Xenopus* oocytes. **(A)** Inward current measured by two-electrode voltage clamp in oocytes co-expressing AipsOR3 and AipsOrco in response to four moth pheromone compounds ($10^{-5}$ M) and four volatile plant compounds ($10^{-4}$ M). Boxes show the median and the first and third quartiles, and whiskers show the minimum and maximum values of the distribution ($n$=12–17). ***$p$<0.001, *$p$<0.05, significantly different from 0 (Wilcoxon signed rank test). **(B)** Representative TEVC recording obtained for a *Xenopus* oocyte co-expressing AipsOR3 and AipsOrco.

(Herre et al., 2022). In *Drosophila*, Orco and the three IR co-receptors extensively overlap in expression (Task et al., 2022). It is plausible that *A. ipsilon* (*Z*)7-12:OAc ORNs express IRs in addition to AipsOR3, yet IRs are not the best candidates to explain VPC detection because several VPCs used in our study are known to be detected by ORs in moths (de Fouchier et al., 2017; Guo et al., 2021).

Although our results suggest that at least one OR is co-expressed with AipsOR3 in *A. ipsilon* ORNs, we cannot rule out other possibilities. First, our study may suffer from a protocol bias, as the stimulation system we used (Pasteur pipettes) differs from that used in Rouyar et al., 2015 (vials). Thus, the amount of heptanal reaching the antennae is difficult to compare. However, in our heptanal background experiment, we used exactly the same stimulation system as in Rouyar et al., 2015 and no response of AipsOR3-expressing *Drosophila* ORNs to 1% heptanal could be noticed at the onset on the background delivery. In addition, we found no effect of this background on the pheromone response of these ORNs, contrary to what has been observed for *A. ipsilon* (*Z*)7-12:OAc ORNs. Another explanation could be that AipsOR3 is in fact less specific *in vivo* than what we observed when expressed in *Drosophila* ORNs. Indeed, it is known that the sensillum environment can affect OR response profiles. For instance, odorant-binding proteins (OBPs) and/or chemosensory proteins (CSPs) are proposed to transport odorants within the sensilla lymph to the ORN membrane, with implications for ORN sensitivity and specificity (Große-Wilde et al., 2007; Forstner et al., 2009; Pelosi et al., 2014). Moreover, it has been shown that some OBPs can modulate olfactory physiology and the behavior that it drives (Xiao et al., 2019). Sensory Neuron Membrane Proteins (SNMPs) may also be part of the pheromone reception pathway (Jin et al., 2008). OBPs, CSPs and/or SNMPs, housed in *Drosophila* at1 sensilla, may strongly differ from those naturally occurring in *A. ipsilon* pheromone sensitive sensilla, and they could have affected the response of ORNs expressing AipsOR3. To test this hypothesis, we used another functional assay to isolate AipsOR3 from components of the sensillum environment (OBPs, CSPs, SNMPs), which consisted of *in vitro* expression in *Xenopus* oocytes coupled to two-electrode voltage clamp. In this system, odorants (pheromones and VPCs) were solubilized in water and carried to the OR *via* the use of dimethyl sulfoxide. Thus, VPCs should not encounter any solubilization nor transport issues from insect OBPs/CSPs/SNMPs to reach the OR, although the *Xenopus* oocyte solution used to solubilize odorants might probably not exactly recapitulate OBP function. By doing so, we confirmed that AipsOR3 responded strongly to (*Z*)7-12:OAc only, and no response was found for any of the four VPCs.

Taken all together, our results demonstrate that activation of (*Z*)7-12:OAc-sensitive ORNs by VPCs in *A. ipsilon* does not occur at the level of the AipsOR3 protein. A compelling explanation is that at least another receptor – which remains to be identified – is co-expressed in these neurons and participate in modulating pheromone responses in the presence of plant odors, but we cannot exclude other hypotheses. For instance, the

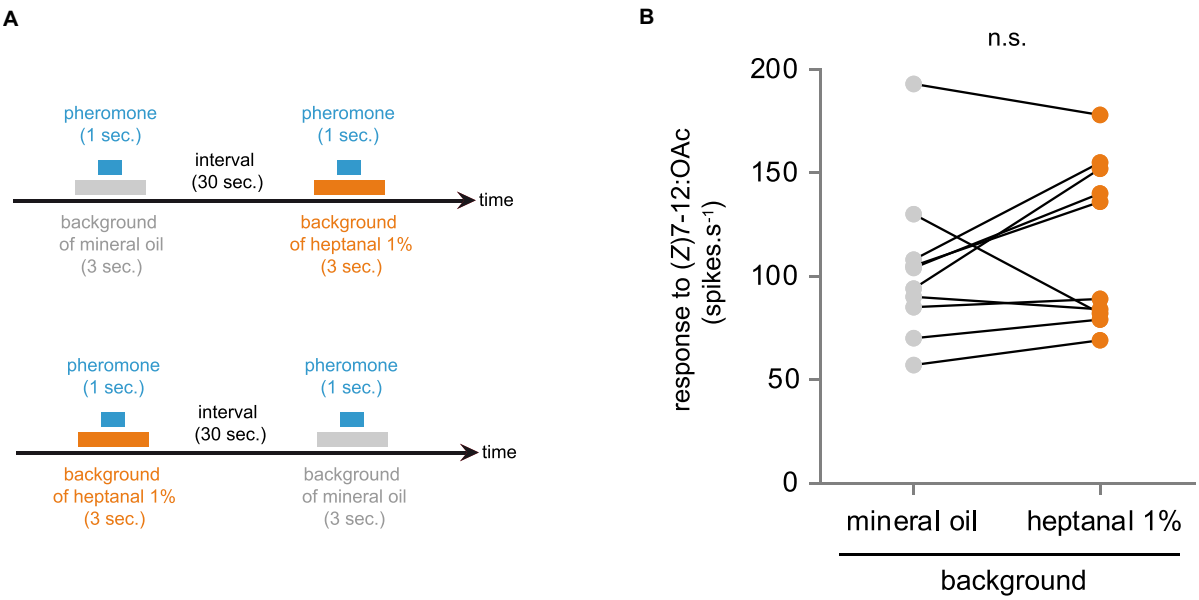


FIGURE 4
Effect of a background of heptanal on AipsOR3 function. **(A)** Protocol used to compare (*Z*)7-12:OAc responses of at1 neurons expressing AipsOR3 with or without a heptanal background. Neurons were alternatively stimulated with the neutral background (mineral oil) first or with the heptanal background first. **(B)** Action potential frequency of at1 neurons expressing AipsOR3 ($n$=10) in response to (*Z*)7-12:OAc (10 μg in the glass vial) in a background of heptanal (1 mL of heptanal 1% in the glass vial). n.s., no significant difference between the two treatments (paired $t$-test, significance threshold set to 0.05).

exact function of OBPs/CSPs is still under study (Rihani et al., 2021) and these proteins may change the OR response threshold (Xiao et al., 2019), making the VPC responses not detectable in our expression systems. Apart OBPs/CSPs, other yet unknown elements of the *A. ipsilon* pheromone sensilla – not present in *Drosophila* at1 sensilla nor in the *Xenopus* oocyte aqueous environment – may also confer responses to heptanal in *A. ipsilon* pheromone ORNs. These could include sensillum shape, cuticle structure or lymph biochemistry, as recently reviewed by Schmidt and Benton, 2020. Alternatively, the response to heptanal in the native system may result from ORN interactions. Such functional interactions between ORNs grouped in the same sensillum have been evidenced earlier in *D. melanogaster* (Su et al., 2012; Zhang et al., 2019; Pannunzi and Nowotny, 2021). However, very careful examination of spike shape and size in hundreds single sensillum recordings on *A. ipsilon* pheromone ORNs revealed a complete recovering of spikes in response to either pheromone or heptanal and a homogenous spontaneous firing in the absence of stimulation. This makes highly improbable the contribution of another ORN type within the pheromone long trichoid sensilla, which are located on antennal branches. In addition, responses of non-pheromonal ORNs to heptanal and other plant volatiles were recorded from another category of olfactory sensilla, short hair sensilla, situated on the antennae stem. Conclusive experiments would come from a genome editing approach in *A. ipsilon* moths, in which AipsOR3 is knocked-out, and testing if the response to heptanal still occurs. Single cell transcriptomics from on *A. ipsilon* (*Z*)7-12:OAc-sensitive ORNs, although challenging, would definitively clarify the second OR identity.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

## Author contributions

PV and RC conducted *Drosophila* single-sensillum recordings and analyzed data. ZL conducted *Xenopus* oocyte TEVC recordings and analyzed data. M-CF performed molecular cloning experiments. MR conceived experiments in odorized backgrounds and supervised single-sensillum recordings. NM and RC carried out bioinformatics and *Drosophila* genetics. EJ-J and NM conceived the study, supervised experiments, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1035252/full#supplementary-material

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