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Expressional and functional interactions of two *Apis cerana cerana* olfactory receptors

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Apis cerana cerana relies on the sensitive olfactory system to perform the foraging activities in the surrounding environment. Olfactory receptors (ORs) are a primary requirement for odorant recognition and coding. However, the molecular recognition of volatile with olfactory receptor in *Apis cerana cerana* is still not clear. Hence, in the present study, we achieved transient transfection and cell surface expression of *Apis cerana cerana* ORs (AcerOr1 and AcerOr2; AcerOr2 is orthologous to the co-receptor) in *Spodoptera frugiperda* Sf9 cells. The results showed that both mRNA and protein levels of AcerOr1 and AcerOr2 were drastically reduced when treated with their respective double stranded (ds) RNA compared to those in the control and double-stranded green fluorescent protein (dsGFP)-treated cells. The response to Ca2+ using 33 volatile odorants indicated that the molecular receptive range of AcerOr2 narrowly responded to N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1, 2, 4- triazol-3-yl) thio) acetamide (VUAA1) whereas AcerOr1 was sensitive to eugenol, lauric acid, ocimene, 1-nonanol, linolenic acid, hexyl acetate, undecanoic acid, 1-octyl alcohol, and nerol, and it revealed distinct changes in the dose-response curve. We discovered ligands that were useful for probing receptor activity during odor stimulation and validated three of them using an electroantennography (EAG) assay. The response increased with the concentration of the odorant. Further, both AcerOr1 and AcerOr2 knockdowns exhibited significantly reduced intracellular Ca2+ levels in response to the corresponding ligands in vitro. Overall, the present study provides insight into the mechanism of olfactory discrimination in *Apis cerana cerana*.

Expressional and functional interactions of two *Apis cerana cerana* olfactory receptors

Short Title: Functions of *Apis cerana cerana* olfactory receptors

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Abstract *Apis cerana cerana* relies on its sensitive olfactory system to perform foraging activities in the surrounding environment. Olfactory receptors (ORs) are a primary requirement for odorant recognition and coding. However, molecular recognition of volatile compounds with olfactory receptor in *Apis cerana cerana* is still not clear. Hence, in the present study, we achieved transient transfection and cell surface expression of *Apis cerana cerana* ORs (AcerOr1 and AcerOr2; AcerOr2 is orthologous to the co-receptor) in *Spodoptera frugiperda* Sf9 cells. The results showed that both mRNA and protein levels of AcerOr1 and AcerOr2 were drastically reduced when treated with their respective double-stranded (ds) RNA compared to those in the control and double-stranded green fluorescent protein (dsGFP)-treated cells. The response to Ca^{2+} using 33 volatile odorants indicated that the molecular receptive range of AcerOr2 narrowly responded to N-(4-ethylphenyl)-2-((4-ethyl-5-(3-pyridinyl)-4H-1, 2, 4- triazol-3-yl) thio) acetamide (VUAA1) whereas AcerOr1 was sensitive to eugenol, lauric acid, ocimene, 1-nonanol, linolenic acid, hexyl acetate, undecanoic acid, 1-octyl alcohol, and nerol, and it revealed distinct changes in the dose-response curve. We discovered ligands that were useful for probing receptor activity during odor stimulation and validated three of them using an electroantennography (EAG) assay. The response increased with concentration of the odorant. Further, both AcerOr1 and AcerOr2 knockdowns exhibited significantly reduced intracellular Ca^{2+} levels in response to the corresponding ligands *in vitro*. Overall, the present study provides insight into the mechanism of olfactory discrimination in *Apis cerana cerana*.

Introduction

The olfactory system of insects performs the complex task of discriminating thousands of

different odorants present at different concentrations. The odorant signal is relayed through a sophisticated olfactory system and is used in processes related to survival and reproduction, food source location, and predator avoidance. Olfaction is mediated by the interplay between volatile chemical odor ligands and a large family of specialized transmembrane G-protein-coupled receptors known as olfactory receptors (ORs). These seven receptors are not related to any other receptor family, and they form heteromers of a neuron-specific OR protein (OrX) and a ubiquitous co-receptor (Orco).

Hundreds of ORs have been found in various insect species, e.g., 49 OR genes were discovered in *Bombyx (Wanner et al., 2007a)*, 57 in *Megacyllene caryae (Mitchell et al., 2012)*, 62 in *Drosophila (Robertson, Warr, & Carlson, 2003)*, 79 in *Anopheles gambiae (Fox et al., 2001; Hill et al., 2002)*, 301 in *Nasonia vitripennis (Robertson, Gaddau, & Wanner, 2010)*, 341 in *Tribolium castaneum (Engsontia et al. 2008)*, more than 400 putative ORs in *Solenopsis invicta (Wurm et al., 2011)*, 119 in *Apis cerana (Park et al., 2015)*, and more than 177 in *Apis mellifera (Robertson & Wanner, 2006; Wanner et al., 2007b)*. However, characterization and/or deorphanization of the majority of novel putative ORs has yet to have been achieved. A quick and reliable method of heterologous expression and functional analysis of ORs is expected to improve our understanding of how insects perceive the environment. Several *in vitro* experiments have been conducted to analyze the function of insect ORs in heterologous systems such as with human embryonic kidney (HEK) cells *(Hamana et al., 2010; Jones et al., 2011; Corcoran et al., 2014; Liu et al., 2016)*, human HeLa cells *(Sato et al., 2010)*, *Xenopus* oocytes *(Sakurai et al., 2004; Wanner et al., 2007b; Mitsuno et al., 2008; Wanner et al., 2010; Nichols,*

Chen, & Luetje, 2011; Leary et al., 2012; Montagne et al., 2012; Liu et al., 2013; Zhang & Löfstedt, 2013; Jiang et al., 2014; Liu et al., 2016; Zhange et al., 2016), cell-free expression systems *(Tegler et al., 2015)*, and *Cercopithecus aethiops* kidney (COS-7) cells (Levasseur et al., 2003) as well as with insect cells such as *Spodoptera frugiperda* Sf9 cells *(Kiely et al., 2007; Smart et al., 2008; Anderson et al., 2009; Jordan et al., 2009; Jordan & Challiss, 2009)*. Unlike other heterologous systems, the main advantages of using insect cells to study insect OR function is that they do not require exogenous factors and that Sf9 cells derived from the moth *S. frugiperda* naturally express the co-receptor protein SfruOrco. Another major advantage of using Sf9 cells is that they provide "native" conditions for the expression and localization of insect olfactory receptors in heterologous cell membranes.

RNAi has been widely used as a tool to study insect physiology. Various studies have been used to inhibit OR co-expression *(Zhang et al., 2007; Zhou et al., 2014; Lin et al., 2015; Franco et al., 2016)* or for complete inhibition of Orco via genome editing *(DeGennaro et al., 2013)*. Previous studies have shown that RNAi regulated gene knockdown through directly feeding dsRNA in *Apis mellifera (Desai et al., 2012; Vélez et al., 2015)*.

In honeybee, functional characterization of ORs has mostly been restricted to floral scent detection. AmOr11, a highly specific OR, binds to the queen pheromone 9-oxo-decenoic acid (9-ODA) *(Wanner et al., 2007b)*. Compared with levels in worker honeybees, AmOr11 is upregulated in drones. Moreover, AmOr11 was expressively downregulated after honeybees were treated with odorants in an olfactory discrimination learning paradigm *(Claudianos et al., 2014)*. These observations suggest that expression of ORs in honeybee might be caste- or task-

dependent. Expression levels of these receptors were significantly different in different tissues of the bees *(Reinhard & Claudianos, 2012)* indicating that their expression might be plastic and correlated with the environment that the worker bees experience.

In the present study, we achieved transient transfection and cell surface expression of *Apis cerana cerana* ORs, namely AcerOr1 and AcerOr2 (AcerOr2 is orthologous to the co-receptor), in Sf9 cells and determined the odorant ligands for these ORs. We screened a panel of 33 odorants and determined the molecular receptive range of AcerOr1 and AcerOr2. We discovered ligands useful for probing receptor activity during odor stimulation and validated these by electroantennography (EAG) assay. Overall, the assessment of the functional properties of AcerOrs improve our understanding of the mechanism of olfactory regulation in *A. cerana cerana*.

Materials and methods

Odors

All odorants used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of the purest grade (> 95% pure). Stock solutions (100 mM) of the odorants were prepared using dimethyl sulfoxide (DMSO) and stored at -20 °C. For each assay, odorant solutions were freshly diluted from the stock solution to the desired concentration in DMSO. Fluo-4-(acetoxymethyl) ester (Fluo-4 AM) (excitation at 494 nm, emission at 516 nm), obtained from Beyotime (Shanghai, China) as a lyophilized powder, was diluted to 1 mM using DMSO and stored at -20 °C. The composition of the calcium assay buffer was as follows: 21 mM KCl, 12 mM NaCl, 18 mM $MgCl_2$, 3 mM $CaCl_2$, 170 mM D-glucose, 1 mM probenecid (Sigma-

97 Aldrich), and 10 mM Piperazine-1,4-bisethanesulfonic acid (PIPES). The pH of the buffer was
98 adjusted to 7.2, and the buffer was filter-sterilized (using a 0.22 µm filter) prior to use.

99 **Vector construction**

100 The pIB-AcerOr1/pIB-AcerOr2 plasmid constructs containing intact open reading frames
101 (ORFs) for the *Apis cerana cerana* ORs, AcerOr1 and AcerOr2, cloned on the multiple cloning
102 site of the pIB/V5-His vector (Invitrogen, Carlsbad, CA, USA) were used to generate the final
103 transformation plasmids by restriction digestion with *Bam*HI and *Eco*RI (NEB, Beverly, MA,
104 USA). The constructs were verified by restriction digestion and subsequent visualization on a 1%
105 agarose gel.

106 **Cell culture and transfection of Sf9 cells**

107 *Spodoptera frugiperda* Sf9 cells (purchased from the Chinese Academy of Sciences) were
108 maintained as an adherent culture in Sf-900 III serum-free medium (SFM; Gibco, Invitrogen,
109 Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sijiqing, Hangzhou, China)
110 and 100 µg mL^{-1} penicillin-streptomycin at a constant temperature of 28 °C in a humidified
111 incubator (Thermo Scientific, Cornelius, OR, USA) in the absence of CO_2 in T-25 tissue culture
112 flasks (Corning Inc., NY, USA). Sf9 cells were grown to approximately 80–90% confluence as
113 observed under a light microscope. Cells were dislodged from the flask by washing with the
114 media contained in the flask. A total of 1×10^6 Sf9 cells were suspended in 2 mL of Sf-900 III
115 SFM in each well of a Nunclone six-well tissue culture plate (Corning Inc., NY, USA).
116 Confluent cells (80–90%) were transiently transfected with 2.0 µg pIB-AcerOr1/pIB-AcerOr2
117 using 8 µL Cellfectin II® reagent (Invitrogen, Carlsbad, CA, USA) in 6-well plates according to

the manufacturer's instructions. The medium containing plasmid DNA and Cellfectin II was removed after incubation of cells with a DNA/Cellfectin II mix for 3–5 h. The cells were washed twice with fresh Sf-900 III SFM and overlaid with 2 mL of fresh SFM. G418 was used to select stable transfected cell lines. After incubation for 48 h to detected the expression, subcellular localization and calcium imaging of ORs.

Western blot and immunofluorescence analysis

Polypeptide antigens (pAb_AcerOr1 and pAb_AcerOr2), designed based on the AcerOr1 and AcerOr2 cDNA sequences for *A. cerana cerana*, were raised against the antigenic peptides AcerOr1 ENTTNYRNIHYKSD (14 aa) and AcerOr2 NARYHQIAVK (10 aa). An antibody made by AbMax (AbMax Biotechnology Co., Ltd. China) was used for western blot analysis and immunostaining to confirm expression of AcerOr1 and AcerOr2. Goat anti-Rabbit IgG, Alexa Fluor® 488/594, and 4',6-diamidino-2-phenylindole (DAPI) (Beyotime) were used to stain AcerOr1/AcerOr2 in transfected Sf9 cells grown on poly-L-lysine-coated coverslips placed in 6-well plates. Thereafter, the medium was removed from wells and the cells were washed with phosphate-buffered saline (PBS), and 1 mL paraformaldehyde (PFA) was added to the wells. Cells were incubated for 30 min. Subsequently, the PFA was removed and the cells were washed with PBS. The cells were then treated with 5% bovine serum albumin (BSA) for 1 h at room temperature to block non-specific binding. The BSA was then removed and pAb (1:2000 dilution) was prepared in 1% BSA and was added. The cells were incubated at 4 °C overnight and then washed with PBS. Thereafter, the secondary antibody (goat anti-Rabbit Alexa Fluor® 488; 1:10000), prepared in 1% BSA, was added and incubated for 2 h at room temperature. Next, cells

were washed with PBS, incubated with 1 mL DAPI (1:10000), and added to each well. Cells were again washed, and the coverslips with the stained cells were removed for analysis using immunofluorescence microscopy. Images were analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

For western blotting, protein was extracted from cells expressing plasmids and transfected with either AcerOr1 or AcerOr2 or co-transfected with AcerOr1 and AcerOr2 using a cell lysis buffer. Total protein was quantified using a BCA Protein Assay Kit (Boster, Wuhan, China), according to the manufacturer's instructions, using BSA as a standard. The extracted proteins (100 μg per sample) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose filter membrane (Boster). Membranes were blocked for 1.5 h at room temperature in 5% skimmed milk (Boster), washed with Tris-buffered saline containing Tween-20 (TBST, pH 8.0), and incubated overnight at 4 °C with rabbit polyclonal anti-AcerOr1, anti-AcerOr2, and mouse-anti-His-tagged [1:1,000 (v/v)] (BioWorld, USA) and mouse anti-β-actin [1:500 (v/v)] (Boster) antibodies. Thereafter, membranes were washed with TBST and incubated with the secondary antibodies, namely horseradish peroxidase-conjugated donkey anti-rabbit IgG [1:5,000 (v/v)] (Boster) and goat anti-mouse [1:2,000 (v/v)] IgG (Boster), respectively, for 2 h at room temperature. Finally, membranes were washed three times with TBST. Bands were detected using Super ECL Plus detection reagent (Boster) and analyzed using Image Lab (Bio-Rad Laboratories, Hercules, CA, USA) and Image J 1.49.

RNA interference and qRT-PCR

To synthesize dsRNA for AcerOr1, the cDNA sequence of a 218 bp fragment of *A. cerana*

cerana AcerOr1 (GenBank accession number JN544932) was amplified by PCR. Primers were designed from the conserved region of AcerOr1 and fused with T7 promoter sequences (underlined) at their 5′-ends. The primer sequences were as follows: F: 5′-TAATACGACTCACTATAGGGCGATACCATTGCCTTATTTGAGC-3′, R: 5′-TAATACGACTCACTATAGGGCGAATCCCGATTATTCCACTTGC-3′. Similarly, a 471 bp fragment of AcerOr2 was amplified using primers: F: 5′-TAATACGACTCACTATAGGGGGACAACACGACTCAGATA-3′, R: 5′-TAATACGACTCACTATAGGGGAGACGGTCACCAATAAAC-3′. Additionally, a 625 bp fragment of the green fluorescent protein (GFP) gene was amplified from a pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) using primers: F: 5′-TAATACGACTCACTATAGGGGGTGCTCAGGTAGTGGTTGTC-3′, R: 5′-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC-3′. Each PCR product was TA-cloned into a pGEM-T easy vector (Promega, Madison, USA). The inserts were PCR-amplified and used as templates for T7-dependent *in vitro* transcription; dsRNAs were synthesized according to the manufacturer's protocol for the T7 RiboMAX Express RNAi System (Promega, Madison, USA). Reaction products were subjected to DNase digestion followed by phenol extraction and ethanol precipitation. The dsRNA product was dissolved in an appropriate amount of nuclease-free water to obtain a concentration of 5 μg μL^{-1}. Purified dsRNAs were quantified by spectroscopy and examined by electrophoresis on a 1% agarose gel to ensure their integrity.

To investigate the efficiency of each dsRNA in knocking down the expression of its target

gene (AcerOr1 or AcerOr2), Sf9 cells transfected with the plasmids were seeded at a density of 5 × 10^5 cells per well in 24-well plates in an Sf-900 III SFM complete medium (without fetal bovine serum and penicillin-streptomycin). After 48 h of incubation at 28 °C, cells were transfected with 10 ng, 50 ng, 100 ng, 500 ng, 1 μg, and 5 μg of dsRNA using Cellfectin II® reagent (Invitrogen, Carlsbad, CA, USA). Transfection with GFP was used as negative controls, and we directly added 5 μg dsGFP.

Total RNA was extracted from the cells after 48 h of RNA interference using the standard TRIzol method according to the manufacturer's instructions (TaKaRa, Japan). The process of RNA reverse transcription was accomplished using a PrimeScript RT Reagent Kit (TaKaRa, Japan). For quantifying transcripts of the corresponding genes, quantitative real-time PCR (qRT-PCR) was performed on a LightCycler® 480 (Roche, Switzerland) using SYBR® Green Premix Ex Taq (Takara, Japan). mRNA expression levels of the target genes were normalized to that of β-actin in the same sample, and the cycle threshold (Ct) values were collected and normalized to that of the housekeeping gene β-actin. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA levels of each target gene. All RT reactions, including those for the β-actin controls, were performed in triplicate. The primers used for AcerOr1 were 5′-ATCTTCTTCGCATTCCACG-3′ and 5′-ATGAAAGTGATTGCCGCTC-3′; those used for AcerOr2 were 5′-GTGTTGTTCTGCTCCTGGCT-3′ and 5′-GGAAGGTGGTCGTGAAGTCG-3′; and those used to amplify β-actin were 5′-TTCCCGTCCATCGTAGGT-3′ and 5′-GTTGGTGATGATACCGTGC-3′. Reactions were performed on a 7,500 real-time PCR system (ABI, Foster City, CA) using SYBR® Select Master Mix.

202 **Ca^{2+} imaging**

203 To identify candidate ligands, we tested 33 compounds (most of which were volatile
204 compounds from host plants, including aldehydes, alcohols, monoterpenes, benzoates, and
205 sesquiterpenes) (at a final concentration of 10^{-6} M) by Ca^{2+} imaging. Thereafter, we determined
206 dose-response curves for ten compounds (selected from the 33 compounds) and calculated their
207 half-maximal effective concentration (EC_{50}) values. Approximately 48 h after transfection with
208 plasmids containing the ORFs of the OR genes, the medium was removed and the cells were
209 washed three times with Hank's Balanced Salt Solution (HBSS) (without Ca^{2+}). The cells were
210 subsequently cultured at 37 °C in the dark for 30 min in the presence of 2 μmol L^{-1} Fluo-4-AM
211 (Beyotime, Shanghai, China) and were stimulated by the chemical odorants. Each test chemical
212 ligand was exposed to Fluo-4 loaded Sf9 cells expressing AcerOr1 at a final concentration of
213 10^{-6} M, and the increase in fluorescence caused by the substrate was measured and expressed as
214 a fraction of the fluorescence elicited by the calcium ionophore, ionomycin. The Ca^{2+}-free
215 solution used was Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.4 mM
216 ethylene glycol tetraacetic acid (EGTA). Fluorescence was measured using excitation and
217 emission wavelengths of 494 and 516 nm, respectively, and the results were recorded by a
218 Synergy H1 microplate reader (BioTek, Winooski, VT, USA). The formula used for calculating
219 the free intracellular Ca^{2+} concentration was as follows: $[Ca^{2+}]i = K_d(\frac{F - F_{\min}}{F_{\max} - F})$, where F_{min} and
220 F_{max} are the minimum fluorescence values under Ca^{2+}-saturating conditions in the presence of 5
221 μM A23187 (a Ca^{2+}-ionophore) and the maximum fluorescence values under zero-Ca^{2+}

conditions when 4 mM EGTA was used in combination with 5 μM A23187, respectively. K_d is the dissociation constant of Fluo-4/Ca^{2+} (360 nM).

Electroantennography (EAG)

Based on the results of the Ca^{2+} assay, three volatile compounds (VUAA1, eugenol, and linolenic acid) were used to record antennal responses. Compounds were dissolved and diluted in liquid paraffin to final concentrations of 0.1, 1, 10, 100, and 500 μg μL^{-1}. Pure liquid paraffin wax was used as a blank, and results were calculated relative to the blank. Antennae were carefully cut at the base and were placed into EAG electrode probes (Syntech, Hilversum, the Netherlands) with a drop of Spectra 360 electrode gel (Parker Lab, Inc. Fairfield, NJ, USA). Filter paper strips (5 mm × 50 mm) were loaded with 20 μL of the different test solutions and inserted into glass Pasteur pipettes and served as sources of stimuli. Humidified airflow was delivered at a constant rate of 700 mL min^{-1} by an air stimulus controller CS-55 (Syntech, Kirchzarten, Germany). Odor stimuli were administered three times at 2 mL s^{-1} for 0.5 s at 30 s intervals. EAG recordings of antennal responses to each stimulus were documented as voltage waveforms using an IDAC-4 computer-operated amplifier controller (Syntech), and the data were analyzed with EAGPro software (Syntech). A newly prepared antenna was used for each recording. A dose-response curve was plotted using the EAG recordings (in mV) for each concentration.

Data analysis and statistics

Data were analyzed with SPSS v17.0 (SPSS Inc., Chicago, IL, USA) and expressed as means ± standard error (SEM). *t*-tests, ANOVAs, and Duncan's multiple range tests were used to

243 determine whether differences in mRNA and protein levels or EAG responses of antennae were
244 significantly different among treatments. In all the cases, statistical significance was tested at the
245 0.05 level.

246 **Results**

247 **Heterologous expression and localization of AcerOr1 and AcerOr2 in Sf9 cells**

248 We successfully constructed the pIB-AcerOr1 and pIB-AcerOr2 plasmid vectors of the
249 expected size (Fig. 1A). Western blotting of Sf9 cell extracts using an anti-AcerOr1 or anti-
250 AcerOr2 antibody revealed a specific band of approximately 52 kDa in Sf9 cells transfected with
251 pIB/V5-AcerOr1 or pIB/V5-AcerOr2, but no specific band was detected in Sf9 cells (negative
252 control) or pIB/V5-His-transfected Sf9 cells (Fig. 1B). Staining of Sf9 cells using anti-AcerOR1
253 or anti-AcerOR2 followed by goat anti-Rabbit Alexa Fluor® 488 or 594, showed that both
254 AcerOR1 and AcerOR2 were expressed and located in the plasma membrane of Sf9 cells (Fig. 2).
255 These results confirmed successful construction of the recombinant plasmids and expression of
256 the corresponding OR in Sf9 cells after *in vitro* transfection.

257 **RNAi knockdown of heterologous expression of AcerOr1 and AcerOr2 in Sf9 cells**

258 Expression of the AcerOr1 and AcerOr2 mRNAs and proteins was drastically reduced in
259 Sf9 cells treated with different concentrations of dsRNA as compared with that in control cells
260 (transfected with 5 μg empty vector) or cells treated with 5 μg dsGFP. (Fig. 3 and 4). When cells
261 were transfected with AcerOr1 and AcerOr2 alone, the addition of 500 ng, 1 μg, and 5 μg
262 dsRNA was associated with significantly reduced target gene expression in Sf9 cells (Fig. 3A
263 and B). In cells co-expressing AcerOr1 and AcerOr2, when one of the genes was knocked down,

the other was not completely knocked down and that this result was greatest when 500 ng, 1 μg, or 5 μg dsRNA were used (Fig. 3C and D). However, co-expression of AcerOr1 and AcerOr2 knocked out both genes simultaneously, and the greatest knockdown effect was observed when 500 ng dsRNA was applied to AcerOr1, while the expression of AcerOr2 was lowest at 5 μg dsRNA (Fig. 3E and F).

As above we can see 500 ng dsRNA can significantly reduced target mRNA expression in Sf9 cells. So, we selected this concentration to detected the protein expression. When transfected with AcerOr1 and AcerOr2 alone, dsRNA significantly reduced protein expression levels (Fig. 4A and B). In cells co-expressing AcerOr1 and AcerOr2, knockout of AcerOr2 with only the corresponding dsRNA had no obvious change, but when AcerOr1 and AcerOr2 were knocked out at the same time, AcerOr1 was significantly reduced; While only knockout of AcerOr1 or both AcerOr1 and AcerOr2 at the same time both can significantly reduced AcerOr2 protein expression (Fig. 4C and D). These results show that the two receptors were indeed knocked down either at the mRNA or protein level.

Identification of odorants activating AcerOr1 and AcerOr2 in Sf9 cells by Ca^{2+} imaging using Fluo-4 AM

Nine of the thirty-three compounds, including eugenol, lauric acid, ocimene, 1-nonanol, linolenic acid, hexyl acetate, undecanoic acid, 1-octyl alcohol, and nerol, elicited responses from AcerOr1-expressing cells when administered at the high concentration of 10^{-6} M. Cells expressing AcerOr2 were the most sensitive to VUAA1, whereas those co-expressing AcerOr1 and AcerOr2 or AcerOr2 alone were sensitive to all nine volatile compounds mentioned above

and VUAA1. Expression levels increased by 10–15% as compared with those in cells expressing AcerOr1 alone (Fig. 5).

We then determined the dose-response curves for the ten abovementioned compounds and calculated half-maximal effective concentration (EC_{50}) values for them. AcerOr1 was sensitive to the volatile constituents of plant volatile linolenic acid [EC_{50} = 8.125 × 10^{-8}]. Cells co-expressing AcerOr1 and AcerOr2 responded to low concentrations of ocimene [EC_{50} = 6.088 × 10^{-8}]. Cells expressing AcerOr2 displayed relatively high sensitivity to VUAA1 [EC_{50} = 6.6 × 10^{-8}] (Fig. 6, Table 1).

In the cells expressing AcerOr1 that were treated with AcerOr1 dsRNA, the average reduction in Ca^{2+} concentration was 1.76, 2.6, 1.76, 2.0, 2.35, 1.4, 2.4, 2.2, and 1.9 times for eugenol, lauric acid, ocimene, 1-nonanol, linolenic acid, hexyl acetate, undecanoic acid, 1-octyl alcohol, and nerol, respectively, as compared with that in the control (Fig. 7 A). In the cells expressing AcerOr2 that were treated with AcerOr2 dsRNA, Ca^{2+} concentration was reduced by approximately three times that of the control group (Fig. 7 C). The concentration of Ca^{2+} in cells co-expressing AcerOr1 and AcerOr2 was reduced by 1.85 times after treatment with dsAcerOr1, although the response to VUAA1 did not change owing to VUAA1-insensitivity (Fig. 7 B). In addition, when treated with dsAcerOr1 and dsAcerOr2 simultaneously, Ca^{2+} was reduced six times that of the control (Fig. 7 B). The residual response likely indicated an incomplete knockdown of the mRNAs.

Electrophysiological response of *Apis cerana cerana* antennae

The three floral volatiles (VUAA1, eugenol, and linolenic acid) caused irritation and

elicited EAG responses (Fig. 8). All three compounds showed a dosage-dependent increase in EAG response, and the most dramatic effect was observed at 500 μg μL^{-1} of compound. These results were consistent with those for the Ca^{2+} imaging.

Discussion

In the present study, we reported the role of AcerOr1 and AcerOr2 (AcerOr2 is an Orco orthologue) of *A. cerana cerana* in olfactory functions when heterologously expressed in Sf9 cells. Orco can form stabilized complexes with other ligand-binding ORs to form a nonselective cation channel and perform important roles in transporting and localizing them to dendritic membranes *(Benton et al., 2006; Sato et al. 2008; Wicher et al., 2008)*. Conventional ORs have mainly been used to detect odorants. The difference among Orco and conventional ORs imply that they affect olfactory functions in different ways. Indeed, we showed that AcerOr1 could respond to odor stimulation in Sf9 cells alone or in the presence of AcerOr2. However, co-expression of AcerOr1 and AcerOr2 produced responses that were significantly different compared with the expression of AcerOr1 alone. AcerOr2 did not respond to odorants other than VUAA1 when expressed independently in Sf9 heterologous systems. In addition, we found that AcerOr1 was expressed in Sf9 cells alone and did not required AcerOr2, suggesting that endogenous AcerOr2-type function factors were present in Sf9 cells and that AcerOr1 could function in heterologous Sf9 cells in the absence of AcerOr2. These results are consistent with previous research that has found that in the absence of Orco, ORs can also be expressed in heterologous cells *(Fox et al., 2001; Hill et al., 2002; Robertson, Warr & Carlson, 2003)*. These results provide further evidence supporting the hypothesis that OrX and Orco or Orco and Orco

form a heteromeric complex, which might act as an odorant-gated cation channel with ionic permeability mostly for Ca^{2+}. This scenario might be caused by stimulation by odorants and transmission of odor signals. Moreover, AcerOr1 responded with different sensitivity to each odor.

There have been previous studies done on the function of ORs in *Bombyx mori* (BmorORs), *Drosophila melanogaster* (DmelORs), and *A. gambiae* (AgamORs) and the respective Orcos that were heterologously expressed using the voltage clamp technique as well as Ca^{2+} imaging *(Benton et al., 2006; Smart et al., 2008; Wicher et al., 2008)*. Even in the absence of odorant induction, the receptor complexes, as well as Orco alone, mediated the activation of intracellular Ca^{2+} influx reminiscent of receptor-dependent spontaneous activity of insect olfactory receptor neurons *(deBruyne and Carlson, 2001; Dobritsa et al., 2003; Hallem, Ho & Carlson, 2004; Hallem, Dahaunkar & Carlson, 2006; Ignatious et al., 2014)*. The functional divergence between conventional OrX and Orco might be correlated with different behaviors. Host plant-seeking behavior may not rely on individual conventional ORs, but depend on the cumulative effects of multiple ORs.

To test the potential functional activity of AcerOr during the olfaction process, odorant ligand binding is essential. A set of 33 compounds *(Dobritsa et al., 2003; Hallem, Ho & Carlson, 2004; Hallem, Dahanukar & Carlson, 2006; Caludianos et al., 2014)* were used for this study. Our results showed that AcerOr1 and AcerOr2 responded to odor stimulation in the Sf9 cells. We tested a range of odorants and found nine AcerOr1-sensitive ligands. AcerOr2 was activated only by VUAA1, which stimulated a response from AcerOr1-sensitive ligands when AcerOr1 and

AcerOr2 were co-expressed. This result confirmed that VUAA1 was an AcerOr2 agonist and that AcerOr1-AcerOr2 heteromers could form ligand-gated ion channels. The activation of Orco at different concentrations of VUAA1 does not change in different species, such as in *Drosophila melanogaster*, *Anopheles gambiae*, or *Culex quinquefasciatus (Elmore et al., 2003; Neuhaus et al., 2005; Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008; Jones, Rinker & Zwiebel, 2011; Pask et al., 2011; Chen & Luetje, 2012)*. VUAA1 binds to Orco directly increasing its ion-channel-opening probability across species *(Elmore et al., 2003; Jones, Rinker & Zwiebel, 2011; Pask et al., 2011; Bohbot & Dickens, 2012; Chen & Luetje, 2012)*.

We found that AcerOr2 did not respond to any of the tested odorants except VUAA1, which is the activator of insect Orco. The result was consistent with that of a previous study on *D. melanogaster* DOR83b, which was found not to respond to any of a large panel of odorants; however, the conventional OR itself had ligand-binding properties.Or151 and Or15 have been shown to respond to 14 common floral odorants, and the best ligand for Or151 is linalool *(Reinhard et al., 2010)*, and another, the highly specific queen pheromone receptor Or11, can specifically bind to 9-ODA *(Wanner et al., 2007b)*. In the present study, AcerOr1 responded to nine common floral odorants. Thus, the functional divergence between conventional ORs might be correlated with different behaviors.

A previous study found that *Orco* in insects that were silenced failed to identify a host, and this effect could reduce contact between insects and vertebrates *(Zhang et al., 2016)*. Using dsRNA-treated *TcOr1* (*Orco*) has been shown to reduce the response to aggregation pheromone in the beetle *Tribolium castaneum* suggesting that *TcOr1* (*Orco*) plays a crucial role in olfactory

activity *(Engsontia et al., 2008)*. In mosquitos, *Orco*-knockdown was found to cause severely reduced behavioral attraction to sugar, and mosquitos in this condition did not respond to human odors in the absence of CO_2 *(DeGennaro et al., 2013)*. A similar disrupted behavior phenomenon has been reported in the coleopteran *Phyllotreta striolata* in which *Orco*-knockdown alters preference and attraction to its host-plant and other cruciferous vegetables *(Zhao et al., 2011)*. Host preference is affected significantly in AalOrco siRNA-injected mosquitoes and Orco mutant mosquitoes *(DeGennaro et al., 2013; Liu et al., 2016)*. In *M. mediator*, *MmedOrco* plays a crucial role in nonanal and farnesene perception *(Li et al., 2012)*, and LdisOrCo-knockdown can reduce the response to sex pheromones of male gypsy moth, *Lymantria dispar (Lin et al., 2015)*. Tmol/Orco silencing can significantly alter the ability to recognize mates in *Tenebrio molitor (Liu et al., 2016)*. To the best of our knowledge, there are no reports of a positive correlation between dsRNA-induced suppression of target genes with the level of mRNA and protein transfected *in vitro* and the best effective silencing dose *in vitro*. The present study showed that AcerOrs expressed in Sf9 cells can be successfully manipulated by RNAi silencing at both the mRNA and protein levels at a suitable concentration and that the expression pattern of mRNA was consistent with protein expression indicating that post-transcriptional processes play a critical role in regulating the protein level during infection. In a stable state, the mRNA level determines the level of protein. Moreover, we found that intracellular calcium levels decreased significantly upon stimulation with odorants after RNAi. When applied to the functional study, this approach should facilitate a significantly improved understanding of AcerOrs in olfaction processes and their regulation at the molecular level *in vitro*.

It has been found that co-expression of AgOrco and AgOR10 genes from *A. gambiae* in HEK293 result in an obvious sensitivity to UVAA1 as compared to that when AgOrco is expressed alone *(Jones, Rinker & Zwiebel, 2011)*. This interesting phenomenon has also been observed in *Culex pipiens pallens* and D. *melanogaster (Chen & Luetje, 2012)*. In the present study, we found consistent results; co-expression of AcerOr1 and AcerOr2 in Sf9 cells made the cells more sensitive to odorants than those expressing AcerOr2 alone. Nevertheless, we found that odorant responses were enhanced when AcerOr1 was co-expressed with AcerOr2, consistent with previous studies *(Neuhaus et al., 2005; Smart et al., 2008),* and might support the hypothesis that when ORs are successfully inserted into the plasma membrane, Orco can form a dimer with OrX and support the function of both for odorant recognition and detection. Changes in the intracellular calcium ion concentration affects the electrical potential inside and outside the cell membrane, and the change in the membrane voltage is signaled via the olfactory receptor neurons to the central nervous system of the insect, which in turn, affects their behavior. The antennae of insects are very sensitive; they have thousands of olfactory receptor neurons that can discriminate minute quantities of odorants within complex chemical compounds in the environment. Scientists have started extracting signals from the antennae of insects using electrodes. Herein, the EAG detection system was used to detect the differential response of honeybees to different stimuli for discrimination and recognition of different odors. Additionally, the sensitivity and selectivity of the insect olfactory signal transduction and recognition processes depended upon interaction with a variety of olfactory-specific proteins, namely, odorant-binding proteins, odorant-enzymes, odorant receptors, and olfactory neuron receptor

membranes to regulate the signal transduction pathway for odor recognition.

Conclution

In summary, we identified the expression and function of the odorant receptors AcerOr1 and AcerOr2 in *A. cerana cerana*. The results improved our understanding of AcerOrs in the olfaction processes and their regulation at the molecular level *in vitro*. Further studies are needed on the molecular mechanisms of AcerOr1 and AcerOr2 regulation of the signal transduction pathway for odor recognition *in vivo* and further study is needed on delivery of this signal to native neurons that regulate behavior (e.g., foraging).

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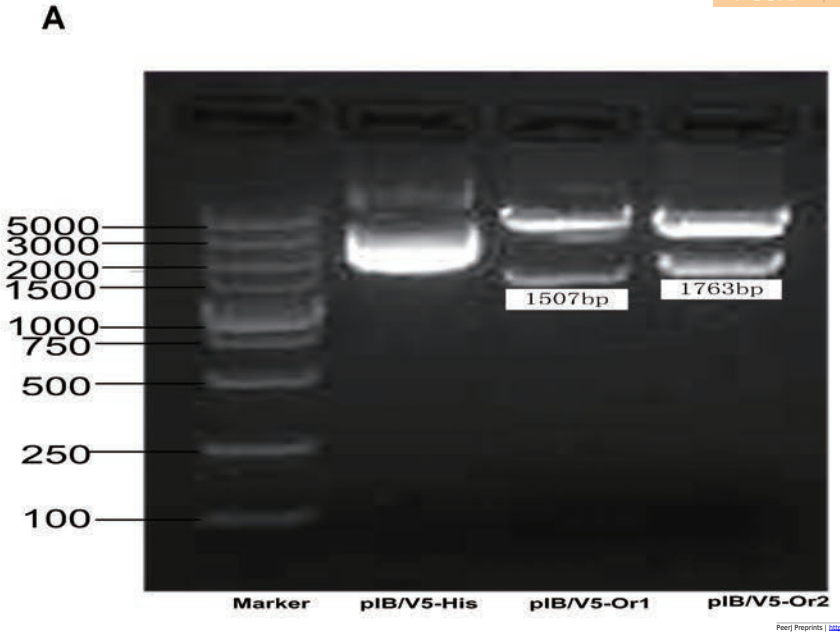
610

Figure 1(on next page)

Detection of recombinant vector and the effects of transfection.

(A) The plasmids pIB-AcerOr1 and pIB-AcerOr2 were detected by 1% agarose gel electrophoresis and verified by restriction enzyme digestion. (B) Recombinant His-tagged AcerOr1 and AcerOr2 vector expression levels were detected by western blotting in non-transfected and transfected AcerOr1 and AcerOr2 and co-transfected Sf9 cells.



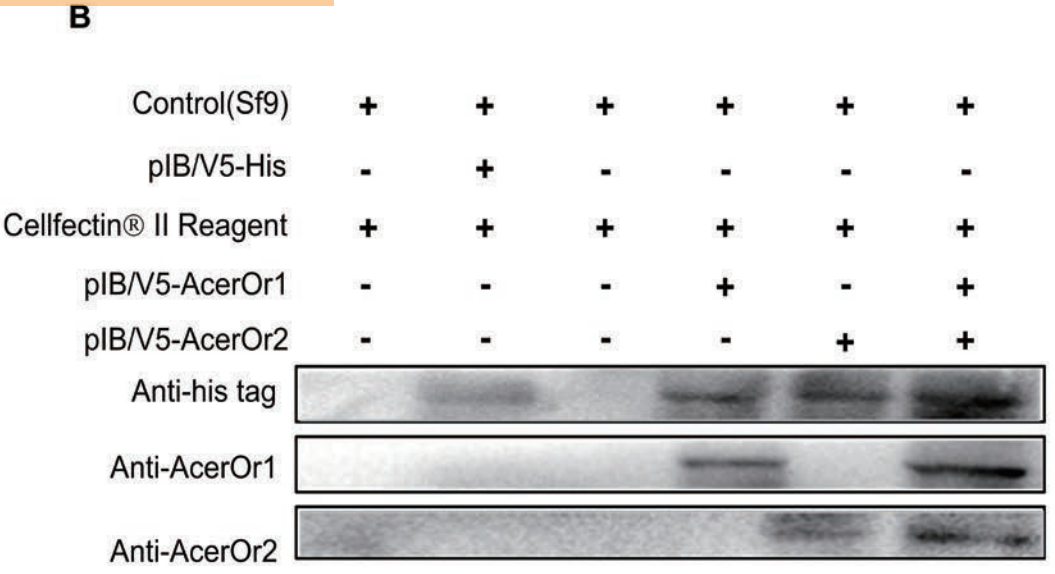


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Figure 2(on next page)

Subcellular localization of AcerOr1 and AcerOr2 expressed in Sf9 cells.

(A) Cells transfected with pIB/V5-His as a control DNA construct. (B) Alexa 488 (Green) staining of cells expressing AcerOr1. (C) Alexa 594 (red) staining of cells expressing AcerOr2. Nuclei were stained with DAPI (blue). Scale bar = 50 µm.

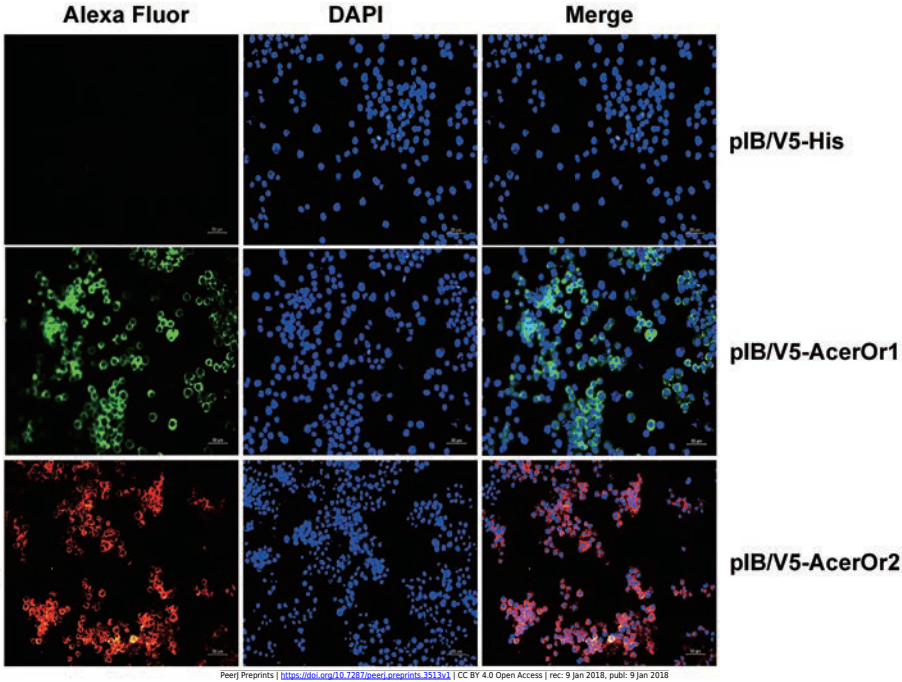

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Figure 3(on next page)

Effect of ds-AcerOr on expression of the *AcerOr* gene in Sf9 cells.

Expression of the target *AcerOr* gene was determined by qRT-PCR after a 48 h dsRNA treatment at different concentrations (10 ng, 50 ng, 100 ng, 500 ng, 1 µg, and 5 µg) . Cells not treated with ds*AcerOr* (pIB-V5-His, 5µg) or dsGFP (5µg) were the control, and relative expression levels were determined with respect to these controls. (A–B) Expression of *AcerOr1* cells treated with different concentrations of dsRNA for *AcerOr1* and expression of *AcerOr2* cells treated with different concentrations of dsRNA AcerOr2 relative to that in the control. (C–D) Co-expression of AcerOr1 and AcerOr 2 in cells treated with different concentrations of dsRNA of AcerOr1 and AcerOr2, respectively. (E–F) Co-expression of *AcerOr1* and *AcerOr2* in cells treated simultaneously with different concentrations of dsRNA for AcerOr1 or AcerOr2 as compared with that in the control. ** $P < 0.01$, * $P < 0.05$. Error bars represent the standard error of the mean (SEM) (n = 9).

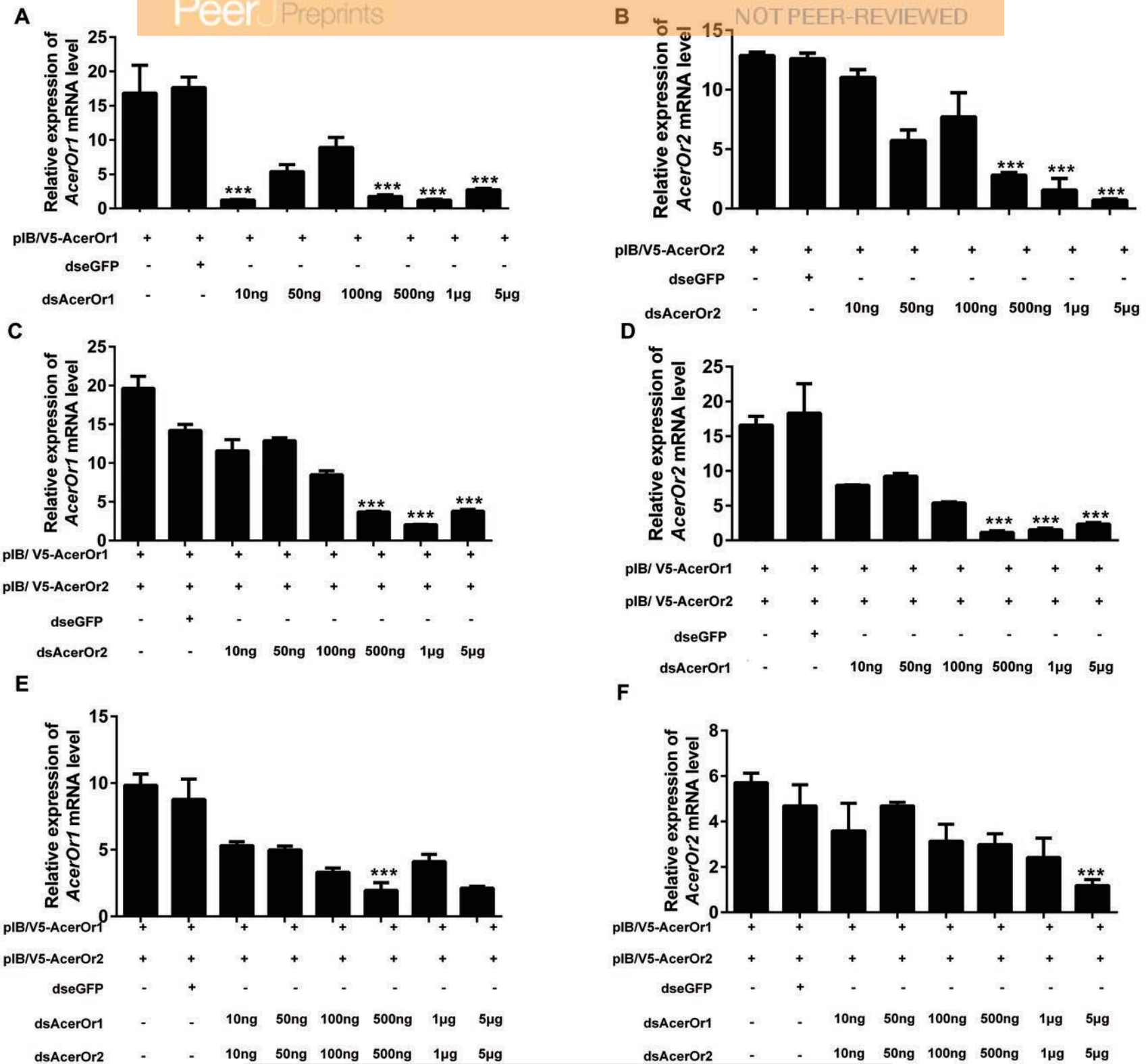
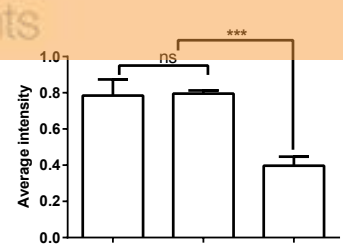


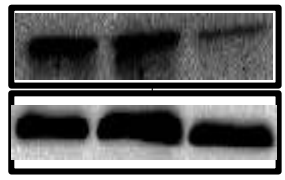
Figure 4(on next page)

Effect of dsAcerOr on AcerOr protein expression in Sf9 cells.

Expression of the AcerOr protein was determined by western blot after a 48 h treatment with 500 ng dsRNA. Cells not treated with ds*AcerOr* (pIB-V5-His, 500 ng) or dsGFP (500 ng) were the control, and relative expression levels were determined with respect to these controls. (A–B) Expression of AcerOr1 cells and AcerOr2 cells treated with 500ng dsRNA relative to that in the control. (C–D) Expression of AcerOrs in co-expression of AcerOr1 and AcerOr 2 in cells treated with one or two of AcerOr1 and AcerOr2 500ng dsRNA as compared with that in the control. ** $P < 0.01$, * $P < 0.05$. Error bars represent standard error of the mean (SEM) based on nine biological replicates.

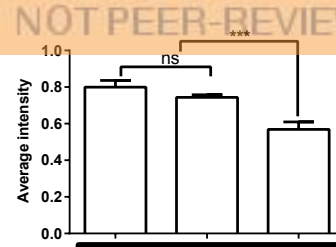
A

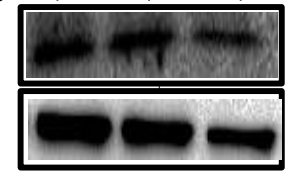




pIB/V5-AcerOr1	+	+	+
dseGFP	-	+	-
dsAcerO1	-	-	+

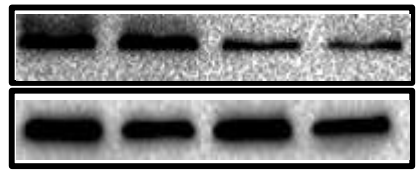
B



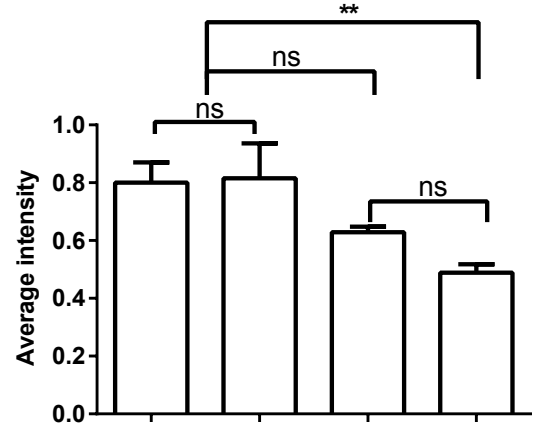


pIB/V5-AcerOr2	+	+	+
dseGFP	-	+	-
dsAcerOr2	-	-	+

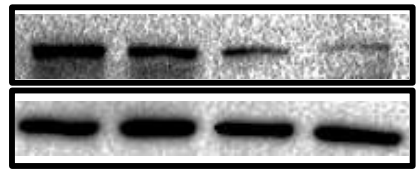
C



pIB/V5-AcerOr1+AcrOr2	+	+	+	+
dseGFP	-	+	-	-
dsAcerOr1	-	-	-	+
dsAcerOr2	-	-	+	+



D



pIB/V5-AcerOr1+AcrOr2	+	+	+	+
dseGFP	-	+	-	-
dsAcerOr1	-	-	+	+
dsAcerOr2	-	-	-	+

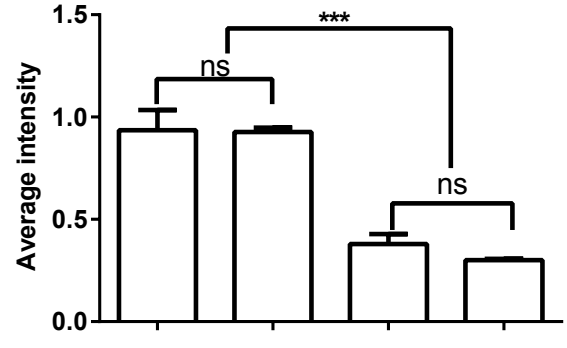


Figure 5(on next page)

Response profile of Fluo-4-loaded Sf9 cells transfected with pIB-AcerOr1 and pIB-AcerOr2 to a various odorants (10^{-6} M) using calcium imaging.

(A) Cells expressing pIB-AcerOr1 or (B) pIB-AcerOr2 or (C) co-expressing pIB-AcerOr1and pIB-AcerOr2 were stimulated by different odorants as indicated. For each Sf9 cell, the value of $[Ca^{2+}]i$ (the concentration of Ca^{2+}) was calculated. This value represented the maximum increase in $[Ca^{2+}]i$ obtained for an odorant minus the $[Ca^{2+}]i$ in the resting state. Bars indicate standard deviation based on three independent experiments. Data points represent means ± SEM.

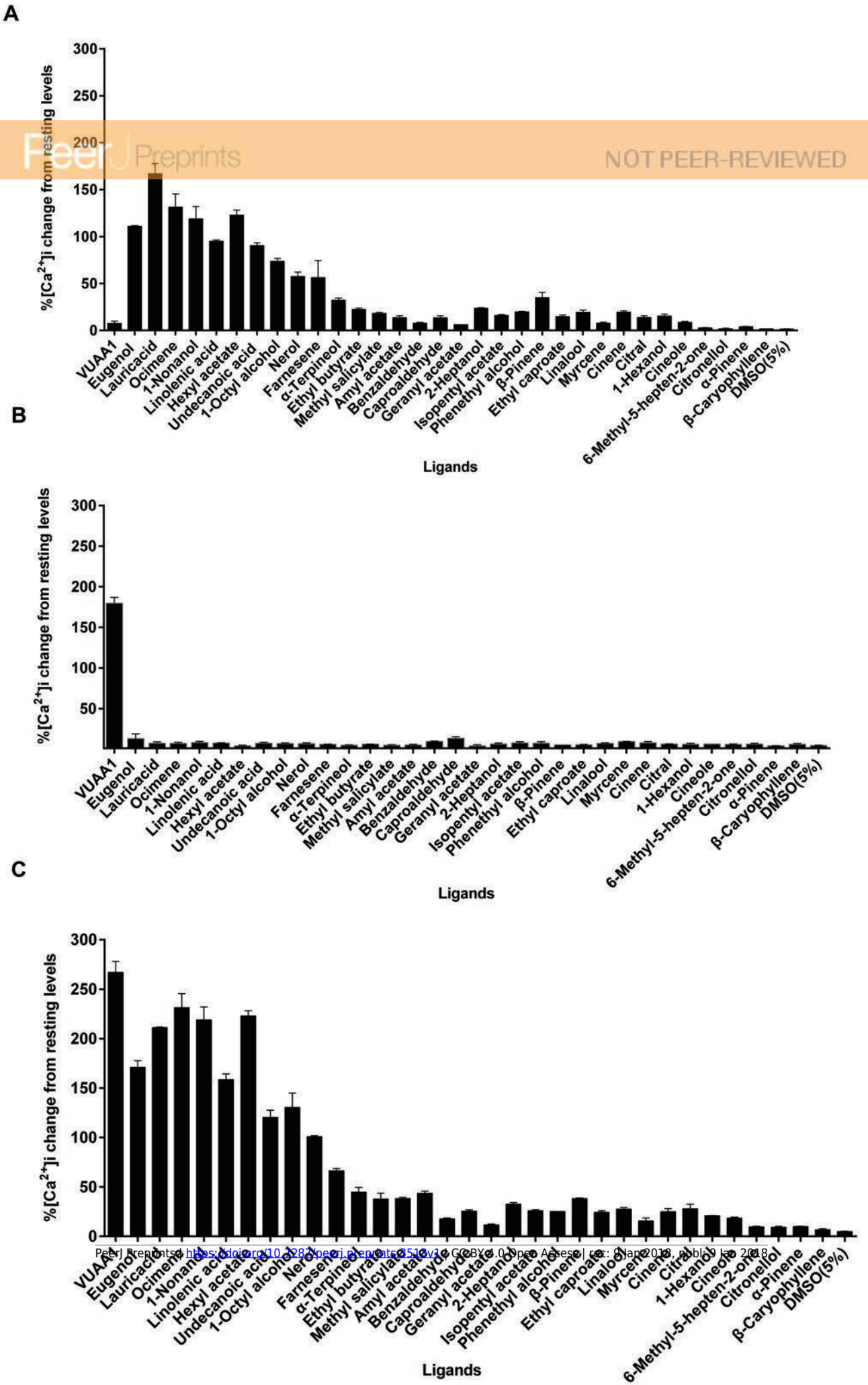
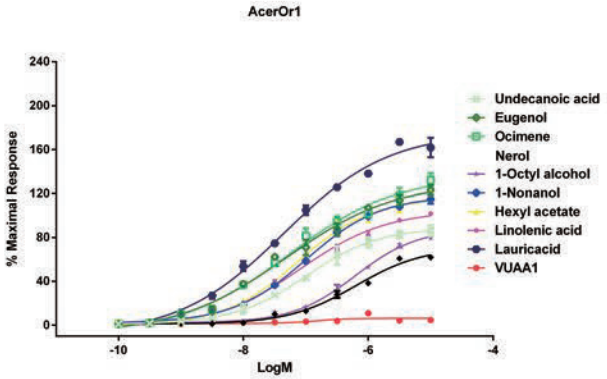


Figure 6(on next page)

Concentration responses curves for AcerOr1 and AcerOr2.

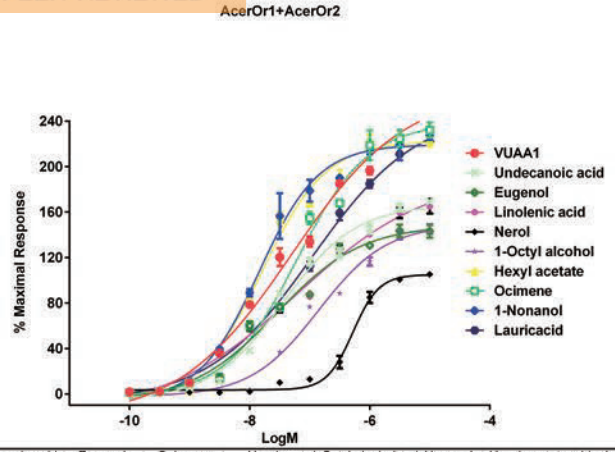
(A) Concentration-response curve of AcerOr1 for nine compounds and that of (B) AcerOr2 with VUAA1 and (C) AcerOr1 + AcerOr2 based on Ca^{2+}-imaging assays. Bars indicate the standard deviation based on three independent experiments. Data points represent means ± SEM .

A



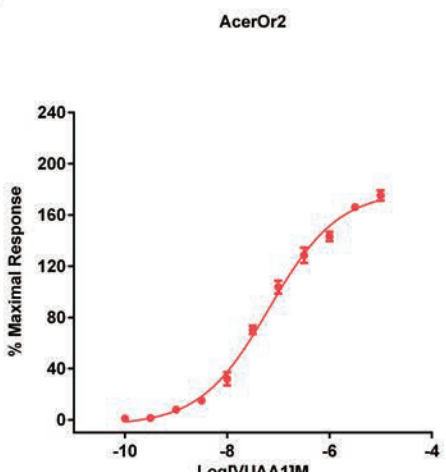
	VUAA1	Undecanoic acid	Eugenol	Ocimene	Nerol	1-Octyl alcohol	1-Nonanol	Hexyl acetate	Linolenic acid	Lauricacid
EC50	1.513e-007	1.020e-007	4.811e-008	5.322e-008	6.327e-007	6.175e-007	1.008e-007	7.357e-008	8.125e-008	4.337e-008

B



	VUAA1	Undecanoic acid	Eugenol	Ocimene	Nerol	1-Octyl alcohol	1-Nonanol	Hexyl acetate	Linolenic acid
EC50	4.975e-008	3.587e-008	2.772e-008	6.088e-008	5.244e-007	1.395e-007	1.407e-008	1.587e-008	5.972e-008

C

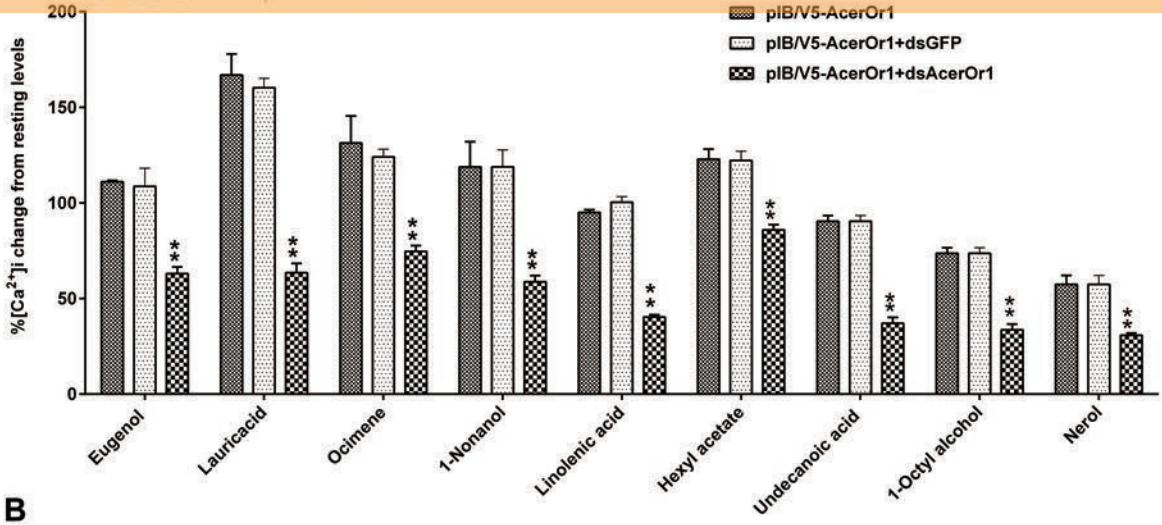


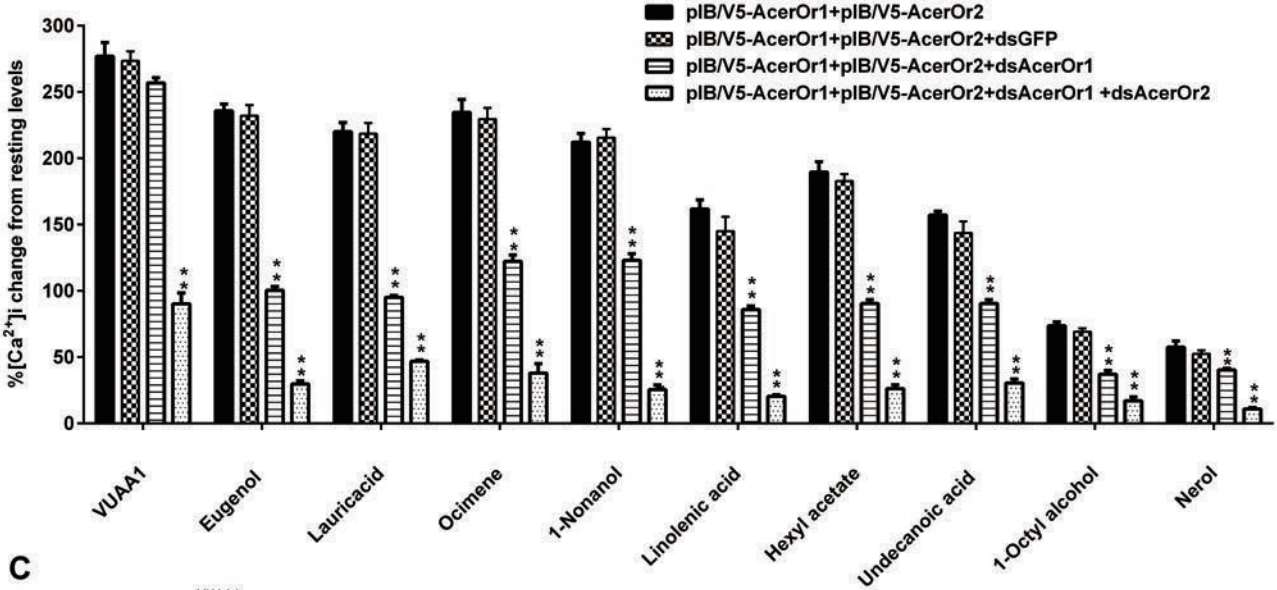
	VUAA1
EC50	6.621e-008

Figure 7 (on next page)

Quantification of intracellular calcium ([Ca^{2+}]i)-reduced AcerOr1 and AcerOr2 mRNA abundance in Sf9 cells expressing AcerOr1 or AcerOr2 or coexpressing AcerOr1 and AcerOr2 stimulated by odorants.

(A) Ca^{2+} assay recording of the responses of cells expressing AcerOr1 or that have experienced knockdown of AcerOr1 by dsRNA in Sf9 cells. (B) Cells co-expressing AcerOr1 and AcerOr2 or with a knockdown of AcerOr1 alone or with a simultaneous knockdown of AcerOr1 and AcerOr2 by dsRNA. (C) Cells expressing AcerOr2 and with a knockdown of AcerOr2 by dsRNA were stimulated by VUAA1. The results shown are representative of six separate experiments. Bars represent the means ± SEM based on a one-way ANOVA. ** $P < 0.01$, * $P < 0.05$.


A
pIB/V5-AcerOr1
pIB/V5-AcerOr1+dsGFP
pIB/V5-AcerOr1+dsAcerOr1
%[Ca2+]i change from resting levels
200
150
100
50
0
**
Eugenol
Lauricacid
Ocimene
1-Nonanol
Linolenic acid
Hexyl acetate
Undecanoic acid
1-Octyl alcohol
Nerol


B
pIB/V5-AcerOr1+pIB/V5-AcerOr2
pIB/V5-AcerOr1+pIB/V5-AcerOr2+dsGFP
pIB/V5-AcerOr1+pIB/V5-AcerOr2+dsAcerOr1
pIB/V5-AcerOr1+pIB/V5-AcerOr2+dsAcerOr1 +dsAcerOr2
%[Ca2+]i change from resting levels
300
250
200
150
100
50
0
**
VUAA1
Eugenol
Lauricacid
Ocimene
1-Nonanol
Linolenic acid
Hexyl acetate
Undecanoic acid
1-Octyl alcohol
Nerol

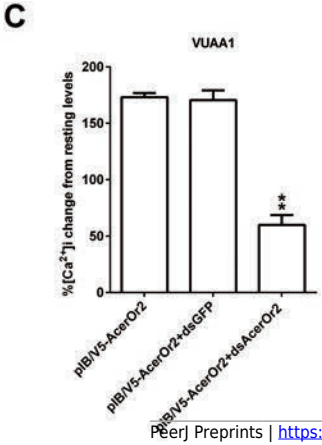
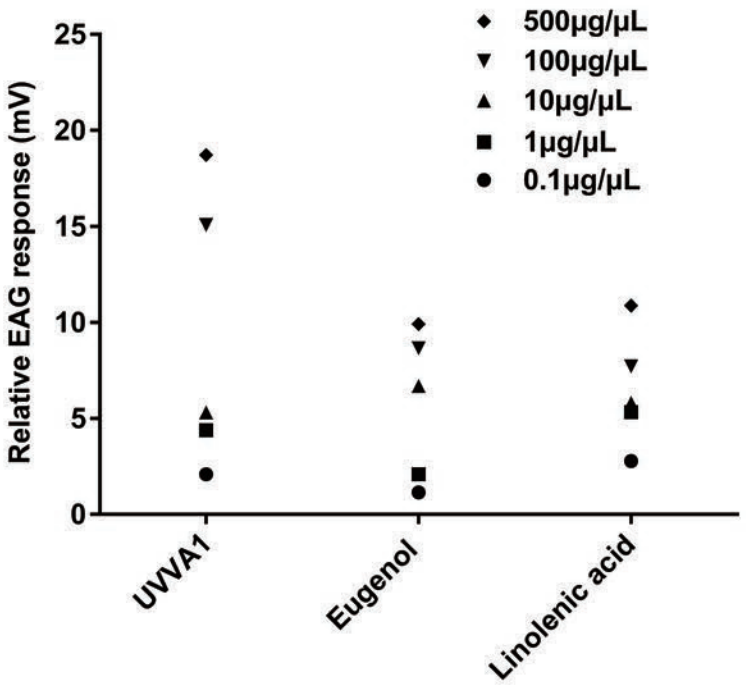
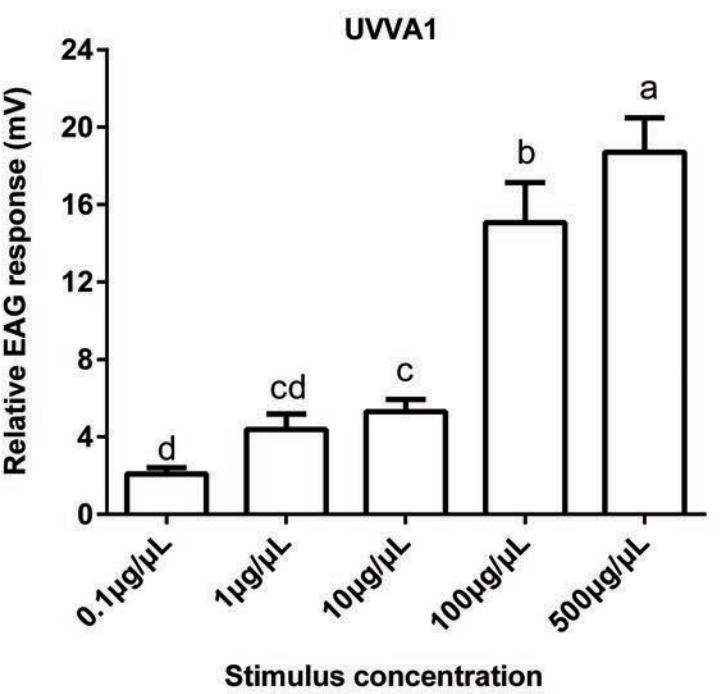

C
VUAA1
%[Ca2+]i change from resting levels
200
150
100
50
0
**
pIB/V5-AcerOr2
pIB/V5-AcerOr2+dsGFP
pIB/V5-AcerOr2+dsAcerOr2

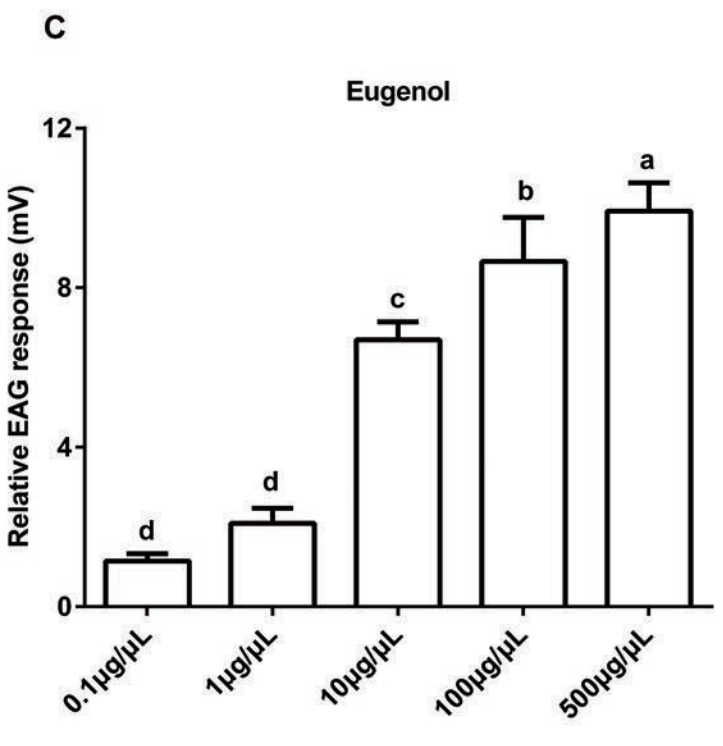
Figure 8(on next page)

Relative electroantennogram (EAG) responses of *Apis cerana cerana* to three volatile odorants at different doses.

Experiments were repeated three times, and EAG recordings from ten antennae per group were obtained. Bars represent the means ± SEM based on a one-way ANOVA with the Duncan's test. Different letters within the same figure indicate significantly different values ($P < 0.05$).







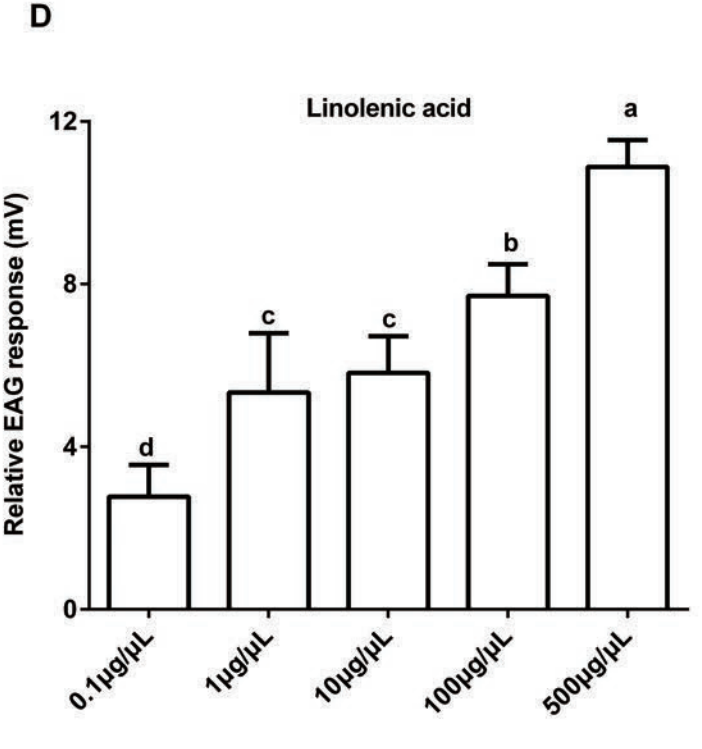


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Table 1(on next page)

EC_{50} values of different odorants for cells expressing AcerOr1 or AcerOr2 or co-expressing AcerOr1 and AcerOr2 (AcerOr1 + AcerOr2).

1

	AcerOr1	AcerOr2	AcerOr1+AcerOr2
	EC50	**EC50**	**EC50**
VUAA1	$(1.513 \pm 1.72) \times 10^{-7}$	$(6.621 \pm 0.64) \times 10^{-8}$	$(4.975 \pm 0.45) \times 10^{-8}$
Eugenol	$(1.02 \pm 0.74) \times 10^{-7}$	NR	$(3.587 \pm 0.72) \times 10^{-8}$
Lauric acid	$(4.811 \pm 0.49) \times 10^{-8}$	NR	$(2.772 \pm 0.61) \times 10^{-8}$
Ocimene	$(5.322 \pm 0.50) \times 10^{-8}$	NR	$(6.088 \pm 0.72) \times 10^{-8}$
1-Nonanol	$(6.327 \pm 0.80) \times 10^{-7}$	NR	$(5.244 \pm 2.0) \times 10^{-7}$
Linolenic acid	$(6.175 \pm 0.86) \times 10^{-7}$	NR	$(1.395 \pm 0.76) \times 10^{-7}$
Hexyl acetate	$(1.008 \pm 0.73) \times 10^{-7}$	NR	$(1.407 \pm 0.86) \times 10^{-8}$
Undecanoic acid	$(7.357 \pm 0.72) \times 10^{-8}$	NR	$(1.587 \pm 0.81) \times 10^{-8}$
1-Octyl alcohol	$(8.125 \pm 0.62) \times 10^{-8}$	NR	$(5.972 \pm 0.39) \times 10^{-8}$
Nerol	$(4.34 \pm 0.52) \times 10^{-8}$	NR	$(1.12 \pm 0.49) \times 10^{-7}$

2 NR = no detectable response.

3

4