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Odorant Receptors from the Light brown Apple Moth (*Epiphyas postvittana*) Recognize Important Volatile Compounds Produced by Plants

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Abstract

Moths recognize a wide range of volatile compounds, which they use to locate mates, food sources, and oviposition sites. These compounds are recognized by odorant receptors (OR) located within the dendritic membrane of sensory neurons that extend into the lymph of sensilla, covering the surface of insect antennae. We have identified 3 genes encoding ORs from the tortricid moth, *Epiphyas postvittana*, a pest of horticulture. Like *Drosophila melanogaster* ORs, they contain 7 transmembrane helices with an intracellular N-terminus, an orientation in the plasma membrane opposite to that of classical GPCRs. EpOR2 is orthologous to the coreceptor Or83b from *D. melanogaster*. EpOR1 and EpOR3 both recognize a range of terpenoids and benzoates produced by plants. Of the compounds tested, EpOR1 shows the best sensitivity to methyl salicylate [$EC_{50} = 1.8 \times 10^{-12}$ M], a common constituent of floral scents and an important signaling compound produced by plants when under attack from insects and pathogens. EpOR3 best recognizes the monoterpene citral to low concentrations [$EC_{50} = 1.1 \times 10^{-13}$ M]. Citral produces the largest amplitude electrophysiological responses in *E. postvittana* antennae and elicits repellent activity against ovipositing female moths. Orthologues of EpOR3 were found across 6 families within the Lepidoptera, suggesting that the ability to recognize citral may underpin an important behavior.

Key words: citral, dose–response, *Epiphyas postvittana*, methyl salicylate, odorant receptor, plant volatile

Introduction

Moths use endogenous chemical signals to communicate both within and between species. They perceive a wide range of exogenous volatile compounds within their environment, including those released by the foliage, flowers, and fruits of plants. Detection of these odors is important for guiding both male and female moths to sources of food and female moths to suitable oviposition sites (Honda 1995; Bruce et al. 2005). Odors are detected by odorant receptors (ORs) located on the dendrites of sensory neurons within specialized sensory hairs (sensilla). The majority of ORs from moths have been isolated from 2 species of macrolepidoptera, *Bombyx mori* (Sakurai et al. 2004; Krieger et al. 2005; Nakagawa et al. 2005; Wanner et al. 2007) and *Heliothis virescens* (Krieger et al. 2002, 2004). Functional characterization of a subset of these ORs has revealed specificity toward components of the female sex pheromone (Sakurai et al. 2004; Krieger et al. 2005; Nakagawa et al. 2005; Große-Wilde et al. 2007, Mitsuno et al. 2008), identifying them as sex pheromone receptors. Electroantennogram (EAG) recordings from the antennae of many species of moth have identified numerous physiologically active compounds produced by plants, especially leaves (Fraser et al. 2003; Das et al. 2007). Furthermore, the specificity of sensory neurons receptive to these compounds, including terpenes, aldehydes, esters, and aliphatics, has been determined using single unit electrophysiology coupled to gas chromatography (reviewed in Bruce et al. 2005; Mustaparta and Stranden

2005). However, to date, the underlying receptors responsible for this reception have yet to be isolated and characterized.

Epiphyas postvittana (Walker) (Lepidoptera: Tortricidae), more commonly known as the light brown apple moth, is a member of the microlepidoptera. Tortricids make up many of the serious lepidopteran horticultural pests of the world, including codling moth (*Cydia pomonella*) and the oriental fruit moth (*Grapholitha molesta*). *Epiphyas postvittana* larvae are highly polyphagous and have been recorded in over 250 species of plants (Danthanarayana 1975; Thomas 1989); consequently, they are a major pest of many economically important horticultural crops. The species is highly mobile; originally from Australia, it has migrated to New Zealand, the United Kingdom, Hawaii, and most recently California. The sex pheromone of *E. postvittana* is a blend of 3 compounds, (*E*)-11-tetradecenyl acetate, (*E,E*)-9,11-tetradecadienyl acetate, and dodecyl acetate, which are blended in a proportion of 20:1:2, respectively (Bellas et al. 1983; Rumbo 1983). EAG recordings have shown that adult males respond to the female sex pheromone and the sex pheromone antagonist (*Z*)-11-tetradecenyl acetate (Rumbo et al. 1993). Both male and female adults respond similarly to a wide range of plant volatiles including esters, terpenes, alcohols, and aldehydes (Suckling et al. 1996). Volatiles released by host plants generally elicit larger EAG amplitudes compared with nonhosts, suggesting that host odors play an important role in the initial host recognition by this moth. Two compounds, citral and nonanol, elicit the largest EAG amplitudes in both sexes, and during behavioral experiments with adult females, these same 2 compounds deter oviposition (Suckling et al. 1996).

Expressed Sequence Tags (EST) have been used successfully to isolate genes from *E. postvittana* expressed in the larval gut (Simpson et al. 2007) and from male antennae (Jordan et al. 2008). Here we characterize 3 ORs identified during antennal EST screening. The ORs show sequence similarities to other moth receptors, 1 being the putative orthologue of Or83b from *D. melanogaster*. This coreceptor forms stabilizing complexes with conventional ORs and performs important roles in the transportation and localization of these ORs to the dendritic membrane (Larsson et al. 2004; Benton et al. 2006). These OR complexes can function as nonselective cation channels that are activated on binding of the appropriate odor ligand independent of traditional G protein signaling cascades (Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008). Cell culture–based functional assays reveal the other 2 ORs recognize important volatile compounds produced by plants.

Materials and methods

Insects and antennal collection

Epiphyas postvittana were from a laboratory colony maintained at HortResearch, Auckland, New Zealand, which was initially collected from the wild in Nelson, New Zealand, in 1967 and maintained in the laboratory thereafter. In 1988, at generation 128, colony females were crossed with wild males. Larvae were reared on a general all-purpose diet (Singh 1983). The pupae were kept at room temperature until eclosion. *Bombyx mori* were from the domesticated Dazao strain and were provided by Professor Qingyou Xia, South West University, China. *Plutella xylostella* was from Dr Nancy Endersby at IHD-DPI (Institute for Horticultural Development, Department of Primary Industries), Victoria, Australia. *Ephestia cautella*, *Plodia interpunctella*, *Leuciris fimbriaria*, and *Helicoverpa armigera* were from laboratory strains held at CSIRO, Canberra, Australia, whereas *Ctenopseustis obliquana* and *Planotortrix excessana* were from laboratory strains held at HortResearch. Tissues were removed from 2- to 3-day-old, cold-anesthetized adults using forceps and immediately frozen and stored at –80 °C.

Bioinformatics, RNA extraction, and Rapid Amplification of cDNA Ends

Genes encoding ORs were identified from the EST sequences described in Jordan et al. (2008) by either similarity to known insect ORs using TBLASTN (Altschul et al. 1990) or by the identification of amino acid sequences predicted to contain 2 or more transmembrane regions in close proximity by hydrophobicity plot analysis.

Total RNA extraction was performed using TRIzol Reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. First strand cDNA was synthesized from approximately 1 μg of total RNA using Superscript III Reverse Transcriptase (Invitrogen) and oligo-dT_{16} primer. RACE PCR was used to amplify the 3′ end of the EposOR3 gene from male antennal cDNA using a primer (EposOR3 F: 5′-ACATCGCCACATTCATTTTCAA-3′) designed from sequence of the EST together with oligo-dT_{16}. 5′ RACE PCR was used to amplify the 5′ end of all 3 EpOR genes. First strand cDNA, for EpOR1, EpOR2, and EpOR3, was synthesized as described above except that a unique gene specific primer "RACEout" was used in place of oligo-dT. The cDNA was purified using the PCR purification kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. A homopolymeric tail of cysteine residues was added to the 3′ end of the cDNA using Terminal Transferase (Roche), and the tailed cDNA was then directly amplified by 2 rounds of PCR using the appropriate gene specific primers (EpOR1, RACEout: 5′-TGTAAGGGCAGCATCGTTCT-3′, RACEnest:5′-CACCGATAGACCTCAGCGTA-3′; EpOR2, RACEout:5′-GGGCTGGTTCTGTCAGGGTAT-3′, RACEnest:5′-GCCGAAGAAGGTTATGGTTA-3′; EpOR3, RACEout: 5′-TCCAGATTGAGGAGTATGAAGGTC-3′, RACEnest: 5′-CGTTGAAAATGAATGTGGCGATGT-3′). For the first round of PCR, products were amplified using the outer gene specific primer RACEout and the Abridged Anchor Primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′). Thermocycling conditions were as follows:

94 °C for 2 min, then 30 cycles of 94 °C for 10 s, 50 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 10 min. One microliter was taken from the first PCR to be used as template for the 2nd round of PCR employing the same reaction conditions, except that a nested gene specific primer "RACEnest" was used together with the Abridged Universal Amplification Primer (5′-GGCCACGCGTCGACTAGTAC-3′).

RT-PCR and sequencing of EpOR3 from other Lepidoptera

RNA was extracted from the closely related tortricid species *C. obliquana* and *P. excessana* as described above. RNA was extracted from antennae of the more distantly related lepidopteran species, *E. cautella*, *P. interpunctella*, *L. fimbriaria*, *H. armigera*, and *P. xylostella* using a modified guanidinium thiocyanate/phenol/chloroform extraction method as described previously (Clyne et al. 1999). Oligo-dT_{16} primed cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen) was performed on total RNA according to the manufacturer's directions. The primers OR3F:5′-ATGGAAGAGACCATCCCAACCTTC-3′ and OR3R:5′-GTTTTCATCAAACACTGACATCACC-3′ were used to amplify full length *EpOR3* homologues from *C. obliquana* and *P. excessana*. For the other species the degenerate primers 5′-GCNGTSACNGTRAARTGGTA-3′ and 5′-CATSACSAGNGTRAARAANSWC-3′ were used in PCR reactions to amplify a C-terminal region of each *EpOR3* orthologue.

Phylogenetics, transmembrane domain predictions, and orientation determination

Protein sequences were aligned with the multiple alignment program ClustalX 1.83 (Thompson et al. 1997). Phylogenetic trees were constructed with the PHYLogeny Inference Package (PHYLIP) v3.6 (Felsenstein 2005) using the FITCH method from Jones–Thorton distances (PROTDIST). Branch support was assessed by bootstrapping with SEQBOOT using 1000 bootstrap replicates. The transmembrane domains of the 3 *E. postvittana* receptors were predicted using TMHMM 2.0 (Sonnhammer et al. 1998; Krogh et al. 2001), TMAP (Persson and Argos 1994), and TMPred (Hofmann and Stoffel 1993).

An N-terminal c-Myc epitope and restriction sites for subcloning were incorporated into the *EpOR1* cDNA sequence using PCR and the primers 5′-CTCGAGATGGAACAAAAGCTTATCTCCGAAGAAGACCTTATGGATGTATTCAATTTA-3′ and 5′-CCGCGGTCACTGATTTGCAAATGTTCT-3′. PCR product was cloned into pGem-T Easy (Promega), sequenced to verify integrity of the construct and shuttled into pIB/V5-His (Invitrogen) using the *Xho* I and *Sac* II sites. C-terminally c-Myc-tagged EpOR1 was created using N-terminally c-Myc tagged EpOR1 as the template in a PCR using the primers 5′-CTCGAGATGGATGTATTCAATTTAAAAT-3′ and 5′-CCGCGGTTAAAGGTCTTCTTCGGAGATAAGCTTTTGTTCCTGATTTGCAAATGTTCTCAG-3′. PCR products were cloned and shuttled as above. Transfection of the plasmid DNA into *D. melanogaster* Schneider S2 cells (Invitrogen) was performed with Escort IV transfection reagent (Sigma, St Louis, MO) following manufacture's instructions. Experimental determinations of the location of the N- and C-termini of the receptor EpOR1 were conducted by detecting the presence of fused c-Myc tags with and without the presence of detergent (saponin) to allow passage of the primary antibody into the cell cytoplasm, as described in Smart et al. (2008). Positive fluorescence images are representative of at least 100 positive-staining cells from duplicate slides from 3 separate experiments. A result was deemed negative when no stained cells were observed in any duplicate slides in experiments repeated at least 3 times. We estimate this amounts to observation of at least 20 000 cells.

Quantitative RT-PCR (qPCR)

Antennal, body, and leg tissues were removed from 2- to 3-day-old male and female adult moths. The separate tissues were snap frozen immediately with liquid nitrogen and stored at –80 °C. RNA extraction, cDNA transcription, and qPCR were all performed as described previously (Jordan et al. 2008). The primer pairs EpOR1, F:5′-CGGTTTACGCTGAGGTCTAT-3′ and R:5′-GTTTGTCCCAAGTCCATGAA-3′; EpOR2, F:5′-CCGACAAGGAGAGCAACGAT-3′ and R:5′-CTGGTAGACGAAAGCCACAAT-3′; EpOR3, F:5′-TCATCTCCTTCGTCGTCTGTT-3′ and R:5′-TCAGTTTCCCACCGCTTTCT-3′ and reference gene primer pairs EpEF1α, F:5′-ACGTCTACAAAATCGGCGGT-3′ and R:5′-GATGTTGGCAGGTGCGAATA-3′; Epαtubulin, F:5′-GACCCTCGCCACGGCAAGTA-3′ and R:5′-GTAGGGCACCAGTCCACGAA-3′ were employed in qPCR. PCRs were performed with the addition of SYBR Green dye (Invitrogen) on an ABI PRISM 7900HT Fast Real-Time PCR System. The cycling conditions were as follows: 94 °C for 2 min, then 40 cycles of 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 15 s. A dissociation curve with the thermal profile 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s, using a ramp rate of 2% for the final step, was performed immediately after the main cycling protocol.

The dissociation curve of each triplicate reaction was used to confirm a single PCR product had been amplified. The amplification efficiency for each set of primers was calculated from the exported clipped data using the program LinRegPCR (Ramakers et al. 2003). The Cycle Threshold (CT) values of all the individual samples were then converted to quantities using a modified delta CT method with correction for primer amplification efficiencies (Pfaffl 2001). The normalization factor for each tissue type was determined from the geometric mean of the housekeeping gene quantities using the software program geNorm (Vandesompele et al.

2002). Relative expression was then calculated for each of the samples using the appropriate normalization factor.

Receptor expression, detection, and functional assays

The open reading frame of *EpOR1* was fused to the 5′ end of Green Fluorescent Protein (GFP) and cloned into the baculovirus expression plasmid pFASTBAC (Invitrogen) and transfected into Sf9 cells using manufacturer's protocols. For comparison, a similar construct expressing GFP only was constructed and also transfected into Sf9 cells. The cellular location of the expressed GFP and EpOR1-GFP fusion was determined by fluorescence microscopy.

N-Terminally c-Myc-epitope-tagged versions of EpOR1 and EpOR3 were cloned into pIB/V5-His (Invitrogen) and transiently expressed in Sf9 cells according to manufacturer's instructions. The expression of the EpOR1-Myc protein by Sf9 cells was confirmed by western blot analysis (data not shown). Sf9 cell culture, transfection, functional assays, and imaging were performed as described previously (Kiely et al. 2007). We note that in Sf9 and S2 cells, the addition of Or83b or orthologue is not required as these cell lines express an endogenous version of Or83b (Kiely 2008; Smart et al. 2008). Briefly, Sf9 cells were transiently transfected with 500 ng pIB-OR DNA using Escort IV in 12-well plates. Transfected cells were incubated for 48 h to allow for expression of the receptor before calcium imaging of responses to ligands. Fluo4 (Invitrogen) was used as the calcium indicator. Fluorescence images were recorded using a Leitz digital still camera. Images were recorded every 10 s for 50 s following the addition of saline (negative control), the test ligand, and ionomycin (to determine maximal fluorescence). Transfected cells were tested with each of the following odorants at a final concentration of 10^{-5} M: β-pinene (99%, racemic, BDH), citral (95%, Sigma, mixture of neral and geranial), geraniol (97%, BDH, West Chester, PA), geranial (96.1%, synthesized according to the method of Dess and Martin 1983), geranyl acetate (98%, Sigma), nerol (97%, Sigma), linalool (97%, racemic, Sigma), (+)-limonene (97%, Sigma), 1,4 cineole (90%, Fluka, Ronkonkoma, NY), myrcene (Sigma), α-terpineol (98%, Sigma), α-farnesene (Sigma), β-caryophyllene (Koch-light labs, Cambridge, MA), citronellol (95%, racemic, Sigma), α-humulene (ABD), octanol (99.5%, Fluka), nonanol (98%, Fluka), hexanol (98%, Fluka), hexanal (98%, Sigma), hexyl acetate (99%, Sigma), methyl salicylate (99%, Sigma), eugenol (BDH), ethyl butyrate (Hopkin and Williams, Chadwell Health, Essex, UK), ethyl hexanoate (99%, Sigma), pentyl acetate (99%, Sigma), and dodecyl acetate (97%, Sigma). Nerol, pentyl acetate, octanol, geranyl acetate, and methyl salicylate were stored at RT, whereas all remaining compounds were stored at 4 °C. Odorants were made up to 0.1 M in dimethyl sulfoxide, and then further dilutions were made in saline. Dose–response data were collected for compounds that elicited repeatable responses at concentrations below 10^{-5} M. Images were analyzed using the Metafluor imaging system and ΔF was calculated as the ratio of change in fluorescence from basal levels (saline) upon the addition of ligand relative to change in fluorescence from basal levels following the addition of ionomycin. ΔF values were calculated from a minimum of 3 responding cells, and cells responding to saline were removed from the analysis. EC_{50} values and Hill's slopes were calculated using Graphpad Prism.

Results

Isolation and phylogenetics of OR genes

Three putative ORs were identified from *E. postvittana* male antennal cDNA libraries described in Jordan et al. (2008). 3′ and 5′ RACE PCR were used to obtain the full coding region of all 3 genes. The predicted translation initiation site and flanking nucleotides of the 3 *E. postvittana* receptor gene sequences show a high degree of similarity with the published *Drosophila* spp. consensus sequence C/A A A A/C ATG (Cavener 1987). All 3 genes have a purine at the -3 position consistent with published observations that eukaryotes have a strong preference for purines (preferentially A) at this position (Cavener and Ray 1991). Together these factors indicate that all 3 receptor sequences encode full length proteins of 415 (*EpOR1*), 474 (*EpOR2*), and 410 amino acids (*EpOR3*), respectively (Figure 1). These sequences have been deposited in Genbank under accession numbers EU791886–8, respectively.

Multiple sequence alignment and phylogenetic analyses were conducted for EpOR1, EpOR2, and EpOR3, together with ORs from other moths (Krieger et al. 2002, 2004, 2005; Sakurai et al. 2004; Wanner et al. 2007; Mitsuno et al. 2008) and the *D. melanogaster* Or83b coreceptor (Vosshall et al. 1999, 2000) (Figure 2). EpOR1 clusters with 19 other ORs from 5 different species of moth. Many of the ORs in this clade are sexually dimorphic with respect to their levels of gene expression, being more highly expressed in male than female antennae (Krieger et al. 2004, 2005; Nakagawa et al. 2005; Mitsuno et al. 2008). Furthermore, this clade includes receptors that respond to female sex pheromone components from *B. mori* (BmOR1 and BmOR3) (Sakurai et al. 2004; Nakagawa et al. 2005), *P. xylostella* (PxOR1), *Mythimna separata* (MsOR1), *Diaphania indica* (DiOR1) (Mitsuno et al. 2008), and *H. virescens* (HR13, HR14, and HR16) (Große-Wilde et al. 2007). EpOR2 is an orthologue of a class of ORs first identified in *D. melanogaster* (Or83b) (Vosshall et al. 1999) that is conserved across all insect orders studied (Krieger et al. 2003).

Full length homologues of *EpOR3* were amplified from antennal cDNA preparations of 2 closely related tortricid species, *C. obliquana* (GenBank accession number FJ668017) and *P. excessana* (GenBank accession number FJ668018), both sharing 88% amino acid identity with EpOR3. A full length orthologue of *EpOR3* was also identified from *B. mori* by TBLASTN searches (Altschul et al. 1990) of the *B. mori*

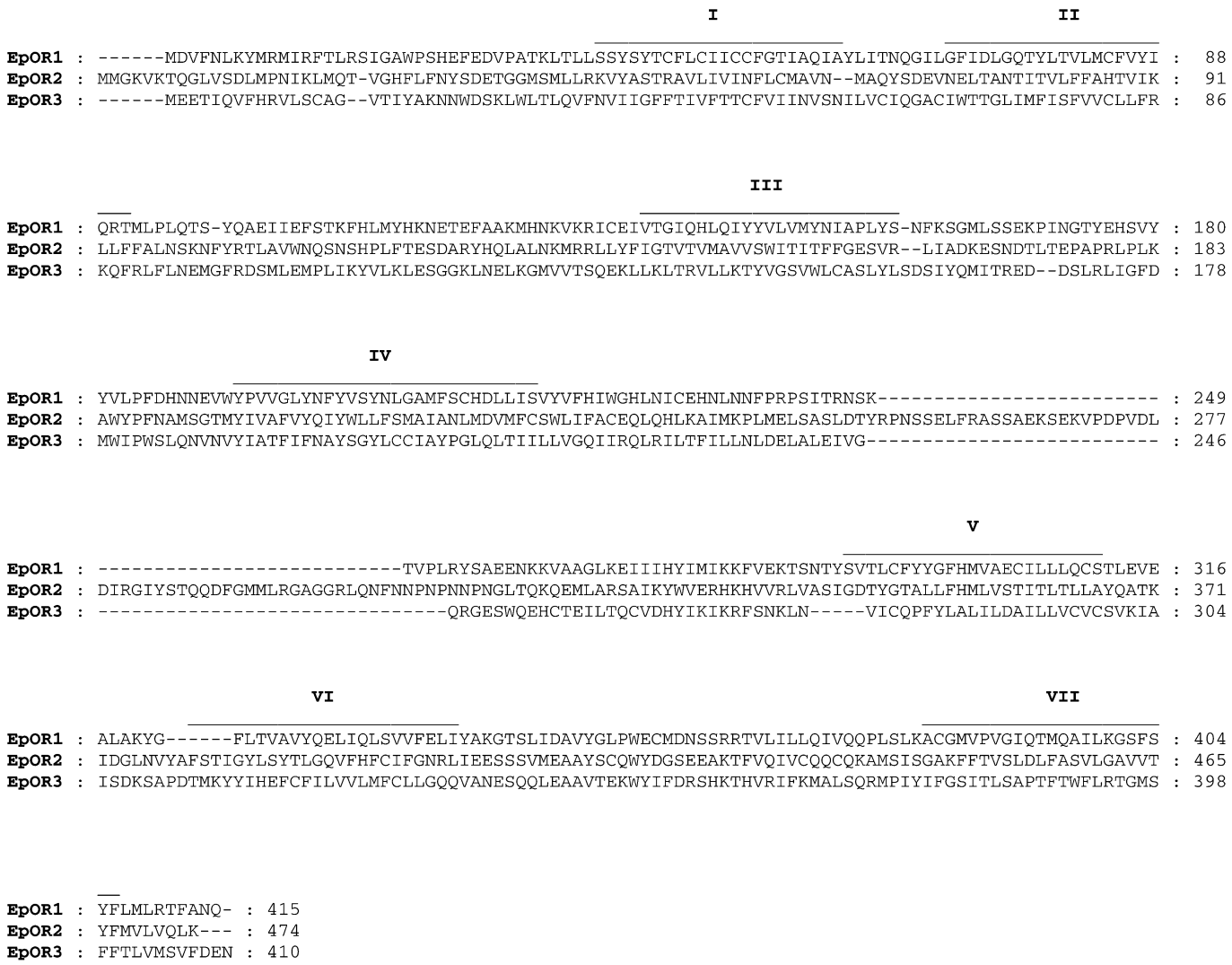


Figure 1 Amino acid sequence alignment of the *Epiphyas* postvittana ORs, EpOR1, EpOR2, and EpOR3. The 7 predicted transmembrane domains are identified with roman numerals (I–VII). Amino acid numbering is given on the right of the alignment. Gaps in the alignment are indicated by a dash.

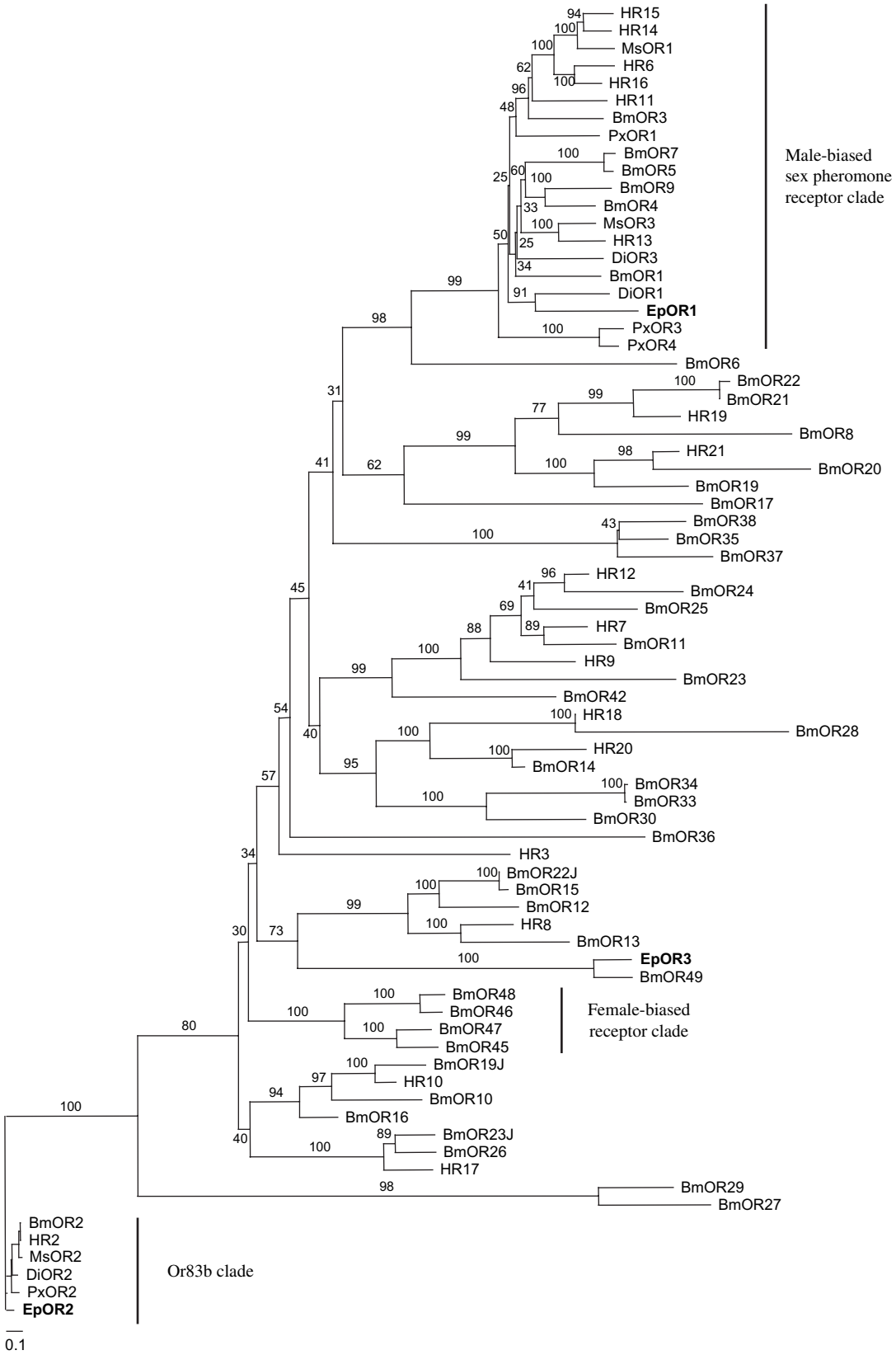
genome databases (Mita et al. 2004; Xia et al. 2004) and was constructed from a Chinese scaffold (CH381065.1—*B. mori* strain Dazao Scaffold001484 genomic scaffold). The predicted *B. mori* orthologue (BmOR49, GenBank accession number EU779802) shares 66% amino acid identity with EpOR3 (see Figure 2 for phylogenetic relationship with other ORs). Similar searches of *D. melanogaster*, *Anopheles gambiae*, and *Apis mellifera* genomes failed to identify further orthologues of *EpOR3*. RT-PCR was used to amplify sequences encoding the 57 C-terminal amino acids of EpOR3 and its orthologues and revealed that this gene is highly conserved over this 3′ region and is expressed in the antennae of members of at least 6 lepidopteran families (Figure 3).

Gene expression profile of receptors

The gene expression profiles of the 3 *E. postvittana* ORs were analyzed by qPCR across 3 tissues (antenna, body, and leg) of adult males and females (Figure 4A). All 3 OR genes are expressed in both adult male and female antennal tissue, but expression is negligible in both adult body and leg tissue. *EpOR2* shows the highest antennal expression being 23× and 13× more abundant than *EpOR1* in male and female antennae, respectively. Similarly *EpOR2* is 57× and 39× more abundant than *EpOR3* in male and female antennae, respectively. The expression of each of the 3 OR genes was also compared between male and female antennal tissue (Figure 4B). *EpOR2* was over 2× more highly expressed in adult male antennae than in female antennae. *EpOR1* and *EpOR3* do not show any sex bias in their expression.

Transmembrane domains and orientation in the plasma membrane

The number and location of the transmembrane domains of EpOR1, EpOR2, and EpOR3 were predicted using 3



```
                              3         3         3         3         3         4
                              5         6         7         8         8         0
                              0         0         0         0         0         0
Epiphyas postvittana          AVTEKWYIFDRSHKTHVRIFKMALSQRMPIYIFGSITLSAPTFTWFLRTGMSFFTLVM
Ephestia cautella             ........H.KR...D.....T............T.P..L......IK...C......
Plodia interpunctella         ........H.KR...D.....T............T.P..L......IK..........
Helicoverpa armigera          .........NKT..VN.L..N.............T....L......IK..........
Bombyx mori                   .........N.A..QN..................T....L......IK..........
Leuciris fimbriaria           ........Y..K..KN.L..S...G.........T....L......I...........
Ctenopseustis obliquana       ...........R..VN..................................... 
Planotortrix excessana        ...........R..VN..................................... 
Plutella xylostella           .......M...T..VN.............................KA..W......
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Figure 3 Alignment of the C-terminal region of EpOR3 (amino acids 347–404) with orthologues from 8 other species of Lepidoptera. Dots indicate identical amino acids to EpOR3.

different transmembrane prediction programs TMHMM 2.0 (Sonnhammer et al. 1998; Krogh et al. 2001), TMAP (Persson and Argos 1994), and TMPred (Hofmann and Stoffel 1993). All 3 receptor sequences were predicted to contain 7 transmembrane domains when submitted to TMHMM 2.0. These transmembrane coordinates were then compared with the predictions of the TMAP and TMPred algorithms. A consensus for the assignment of transmembrane domains was compiled from the 3 predictions (Figure 1) and are similar to those predicted for the nine *H. virescens* putative ORs published in 2002 (Krieger et al. 2002).

Orientation of the *D. melanogaster* ORs, Or83b (Benton et al. 2006; Lundin et al. 2007), and Or22a (Smart et al. 2008) in the plasma membrane is distinct from that of members of the GPCR superfamily, with these ORs having an intracellular N-terminus. For the 3 *E. postvittana* ORs, 2 transmembrane prediction algorithms suggested an intracellular N-terminus with the exception of TMPred, which predicted an extracellular N-terminus for EpOR1 and EpOR3 but not EpOR2. The orientation of EpOR1 was determined experimentally in *D. melanogaster* S2 cells (Figure 5A). The c-Myc-epitope-tagged N-terminus of EpOR1 was only accessible to an anti-c-Myc antibody in the presence of 0.1% saponin, implying that this domain is located on the cytoplasmic side of the membrane, whereas the c-Myc-epitope-tagged C-terminus was accessible regardless of the presence or absence of saponin. No antibody labeling was seen in S2 cells transfected with the vector only (data not shown). In functional assays, the N-terminal fusion version of EpOR1-Myc, but not the C-terminal fusion, displayed ligand-dependent activation as observed for similar fusions of *D. melanogaster* Or22a (data not shown; Smart et al. 2008).

Olfactory receptor function

To test whether expression of ORs from *E. postvittana* would be properly targeted to the membrane of Sf9 cells, we produced a recombinant fusion protein of GFP and EpOR1 in Sf9 cells using baculovirus. Fluorescence microscopy showed that the fusion protein was located in the plasma membrane, compared with a GFP alone construct, which was cytoplasmic, indicating proper expression and targeting of the *E. postvittana* OR to the plasma membrane (Figure 5B).

Putative ligands for EpOR1 and EpOR3 were screened using a functional assay developed for insect ORs using calcium imaging of transfected Sf9 cells (Kiely et al. 2007). A range of plant volatiles known to elicit electrophysiological responses in antennae of *E. postvittana* (Suckling et al. 1996) were tested against EpOR1 and EpOR3 expressed transiently in Sf9 cells. Ten of the 26 compounds elicited a response from EpOR1-expressing cells at the high concentration of 10^{-5} M, including several monoterpenes (geraniol, geranial, geranyl acetate, nerol, citral, and 1,4 cineole); a sesquiterpene (α-faresene); an alcohol (octanol); an ester (pentyl acetate); and a benzoate (methyl salicylate; Table 1). Fifteen of the compounds, including many monoterpenes, a sesquiterpene, and a range of alcohols, aldehydes, and esters elicited a response in cells expressing EpOR3. EC_{50} values were estimated for a subset of the compounds that were able to elicit responses at lower concentrations (Table 2). EpOR1 was sensitive to the plant volatile methyl salicylate [$EC_{50} = 1.8 \times 10^{-12}$ M] (Figure 6A), but also responded to low concentrations of geraniol [$EC_{50} = 5.8 \times 10^{-11}$ M] (Figure 6B). EpOR3 responded to very low concentrations of the monoterpene citral (a 50:50 mixture of the cis–trans isomers, neral and geranial, respectively) [$EC_{50} = 1.1 \times 10^{-13}$ M] (Figure 6C). EpOR3 was less sensitive to geranial than to citral, suggesting a preference for neral over geranial.

Discussion

Three gene sequences encoding novel ORs were isolated from the antennae of the tortricid moth *E. postvittana*

Figure 2 Phylogenetic tree of lepidopteran odorant and pheromone receptors with EpORs1–3 from *Epiphyas postvittana* in bold. The tree was constructed using the FITCH method from Jones–Thorton distances and rooted with the clade of Or83b orthologues. Bootstrap values calculated from 1000 bootstrap replicates are given, wherever possible, to the right of each corresponding node in the tree as a percentage value. GenBank accession numbers for BmOR1–48 from *Bombyx mori* are listed in Wanner et al. (2007) and BmOR19a is AB234351, BmOR23a is AB234355, and BmOR49 is EU779802. Accession numbers for HR1-21 from *Heliothis virescens* are AJ487476–AJ487484 and AJ48325–AJ748336. Accession numbers for receptors from *Plutella xylostella*, *Mythimna separata*, and *Diaphania indica* are listed in Mitsuno et al. (2008). Accession numbers for EpOR1–3 are EU791886–8.

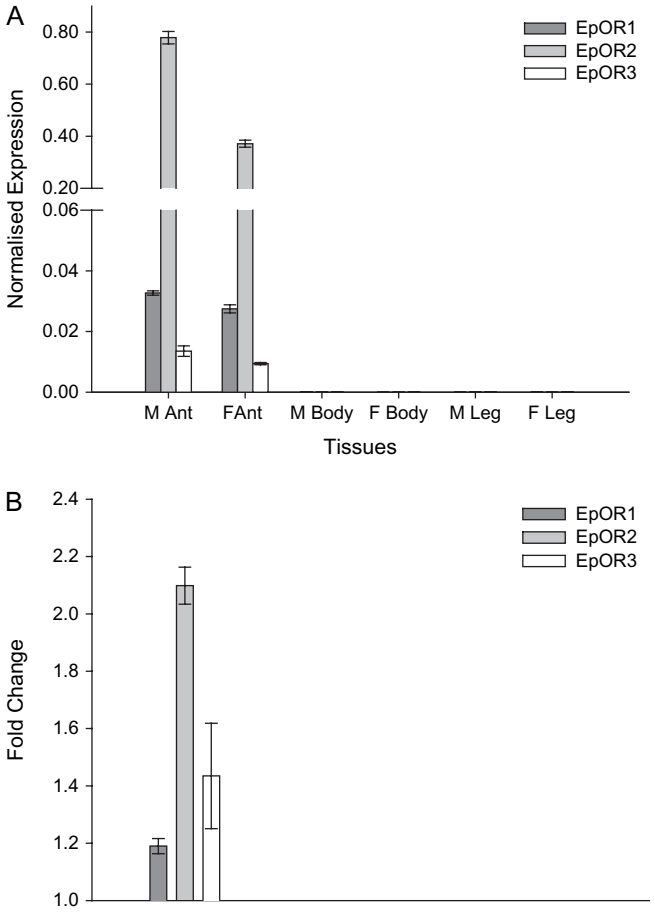

Figure 4 Normalized expression levels of *EpOR1*, *EpOR2*, and *EpOR3* in antennal, body, and leg tissues from adult males and females assessed by quantitative RT-PCR **(A)**. Ant = antennae. Error bars represent standard errors generated from at least 3 replicates. Differential expression of *EpOR1*, *EpOR2*, and *EpOR3* in male antennae compared with female antennae **(B)**. Relative expression above 1 indicates that the gene is more highly expressed in male antennae. Error bars represent standard errors generated from at least 3 replicates.

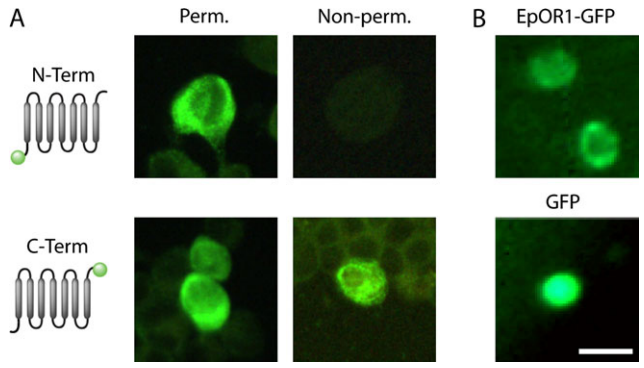

Figure 5 Representative S2 cells, expressing EpOR1 tagged with a c-Myc epitope at the N- and C-termini, antibody labeled in the presence (Perm.) and absence (Nonperm.) of 0.1% saponin **(A)**. Baculovirus-expressed EpOR1-GFP fusion protein and GFP alone in Sf9 cells **(B)**. Scale bar = 20 μm.

Table 1 Change in fluorescence (ΔF) in response to 10^{-5} M odor for EpOR1 and EpOR3

Compound class	Compound name	EpOR1	EpOR3
Monoterpene	(+)-Limonene[a]	NR[b]	NR
	Myrcene	NR	NR
	β-Pinene	NR	NR
	Linalool	NR	NR
	Citronellol	NR	NR
	α-Terpineol	NR	0.21 ± 0.10
	1,4 Cineole	0.43 ± 0.25	0.12 ± 0.06
	Nerol	0.41 ± 0.14	0.14 ± 0.04
	Geraniol	0.31 ± 0.03	0.30 ± 0.13
	Citral	0.32 ± 0.02	0.29 ± 0.16
	Geranial	0.36 ± 0.02	0.34 ± 0.23
	Geranyl acetate	0.32 ± 0.02	0.26 ± 0.17
Sesquiterpene	α-Farnesene	0.41 ± 0.12	NR
	β-Caryophyllene	NR	0.38 ± 0.10
	α-Humulene	NR	NR
Alcohol	Hexanol	NR	0.18 ± 0.02
	Octanol	0.25 ± 0.21	0.37 ± 0.14
	Nonanol	NR	0.13 ± 0.01
Aldehyde	Hexanal	NR	0.45 ± 0.02
Ester	Ethyl butyrate	NR	0.27 ± 0.02
	Ethyl hexanoate	NR	0.15 ± 0.02
	Pentyl acetate	0.37 ± 0.31	NR
	Hexyl acetate	NR	0.11 ± 0.09
	Dodecyl acetate	NR	NR
Benzoate	Eugenol	NR	NR
	Methyl salicylate	0.31 ± 0.03	NR

[a] Unless otherwise specified, compounds containing cis–trans and optical centers are racemic mixtures.
[b] NR = no detectable response.

(EpOR1, EpOR2, and EpOR3). Sequence analyses predicted that all 3 ORs contain 7 transmembrane helices, like ORs from other insects. Mounting evidence suggests that all *D. melanogaster* ORs (Benton et al. 2006; Lundin et al. 2007; Smart et al. 2008) exhibit an orientation in the plasma membrane with an intracellular N-terminus and extracellular C-terminus, which is distinct from classical GPCRs. The similarity in membrane orientation of EpOR1 with ORs from flies suggests that this inverted-GPCR topology may be widespread among insect ORs, and is consistent with insect ORs forming a functionally and structurally coherent gene family that is distinct from GPCRs and indeed mammalian ORs.

Table 2 EC_{50} and Hill slope values for EpOR1 and EpOR3

	EpOR1		EpOR3	
	EC_{50} (M)	Hill slope	EC_{50} (M)	Hill slope
Methyl salicylate	1.8×10^{-12}	0.35	NR[a]	NR
Citral	1.3×10^{-9}	0.33	1.1×10^{-13}	16.4
Geranial	5.3×10^{-8}	0.61	6.0×10^{-12}	ND[b]
Geraniol	5.8×10^{-11}	3.2	2.1×10^{-9}	5.0
Geranyl acetate	2.8×10^{-8}	0.83	1.4×10^{-8}	2.8

[a] NR = no detectable response.
[b] ND = could not be determined.

One of the 3 *E. postvittana* ORs, EpOR2, is orthologous to Or83b from *D. melanogaster*. Or83b forms stabilizing complexes with other conventional ligand-binding ORs and performs important roles in transporting and localizing them to the dendritic membrane (Benton et al. 2006). More recently, Or83b, in complex with a ligand-binding OR, has been shown to form a nonselective cation channel in heterologous cells (Sato et al. 2008; Wicher et al. 2008). Orthologues of Or83b are expressed at a high level because of their presence in the majority of sensory neurons (Vosshall et al. 1999; Krieger et al. 2002, 2003, 2005; Jones et al. 2005; Malpel et al. 2008). In *E. postvittana*, the expression of *EpOR2* was significantly higher (13–57×) than the expression levels of both *EpOR1* and *EpOR3* in male and female antennae, consistent with EpOR2 being present in the majority of OR-expressing sensory neurons. *EpOR2* expression in male antennae was twice that of female antennae. Such a sexual bias in expression has not been documented previously; in a similar qPCR study, the OR2 orthologues from *B. mori* showed equal expression between males and females (Wanner et al. 2007). The male-biased expression of EpOR2 in *E. postvittana* is perhaps due to the higher number of sex pheromone–detecting long trichoid sensilla on the antennae of males compared with females (Jordan et al. 2008), resulting in the higher amounts of EpOR2 required for coexpression with the pheromone receptors of *E. postvittana.*

For *EpOR1* and *EpOR3*, we found no significant difference in the expression levels of the genes between males and females. Sexually dimorphic expression of an OR is likely to indicate a role in a sex-specific behavior. Male-biased expression of ORs is often associated with their involvement in the recognition of the female-produced sex pheromone components (Krieger et al. 2004; Sakurai et al. 2004; Nakagawa et al. 2005; Große-Wilde et al. 2007). *EpOR1* does not show sex-biased expression, although phylogenetic analysis places it in the sex pheromone clade; some other receptors in this clade, BmOR9 and HR6 (Krieger et al. 2005; Wanner et al. 2007), also express at similar levels between the 2 sexes. In functional assays, EpOR1 did not respond to the *E. postvittana* female sex pheromone blend (data not shown), suggesting that this receptor is not a pheromone receptor.

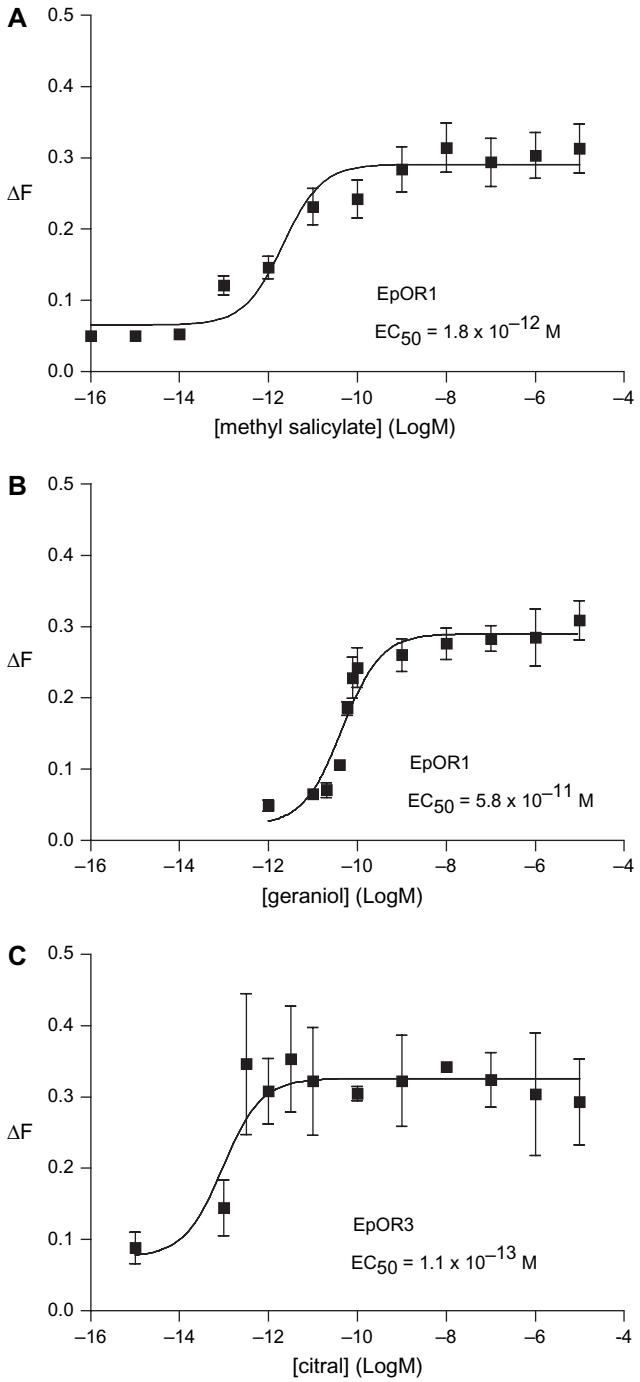

Figure 6 Dose–response curves for EpOR1 with methyl salicylate **(A)** and geraniol **(B)** and EpOR3 with citral **(C)**. ΔF is the relative increase in normalized fluorescence after the addition of ligand (maximum over the 6 × 10 s time points) compared with before ligand addition.

EpOR1 and EpOR3 responded to some, but not all of the plant compounds found by Suckling et al. (1996) to elicit an EAG response from the antennae of *E. postvittana*. At high concentrations of odorant, receptors responded to terpenes, alcohols, and esters but not to the benzoate, eugenol, which generates a large response in EAG (Suckling et al. 1996). The

2 receptors favor different size and shape compounds. EpOR1 responded to compounds of carbon length 8–15, whereas EpOR3 was able to respond to a wider range of compounds at 10^{-5} M, including smaller molecules down to 6 carbons in size. EpOR1 and EpOR3 responded to both cyclic and acyclic compounds, suggesting that the acyclic ligands of these receptors may bind to the receptor in a cyclic form.

Although EpOR1 and EpOR3 were able to respond to a range of different compounds delivered at a high concentration, they responded to far fewer compounds at lower concentrations to allow dose–response curves to be produced. For both receptors, the lowest EC_{50} values were obtained for monoterpenes and benzoates produced by plants. EpOR1 best recognizes methyl salicylate, based on its EC_{50} value of 1.8×10^{-12} M. An OR from *D. melanogaster*, Or10a, is also tuned to methyl salicylate (Hallem et al. 2004); however, EpOR1 and Or10a are not similar at the amino acid level, suggesting that the ability to detect this compound may have evolved more than once within the insect ORs. Interestingly, EpOR1 exhibits different slope dose–response curves for different compounds. The slope of the dose–response curve for geraniol (Hill slope = 2.25) suggests a higher level of cooperativity than for methyl salicylate (Hill slope = 0.38). EpOR3 is most sensitive to citral, a mixture of the cis–trans isomers neral and geranial, with an EC_{50} value of 1.1×10^{-13} M. Overall, these EC_{50} values are lower than those described for *D. melanogaster* receptors to date, such as for Or22a (Pelz et al. 2006; Kiely et al. 2007). However, they are similar to those of the *H. virescens* pheromone receptor, HR13, for the sex pheromone component Z11-16:Al, which in the presence of the appropriate pheromone-binding protein (PBP2) exhibits an EC_{50} value of approximately 2×10^{-13} M (Große-Wilde et al. 2007). It will be interesting to test whether the addition of odorant binding proteins to the assay further reduces the EC_{50} values of the *E. postvittana* ORs to these plant odorants.

Methyl salicylate and citral are both important plant semiochemicals. Methyl salicylate is a common plant stress signal elicited in response to abiotic and biotic factors such as damage by insect herbivores and pathogens and is also the airborne version of salicylic acid, used by plants as a signal to propagate systemic acquired resistance (Shulaev et al. 1997; Park et al. 2007). In the moth, *Mamestra brassicae,* methyl salicylate is a strong deterrent of oviposition (Ulland et al. 2008), likely acting as a signal to warn females that a plant has already been colonized and that the plant's defences have been primed. Terpenes such as citral make up a large proportion of all volatile compounds produced by plants (Dudareva et al. 2004), and have been implicated as important oviposition cues in a number of moth species, including the pest species *Cydia pomonella* and *H. armigera* (Jallow et al. 1999; Witzgall et al. 2005). In adult *E. postvittana*, citral elicits the greatest electrophysiological responses by EAG in both males and females, and in behavioral studies is an oviposition repellent to females (Suckling et al. 1996). The high level of sequence conservation of EpOR3 together with this receptors sensitivity to citral suggests that this ability may be important across the Lepidoptera. Furthermore, the orthologue of EpOR3 from *B. mori*, BmOR49, also responds to citral in cell assays (data not shown). However, as yet, the conserved functional significance of the ability to detect citral by moths is not known. Finally, this level of conservation opens the way to use such receptors as potential targets for the design or screening of new attractants or repellents that might have activity across a wide range of economically significant species of moth.

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