Chem. Senses 34: 305–316, 2009

doi:10.1093/chemse/bjp002
Advance Access publication February 2, 2009

A Candidate Pheromone Receptor and Two Odorant Receptors of the Hawkmoth *Manduca sexta*

Harland M. Patch[1], Rodrigo A. Velarde[2], Kimberly K.O. Walden[3] and Hugh M. Robertson[3]

[1]Department of Entomology, Center for Chemical Ecology, The Pennsylvania State University, University Park, PA 16802, USA, [2]Department of Biology, Wake Forest University, Winston-Salem, NC 27109, USA and [3]Department of Entomology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Correspondence to be sent to: Harland Patch, Department of Entomology, Center for Chemical Ecology, The Pennsylvania State University, University Park, PA 16802, USA. e-mail: hmpatch@psu.edu

Abstract

In this study, we cloned and characterized three *Manduca sexta* odorant receptors (ORs). One receptor is a putative pheromone receptor expressed exclusively in a cell associated with male-specific type-I trichoid sensilla. We describe the results of real-time PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) experiments that show *MsextaOR1* is expressed only in male antennae. In situ hybridization labels a single cell associated with type-1 trichoid sensilla, which houses two neurons that have been previously determined to respond to the major components of the pheromone blend. The second receptor, *MsextaOR2*, was discovered using degenerate primers designed to conserved motifs of a unique group ORs that share as much as 88% identity. Comparison of RT-PCR, qRT-PCR, and in situ hybridization results with those of ORs in the *Drosophila melanogaster* Or83b subfamily shows a strong sequence and expression pattern similarity. The third receptor, *MsextaOR3*, was found by 5′-end sequencing of a normalized and subtracted cDNA library from male *M. sexta* antennae. RT-PCR and qRT-PCR show that this receptor is expressed only in male and female antennae. These are the first ORs, including a putative pheromone receptor, to be described from *M. sexta*.

Key words: insect, olfaction, pheromone, receptor

Introduction

Insects encounter an environment composed of thousands of potential odorant signals that they detect and decode with specialized olfactory systems. These signals are relevant to many aspects of insect life strategies—reproduction, host finding, avoidance of predators, identification of conspecifics, and social regulation. The sensory system of the hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae) has been one of the most extensively investigated olfactory systems of any insect species (Christensen and Hildebrand 1987; Hansson 1995; Hildebrand 1995, 1996; Hildebrand et al. 1997; Homberg et al. 1989). A primary focus of these investigations has been the peripheral detection and neural integration of sex pheromone sensory information in the central nervous system (Christensen et al. 1989; Kanzaki et al. 1989; Kanzaki et al. 1991; Hildebrand 1996). Pheromones, chemicals released by an individual that modify the behavior or physiological state of a conspecific, are either single chemicals or, as for moth sex pheromone, a blend of chemical compounds produced by the female pheromone gland (Karlson and Luscher 1959; Tillman et al. 1999). Twelve compounds were identified in *M. sexta* pheromone gland rinses; of these, eight C_{16} aldehydes are shown to be behaviorally active or neurophysiologically relevant (Starratt et al. 1979; Christensen et al. 1989; Tumlinson et al. 1989, 1994). Two components (*E,Z*)-10,12-hexadecadienal (bombykal) and (*E,E,Z*)-10,12,14-hexadecatrienal (EEZ), in combination are required for complete male mating behavior in wind tunnel bioassays (Tumlinson et al. 1989).

The sexually dimorphic antennal flagellum is the primary pheromone sense organ in *M. sexta* (Tumlinson et al. 1989; Kalinová et al. 2001). The sensory region (S) is rich in sensilla and is oriented "windward" in the flying adult; the "lagging" region is covered with thick scales (L) (Figure 1). The sensory regions contain many types of olfactory sensilla that likely detect plant-associated compounds (Lee and Strausfeld 1990; Anderson et al. 1995; Anderson et al. 1996; Shields and Hildebrand 1999a, 1999b; Shields and Hildebrand 2001). Male *M. sexta* antennae are easily distinguished from female antennae by the presence of long type-I trichoid sensilla distributed on the dorsal and ventral sides of each

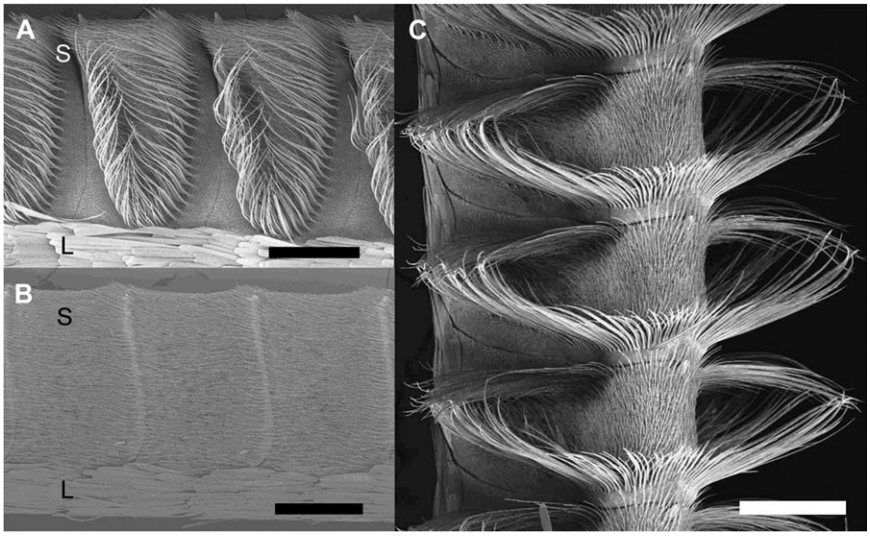

Figure 1 Scanning electron micrographs of male and female *Manduca sexta* antennae. The sensory region (S) is covered with sensilla and is oriented windward in the flying adult. The lagging region (L) is covered with thick scales in both males and females. **(A)** Region of the male *M. sexta* antennae. The male antenna is distinguished by pheromone detecting type-I trichoid sensilla distributed on the dorsal and ventral sides of each annulus in a characteristic U-shape (scale bar = 200 μm). **(B)** Region of the female *M. sexta* antenna. The sensory region (S) of each annulus is covered in short basiconic and trichoid sensilla (scale bar = 200 μm). **(C)** Windward view of adult male antennal flagellum. Note the long male-specific trichoid sensilla forming symmetrical U-shaped phalanxes above shorter basiconic and trichoid sensilla (scale bar = 200 μm).

annulus in a characteristic U-shape (Figure 1) (Lee and Strausfeld 1990; Shields and Hildebrand 1999a, 1999b; Shields and Hildebrand 2001). The cuticle of the male-specific sensilla is permeated by small pores that connect the external environment to an aqueous lumen that bathes the dendrites of two odorant neurons (Sanes and Hildebrand 1976; Keil 1989).

Recordings from the type-1 trichoid sensilla found in the antennal phalanx identified four cell types specialized to detect distinct components of the pheromone blend. The most prominent two-neuron combination revealed by tip recordings of type-1 trichoid sensilla shows one of the receptor cells responded to bombykal, and the other cell responded to EEZ (Kaissling et al. 1989). Recordings from 3 of 20 trichoid sensilla showed the second cell detected the isomer of EEZ—the C_{16} trienol (*E*,*E*,*E*)-10,12,14-hexadecatrienal (EEE). Kalinová et al. (2001) not only confirmed these results but also found that, in 26 sensilla tested, one neuron in the trichoid sensilla was tuned to bombykal, and the second cell was tuned to (*E*,*E*)-10,12-hexadecadienal (EE). This high degree of cellular specificity is likely to be achieved by a narrowly tuned odorant receptor (OR) expressed in the membrane of one of these male-specific sensory neurons, but not in female antennae.

Recent studies have characterized chemoreceptors located in specialized sensilla that detect insect pheromones (Bray and Amrein 2003; Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2007; Kurtovic et al. 2007; Wanner, Nichols, et al. 2007; Mitsuno et al. 2008). Pheromone receptors in *M. sexta* should have a similar distribution and should bind one of the four compounds that activate sensory neurons in male-specific trichoid sensilla.

Methods to find insect chemoreceptors depended largely, although not exclusively, on bioinformatic techniques owing to the high divergence of the genes in this superfamily (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999; Clyne et al. 2000; Vosshall et al. 2000; Dunipace et al. 2001; Scott et al. 2001; Warr et al. 2001; Robertson et al. 2003). In this study, we employed three techniques to clone and characterize ORs from the antennae of *M. sexta*. We used differential screening to isolate a putative sex pheromone receptor from the antennae of male moths. *MsextaOR1* belongs to a small subgroup of moth receptors that are predominantly male specific and have been shown to respond to pheromones. RT-PCR and qRT-PCR indicate *MsextaOR1* is exclusively expressed in male antennae at high levels. Furthermore, in situ hybridization revealed that this OR localizes to single cells associated with long type-I trichoid sensilla. To find a second OR, we employed homology cloning. We identify and characterize MsextaOR2 as an ortholog of a highly conserved subgroup of insect ORs. These are essential proteins that are coexpressed with a ligand-binging protein in most olfactory sensory neurons. (Larsson et al. 2004; Benton et al. 2006; Sato et al. 2008). We also normalized and subtracted a library made from male antennal whole RNA to identify other ORs. In this screen, we found one OR- *MsextaOR3* is expressed at higher levels in female antennae.

Materials and methods

Colony

Manduca sexta from a colony maintained at the Department of Entomology at the University of Illinois at

Urbana-Champaign were reared on a standard artificial diet at a constant temperature of 26 °C and a photoperiod light regime of 18:6 h light:darkness. For RNA samples, tissues were pooled from 3- to 4-day-old adults collected between 600 and 100 h.

Scanning Electron Microscopy

Specimens were mounted on aluminum stubs using double-stick carbon tape, grounded with colloidal silver paint, and coated with ca. 4 nm of gold/palladium using a Denton (Moorestown, NJ) Desk-2 turbo sputter coater. They were imaged using an FEI (Hillsboro, OR) XL30 environmental scanning electron microscope with a field-emission electron gun (ESEM-FEG) in HiVac (normal SEM) mode at 5 kV and a spot size of 2.1 nm. The microscope is housed in the Microscopy Suite, part of the Imaging Technology Group at the Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign.

Isolation of *MsextaOR1* with differential screening

To identify male-specific ORs, we differentially screened a previously described male *M. sexta* antennal library (Uni-ZAP XR library; Strategene, La Jolla, CA) (Robertson et al. 1999). Isolation of individual pBluescript phagemids from the Uni-ZAP XR Vector was done by in vivo mass excision in SOLR cells following the Uni-ZAP Instruction Manual (Revision #087001d). Ten plates were made, and these colonies were picked randomly and placed on seven plates in a 150-block grid. Besides the random clones, we also plated nine previously identified and highly represented clones to subtract from the library in four pools. These also acted as positive controls. They are a 16S rRNA protein (B49, no accession number), ABPX (B68, AF117577), SAP2 (C23, AF117592), ABP1 (C85, AF117591), a putative cuticle protein (D56, AF117600), PBP1 (D116, AF117953), a chitinase (D176, no accession number), glutathione S-transferase (D182, AF117596), a second putative cuticle protein (E58, AAO32819), and a third putative cuticle protein called E240 (AF117571).

Four replicate lifts were made with velveteen squares from each of the seven plates, transferred to 28 plates and grown overnight at 37 °C. Then the bacterial colonies were transferred to uncharged nylon membranes (Roche, Mannheim, Germany) for hybridization. After denaturing, neutralization and proteinase K treatment, the membranes were dried overnight. Membranes were placed in four hybridization bottles and prehybridized at 42 °C for 1 h in a Hybaid rotisserie oven (Thermo Hybaid, Ashford, UK). The four-probe mixes (80 ng probe/10 mL) were denatured at 96 °C and hybridized overnight at 68 °C in the Hybaid oven. DNA probes were labeled with digoxigenin-11-dUTP (DIG) using PCR (Roche). The following day the nylon membranes were washed for 5 min in 2× saline sodium citrate (SSC) at 25 °C, then in two subsequent washes in 0.5× SSC at 42 °C. Blocking and detection were performed with the DIG Wash and Block Buffer Set (Roche) following the standard protocol. DIG-labeled probe was detected with antidigoxigenin-AP Fab fragments (Roche) and visualized with NBT-BCIP (Amresco, Solon, OH). From the seven plates, 644 random clones did not hybridize to the probes. These clones were sequenced at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign using ABI automation.

The chromatograms of these sequences were edited by eye using Editview v1.0.1 (Applied Biosystems, Foster City, CA) and examined in DNA Strider v1.1 (Ch. Mrack & C.E.) for open reading frames (ORFs) and then searched against Genbank databases for DNA matches using BLASTN and translated proteins using BLASTX (Altschul et al. 1997). Translated proteins were examined using DNA Strider for possible transmembrane domains using Kyte–Doolittle hydropathy plots (Kyte and Doolittle 1982). From these edited sequences, we made specific primers for sequences that met our criteria for putative ORs to generate full-length DIG-labeled probes to screen the male and female *M. sexta* cDNA libraries (Robertson et al. 1999).

Isolation of *MsextaOR2* using sequence homology and PCR

Primers were designed against conserved regions of three closely related genes, *Or83b* (AAF52031) (Clyne et al. 1999; Vosshall et al. 1999), an *Anopheles gambiae* gene, *AgOr7* (AY363725) (Hill et al. 2002), and a gene from a ±21,000 clone *Apis mellifera* EST library *AmOr3* (AJ555537) (Whitfield et al. 2002). The final sequence for the *M. sexta* an ortholog of *Or83b* was amplified with the primers AmOr3-F7-5′-TIGTIGCI-GAYYTIATGCCIAAYATI-3′ and AmOr3-R2-3′-ACY-TAYTTYATGGTNCTGGTGCAGCT-5′.

Isolation of *MsextaOR3* from a normalized and subtracted antennal library

The poly(A)+ mRNA from antennae was converted to double-stranded cDNA using the Superscript Choice System kit (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was primed with a modified oligo (dT) primer, designed to anchor initiation at the 5′ end of the poly(A)+ tail and enable directional cloning (V18(T)AGCCACGCCGGC-GCTTAAGAAGGTCAA). Complementary DNAs (cDNAs) longer than 400 bp were selected by agarose gel electrophoresis. *Eco*RI adaptors (Invitrogen) were ligated to both ends of the cDNAs, which were then digested with *Not*I and directionally cloned into the *Eco*RI and *Not*I sites of pGEM 11Z F(+) (Promega, Madison, WI). Cloned cDNAs were transformed into DH12S electrocompetent cells (Invitrogen).

The primary cDNA library was then subtracted as previously described (Bonaldo et al. 1996). A single-stranded version of the primary library was created by superinfection with M13K07 phage. Contaminating double-stranded DNA was removed by hydroxyapatite (HAP) chromatography.

The purified plasmid DNA from previously sequenced clones was used as a template for PCR amplification using the T7 and SP6 priming sites that flank the cloned cDNA inserts. The purified PCR products were used as a driver for subtractive hybridization. PCR-amplified cDNA inserts (2.5 mg) were denatured and mixed with 100 ng of purified single-stranded circles, as well as 40 mg each of 5′ and 3′ blocking oligonucleotides as previously described (Bonaldo et al. 1996). The resulting solution (50% formamide, 0.12 M NaCl, and 1% SDS) at a final volume of 20 mL was overlaid with mineral oil, and subtractive hybridization was carried out for 88 h at 30 °C. Unhybridized single-stranded DNA circles were separated from hybridized DNA duplexes by HAP. Purified single-stranded circles were rendered partially double stranded by M13 reverse primer extension and were electroporated into DH12S cells (Invitrogen) to generate the normalized library. The library was plated, and 192 (two 96-well plates) clones were picked and sequenced to determine redundancy. The sequence of *EcoR*I adaptors at 5′ end of sequences: 5′-AATTCCATTGTGTTGGG-3′.

After the library normalization, four 96-well plates were sequenced, and a total of 130 clones from these plates that represented ubiquitous genes were subtracted to enrich for rare transcripts. After subtraction, eight new plates were sequenced, and the chromatograms edited by eye using Editview v1.0.1 (Applied Biosystems) and examined in DNA Strider v1.1 (Ch. Mrack & C.E.) for ORFs. Sequences with an ORF over ~500 bp were batch BLAST searched using the NCBI BLAST network client (Blastcl3) against Genbank databases for peptide similarity using BLASTX (Altschul et al. 1997). This subtraction and normalization strategy resulted in one full-length OR we designated *MsextaOR3*.

In situ hybridization

Antennae from 1- to 5-day-old adult *M. sexta* males and females were harvested by anesthetizing adults and dissecting antennae in 1% Triton X-100 (Sigma, St Louis, MO) in 1× phosphate-buffered saline (PBS) with diethyl pyrocarbonate-treated water. Dissected antennae were embedded in Tissue-Tek OCT Compound (Sakura, Tokyo, Japan) on aluminum cryostat chucks on dry ice. Sections (12 μm) were prepared on Fisherbrand Superfrost/Plus microscope slides (Fisher, Pittsburgh, PA) at –16 °C on a Bright OTF cryostat (Huntingdon, UK). Slides were allowed to air-dry for 10 min and were dried overnight in an oven at 35 °C.

The sections were then delipidized in chloroform with an RNase inhibitor, rinsed, and allowed to dry overnight at room temperature (RT) to remove all residual chloroform. The tissue was fixed at RT in fresh 4% paraformaldehyde for 15 min. Sections were then washed in PBS at RT. Next, the slides were dehydrated for 2 min in 70%, 95%, and finally 100% EtOH. Slides were then air-dried for about 30 min until no moisture was evident.

Immediately following drying, the antennal tissue was acetylated, rinsed, and dehydrated. Tissue was then hybridized in 50-mL hybridization solution (50% formamide, 10 mM dithiothreitol, 1 mg/mL BSA, 1× Denhardt's solution, 10% dextran sulfate, 2× SSC, and 1000 ng/mL DIG-labeled RNA probe) in a sealed chamber humidified with 50% formamide/2× SSC overnight (16 h) at 50 °C. Stringency washes and RNase treatment were performed in hot water baths. After hybridization solution was added to tissue, a coverslip of Parafilm was placed over the sections. The next day, the Parafilm was removed by floating each slide in 5× SSC at 50 °C. Slides were then transferred to a 50% formamide solution at 50 °C for 2 h and then subjected to an RNase treatment. The slides were prehybridized at 37 °C, and then 200 mL of 10 mg/mL RNAse A was added for incubation. The final three stringency washes were at 50 °C for 20 min.

To visualize the DIG-labeled probes, slides were washed with shaking in washing buffer followed by a 1-hincubation in blocking solution at RT. Slides were incubated in fresh blocking solution containing 1:500 dilution of sheep anti-DIG-alkaline phosphatase (Anti-Digoxigenin-AP, Fab fragments, Roche) for 2 h in a humid chamber followed by another 2× wash in washing buffer for 10 min. Next, sections were incubated in 1× detection buffer (pH 9.5). For visualization of DIG, we used the Vector NBT-BCIP kit (Amresco) with 1 mM levamisole. After development, slides were dipped in water to terminate the detection reaction. Sections were mounted in Crystal/Mount of 70% glycerol in PBS.

Images of in situs were taken on Nikon Eclipse E600 (Kanagawa, Japan) with a Diagnostic Instruments Spot Insight V3.4 Camera (Sterling Heights, MI) using Image Pro-Plus V4.5 software (Media Cybernetics, Silver Spring, MD).

Probes for in situ

Probe templates were made with T3 and T7 adapter ends from *MsextaOR1* and *MsextaOR2* plasmids. Templates for both ORs were made with a standard PCR reaction using the following primer pairs: The primer pairs used to make a 310 bp probe for *MsextaOR1* were MsextaOR1(is)-F2 (5′-AACATTGAGCCGTAGCATCA-3′)/MsextaOR1(is)-R2b-T7 (5′-TTCAAAACCCCAACAAAATTCTcctatagtgagtcgtatta-3′) and MsextaOR1(is)-F2-T3(5′-aattaaccctcactaaaggAACATTGAGCCGTAGCATCA-3′/MsextaOR1(is)-R2 (5′-TTCAAAACCCCAACAAAATTCT-3′). The primer pairs used to make a 303-bp probe for *MsextaOR2* were MsextaOR2(is)-F1(5′-CTGGAATGACCATGCTTCTG-3′)/MsextaOR2(is)-R1b-T7(5′-GCTCTTACCAAAATGAGAAGGCTcctatagtgagtcgtatta-3′) and MsextaOR2(is)-F1-T3(5′aattaaccctcactaaaggCTGGAATGACCATGCTTCTG-3′)/MsextaOR2(is)-R1(5′-GCTCTTACCAAAATGAGAAGGCT-3′). For a 325-bp probe for *PBP1*, the primers PBP1(is)-F1(5′-CGCTAACTTCTGGGTTGAGG-3′)/PBP1(is)-R1-T7(5′-ATCCACAAGTTGAACTGGGCcctatagtgagtcgtatta-3′) and PBP1(is)-F1-T3-(5′-aattaaccctcactaaaggCGCTAACTTCTGGGTTGAGG-3′)/PBP1(is)-R1(5′-ATCCACAAGTTGAACTGGGC-3′) were used.

The templates were run out on 1% Seakem gels, excised, and purified with a QIAquick Extraction Kit (Qiagen, Valencia, CA). The templates were then further purified on a CENRI-SEP spin column (Princeton Separations, Adelphia, NJ). T3 and T7 polymerases were used for in vitro transcription with digoxigenin–UTP following the directions of the DIG RNA labeling kit (Roche).

Reverse transcriptase-PCR

Approximately 100 mg of tissue was homogenized in 1 mL TRIzol reagent (Invitrogen) with a tissue grinder and spun through a Quiashredder column (Qiagen). The pellet was suspended in Sigma water (Sigma) and further purified, to remove scales, on a CENRI-SEP spin column (Princeton Separations, Adelphia, NJ).

To make cDNA, 1 μg of total RNA was reverse-transcribed with random decamers or oligo-dT_{18} primers using a RETROscript kit (Ambion) for comparison of template in amplification quality. DNA was removed, and the pellet was suspended in Sigma water (Sigma) and further purified on a CENRI-SEP spin column (Princeton Separations, Adelphia, NJ). Tissues (100 mg) from pooled male antennae, female antennae, male heads, male proboscis, male forelegs, male thorax, male abdomens, male palps, and female palps were used to determine if *MsextaOR1*, *MsextaOR2*, and *MsextaOR3* were present. Following PCR using standard conditions (94 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min) for 40 cycles the products were visualized on 2% agarose gels. The same conditions were used for nested PCR. The primers pairs were as follows: MsextaOR1-RTPCR-F (5′-TCAGAAGCTTTCGGTCCGAT-3′)/MsextaOR1-RTPCR-R (5′-ACCGGCTATCTGAAGGATGCT-3′) 206-bp product, MsextaOR2-RTPCR-F (5′-TCAGAAGCAAGAGATGCTGGC-3′)/MsextaOR2-RTPCR-R (5′-AATGTGTACGCGTTCAGCACA-3′) 204-bp product, MsextaOR3-RTPCR-F (5′-ATCAGTGCAAGACGCAGCTCA-3′)/MsextaOR3-RTPCR-R (5′-AGTTCGCGTCAACGACTCTGT-3′) 296-bp product, and RPS3-RTPCR-F (5′-TGCAATCATGGCGGTGAAC-3′)/RPS3-RTPCR-R (5′- TCGTGCAGAAGCGGTTCAA-3′) 245-bp product.

qRT-PCR with SYBR Green

Four RT-PCR, primers were designed using Primer Express software and subsequently checked for fidelity with Amplify v1.2 (Engels 1993). Primers are as follows: MsOR1-TaqmanF2 (5′-GGAACAGTGACCGGCTATCTG-3′)/MsOR1TaqmanR2 (5′ACATGGACACACAGGACAGGAA-3′), MsOR2TaqmanF2- (5′-CGGCGAGTCAGTGCGTATG-3′)/MsOR2TaqmanR2- (5′-TGACAGAACCCGCGCC-3′), and MsOR3TaqmanF1- (5′-TGGCCGCTTACCAGATTGTC-3′)/MsOR3TaqmanR1 (5′-TCTGTTCATCGTGTGCATCCTC-3′).

The cDNA template for each tissue was the same as above for RT-PCR. For this experiment, oligo-dT nucleotides were used to make the template cDNA. The qRT-PCR was carried out using SYBR Green (Applied Biosystems) on an ABI Prism HT7900 Sequence Detection System (Applied Biosystems). Each sample was tested in triplicate reactions. For the endogenous control, the housekeeping gene 16S *M. sexta* ribosomal protein RPS3 was used (Jiang et al. 1996). The exogenous control was a cysteine protease from *Arabidopsis thaliana* (BT004822) Cyst-Prot-142R (TGAGTTTGTCATGAGATTCCAAAT) Cyst-Prot-74 (5′-TCGCTTCTTCCCACGATTAC-3′). Negative control reactions without total cDNA were used to ensure primer specificity and lack of contamination. The $2^{-\Delta\Delta Ct}$ method was used to measure relative expression levels across the samples (Livak and Schmittgen 2001). The expression ratios of individual samples were first normalized to the endogenous control (RPS3) and then normalized to foreleg within each trial.

Phylogenetic analysis

Sequences of 68 moth receptors as previously described (Krieger et al. 2004; Sakurai et al. 2004; Krieger et al. 2005; Nakagawa et al. 2005;Wanner, Anderson, et al. 2007; Mitsuno et al. 2008) were aligned with MsextaOR1 (FJ546086), MsextaOR2 (FJ546087), and MsextaOR3 (FJ546088), using CLUSTAL W. The neighbor joining tree (Figure 2) was constructed using MEGA4.0.2 (Tamura et al. 2007). The tree is rooted with lepidopteron members of the Or83b lineage based on the basal position of this lineage in the dipteran ORs (Robertson et al. 2003). Pairwise deletion was used because distances could not be calculated for all receptor pairs and a number of proteins lacked complete sequence. The aligned sequences were Poisson corrected using the distance function of MEGA4.0.2 (Tamura et al. 2007).

Results

MsextaOR1

To identify a putative pheromone receptor, we differentially screened a male antennal cDNA library by subtraction of ubiquitous clones. Of 644 nonhybridizing clones, we found one clone, *MsextaOR1*, that met the criteria for a seven-transmembrane chemoreceptor. It was a full-length cDNA clone (1528 bp, 431 aa) with seven transmembrane domains revealed by Kyte–Doolittle hydropathy plot (Kyte and Doolittle 1982) but with only weak BLAST matches to those found in the completed *Drosophila melanogaster* genome (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999). Subsequent comparison with *D. melanogaster* (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999) and *A. gambiae* (Fox et al. 2001) and annotated *Heliothis virescens* (Krieger et al. 2002, 2004), *Bombyx mori* (Sakurai et al. 2004; Wanner, Anderson, et al. 2007; Wanner, Nichols, et al. 2007) *Plutella xylostella*, *Mythimna separata*, and *Diaphania indica* (Mitsuno et al. 2008) ORs reveals a subgroup of related

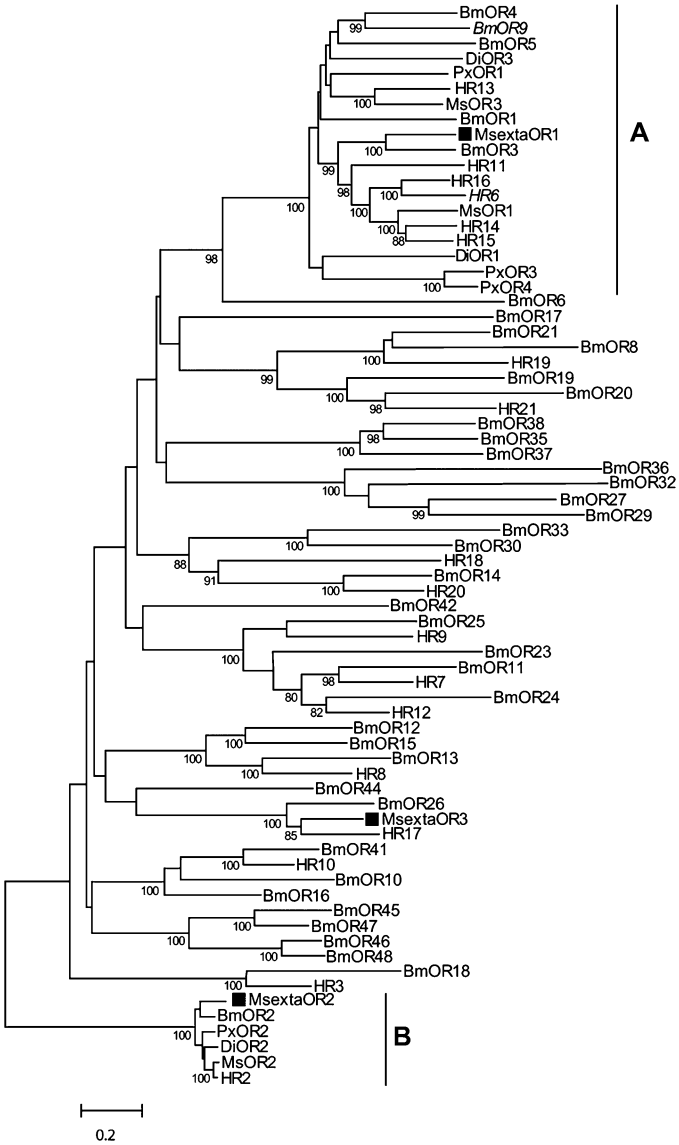

Figure 2 Neighbor joining phylogenetic tree with Poisson corrected distances of moth ORs. Values are shown above relevant branch points only for clades supported by ≥80% (2000 replicates) bootstrap values. The tree is rooted **(B)** with lepidopteron members of the Or83b lineage (the *Bombyx mori* OR BmOR2, HR2 from *Heliothis virescens*, PxOR2 from *Plutella xylostella*, MsOR2 from *Mythimna separata*, and a receptor from *Diaphania indica* DiOR2) including MsextaOR2, based on the basal position of this lineage in the dipteran ORs as shown by Robertson et al. (2003). As with other insect receptor phylogenies, the interior branches have weak bootstrap support. MsextaOR1 belongs to the putative pheromone receptor lineage **(A)** and has the strongest identity (61%) to *B. mori* bombykal-binding protein BmOR3. Unlike other members of this group, BmOR9 and HR6 are expressed in both male and female antennae (Wanner, Anderson, et al. 2007; Wanner, Nichols, et al. 2007). MsextaOR3 is most closely related to HR17 (62% identity) and belongs to the small subgroup of ORs including BmOR26 (55% identity). The other lepidopteron receptors represented here have been previously described (Sakurai et al. 2004; Krieger et al. 2005; Nakagawa et al. 2005; Wanner, Anderson, et al. 2007; Wanner, Nichols, et al. 2007; Mitsuno et al. 2008).

moth-specific receptors (Figure 2). MsextaOR1 has the strongest identity (61%) with the male antennae-expressed *B. mori* receptor BmOR3 that responds to bombykal (Nakagawa et al. 2005) (see supplemental Figure 1 for alignment). It has a 41% identity with the functionally described bombykol receptor BmOR1 (Sakurai et al. 2004) but somewhat stronger protein identities to *H. virescens* receptors (~46%), which, with the exception of HR6, are expressed exclusively in male antennae (Krieger et al. 2004). Phylogenetic analysis of MsextaOR1 using a neighbor joining tree with corrected distances to other moth receptors shows MsextaOR1 falls within the pheromone receptor subfamily of chemoreceptors (Figure 2). This subgroup was also identified in previous studies where the sequences share at least 34% of their amino acids (Krieger et al. 2003; Wanner, Anderson, et al. 2007; Mitsuno et al. 2008).

The expression pattern of *MsextaOR1* is shown by in situ hybridization at the dorsal edge of a male annulus (Figure 3A). Expression of *MsextaOR1* was limited to peripheral regions of the annulus where type-I trichoid sensilla are distributed and was not detected in any other cells. This distribution is correlated with the expression pattern of *M. sexta PBP1*, a male-specific pheromone binding protein (Figure 3C). *PBP1* was limited to the basal region of the epithelium just below type-I trichoid sensilla (Györgyi et al. 1988; Vogt et al. 1991). Vogt et al. (1991) interpreted this region to correspond to the area of the trichogen support cell shown in Sanes and Hildebrand (1976) to be apical to the neuronal somata of the sensilla. The heavy staining of these cells may be attributed to cellular density, similar to Vogt et al. (2002), and to expression levels of mRNA in the trichogen secretory cells. In all of our male sections, the expression pattern of *PBP1* was limited to the annular periphery, a pattern markedly different from that for *PBP2* and *PBP3* reported by Nardi et al. (2003) localized to the midannular region. Comparison of in situ expression patterns of *MsextaOR1* and *PBP1* indicate *MsextaOR1* localizes to cells between the trichogen support cell and the cuticle. We did not detect transcripts of *MsextaOR1* in female annular sections (Figure 3B).

MsextaOR2

A second *M. sexta* OR was discovered using degenerate inosine primers to amplify a 714-bp fragment from male antennal cDNA with a hydropathy plot showing seven transmembrane domains. The primers were designed against conserved regions of three unusually closely related proteins, Or83b from *D. melanogaster* (Clyne et al. 1999; Vosshall et al. 1999), an *A. gambiae* gene, AgOr7 (Hill et al. 2002), and a gene from a ±21 000 clone *A. mellifera* EST library AmOr3 (Whitfield et al. 2002). MsextaOR2 has a high sequence identity to Or83b (63%), *A. gambiae* AgOr7 (65%), *A. mellifera* AmOr3 (72%), and *H. virescens* HR2 (85%) and others including other lepidopteron species (Hill et al.

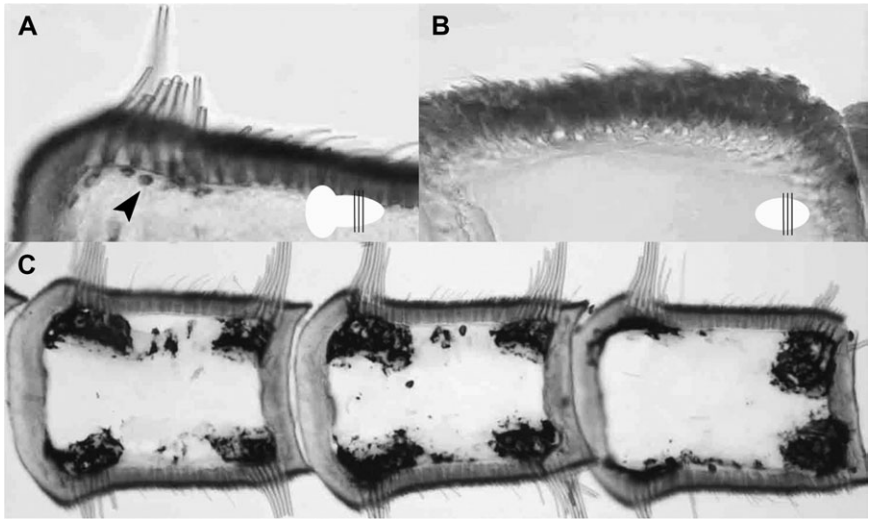

Figure 3 Male and female annuli with Dig-labeled probe to *MsextaOR1*. **(A)** Male annulus. The arrow indicates labeled single cells associated with different type-I trichoid sensilla. (40×) Inset indicates section. Single cell labeling associated with the male-specific sensilla and the absence of such labeling in female annuli suggests MsextaOR1 is a pheromone receptor. **(B)** Female annulus with no labeling. (40×) **(C)** Sagittal section of male annuli with Dig-labeled probe to *PBP1* in tricogen cells associated with male-specific type-I trichoid sensilla. (10×) The mass of PBP mRNA makes distinction of individual secretory cells difficult, but they are clearly associated with the long pheromone-sensitive sensilla at either end of segments.

2002; Krieger et al. 2002, 2003; Pitts et al. 2004; Jones et al. 2005; Mitsuno et al. 2008). To obtain an ortholog of Or83b, fully degenerate inosine primers designed to conserved regions were used to produce amplicons and partial sequences. *MsextaOR2* is a partial clone (1323 bp, 441 aa) that lacks a 5′ and 3′ end. It is about 20 amino acids shorter when compared with other receptors and 10 amino acids shorter on the 3′ end. In situ hybridization with DIG-labeled antisense probes to *MsextaOR2* in male and female antennae reveals a topographic pattern similar to that found in other studies on the members of this highly conserved group of receptors (Figure 4) (Vosshall et al. 1999; Krieger et al. 2003; Pitts et al. 2004; Nakagawa et al. 2005). Characteristically, the Or83b ortholog, *MsextaOR2* positive cells are associated with a range of sensilla in male and female antennae including long trichoid sensilla. Our level of resolution does not allow the identification of the sensilla types, but positive cells are clearly visible throughout the sensory portion of the annuli (Figure 4).

MsextaOR3

Because the *M. sexta* genome is yet to be sequenced, we made a normalized male antennal cDNA library and subtracted the most abundant clones to enrich for rare transcripts. From the subtracted library, we sequenced 768 clones, and one was a full-length OR (1259 bp, 395 aa). Analysis by Kyte–Doolittle hydropathy (Kyte and Doolittle 1982) revealed seven transmembrane domains characteristic of ORs. Comparison of the protein sequence with *D. melanogaster* and *A. gambiae* ORs shows weak (<25%) overall identity, but MsextaOR3 has 62% identity with *H. virescens* receptor, HR17 and 55% identity with BmOR26. This small group with a relatively high degree of protein similarity represents a separate, perhaps nonpheromone group, of ORs in moths (Figure 2).

RT-PCR analysis

To assess the tissue-specific expression patterns of ORs in *M. sexta,* RT-PCR experiments were performed using sequence-specific primers that amplify ~200-bp sequences from cDNA produced from 1 μg of total RNA. All PCR bands were of the expected size for each receptor and for the control gene *RPS3* (Figure 5). *MsextaOR1* was limited to expression in male antennae suggesting, a male-specific function such as pheromone detection (Sakurai et al. 2004; Nakagawa et al. 2005). For *MsextaOR2*, the Or83b ortholog, bands are strongly expressed in male and female antennae with somewhat more expression in the female antennae. The bands for *MsextaOR3* were much fainter than for other receptors. Although the brightest bands were the expected 296 bp, there are three bands of smaller PCR products, which suggests inefficient primer design under the conditions used. Because nested PCR was necessary for adequate visualization of PCR, we cannot compare the relative amounts with much assurance.

qRT-PCR

The difficulty in resolving the tissue specificity of *MsextaOR3* led to a second group of experiments using

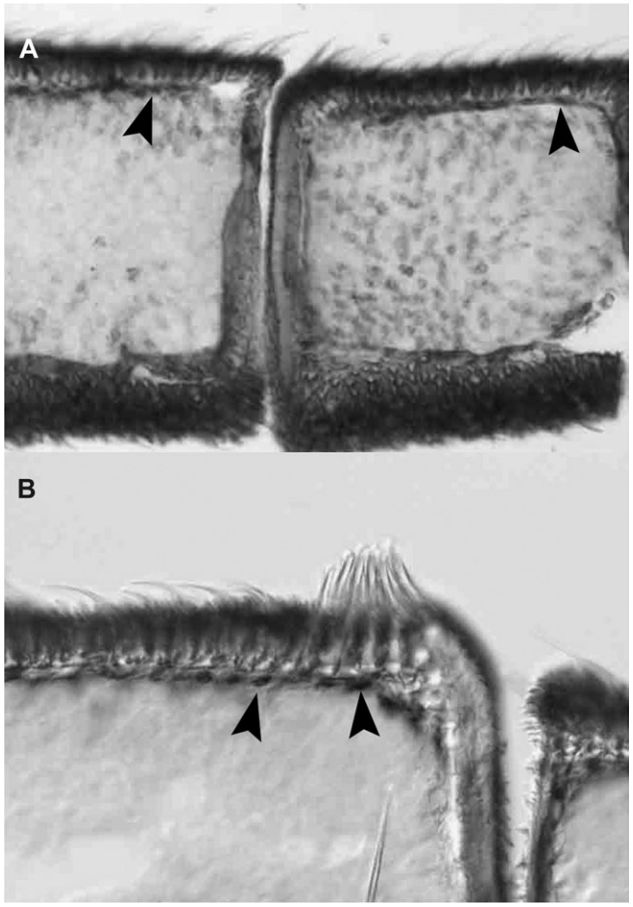

Figure 4 Ubiquitous expression of Or83b ortholog *MsextaOR2* in female and male antennae. **(A)** Proximal sagittal section of female annuli with Dig-labeled probe to *MsextaOR2*. Note expression in many cells associated with different types of sensilla along the epidermal edge. These sections were made close to the cuticle and reveal cells in central somata associated with sensilla on windward edge. (40×) **(B)** Sagittal section of male annuli with Dig-labeled probe to *MsextaOR2*. Expression in this section is only noticeable at the top edge of the annuli, indicated by the arrows. Male-specific type-I trichoid sensilla are to the right of the center. *MsextaOR2* is associated with many types of sensilla including male-specific type-I trichoid sensilla, a pattern typical of Or83b-related receptors (40×).

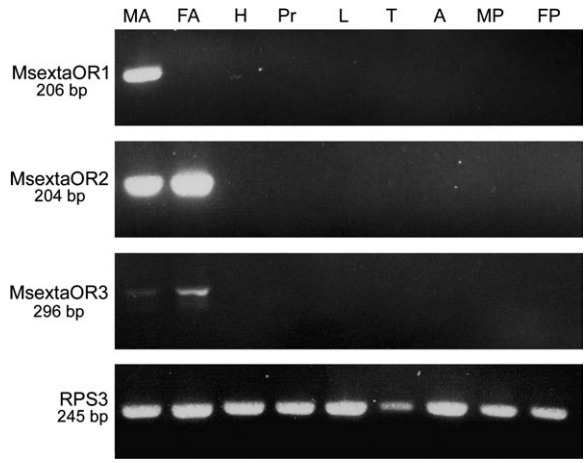

Figure 5 RT-PCR tissue-specific expression patterns of ORs in *Manduca sexta*. *MsextaOR1* is expressed only in male antennae as would be expected for a pheromone receptor. *MsextaOR2* is expressed at high levels in only male and female antennae. Similar patterns have been described for nonORs in the Or83b subfamily. There are low levels of expression of *MsextaOR3* in male and female antennae. Our inability to produce satisfactory in situs for this receptor could be due to its low expression levels or its expression in only a few, and perhaps hard to experimentally identify, cells. Samples are pooled, and all tissue expression is compared with the control gene *RPS3*. Nested PCR was necessary to visualize expression on an agarose gel with EtBr staining. MA, male antennae; FA, female antennae; H, male head without palps, proboscis, antennae; Pr, male proboscis; L, male foreleg; T, male thorax; A, male abdomen; MP, male maxillary palp; and FP, female maxillary palp.

qRT-PCR. For qRT-PCR, primers were designed to amplify small ~100-bp PCR products. In these experiments, primers were not designed across introns, so there was the possibility of DNA contamination. This is most likely the reason for small levels of amplification in male thorax and abdomen for all the genes tested. The RT-PCR results for *MsextaOR1* were similar to the results for RT-PCR in that only male antennae showed expression of *MsextaOR1* RNA (Figure 6A). The relative levels of expression of *MsextaOR1* are 4150-fold higher in male antennae than in any other tissue. qRT-PCR results for *MsextaOR2* (Figure 6B) show expression levels in the female antennae to be 6400 times that of male antennae, and no expression is observed in the male proboscis. However, in the RT-PCR experiments, the expression in male antennae is not low when visualized on an agarose gel with EtBr staining. This can be explained by the nested PCR, which was necessary to produce a clearly distinguishable band using this method. qRT-PCR allowed for better resolution of expression levels and tissue specificity for *MsextaOR3* (Figure 6C) than did RT-PCR. Expression of this receptor in female antennae was more than 450 times greater than in male antennae.

Discussion

MsextaOR1

To clone and characterize a pheromone receptor from *M. sexta*, we hypothesized this receptor would be exclusively expressed in antennae of male moths. Differential screening of a previously described cDNA library (Robertson et al. 1999) resulted in a 431 amino acid OR that we designated *MsextaOR1*. Several lines of evidence suggest this OR is a pheromone receptor. Similar to the male antennae-specific pattern of the bombykol and bombykal receptors in *B. mori*, *BmOR1*, and *BmOR3*, respectively, *MsextaOR1* is limited to expression in male antennae (Figure 3), suggesting a male-specific function, such as pheromone detection (Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2007; Mitsuno et al. 2008). qRT-PCR results indicate that *MsextaOR1* is expressed exclusively and at higher levels

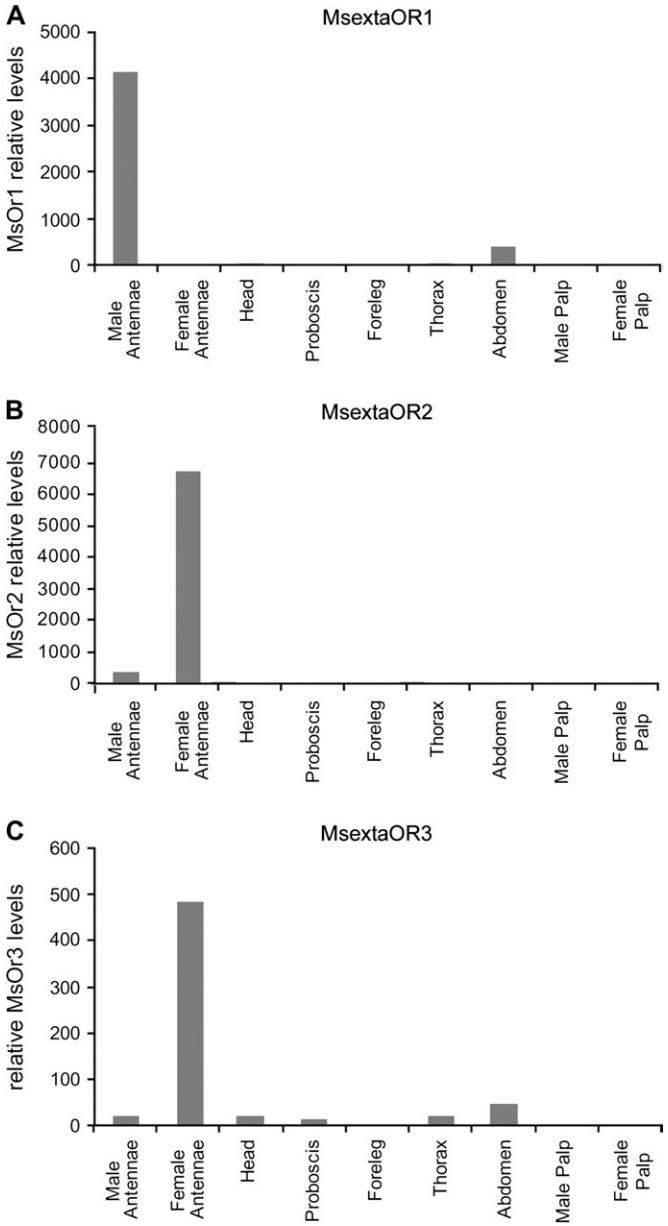

Figure 6 qRT-PCR comparing tissue-specific expression patterns for ORs of *Manduca sexta*. **(A)** Expression patterns for *MsextaOR1* were similar to the results for RT-PCR in that only male antennae showed expression of *MsextaOR1* RNA. The relative levels of expression of *MsextaOR1* are 4150-fold higher in male antennae than in any other tissue. **(B)** qRT-PCR results for *MsextaOR2* show expression in both male and female antennae. The reason for the higher levels of expression in females is not clear, although this result was repeatable. **(C)** qRT-PCR allowed for better resolution of expression levels and tissue specificity for *MsextaOR3* than did RT-PCR. Expression of this receptor in female antennae was more than 450 times greater than in male antennae. Primers were not designed across introns to avoid DNA contamination; therefore, low levels of expression in other tissues, not reflected in the RT-PCR results, could be due to contamination. The $2^{-\Delta\Delta Ct}$ method was used to measure relative expression levels across the samples (Livak and Schmittgen 2001). The expression ratios of individual samples were first normalized to the endogenous control *RPS3* and then normalized to foreleg within each trial.

(>4000-fold) in male antennae than in any other tissue examined (Figure 6A). This expression level is similar in magnitude to that observed for *D. melanogaster* receptor *Or83b*, which is coexpressed in >75% of olfactory neurons in *D. melanogaster* (Larsson et al. 2004). In male *M. sexta*, 40% of the antennal neurons arise from male-specific long type-I trichoid sensilla (Keil 1989). Given the relative abundance of mRNA, it is likely to be expressed in ubiquitous neurons such as those associated with male sensilla. Phylogenetic analysis (Figure 2) reveals MsextaOR1 is most similar to a subgroup of ORs shown to respond to a pheromone ligand (Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2007; Mitsuno et al. 2008). Furthermore, MsextaOR1 is most similar (61% identity) to *B. mori* bombykal-binding OR BmOR3 (Nakagawa et al. 2005) suggesting a similar function, although this deduction does not hold true for other pheromone receptors (see Mitsuno et al. 2008). For example, BmOR6 and HR6 also belong phylogenetically to the group of moth pheromone receptors although they are not expressed exclusively in male antennae (Wanner, Anderson, et al. 2007). This raises the possibility that although these ORs shared a common lineage, they may not be functionally homologous.

To date, six moth pheromone receptors (the *B. mori* ORs BmOR1 and BmOR3, HR13 from *H. virescens*, and PxOR1 from *P. xylostella*, MsOR1 from *M. separata*, and a receptor from *D. indica* DiOR1) have been functionally described (Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2007; Mitsuno et al. 2008). All of these are expressed in male antennae in cells in close proximity to cells that expressed pheromone binding protein (PBP). In *M. sexta*, electrophysiological recordings reveal that three types of type-1 trichoid sensilla, each with two neurons tuned to different constituents of the pheromone blend: bombykal and EE, bombykal and EEE, or bombykal and EEZ, which make up more than 85% of the sensilla surveyed (Kaissling et al. 1989; Kalinová et al. 2001). Because differential screening is likely to find the most ubiquitous pheromone OR and given the high relatively level of MsextaOR1 expression in male antenna as revealed by qRT-PCR it is likely that MsextaOR1 detects the major pheromone bombykal but does not exclude the possibility that this receptor could be tuned to other components of the pheromone blend. The high degree of sequence similarity between MsextaOR1 and the bombykal receptor BmOR3 further strengthens this view.

We attempted to use the GAL4-UAS system to misexpress *MsextaOR1* in *D. melanogaster* antenna (Brand and Perrimon 1993; Dobritsa et al. 2003; Hallem et al. 2004). Tests with constituents of the pheromone blend including bombykal and C-15, a stable mimic of EEZ, did not result in identification of the ligand that binds MsextaOR1. Recent studies in *D. melanogaster* (Xu et al. 2005) and moths (Pophof 2004; Syed et al. 2006) suggest that in least some instances, PBPs may be necessary for pheromone–ligand binding in the context of olfactory sensilla.

MsextaOR2

A second *M. sexta* OR, *MsextaOR2*, was discovered by using sequence homology to isolate a 441 amino acid peptide fragment from male antennal cDNA. MsextaOR2 is a member of a unique subgroup of genes closely related to Or83b. This subgroup can be distinguished in that they are significantly diverged from odorant binding ORs, they share between 60% and 80% amino acid identity, they are larger in size (e.g., Or83b, 486 aa), and they are coexpressed in ~70% to 80% of odorant neurons on the antennae, legs, maxillary palps, and proboscis of many insects (Clyne et al. 1999; Vosshall et al. 1999; Vosshall et al. 2000; Krieger et al. 2003; Larsson et al. 2004; Melo et al. 2004; Pitts et al. 2004; Benton et al. 2006; Sato et al. 2008). RT-PCR and qRT-PCR show that *MsextaOR2* is expressed in male and female antennae but not in any other tissue in this study (Figures 5 and 6). The high relative expression level in female antennae is difficult to account for. Estimates of numbers of chemosensory neurons are not accurate enough to make an absolute assessment, but it is unlikely that the differences would be orders of magnitude (Shields and Hildebrand 1999a, 1999b). In situ results (Figure 4) are similar to those in other studies where this receptor type is expressed in a wide range of sensilla across the sensory organ (Vosshall et al. 2000; Krieger et al. 2003; Melo et al. 2004; Pitts et al. 2004).

Extensive experimentation has established that Or83b is necessary for olfactory response in *Drosophila* (Larsson et al. 2004; Benton et al. 2006; Wicher et al. 2008). In odorant sensing neurons, it forms a heteromeric receptor complex with a variable ligand-binding OR (Larsson et al. 2004; Benton et al. 2006; Sato et al. 2008; Wicher et al. 2008). OR complexes have been described as ligand-activated nonselective cation channels (Sato et al. 2008). On stimulation with an odorant heterologous cells expressing the *Drosophila* OR/Or83b complex show an influx of Ca^{2+} and cation-non-selective conductance (Sato et al. 2008; Wicher et al. 2008). These authors have concluded that the complex of OR and Or83b itself is responsible for odorant gated-channel activity (Sato et al. 2008; Wicher et al. 2008). Similar results were shown when *B. mori* and *A. gambiae* ORs were expressed with their Or83b ortholog in the heterologous expression system suggesting a universal function for OR coreceptors in insects (Sato et al. 2008). More evidence for an evolutionarily conserved function for Or83b-like genes was provided by transgene experiments where wild-type function could be rescued by transgene misexpression from three pest species (Jones et al. 2005). The relatively high 63% identity and the conserved motifs of MsextaOR2, as well as a similar expression pattern, suggest that this receptor is an ortholog of Or83b and related receptors.

MsextaOR3

In an attempt to identify another pheromone receptor by enrichment of male antennal cDNA library through normalization and subtraction of common clones, we identified a third receptor—*MsextaOR3*. Kyte–Doolittle hydropathy plots shows a characteristic seven-transmembrane motif expected for a pheromone receptor but RT-PCR and qRT-PCR show that this OR is expressed in both male and female antennae. The low level of expression of this receptor is clear in the RT-PCR results (Figure 5), but the 460-fold greater expression of this gene in female antennae suggests a predominantly female role for this receptor (Figure 6C). MsextaOR3 belongs to a subgroup of moth ORs (Figure 2) that are distinct from the "pheromone" subgroup of MsextaOR1 and BmOR1, further suggesting a biologically different function for this gene. Plant-associated compounds may be ligands for MsextaOR3. A few studies focused on the peripheral neuronal sensitivity of these compounds *M. sexta* (Shields and Hildebrand 2000; Kalinová et al. 2001). In addition to these odorants, the minor pheromone component Z11 has been shown to stimulate neurons on male and female sensilla (Kalinová et al. 2001).

Conclusion

We used three cloning strategies to identify diverse ORs in the antennae of *M. sexta*. *MsextaOR1* is related to a lineage of receptors that include the known moth pheromone ORs. We hypothesized, based on sequence identity, expression levels and topographic distribution, that MsextaOR1 may bind bombykal. Further functional studies will be necessary, perhaps with a solubilizing protein, to determine the ligand that binds MsextaOR1, but a comparison of these receptors and others in the subgroup will allow for analysis of the evolutionary relationship between ligand, peripheral detection and higher-order processing.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

Funding

NSF Sensory Systems Board and the Research Board and Critical Research Initiatives at the University of Illinois at Urbana-Champaign.

Acknowledgements

Nate Hutchens assisted with library screens for *MsextaOR1* and similar ORs. Greg New conducted sequencing and identification of insect orthologs to Or83b that led to the discovery of *MsextaOR2*. Lauren Kent was invaluable in helping with the transgenic GAL4-UAS project. Scott Robinson and the Imaging Technology Group at the Beckman Institute at UIUC provided skilled assistance with ESEM imaging. Alvaro Hernandez at the W. M. Keck Center for Comparative and Functional Genomics assisted with the EST library for *MsextaOR3*. Christina Grozinger, Adrienne Moran Lauter, and Tom Newman assisted with the qRT-PCR analysis.

May Berenbaum, Jim Nardi, Stewart Berlocher, and Christina Grozinger provided comments on early versions of the manuscript.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Anderson P, Hansson BS, Löfqvist J. 1995. Plant-odour-specific receptor neurones on the antennae of female and male *Spodoptera littoralis*. Physiol Entomol. 20:189–198.

Anderson P, Larsson M, Löfqvist J, Hansson BS. 1996. Plant odour receptor neurones on the antennae of two moths *Spodoptera littoralis* and *Agrotis segetum*. Entomol Exp Appl. 80:32–34.

Benton R, Sachse S, Michnick SW, Vosshall LB. 2006. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. PLoS Biol. 4:e20.

Bonaldo MF, Lennon G, Soares MB. 1996. Normalization and subtraction: two approaches to facilitate gene discovery. Genome Res. 6:791–806.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118:401–415.

Bray S, Amrein H. 2003. A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. Neuron. 39:1019–1029.

Christensen TA, Hildebrand JG. 1987. Functions, organization, and physiology of the olfactory pathways in the Lepidopteran brain. In: Gupta AP, editor. Arthropod brain: its evolution, development, structure and functions. New York: John Wiley and Sons. p. 457–484.

Christensen TA, Hildebrand JG, Tumlinson JH, Doolittle RE. 1989. Sex pheromone blend of *Manduca sexta*: responses of central olfactory interneurons to antennal stimulation in male moths. Arch Insect Biochem Physiol. 10:281–291.

Clyne PJ, Warr CG, Carlson JR. 2000. Candidate taste receptors in *Drosophila*. Science. 287:1830–1834.

Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR. 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron. 22:327–338.

Dobritsa AA, van der Goes van Naters W, Warr CG, Steinbrecht RA, Carlson JR. 2003. Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. Neuron. 37:827–841.

Dunipace L, Meister S, McNealy C, Amrein H. 2001. Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system. Curr Biol. 11:822–835.

Engels WR. 1993. Contributing software to the internet: the Amplify program. Trends Biochem Sci. 18:448–450.

Fox AN, Pitts RJ, Robertson HM, Carlson JR, Zwiebel LJ. 2001. Candidate odorant receptors from the malaria vector mosquito *Anopheles gambiae* and evidence of down-regulation in response to blood feeding. Proc Natl Acad Sci USA. 98:14693–14697.

Gao Q, Chess A. 1999. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. Genomics. 60:31–39.

Grosse-Wilde E, Gohl T, Bouche E, Breer H, Krieger J. 2007. Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. Eur J Neurosci. 25:2364–2373.

Györgyi TK, Roby-Shemkovitz AJ, Lerner MR. 1988. Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: a tissue-specific developmentally regulated protein. Proc Natl Acad Sci USA. 85:9851–9855.

Hallem EA, Ho MG, Carlson JR. 2004. The molecular basis of odor coding in the *Drosophila* antenna. Cell. 117:965–979.

Hansson BS. 1995. Olfaction in Lepidoptera. Experientia. 51:1003–1027.

Hildebrand JG. 1995. Analysis of chemical signals by nervous systems. Proc Natl Acad Sci USA. 92:67–74.

Hildebrand JG. 1996. Olfactory control of behavior in moths: central processing of odor information and the functional significance of olfactory glomeruli. J Comp Physiol [A]. 178:5–19.

Hildebrand JG, Rossler W, Tolbert LP. 1997. Postembryonic development of the olfactory system in the moth Manduca sexta: primary-afferent control of glomerular development. Semin Cell Dev Biol. 8:163–170.

Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, Chrystal MA, Cravchik A, Collins FH, Robertson HM, Zwiebel LJ. 2002. G protein-coupled receptors in *Anopheles gambiae*. Science. 298:176–178.

Homberg U, Christensen TA, Hildebrand JG. 1989. Structure and function of the deutocerebrum in insects. Annu Rev Entomol. 34:477–501.

Jiang H, Wang Y, Kanost MR. 1996. Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. Insect Mol Biol. 5:31–38.

Jones WD, Nguyen TA, Kloss B, Lee KJ, Vosshall LB. 2005. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. Curr Biol. 15:R119–R121.

Kaissling KE, Hildebrand JG, Tumlinson JH. 1989. Pheromone receptor cells in the male moth *Manduca sexta*. Arch Insect Biochem Physiol. 10:273–279.

Kalinová B, Hoskovec M, Liblikas I, Unelius CR, Hansson BS. 2001. Detection of sex pheromone components in *Manduca sexta* (L.). Chem Senses. 26:1175–1186.

Kanzaki R, Arbas EA, Hildebrand JG. 1991. Physiology and morphology of descending neurons in pheromone-processing olfactory pathways in the male moth *Manduca sexta*. J Comp Physiol [A]. 169:1–14.

Kanzaki R, Arbas EA, Strausfeld NJ, Hildebrand JG. 1989. Physiology and morphology of projection neurons in the antennal lobe of the male moth *Manduca sexta*. J Comp Physiol [A]. 165:427–453.

Karlson P, Luscher M. 1959. Pheromones: a new term for a class of biologically active substances. Nature. 183:55–56.

Keil TA. 1989. Fine structure of the pheromone-sensitive sensilla on the antenna of the hawkmoth, *Manduca sexta*. Tissue and Cell. 21: 139–151.

Krieger J, Grosse-Wilde E, Gohl T, Breer H. 2005. Candidate pheromone receptors of the silkmoth *Bombyx mori*. Eur J Neurosci. 21:2167–2176.

Krieger J, Grosse-Wilde E, Gohl T, Dewer YM, Raming K, Breer H. 2004. Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*). Proc Natl Acad Sci USA. 101:11845–11850.

Krieger J, Klink O, Mohl C, Raming K, Breer H. 2003. A candidate olfactory receptor subtype highly conserved across different insect orders. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 189:519–526.

Krieger J, Raming K, Dewer YME, Bette S, Conzelmann S, Breer H. 2002. A divergent gene family encoding candidate olfactory receptors of the moth Heliothis virescens. Eur J Neurosci. 16:619–628.

Kurtovic A, Widmer A, Dickson BJ. 2007. A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. Nature. 446:542–546.

Kyte J, Doolittle RF. 1982. A simple method for displaying the hydropathic character of a protein. J Mol Biol. 157:105–132.

Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. 2004. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron. 43:703–714.

Lee JK, Strausfeld NJ. 1990. Structure, distribution and number of surface sensilla and their receptor cells on the olfactory appendage of the male moth *Manduca sexta*. J Neurocytol. 19:519–538.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 25:402–408.

Melo AC, Rutzler M, Pitts RJ, Zwiebel LJ. 2004. Identification of a chemosensory receptor from the yellow fever mosquito, *Aedes aegypti*, that is highly conserved and expressed in olfactory and gustatory organs. Chem Senses. 29:403–410.

Mitsuno H, Sakurai T, Murai M, Yasuda T, Kugimiya S, Ozawa R, Toyohara H, Takabayashi J, Miyoshi H, Nishioka T. 2008. Identification of receptors of main sex-pheromone components of three *Lepidopteran* species. Eur J Neurosci. 28:893–902.

Nakagawa T, Sakurai T, Nishioka T, Touhara K. 2005. Insect sex–pheromone signals mediated by specific combinations of olfactory receptors. Science. 307:1638–1642.

Nardi JB, Miller LA, Walden KK, Rovelstad S, Wang L, Frye JC, Ramsdell K, Deem LS, Robertson HM. 2003. Expression patterns of odorant-binding proteins in antennae of the moth *Manduca sexta*. Cell Tissue Res. 313:321–333.

Pitts RJ, Fox AN, Zwiebel LJ. 2004. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci USA. 101:5058–5063.

Pophof B. 2004. Pheromone-binding proteins contribute to the activation of olfactory receptor neurons in the silkmoths antheraea polyphemus and *Bombyx mori*. Chem Senses. 29:117–125.

Robertson HM, Martos R, Sears CR, Todres EZ, Walden KK, Nardi JB. 1999. Diversity of odourant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. Insect Mol Biol. 8:501–518.

Robertson HM, Warr CG, Carlson JR. 2003. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. Proc Natl Acad Sci USA. 100(Suppl 2):14537–14542.

Sakurai T, Nakagawa T, Mitsuno H, Mori H, Endo Y, Tanoue S, Yasukochi Y, Touhara K, Nishioka T. 2004. Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. Proc Natl Acad Sci USA. 101:16653–16658.

Sanes JR, Hildebrand JG. 1976. Origin and morphogenesis of sensory neurons in an insect antenna. Dev Biol. 51:300–319.

Sato K, Pellegrino M, Nakagawa T, Vosshall LB, Touhara K. 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature. 452:1002–1006.

Scott K, Brady RJr, Cravchik A, Morozov P, Rzhetsky A, Zuker C, Axel R. 2001. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. Cell. 104:661–673.

Shields VD, Hildebrand JG. 2000. Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. J Comp Physiol [A]. 186: 1135–1151.

Shields VD, Hildebrand JG. 2001. Recent advances in insect olfaction, specifically regarding the morphology and sensory physiology of antennal sensilla of the female sphinx moth *Manduca sexta*. Microsc Res Tech. 55:307–329.

Shields VDC, Hildebrand JG. 1999a. Fine structure of antennal sensilla of the female sphinx moth, *Manduca sexta* (Lepidoptera: Sphingidae) I. trichoid and basiconic sensilla. Can J Zool. 77:290–301.

Shields VDC, Hildebrand JG. 1999b. Fine structure of antennal sensilla of the female sphinx moth, Manduca sexta (Lepidoptera: Sphingidae) II. auriculate, coeloconic, and styliform complex sensilla. Can J Zool. 77:302–313.

Starratt AN, Dahm KH, Allen N, Hildebrand JG, Payne TL, Röller H. 1979. Bombykal, a sex pheromone of the sphinx moth *Manduca sexta*. Z Naturforsch. 34C:9–12.

Syed Z, Ishida Y, Taylor K, Kimbrell DA, Leal WS. 2006. Pheromone reception in fruit flies expressing a moth's odorant receptor. Proc Natl Acad Sci USA. 103:16538–16543.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24: 1596–1599.

Tillman JA, Seybold SJ, Jurenka RA, Blomquist GJ. 1999. Insect pheromones—an overview of biosynthesis and endocrine regulation. Insect Biochem Mol Biol. 29:481–514.

Tumlinson JH, Brennan MM, Doolittle RE, Mitchell ER, Brabham A, Mazomenos BE, Baumhover AH, Jackson DM. 1989. Identification of a pheromone blend attractive to *Manduca sexta* (L.) males in a wind tunnel. Arch Insect Biochem Physiol. 10:255–271.

Tumlinson JH, Mitchell ER, Doolittle RE, Jackson DM. 1994. Field tests of synthetic *Manduca sexta* sex pheromone. J Chem Ecol. 20:579–591.

Vogt RG, Prestwich GD, Lerner MR. 1991. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. J Neurobiol. 22:74–84.

Vogt RG, Rogers ME, Franco MD, Sun M. 2002. A comparative study of odorant binding protein genes: differential expression of the PBP1-GOBP2 gene cluster in Manduca sexta (Lepidoptera) and the organization of OBP genes in Drosophila melanogaster (Diptera). J Exp Biol. 205(6):719–744.

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R. 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell. 96: 725–736.

Vosshall LB, Wong AM, Axel R. 2000. An olfactory sensory map in the fly brain. Cell. 102:147–159.

Wanner KW, Anderson AR, Trowell SC, Theilmann DA, Robertson HM, Newcomb RD. 2007. Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. Insect Mol Biol. 16:107–119.

Wanner KW, Nichols AS, Walden KK, Brockmann A, Luetje CW, Robertson HM. 2007. A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. Proc Natl Acad Sci USA. 104:14383–14388.

Warr C, Clyne P, de Bruyne M, Kim J, Carlson JR. 2001. Olfaction in *Drosophila*: coding, genetics and e-genetics. Chem Senses. 26:201–206.

Whitfield CW, Band MR, Bonaldo MF, Kumar CG, Liu L, Pardinas JR, Robertson HM, Soares MB, Robinson GE. 2002. Annotated expressed sequence tags and cDNA microarrays for studies of brain and behavior in the honey bee. Genome Res. 12:555–566.

Wicher D, Schafer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS. 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature. 452:1007–1011.

Xu P, Atkinson R, Jones DN, Smith DP. 2005. *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron. 45:193–200.

Accepted January 2, 2009