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Identification of a Chemosensory Receptor from the Yellow Fever Mosquito, *Aedes aegypti*, that is Highly Conserved and Expressed in Olfactory and Gustatory Organs

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Abstract

Aedes aegypti is a highly anthropophilic mosquito responsible for the transmission of dengue and yellow fever around the world. Like other mosquitoes, the biting and host preference behaviors of this disease vector are largely influenced by its sense of smell, which is presumably facilitated by G protein-coupled receptor signaling cascades. Here, we report the identification and characterization of *AaOr7*, the first candidate odorant receptor from *Ae. aegypti*. This receptor displays extremely high primary amino acid conservation with *AgOr7* another candidate odorant receptor identified in the Aftrotropical malaria vector, *Anopheles gambiae* as well as several previously identified candidate odorant receptors in *Drosophila melanogaster* and other insects. Its transcript is expressed in adult chemosensory tissues and during several stages of *Ae. aegypti* development. Within the adult olfactory system, AaOr7 protein is found specifically within most antennal and maxillary palp sensilla, as well as in a subset of proboscis sensilla. These results are consistent with a role for *AaOr7* in olfaction and gustation supporting the hypothesis that *AaOr7* and its orthologs may be of general importance to chemosensory processes throughout the lifetime of an insect.

Key words: host preference, mosquito, olfaction, receptor, vector

Introduction

The control of arthropod-borne diseases presents a great challenge for global public health officials. Every year hundreds of millions of people suffer from malaria, dengue fever, yellow fever and encephalitis, according to the World Health Organization (for more information, visit http://www.who.int). Female mosquitoes, requiring a vertebrate blood meal in order to complete their gonodatrophic reproductive cycle, are responsible for the transmission of each of those diseases. Olfaction has the greatest influence on the host preference behavior of mosquitoes (Takken and Knols, 1999). As such, this sensory mode makes a significant contribution to the mosquitoes' ability to transmit disease, a quantifiable characteristic known as vectorial capacity (Macdonald, 1957). Similarly, olfaction contributes to the impact of many other insects of economic and medical importance (Zwiebel and Takken, 2004). As part of a general study of olfaction in disease vector mosquitoes, we have used molecular and informatics based approaches to search for genes that are potentially active in olfactory signal transduction in *Aedes aegypti*.

The first *Ae. aegypti* odorant receptor identified, named *AaOr7*, is a member of a group of extremely well conserved, single gene orthologs from other insects including *Anopheles gambiae* (Hill *et al.*, 2002), *Drosophila melanogaster* (*DOr83b*; Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999), *Heliothis virescens* (*HvirR2*; Krieger *et al.*, 2002), *Apis mellifera* (*AmelR2*) and others (Krieger *et al.*, 2003). Each of these genes is expressed broadly in olfactory tissues, in contrast to other insect odorant receptors that are characteristically divergent and display restricted expression in a subset of olfactory neurons (Clyne *et al.*, 1999; Vosshall *et al.*, 1999; Krieger *et al.*, 2002). In this report we demonstrate that *AaOr7* is widely expressed in olfactory and gustatory organs of adults and during immature stages. The high level of conservation of the primary amino acid sequence and the broad expression profile of *AaOr7* in olfactory and

gustatory tissues suggests that it may play a significant and generalized role in chemosensory signal transduction in mosquitoes.

Materials and methods

Mosquito rearing

Ae. aegypti (Costa Rica strain) were reared as previously described (Fox *et al.*, 2001) For stock propagation, 4- to 5-day-old female mosquitoes were blood-fed for 20–30 min using anesthetized mice, following guidelines set by the Vanderbilt Institutional Animal Care and Use Committee.

Isolation of AaOr7

A pair of degenerate primers (*AaOr7*.5′2F, 5′-GCNATH-GGNGAYCANTAYGGN-3′ and *AaOr7*.3′2R, 5′-RTCRTACCARTGRCANGARTA-3′) were synthesized based on the conserved carboxy-terminal end of the deduced amino acid compositions of *AgOr7* and *DOr83b* and were used to amplify a 240 base pair product from antennal cDNA of *Ae. aegypti* under the following conditions: 50 mM $MgCl_2$, 10μm of each primer, 5 U/μl Taq DNA polymerase, annealing temperature of 50°C for five cycles, 52°C for five cycles, 54°C for five cycles and 56°C for 25 cycles. A near full length *AaOr7* was obtained by amplifying from *Ae. aegypti* antennal cDNA as above using one degenerate primer designed from the alignment of *AgOr7*, *DOr83b* and *Hvir*R2 (the kind gift of Dr Hugh Robertson, University of Illinois, Champagne/Urbana) and a specific primer designed from the short *AaOr7* sequence as follows: *Am*Or3-F4, 5′-GGIYTIGTIGCIGAYYTIATGCC-3′ and *AaOr7*.5′new, 5′-TCACTGATGAACTCTCTTCA-3′. A partial genomic clone was isolated by amplifying a fragment from *Ae. aegypti* genomic DNA using the primers: *AaOr7*.3′new, 5′-TGTTGCTTCACATGTTGA-3′; and *AaOr7*.rev1, 5′-TTATTTCAACTGCACCAACACCAT-3′. All PCR fragments were subcloned into the TOPO-TA cloning vector (Invitrogen) and sequenced using BigDye™ chemistry prior to analysis on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

Alignments

Primary amino acid sequences of the following genes were aligned using the Clustal X software package (Swofford, 1998). GenBank accession numbers are indicated in parentheses: *D. melanogaster* Or83b (NM079511), *H. virescens* R2 (AJ487477), *An. gambiae* Or7 (AY363725, AY363726), *Ae. aegypti* Or7 (AY582943), *A. mellifera* R2 (AJ555537), *Calliphora erythrocephala* R2 (AJ555538), *Tenebrio molitor* R2 (AJ555539), *Bombyx mori* R2 (AJ555487) and *Antheraea pernyi* R2 (AJ555486).

RNA expression

Total RNA was isolated from *Ae. aegypti* tissues using the RNeasy® kit (Qiagen): embryos (morning of laying), early instar larvae (2–4 days old), late instar larvae (10–14 days old), pupae, or adult tissues (4–6 days old). First strand cDNA synthesis was carried out using ~0.5μg RNA and SuperScript II Reverse Transcriptase (RNaseH-) according to the manufacturers protocol (Invitrogen). PCR was performed on each cDNA sample under the following conditions. Intron-spanning primers: *AaOr7*.3′new, 5′-TGTTGCTTCACATGTTGA-3′; *AaOr7*.rev1, 5′-TTATT-TCAACTGCACCAACACCAT-3′. The ribosomal protein 6 (*rps*6) gene (Hernandez and Fallon, 1999) was amplified in tandem from each sample as a control for cDNA integrity using the following primer pair: *rps*6.5′, 5′-ATCTCGGA-GACGAATGGAA-3′ and *rps*6.rev1, 5′-CTGCCTTCT-TCGGTTCAG-3′. Optimal annealing temperature was 56°C. RT–PCR products were gel purified using the QIAquick gel extraction kit (Qiagen), subcloned into the TOPO-TA cloning vector and sequenced as described above.

Antibody

Polyclonal antibodies were generated by immunizing white rabbits with a synthetic peptide designated Or7a, corresponding to amino acids 268–281 of *AgOr7* (NH_2-KSELIINEEKDPDV-COOH) that was coupled to keyhole limpet hemacyanin (KLH; Sigma-Genosys). IgGs were purified from crude sera using protein-G sepharose (Sigma) according to the manufacturers protocol, followed by subtraction against a KLH affigel-10 column (BioRad) prior to use in immunolocalizations.

Immunofluorescence

Immunofluorescence was performed as described (Dobritsa *et al.*, 2003) with the following modifications: 0.1% Triton X-100 was added to the 4% paraformaldehyde fixing buffer. As a secondary antibody, donkey anti-rabbit biotin conjugate, (1:500 in blocking solution: 1× PBS, 5% normal goat serum, 1% BSA, 0.01% Triton X-100) was applied for 2 h at 20°C, followed by incubation with streptavidin-Cy3 conjugate (1:1800 in blocking solution; both from Jackson Immunoresearch, West Grove, PA) for 1 h. In parallel control experiments, Or7 immunolocalizations were carried out using pre-immune sera as well as anti-OR7 antisera pre-incubated for 15 min on ice with a 100-fold molar excess of the Or7a peptide used for rabbit immunizations. Neurons were labeled with goat anti-horseradish peroxidase (HRP) at 1:500 in blocking solution. Nuclear counterstaining was performed using TOTO-3 (Molecular Probes Eugene, OR) diluted 1:5000 in PBS at RT for 20 min, followed by brief rinsing in H_2O. Slides were mounted with Vectashield fluorescent medium (Vector Laboratories Inc., Burlingame, CA). Confocal images were captured using a Zeiss Axioplan fluorescent microscope with the LSM 510 META system.

Other images were captured using a DP70 CCD camera (Olympus) attached to an Olympus BX-60 fluorescent microscope.

Results

AaOr7 gene

Using degenerate primers based on an alignment of the *AgOr7* and *DOr83b* peptide sequences, a small C-terminal fragment of *AaOr7*, was initially amplified from antennal cDNA and confirmed by DNA sequencing. In this manner, a gene-specific primer was designed and used in combination with a N-terminal degenerate primer to amplify a large fragment of the coding region from *Ae. aegypti* antennal cDNA. This clone encodes a partial peptide of 468 amino acids in length that is missing the N-terminus, a sequence that is likely to be <10 amino acid residues in length (Figure 1). A small C-terminal genomic fragment of *AaOr7* was also isolated and confirmed by sequencing.

The predicted *AaOr7* amino acid sequence was aligned with odorant receptor sequences of other insects and specifically those closely related to *AgOr7*. As shown in the alignment in Figure 1, all of the deduced amino acid sequences are very similar both in terms of their primary sequences and, for the complete sequences, in their lengths. Overall, the five full-length sequences, plus the presumably nearly full length *AaOr7* sequence, share >60% identity and >80% similarity. Most remarkable is the extreme conservation of sequence in the C-terminal one-third of the peptides, where all nine proteins are nearly 90% identical (Figure 1). Specifically, the *AaOr7* peptide shares 86% identity and 92% similarity overall with the *AgOr7* peptide and 95% identity in the terminal 160 amino acids.

RNA expression

Odorant receptors are expressed in sensory neurons in olfactory tissues of insects, including the antennae and maxillary palps (Vosshall *et al.*, 2000). The expression pattern of *AaOr7* was examined by non-quantitative RT-PCR analysis of tissues isolated from several developmental stages. The gene product was readily detectable in larvae as with the most robust expression observed in RNA isolated from larval heads (Figure 2). In contrast to its expression throughout the larval stages, *AaOr7* expression was less robust in pupae (Figure 2). In the adult, the expression of *AaOr7* was observed in the antennae, maxillary palps, organs that constitute the major olfactory tissues in adult mosquitoes (Clements, 1999), as well as in proboscises and legs (Figure 2).

Protein expression

To investigate the localization of the AaOr7 protein, we used an antibody that was raised against a 14 amino acid polypeptide domain corresponding to the Or7a peptide of

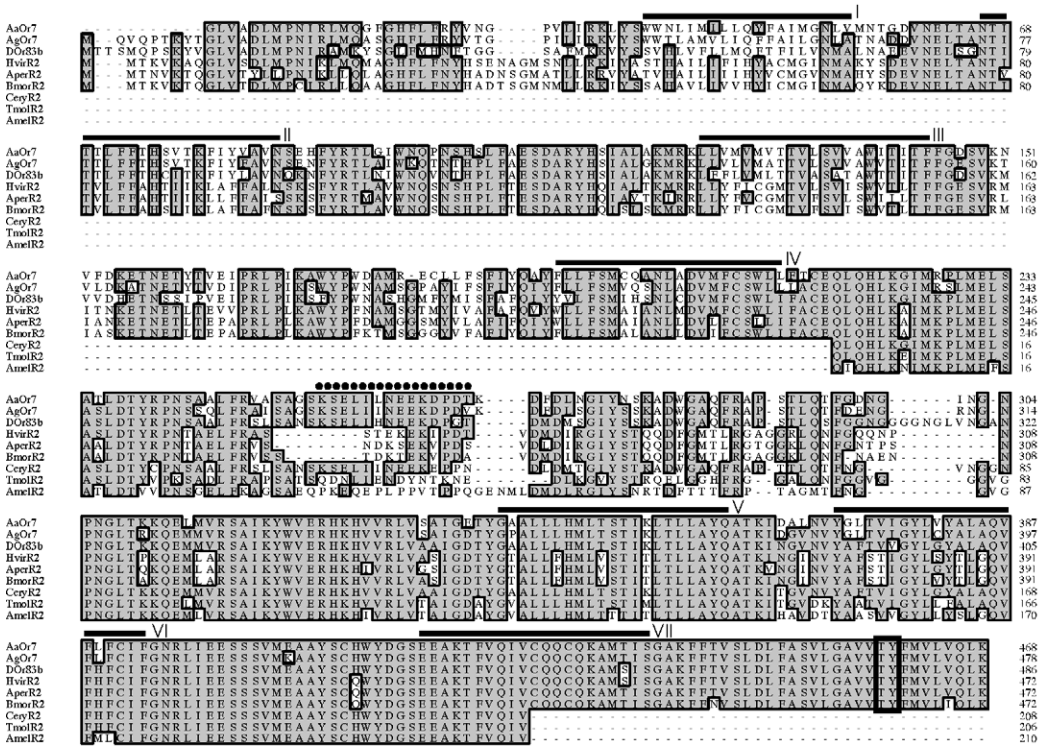

Figure 1 Alignment of AaOr7 ortholog peptides using the single amino acid code. Identical residues are shaded and boxed. Residue numbering is to the right. TMs I–VII are indicated with black bars. Threonine (T) and tyrosine (Y) residues that are sites of potential phosphorylation are enclosed in a heavy black box. Dotted line indicates peptide used for generating antiserum. For a list of genes and accession numbers see Materials and Methods.

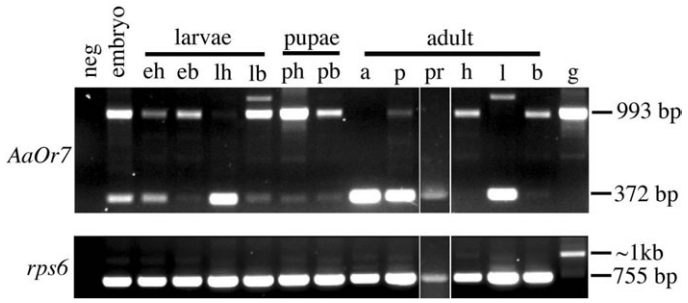

Figure 2 Expression of the AaOr7 gene in Aa. aegypti by RT–PCR. RNA samples were isolated from several developmental stages and tissues. Lanes are: neg (no DNA control); embryo (freshly laid embryos post bloodfeed); eh (early stage larval heads); eb (early stage larval bodies); lh (late stage larval heads); lb (late stage larval bodies); ph (pupal heads); pb (pupal bodies); a (antennae); p (maxillary palps); pr (proboscises); h (heads); l (legs); b (bodies); g (genomic DNA control). Proboscises RT–PCR was performed in a separate experiment and added to the figure. Numbers indicate expected sizes of genomic and cDNA products in base pairs (bp). The ribosomal protein gene, rps6, was also amplified from each sample as a control for RNA integrity.

the AgOr7 protein. Despite the fact that AaOr7 has two amino acid substitutions in this domain, the polyclonal serum specifically labeled neurons in proximal, intermediate and distal segments of the female *Ae. aegypti* antenna (Figure 3). Importantly, no labeling was observed in control immunolocalization studies using either pre-immune serum or OR7 anti-serum pre-adsorbed with a molar excess of Or7a peptide (data not shown). While we were not able to obtain a single immunolabeled section that comprised all 13 segments, we frequently obtained overlapping sections that consisted of five to seven consecutive segments. Since the thicker and shorter proximal segments can be distinguished reasonably from the longer and thinner distal segments we conclude that we observed AaOr7-specific labeling in all flagellar segments.

Interestingly, AaOr7 labeling was observed mainly within sensilla trichodea (Figure 3D,E), which represent the largest group of sensory hairs (~650) on the antenna of the female yellow fever mosquito (McIver, 1982). We identified specific labeling in 97.5% of these sensilla ($n = 79$). Typically, each of these sensilla is innervated by two neurons, which respond to a wide variety of olfactory stimuli, including host related odors, oviposition attractants and repellents with characteristic specificities (Lacher, 1967; Davis and Rebert, 1972; Davis, 1977; Davis and Takahashi, 1980). Furthermore, a large number of neuronal cell bodies were labeled for AaOr7 protein (Figure 3B–E). Concomitant labeling with an anti-HRP antibody, which has been used to identify neurons in insects (Jan and Jan, 1982; Sun and Salvaterra, 1995) indicated that ~20–30% of all neuronal cell bodies were labeled for AaOr7. In some cases, dendritic projections from cell bodies into sensilla could be observed (not shown).

It is noteworthy that within the first flagellar segment AaOr7 labeling was not observed in the proximal part (Figure 3B,C). Unlike the 12 distal segments, this segment is not surrounded by a whorl of sensilla chaetica at its base (Slifer and Sekhon, 1962). In contrast, such sensilla are emerging from the proximal half of the first segment. This area is also covered with scales and microtrichia, which we do not observe on the distal third of the first segment and are absent on all other flagellar segments (Slifer and Sekhon, 1962). We did, however, observe AaOr7 labeling in the distal third of the first segment, which is carrying hairs that resemble sensilla trichodea of narrow diameter. Ismail (1964) described a total of 31 sensilla trichodea on the first segment of the female *Ae. aegypti* antenna. Along the remainder of the length of the antennae, AaOr7 labeling was usually absent in grooved peg sensilla, which represent a second type of sensilla that have been described as having an olfactory function (Lacher, 1967; Kellogg, 1970). Occasionally, faint labeling was associated with the base of the latter hair type. Labeling of the dendrite with anti-HRP indicated that the structure was accessible to antibody. Importantly, AaOr7 labeling was never observed within scales, microtrichia or sensilla chaetica. Of these structures only the sensilla chaetica, which presumably function in mechanosensation, are innervated at their base (McIver, 1972).

The maxillary palp of female culicines consists of five segments, where the fifth, most distal, segment is reduced to a knob (McIver, 1982). A total of four types of hair structures are found on *Ae. aegypti* maxillary palps: non-innervated microtrichia and scales as well as mechanosensory sensilla chaetica and thin walled capitate peg sensilla. Of the latter, 29 are found on each of the palps of female *Ae. aegypti*, where they are restricted to segment 4. We find AaOr7 labeling in all capitate peg sensilla. In contrast to our observation, that a number of cell bodies were labeled with AaOr7 specific antibody in the antenna of female *Ae. aegypti*, we found few labeled cell bodies in the maxillary palp. Again, mechanosensory and non-innervated hair structures (microtrichia and scales) remained unlabeled (Figure 4A,B).

Two types of sensilla trichodea have been described on the labellum of female *Ae. aegypti* reviewed by McIver (1982). Based on the morphology, one sensillum type was considered a tactile receptor. The other sensillum type is apparently the equivalent of the T1 (type 1) sensillum, described by Pappas and Larsen (1976) on the labellum of the mosquito *Culiseta inornata*. Additionally, these authors described two other innervated sensillum types at the labellum of *Cs. inornata*, which where termed T2 and T3 sensilla (Pappas and Larsen, 1976). We did not identify T3 sensilla on the *Ae. aegypti* labellum, which are located on the oral surface of *Cs. inornata*. Yet, on the outer surface, we observed about 20 hairs on each half of the labellum matching the description of T2 sensilla. These resemble short hairs of ~7 μm length that reside in a socket (Figure 4D, inset). We found that AaOr7 labeling on the labellum was specific to T2 sensilla (Figure 4C,D), but not to T1

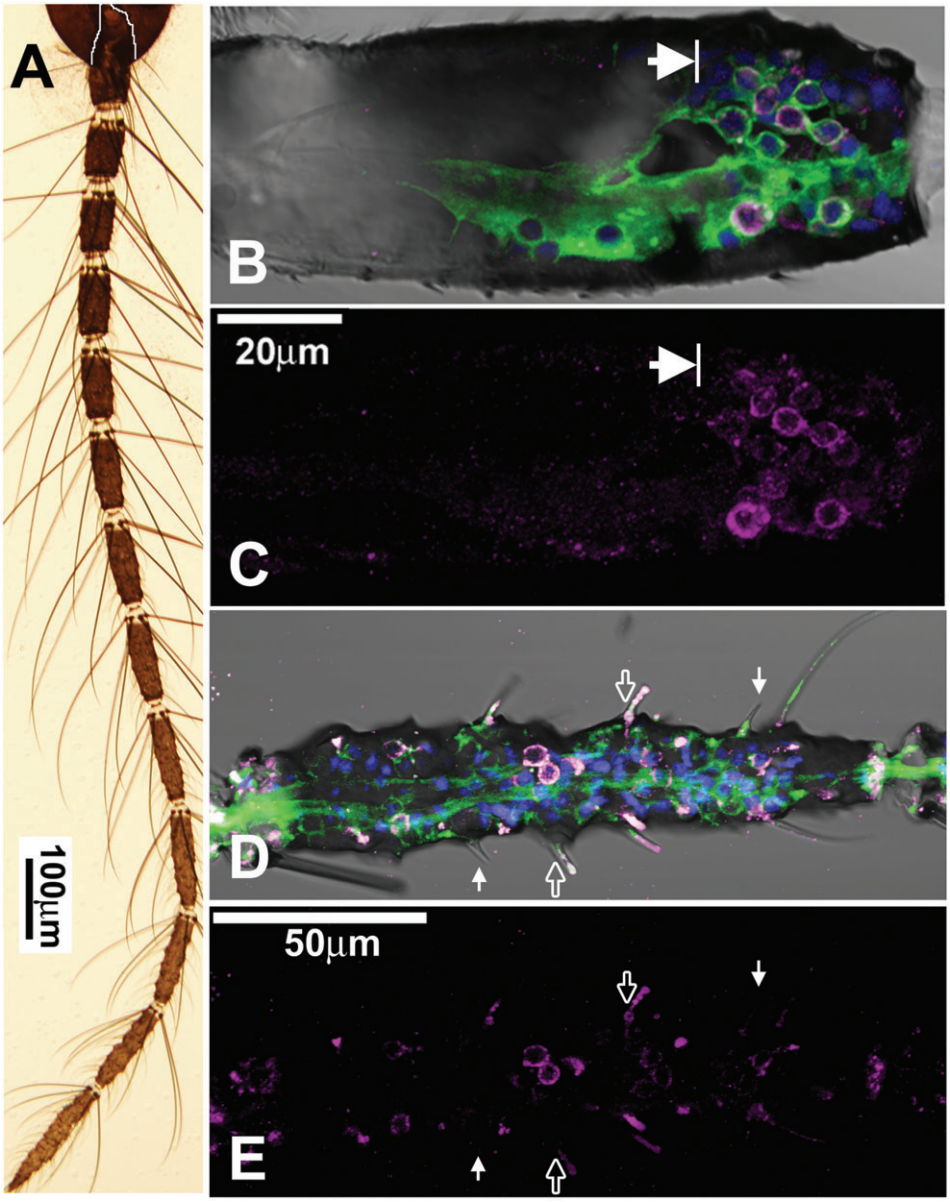

Figure 3 Overview of a female Ae. aegypti antenna (A). In the first flagellar segment of the female antenna, AaOr7 specific labeling is restricted to the distal third (right, indicated by arrow), where a number of labeled neuronal cell bodies and projecting dendrites are visible (B, C) AaOr7 specific labeling was observed along the whole antenna, an examples for a distal segment, presumably 9–11 (D, E), is presented. AaOr7 labeling is visible in dendrites innervating trichodic (framed arrows) but absent in grooved peg (filled arrows) sensilla. Composite of light-microscopic image, AaOr7 (magenta), neuronal (green) and nuclear (blue) specific labeling (B, D) and corresponding single image of AaOr7 specific labeling (C, E).

sensilla or microtrichia. Labeling of T2 sensilla was mostly confined to the base of each sensillum, but weak labeling resembling a dendritic structure innervating this sensillum type was observed occasionally (Figure 4D). Rarely were we able to identify the very weakly labeled outline of a neuronal cell body (not shown).

Discussion

In this report we have identified *AaOr7*, a candidate chemoreceptor in *Ae. aegypti*, and characterized its expression pattern. *AaOr7* is extremely well conserved in its primary sequence with a group of receptors identified from diverse insect taxa (Vosshall *et al.*, 1999; Krieger *et al.*, 2003). These genes are apparent orthologs based on the conservation of their peptide sequences (Figure 1) as well as conservation of gene structures within *D. melanogaster*, *An. gambiae* and *Ae. aegypti* (data not shown). Considerable selective pressure must have been placed on these organisms, which are separated by millions of years of evolutionary distance, to maintain the primary sequence of this gene, especially in the C-terminus where residue identity is highest. The observation reported here that *AaOr7* is robustly expressed in larval

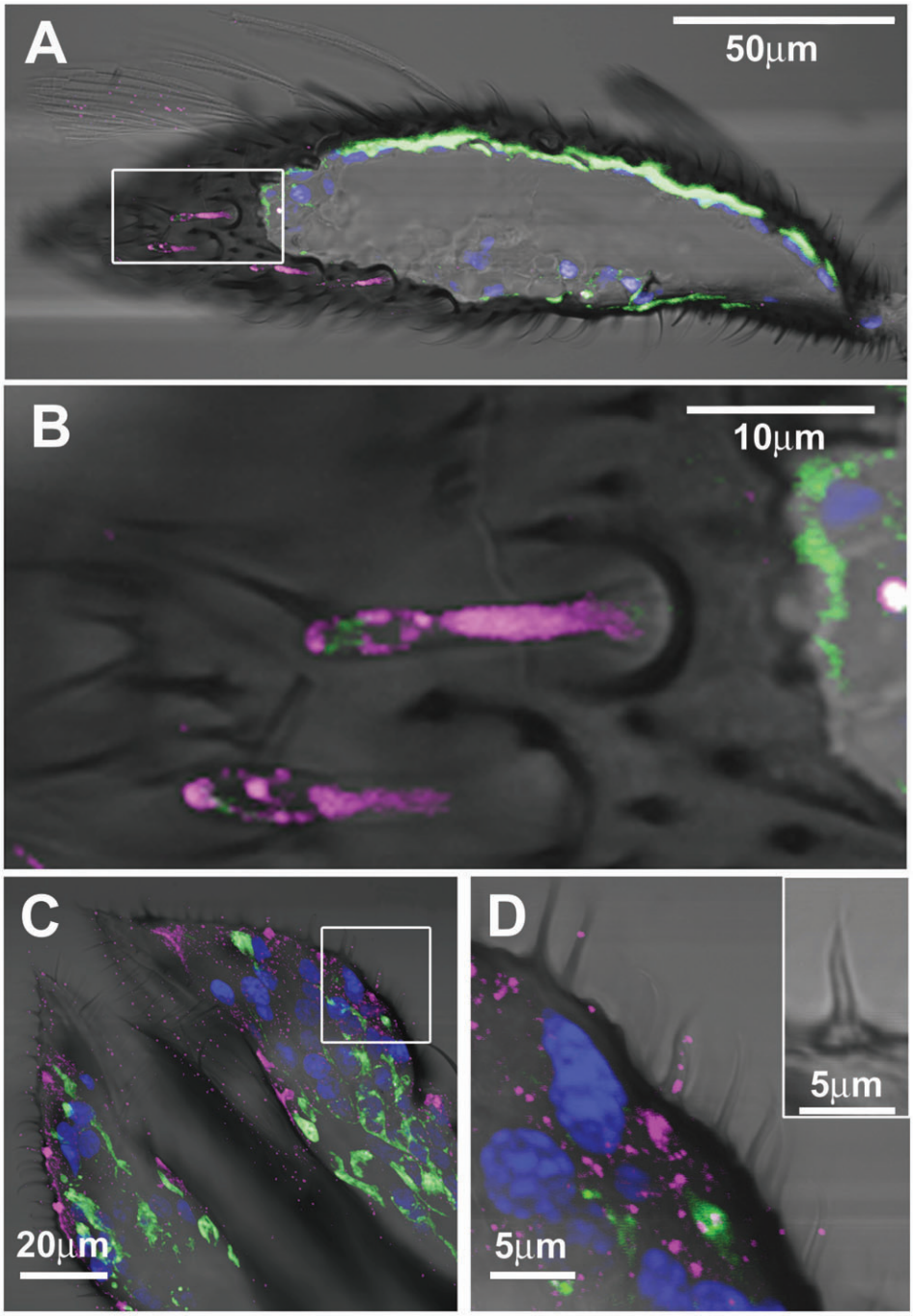

Figure 4 AaOr7 specific labeling on the female maxillary palp capitate peg sensilla (A, B) and labial palp T2 sensilla (C, D). (A) Composite image, AaOr7 (magenta), neuronal (green) and nuclear (blue) labeling. (B) Magnified view of box depicted in (A). (C) Composite image of 2 μm optical section of the tip of a female labial palp where labeling reminiscent of dendritic structures in these sensilla is very weak but reproducibly observed, AaOr7 (magenta), neuronal (green) and nuclear (blue) labeling. (D) Magnified view of box depicted in (C).

stages (Figure 2), is in agreement with the expression of *DOr83b*, the only *D. melanogaster* odorant receptor reported to be expressed in larvae (Vosshall *et al.*, 1999). Moreover, the expression of *AaOr7* in the larval head, the location of the larval antennae (Clements, 1999), suggests a chemosensory role for the *AaOr7* gene in this developmental stage.

A polyclonal antiserum against a 14-residue domain of the AgOR7 protein was utilized to immunolocalize the AaOR7 protein. While for the present, the absence of complete sequence information for the *Ae. aegypti* proteome precludes a definitive comparison, it should be noted that the peptide used to generate this antiserum is unique to the AgOR7 protein and not found in the primary amino acid

sequence of any other AgOR. Indeed, the specificity of this antiserum is further supported by a lack of labeling in both pre-immune and pre-adsorbtion controls against *Ae. aegypti* tissue. In adults, we observed *AaOr7* expression in three types of sensilla in female *Ae. aegypti*. Of these, two have been described to function in the perception of a variety of volatiles including host related odorants, oviposition attractants, and carbon dioxide, while the third has been implicated in contact chemosensation and mechanosensation. Trichodic sensilla on the antenna of *Ae. aegypti* have been described as containing receptor neurons responding to a variety of olfactory cues, including fatty acids, oviposition attractants, plant odors and repellents (Lacher, 1967; Davis and Rebert, 1972; Davis, 1977; Davis and Takahashi, 1980). Within female *Ae. aegypti* antenna, according to a long-standing report (McIver, 1982), ~70% of all neurons innervate trichodic sensilla. Our result, that 97.5% of these sensilla are labeled for AaOR7 protein, suggests that most or all sensilla of this type are innervated by at least one neuron expressing *AaOr7*.

Interestingly, we observed that a large number of cell bodies were labeled using the anti-OR7 serum. In some cases it was possible to observe a dendrite within one section that was connected to a corresponding cell body. However, the number of AaOr7 labeled cell bodies was considerably lower than the number of neurons innervating trichodic sensilla. This may indicate that only one of the two neurons typically innervating a trichodic sensillum is expressing AaOR7. In contrast it has been reported that *DOr83b*, the *D. melanogaster* ortholog of *AaOr7*, is expressed in all olfactory neurons or two thirds of all neurons (Vosshall *et al.*, 1999; Kalidas and Smith, 2002). Alternatively, AaOR7 labeling in cell bodies may fluctuate as a result of intermediate states in protein production or recycling, accounting for the variability in its observation. It was also surprising to observe that grooved peg sensilla, which represent the second type of thin-walled sensilla on the *Ae. aegypti* antenna functioning in olfaction (Lacher, 1967; Kellogg, 1970), were not labeled with anti-OR7 serum. Importantly, HRP-specific immunoreactivity was readily observed for these sensilla, implying that the structure was accessible to antibody. We cannot, however, exclude the possibility that AaOR7 is expressed at a very low level in this sensillum-type.

Based on the results of RT-PCR experiments where we found *AaOr7* expression in the maxillary palps and proboscises of *Ae. aegypti* (Figure 2), we investigated localization of the AaOR7 protein in these organs. We observed AaOR7 labeling in the capitate pegs of the maxillary palp (Figure 4), the single olfactory sensillar type in this organ (Sutcliffe, 1994). Capitate pegs have been demonstrated to respond to *n*-heptane, amyl-acetate, acetone and carbon dioxide (Kellogg, 1970). The expression of AaOR7 in these sensilla is consistent with an olfactory function for this receptor in capitate pegs. In the case of the proboscis, for which a contact chemosensory function has been described in mosquitoes (Elizarov and Sinitsyna, 1974; Pappas and Larsen, 1976) we found AaOR7 labeling in a sensillum-type that has not been described in *Ae. aegypti*. These sensilla resemble the T2 labellar sensilla of *Cs. inornata* that are innervated by two salt-sensitive neurons and one mechanoreceptor (Pappas and Larsen, 1976). This observation suggests, that the AaOR7 protein may be a component of multiple sensory pathways, particularly since *AaOr7* homologues show similar primary sequence homology with both odorant and gustatory receptor (GR) families (Hill *et al.*, 2002).

In *H. virescens* candidate odorant receptors, including R2, are expressed in the antennae and proboscis (Krieger *et al.*, 2002). *AaOr7* is expressed not only in the proboscis but also in the legs of *Ae. aegypti* (Figure 2). It is important to note that both of these organs are known only as having a gustatory function. For example, morphological and electrophysiological studies of the labellum of *Ae. aegypti* have shown that the sensilla residing there are of the contact chemosensory type (for reviews, see McIver, 1982; Sutcliffe, 1994). Likewise, the labella of other dipterans function in gustation (Mitchell *et al.*, 1999; Dahanukar *et al.*, 2001). Furthermore, GRs are the only chemosensory receptors observed in the legs of *D. melanogaster* (Dunipace *et al.*, 2001; Scott *et al.*, 2001) the location of gustatory sensilla. It is possible that in addition to their gustatory function, these tissues may have an uncharacterized olfactory capability. Alternatively, it is reasonable to speculate that *AaOr7*, like other members of this OR sub-family, may act broadly in chemosensory tissues, possibly having a similar function in both olfactory and gustatory signal transduction.

Perhaps *AaOr7* and its othologs act independently of odorant/tastant binding *per se*, but are required for both OR and GR function, possibly as general partners for their hetero-dimerization as has been suggested previously (Vosshall *et al.*, 2000; Krieger *et al.*, 2003). Further study will be needed to distinguish these hypotheses and to provide a greater general understanding of olfaction in mosquitoes. This knowledge may, in time, facilitate the development of more sophisticated insect control strategies that rely specifically upon olfactory methodologies.

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