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Molecular identification and characterization of two new Lepidoptera chemoreceptors belonging to the *Drosophila melanogaster* OR83b family

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Abstract

In insect antennae, olfaction depends on olfactory receptors (ORs) that function through heterodimerization with an unusually highly conserved partner orthologue to the *Drosophila melanogaster* DOR83b. Here, we report the identification of two cDNAs encoding new DOR83b orthologues that represent the first members, although nonconventional, of the OR families of two noctuid crop pests, the cotton leafworm *Spodoptera littoralis* and the cabbage armyworm *Mamestra brassicae*. They both displayed high protein sequence conservation with previously identified DOR83b orthologues. Transcripts were abundantly detected in adult chemosensory organs as well as in fifth instar larvae heads. In adult antennae, the expression patterns of both genes revealed common features with other members of the OR83b subfamily: they appeared to be expressed at the bases of numerous olfactory sensilla belonging to different functional categories, suggesting that both receptors may be co-expressed with yet unidentified conventional ORs. Bioinformatic analyses predicted the occurrence of seven transmembrane domains and an unusual topology with intracellular N-termini and extracellular C-termini, extending to Lepidoptera the hypothesis of an inverted topology for DOR83b orthologues, demonstrated to date only in *D. melanogaster*.

Keywords: olfactory receptor, olfaction, *Mamestra brassicae*, *Spodoptera littoralis*.

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Introduction

Deciphering the molecular mechanisms involved in insect olfaction has been a particular challenge for several decades, especially in disease vectors and crop pest species, because this sensory modality contributes greatly to insects' ability to locate and recognize their hosts as well as their sexual partners. In the antennae, odorant recognition relies upon the intervention of specific proteins, including the olfactory receptors (ORs) that are expressed at the dendritic membrane of the olfactory sensory neurones (OSNs). Insect ORs constitute a large family of seven transmembrane domain (TM) receptors. The family is unrelated to the vertebrate OR family (Benton, 2006). Insect ORs display a high divergence (only 20–40% identities) in their sequences among and within species. Thus, because of unsuccessful homology-based strategies, insect ORs have so far only been identified in species for which genomic data have been available (Clyne *et al*., 1999; Gao & Chess, 1999; Vosshall *et al*., 1999; Hill *et al*., 2002; Krieger *et al*., 2002, 2004; Melo *et al*., 2004; Robertson & Wanner, 2006; Abdel-Latief, 2007; Bohbot *et al*., 2007; Wanner *et al*., 2007; Kent *et al*., 2008). As an exception, one particular OR is remarkably conserved among insect species. First discovered in *Drosophila melanogaster* and referred to as DOR83b, its conservation allowed the isolation of its counterparts in other Diptera (referred to as OR7; Melo *et al*., 2004; Pitts *et al*., 2004; Xia & Zwiebel, 2006), several Lepidoptera (referred to as OR2 or R2), Coleoptera and the honey bee (Krieger *et al*., 2003), defining a unique family of receptor subtypes.

Classically, only one type of OR is expressed in one OSN and a defined functional type of OSN expresses the same OR. DOR83b and its orthologues appear to be exceptions. In *D. melanogaster*, DOR83b is expressed in almost all the

olfactory sensilla, in association with one (sometimes two) particular OR(s) expressed in the same neurone (Vosshall *et al*., 1999, 2000; Dobritsa *et al*., 2003; Elmore *et al*., 2003; Larsson *et al*., 2004). The widespread expression of this receptor has also been observed in other species (Krieger *et al*., 2002; Pitts *et al*., 2004; Nakagawa *et al*., 2005). These observations, together with the high level of conservation of DOR83b-related proteins among insect species, suggest that the family of DOR83b orthologues represents an OR subfamily that may play a critical role in insect olfaction. It is unlikely that this receptor alone has odour-binding properties (Elmore *et al*., 2003). It is nevertheless important for olfactory reception in general because odour-evoked responses are impaired in *DOR83b* mutant flies (Larsson *et al*., 2004) or after RNA interference (Neuhaus *et al*., 2005). *DOR83b* orthologues from diverse insect species can functionally substitute for *DOR83b* in *DOR83b* mutants, confirming that this receptor family has an essential function in olfaction that has been conserved through insect evolution (Jones *et al*., 2005).

DOR83b is currently the best functionally characterized receptor of this subfamily. An abnormal cytoplasmic aggregation of co-expressed receptors is observed in *DOR83b* mutants (Larsson *et al*., 2004), suggesting a role in membrane targeting of conventional ORs. *In vitro* and *in vivo* experiments have shown that DOR83b can form heteromeric complexes with conventional ORs (Neuhaus *et al*., 2005; Benton *et al*., 2006), and its function as a dimerization partner could be essential for correct targeting and/or functionality of ORs. *In vitro*, co-expression of this receptor enhances the electrophysiological responses of conventional ORs when expressed in cultured cells (Human Embryonic Kidney (HEK) cells) (Neuhaus *et al*., 2005) and in *Xenopus* oocytes (Nakagawa *et al*., 2005), although the responses of transfected cells can also be observed without co-expression in insect cells (Sf9) (Kiely *et al*., 2007) and HEK (Grosse-Wilde *et al*., 2006, 2007).

Recently, an important and interesting controversy has arisen about the way in which DOR83b, and more generally insect ORs, function. Since insect ORs were identified, they have been considered to be G-protein coupled receptors (GPCRs), as are vertebrate ORs. However, on the basis of bioinformatic and experimental arguments, it is now proposed that, at least in *D. melanogaster*, ORs have an inverted topology compared with already known GPCRs, with their N-terminus inside the cell and their C-terminus outside (Benton *et al*., 2006). This inverted topology has been confirmed for DOR83b by a second study (Lundin *et al*., 2007). One major implication of this hypothesis would be that as yet unknown mechanisms could trigger signal transduction, in a possible G-protein independant manner. Insect ORs may constitute, with insect gustatory receptors, a new super-family of receptor proteins whose analogy with GPCRs (seven TM receptors) would only be because of convergent evolution (Benton, 2006; Wistrand *et al*., 2006). However, information on other insect species is required to confirm this hypothesis.

Among Lepidoptera, the noctuid family includes the most devastating pests on the planet. Deciphering the mechanisms of olfaction may lead to the discovery of new target genes for attenuating pest damage. However, ORs have been identified to date in only two lepidopteran species, the silkworm moth *Bombyx mori* (Sakurai *et al*., 2004; Krieger *et al*., 2005; Wanner *et al*., 2007) and the noctuid *Heliothis virescens* (Krieger *et al*., 2002, 2004). In a first attempt to identify new lepidopteran ORs, we report here the isolation and the molecular characterization of new DOR83b orthologues in two important crop pests, the cotton leafworm *Spodoptera littoralis* and the cabbage armyworm *Mamestra brassicae*. These two receptors represent the first members, although nonconventional, of the OR families of these two noctuid species. The full-length cDNAs were cloned through homology cloning and rapid amplification of cDNA ends (RACE) PCR-based strategies. Their spatial and temporal expression patterns were then determined by reverse transcription-PCR (RT-PCR) and *in situ* hybridization, revealing common features with other members of this subfamily. Bioinformatic analyses supported intracellular N-termini and extracellular C-termini for these two receptors, extending to Lepidoptera the hypothesis of OR inverted topology, demonstrated to date only in *D. melanogaster*.

Results and discussion

Identification of two new DOR83b orthologues in Lepidoptera

With degenerate primers based on the sequence of HR2 (Krieger *et al*., 2003), we amplified internal fragments of *DOR83b*-related sequences from both *S. littoralis* and *M. brassicae* antennal cDNAs. Full-length cDNAs were obtained by RACE-PCR and were called *SlitR2* (*S. littoralis*: GenBank accession number: EF395366) and *MbraR2* (*M. brassicae*: AY485222), in agreement with previous denomination of lepidopteran DOR83b orthologues (R2). The deduced proteins contained 473 (SlitR2) and 472 (MbraR2) amino acids and their sequences shared very high levels of conservation (> 80% similarity and > 60% identity) with the other members of the DOR83b subfamily (Fig. 1). For instance, SlitR2 is almost identical to *Spodoptera litura* OR2 (99.6% identity) and MbraR2 is closely related to other noctuid orthologues (eg 95% identity with R2 from *Helicoverpa* spp.). Alignment of various DOR83b-related proteins, including ours, revealed a very high level of conservation in the C-terminus region (Fig. 1). In particular, the loop between TMs VI and VII is totally conserved among species (100% identity among most of the sequences, 100% similarity with one conservative substitution Ser/Thr for five sequences). This loop is of particular interest

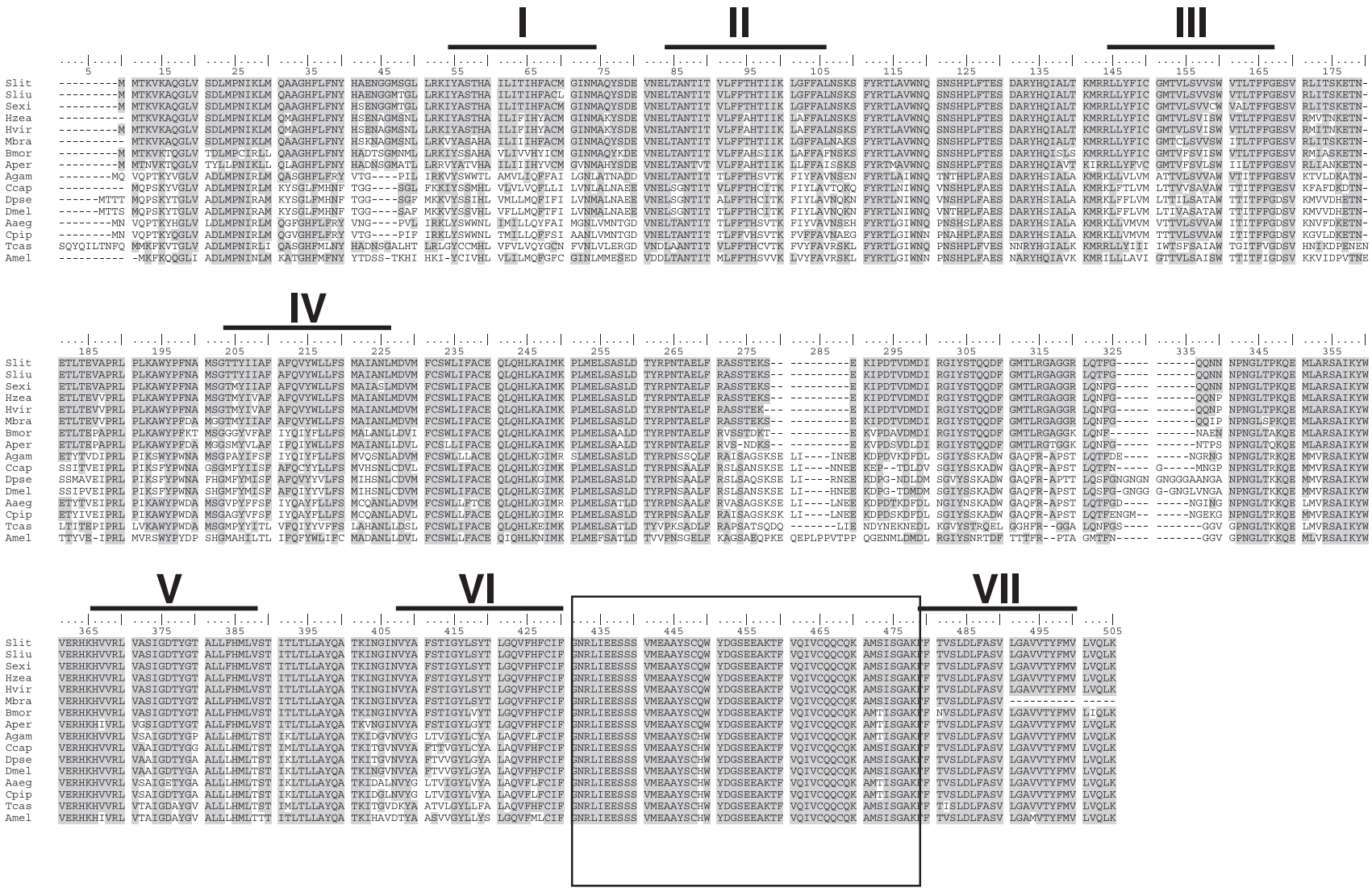

Figure 1. Alignment of *Spodoptera littoralis* olfactory receptor 2 (SlitR2) and *Mamestra brassicae* R2 (MbraR2) with selected members of the DOR83b subfamily. Identical residues are in grey. Transmembrane domains (as determined by TMHMM 2.0) are indicated with black bars and numbered from I to VII. Slit, *Spodoptera littoralis* (accession no.: EF395366); Sliu, *Spodoptera litura* (ABH10019); Sexi, *Spodoptera exigua* (AAW52583); Hzea, *Helicoverpa zea* (AAX14773); Hvir, *Heliothis virescens* (CAD31851); Mbra, *Mamestra brassicae*, (AAS49925); Bmor, *Bombyx mori* (NP_001037060); Aper, *Antheraea pernyi* (CAD88205); Agam, *Anopheles gambiae* (AAR14938); Ccap, *Ceratitis capitata* (AAX14775); Dpse, *Drosophila pseudoobscura* (EAL28510); Dmel, *Drosophila melanogaster* (AAT71306); Aaeg, *Aedes aegypti* (EAT42706); Cpip, *Culex pipiens quinquefasciatus* (ABB29301); Tcas, *Tribolium castaneum* (XP_973196); Amel, *Apis mellifera* (XP_001121145). The boxed region represents the highly conserved loop, part of the putative receptor/receptor interaction (Benton *et al*., 2006).

because it has been demonstrated in *D. melanogaster* to be part of the interaction domain between conventional ORs and DOR83b (Benton *et al.*, 2006). This conservation across insect families suggests a conserved function for DOR83b-related proteins in heterodimerization with conventional ORs, supported by the successful rescue of *DOR83b* mutant flies with DOR83b orthologues from other species (Jones *et al.*, 2005). The 100% identity among SlitR2, MbraR2 and DOR83b in this particular region suggests that the two newly identified receptors possess the capacity to interact with other conventional ORs.

SlitR2 *and* MbraR2 *are expressed in adult chemosensory tissues of both sexes and in different developmental stages*

We determined the expression patterns of *SlitR2* and *MbraR2* transcripts by RT-PCR on cDNAs from different adult tissues and developmental stages (Fig. 2). In adults, *R2* expression was restricted to the chemosensory tissues. In both species, PCR products of the expected sizes were observed in antennae of both sexes, as observed in other species (Krieger *et al.*, 2002, 2003; Pitts *et al.*, 2004; Xia & Zwiebel, 2006). In addition, faint expression could be observed in the *M. brassicae* proboscis (Fig. 2A). Although DOR83b is not expressed in the *D. melanogaster* proboscis, DOR83b orthologues from several mosquito species (Melo *et al.*, 2004; Pitts *et al.*, 2004; Xia & Zwiebel, 2006) and the Lepidoptera *H. virescens* (Krieger *et al.*, 2002) have been described as being expressed in gustatory tissues. This receptor thus could have a dual function in both olfaction and taste, depending upon the species. However, further studies revealed that the *Anopheles gambiae* proboscis carries some olfactory neurones that co-express AgOR7 and conventional ORs, and that are responsive to a small spectrum of human-related odours (Kwon *et al.*, 2006). *MbraR2* expression in the proboscis thus suggests that, either MbraR2 is involved in both taste and olfaction, or that uncharacterized olfactory sensilla may also exist on the *M. brassicae* proboscis. The absence of *MbraR2* expression in female ovipositors (known to carry contact chemosensilla) (Fig. 2A) and in taste sensilla carried by the antennae (the chaetic sensilla, see paragraph below), supported this second hypothesis.

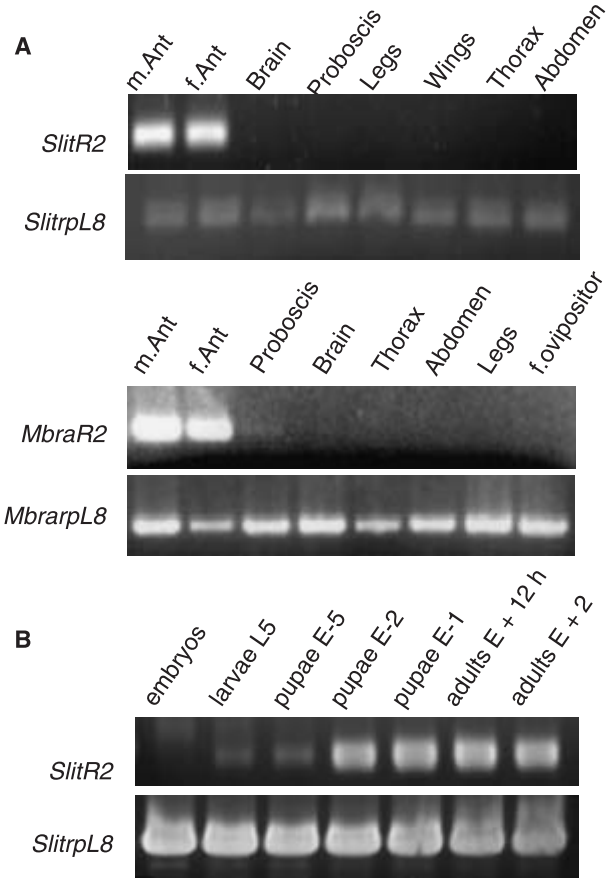

Figure 2. Expression studies by reverse transcription-PCR. (A) Expression of *Spodoptera littoralis* olfactory receptor 2 (*SlitR2*) and *Mamestra brassicae* R2 (*MbraR2*) in different adult tissues. m, males; f, females; Ant, antennae. (B) Developmental study of *SlitR2*. Larvae L5, fifth instar larvae; pupae E-X, pupae collected X days before eclosion; adults E + 12 h, adults 12 h after eclosion; adults E + 2, 2-day-old adults.

We also analysed *SlitR2* expression by RT-PCR during different developmental stages: embryos, fifth instar larvae, pupae and adults of different ages (Fig. 2B). No expression was observed in embryos. *SlitR2* was weakly detected in heads of fifth instar larvae, in agreement with previous observations in *D. melanogaster* (Vosshall *et al.*, 1999; Kreher *et al.*, 2005) and mosquito (Melo *et al.*, 2004; Pitts *et al.*, 2004; Xia & Zwiebel, 2006) larval stages. Our observation suggests that chemoreception may function through similar mechanisms in larvae and adults in Lepidoptera. A detailed study of the expression pattern in larvae heads is now needed to confirm R2 expression in caterpillar antennae and/or maxillae. In pupae, faint expression was observed 5 days before eclosion, which increased to reach its maximum in pupae antennae 2 days before eclosion, then maintaining a high level of expression until and during adulthood. This expression kinetic in pupal development was similar to that of previously characterized olfactory genes in Lepidoptera antennae (Gyorgyi *et al.*, 1988; Rogers *et al.*, 1997) and thus supported the possible involvement of SlitR2 in conventional OR targeting and/or functioning.

To investigate further the expression pattern of *SlitR2* and *MbraR2* in adult antennae, we performed *in situ* hybridization (Fig. 3). In both species, antennae are filiform and segmented. The olfactory sensilla are located on the ventral part, whereas the dorsal part is covered with scales (Fig. 3A,F). In both species, labelling was restricted to the sensilla side of the antennae, consistent with an olfactory

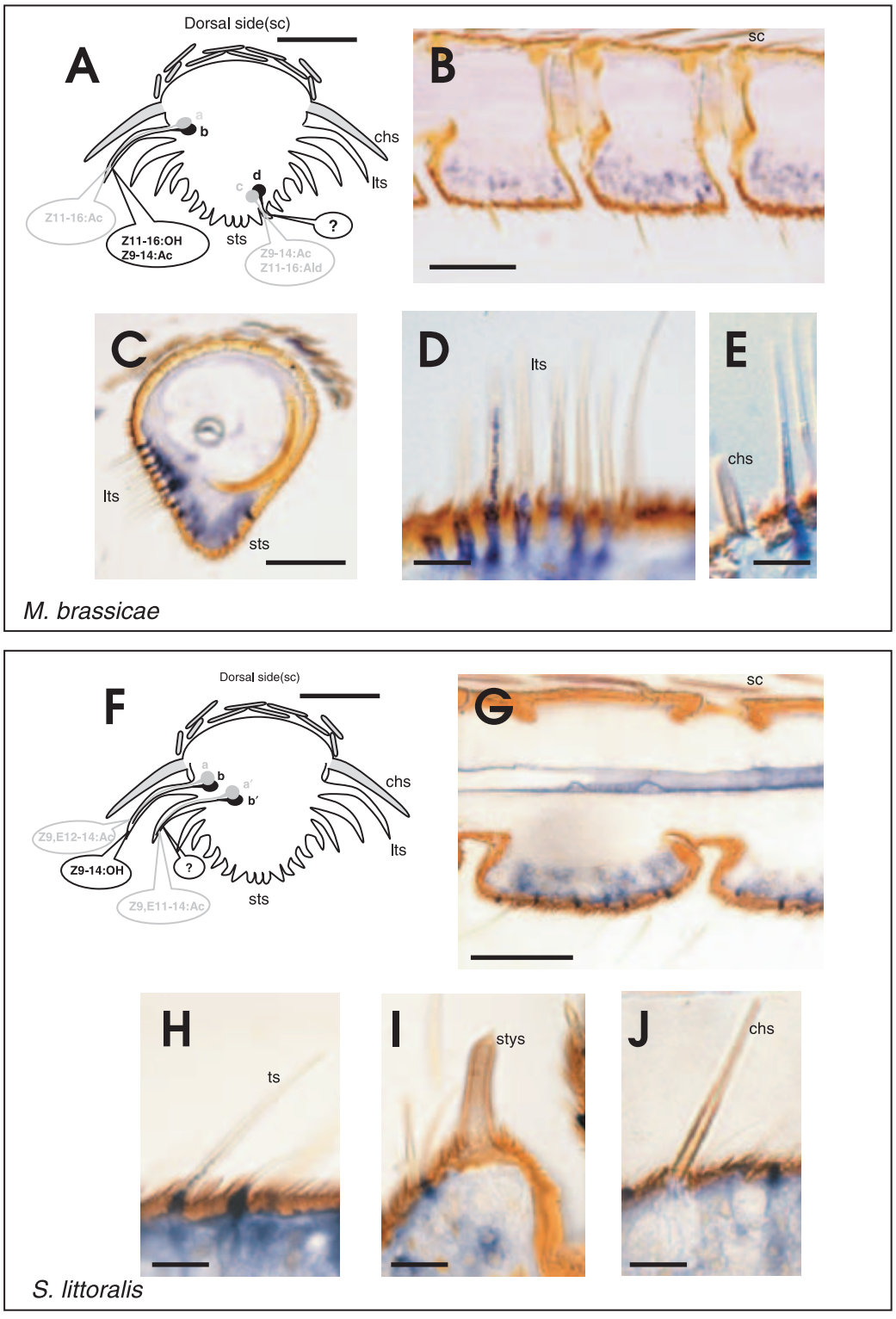

Figure 3. Expression pattern of *Spodoptera littoralis* olfactory receptor 2 (*SlitR2*) and *Mamestra brassicae* R2 (*MbraR2*) in adult antennae by *in situ* hybridization. (A, F) Schematic representation of transverse sections through the antennae of *M. brassicae* and *S. littoralis*. Different morphological and functional types of olfactory sensilla are localized (chs, chaetic sensilla; lts, long trichoid sensilla; sts, short trichoid sensilla; sc, scales). a, *a′*, b, b′, c, d, different functional types of olfactory sensory neurones and the known pheromone components/behavioural antagonists that they respond to (Ljungberg *et al.*, 1993; Renou & Lucas, 1994). (B, C, D, E) longitudinal sections (B) and cross sections (C, D, E) of *M. brassicae* male antenna. (G, H, I, J) longitudinal sections of *S. littoralis* male antenna. Staining is restricted to the ventral side (no labelling on the dorsal side) (A, F) (B, G), associated with trichoid sensilla (ts) (C, D, H), long (lts) and short (sts) ones, but not chaetic (chs) (E, J) nor styloconic (stys) (I) sensilla. Scale bars: A, B, C, F, G: 50 μm; D, E, H, I, J: 10 μm.

function for R2 (Fig. 3B,G). Among the olfactory sensilla, different morphological and functional types of trichoid sensilla have been described in *S. littoralis* and *M. brassicae* (Ljungberg *et al*., 1993; Renou & Lucas, 1994) (Fig. 3A,F). In *M. brassicae*, long trichoid sensilla are arranged in parallel rows on the lateral sides of the antennae and short trichoid sensilla are situated on the ventral side. Long and short trichoid sensilla have different functional properties. They house two neurones, each responding to specific compounds (Fig. 3A). On transverse sections of the antennae, long and short sensilla are easily distinguishable according to their position. *MbraR2* labelling was observed at the bases of both morphological and functional types of olfactory sensilla (Fig. 3C). In *S. littoralis*, long sensilla trichodea are not arranged in parallel rows and are often intermingled with short sensilla, making it difficult to distinguish between these two kinds of sensilla on optical sections. However, as in *M. brassicae*, labelling could be observed at the bases of almost all the sensilla (Fig. 3G), suggesting that R2 is likely to also be expressed in different kinds of sensilla. Such a pattern within the antennae is different to what might be expected for conventional ORs whose expression would be restricted to only one functional type of sensilla. Rather, the expression of *SlitR2* and *MbraR2* in numerous olfactory sensilla is consistent with their probable co-expression with conventional ORs. Apart from in *D. melanogaster*, such co-expression has been demonstrated to date only in *B. mori* antennae, in which BmorR2 is co-expressed with pheromone receptors (Nakagawa *et al*., 2005). Although moth olfactory sensilla usually house two receptor neurones (Fig. 3A,F), it was not possible to tell whether the labelling observed in our *in situ* experiments corresponded to one or two OSNs because the labelling is spread and the cell bodies were not clearly visible. Interestingly, we did not observe any staining at the base of chaetic (Fig. 3E,J) and styloconic sensilla (Fig. 3I), which are known to be involved in mechano/contact chemoreception and hygro/thermoreception, respectively (Keil, 1999), suggesting that *SlitR2* expression is restricted to olfactory sensilla.

SlitR2 *and* MbraR2 *were predicted to have seven transmembrane domains and an inverted topology*

Recent work on *D. melanogaster* suggests that DORs, and in particular DOR83b, present an inverted topology with an intracellular N-terminus and an extracellular C-terminus (Benton *et al*., 2006; Lundin *et al*., 2007), in accordance with theoretical predictions based on hidden Markov models (HMM) (Wistrand *et al*., 2006). In order to verify whether such a topology can occur in Lepidoptera, we analysed SlitR2 and MbraR2 sequences by the Sfinx metaserver that regroups different HMM-based algorithms (Sonnhammer & Wootton, 2001) (Fig. 4) and that has been experimentally approved using the DOR83b sequence (Lundin *et al*., 2007). In our study, four out of the five algorithms (Phobuis, TMHMM 2.0, HMMTOP2.1, TOPPRED) predicted the occurrence of seven TMs in both receptors, with positions similar to those described in other DOR83b orthologues, and defining a large fourth loop between TM IV and V (Krieger *et al*., 2003) (Figs 1 and 4) whose function remains unknown. In addition, all five algorithms (same as above + PHDhtm), predicted a N_{in}-C_{out} topology for both SlitR2 and MbraR2. On the contrary, the recent GPCRHMM specifically tuned to GPCR identification (Wistrand *et al*., 2006) did not predict such an inverted topology for both proteins. However, when we used this algorithm with DOR83b (not shown), a classical GPCR topology (N_{out}-C_{in}) was also predicted, contrary to experimental data obtained on this receptor (Benton *et al*., 2006; Lundin *et al*., 2007). This algorithm thus did not appear to be adapted for DOR83b prediction. Through bioinformatic predictions, our work thus extends to the Lepidoptera order the hypothesis of an inverted topology of at least the DOR83b orthologues, demonstrated to date only in *D. melanogaster*. The question of the mechanism of the transduction pathway with such an inverted topology compared to classical GPCR has been addressed. In Lepidoptera, accumulating data strongly support the intervention of a G-protein transduction cascade in the olfactory process, at least in pheromone reception (Boekhoff *et al*., 1990, 1993; Stengl *et al*., 1992; Lucas & Shimahara, 2002). The possible inverted topology of Lepidoptera DOR83b orthologues may still allow interaction with a G-protein. Alternatively, as insect ORs function through heterodimers with DOR83b orthologues, it is possible that the involvement of a G-protein in lepidopteran pheromone reception arises from the conventional OR partner, whose topology has not yet been addressed in Lepidoptera.

Taken together, our data clearly show that SlitR2 and MbraR2 possess all the characteristics of new DOR83b orthologues. They were highly conserved with their counterparts in other species; they were largely and specifically expressed in the moth olfactory sensilla, probably co-expressed with as yet unidentified conventional ORs. In addition, our work presented new data that probably support the hypothesis that the inverted topology of DOR83b could be extended to other insect orders. Further identification of conventional ORs is now in progress in both *S. littoralis* and *M. brassicae* that, together with the identification of the two new lepidopteran DOR83b orthologues described in this study, will offer new opportunities to investigate OR functioning in Lepidoptera.

Experimental procedures

Insect rearing and tissue collection

Insects were reared on semiartificial diet in the laboratory at 24 °C, 60–70% relative humidity under a 16 : 8 light : dark cycle. Tissues

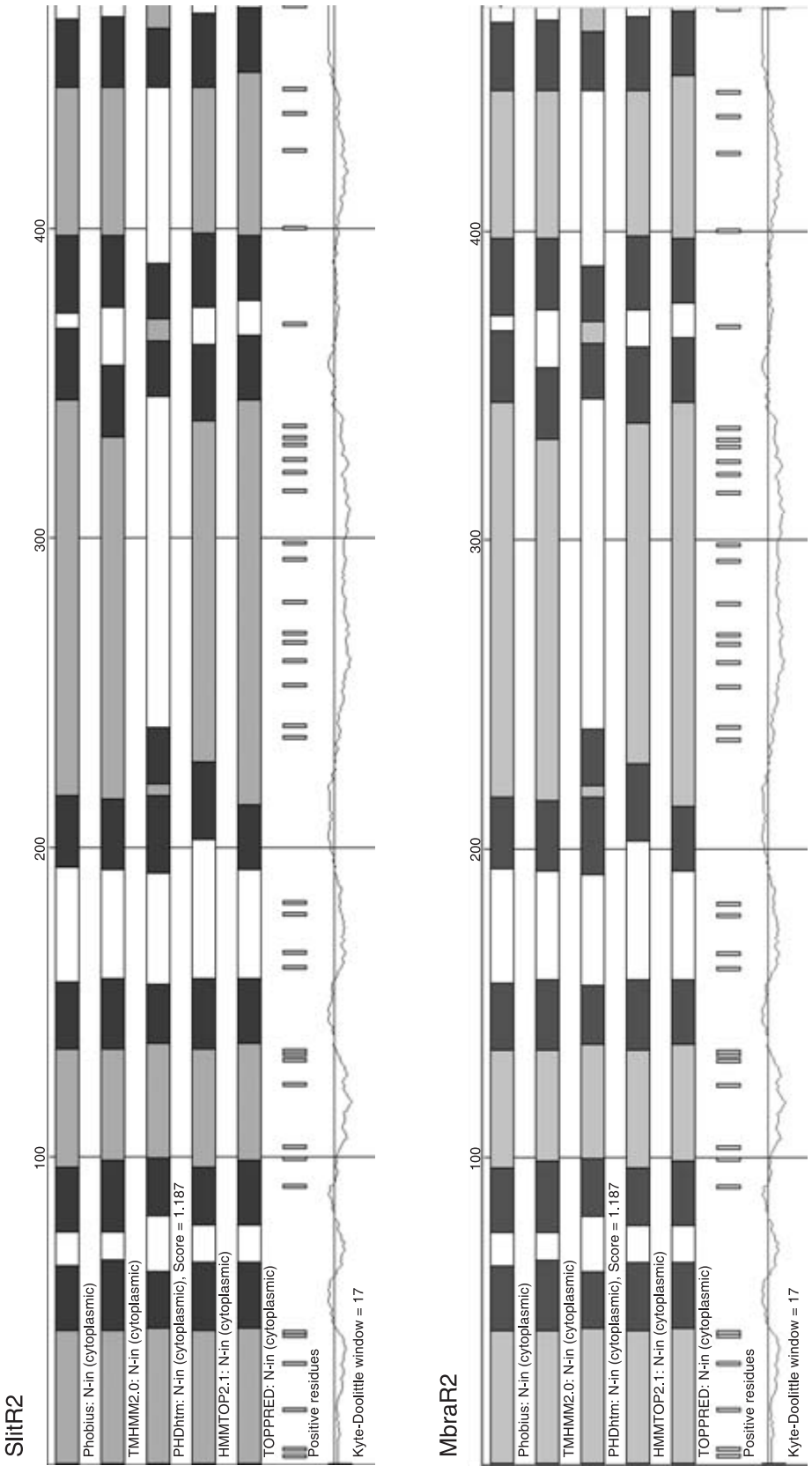

Figure 4. Prediction of *Spodoptera littoralis* olfactory receptor 2 (SlitR2) and *Mamestra brassicae* R2 (MbraR2) transmembrane topology, adapted from the Sfinx metaserver using default parameters (http://sfinx.cgb.ki.se) (Sonnhammer & Wootton, 2001). Different algorithms were used (Phobius, TMHMM 2.0, PHDhtm, HMMTOP 2.1, TOPPRED). Positions of positively charged residues and a Kyte–Doolittle hydrophobicity curve are also presented. Predicted transmembrane domains are in black, cytoplasmic regions are in grey and extracellular regions are in white, according to the different prediction methods. All five algorithms predicted an inverted topology for both proteins.

from fifth instar larvae (heads), different pupal stages (antennae) and adults (male and female antennae, proboscis, brains, legs, wings, thoraxes, abdomens) were dissected and used directly for total RNA isolation. For *in situ* hybridization, male antennae were cut into pieces and fixed overnight in 4% paraformaldehyde at 4 °C, then dehydrated in methanol and stored at −20 °C until use.

RNA extraction and cDNA synthesis

Total RNAs were extracted from the different tissues with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase1 (Promega, Madison, WI, USA). Single-stranded cDNAs were synthesized from 1 μg total RNAs with M-MLV reverse transcriptase (Clontech, Mountain View, CA, USA) using buffer and protocol supplied in the Advantage™ RT-for-PCR Kit (Clontech). For the 3′ and 5′ RACE, cDNAs were synthesized from 1 μg male antennae total RNA at 42 °C for 1.5 h using the SMART™ RACE cDNA Amplification Kit (Clontech) with 200 U of Superscript II (Gibco BRL, Invitrogen), 3′ cDNA synthesis (CDS)-primer and SMART II oligonucleotide, according to the manufacturer's instructions.

Molecular cloning of S. littoralis *and* M. brassicae *DOR83b-related cDNAs in male antennae*

Antennal cDNAs were used in PCRs with two degenerate primers, ORf: TGYGARCARYTICARCA and ORr: IACIGTRAARIAYTTIGC, designed from the sequence of *HR2* (AJ487477, Krieger *et al*., 2002). PCRs consisted of 40 cycles at 94 °C for 30 s, 40 °C for 30 s and 72 °C for 1 min. The generated 660 bp fragments were gel-purified (GenElute™, Sigma, St Louis, MO, USA) and cloned into pCR® II-TOPO® plasmid (Invitrogen). Recombinant plasmids were isolated by mini preparation (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and sequenced (Genome Express, Grenoble, France). Gene sequence analyses and database comparisons were performed using the BLAST program (Altschul *et al*., 1990) and alignments were carried out using CLUSTALW (NPS@IBCP). Several clones were sequenced and presented 100% identity.

The 3′ and 5′ regions of the cDNAs were obtained by 3′ and 5′ RACE-PCRs, according to the SMART™ RACE kit instructions (Clontech) using Universal Primer Mix vs. gene-specific primers: 5′Race (used for both species): CCCTCCAGCTCCTCGCAGAGTCATGCCG; Mbra3′Race: GCCAATGGTATGATGGCTCCGAGGAAGCC; Slit3′Race: CGTGCAGATCGTGTGCCAACAGTGCCAG. Touchdown PCRs were performed as follows: after 1 min at 94 °C, five cycles of 30 s at 94 °C and 3 min at 72 °C, then five cycles of 30 s at 94 °C, 30 s at 70 °C and 3 min at 72 °C, then 30 cycles of 30 s at 94 °C, 30 s at 68 °C and 3 min at 72 °C, then 10 min of a final elongation step at 72 °C. The PCR products were cloned, sequenced and analysed as described above. By merging the overlapping sequences obtained by 3′ and 5′ RACE, two cDNAs of 2462 and 1772 bp, called *SlitR2* and *MbraR2*, respectively, were generated and identified as putative *DOR83b* orthologues after sequence analyses.

Tissue expression and developmental studies

SlitR2 and *MbraR2* cDNAs were amplified in different tissues with the following pair of specific primers: SlitR2f: TGGACAGCAGAACAACAACC/SlitR2r: AGCCTGATAAGCAAGTAGAGTG (53 °C) (200 bp fragment), MbraR2f: TGCTTCTCTGGATACTTACA/MbraR2r: TCGGAGCCATCATACCATTG (50 °C) (500 bp fragment). The ribosomal protein L8 gene (*rpL8*) was used as an RNA extraction control: (rpL8f: GAGTCATCCGAGCTCARMGNAARGG; rpL8r: CCAGCAGTTTCGCTTNACYTTRTA; 54 °C), generating a 508 bp fragment for each species, as already described (Maïbeche-Coisné *et al*., 2004). The developmental study was performed only on *S. littoralis* larvae and adults with the same primers as described above. Amplification products were loaded on 2% agarose gels and visualized with ethidium bromide.

In situ *hybridization*

Digoxigenin-labelled RNA sense and antisense probes (600 b long for both *SlitR2* and *MbraR2*) were *in vitro* transcribed from PCR fragments amplified from the recombinant plasmids *SlitR2*-pCR®II-TOPO and *MbraR2*-pCR®II-TOPO with M13 Forward and M13 Reverse primers. The transcriptions were performed using T7 and SP6 RNA polymerases (Promega) following the recommended protocol to generate both *SlitR2* and *MbraR2* sense and antisense probes. The probes were purified with RNA G50 sephadex columns (Quick Spin columns; Roche, Basel, Switzerland). The hybridization protocol was performed on whole-mount pieces of antennae, as previously described (Jacquin-Joly *et al*., 2000). After hybridization, longitudinal and transverse sections were performed at 6 μm and counter-stained with acridine orange. Sections were photographed and pictures were digitized and processed using Adobe Photoshop 7.0 (Adobe, San Jose, CA, USA).

HMM topology and transmembrane domain analyses

Default prediction of SlitR2 and MbraR2 transmembrane topology was analysed using the Sfinx metaserver (http://sfinx.cgb.ki.se; Sonnhammer & Wootton, 2001), that included unconstrained prediction from Phobius (Kall *et al*., 2004), TMHMM 2.0 (Krogh *et al*., 2001), HMMTOP2.1 (Tusnady & Simon, 2001), PHDhtm (Rost *et al*., 1996), TOPPRED (von Heijne, 1992), position of positive residues (preferentially located on the cytoplasmic side) and a Kyte–Doolittle hydrophobicity curve (Kyte & Doolittle, 1982). The new GPCRHMM (Wistrand *et al*., 2006) algorithm was also used for comparison, as it predicted the insect OR and GR families as non-GPCRs (Wistrand *et al*., 2006).

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References

Abdel-Latief, M. (2007) A family of chemoreceptors in *Tribolium castaneum* (Tenebrionidae: Coleoptera). *PLoS ONE* **2**: e1319.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Benton, R. (2006) On the ORigin of smell: odorant receptors in insects. *Cell Mol Life Sci* **63**: 1579–1585.

Benton, R., Sachse, S., Michnick, S.W. and Vosshall, L.B. (2006) Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLoS Biol* **4**: e20.

Boekhoff, I., Raming, K. and Breer, H. (1990) Pheromone-induced stimulation of inositol-triphosphate formation in insect antennae is mediated by G-proteins. *J Comp Physiol A* **160**: 99–103.

Boekhoff, I., Seifert, E., Goggerle, S., Lindemann, M., Kruger, B.-W. and Breer, H. (1993) Pheromone-induced second-messenger signaling in insect antennae. *Insect Biochem Mol Biol* **23**: 757–762.

Bohbot, J., Pitts, R.J., Kwon, H.W., Rutzler, M., Robertson, H.M. and Zwiebel, L.J. (2007) Molecular characterization of the *Aedes aegypti* odorant receptor gene family. *Insect Mol Biol* **16**: 525–537.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J. and Carlson, J.R. (1999) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* **22**: 327–338.

Dobritsa, A.A., van der Goes van Naters, W., Warr, C.G., Steinbrecht, R.A. and Carlson, J.R. (2003) Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* **37**: 827–841.

Elmore, T., Ignell, R., Carlson, J.R. and Smith, D.P. (2003) Targeted mutation of a *Drosophila* odor receptor defines receptor requirement in a novel class of sensillum. *J Neurosci* **23**: 9906–9912.

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

Grosse-Wilde, E., Svatos, A. and Krieger, J. (2006) A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor in vitro. *Chem Senses* **31**: 547–555.

Grosse-Wilde, E., Gohl, E.T., Bouche, E., Breer, H. and 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.

Gyorgyi, T.K., Roby-Shemkovitz, A.J. and Lerner, M.R. (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.

von Heijne, G. (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225**: 487–494.

Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A. *et al.* (2002) G protein-coupled receptors in *Anopheles gambiae. Science* **298**: 176–178.

Jacquin-Joly, E., Bohbot, J., Francois, M.C., Cain, A.H. and Nagnan-Le Meillour, P. (2000) Characterization of the general odorant-binding protein 2 in the molecular coding of odorants in *Mamestra brassicae. Eur J Biochem* **267**: 6708–6714.

Jones, W.D., Nguyen, T.A., Kloss, B., Lee, K.J. and Vosshall, L.B. (2005) Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr Biol* **15**: R119–R121.

Kall, L., Krogh, A. and Sonnhammer, E.L. (2004) A combined transmembrane topology and signal peptide prediction method. *J Mol Biol* **338**: 1027–1036.

Keil, T.A. (1999) Morphology and development of the peripheral olfactory organs. In *Insect Olfaction* (Hansson, B.S., ed.), pp. 5–47. Springer, Berlin.

Kent, L.B., Walden, K.K. and Robertson, H.M. (2008) The Gr Family of Candidate Gustatory and Olfactory Receptors in the Yellow-Fever Mosquito *Aedes aegypti Chem Senses* **33**: 79–93.

Kiely, A., Authier, A., Kralicek, A.V., Warr, C.G. and Newcomb, R.D. (2007) Functional analysis of a *Drosophila melanogaster* olfactory receptor expressed in Sf9 cells. *J Neurosci Methods* **159**: 189–194.

Kreher, S.A., Kwon, J.Y. and Carlson, J.R. (2005) The molecular basis of odor coding in the *Drosophila* larva. *Neuron* **46**: 445–456.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**: 567–580.

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

Krieger, J., Klink, O., Mohl, C., Raming, K. and Breer, H. (2003) A candidate olfactory receptor subtype highly conserved across different insect orders. *J Comp Physiol A* **189**: 519 – 526.

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

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

Kwon, H.W., Lu, T., Rutzler, M. and Zwiebel, L.J. (2006) Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae. Proc Natl Acad Sci USA* **103**: 13526 – 13531.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**: 105–132.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H. and Vosshall, L.B. (2004) Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43**: 703–714.

Ljungberg, H., Anderson, P. and Hansson, B.S. (1993) Physiology and morphology of pheromone-specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J Insect Physiol* **39**: 253–260.

Lucas, P. and Shimahara, T. (2002) Voltage- and calcium-activated currents in cultured olfactory receptor neurons of male *Mamestra brassicae* (Lepidoptera). *Chem Senses* **27**: 599–610.

Lundin, C., Kall, L., Kreher, S.A., Kapp, K., Sonnhammer, E.L., Carlson, J.R. *et al.* (2007) Membrane topology of the *Drosophila* OR83b odorant receptor. *FEBS Lett* **581**: 5601–5604.

Maïbeche-Coisné, M., Merlin, C., François, M.-C., Queguiner, I., Porcheron, P. and Jacquin-Joly, E. (2004) Putative odorant-degrading esterase cDNA from the moth *Mamestra brassicae*: cloning and expression patterns in male and female antennae. *Chem Senses* **29**: 381–390.

Melo, A.C., Rutzler, M., Pitts, R.J. and Zwiebel, L.J. (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.

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

Neuhaus, E.M., Gisselmann, G., Zhang, W., Dooley, R., Stortkuhl, K. and Hatt, H. (2005) Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster. Nat Neurosci* **8**: 15–17.

Pitts, R.J., Fox, A.N. and Zwiebel, L.J. (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.

Renou, M. and Lucas, P. (1994) Sex pheromone reception in *Mamestra brassicae* L. (Lepidoptera): responses of olfactory receptor neurones to minor components of the pheromone blend. *J Insect Physiol* **40**: 75–85.

Robertson, H.M. and Wanner, K.W. (2006) The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res* **16**: 1395–1403.

Rogers, M.E., Sun, M., Lerner, M.R. and Vogt, R.G. (1997) Snmp-1, a novel membrane protein of olfactory neurons of the silk moth *Antheraea polyphemus* with homology to the CD36 family of membrane proteins. *J Biol Chem* **272**: 14792–14799.

Rost, B., Fariselli, P. and Casadio, R. (1996) Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci* **5**: 1704–1718.

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

Sonnhammer, E.L. and Wootton, J.C. (2001) Integrated graphical analysis of protein sequence features predicted from sequence composition. *Proteins* **45**: 262–273.

Stengl, M., Hatt, H. and Breer, H. (1992) Peripheral processes in insect olfaction. *Annu Rev Physiol* **54**: 665–681.

Tusnady, G.E. and Simon, I. (2001) The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**: 849–850.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A. and Axel, R. (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* **96**: 725–736.

Vosshall, L.B., Wong, A.M. and Axel, R. (2000) An olfactory sensory map in the fly brain. *Cell* **102**: 147–159.

Wanner, K.W., Anderson, A.R., Trowell, S.C., Theilmann, D.A., Robertson, H.M. and Newcomb, R.D. (2007) Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. *Insect Mol Biol* **16**: 107–119.

Wistrand, M., Kall, L. and Sonnhammer, E.L. (2006) A general model of G protein-coupled receptor sequences and its application to detect remote homologs. *Protein Sci* **15**: 509–521.

Xia, Y. and Zwiebel, L.J. (2006) Identification and characterization of an odorant receptor from the West Nile virus mosquito, *Culex quinquefasciatus Insect Biochem Mol Biol* **36**: 169–176.