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Molecular Characterization of MbraOR16, a Candidate Sex Pheromone Receptor in *Mamestra brassicae* (Lepidoptera: Noctuidae)

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Abstract

Sex pheromone communication in Lepidoptera has long been a valuable model system for studying fundamental aspects of olfaction and its study has led to the establishment of environmentally-friendly pest control strategies. The cabbage moth, *Mamestra brassicae* (Linnaeus) (Lepidoptera: Noctuidae), is a major pest of Cruciferous vegetables in Europe and Asia. Its sex pheromone has been characterized and is currently used as a lure to trap males; however, nothing is known about the molecular mechanisms of sex pheromone reception in male antennae. Using homology cloning and rapid amplification of cDNA ends-PCR strategies, we identified the first candidate pheromone receptor in this species. The transcript was specifically expressed in the antennae with a strong male bias. In situ hybridization experiments within the antennae revealed that the receptor-expressing cells were closely associated with the olfactory structures, especially the long trichoid sensilla known to be pheromone-sensitive. The deduced protein is predicted to adopt a seven-transmembrane structure, a hallmark of insect odorant receptors, and phylogenetically clustered in a clade that grouped a majority of the Lepidoptera pheromone receptors characterized to date. Taken together, our data support identification of a candidate pheromone receptor and provides a basis for better understanding how this species detects a signal critical for reproduction.

Key words: sex pheromone, odorant receptor, in situ hybridization, crop pest

Thousands of volatile compounds hover in our environment. For a nocturnal moth, some of these carry crucial information about the host plants or conspecific mates. In moths, as in many insects, mate recognition usually relies on sex pheromone emission and reception by the corresponding partners (Tamaki 1985). Moth sex pheromones are classified into different types, based on the chemical features and biosynthetic pathways of the molecules (Löfstedt et al. 2016). These molecules are present only in picomolar concentrations in the atmospheric mixture, necessitating a highly sensitive and specific detection system (Kaissling 2004, Jones et al. 2005). PRs tuned to type I pheromone components all belong to the same OR subfamily, but some PRs tuned to type 0 and type II pheromone components have been recently described in other lepidopteran OR subfamilies (Li et al. 2017, Yuvaraj et al. 2017).

Pheromones have been used for decades for environmental-friendly pest management and control (Witzgall et al. 2010); thus, a better understanding of the molecular process of pheromone detection would help improve these strategies. Especially, identification of sex PRs in crop pests opens the way to act as early as the reception step for signal disruption, via the search of receptor activators and/or inhibitors that would interfere with the receptor (ORs) termed pheromone receptors (PRs). The PRs are delicately tuned to detect the components of the pheromone blend emitted by the conspecific female (Zhang and Löfstedt 2015). As ORs, PRs are also seven transmembrane domain receptors and form heteromeric complexes of unknown stoichiometry with the OR co-receptor (Orco) that is conserved among insects (Larsson et al. 2004, Jones et al. 2005). PRs tuned to type I pheromone components are seven-transmembrane domain receptors and form heteromeric complexes of unknown stoichiometry with the OR co-receptor (Orco) that is conserved among insects (Larsson et al. 2004, Jones et al. 2005). PRs tuned to type I pheromone components all belong to the same OR subfamily, but some PRs tuned to type 0 and type II pheromone components have been recently described in other lepidopteran OR subfamilies (Li et al. 2017, Yuvaraj et al. 2017).

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response. More avenues of effective mating disruption could become available with the identification, via OR functional screening, of behavior modifying chemicals such as host plant volatiles for mating sites (push-pull strategies) (Cook et al. 2007) or intervening in host seeking strategies for feeding and/or egg laying (Conchou et al. 2017).

The cabbage moth (Mamestra brassicae [Linnæus] [Lepidoptera: Noctuidae]) is the major pest of Cruciferous vegetable plants in Eurasia (Hill 1987). Its sex pheromone has been characterized (Artygalle et al. 1987) and is currently used as a lure to trap males, but very little is known about the molecular mechanisms of sex pheromone reception in male antennae. Although these mechanisms are well studied in many moths as reviewed (Zhang and Lofstedt 2015), it is surprising that no PR has yet been characterized in M. brassicae. So far, only Orco (Malpel et al. 2008) and two PBPs have been cloned in this species (Maïbèche-Coisné et al. 1998). To extend the sex pheromone reception cascade in M. brassicae, here we present multiple lines of evidence to suggest that MbraOR16 is a PR expressed in the olfactory sensilla of male M. brassicae antennae.

**Materials and Methods**

**Insect Rearing and cDNA Synthesis**

Insects were reared on a semiartificial diet (Poitout and Buès 1974) at 20°C, 60–70% relative humidity and under a 16:8 light:dark cycle. For cDNA synthesis, various tissues (male and female antennae, proboscis, brain-subesophageal ganglion complex, thorax, abdomens, legs, and wings) were dissected from 3-d-old adults. For in situ hybridization, male antennae were cut into pieces and fixed in 4% paraformaldehyde (PFA) at 4°C, then dehydrated in methanol, and stored at −20°C until use. Total RNAs were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Single-stranded cDNAs were synthesized from 1-μg total RNA for each sample after DNaseI treatment (Promega, Madison WI) with M-MLV reverse transcriptase and oligo(dT) primer using protocol supplied in the Advantage RT-for-PCR kit (Clontech, Mountain View, CA) and were used as template for PCR reactions. For 5′ and 3′ rapid amplification of cDNA ends (RACE), 3′- and 5′-cDNAs were synthesized from 1-μg total RNA from male antennae by using the SMART RACE cDNA Amplification kit (Clontech) according to the manufacturer’s instructions.

**Molecular Cloning and RT–PCR Analysis**

Antennal cDNA was used in PCRs (hot-start at 95°C for 1 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 68°C for 30 s, and a final step at 68°C for 3 min) with Titanium Taq (Clontech) and two primers designed from conserved regions (Zhang and Lofstedt 2015). The MbraOR16 sequence was aligned with 66 PR sequences from 16 Lepidoptera species (details in Fig. 3) using MAFFT v.7 (Katoh and Standley 2013). The 66 sequences included in the dataset belong to the so-called ‘pheromone receptor clade’, which includes most of the PRs identified to date that are tuned to type I pheromone components. Phylogenetic reconstruction was performed with PhyML 3.0 (Guindon et al. 2010) using the maximum likelihood method with the JTT + I + G + F substitution model (Jones et al. 1992) and both SPR (Subtree Pruning and Regrafting) and NNI (Nearest Neighbor Interchange) methods for topology improvement. Rate heterogeneity was set at four categories, and values calculated by ProtTest were used for the gamma distribution parameter and the proportion of invariable sites. Node support was estimated using a hierarchical likelihood-ratio test (Anisimova and Gascuel 2006).

**In Situ Hybridization**

Dig-labeled RNA sense and antisense probes were in vitro transcribed from PCR fragments amplified (PCR: 30 cycles, 65°C) from male antennal cDNA using GSPs flanked with T7 (for the antisense probe) and SP6 (for the sense probe) sequences: OR16F 5′-GGTGTAACCGGTGTTGGCATGATTGGA-3′ and OR16TE-low 5′-CATGTAATACAGAACGACATCGGTTCC-3′ that generated a 765-bp product. The rpfl gene (508 bp) of M. brassicae was used as a control as described previously (Maïbèche-Coisné et al. 2004).

**Results**

**Molecular Cloning of MbraOR16 Full-Length cDNA**

With primers designed to conserved regions of noctuid PRs, we were able to amplify a fragment of a M. brassicae OR (Fig. 1). Subsequent RACE PCRs facilitated identification of a full-length cDNA encoding a 430-amino acid protein with high homology (72% identity and 84% similarity on average) to previously identified ORs annotated as PRs in various moth species (Fig. 2). The cDNA sequence has been deposited in GenBank (accession number: MF431269). As expected for insect ORs, the sequence was predicted to have seven
transmembrane domains (Fig. 1). The C-terminal region known to be conserved in moth PRs was present as were the three highly conserved PR motifs (Fig. 2) (Zhang and Löfstedt 2015).

Phylogenetic Analysis

We constructed a maximum-likelihood phylogeny of the OR subfamily members that have been reported to be tuned to type I pheromones. In this tree, sequences grouped within five distinct clades (named A to E in Fig. 3) that were highly supported by the likelihood-ratio test. PRs from Noctuidae clustered only in clades D and E. MbraOR16 belonged to a particular lineage within clade E that included PR sequences (OR6 and OR16) from *H. armigera*, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), and *Agrotis segetum* (Schiff.) (Lepidoptera: Noctuidae) that are tuned to type I pheromones of different natures. Due to moderate support at several nodes within this lineage, the precise phylogenetic position of MbraOR16 could not be determined.

Fig. 1. Full length sequence of *MbraOR16* transcript and deduced amino acid sequence. Transmembrane domains (TMs) were predicted using HMMTOP2.1 (Tusnády and Simon 2001); amino acids corresponding to the predicted TM 1–7 are shown in gray boxes.
Tissue-Specificity by RT–PCR Analyses

MbraOR16 was only amplified from antennal cDNAs (Fig. 4, upper part). Although RT–PCR is not a quantitative method, we noticed a markedly higher amplification in male antennal cDNA relative to female (Fig. 4). The integrity of all cDNAs was confirmed by rp18 amplification, which exhibited little variation.

Fig. 2. MbraOR16 amino acid alignment with other moth pheromone receptors. Multiple sequence alignment was done with MULTALIN (Combet et al. 2000). In the alignment only conserved amino acids are shown, nonconserved ones are represented with dots. Sequences included those from Spodoptera littoralis (Slit) (Legeai et al. 2011), Spodoptera litura (Slitu) (Zhang et al. 2015), Spodoptera exigua (Sexi) (Liu et al. 2013a), Agrotis segetum (Aseg) (Zhang and Löfstedt 2013), Athetis dissimilis (Adis) (Dong et al. 2016), Heliothis virescens (Hvir) (Krieger et al. 2004), and Helicoverpa armigera (Harm) (Liu et al. 2013b). The three C-terminal conserved domains characteristic of moth PRs (Zhang and Löfstedt 2015) are boxed.

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in expression across the various tissues investigated (Fig. 4, lower part).

Antennal Expression Pattern via In Situ Hybridization

*M. brassicae* antennae are filiform and segmented, and their dorsal part is covered with scales, whereas the ventral part has numerous trichoid sensilla (Jacquin-Joly et al. 2000) devoted to olfaction. Although no signal was generated with the sense probe (Fig. 5A), antisense probe labeling was restricted to the ventral side (the olfactory side) of the male antennae (Fig. 5B). More precisely, *MbraOR16* transcripts were localized to olfactory sensilla bases in the male antenna, which likewise house olfactory neuron cell bodies. Expression in particular could be seen at the bases of the long trichoid sensilla, which are known to be involved in pheromone reception and are easily recognizable in the transversal sections of the antennal segments (Fig. 5C).

**Discussion**

Our results suggest that we cloned a full-length mRNA encoding *MbraOR16*, which represents the first candidate PR of the crop pest moth *M. brassicae*. The encoded protein possessed the hallmarks of insect ORs, such as seven transmembrane domains and specific expression in the antennae that in situ hybridization showed was restricted to olfactory sensilla (Fig. 5). Additional findings support classification of this receptor as a PR: 1) the transcript was male enriched, as usually observed for moth PRs (Zhang and Löfstedt 2015, Zhang et al. 2015); 2) the deduced protein possessed the conserved C-terminal signature motifs typical of moth PRs (Zhang and Löfstedt 2015); and 3) *MbraOR16* clustered in a lineage containing noctuid PRs tuned to type I pheromone components. Taken together, our findings suggest that *MbraOR16* is also a type I PR, whereas *M. brassicae* pheromone blend consists of type I components. However, the precise position of *MbraOR16* within the clade could not be determined. Furthermore, because PR response spectra evolved rapidly, it is difficult to infer a putative ligand for *MbraOR16* based on the phylogeny. Nevertheless, several lines of evidence let us propose that we have identified the receptor for *M. brassicae* behavioral antagonists Z11-16:OH and/or Z9-14:Ac (Descoins et al. 1978), compounds found in the sex blends of heterospecific females that prevent unspecific attraction between species with similar sex pheromone blends. The in situ hybridization performed on male antennae indicates that *MbraOR16* transcripts were localized in long trichoid sensilla, that are known to house two neurons, one tuned to the main pheromone component Z11-16:Ac, and the other one tuned to the behavioral antagonists Z11-16:OH and Z9-14:Ac (Renou 1991, Renou and Lucas 1994). In the phylogeny, *MbraOR16* did not cluster with moth PRs tuned to Z11-16:Ac, such as *H. virescens* OR14 (Wang et al. 2011) and *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) OR1 (Mitsuno et al. 2008), but rather clustered with receptors tuned to Z11-16:OH, such as *H. armigera* and *H. virescens* OR16 (Wang et al. 2011, Liu et al. 2013b), and to Z9-14:Ac, such as *A. segetum* OR10 (Zhang and Löfstedt, 2013). However, unequivocal demonstration of ligand tuning will require further functional studies for a better understanding of *M. brassicae* sex pheromone reception.

**Fig. 4.** RT–PCR expression study of *MbraOR16* in samples derived from different tissues. PCR products are visualized by ethidium bromide after electrophoresis on a 1.5% agarose gel. Tissues examined included male antennae, female antennae, proboscis, Br-SOG (brain–subesophageal ganglion complex), thorax, abdomens, legs, and wings. *MbraOR16* amplification led to a 765 bp product. A 508-bp fragment of ribosomal protein 8 (rpl8) was amplified in each sample and used as a cDNA integrity control. Ladder: 1kb DNA ladder (Invitrogen). Only the 500-bp band is visible.

**Fig. 5.** Expression pattern of *MbraOR16* in male antennae of *M. brassicae*. Male antennae longitudinal (A and B) and transversal (C) sections (6 µm) counterstained with acridine orange after whole-mount hybridization with a DIG-labeled sense probe (A, control) or a DIG-labeled antisense probe (B and C). Black triangles: trichoid sensilla. White triangles: long trichoid sensilla. Small arrows: labeled structures.
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