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Short Communication

# 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 environmental-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 them 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, Vogt 2005). This system consists of pheromone-sensitive sensory neurons housed in sensilla located on the antennae (Montagné et al. 2015). Most moth sex pheromone components are hydrophobic and are thought to be transported from the air through the sensillum lymph to the neuron by dedicated proteins, the pheromone-binding proteins (PBPs) (Vogt 2005, Pelosi et al. 2014). Reaching the neuron dendritic membrane, the pheromone components are detected by specific subtypes of odorant receptors (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 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

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* [Linnaeus] [Lepidoptera: Noctuidae]) is the major pest of Cruciferous vegetable plants in Eurasia (Hill 1987). Its sex pheromone has been characterized (Attygalle 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 Löfstedt 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-suboesophageal 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 overnight 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 of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) PRs: OR16up 5′-ATGGACTACAAATATATGAAAGT-3′ and OR16low 5′-TAGTTGTTGTACATGGGTATC-3′. The 3′ and 5′ regions of the cDNAs were obtained by 3′ and 5′ RACE-PCR using the SMART RACE kit with a Universal Primer Mix versus gene-specific primers (GSPs), OR16-5′-GSP: 5′-ACGTTGGTGATCGTCGGCTCTTGGCCCAG-3′ and OR16-3′-GSP: 5′-GGTGTAACCGGTGTTGGCATGATTGGAGCG-3′. These GSPs were designed to overlap the positions of the initial degenerated primers, allowing sequence verification. The generated fragments were cloned into pCR II-TOPO (Invitrogen) and sequenced (Biofidal, Vaux-en-Velin, France). Several clones were sequenced for each PCR product with all exhibiting 100% identity. By merging all sequences obtained, a cDNA with a CDS of 1290 bp, referred to as *MbraOR16*, was identified as a putative *OR16* homolog based on BLAST (Altschul et al. 1990) sequence analyses and multiple sequence alignments with Lepidoptera PRs using MULTALIN (Combet et al. 2000). Sequence integrity was verified by PCR amplification of the whole open reading frame and sequencing of both strands. Default prediction of the MbraOR16 transmembrane topology was done using HMMTOP2.1 (Tusnády and Simon 2001). For tissue expression analyses, PCR profiling (40 cycles, 60°C) was done using the different tissue templates and primers (OR16TE-up 5′-GGTGTAACCGGTGTTGGCATGATTGGA-3′; OR16TE-low 5′-CATGTAATACACGAACAGCATCGGTCC-3′) that generated a 765-bp product. The *rpl8* gene (508 bp) of *M. brassicae* was used as a control as described previously (Maïbèche-Coisné et al. 2004).

### Phylogeny

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 OR16R-T7 5′-ATTG TAATACG ACTCACTATAGGGC ATGTAATACACGAAC AGCATCGGTCC-3′; OR16F-SP6: 5′-AGCTATTTAGGT GACACTATAGGGTGTAAC CGGTGTTGGC ATGATTGGA-3'and OR16R 5'-CATGTAAT ACACGAACAG CATCGGTCC-3′. Transcriptions were performed using T7 and SP6 RNA polymerases (Promega) according to the recommended protocol. Hybridization and tissue sectioning (longitudinal and transversal) were performed as described previously (Jacquin-Joly et al. 2000).

## 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

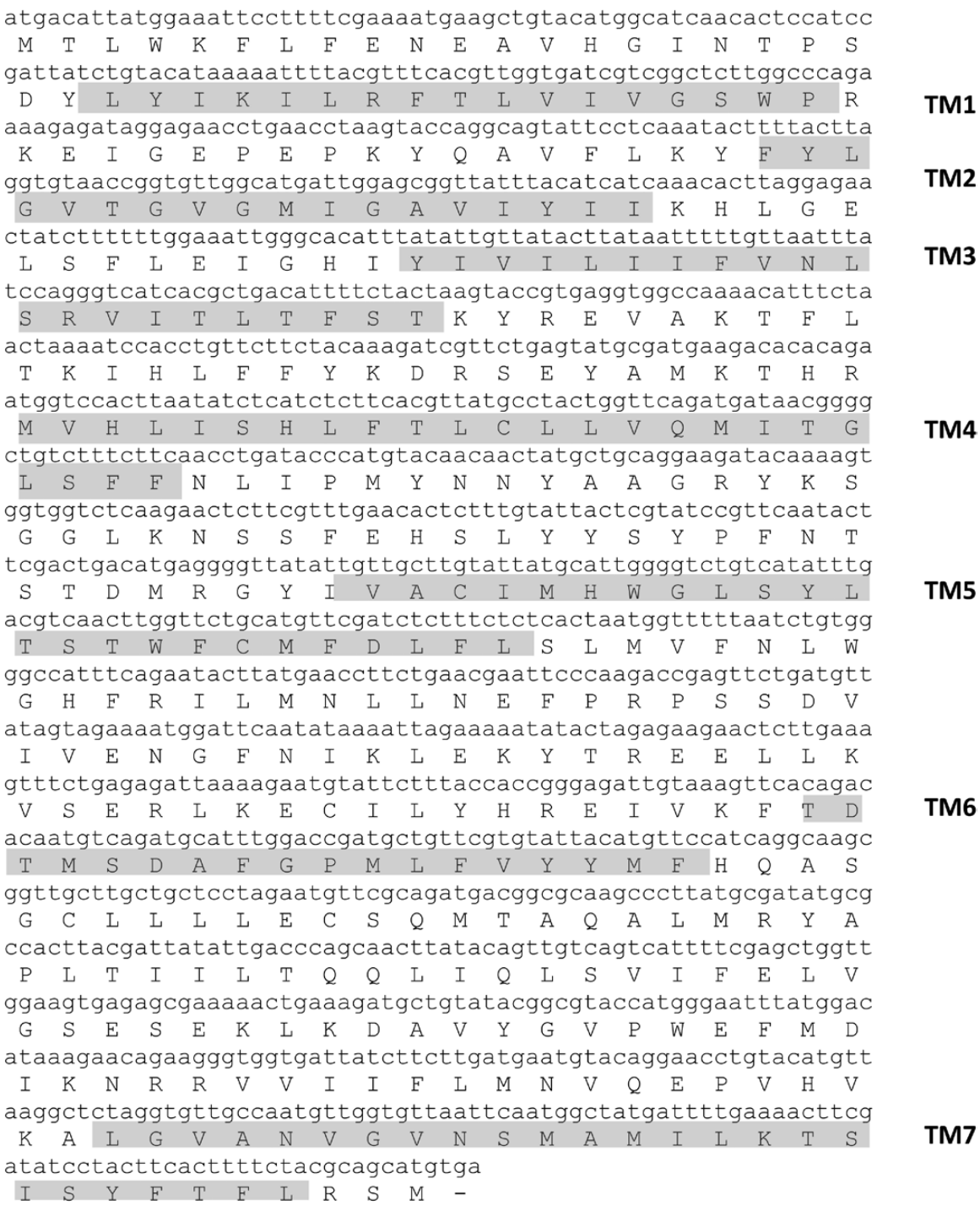


**Fig. 1.** Full length sequence of *MbraOR16* transcript and deduced amino acid sequence. Transmembrane domains (TMs) were predicted using HMMTOP2.1 (Tusnády and Simon 2001); amino acids corresponding to the predicted TM 1–7 are shown in gray boxes.

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.

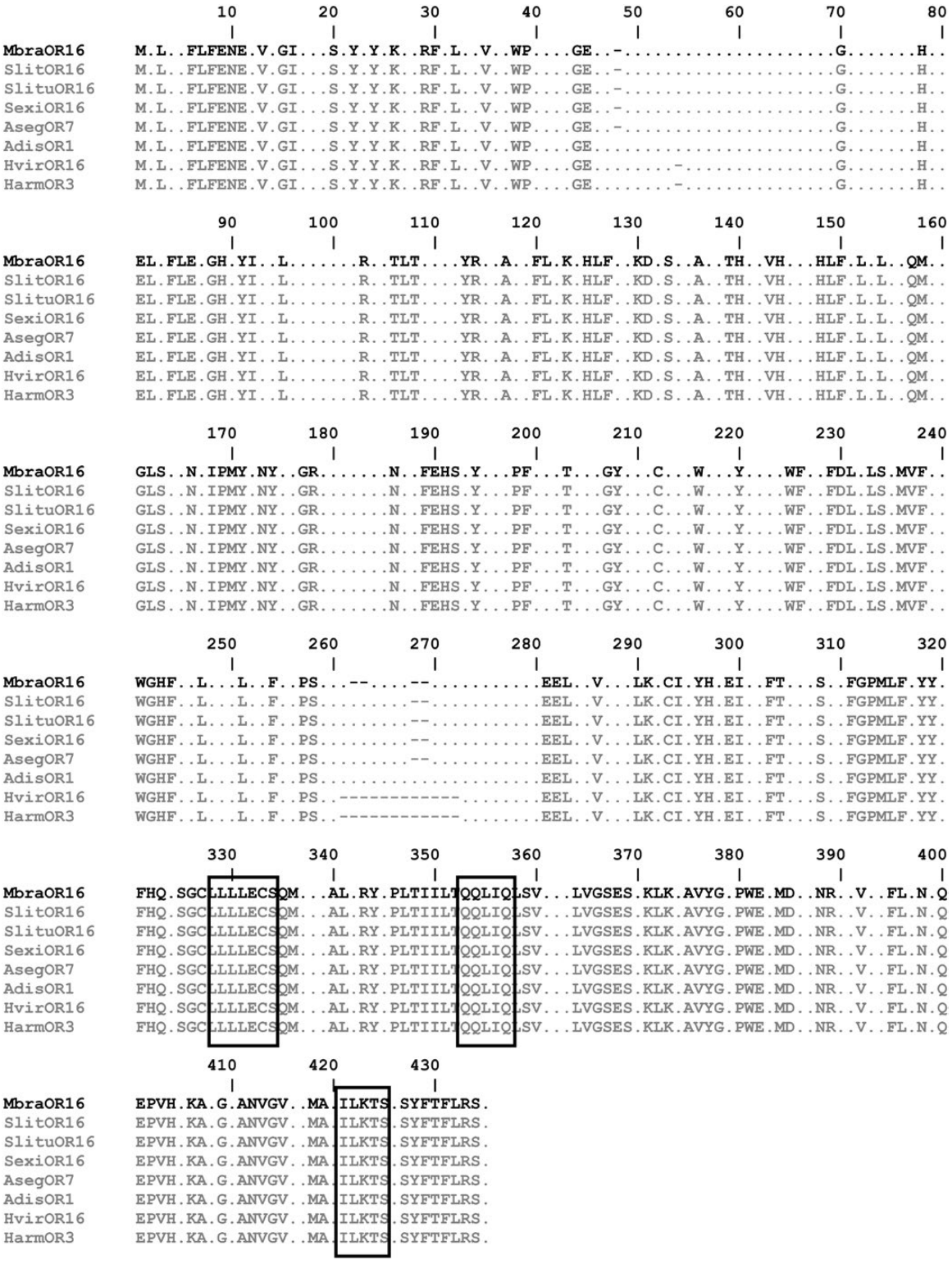


**Fig. 2.** MbraOR16 amino acid alignment with other moth pheromone receptors. Multiple sequence alignment was done with MULTALIN (Combet et al. 2000). In the alignment only conserved amino acids are shown, nonconserved ones are represented with dots. Sequences included those from *Spodoptera littoralis* (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.

## 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

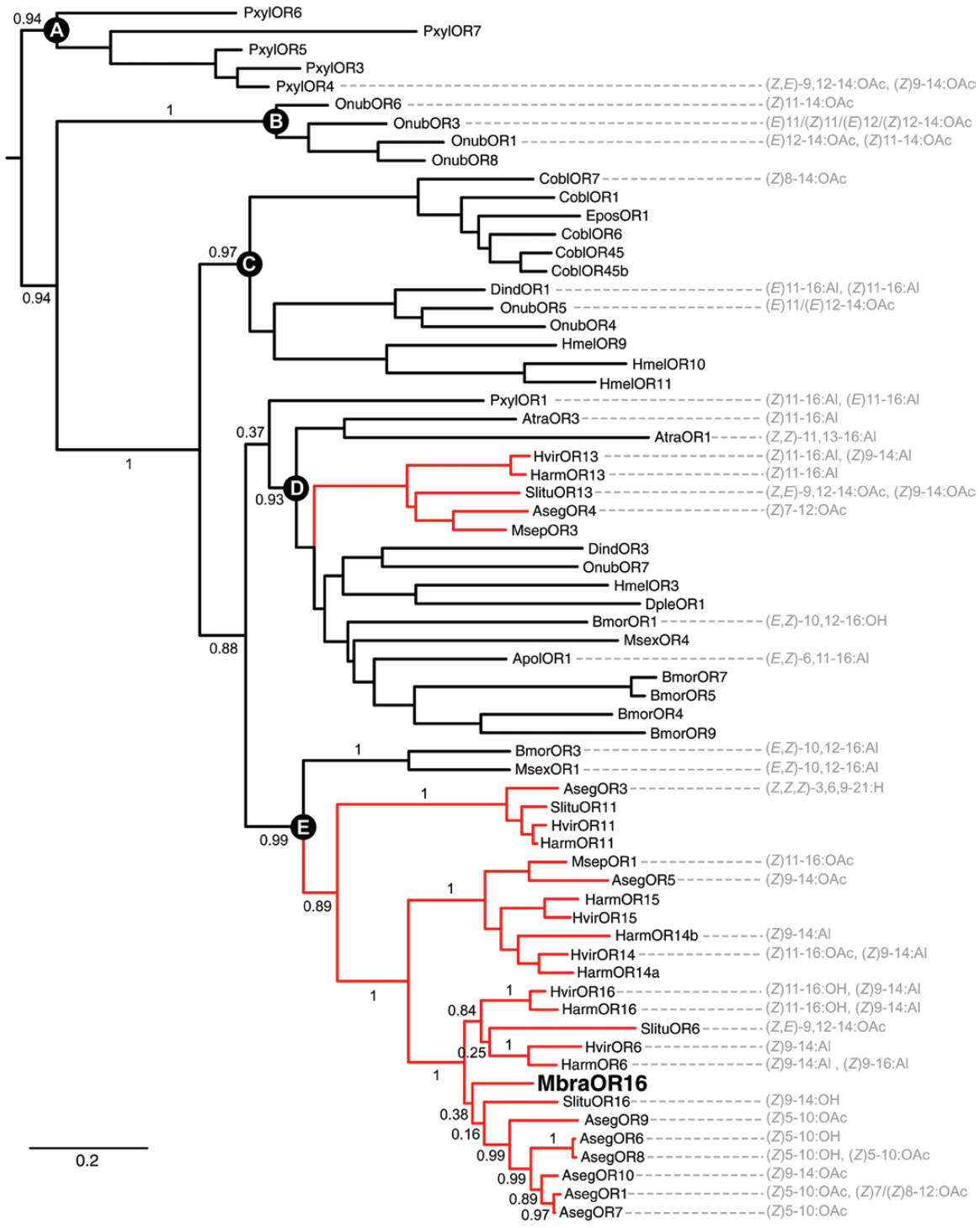


**Fig. 3.** Maximum likelihood tree of Lepidopteran candidate pheromone receptors. The phylogenetic tree was constructed using the MbraOR16 sequence identified in this study and pheromone receptor sequences identified in *Agrotis segetum* (Aseg) (Zhang and Löfstedt 2013), *Amyelois transitella* (Atra) (Xu et al. 2012), *Antheraea polyphemus* (Apol) (Forstner et al. 2009), *Bombyx mori* (Bmor) (Krieger et al. 2005, Nakagawa et al. 2005, Tanaka et al. 2009), *Ctenopseustis obliquana* (Cobl) (Steinwender et al. 2015), *Danaus plexippus* (Dple) (Zhan et al. 2011), *Diaphania indica* (Dind) (Mitsuno et al. 2008), *Epiphyas postvittana* (Epos) (Jordan et al. 2009), *Heliconius melpomene* (Hmel) (The Heliconius Genome Consortium 2012), *Helicoverpa armigera* (Harm) (Liu et al. 2013b, Jiang et al. 2014, Liu et al. 2014), *Heliothis virescens* (Hvir) (Krieger et al. 2002, Krieger et al. 2004, Wang et al. 2011), *Manduca sexta* (Msex) (Patch et al. 2009, Grosse-Wilde et al. 2010), *Mythimna separata* (Mspe) (Mitsuno et al. 2008), *Ostrinia nubilalis* (Onub) (Miura et al. 2010, Wanner et al. 2010, Yasukochi et al. 2011, Leary et al. 2012), *Plutella xylostella* (Pxut) (Mitsuno et al. 2008, Sun et al. 2013), and *Spodoptera litura* (Slitu) (Zhang et al. 2015). When identified, the corresponding ligands are indicated on the right. Numbers on the branches are support values (approximate likelihood ratio-test) for basal nodes and for nodes within the clade containing MbraOR16. Letters A to E indicate the five distinct clades that were highly supported by the likelihood-ratio test. The scale bar represents the expected number of amino acid substitutions per site. In the color figure of the online version, red branches represent sequences from the Noctuidae family.

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).

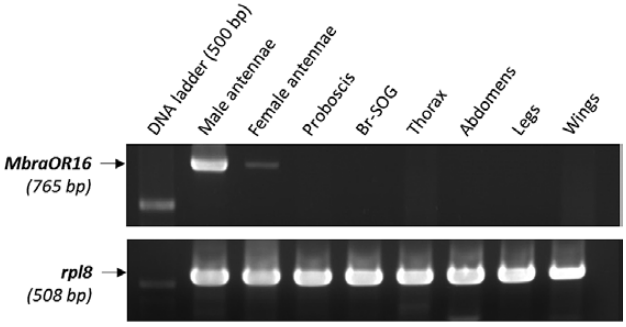


**Fig. 4.** RT–PCR expression study of *MbraOR16* in samples derived from different tissues. PCR products are visualized by ethidium bromide after electrophoresis on a 1.5% agarose gel. Tissues examined included male antennae, female antennae, proboscis, Br-SOG (brain–subesophageal ganglion complex), 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.

## 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.

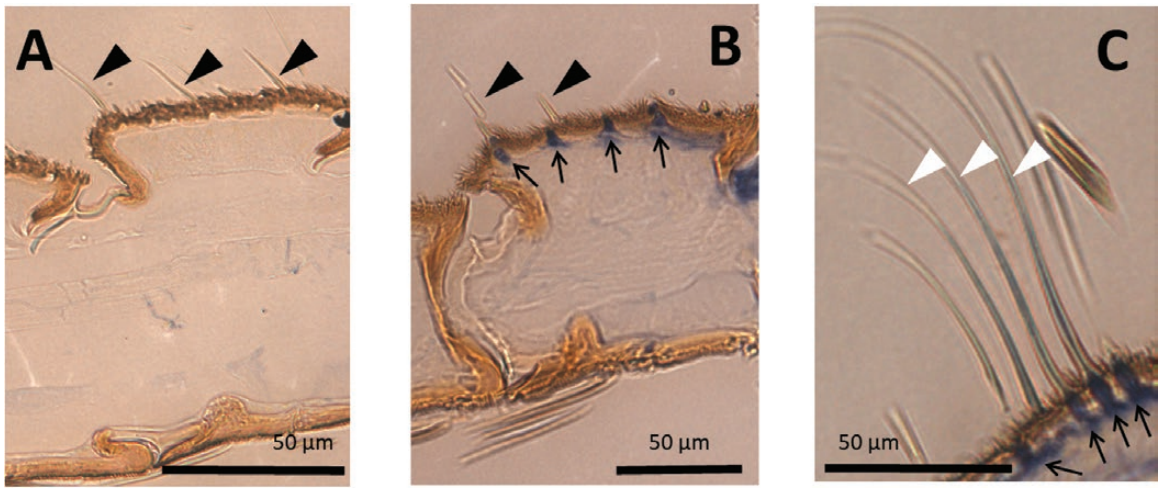


**Fig. 5.** Expression pattern of *MbraOR16* in male antennae of *M. brassicae*. Male antennae longitudinal (A and B) and transversal (C) sections (6 µm) counterstained with acridine orange after whole-mount hybridization with a DIG-labeled sense probe (A, control) or a DIG-labeled antisense probe (B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

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