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Biologisch-Pharmazeutische Fakultät

Diploma thesis

Flies smelling like a moth - Expressing *Manduca sexta* odorant receptors in the "empty neuron system"

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for Chemical Ecology

submitted by

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Declaration of original authorship

I hereby declare that the work submitted is my own and that all passages and ideas that are not mine have been fully and properly acknowledged.

Jena, 01.11.2011 .....

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1. Introduction

The sense of smell allows insects to detect and assess volatile cues from the environment. Odor information is crucial for many important behaviors, e.g. orientation to food sources, communication between individuals and location of suitable oviposition sites (Baker 1989; Renwick 1989). The first volatile compound involved in intraspecific communication, was identified by Butenandt et al., (1959) in *Bombyx mori* and named bombykol. In the same year a new term for substances active involved in this kind of communication was introduced; "pheromone" derived from the Greek "pherein", to transfer (Karlson et al. 1959). Therefore, in the following decades olfactory-guided behaviors of insects were studied intensely. One focus were sex pheromones emitted by females to attract potential mating partners (Tumlinson et al. 1989; Baker 1989). Since *Bombyx mori* is domesticated and of limited use for studies in the context of ecology, a multitude of other lepidopteran species were analyzed as well. Especially the giant sphinx moth *Manduca sexta* has become one of the best described model organism in the field of olfaction due to its complex natural behavior.

A focus were plant-emitted odors; it was shown for example that female *Manduca sexta* use olfactory cues for orientation towards their hostplants (Yamamoto et al. 1969). Olfactory cues are required for the detection of oviposition sites and for seeking floral nectar sources on which both sexes feed (Raguso et al. 2002). Volatile fruit compounds were shown to be attractant for females (Cossé et al. 1994) as well as volatiles released by flowers (Haynes et al. 1991). *Manduca sexta* preferentially selects *Solanacae* e.g. tobacco plants (*Nicotiana spec.*), tomato leafs *(Solanum lycopersicum)* (Mechaber et al. 2002) and *Datura wrightii* (Raguso et al. 2003; Riffell et al. 2008) for oviposition and larval feeding. Investigation of odor bouquets emitted by nine *Nicotiana* species identified 125 volatile compounds, including several compounds for which emission levels change during the day (Raguso et al. 2003). Hostplants emit large numbers of volatile compounds, but only a minority is behaviorally important

(Reisenman et al. 2009; Fraser et al. 2003). Besides the identified green leaf volatiles (GLVs) and flower odors, it has been shown that herbivore-induced plant volatiles (HIPV) that are released after herbivore damage are attractant to predators of herbivores (Hare 2010; Rasmann et al. 2005; Köllner et al. 2008), and that female moths avoid oviposition on plants which are herbivore damaged (Reisenman et al. 2009). All this indicates that a highly dynamic olfactory environment crucial to fitness is perceived by the animals.

The primary olfactory organ of *Manduca sexta* is the antenna; the antennal flagellum is subdivided into 80 annuli and bears ca. 100,000 hair-like sensilla that house ca. 250,000 olfactory peripheral sensory neurons (OSN). The OSNs detect odorant molecules (Sanes et al. 1976; Lee et al. 1990). Several types of sensilla were identified: two types of sensilla trichoidea, two types of sensilla basiconica and one type of sensilla coeloconica (Sanes et al. 1976; Lee et al. 1990).

The *Manduca sexta* antenna shows a pronounced sexual dimorphism (Figure 1, taken from Shields et al. 1999); the male antennal flagellum is keyhole shaped in cross-section and enlarged (Figure 1B, 1D, 1F), the female antenna is in comparison considerably shorter and oval shaped (Figure 1A, 1C, 1E). The male antenna contains ca. 40,000 long trichoid sensilla housing two pheromone-sensitive OSNs (Sanes et al. 1976; Keil 1989).

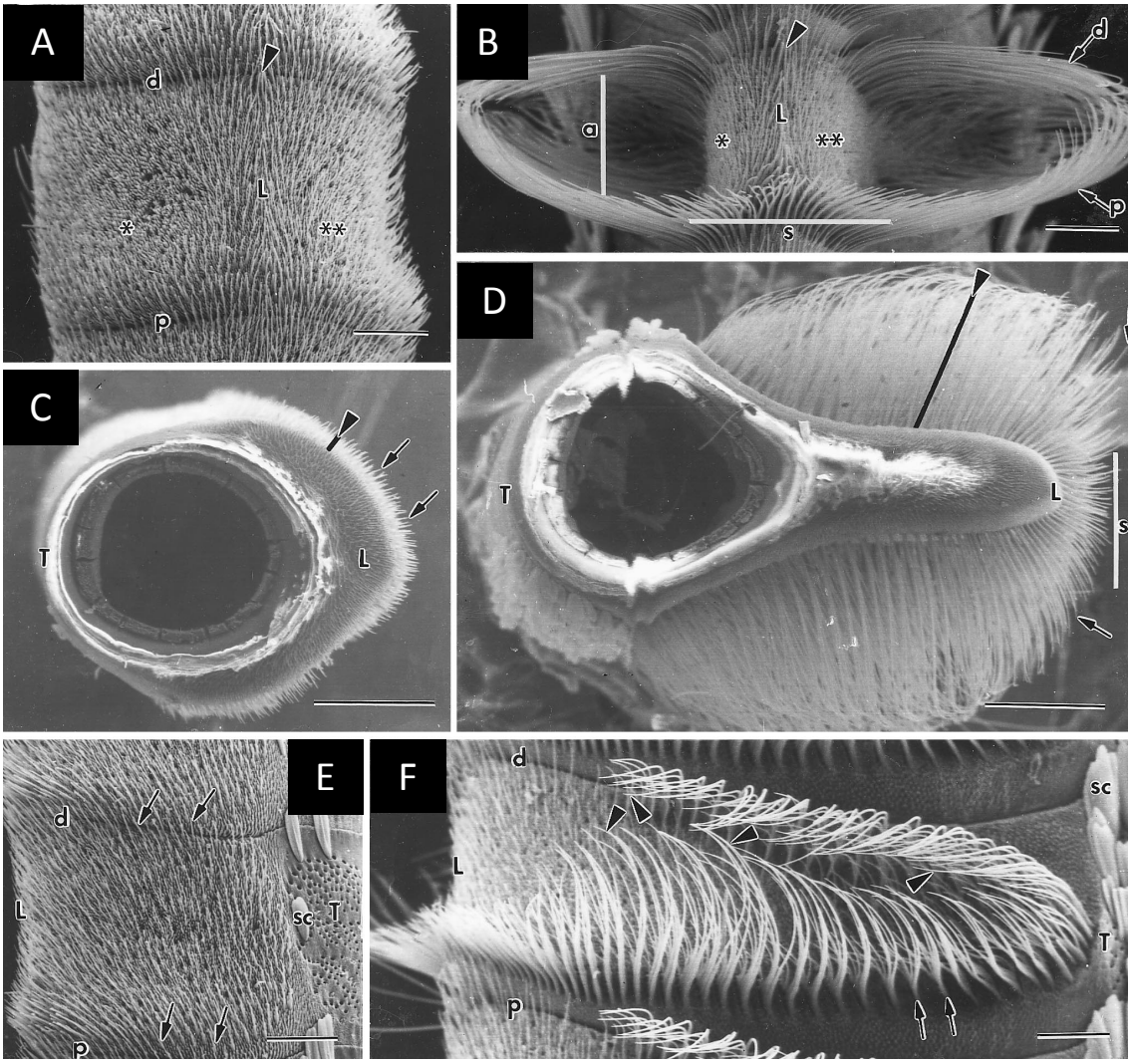


Figure 1: Scanning electron micrographs of adult female and male Manduca sexta antenna.
Taken from Shields and Hildebrand 1999a. **A)** Female flagellar annulus. Scale bar = 100 µm **B)** Male flagellar annulus. Notable are the long male-specific trichoid sensilla. Scale bar = 100 µm **C)** Cross section of the female flagellar annulus present their oval profile. Scale bar = 250 µm. **D)** Cross-sectional view of a male flagellar annulus. Notable are the keyholed shape and the log trichoid sensilla type A (arrow). Scale bar = 250 µm. **E)** Ventral view of a female flagellar annulus. The flagellum is much narrower than in the male. Arrows show shorter Type A trichoid sensilla than in male. Scale bar = 100 µm. **F)** Ventral view of a male flagellar annulus demonstrates the typical u-shaped order of trichoid type A sensilla. Scale bar = 100 µm.

Electrophysiological recordings of the pheromone-sensitive trichoid sensilla revealed that the majority of the trichoid OSN subtypes is tuned to bombykal (50% short vs. 46,2% long (Kaissling et al. 1989; Kalinová et al. 2001)), the others to either of the minor pheromone components E10, E12, Z14 hexadecatrienal (EEZ) (42,5% short vs. (Kaissling et al. 1989; Kalinová et al. 2001)); E10, E12, E14-hexadecatrienal (EEE) (6% short vs. 0,9% long (Kaissling et al. 1989; Kalinová et al. 2001)); E10, E12-

hexadecadienal (EE) (4,71% long (Kalinová et al. 2001)) and Z11- hexadecanal (0% long (Kalinová et al. 2001)). Furthermore, it was shown that pheromone-specific OSNs of the trichoid sensila project to the sexual dimorphic macroglomerular complex (MGC) in the antennal lobe, the first information processing center in the CNS (Christensen et al. 1995). Different MGC glomeruli were innervated by the main OSN subtypes that respond to bombykal and EEZ, respectively (Hansson et al. 1991). Stimulation of female antennae using pheromones did not elicit a response (Hansson et al. 2003). Therefore female *Manduca sexta* generally lack OSNs tuned to the female-produced pheromone. However, it has been shown that a small number of M*anduca* females respond to the minor pheromone component Z11- hexadecanal (Kalinová et al. 2001). The sexual dimorphism reflects male adaptations in detection and processing of sex-pheromonal information. Short sensilla recordings revealed sensitivity to plant odors in both sexes (Kalinová et al. 2001). Plant odors activate a glomerulus in a similar position in both sexes, which indicates similar plant odor processing (Hansson et al. 2003). Stimulation with the plant-odor linalool leads to activation of the lateral large female glomerulus (latLFG) (King et al. 2000) via a specific OSN population. Furthermore Reisenman et al. (2004) described a second distinct glomerulus, with the latLFG and this glomerulus responding independently to one of the two enantiomers of linalool. This indicates a possible function for selection of appropriate host plants for oviposition.

These observations indicate a very specific molecular recognition mechanism. At the molecular level, in recent decades a small number of gene families involved in the olfactory system have been identified:

The identification of the main protagonist in odor detection, the odorant receptors (ORs), was challenging. Initially, putative odorant receptors genes had been identified using genomic data of *Drosophila melanogaster* (Clyne et al. 1999; Gao et al. 1999; Vosshall et al. 1999). Odorant receptors embedded in the dendrites of OSN in the antenna interact specifically with odorants and determine the ligand specificity of distinct neurons (Hallem et al. 2004). ORs are predicted to have seven transmembrane

domains, indicating a possible identity as G protein-coupled receptors (GPRC). However, their transmembrane topology is inverse in comparison to other GPCRs, with an intercellular N-terminus (Benton et al. 2006). ORs show remarkably high sequence variety and low conservation in insects. Therefore, sequence similarity analysis failed to facilitate identification of ORs in other species and methods employing genomic databases were used to identify putative ORs in *Lepidoptera.*

Nevertheless recent studies revealed an extensive set of putative members of olfactory gene families in *Manduca sexta* (Patch et al. 2009; Grosse-Wilde et al. 2010, 2011). The *Manduca sexta* homolog of the insect olfactory coreceptor ORCo (Vosshall et al. 2011) was found, which shows a high degree of conservation across insect species (Patch et al. 2009; Grosse-Wilde et al. 2010; Krieger et al. 2003; Larsson et al. 2004). ORCo is necessary as a chaperon for the transfer of ORs to the OSN dendrites (Benton et al. 2006) and forms a heteromultimer with ORs that acts as an ion channel (Wicher et al. 2008; Sato et al. 2008). Members of a comparatively conserved group (Krieger et al. 2004) of male-specific lepidopteran pheromone receptors (Sakurai et al. 2004; Nakagawa et al. 2005; Krieger et al. 2005) have been identified in *M.sexta* (Patch et al. 2009; Grosse-Wilde et al. 2010). Especially the recently published antennal transcriptome data of *Manduca* is essential for further studies to identify members of the main olfactory gene families (Grosse-Wilde et al. 2011). Compared to 73 glomeruli in the antenna lobe, 54 putative receptor genes (47 ORs) have been identified in *Manduca sexta* (Grosse-Wilde et al. 2011). Due to the 1:1 correlation observed in other insect species between ORs and glomeruli (de Bruyne et al. 2008), unidentified receptor genes are expected. However, to verify the identity of the identified genes as OR-coding functional analysis is needed. Additionally, this will allow association of single genes with specific behaviors.

Due to the involved work we focused on a subset of receptors. The male-specific putative pheromone receptors MsexOR-1 and MsexOR-4 were chosen as good candidates for deorphaning; furthermore we chose the female specific MsexOR-5 and -6 that are related to the linalool receptor BmOR-19 in *Bombyx mori,* since they seem

to be candidates to detect enantiomers of linalool (Reisenman et al. 2004). A more unusual candidate was MsexOR-31, which seems to be a highly conserved OR subtype specific to Lepidoptera, indicating a special but yet unknown function.

The well studied model organism *Drosophila melanogaster* has become the most important genetic tool for manipulation. The utility offered by the UAS-Gal4-System (Brand et al. 1993) for targeted gene expression allows using *Drosophila melanogaster* as heterologous expression system. Compared to the in vitro heterologous expression techniques using *Xenopus* oocytes or human embryonic kidney cells, the advantage of this approach is that ORs are expressed in a natural insect system, allowing rapid de-orphanization using electrophysiological methods (Figure 2.).

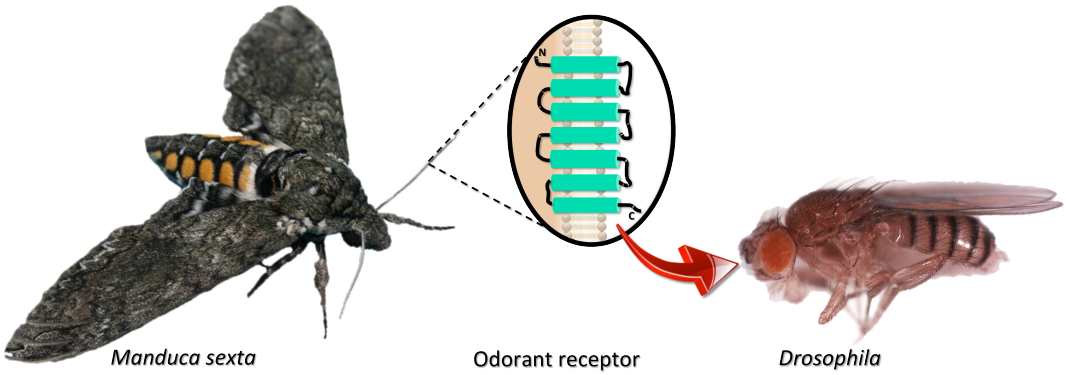


Figure 2: The "empty neuron system".

The "empty neuron system" makes use of the Δhalo mutant, i.e. flies with a deletion of DmelOR22a and DmelOR22b. The upstream promoter region of DmelOR22a is used to drive expression of *Manduca sexta* ORs in ab3A receptor neurons (which in wild type flies express DmelOR22a) using the Gal4-UAS-System (van der Goes van Naters et al. 2007; Hallem et al. 2004; Dobritsa et al. 2003).

Functional analysis of *Drosophila melanogaster* ORs by expression in the "empty neuron" revealed that with only few exceptions antennal OSNs express only one functional OR (Hallem et al. 2004). Functional analysis *Anopheles gambiae* odorant receptor (AgOr) repertoire, employing the "empty neuron" as heterologous expression

system, revealed receptors that respond to human-emitted volatiles and seem to be involved in identifying its human hosts (Carey et al. 2010). Furthermore the pheromone sensitive odorant receptor BmOR-1 of *Bombyx mori* was expressed in the Δhalo-mutant flies. BmOR-1 expressing neurons respond to the *B. mori* pheromone bombykol (Syed et al. 2006).

We used *Drosophila melanogaster* as heterologous expression system to functionally express MsexORs using the Δhalo approach, verifying successful transgene integration and expression levels. Finally, we assessed MsexOR function in electrophysiological measurements, which enables us to predict possible functions for several analyzed receptors.

2. Material and Methods

2.1. General Chemicals

The following chemicals were manufactured by Sigma Aldrich (St. Lois, MO): wheat germ, cholesterol, ascorbic acid, sorbic acid, methyl paraben, nicotinic acid, riboflavin, thiamine, pyridoxine, folic acid, biotin, Tri Reagent, 1-bromo-3-chloropropan, trans-2-hexenyl acetate, ethyl butyrate and trans-caryophyllene. Bombykol and bombykal were kindly provided by Dr. A. Svatos (Max Planck Institute for Chemical Ecology, Jena). Biozym (Hessisch Oldendorf, Germany) supplied the LE Agarose and MP Biomedicals (Costa Mesa, CA) manufactured the Salt Mix. Sugar and linseed oil were purchased via Rewe (Köln, Germany). Rapunzel (Legau, Germany) provided corn meal and soy flour. The manufacturer Roth (Karlsruhe, Germany) delivered all other used general chemicals. Double distilled water (ddH2O) was used in all experiments. Diethylpyrocarbonate (DEPC) treated water was gained by incubating 1 ml of 0.1% DEPC and 1000 ml ddH2O at room temperature over night followed by autoclaving the solution at 121°C for 20 min.

2.2. Special Chemicals for Molecular Biology

The Taq DNA Polymerase Kit from Qiagen (Hilden, Germany) was used for PCR reactions. Furthermore Qiagen manufactured the Rotor Gene SYBR Green PCR Kit. Clontech (Mountain View, CA) produced the Advantage 2 PCR Enzyme System.

New England Biolabs (Ipswich, MA) manufactured 2log DNA ladder and all restriction enzymes used with associated buffers, Invitrogen (Carlsbad, CA) delivered SuperScript III First Strand System for RT-PCR including DEPC treated water, oligo dt, dNTPs, 10X Reaction buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNAse out, SuperScript RT III and RNAse H. Additionally, Invitrogen supplied the Topo TA Dual Promotor Cloning Kit (comprising pCRII-Topo Vector, Salt Solution) and One shot Top10 chemical competent *E. coli* cells (containing SOC Medium, *E. coli* cells). TURBO-DNAse was provided by Applied Biosystems/ Ambion (Austin, TX). EZNA (Norcross, GA) supplied the Gel Extraction Kit

(comprising Binding Buffer, Elution Buffer, Wash Buffer with Ethanol added as written in the manual) and the Plasmid Mini Prep Kit (including Solution I (with RNAse A as written in the manual), II and III as well as Equilibration Buffer, Buffer HB, Elution Buffer and DNA Wash Buffer with Ethanol added as written in the manual). Roth delivered dNTPs for RT PCR. All used primers were provided by MWG (Ebersberg, Germany).

2.3. Devices

Device	Name/Type	Manufacturer
Autoclave	MLS 3781 L	Sanyo (Moriguchi City, Japan)
Centrifuge	Centrifuge 5415 R	Eppendorf (Hamburg, Germany)
Cryostat	Microm HM 560	Thermo Fisher Scientific
Distiller	Mono Dest 3000E	Lenz Laborglas (Wertheim, Germany)
Electrophoresis chamber	Mupid One	Advance (Tokyo, Japan)
Heater	Thermomixer comfort	Eppendorf (Hamburg, Germany)
Climate chamber	Economic Premium	Snijders (Drogenbos, Belgium)
Photometer	BioPhotometer	Eppendorf (Hamburg, Germany)
Pipettes	Pipetman	Gilson (Middleton, WI)
UV - chamber	BioVision 3026	Peqlab (Erlangen, Germany)
Shaker	AI82K	Infors (Bottmingen, Switzerland)
Ceramic beads	2,8 mm	Peqlab (Erlangen, Germany)
Thermocycler	PCR System 9700	Applied Biosystems (Carlsbad, CA)
Tissuelyzer	Tissuelyzer LT	Qiagen (Hilden, Germany)
Vortex shaker	Vortex Genie 2	Scientific Industries (Bohemia, NY)
Water bath	SW22	Julabo (Seelbach, Germany)
Binocular microscope	S8APO	Leica (Solms, Germany)
Cold light source	KL200	Olympus (Hamburg, Germany)

Real time cycler	Rotor Gene Q	Qiagen (Hilden, Germany)
Spectrophotometer	NanoDrop ND-2000	Peqlab (Erlangen, Germany)
Manipulator	SM-59	Luigs & Neumann (Ratingen, Germany)

Table 1: List of the used devices.

2.4. Cloning of putative MsexOR genes

2.4.1. Rapid Amplifying of cDNA Ends-Polymerase Chain Reaction (RACE-PCR)

Performing RACE-PCR, components of MARATHON RACE cDNA Amplification Kit and Advantage 2 PCR Enzyme System were used. cDNA-Synthesis was provided The 5'-RACE PCR reaction consists of 18µl ddH_2O; 2.5 µl 10x cDNA PCR reaction buffer; 0.5 µl 10mM dNTP mix; 2.5 µl adaptor ligated cDNA; 0.5 µl AP1 primer; 0.5 µl of reverse primer listed in the table and 0.5 µl Advantage 2 Polymerase Mix. The following PCR program was used: 1 cycle 94°C for 30 s; 5 cycles 94°C for 5 s, 72°C for 4 min; 5 cycles 94°C for 5 s, 70°C for 4 min; 25 cycles 94°C 5 s, 68°C 4 min. The resulting amplified products were visualized using gel electrophoresis.

Gene	Primer name	Primer sequence (5'-3')	TM in °C
MsexOR-34	35ORFrev	TTAGAATGATGATGACCCAAATATCTTGTGTGG	64.5
MsexOR-16	112ORFrev	TCAATCTTCGACGCTCTGCAGTACGG	66.4
MsexOR-6	OR-6rev	TTAGTGAAATACTGAGATTAAAGAATACGCAGATTGA	63.9
	AP1	CCATCCTAATACGACTCACTATAGGGC	71

Table 2: Oligonucleotides used for RACE-PCR.
AP1=adaptor primer 1**.**

2.4.2. Agarose- Gel Electrophoresis

Gel Electrophoresis was performed in Agarose (1%) - TAE buffer (0.04 M tris, 0.04 M vinegar acid, 1 mM EDTA) system. To allow detection of DNA ethidium bromide was added to the gel to a final concentration of 0.005%. While necessary 6X loading dye (30% Glycerol, 70% dH_2O, 2.5 mg bromphenol blue) was applied to samples, 2log Ladder (New England Biolabs, Ipswich, MA) was used as a marker. Gelelectrophoresis was performed at 135 V for 30 min in TAE buffer. Gels were observed by UV chamber to visualize PCR products for extracting. DNA bands of the expected fragment size were separated from the preparative gel with a sterile scalpel blade.

2.4.3. Gel Extraction

Gel extraction was performed using E.Z.N.A. MicroElute Gel Extraction Kit (Omega Bio-Tek, Norcross, GA). The separated gel slices were placed in a 1.5 ml tube along with 300 µl Binding Buffer. The Gel was molten by incubation at 60°C for 7 min, vortexing briefly every 2 min. Solved DNA fragments were transfer to DNA Mini Columns, which were placed in 2 ml collection tubes. After centrifuging at 10,000 x g for 1 min the liquid flow-through was discarded and 300 µl Binding Buffer added to the column, followed by another centrifugation step at 10,000 x g for 1 min to wash the column. The flow-through was chucked and the column was washed two times with 700 µl Washing Buffer each followed by centrifugation at 10,000 x g for 1 min. The columns were centrifuged for 2 min at 16,000 x g to dry the column matrix. 30 µl Elution buffer was applied directly onto the column matrix. After an incubation step for 1 min at room temperature the samples were centrifuged for 1 min at 16,000 x g to elute the DNA.

2.4.4. Cloning

The Topo TA Dual Promotor Cloning Kit (Invitrogen) with One Shot Top10 chemical competent cells was used for cloning. 2 µl of the extracted PCR product were combined with 0.5 µl salt solution and 0.5 µl pCR2.1-TOPO Vector into a 1.5 ml tube, followed by incubation for 5 min at room temperature, 25 µl chemical competent cells were thawed for 5 min on ice, 2 µl reactions added carefully and incubated for 15 min on ice. The cells were exposed to 42°C for 45 s and kept on ice for 5 min immediately, afterwards 150 µl SOC Medium were applied to the cells and placed on a shaker for 1 h at 37°C. The cells were plated on LB agarose plates with 100 µg/ml ampicillin and 60 ng/ml X-gal and stored in the incubator at 37°C over night.

2.4.5. Colony – PCR

Colony - PCR was performed to confirm that cloning was successful. The used cloning vectors are suitable for ß-Gal based blue white screening. White colonies were sampled with a sterile toothpick and a small quantity of cells transferred into a starter culture (50 µl of LB medium and ampicillin), which was grown for 1 h. The following components were pipetted into PCR-tubes for a combined total of 25 µl: 19 µl ddH_2O, 2.5 µl 10x CL PCR buffer (Qiagen Inc., Valencia, CA), 0.5 µl 10 mM dNTP mix, 1 µl 10 µM M13-forward primer, 1 µl 10 µM M13-reverse primer and 0.125 µl Taq polymerase (Qiagen Inc., Valencia, CA). 2 µl of the starting culture were added to the samples as template, mixed thoroughly by vortexing and run in a PCR-Cycler. PCR was performed using the following steps: 94°C for 2 min, 34 Cycles 94°C for 15 s, 57°C for 30 s and 72°C for 90 s). The terminal cycle was followed by 5 min at 72°C. In order to analyze the fragment size, an analytic gel electrophoresis was performed. Starter cultures of the probes with the expected fragments were allowed to grow over night in 5 ml LB medium with 10 µl Ampicillin.

Primer name	Sequence (5‘-3‘)
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC

Table 3: List of the used primers for Colony-PCR primers.

2.4.6. Plasmid Mini Preparation and Sequencing

Plasmid Mini Preparation was performed using E.Z.N.A. Plasmid Miniprep Kit (Omega Bio-Tek, Norcross, GA). The bacterial cultures were centrifuged at 4,000 x g for 15 min. The supernatant was discarded and the bacterial pellet resuspended in 250 µl Solution I/ RNaseA solution. The suspension was transferred to a 1.5 ml tube, 250 µl Solution II added and inverted until a clear lysate formed, followed by incubation for 2 min. The solution was neutralized with 350 µl Solution III and mixed by inverting until a flocculent white precipitate appeared. Afterwards, probes centrifugated at 13,000 x g for 10 min. Simultaneously, DNA Miniprep columns placing in collection tubes were prepared with 100 µl Equilibration Buffer and centrifuged at 13,000 x g for 1 min. The cleared supernatant of the cell lysate was transferred to the columns and centrifuged for 1 min at 13,000 x g.

Columns were washed once with 500 µl HB Buffer and twice with 700 µl DNA Wash Buffer followed by centrifugation step for 1 min at 13,000 x g. Then the columns were dried by centrifugation at 13,000 x g for 2 min and placed in new 1.5 ml tubes.

DNA was eluted from the column with 30 µl Elution Buffer by centrifugation at 13,000 x g for 1 min. The DNA concentration was determined photometrically and samples sent to MWG Eurofins for sequencing.

2.4.7. Sequence Analysis

Sequences of putative ORs of *Manduca sexta* were truncated to remove bad base calls of vector sequences, and assembled using SeqMan with standard settings. Contigs were analyzed of existence of open reading frames (ORF) using SeqBuilder (DNAStar,

Madison, WI, USA). Consensus sequences were aligned with transcriptom analysis data (Grosse-Wilde et al. 2011) to identify putative ORs to full length.

2.4.8. Sequence alignment and maximum-likelihood analysis

Putative OR amino acid sequences were aligned to other known lepidopteran OR proteins by using MAFFT (http://mafft.cbrc.jp/alignment/server/), (Katoh et al. 2010). Maximum-likelihood analysis and the dendrogram creation were performed using MEGA5 (Tamura et al. 2011). Dendrogram was colored utilizing FigTree v1.3.1 and Adobe Illustrator (Adobe Systems).

2.4.9. Topology analysis

The amino acid sequences of putative OR's were analyzed using the transmembrane prediction program TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), (Krogh et al. 2001) to determine putative topology.

2.5. Touchdown - PCR to insert restriction sites

The Advantage 2 PCR Enzyme System was used to amplify full length OR coding ORF combined in the pCR2.1-TOPO Vector, using primer-based site directed mutagenes to allow for subcloning with restriction sites. Primers were designed to contain recognition site sequences of NotI- (forward) and XhoI (reverse) restriction enzymes. (Table 4) PCR reactions consisted of 20 µl ddH_2O, 2.5 µl 10x Advantage 2 PCR buffer, 0.5 µl 10 mM dNTP mix, 0.5 µl 10 µM primer 1, 0.5 µl 10 µM primer 2, 1 µl DNA, 0.5 µl DNA-template and 0.5 µl Advantage 2 polymerase with a final volume of 25 µl. Touchdown PCR was performed using the following steps: 94°C for 2 min, 20 Cycles 94°C for 45 s, 70°C for 1.30 min and 68°C for 45 s. The annealing temperature of 70°C was degreased each round by 0.5°C. Afterwards 10 cycles with 94°C for 45 s, 60°C for

1.30 min and 68°C for 45 s were connected. The terminal cycle was run by 68°C for 7 min and dropped finally to 4°C for cooling.

Gene	Primer name	Sequence (5‘-3‘)	TM in °C
MsexOR-16	112NOTF	aagcggccgcATGGGAATTTTCGTGCAAAATGTCAA	71,6
MsexOR-16	112XHOR	aacctcgagTCAATCTTCGACGCTCTGCAGTAC	70,7
MsexOR-22	16NOTF	aagcggccgcATGGGTTGGATCGAGAGAATAAAGG	73
MsexOR-22	16XHOR	aacctcgagTTAATTACTGTTCCTCAGCATGTTGAAATAA	67,4
MsexOR-31	29NOTF	aagcggccgcATGGCTCAAAACACAGAATTATTTTTGGGT	71,5
MsexOR-31	29XHOR	aacctcgagTTATGTGTTCGCCCTGTTGAGGAC	70,7
MsexOR-34	35NOTF	aagcggccgcATGAAGATATTCATYSACAACGCCAAC	72,2
MsexOR-34	35XHOR	aacctcgagTTAGAATGATGATGACCCAAATATCTTGTG	68,4
MsexOR-1	OR1NOTF	aagcggccgcATGATATTTATGGACGATCCTCTATCAAAG	71,5
MsexOR-1	OR1XHOR	aacctcgagTTAGTTAGAAACGGTGCGAAGAAATGC	69,5
MsexOR-4	OR4NOTF	aagcggccgcATGAAGTTTTTTGTAGACGGCAGCGAA	72,8
MsexOR-4	OR4XHOR	aacctcgagTTAGCTCTCATCTTTGGCGATTGTTTG	69,5
MsexOR-5	OR5NOTF	aagcggccgcATGAAGGTACCTCTAAAAAAATTCAGGC	71,6
MsexOR-5	OR5XHOR	aacctcgagTTAGTAAAGTACTGAGAACACCGAATATG	68,4
MsexOR-6	OR6NOTF	aagcggccgcATGGAAGAGACAAAAAAATACACACCAAC T	71,5
MsexOR-6	OR6XHOR	aacctcgagTTAGTGAAATACTGAGATTAAAGAATACGCA G	68,4

Table 4: List of the used Oligonucletides.
Underlined are the binding sites for the restriction enzymes.

2.6. Plasmid Subcloning

2.6.1. Restriction digestion

Double digestion was performed using the following components: 2.5 µl 10x buffer 4, 0.5 µl BSA, 1 µl restriction enzyme 1 (NotI-HF), 1 µl restriction enzyme 2 (XhoI), 1 µg/ml plasmide containing the mutated OR-coding ORF and ddH_2O to a combined total volume of 25 µl. The target pUAST vector was also digested using the same enzymes and addionally treated with 1 µl of calf intentestinal alkaline phosphatase (CIP) at 37°C. Following a 2 hour of incubation at 37°C, the reaction mixtures were analyzed after loading onto a preparative gel. DNA bands of the expected fragment size were separated and purified from the gel.

2.6.2. Ligation and transformation

Ligation was used to insert the OR-coding sequences into the pUAST vector multicloning site. The 20 µl ligation reaction consisted of 2 µl 10x ligation buffer, 2 µl digested pUAST vector, 15 µl digested OR-gene and 1 µl T4-ligase. The samples were incubated over night at 4°C. One Shot Top10 chemical competent cells were transformed using the ligated DNA, by first adding 5 µl of the ligation mixture to 25 µl of chemical competent cells and incubating on ice for 15 min. Then cells were exposed to heat shock at 42°C for 45 s and stored on ice for 2 min immediately. Afterwards 150 µl SOC Medium was applied to the cells and placed on a shaker for 1 h at 37°C. The cells were plated on LB agarose plates with 100 µg/ml ampicillin and kept in the incubator at 37°C over night.

2.6.3. Colony-PCR, Plasmid Mini Preparation and Sequencing

Colony-PCR, Plasmid Mini Preparation and sequencing were performed as described above.

2.6.4. Plasmid Midi Preparation

Plasmid Midi Preparation was performed using Plasmid Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Bacterial cultures were centrifuged at 4,000 rpm for 15 min at 4°C. The medium was discarded and the pellet resuspended in 4 ml buffer P1, containing RNAse A and LyseBlue reagent. 4 ml of buffer P2 were added to the suspension. The suspension was mixed by inverted until turning blue, and was incubated at RT for 5 min. Suspension was neutralized with 4ml of pre-chilled buffer P3 and mixed by inverting until a fluffy white precipitate appeared. The lysate was transferred immediately into a filter cartridge and incubated for 10 min. To equilibrate the column, 4 ml of buffer QBT were applied and allowed to empty by gravity flow. The plunger was inserted into the filter cartridge after the cap was removed and the lysate was filtered onto the previously equilibrated column and allowed to enter the resin by gravity flow. The column was washed twice with 10 ml of buffer QC and the DNA eluted using 5 ml of buffer QF. The flowthrough was collected and for DNA precipitation 3.5 ml of isopropanol were added, followed by mixing and centrifuging at 4,000 rpm for 60 min at 4°C. The supernatant was carefully removed and the pellet washed with 70% ethanol. After centrifuging at 4,000 rpm for 5 min, the DNA pellet was air dried for 10 min and redissolved by adding 100 µl of TE buffer. The DNA concentration was measured photometrically and the samples sent to MWG Eurofins for sequencing to verify correct insertion in the pUAST vector.

2.6.5. Fly transgenesis

Confirmed constructs of MsexOR-1, -4, -5, -6, -16, -22, -31 and -34 in the pUAST vector were diluted to a concentration of 1 µg/µl and sent to University of Cambridge, Department of Genetics for germline transformation of *Drosophila melanogaster* embryos. Balanced lines were established with insertions of UAS-MsexOR-X for each of the chromosomes X, 2 and 3. Flies containing insertion of UAS-MsexOR-X on the third chromosome were selected and used to establish fly lines carrying the UAS-MsexOR-X

in a Δhalo background. They were crossed with Δhalo strains using the DmelOR22a-Gal4 as driver to produce experimental flies with the following genotype: w; Δhalo; UAS-MsexOR/OR22a-Gal4 (see Results for crossing scheme). Transgenic flies with co-expressed DmelOR22a and Msex-OR-X were generated as well with the following genotype w; Δhalo/+; UAS-MsexOR/DmelOR22a-Gal4.

2.7. Experiments performed on *Drosophila*

2.7.1. Fly Rearing and stocks

Flies were reared continuously in plastic vials containing fly food prepared to the following recipe: While 909 ml of water were boiled, 870 g molasses and 49 g yeast were added along with 18.3 g agar, dissolved in 1215 ml of cold water. Corn meal (426 g) is dissolved in 1215 ml of hot water and applied to the boiling mixture. After rinsing with 306 ml of water the blend was brought to the boil. Finally 11 ml of propionic acid with 456 ml water were added to the boiling mixture. The blend was allowed to cool down slowly and 15 ml Nipagin (30 g in 100 ml Ethanol) were added before dispensing into new vials. The flies were turned over every 14-17 days if stored in a climate chamber at 25°C with 70% humidity and a 12 h light/12 h dark cycle. For crossing, vials were cleared of adult flies in the morning and were kept 6 h at 25°C. The enclosed females in the meantime are virgins and were introduced to their selected mates.

Identification	x Chr.	2nd Chr.	3rd Chromosome	Source
OR-1 5/TM6c	+	+	UAS-MsexOR1/Tm6c	Cambridge
OR-4 4/TM6c	+	+	UAS-MsexOR4/Tm6c	Cambridge
OR-5 11/TM6c	+	+	UAS-MsexOR5/Tm6c	Cambridge
OR-6 3/TM6c	+	+	UAS-MsexOR6/Tm6c	Cambridge
MS112 4/TM6c	+	+	UAS-MsexOR16/Tm6c	Cambridge
MS16 6/TM6c	+	+	UAS-MsexOR22/Tm6c	Cambridge
MS29 1/TM6c	+	+	UAS-MsexOR31/Tm6c	Cambridge

MS35 4/TM6c	+	+	UAS-MsexOR34/Tm6c	Cambridge
Δhalo	w1118	Δhalo/CyO	+	
DB	yw	CyO/Bl	TM2/TM6B	BL 3704
Or22aGal4	w[*]	Δhalo/CyO	Or22a-Gal4/Or22a-Gal4	Carlson

Table 5: List of the used transgenic fly lines .Chr.= chromosome

2.7.2. Genomic-DNA Purification

Genomic DNA from flies was extracted utilizing purification of total DNA from insects using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA). Five previously collected adult flies per sex were placed in tubes prefilled with ceramic beads (Peqlab, Erlangen, Germany) and 180 µl PBS. Animals were homogenized using a TissueLyser LT (Qiagen, Valencia, CA) at 50 Hz for 15 min and mixed thoroughly by vortexing with 20 µl proteinase K and 200 µl Buffer AL (without added ethanol), followed by incubation at 56°C for 2 h. After adding 200 µl ethanol (96–100%) and mixing thoroughly by vortexing, the samples were centrifuged for 1 min at ≥ 6,000 x *g.* The homogenous mixture including any precipitate was pipetted into the mini spin columns placed in a 2 ml collection tube and centrifuged at ≥ 6000 x *g* for 1 min. Flow-through and collection tube were discarded. The mini spin columns were placed in a new 2 ml collection tube, 500 µl Buffer AW1 added and centrifuged for 1 min at ≥ 6,000 x *g*. Flow-through and collection tube were discarded. The mini spin columns were placed in a new 2 ml collection tube and mixed with 500 µl Buffer AW2. The samples were centrifuged for 3 min at 20,000 x g to dry the membrane. The centrifugation step prevents residual ethanol to be carried over during the following elution. To avoid the contact between column and flow-through, the mini spin columns were removed carefully, and placed in a clean 1.5 ml tube. Flow-through and collection tube were discarded. The membrane was suffered with 100 µl Buffer AE and incubated at room temperature for 2 min. Finally the samples were centrifuged for 1 min at ≥ 6,000 x g to elute the DNA. The extracted DNA was analyzed in gel electrophoresis to check the results of the purification.

2.7.3. Amplification of specific DNA sequences using PCR

The following components were placed in PCR-tubes to a combined total of 25 µl to start the PCR reaction: 20 µl ddH_2O, 2.5 µl 10x CL PCR buffer (Qiagen Inc., Valencia, CA), 0.5 µl 10 mM dNTP mix, 1 µl 10 µM primer 1, 1 µl 10 µM primer 2, 1 µl purified genomic fly DNA and 0.2 µl Taq polymerase (Qiagen Inc., Valencia, CA). The samples were mixed thoroughly by vortexing and placed in PCR-Cycler. Touchdown PCR was performed using OR gene-specific primers, shown in the table above and the purified DNA as template. The probes run by 94°C for 2 min, 20 cycles 94°C for 45 s, 70°C for 1.30 min and 68°C for 45 s. The annealing temperature of 70°C dropped each round by 0.5°C. Afterwards 10 cycles with 94°C for 45 s, 60°C for 1.30 min and 68°C for 45 s were connected. The terminal cycle was run by 68°C for 7 min and dropped finally to 4°C for cooling.

2.7.4. Isolation of total RNA from *Drosophila* Antennae

Experimental flies of both sexes were collected in 15 ml Falcon tubes and placed in liquid nitrogen for 5 min. Every 30 s the tubes were shaken vigorously and kept back into liquid nitrogen. This was repeated 5 times and the tubes placed on dry ice followed by adding 5-10 ml of precooled 100% acetone. The acetone mixture was passed through increasingly dense meshes to separate palps and antennae from body parts and heads. The eluted palps and antennae were centrifuged at 6.000 rpm for 8 min and transferred into 1.5 ml tubes. After completely remove of the acetone, 600 µl TRI reagent were added and the tubes transferred onto dry ice to freeze the solution solid. The tubes were melted by hand and incubated at room temperature for 10 min to rehydrate, followed by homogenization with RNAse free pestles. The probes were incubated at room temperature for 20 min and centrifuged at 11.400 rpm for 10 min at 4°C to pellet insoluble debris. The upper aqueous phase was relocated into a fresh tube and 72 µl of 1-bromo-3-chloropropan added. After shaking vigorously by hand, samples were incubated 20 min on ice and centrifuged at 10.000 rpm for 15 min at 4°C to induce phase separation. The upper aqueous phase was transferred to a new tube

and 50-80 µl of 10x DNAse buffer and 1 µl of Turbo DNAse (Ambion) added to start DNAse treatment. After incubating for 30 min at 37°C, 600 µl TRI reagent was added and mixed by pipetting. The solution was gently mixed with 72 µl of 1-bromo-3-chloropropan and placed on ice for 20 min, followed by centrifugation at 10.000 rpm for 15 min at 4°C. Subsequently, the upper aqueous phase was transferred to a new tube, 500 µl of 100% isopropanol added and incubated for 10 min at room temperature. After an over night incubation step at -20°C, the samples were centrifuged at 11.400 rpm for 30 min. The supernatant was discarded and the pellet washed with 800 µl of 70% ethanol followed by centrifugation for 10 min at 11.400 rpm. After complete removing of the ethanol residue, the pellet was air-dried for 10 min and finally the pellet was dissolved in 25 µl RNAse free water. The samples were analyzed by gelelectrophoresis as described in 2.4.3. The concentration of RNA was measured photometrically.

2.7.5. cDNA Synthesis

Messenger RNA (mRNA) was used as template for cDNA using SuperScript III First Strand System for RT-PCR. 250 ng RNA, isolated from *Drosophila* antennae, were filled up to 8 µl of DEPC treated H2O. After adding 1 µl dNTPs and 1 µl oligo dT primer, the probes were incubated at 65°C for 5 min and placed 1 min on ice. The following components were added to the mixture: 2 µl 10x RT-buffer, 4 µl $MgCl_2$ 25 mM, 2 µl DTT 0.1 M, 1 µl RNAse out and 1 µl SuperScript III RT. The preparation was incubated at 50°C for 50 min followed by termination by inactivation at 85°C for 5 min. The template RNA was depleted by adding 1 µl RNAse H at 37°C for 20 min.

2.7.6. Real Time PCR (qPCR)

Real time PCR was performed using a Rotor Gene SYBR Green PCR Kit. The qPCR reaction contained 12.5 µl of SYBR Green Master Mix, 9.5 µl dH2O, 1 µl cDNA, 1 µl Primer 1, 1 µl Primer 2 for a combined total of 25 µl. Primers were designed for amplifying fragments of 170-190 bp of length. Sequences of used primers are presented in the table below. Samples ran at 94°C (5 min), 40 cycles 94°C (10 s), 60°C (10 s), while cycling fluorescence intensity of SYBR Green dye was measured at the end of each elongation cycle (72°C for 10 s). This was followed by meltingcurve analysis, heating from 50°C to 99°C with 1°C per 5 s. This allows evaluating the specificity of the amplifications. Data was analyzed using Rotor Gene Q cycler software (Qiagen Inc., Valencia, CA) to compare relative expression of samples to the housekeeping gene (HKG). ß-actin5c was used as HKG, amplified and subsequently gel extracted. The concentration of the product was measured and serial dilutions ($^1/_4$, $^1/_{16}$, $^1/_{32}$, $^1/_{64}$ and $^1/_{256}$) used as standard in the same qPCR run. Each sample was measured in duplicate. Negative control reactions without cDNA were run to confirm primer specificity and lack of contamination.

Gene	Primer name	Primer sequence (5'-3')	Length in bp	TM in °C
OR-4	OR-4_for	GAAGCGGATCCGAACAAA	191	59.73
	OR-4_rev	TCCAGCAACAGCAAGCAG	191	59.84
OR-31	OR-31_for	TGCAGAAGGTGCGTGAAG	183	59.68
	OR-31_rev	TAGCAACCGTGCCACTCA	183	59.98
OR-34	OR-34_for	TGCATTGGAAACCCATGA	171	59.39
	OR-34_rev	GGAATTGGGCACGCATAC	171	59.89
ß-Actin5c	Actin_for	GCCCTCGTTCTTGGGAAT	170	60.01
	Actin_rev	GGGCGGTGATCTCCTTCT	170	60.16

Table 6: List of the used Oligonucleotides for qPCR

2.7.7. Electrophysiology

A fly was mounted in a truncated pipette tip with the head facing the narrow end. The tip was truncated and a small amount of wax placed into the back to prevent backward movement of the fly, on a microscope slide an antenna was fixed on a cover slip using a glass electrode placed. A tungsten electrode was pushed into the eye for grounding; the recording electrode was brought into contact with the base of the sensillum using a Luigs and Neumann SM-59 manipulator.

2.7.8. Odor stimulation

The odorants were diluted in hexane. 10 µl of 1 µg/µl dose of each odorant was loaded onto a 1 cm diameter filter paper and placed in a Pasteur pipette. Synthec stimulus air controller filtered and humidified air to be passed over the antenna at approximately 1 l min^{-1}. During stimulation, airflow switched to a complementary air stream (0.5 l min^{-1} during 0.5 s) passing through the stimulus pipette and onto the antenna.

3. Results

3.1. Cloning of putative MsexOR genes

The aim of my studies was to characterize olfactory receptors (ORs). Recently published antennal transcriptome data (Grosse-Wilde et al. 2011) were the basis for identification of members of the main olfactory gene families, including fragments of ORs. To faciltate functional analysis of MsexORs, fragments of candidate genes needed to be extended to full-length. Using rapid amplification of cDNA ends PCR (RACE-PCR), unknown 3' and 5'-ends of a gene can be amplified (Chenchik et al. 1996) (Figure 3).

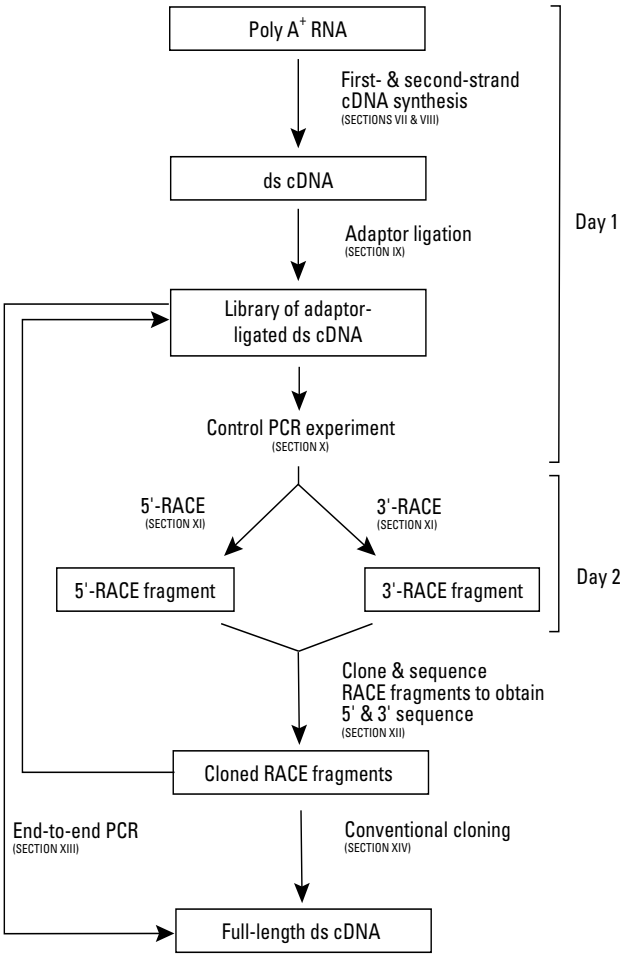


Figure 3: Overview of Marathon RACE-PCR procedure.
Taken from Marathon® cDNA Amplification Kit User Manual, Clontech Laboratories, Inc.

Previously to this study, 3'ends of putative *Manduca* ORs had been identified; I used RACE-PCR to clone the corresponding 5'ends. The 5' RACE-PCR used a gene-specific-primer (GSP), the adaptor primer (AP1) and adaptor-ligated dscDNA that does not contain a binding site for AP1. Elongation of the GSP to the end of the adaptor generates an AP1 binding site at the 5' terminus of the cDNA.

Accordingly both AP1 and GSP can bind to afford exponential amplification of the cDNA of the odorant receptor (Chenchik et al. 1996). To characterize RACE products and confirm that the targeted odorant receptor gene was amplified, PCR products were subject to gel electrophoresis and products extracted from the gel. The extracted amplificate were cloned and sequenced. Analysis of the data yielded putative complete open reading frame (ORF) sequences of MsexOR-6, MsexOR-22 and MsexOR-34. ORF sequences can be seen in appendix.

3.1.1. Sequence similarity analysis

To analyze relatedness of the putative transcripts to other known lepidopteran OR proteins, predicted amino acid sequences were aligned using MAFFT, maximum likelihood analysis performed and the results visualized as a dendrogram (Figure 4).

According to recently published data of the antennal transcriptome of *Manduca sexta* (Grosse-Wilde et al. 2011) MsexOR-6 is a homolog of the female specific MsexOR-5 and closely related to BmorOR-19, a linalool plant odor responding receptor of *Bombyx mori* (Anderson et al. 2009; Grosse-Wilde et al. 2010).

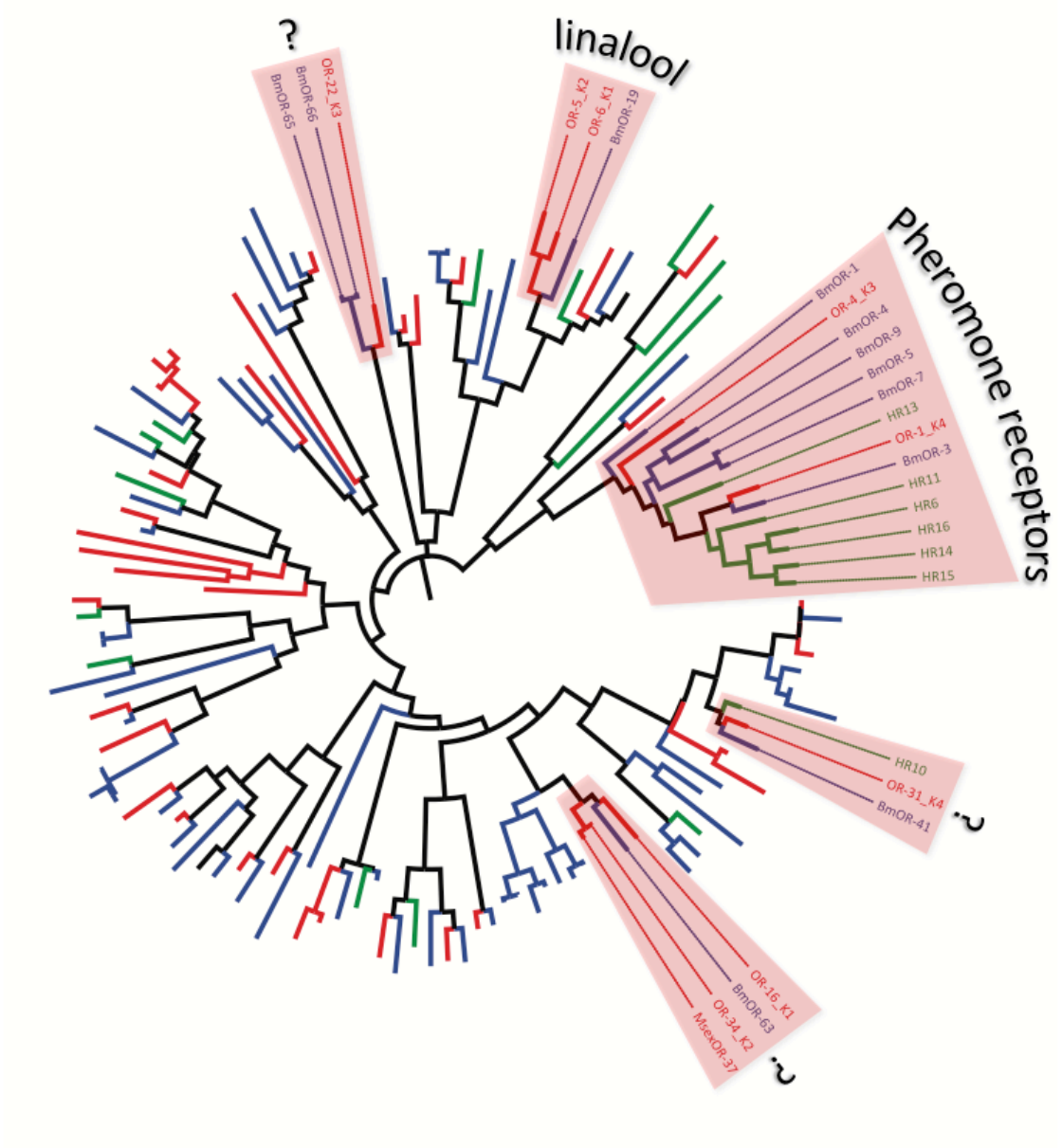
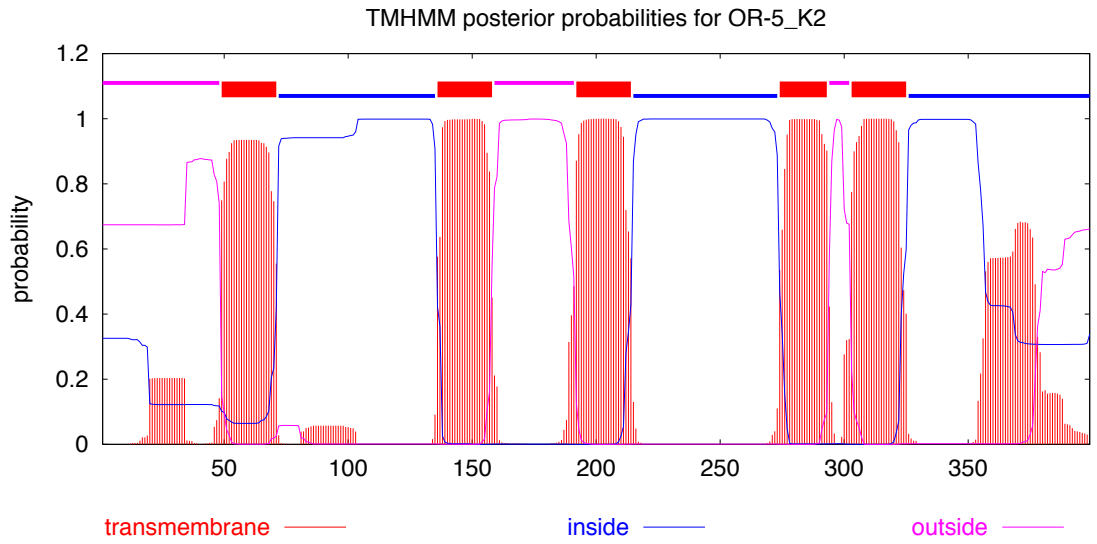


Figure 4: Maximum-likelihood-dendrogram.
Maximum-likelihood-dendrogram of amino acid sequences of putative OR's of *Manduca sexta* (red*)*, *Bombyx mori* (blue) and *Heliothis virescens* (green). Highlighted are the full-length cloned OR's and relatives in other lepidopteran species, including male specific pheromone receptors and the female specific linalool detecting subgroup.

3.1.2. Transmembrane topology

Further evidence for MsexOR-6 as an odorant receptor is the prediction of the transmembrane domain structure of the full ORF shown in Figure 5B.

A



B

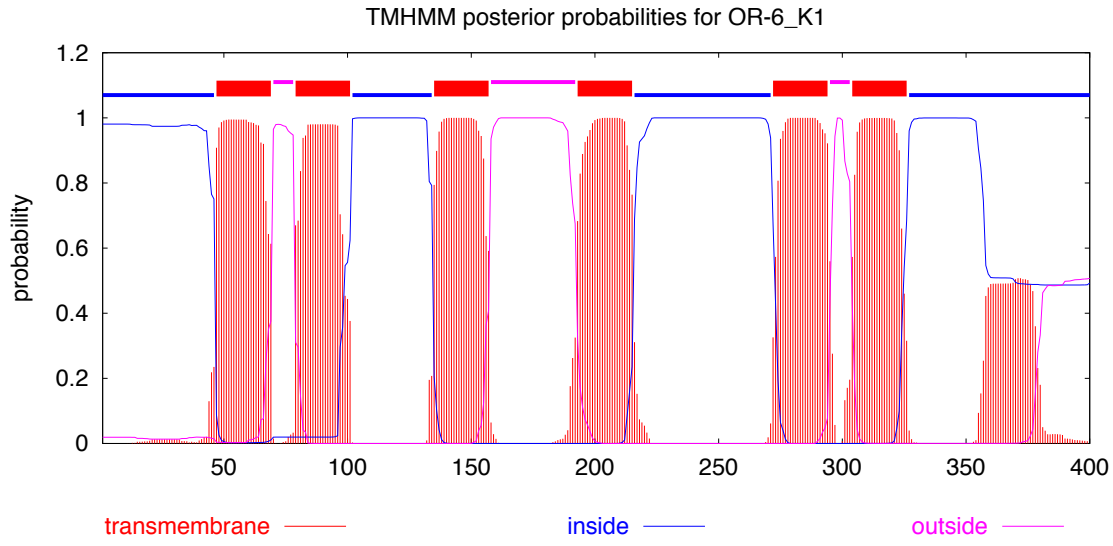
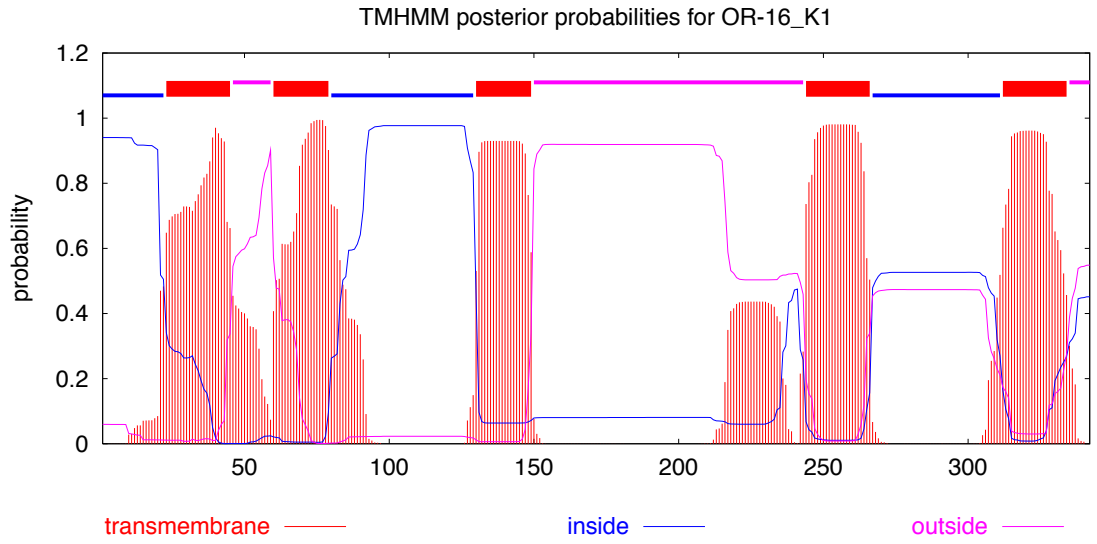


Figure 5: Predicted transmembrane topologies of MsexOR-5 (A) and MsexOR-6 (B).
Red bars and peaks correspond to positions predicted to be putative transmembrane domains. Five segments of both receptors indicate similarities due to their homology.

The program TMHMM 2.0 was used to predict transmembrane topology of the corresponding protein. A pattern of five to nine membrane spanning helices is typical for insect odorant receptors. Six domains in the 400 amino acid sequence of MsexOR-6 show high probability of being transmembrane helices (Figure 5B), with one additional

peak exhibiting lesser support. MsexOR-5 contains 399 amino acids (Figure 5A), five predicted TM domains and three regions with less certain prediction.

A



B

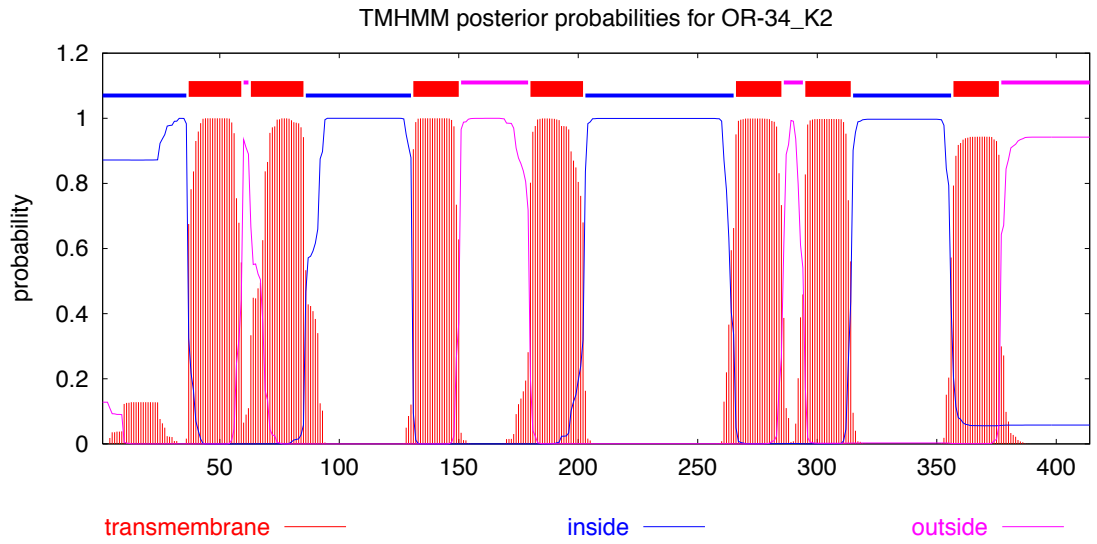


Figure 6: Predicted topology of transmembrane structures of MsexOR-16 (A) and MsexOR-34 (B). The numbers present the probability for a TM (y-axis) and amino acid position (x-axis). **A)** MsexOR-16 displays five regions with high probability for being membrane spanning helices. The TMHMM algorithm for MsexOR-34 (**B**) predicts seven membrane spanning regions (red bars), characteristically for odorant receptors.

Analysis of MsexOR-34 predicts a topology of 7TM within the 414 amino acids residues (Figure 6B), indicating that MsexOR-34 is cloned in full length. Predicted membrane topology of the recently published MsexORs: MsexOR-1, MsexOR-4, MsexOR-22 and MsexOR-31 (Grosse-Wilde et al. 2011) are shown in Figure 7.

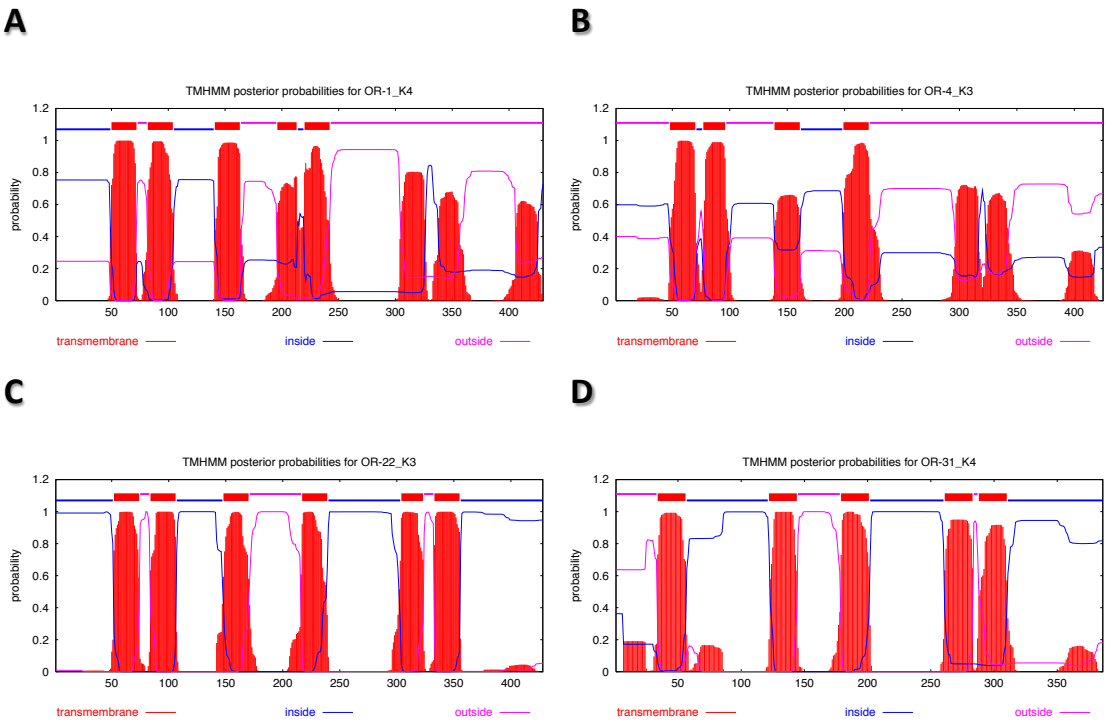


Figure 7: Predicted transmembrane domains of MsexOR-1 (A), MsexOR-4 (B), MsexOR-22 (C) and MsexOR-31 (D). MsexOR-1 and MsexOR-31 display five regions with high probability for being membrane spanning helices. TMHMM predicts four transmembrane domains for MsexOR-4 and six segments of MsexOR-22 show high likelihood of being transmembrane helices.

Based on the TMMHM predictions, all putatively OR coding ORFs seem to be complete. This facilitates further analysis of the cloned MsexORs.

3.2. Heterologous expression

We used *Drosophila melanogaster* as heterologous expression system, employing the ‘‘empty neuron’’ technique. Putative receptor coding ORFs were cloned into the pUAST vector to prepare for p-element insertion into the genomes of *Drosophila melanogaster* embryos, performed by University of Cambridge, Department of

Genetics. The resultant heterozygeous lines contain an insertion of UAS-MsexOR-X for one of the chromosomes X, 2 or 3, with the corresponding chromosome a marked balancer chromosome. Flies with third chromosome insertions of UAS-MsexOR-X were used to prepare genomic DNA.

3.2.1. Verification of transgene integration

To verify the integration of MsexOR genes in the genome of the transgenic flies, we performed PCR with gene specific primers for MsexOR-1, -4, -5, -6, -16, -22, -31 and -34 and the purified genomic DNA as template. The resultant amplified products were subjected to gel electrophoresis and visualized by ethidium bromide staining and UV-illumination, as shown for MsexOR-34 and MsexOR-16 in Figure 8.

Additionally, PCR using gene specific primers for cytochrome c oxidase subunit II (CoxII) was performed as positive control experiment (Figure 9). Genomic DNA of the transgenic flies and wild type respectively was used as template to assess presence of CoxII in every animal. Products of the expected size of 780bp were amplified when using genomic DNA of transgenic and wild type flies as well.

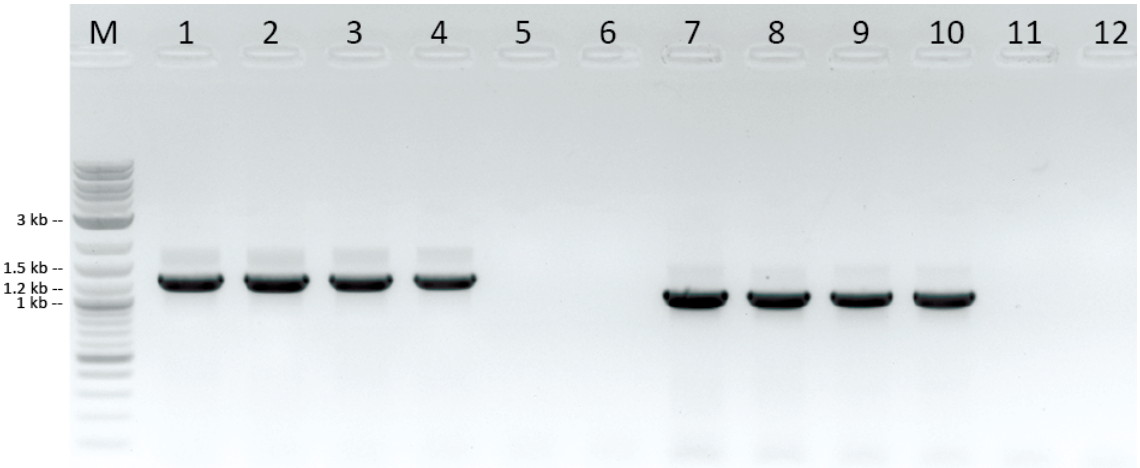


Figure 8: Agarose gel of PCR using genomic DNA of transgenic flies and gene specific primer for MsexOR-34 (lanes 1-4) and MsexOR-16 (lanes 7-10). Bands of both receptors show the expected size of 1248bp for MsexOR-34 and 1032bp for MsexOR-16. The control experiment with genomic DNA of wild-type flies (lane 5 and 11) displayed the absence of a band as well as the water control (lane 6 and 12). M, marker

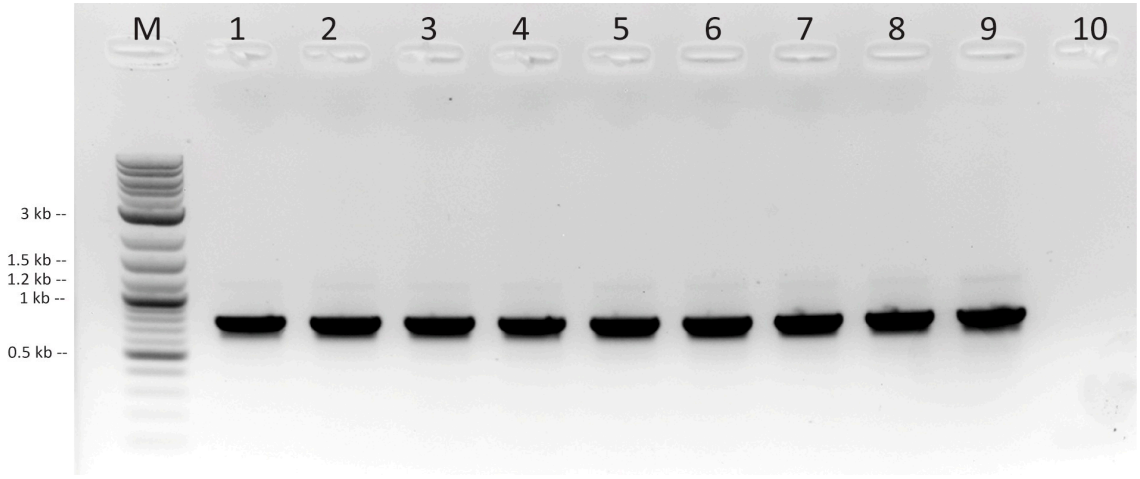


Figure 9: Agarose gel of PCR as positive control experiment using gene specific primers for CoxII.
Bands of MsexOR-34 (lanes 1-4), MsexOR-16 (lanes 5-8) and wild-type (lane 9) show the expected size of 780bp.

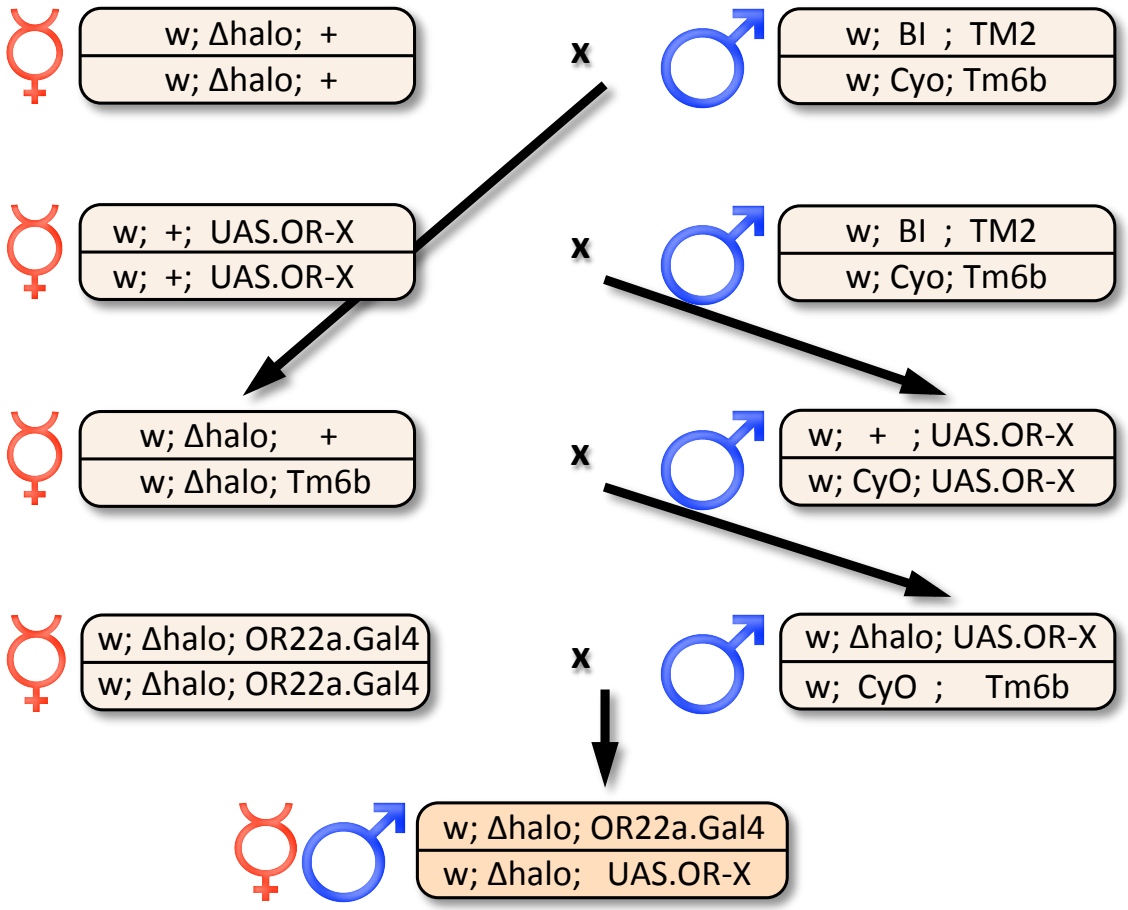
The bands in each of four different purified DNAs were of the expected size (of 1248bp for MsexOR-34 (lanes 1-4)), (1032bp for MsexOR-16 (lanes 7-10)) (Figure 8). As control we used genomic DNA of wild-type flies; as expected neither control led to amplification (lanes 5 and 11); CoxII was amplified for WT-flies. This indicates the specificity of both the primers and the reaction conditions. The PCRs performed for other *Manduca sexta* odorant receptor genes displayed similar results (data not shown). This verified integration of the MsexOR-X genes into the genome of the respective fly lines.

3.2.2. Crossing scheme

The "empty neuron" system is an *in vivo* heterologous expression system in *Drosophila melanogaster* making use of the Δhalo mutant, i.e. flies with a deletion of OR22a and DmelOR22b The promoter region upstream of DmelOR22a is then used to drive expression of other ORs, in our case from *Manduca sexta,* in the ab3A OSN, effectively replacing OR22a (van der Goes van Naters et al. 2007; Hallem et al. 2004; Dobritsa et al. 2003).

A crossing scheme (Figure 10) was devised and followed for two different transgenic fly lines per receptor. On one hand we generated experimental flies expressing MsexOR-X in the "empty neuron" with the genotype: w; Δhalo; UAS-MsexOR-X/DmelOR22a-Gal4 (Figure 10A). Due to the fact that DmelOR22a and -b are only deleted when Δhalo is homocygotic on the second chromosome, flies were generated co-expressing both OR22a and MsexOR (Figure 10B). Test flies (w; Δhalo/+; UAS-MsexOR-X/DmelOR22a-Gal4) were obtained by crossing transgenic lines (w; +; UAS-MsexOR-X and w; Δhalo/Δhalo; DmelOR22a-Gal4) for rapid assessment of gene expression efficiency.

A



B

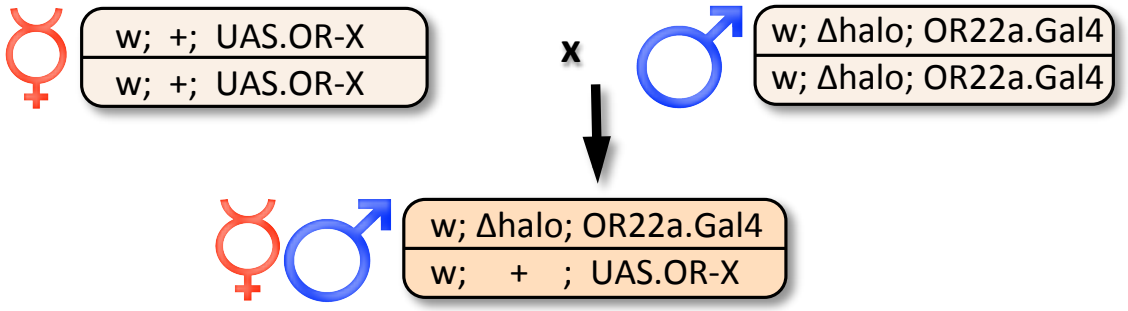


Figure 10: Summary of the crossing scheme to generate transgenic flies expressing MsexOR-X in the "empty neuron" (A) and co-expressing both DmelOR22a and MsexOR-X (B). *W* indicates the *white* – gen that is used for transport of eye-pigment precursor. Used balancer chromosomes are: the *Curley of Oster (CyO)* carrying a curly-wing dominant visible marker; *Bristle (Bl)* carrying a dominant stubbly bristle phenotype; *TM2 (third multiple two)* displayed slightly enlarged halteres; *Tm6b (third multiple six b)* showing 3-5 bristles in the humerus. + = wild-type

3.2.3. Expression analysis

The expression levels of the *Manduca* receptor genes in the transgenic flies were investigated using quantitative real-time PCR. We used fluorogenic SYBR Green dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding double-stranded DNA. The amount of emitted fluorescence increases proportionally to the presence of double-stranded DNA and therefore generated PCR products. This allows following the time-course of the reaction, which is dependent on the initial concentration of the template. By these means the method allows quantification of the gene of interest within a given sample. Specificity of amplifications was evaluated by performing melt-curve analysis to differentiate between DNA-products and unspecific primer-dimer formations.

Quantitative analysis was performed using a relative method relating the expression of the target gene to a housekeeping gene. We used ß-actin5c as housekeeping gene. The dimension of quantification is the threshold cycle (C_T), defined as the PCR cycle number, at which the amplification curve crosses a threshold of detection, indicating that the PCR is in the exponential phase of amplification. The numerical value of C_T is

inversely related to the amount of PCR product (Schmittgen et al. 2008). Ideally the amount of PCR product is doubling with each cycle correlating an amplification efficiency of 1 (100%). In reality these values are rarely achievable. Comparison of C_T values requires similar amplification efficiencies in each run; therefore we normalized C_T values against amplification efficiency.

The normalized C_T amounts of MsexOR-X and ß-Actin5c were determined for each sample and the difference was calculated, using the equation (Figure 11):

$$\Delta C_T = C_{T\ \text{MsexOR-X}} - C_{T\ \text{ß-Actin}}$$

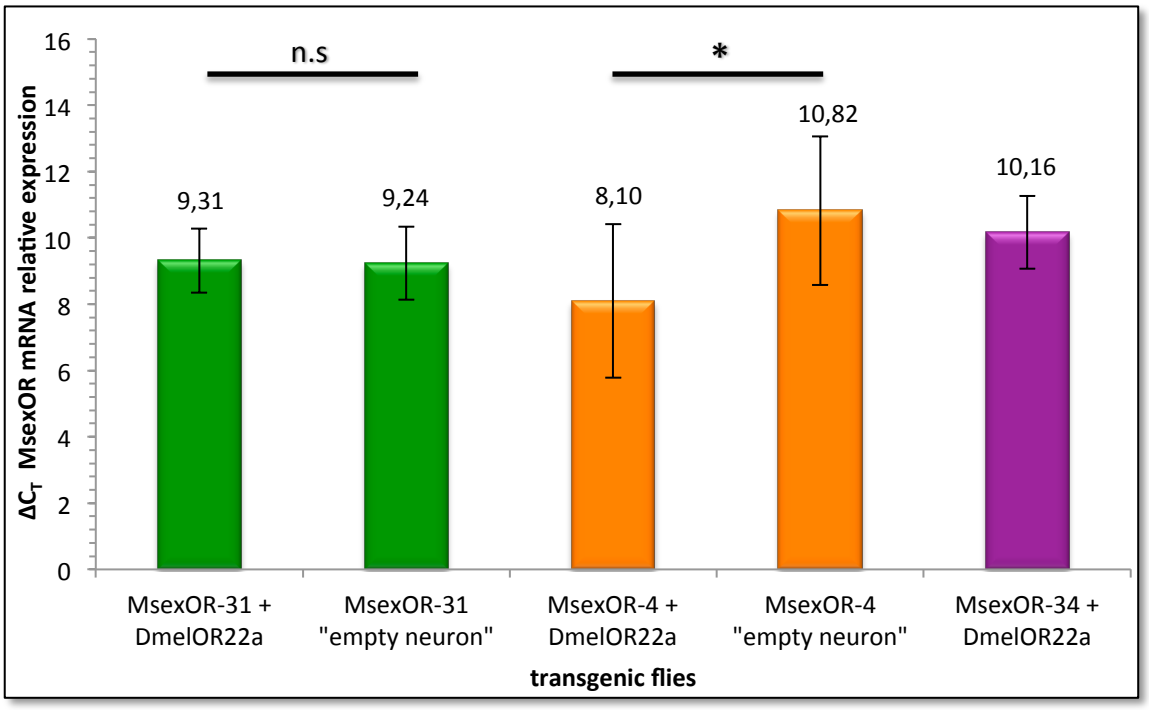


Figure 11: Comparison of MsexOR-31 (green), MsexOR-4 (orange) and MsexOR-34 (violet) relative expression levels normalized to the control gene *ß-Actin* concerning flies with or without DmelOR22a. T-test indicates significantly difference between MsexOR-4 "empty neuron" and MsexOR-4 + DmelOr22a (p-value= 0,0396). In contrast, t-test of MsexOR-31 "empty neuron" and MsexOR-31 + DmelOR22a revealed no significant difference (p-value= 0,8910). (n.s. = nonsignificant; *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

C_T values of target and control genes were measured for two - (MsexOR-4 + DmelOR22a; MsexOR-4 "empty neuron" and MsexOR-31 "empty neuron") respectively one – (MsexOR31 + DmelOr22a and MsexOR-34) insect cohorts (biological replicates) and each sample examined in two (MsexOR-4+ DmelOR22a; MsexOR-4 "empty

neuron" and MsexOR-31 "empty neuron") respectively three (MsexOR31 + DmelOr22a and MsexOR-34) different runs (technical replicates).

The ΔC_T calculation shows that both MsexOR-4 and MsexOR-31, expressed in the respective transgenic fly lines. Expression of MsexOR-4 "empty neuron" mRNA was significantly increased in comparison to flies co-expressing MsexOR-4 with DmelOR22a. In contrast, comparison of MsexOR-31 expression levels in either an "empty neuron"-background or a DmelOR22a background indicates no significant difference (Figure 11).

The comparative quantitation method $2^{-\Delta\Delta CT}$ was chosen to present real-time PCR data as "fold change" in expression (Livak et al. 2001; Schmittgen et al. 2008), using the equation: $2^{-(\Delta CT\ MsexOR\text{-}X+DmelOR22a\ -\ \Delta CT\ MsexOR\text{-}X\ "empty\ neuron")}$ = fold change.

This assumes that the PCR efficiency of the target gene is similar to the internal control gene. Thus, we normalized C_T values against amplification efficiency. The expression of MsexOR-31 in the empty neuron is slightly reduced by 1.13-fold compared with co-expressed. In contrast, the fold change in expression of MsexOR-4 "empty neuron" is 5-fold higher than in co-expression with DmelOR22a.

3.3. Electrophysiological analysis

Electrophysiological analysis of the antennal large basiconic sensilla of the transgenic flies was performed to verify expression of MsexOR-X in ab3A neuron lacking DmelOR22a.

We assayed the odorant response of sensilla containing neurons expressing MsexOR-X by single-sensillum recordings (SSR). A typical recording from flies expressing MsexOR-4 is shown in Figure 12.

Figure 12: Single-sensillum recordings of ab3 sensillum showing ab3A and B OSN in trangenic flies expressing MsexOR-4.

Two different OSNs (A and B) can be differentiated due to their action potential amplitude (spike). The cell with the largest spike amplitude is denoted A, the lower B. The ab3A neuron displayed irregular spontaneous spiking activity; irregular bursts are typical in flies with Δhalo background (Syed et al. 2006). Dobritsa et al. (2003) showed that DmelOR22a and DmelOR22b are co-expressed specifically in the ab3A antennal neuron and are highly sensitive to ethyl butyrate (de Bruyne et al. 2001; Dobritsa et al. 2003).

Therefore we used ethyl butyrate to confirm the Δhalo background. In the case of putative pheromone receptors of *Manduca sexta* (MsexOR-1 and MsexOR-4, respectively) pheromone compounds were applied to confirm their identity as pheromone receptors. Action potential traces of ab3 neurons from transgenic flies expressing MsexOR-1 are shown in Figure 13.

The response of MsexOR-1 if stimulated with ethyl butyrate revealed no increment of spontaneous firing activity of the ab3A neuron (third trace in Figure 13), proving the absence of DmelOR22a. In contrast, the second trace in Figure 13 showed a strong response to stimulation with bombykal, increasing in spike frequency. Stimulation with bombykol (first trace in Figure 13) results in a weaker response in comparison to bombykal.

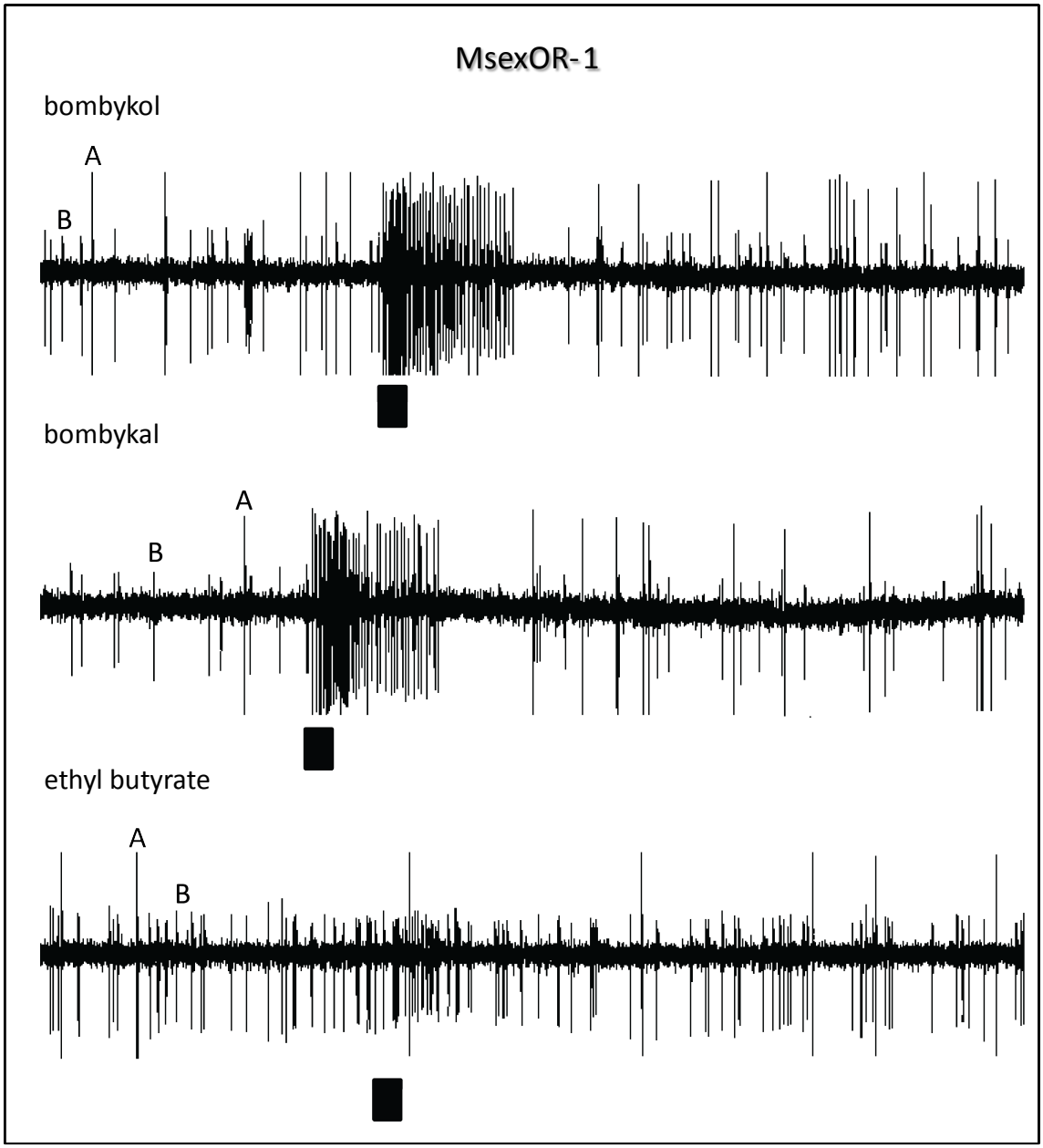


Figure 13. Physiological responses from ab3 OSN of transgenic flies expressing MsexOR-1. The first and second trace show response of the ab3 cells after stimulation with pheromone compounds bombykol and bombykal, respectively. The third trace response to ethyl butyrate. The ab3 sensillum contains two neurons; the characteristically smaller spike amplitude (labeled B) and the larger (A).

The endogenous DmelOR22a has been replaced by the heterologously expressed MsexOR-1, which actually responds to the main pheromone component of *Manduca sexta*, albeit at a high concentration.

Single-sensillum recordings (SSR) of ab3A Neurons expressing MsexOR-4 are shown in Figure 14.

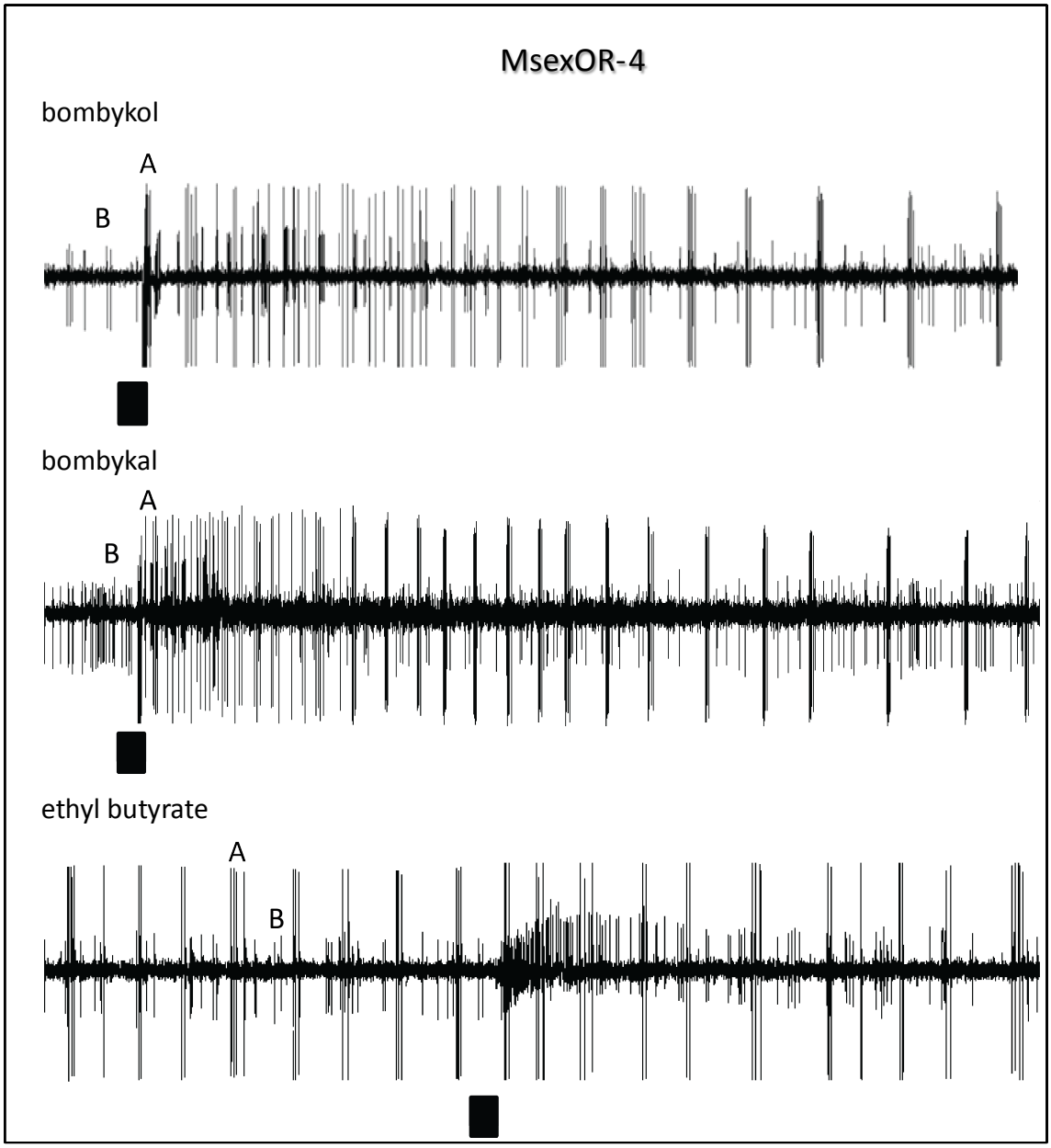


Figure 14: Action potentials recorded from ab3 sensilla of transgenic flies expressing MsexOR-4. The profiles showed response after stimulation with the pheromone compound bombykol (upper trace) and bombykal (middle trace). Stimulation with ethyl butyrate indicates response in the smaller B neuron and no activity in A neuron.

Stimulation with bombykol (1 ug/ul) (upper trace) and bombykal (middle) elicited responses in the ab3 cells expressing MsexOR-4. The neuron labeled A displayed increased firing activity. The lower trace indicates no response from the DmelOR22a (labeled A) expressing neuron to the stimulation with ethyl butyrate. This shows that DmelOr22a is replaced with MsexOR-4. Beyond the subgroup of putative pheromone receptors, we performed SSR from ab3 neurons expressing MsexOR-31 (Figure 15).

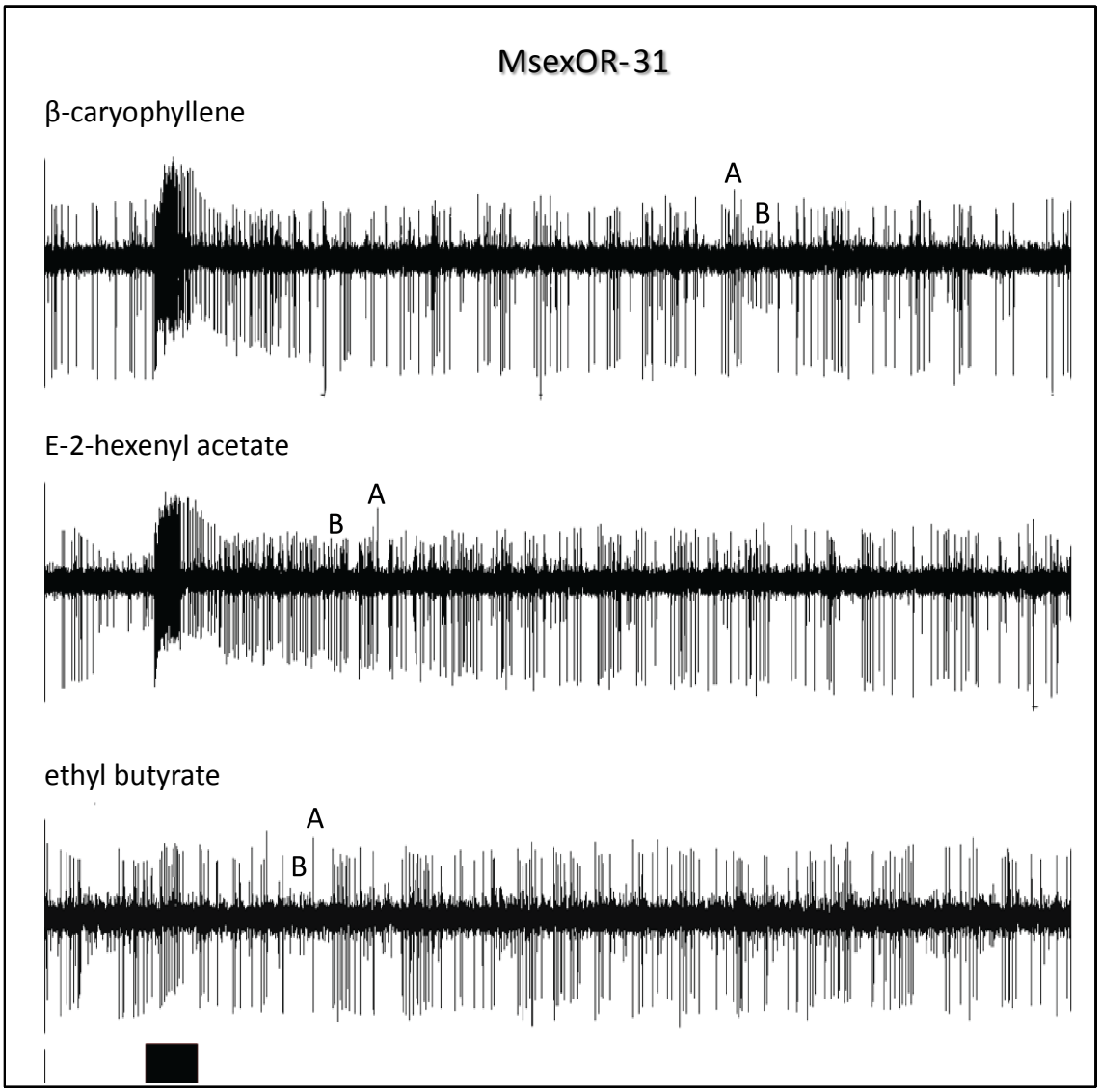


Figure 15: Physiological responses from ab3 OSN of transgenic flies expressing MsexOR-31. The first and second trace show response from ab3 cells after stimulation with the GLVs β-Caryophyllene (1µg) and E-2-hexenyl acetate (1µg), respectively. The third trace show response to ethyl butyrate.

Again, stimulation with ethyl butyrate elicited no response from ab3 sensilla, demonstrating the absence of DmelOR22a. Interestingly, MsexOR-31 showed strong response by increased firing activity after stimulation with the green-leaf volatiles (GLV) β-Caryophyllene (upper trace) and E-2-hexenyl acetate (middle trace). The list of all used compounds can be seen in the appendix.

4. Discussion

Initially we were able to identify complete open reading frames of three putative odorant receptors of *Manduca sexta* (MsexOR-6, MsexOR-22 and MsexOR-34). Beyond that we employed the "empty neuron" technique to scrutinize function of putative *Manduca sexta* ORs, using *Drosophila melanogaster* as *in vivo* heterologous expression system. Previously it had been shown that the „empty neuron" technique is a useful system for testing/ deorphaning of antennal ORs allowing rapid de-orphanization using electrophysiological methods. (Hallem et al. 2004; Syed et al. 2006; Carey et al. 2010)

Our analysis presents the first deorphaning of a non-pheromone sensitive OR of a non–dipteran species using this "empty neuron" system, demonstrating its broad usefulness.

The first receptors we deorphaned were MsexOR-1 and -4. The male-specifically expressed MsexOR-1 (Patch et al. 2009) and MsexOR-4 (Grosse-Wilde et al. 2010) belong to the subgroup of pheromone receptor coding genes (Figure 4). Members of this group in other Lepidoptera respond to pheromone compounds and are involved in the detection of female-emitted pheromones (Sakurai et al. 2004; Nakagawa et al. 2005; Grosse-Wilde et al. 2010).

Three different pheromone-sensitive types of olfactory sensory neurons (OSNs) are located in the male *Manduca sexta* antenna. One of the two OSN types which innervate each trichoid sensillum, responds to the main pheromone component E10, Z12 hexadecadienal (bombykal). The other two subtypes detect the minor pheromone components E10, E12, Z14 hexadecatrienal (EEZ); E10, E12, E14-hexadecatrienal (EEE); E10, E12-hexadecadienal (EE) and Z11- hexadecanal (Kaissling et al. 1989; Kalinová et al. 2001). The distribution of pheromone-specific OSNs corresponds to the gene expression pattern of MsexOR-1 and MsexOR-4 (Grosse-Wilde et al. 2010). Both are expressed in neighboring cells close to the majority of trichoid sensilla (Grosse-Wilde et al. 2010).

Our data provide support to the notion that either MsexOR-1 or -4 is responsible for detecting the main pheromone component bombykal. Transgenic flies expressing

MsexOR-1 in the “empty neuron” respond to bombykal at high concentrations (Figure 13). This matches to the close sequence similarity of MsexOR-1 to the bombykal-detecting receptor BmorOR-3 of *Bombyx mori* (Nakagawa et al. 2005; Grosse-Wilde et al. 2006). Interestingly we could ascertain that MsexOR-4 as well respond to the main pheromone component bombykal albeit at equally high concentrations and with weaker firing activity (Figure 14). Since 70% of the pheromone-sensitive sensilla on the male *M.sexta* antenna contain a pairing of OSNs tuned to bombykal and EEZ (Kaissling et al. 1989; Kalinová et al. 2001), we speculate that MsexOR-4 probably detects EEZ. We did not test if MsexOR-4 responds to EEZ (Grosse-Wilde et al. 2010).

It has been reported that BmOR-1, the closest relative of MsexOR-4 in *Bombyx mori*, responds to bombykol but not to bombykal when heterologously expressed in *Xenopus* oocytes (Sakurai et al. 2004; Nakagawa et al. 2005). In contrast, BmOR-1-expressing HEK cells were activated by both bombykol and bombykal (Grosse-Wilde et al. 2006). It is remarkable that BmOR-1 is the single lepidopteran pheromone receptor expressed so far in the “empty neuron” of Drosophila melanogaster; single-sensillum recordings revealed that BmorOR-1 respond to bombykol, and to a lesser extent to bombykal (Syed et al. 2006). This is interesting since all ORs expressed up to know in the “empty neuron” of Drosophila retained the odorant specificity of the corresponding wild-type neuron, indicating no additional influence of odorant-binding proteins, odorant-degrading enzymes or other proteins involved in the olfactory system (Hallem et al. 2004). It is assumed that ab3 sensilla in transgenic flies contain multiple odorant-degrading enzymes but lack of odorant-degrading enzymes that can rapidly degrade bombykol (Syed et al. 2006) which might explain this.

MsexOR31 is another receptor scrutinized in our study. Sequence similarity analysis revealed that MsexOR-31 is seemingly conserved across the lepidopteran lineage. Homologues were found exclusively in lepidopteran species e.g. *Bombyx mori* and *Heliothis virescens* (BmorOR-41 and HR-10 respectively) (Figure 4). The degree of conservation indicates a very special function of this receptor in lepidopteran behavior. Electrophysiological recordings revealed neurons in mutant flies expressing

MsexOR-31 responding to green leaf volatiles, mainly to β-caryophyllene (Figure 15). This fits well to a previous study using electrophysiological recordings of short sensilla on antennae of *Manduca sexta* identified ß-caryophyllene-sensitive OSNs in males and females (Kalinová et al. 2001). Later studies using calcium-sensitive optical imaging of neural activity in *M. sexta* antennal lobe (AL) showed that the sesquiterpene ß-caryophyllene activates a glomerulus in a similar position in both sexes, but located in a different area than terpenes or aromatics (Hansson et al. 2003). β-caryophyllene has been described as emitted by various plants, mainly in *Solanaceae* e.g. tobacco flowers (*Nicotiana*) (Loughrin et al. 1990). ß-caryophyllene was also detected in tomato leafs *(Lycopersicon),* (Buttery et al. 1987) which are hostplants for oviposition of *Manduca* (Mechaber et al. 2002) (Yamamoto et al. 1969, 1972). It thus can be speculate that MsexOR-31 is involved in identification and localization of appropriate oviposition sites. A similar function can be expected in the context of the plant-pollinator relationship between *Datura wrightii* and *Manduca sexta* (Raguso et al. 2003; Riffell et al. 2008). In this context, it is known that ß-caryophyllene belongs to a group of herbivore-induced plant volatiles (HIPV) (Hare 2007), which are emitted by *Datura wrightii* as indirect defense after herbivore damage and attract natural enemies (Hare 2010). A similar mechanism is found in maize, where ß-caryophyllene attracts predators of both herbivores: *Spodoptera littoralis* larvae damaged leafs and roots attacked by *Diabrotica virgifera virgifera*. (Rasmann et al. 2005; Köllner et al. 2008). It is conceivable that MsexOR-31 involved in a general mechanism of plant odor recognition and detection.

Our recordings of MsexOR-31 expressing ab3 cells revealed responses not only to β-caryophyllene, but also to the green-leaf volatile (GLV) (E)-2-hexenyl acetate (Figure 15). In a recent study it was shown that when *Manduca sexta* larvae were feeding on *Nicotiana attenuata,* (E)-isomers of GLVs were released instead of (Z)-isomers (produced after mechanical damage) to attract the hemipteran predator *Geocoris* spp*.* The isomeric change is triggered by a heat-labile constituent of caterpillar's oral secretion and leads to triple foraging efficiency of carnivores to locate hatched caterpillar babies and eggs laid by the female moths (Allmann et al. 2010). Altogether

it seems very likely that MsexOR-31 is a conserved lepidopteran receptor involved in the assessment of the quality of potential oviposition sites.

Other putative OR coding genes we expressed are MsexOR-5, -6, -16, -22 and -34. So far we mainly used these flies to assess the validity of the approach; functional assays have not yet been performed. Transgene presence in the genomic DNA of the transgenic flies was verified by PCR using gene specific primers. The flies expressing MsexOR-X in the "empty neuron", with distinct lines either co-expressing both MsexOR-X and DmelOR22a for rapid assessment of gene expression efficiency, or MsexOR-X in the empty neuron (without DmelOR22a) for functional analysis of the *M.sexta* receptor. Expression levels of the *Manduca* receptor genes in the transgenic flies were investigated using quantitative real-time PCR. ΔC_T calculations show that both MsexOR-4 and MsexOR-31 expressed in their respective transgenic fly line, demonstrating trans-gene insertion and correct transcription (Figure 11). Our ΔC_T calculations are comparable within fly lines expressing the same receptor, e.g. MsexOR-X with or without DmelOR22a. In order to enhance comparability of the ΔC_T values, amplification efficiencies of each run were included in calculation. In reality reaction efficiency vary between 90% and 110% (Rutledge et al. 2003). Values beyond this range indicate problems with qPCR e.g. suboptimal annealing temperature, contaminating enzyme, primer-dimers, and nonspecific amplicons with secondary structures can yield misleading results (Bustin et al. 2009; Taylor et al. 2009). Since our R^2-values of the standard curves were consistently at 0.99 (indicating that our experimental data matches very well to the regression line (Taylor et al. 2009) we normalized ΔC_T values against reaction efficiency.

The study of (Dobritsa et al. 2003) demonstrated functionally that expression of DmelOR-47a does not interfere with the expression and function of DmelOR22a. It can be derived that both MsexOR-X and DmelOR22a can be expressed in the ab3 sensilla without reciprocal interference. To prove this we investigated if expression levels of MsexOR-X are affected by co-expression of DmelOR22a.

The expression of MsexOR-4 mRNA in the “empty neuron” background is significantly increased in comparison to co-expression of MsexOR-4 and DmelOR22a (Figure 11). Obviously, lack of DmelOR22 leads to a more beneficial background for the expression of MsexOR-4. In contrast, there is no significant influence of Dmel22a co-expression on MsexOR-31 expression levels. It thus can be speculated that the influence of receptor co-expression varies between receptor pairs, indicating possible competitive interaction. This result is supported by using comparative quantitation $2^{-\Delta\Delta CT}$ expression analysis (Livak et al. 2001; Schmittgen et al. 2008). MsexOR-4 “empty neuron” is 5-fold higher expressed than in co-expression with DmelOR22a. As seen before the expression of MsexOR-31 in the “empty neuron” is approximately equal in comparison to expression in front of a DmelOR22a-background. However, it should be mentioned that amplification efficiency of reference and target gene requires being approximately equal to allow use of this analysis. This requirement is not fulfilled in our case. Therefore we normalized the C_T values with amplification efficiency of each run for the reasons mentioned above. In addition, studies revealed that amplification efficiency is often not close to 1 or always approximately equal and vary mostly from gene to gene and sample to sample (Liu et al. 2002). Several methods can be used to analyze real-time PCR data e.g. efficiency correction method (Pfaffl et al. 2001) or sigmoidal curve fitting (SCF) methods (Liu et al. 2002; Rutledge 2004). All methods have their strength and weaknesses in relation to fulfill the objectives and data presentation (Swillens et al. 2008; Schmittgen et al. 2008; Ramakers et al. 2003; Ruijter et al. 2009). The ease of use and the presentation as ‘fold change’ in expression were crucial using $2^{-\Delta\Delta CT}$ expression analysis (Schmittgen et al. 2008). .

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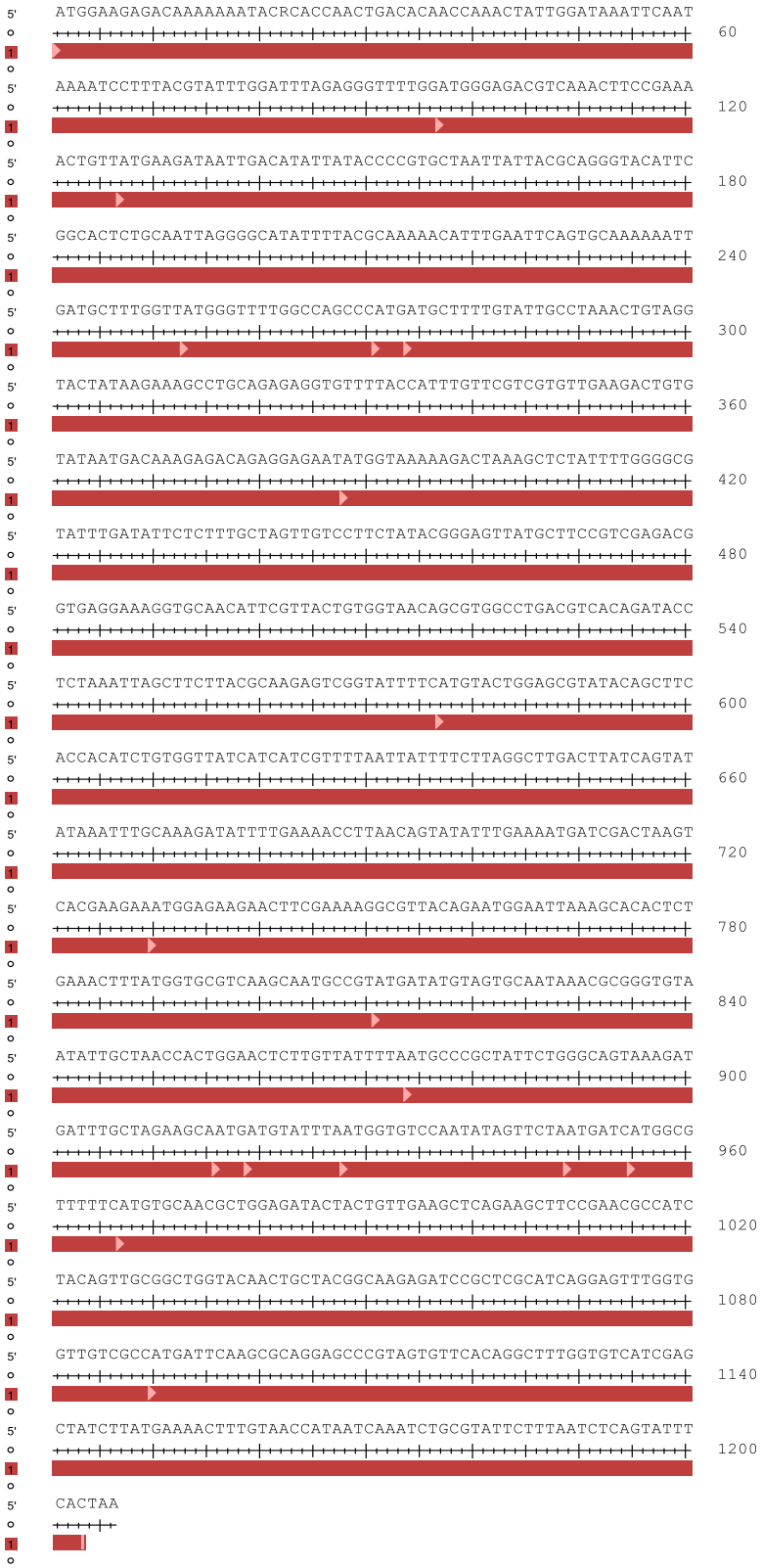
7. Appendix

7.1. List of Compounds used for SSR

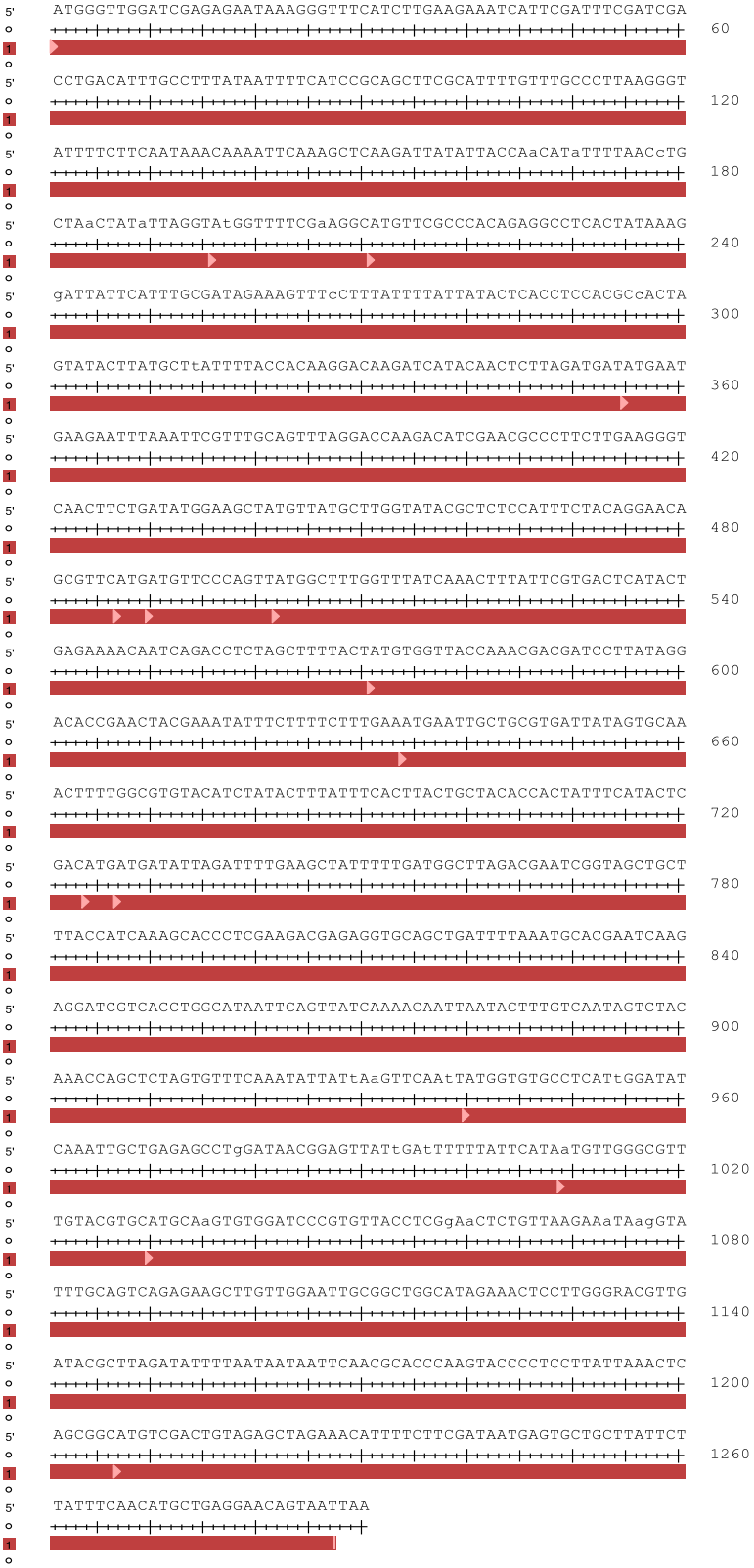
Hexane; Ethyl Butyrate; PAA; 2-Heptanone; B-myrcene; citral; cis-3-hexenyl acetate; methyl salicylate; benzyl alcohol; 2-phenyl ethanol; 4-methyl pentanol; decanal; 6-methyl-5-heptan-2-one; 3-octanol; ethyl benzoate; benzyl salicylate; bemzaldehyde; benzyl ptopionate; cis-3-hexenyl benzoate; nerol; acetophenone; geraniol; geranyl acetate; ocimene; trans-2-henen-1-ol; linalool (racemic); hexanoic acid; decanoic acid; geranyl acetone; trans-2-hexenyl acetate; methyl benzoate; benzyl acetate; E-nerolidol; 4-ethyl guaiacol; Z-11-16-al; propyl benzoate; nonanal; cis-3-hexenyl propionate; cis-3-hexenal; 3-methyl-2-butenyl-3-methylbutanoate; 3-methyl-2-butenylbutanoate; cis-jasmone; methyl hexanoate; prenol; cis-2-penten-1-ol acetate; cis-3-hexenol; 1,2,4-trimethyl benzene; b-caryophyllene; 4-methyl pentanol; cis-3-hexenyl valerate; trans-2-hexenal; hexenal; R-+-limonene; S---limonene; farnesol; cis-3-hexenyl butyrate; pyrrolidine; 6,10,14-trimethyl-2-pentadecanone; butyl acetate; benzyl butyrate; m-pyrol; 3-propyl toluene; 4-pentenyl butanoate; (-)-menthone; triazetin; cis-2-pentenol

7.2. Sequences of putative ORFs

7.2.1. MsexOR-6



7.2.2. MsexOR-22



7.2.3. MsexOR-34