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The Identification of Pheromone Receptors from the Lightbrown Apple Moth, *Epiphyas postvittana*

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A thesis submitted in the fulfillment of the requirements of
Doctor of Philosophy in Biological Science

Ken and Bonnie, this one's for you…

Abstract

The lightbrown apple moth, *Epiphyas postvittana* is a horticultural pest native to Australia that has spread to New Zealand, California and Europe. While modern mating disruption tactics have proven to be efficacious for controlling the pest they have not become well adopted, primarily due to health concerns related to releasing large amounts of pheromones into the environment. A better understanding of odorant and pheromone reception in the moth could facilitate the development of novel pest control tools targeting the moth's olfactory system that could be used in place of pheromones for mating disruption.

Here we undertake a bioinformatic approach to identify odorant receptors (ORs) and other genes associated with olfactory reception in *E. postvittana*. Of the 70 OR genes that we identified, eight are phylogenetically related to known pheromone receptors from other moths. In addition, we found two male-biased ORs that did not group with previously described pheromone receptors.

The development of rapid and reliable assays to characterize insect ORs and pheromone receptors (PRs) remains a challenge for the field. Typically insect ORs and PRs are functionally characterized either *in vivo* in transgenic *Drosophila* or *in vitro* through expression in *Xenopus* oocytes. We have developed a Human Embryonic Kidney (HEK) cell-based assay system for the expression and functional characterization of ORs in 96-well plates using a fluorescent spectrophotometer.

Here, we express candidate EposPRs in HEK293 cells along with EposOrco and EposSNMP1 and test for responsiveness to a panel of 62 pheromone-related compounds. We found that EposOR1, EposOR6 and EposOR45 all respond to certain *E. postvittana* pheromone components and to pheromones used by other moths, some of which have known function as behavioral antagonists in this species. Finally, we show that activation of EposOR6 with the major pheromone component, E11-14:OAc, or the behavioral antagonist, Z11-14:OAc, prevents subsequent re-activation by either compound in this cell-based system, implying a pheromone-degrading enzyme may be required to reset the system. These results lead us to propose that in *E. postvittana* behavioral agonism and antagonism may be mediated through some sort of peripheral molecular mechanism.

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They say it takes a village to raise a child. I think this saying could apply to PhD students as well. While I'm the one that's going to get credit for the work that is presented in this thesis, the truth is that none of this work would have been possible without the support of those I've been surrounded by for the last three years. Sure, there have been certain key persons involved that without none of this would have ever started, but it certainly would have stopped somewhere along the way without the momentum gained from my friends, family and colleagues.

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The undersigned hereby certify that:

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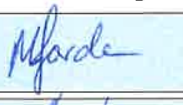
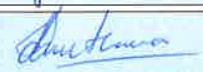
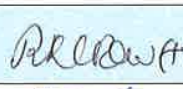
Jordan, M.		24/02/2014
Thrimawithana, A.H.		24/02/2014
Crowhurst, R.		24/02/2014
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Carraher, C.	assisted in data collection; edited manuscript
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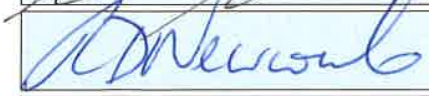
Newcomb, R.		21/02/2014
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Newcomb, R.	assisted with experimental design; facilitated research collaboration; edited manuscript

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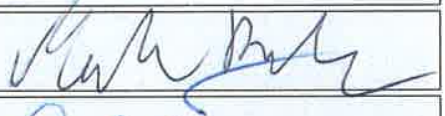
Andersson, M.N.		Click here 2014-02-25
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I

General Introduction

Insects are the most diverse and abundant group of animals on the planet and have evolved to occupy a wide variety of ecological niches. Insects have the ability to sense their environment through visual, audio, tactile, mechanical (i.e., gravity, pressure) and chemical (i.e., taste, olfaction) cues. While some insects have evolved to utilize certain sensory systems more than others, most insects rely on their sense of olfaction much more than any other sensory system (Gullan, 2010).

Insects use their sense of olfaction to detect chemical cues in their environment to find food sources, mates for reproduction and places to lay their eggs. For example, plants release a wide variety of volatile compounds (odorants) that insects can detect from incredibly far distances. While certain odorants are produced by various types of plants, the complete complement of odorants emitted by a particular plant creates a specific chemical signature that allows insects to differentiate host plants from non-host plants using their sense of smell. Similarly, insects release blends of volatile sex pheromones into the environment to attract conspecific mates for reproduction. Multiple species utilize the same pheromone components in their pheromone blends but use them in different proportions, creating unique species-specific blends that can be differentiated by insects. If the correct blend is detected it will be interpreted as a reproductive mate and the insect will be attracted to the source. If the blend is not exactly right the insect will not be attracted to the source, despite the similarities of the individual blend constituents (Wyatt, 2003).

In insects the primary olfactory organ are the antennae, however other structures have been shown to detect odorants (de Bruyne et al., 1999, Kwon et al., 2006). Insect antennae are covered in hair-like structures called sensilla that contain neurons that are stimulated by chemical cues including odorants and pheromones (Figure 1). These olfactory receptor neurons (ORNs) typically contain a single type of olfactory receptor that is activated by specific volatile compounds (Vosshall et al., 2000, Ray et al., 2008). Olfactory receptors that detect odorants are known as odorant receptors (ORs) and are usually housed in ORNs present in specific sensilla called sensilla basiconica (Clyne et al., 1997). Olfactory receptors that detect pheromone molecules are known as pheromone receptors (PRs) and these receptors are typically expressed in ORNs present in specific sensilla called sensilla trichodea (Krieger et al., 2005, Nakagawa et al., 2005, Krieger et al., 2009).

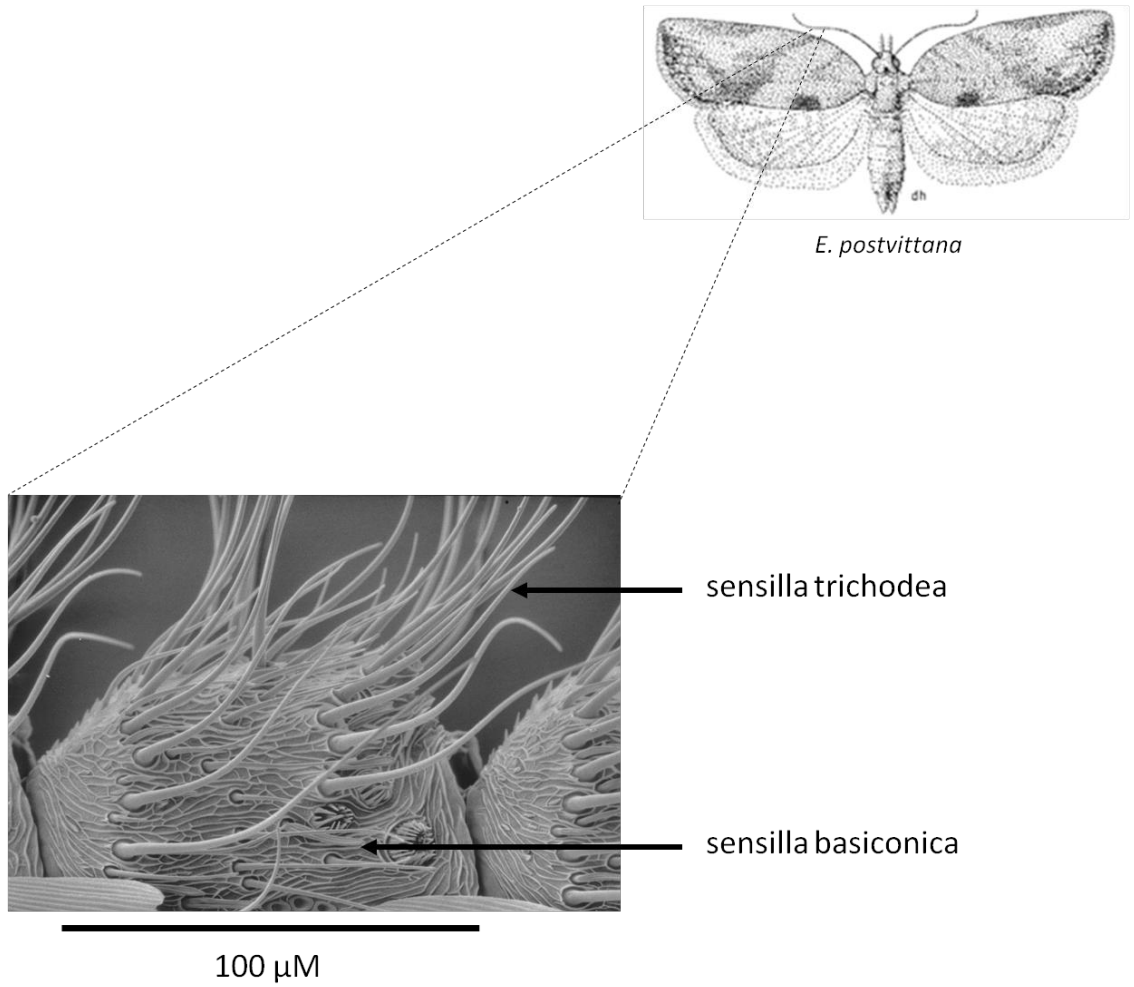

Figure 1. Olfactory receptors are housed in ORNs present in specialized hair-like structures on insect antennae. ORs and PRs are typically expressed in ORNs present in sensilla basiconica and sensilla trichodea, respectively. Scanning electron micrograph of male Epiphyas postvittana antennae courtesy of Melissa Jordan.

Odorant and pheromone receptors each form a heteromeric complex with a common OR co-receptor (Orco) in the ORN membrane (Nakagawa et al., 2005, Benton et al., 2006), however the exact role of Orco in OR and PR signaling and the interactions between the sub-units remain unclear. *In situ* hybridization experiments have shown that ORNs that express ORs and PRs always express Orco and genetic knock down of Orco *in vivo* results in lack of response of ORNs to odorants and pheromone molecules (Larsson et al., 2004). However, ORs and PRs have been shown to be functional *in vitro* using heterologous expression systems without the co-receptor present (Sakurai et al., 2004, Groβe-Wilde et al., 2006). Sensory Neuron Membrane Protein-1 (SNMP1) is a CD36-like membrane protein that is co-expressed with PRs in ORNs (Rogers et al., 2001). In *Drosophila*, endogenous PRs, as well as heterologously expressed moth PRs, only respond to pheromone compounds when SNMP1 is present, indicating that it is required for PR signaling (Benton et al., 2007, Syed et al., 2010). However, moth PRs have been shown to respond well to pheromone compounds when expressed *in vitro* using heterologous expression systems without SNMP1 (Nakagawa et al., 2005, Mitsuno et al., 2008).

Odorants and pheromones enter the sensilla through pores in the cuticular membrane and are transported through the sensillum lymph to olfactory receptors by odorant binding proteins (OBPs) and pheromone binding proteins (PBPs) (Vogt, 2005). Two models have been proposed depicting how OBP-odorant or PBP-pheromone complexes interact with olfactory receptors. In *D. melanogaster* a pheromone binding protein, LUSH, has been shown to have a critical role in neuronal response to the pheromone compound 11-cis-vaccenyl acetate (cVA), however the exact mechanism remains unclear. In 2008, investigators showed that binding of cVA to LUSH created a conformational change in the PBP. The investigators then made a mutation to LUSH that created a structural change that mimicked the conformational change created by cVA binding. This mutated form of LUSH was found to activate OR67d-expressing neurons in the absence of cVA, suggesting the "conformationally-activated" form of LUSH was the ligand for OR67d, not cVA itself (Laughlin et al., 2008). However, in 2013, another group of investigators were unable to replicate these previous findings. Instead, they found that while LUSH does bind to cVA and undergo a conformational change in the process, the "conformationally-active" form of LUSH is not capable of activating OR67d. In addition, these investigators found that cVA was capable of activating OR67d in the absence of LUSH, suggesting that cVA itself is the ligand of OR67d, not LUSH or the pheromone/PBP complex (Gomez-Diaz et al., 2013). So while these studies both support the theory that PBPs bind pheromone compounds and transport them through the sensillum lymph, the exact mechanism through which pheromones and/or PBPs interact with and activate pheromone receptors in the neuronal membrane remains unclear.

Finally, the sensillum lymph surrounding ORNs is full of enzymes capable of degrading odorants and pheromones (ODEs and PDEs, respectively) (reviewed in Vogt, 2005). These enzymes have been suggested to have a role in terminating the ligand-induced receptor activation by removing the odorant or pheromone molecule from the receptor or capturing it after it is released (Figure 2).

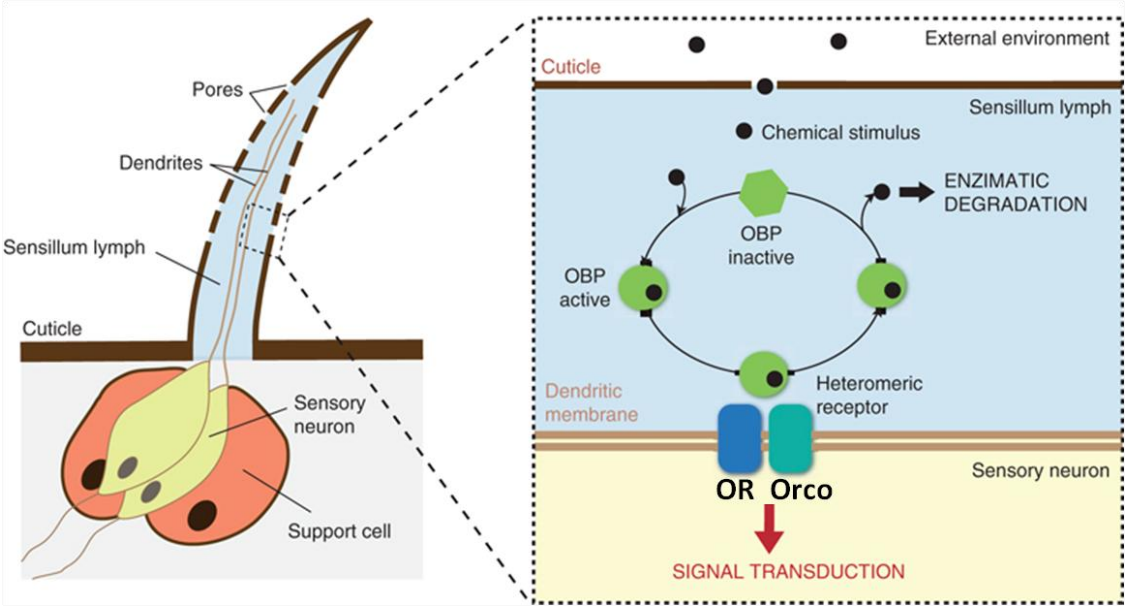

Figure 2. Cartoon depicting insect olfactory sensillum (left panel) and role of molecular entities known to be involved in olfactory reception in insects (right panel). Image modified from Sanchez-Gracia et al. (2009).

When an odorant or pheromone molecule binds to an olfactory receptor and activates the ORN it leads to a depolarization of the neuron and an impulse travels to the macroglomerular complex in the antennal lobe of the insect brain (Hansson et al., 1992, Ochieng et al., 1995). The stimulation of a specific ORN will be interpreted by higher structures of the brain as a specific signal and be translated into a specific behavioral response. Because ORNs typically house only one specific type of receptor, the behavioral response of an insect to the firing of an ORN is governed by the specific receptor that is houses. For example, the moth *Bombyx mori* has specific ORNs that house a receptor that detects the moth's pheromone component, (*E,Z*)-10-12-hexadecadien-1-ol. When this ORN in *B. mori* was genetically modified to express a PR from the moth *Plutella xylostella,* the ORN was no longer stimulated by (*E,Z*)-10-12-hexadecadien-1-ol. Instead, the ORN was stimulated by a *P. xylostella* pheromone component, (Z)-11-hexadecenyl acetate, and *B. mori* moths displayed an attractive behavioral response to the 'wrong' pheromone (Sakurai et al., 2011).

Modern analytical devices (i.e., GC-MS) make it relatively easy to identify the volatile compounds that are emitted by plants and the pheromone compounds that are released by insects. Similarly, electrophysiological techniques such as electroantennogram (EAG) and single-sensillum recording (SSR) devices allow for the identification of compounds that stimulate ORN firing in insects. Because of this, a considerable amount is known regarding what sorts of volatile odorants and pheromones are present in the environment and which compounds an insect is capable of detecting. However, the molecular entities and mechanisms present in the insect that facilitate the detection of these compounds are not totally understood. It is clear that insects use

olfactory receptors to detect odorants and pheromones, but the exact mechanisms of how these compounds are transported to and interact with receptors, receptor specificity, the signaling pathways used, and how ORN firing is interpreted by the insect brain remain the current focus of research into understanding olfactory perception in insects.

The first insect ORs were identified in 1999 from the genome of the model organism, *Drosophila melanogaster* (Dmel) (Clyne et al., 1999, Gao and Chess, 1999). Since then, using various bioinformatic approaches (described later), ORs have been identified and functionally characterized from dozens of insects, including flies (Hallem and Carlson, 2004, Hallem and Carlson, 2006), bees (Wanner et al., 2007), mosquitoes (Carey et al., 2010) and various species of moths (Wanner et al., 2010, Liu et al., 2013, Zhang and Löfstedt, 2013). Using standard molecular biology techniques, investigators have been able to clone olfactory receptor genes and study them using *in vivo* and *in vitro* expression systems (described later). Over the last 15 years, a considerable amount has been learned about insect ORs. Perhaps the most interesting general characteristic of insect olfactory receptors is that they are not closely related to mammalian olfactory receptors; mammalian olfactory receptors are G protein-coupled receptors (GPCRs) (Buck and Axel, 1991) and insect olfactory receptors are not (Benton, 2006). Both mammalian and insect olfactory receptors have seven transmembrane regions, however insect ORs have an inverted topology compared to mammalian olfactory receptors and have been shown to function as heteromeric ligand-gated ion channels (Smart et al., 2008, Sato et al., 2008, Wicher et al., 2008).

Functional studies using DmelORs have shown that some receptors are narrowly tuned to specific compounds, while others are more broadly tuned to multiple compounds (de Bruyne et al., 2001, Hallem and Carlson, 2004, Hallem et al., 2004). Because ORs displayed overlapping response profiles to odorants, it was proposed that insects use a combinatorial system for odorant detection (Hallem and Carlson, 2006). More recently, it has been shown that while insect ORs respond to multiple odorants at high concentrations, they tend to only respond to specific odorants at low concentrations (Bohbot and Dickens, 2012, Mathew et al., 2013). The first insect PRs were identified from the moth *Bombyx mori* in 2004 through functional testing of BmorORs in *in vitro* heterologous expression systems (discussed later) (Sakurai et al., 2004). Since then, PRs have been identified from only a few insects including *Drosophila* (Ha and Smith, 2006), the honeybee, *Apis mellifera*, (Wanner et al., 2007) and several moth species (Groβe-Wilde et al., 2007, Mitsuno et al., 2008, Forstner et al., 2009, Miura et al., 2010). Many moth species are horticultural pests and because of this, the vast majority of current research on insect PRs is focused on the identification of PRs from lepidopteran pests.

Traditionally pest moths have been controlled using insecticides and natural enemies; more recently, control strategies have incorporated the use of the moth's own pheromones to control populations through mass trapping and mating disruption tactics (Borchert and Walgenbach, 2000, Kovanci et al., 2005, Suckling et al., 2011). While mating disruption has proven to be an effective tool for controlling pest moths, it is not particularly efficient, requiring the release of enormous quantities of pheromones to be effective in the field (Witzgall et al., 2010). The relatively high costs associated with producing large quantities of synthetic moth pheromones, as well as health and environmental concerns regarding the release of pheromones into the environment (Garvey, 2008) have prevented widespread adoption of mating disruption campaigns to control these pests. A better understanding of pheromone reception in moths would allow for the identification and development of novel compounds that could potentially be used as mating disruption tools. Ideally, compounds would be identified that somehow affected pheromone reception or perception in a more efficient and effective manner than currently existing tools. Theoretically speaking, these compounds could be inhibitory compounds targeting molecular entities in the peripheral olfactory system or modified versions of pheromones that had different biochemical properties (i.e., affinities, degradation rates) that could somehow alter pheromone perception. The critical requirement however to exploiting a moth's olfactory system as a control measure would be to have a thorough understanding of pheromone reception and perception in a pest moth, and the first step in achieving that understanding would be to identify the moth's PRs.

The lightbrown apple moth, *Epiphyas postvittana*, is a horticultural pest native to Australia. Over the last few decades it has subsequently spread to New Zealand, California and parts of Europe (Danthanarayana, 1975, Tooman et al., 2011, He et al., 2012). *E. postvittana* is a member of the leaf roller family, Tortricidae, and as this name implies, its larvae damage the leaves of apple trees as well as various other horticultural crops (Brockerhoff et al., 2011). The damage that the larvae cause to the fruit is mainly aesthetic, however significant infestations can cause enough damage to the leaves of the plant to reduce crop yields. More importantly, the moth is a quarantine pest, which means that its mere presence will prevent the exportation of harvested fruit from growing regions. As the New Zealand and California economies both rely heavily on agricultural exportation, there is tremendous incentive to develop efficient and effective control tools for this lepidopteran pest.

Investigators have been studying the olfactory system of *E. postvittana* for over 40 years, and during this time much has been learned about the pheromone compounds used and what compounds the animal is capable of responding to. Using crude extracts prepared from the pheromone glands of female moths, Bartell et al. were able to show that compounds present in

extracts elicited electrophysiological responses in male moth antennae (Bartell, 1969, Bartell, 1977). In 1983, three electrophysiologically active compounds were identified, with two being present in extracts from pheromone glands, (*E*)-11-tetradecenyl acetate (E11-14:OAc) and (*E,E*)-9,11-tetradecadienyl acetate ((E,E)9,11-14:OAc). A third compound, (*Z*)-11-tetradecenyl acetate (Z11-14:OAc) was identified as being electrophysiologically active but was not present in the female pheromone gland (Bellas et al., 1983). Behavioral studies conducted in wind tunnels later revealed that Z11-14:OAc inhibited the attractive behavioral response of male *E. postvittana* to the female pheromone blend. Later, it was shown that the addition of Z11-14:OAc to pheromone traps in the field reduced the number of male *E. postvittana* entering the trap, and the compound was re-confirmed as an ORN stimulant and not an ORN inhibitor (Stephens et al., 2008). Since these early experiments into *E. postvittana* pheromone production and antennal responsiveness, the vast majority of research being conducted on the moth has centered on using its own pheromones to control populations through mass trapping and mating disruption (Suckling and Brockerhoff, 1999, Suckling et al., 2007, Suckling et al., 2012). Finally, in 2011, two more compounds, (*E*)-11-tetradecen-1-ol (E11-14:OH) and (*E*)-11-hexadecenyl acetate (E11-16:OAc) were identified in the pheromone gland of female *E. postvittana* and shown to elicit responses in male antennae and to increase trap catches in the field when combined with E11-14:OAc and (E,E)9,11-14:OAc (El-Sayed et al., 2011). Electrophysiological studies continue, as investigators are still characterizing the responsiveness of *E. postvittana* antennae to various odorants and pheromone compounds (Kye-Chung Park, pers. comm.).

Compared to what is known about pheromone production and antennal and behavioral responsiveness to odorants and pheromones, relatively little is known about the molecular entities and mechanisms involved in olfactory reception in *E. postvittana*. In the last decade, only a handful of olfactory-related genes have been identified from the moth. Newcomb et al. (2002) were able to purify four odorant binding proteins from moth antennae and found that two of them were capable of binding the major pheromone component, E11-14:OAc. Later, using a bioinformatic approach, Jordan et al. (2008) identified several more olfactory-related genes from moth antennal Expressed Sequence Tag (EST) libraries, including OBPs, ODEs, SNMPs and chemosensory proteins (discussed later), as well as the *E. postvittana* Orco orthologue and two ORs that responded to plant volatiles in functional assays (Jordan et al., 2009). To date, the olfactory receptors responsible for detecting pheromone compounds in *E. postvittana* antennae remain elusive. Until these PRs are identified and functionally characterized, it will be difficult to develop novel pest control tools that target the insect's pheromone reception system.

Research Aims

The ultimate goal of this PhD research was to identify and functionally characterize PRs from the pest moth, *E. postvittana*. From the onset of this research, this goal was divided, both chronologically and logistically, into three distinct sub-goals: 1) the identification of candidate PR genes from *E. postvittana* antennae using bioinformatic and molecular biology techniques, 2) the development of an assay system optimized for functionally characterizing PRs in a relatively high-throughput manner, and 3) the functional characterization of candidate PR genes using the newly developed assay system. An introduction to the theories and methods used to pursue these three sub-goals, as well as the results achieved for each, are described in the following chapters. Finally, chapter five provides a summary of the achievements made during the course of this PhD research, how these achievements have added to our understanding of olfactory biology in *Epiphyas postvittana*, and what the next steps should be to continue adding to this understanding.

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II

The peripheral olfactory repertoire of the lightbrown apple moth, *Epiphyas postvittana*

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Abstract

The lightbrown apple moth, *Epiphyas postvittana* is an increasingly global pest of horticultural crops. Like other moths, *E. postvittana* relies on olfactory cues to locate mates and oviposition sites. To detect these cues moths have evolved families of genes encoding elements of the peripheral olfactory reception system, including odor carriers, receptors and degrading enzymes. Here we undertake a transcriptomic approach to identify members of these families, describing open reading frames encoding 33 odorant binding proteins, 13 chemosensory proteins, 70 odorant receptors (OR), 19 ionotropic-like receptors, 9 gustatory receptors, 2 sensory neuron membrane proteins, 24 carboxylesterases, 22 glutathione-S-transferases and 51 cytochrome p450s. For the ORs, quantitative RT-PCR corroborated RNAseq count data on steady state transcript levels. Of the eight ORs that group with pheromone receptors from other moths, two displayed significant male-biased expression patterns, one displayed significant female-biased expression pattern and five were expressed equally in the antennae of both sexes. In addition, we found two male-biased ORs that did not group with previously described pheromone receptors. This suite of olfaction-related genes provides a powerful resource for the functional characterization of this signal transduction system and the development of odor-mediated control strategies for tortricid moths.

Introduction

In moths the ability to detect and respond to odors is essential for finding potential mates, food, and hosts on which to lay their eggs. For moths, odor and pheromone reception predominantly takes place within specialized hairs known as sensillae on their antennae. Each sensillum contains one to three sensory neurons that extend dendrites into the lymph within the hair. Odorants enter this lymph through wax-filled pores in the external surface of the sensillum and are transported through the lymph to the receptors on the surface of the dendrites before being broken down to reset the signaling system.

Proteins involved in these peripheral signaling events have been isolated from moths, and include families of proteins involved in binding and transporting odorants, reception and signaling, and system resetting. Members of all these families have been mooted as possible targets against which to design novel, olfactory-mediated pest control strategies. Odorant Binding Proteins (OBPs) form a large family of small (~15 kDa) hydrophilic carrier proteins that contain 2-4 disulphide bridges. Within the OBPs are subfamilies specialized in carrying general odorants (General Odorant Binding Proteins, GOBPs; Antennal Binding Protein Xs, ABPXs) and sex pheromone components (Pheromone Binding Proteins, PBPs). Other families of carrier proteins found in the antennae include the smaller Chemosensory Binding Proteins (CSPs) and the larger Takeout proteins (TOs). In the antennae members of these families are thought to be expressed in the accessory cells and secreted into the sensillum lymph. Structural and binding studies have demonstrated that members of these families are capable of binding small molecules including many odorants and pheromones (Pelosi and Maida, 1995, Xu et al., 2005, Damberger et al., 2013)

Three different families of integral membrane proteins have been implicated in the reception of odors. These include the members of the odorant receptor (OR) and ionotropic receptor (IRs) families, which are both ligand-gated ion channels, and the sensory neuron membrane proteins (SNMPs). Within moths the ORs and IRs both form large families of proteins, whereas only two SNMPs are typically present. ORs are seven transmembrane non-selective cation channels with intracellular N-termini (Smart et al., 2008). They include a common obligate co-receptor known as Orco that is required for reception but is not tuned to odors. This co-receptor pairs with tuning receptors to detect a range of different odorants. These OR-Orco complexes are present in the dendritic membrane of sensory neurons housed in sensilla trichodea and basiconica. In comparison IRs are three-transmembrane-domain proteins that typically detect volatile acids and similar compounds and are found in sensory neurons within sensilla coeloconica (Rytz et al., 2013). They too form multimeric receptor structures comprised

of a co-receptor and ligand-binding IR to form functional receptor complexes. SNMPs are members of the CD36 class of membrane proteins and are predicted to contain two transmembrane regions. The exact role of the two SNMPs is not absolutely clear.

Pheromone receptors (PRs) in moths form a specialized subset of ORs. PRs have been isolated from many species of moths, mainly from members of the Noctuidae (Wang et al., 2011, Groβe-Wilde et al., 2007), Bombycidae (Sakurai et al., 2004, Groβe-Wilde et al., 2006), Saturniidae (Forstner et al., 2009) and Crambidae (Wanner et al., 2010, Miura et al., 2010). To date all PRs fall into a single phylogenetic clade within the OR family and generally they display higher levels of expression in the antenna of males compared to those of females at the RNA level (male-biased expression). Compared with other ORs, receptors from this clade seem to evolve faster, especially compared to Orco (Carraher et al., 2012). Within the genome, members of the PR subfamily have been shown to be physically clustered (Yasukochi et al., 2011).

Members of three families of enzymes have been implicated in the removal of odorant and pheromone compounds in the sensillum lymph to reset the odorant reception system. These odorant degrading enzymes (ODEs) include carboxylesterases (CXEs), glutathione-S-transferases (GSTs) and cytochrome p450s (CYPs). Members of all three families have been shown to be expressed within moth antennae (Vogt, 2005). In particular certain CXEs have been shown to be capable of hydrolyzing acetate sex pheromone components of *Antheraea polyphemus* at an exceptionally fast rate (Ishida and Leal, 2005).

The methods used to isolate members of these families have improved significantly with the advent of so-called “Next Generation” sequencing technology both in sequencing cDNA and also directly sequencing genomes. More and more projects are using these technologies to isolate and sequence transcripts from moth antennae, while the complete genomes of an increasing number of species within the Lepidoptera are also being sequenced (You et al., 2013, Zhan et al., 2011, Heliconius Genome Consortium, 2012, International Silkworm Genome, 2008).

The lightbrown apple moth, *Epiphyas postvittana*, is a horticultural pest in Australia and New Zealand. *E. postvittana* is a member of the leafroller family Tortricidae which also contains a number of other horticultural pests including codling moth (*Cydia pomonella*) and the oriental fruit moth (*Grapholita molesta*). Originating from Australia, *E. postvittana* has subsequently become established in New Zealand, Hawaii, California and parts of Europe. The moth is primarily a pest of pipfruit, but is also found in high numbers in vineyards (Suckling and Brockerhoff, 2010). As well as insecticides, odor-mediated strategies such as mating disruption have been employed to control its numbers. The sex pheromone for *E. postvittana* has been identified as largely comprising (*E*)-11-tetradecenyl acetate and (*E*,*E*)-9,11-tetradecadienyl acetate (Bellas et al., 1983), with further minor components also being identified more recently

(El-Sayed et al., 2011). Electrophysiological studies have shown that, as well as the major pheromone components (Rumbo, 1983), antennae of *E. postvittana* can detect many terpenes, esters, alcohols and aldehydes (Suckling et al., 1996).

Some of the molecular machinery have already been isolated and characterized from the periphery of *E. postvittana* antennae (Jordan et al., 2008, Jordan et al., 2009, Begum, 2011). These include PBPs, GOBPS, ABPXs and CSP carrier proteins, ORs, and ODEs from the CXE, GST and CYP families. Pheromone Binding Proteins 1 and 3 from *E. postvittana* (EposPBP1, 3) are more highly expressed in male antennae compared with female antennae while EposPBP2 shows opposite bias (Jordan et al., 2008). EposPBP1 is capable of binding the major sex pheromone component and displays high levels of allelic diversity (Newcomb et al., 2002). Also showing male-biased expression in *E. postvittana* antennae is the takeout protein EposTO1 (Jordan et al., 2008), however a volatile ligand has yet to be identified for this carrier (Hamiaux et al., 2009, Hamiaux et al., 2013). Three ORs have been isolated from *E. postvittana* (Jordan et al., 2009). These include the Orco ortholog EposOR2, a receptor with high affinity for citral and related monoterpenes (EposOR3) that is conserved across the Lepidoptera and a third (EposOR1) that falls within the pheromone receptor clade but does not bind the major sex pheromone component, but has been shown to be capable of responding to the plant signaling compound methyl salicylate (Jordan et al., 2009).

Here we use next generation sequencing technologies and bioinformatic analyses to isolate further candidate elements of the peripheral olfactory repertoire from *E. postvittana*. We identify large families of carrier proteins, receptors and ODEs. Within the ORs we identify two groups of male-biased receptors with one found outside the pheromone receptor clade.

Methods

Insect Rearing

E. postvittana were obtained from a colony maintained at the New Zealand Institute for Plant and Food Research Ltd, Auckland, New Zealand. Larvae were reared on a general all-purpose diet (Singh, 1974). Moth antennae and bodies (no head or wings) were removed from two to three-day-old, cold-anesthetized adults using forceps and immediately frozen in liquid nitrogen and stored at -80°C.

Next Generation Sequencing

Total RNA was extracted from pools of 100 pairs of male and female moth antennae using TRIzol RNA extraction reagent (Life Technologies) according to the manufacturer's protocol. RNA was

extracted from three independent pools of male and female antennae for use as biological replicates. RNA quality and quantity was determined using an Agilent 2100 bioanalyzer (Agilent Technologies). High quality total RNA isolated from male and female antennae was used to make libraries for paired-end sequencing on an Illumina HiSeq2000 (Axeq Technologies).

Read pair quality control check was carried out using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Sequence data was then pre-processed by the removal of adapters and trimming to a minimum quality threshold of 20 bp and minimum length of 95 bp using fastq-mcf from the ea-utils package (http://code.google.com/p/ea-utils/wiki/FastqMcf). Thereafter, duplicate reads were removed and trimmed by 15 bases from the five prime end and reads containing Ns or mononucleotides were culled using in-house Perl scripts. *De novo* assembly of the individual libraries was then performed by trans-ABySS (version 1.3.2) (Robertson et al., 2010), where a k-mer series from k=31 to k=75 with an increment of two was used.

Gene identification & Phylogenetics

BLAST searchable databases consisting of transcriptomic contigs from male and female antennae were used to identify candidate chemosensory genes. Tblastn searches were performed with publically available lepidopteran sequences including previously identified chemosensory genes from *E. postvittana* (Jordan et al., 2009, Begum, 2011, Jordan et al., 2008). Identified transcripts were imported into Geneious v6.0.5 (Biomatters, http://www.geneious.com) and annotated for open reading frames, start and stop codons and five and three prime untranslated regions. The longest contigs identified for a particular gene from male and female antennal transcriptomes were aligned to confirm sequence accuracy.

Predicted amino acid sequences of *E. postvittana* chemosensory genes were used in multiple sequence alignments and phylogenetic analyses to confirm their annotation. Datasets for each chemosensory gene family were compiled using publically available sequences from Genbank, together with sequences from *B. mori* (Sakurai et al., 2004, Krieger et al., 2005, Wanner et al., 2007, Tanaka et al., 2009), *C. pomonella* (Bengtsson et al., 2012) and *H. virescens* (Krieger et al., 2004). Amino acid sequences were aligned using Muscle as implemented in MEGA5.2 (Tamura et al., 2011). Maximum likelihood trees were constructed using MEGA5.2, employing the Jones Taylor-Thornton substitution model. Node support was assessed using one thousand bootstrap replicates.

Gene expression analyses

Cleaned RNASeq reads were mapped to the built set of olfactory receptor genes using Bowtie (version 2.1.0) (Langmead and Salzberg, 2012). The alignment was then used to obtain expected read counts using multiBamCov of bedtools package (v2.16.2) (Quinlan and Hall, 2010) and cufflinks (v2.1.1) (Trapnell et al., 2010) was used to obtain Fragments Per Kilobase of transcript per Million (FPKM) values.

To confirm RNAseq counting results and compare the expression of candidate ORs in male and female antennae, qPCR was performed using cDNA prepared from male and female antennae and bodies. RNA extracted from each sample was treated with DNAse I (Life Technologies) and converted to cDNA using iScript cDNA Synthesis Kit (BioRad). cDNA was synthesized from each sample with and without reverse transcriptase to allow for detection of genomic DNA contamination by PCR. cDNA samples that tested negative for genomic DNA contamination were used in qPCR experiments.

Forward and reverse primers for each of the 70 putative *E. postvittana* OR genes, as well as the housekeeping genes actin, α-tubulin and elongation factor-1 (EF-1), were designed using Geneious software (Biomatters, http://www.geneious.com). Primer pairs were designed to amplify products of 100-200 bp in length, have TMs of 60°C (+/- 2°C), have a G/C content of 40-60% and to have two to three G or C nucleotides on the three prime end.

Quantitative real-time PCR was conducted on a LightCycler 480 II apparatus using Syber Green Master Mix (Roche Diagnostics) under the following reaction conditions: an initial 2 minute incubation at 94°C followed by 45 cycles of 94°C for 15 seconds, then 60°C for 30 seconds, then 72°C for 30 seconds. Each primer pair was tested in triplicate against three biological replicates of each cDNA sample as well as to a no template control on a single 384-well microtiter plate. The efficiency of each primer pair in each cDNA sample and the Cycle Threshold (Ct) value for each PCR reaction was determined using LinRegPCR (v11) software (http://www.LinRegPCR.nl). Amplification of single products was verified by melting curve analysis and electrophoresis. Amplification of target sequences was verified by Sanger sequencing of qPCR products.

Housekeeping gene expression levels have been shown to vary between moth species as well as between tissues from the same moth (Teng et al., 2012). Because of this, three different housekeeping genes were used to normalize the expression levels of *E. postvittana* OR genes. Previously, gene expression analyses conducted on *E. postvittana* tissues evaluated the expression levels of commonly used housekeeping genes (Sirey, 2006). In these experiments, the genes actin, α-tubulin and EF-1 were identified as having the highest and most consistent expression levels across tissues. In the current qPCR experiments, actin, α-tubulin and EF-1 were

used as housekeeping genes and were tested in triplicate against each biological replicate of each cDNA sample, as well as a no template control, on each 384-well microtiter plate. The variation of expression levels of the three housekeeping genes in each sample was analyzed post-hoc using GeNorm software (Vandesompele et al., 2002). The housekeeping gene with the greatest variation in expression levels between biological replicates for each sample type was eliminated from normalization analysis.

The relative expression of each gene was calculated using a modified version of the ΔCt method (Pfaffl, 2001, Ramakers et al., 2003). Because the efficiency of a given primer set varied between cDNA samples, resulting Ct values for a particular gene and sample were corrected using the formula $(E_{MAX})^{Ct_{corrected}} = (E_{sample\ 'X'})^{Ct_{sample\ 'X'}}$, where E_{MAX} equals the highest efficiency for a primer pair from all samples, $E_{sample\ 'X'}$ equals the efficiency of that primer pair in sample 'X', $Ct_{sample\ 'X'}$ equals the measured Ct value for sample 'X', and $Ct_{corrected}$ equals the corrected Ct value for sample 'X'. A normalization factor was determined for each sample by averaging the corrected Ct values for the two least variable housekeeping genes from that sample. The relative expression of each gene to the normalization factor for each sample was calculated using the formula $(E_{MAX})^{(\Delta Ct)}$, where E_{MAX} equals the highest efficiency for a particular primer set and ΔCt equals the difference between the Ct of the primer set in that sample and the normalization factor for that sample. The average relative expression was calculated by averaging the relative expression of each gene in three biological replicates. Significant differences between the relative expression of a particular gene between male and female antennae was determined using a Welch two sample T-test.

Results

Transcriptome assembly

Individual transcriptome assemblies were generated for female and male antennae. The female antennal assembly generated 270,708 transcripts with an N50 of 1,319 bp and maximum transcript size of 16,438 bases, while the male antennal assembly yielded 266,710 transcripts with an N50 of 1,277 and maximum transcript size of 14,723 bases.

Identification of chemosensory genes and phylogenetics

In total, 19 IRs, nine GRs, two SNMPs, 33 OBPs, 13 CSPs, 24 CXEs, 22 GSTs and 51 CYPs were identified from male and female *E. postvittana* antennal transcriptomes. The sequences of these genes were confirmed using the draft genome and have been deposited into Genbank.

A total of 65 candidate ORs were identified and assembled from the two transcriptome databases, with an extra five identified from a draft genome of *E. postvittana* (unpublished data). The sequences of the 70 *E. postvittana* OR genes were confirmed using the draft genome and have been deposited into Genbank, including the three previously identified EposORs from Jordan et al (2009) which have been updated.

In the phylogenetic analysis of the 70 *E. postvittana* ORs, eight fall into a well supported clade that contains pheromone receptors from other moth species including BmorOR1, BmorOR3, HvirOR6, HvirOR13, HvirOR14 and HvirOR16 (Sakurai et al., 2004, Nakagawa et al., 2005, Krieger et al., 2004, Groβe-Wilde et al., 2007, Wang et al., 2011). The remaining 62 ORs are dispersed throughout the phylogeny often forming orthologous sets with other moth ORs. There are only a few instances of radiations of ORs within *E. postvittana* including one group of five ORs (OR30, 31, 33, 34 and 36), one of four ORs (OR1, 41, 43 and 45) that falls within the clade containing pheromone receptors from other species, and another of three ORs (OR14, 15 and 20) (Figure 1).

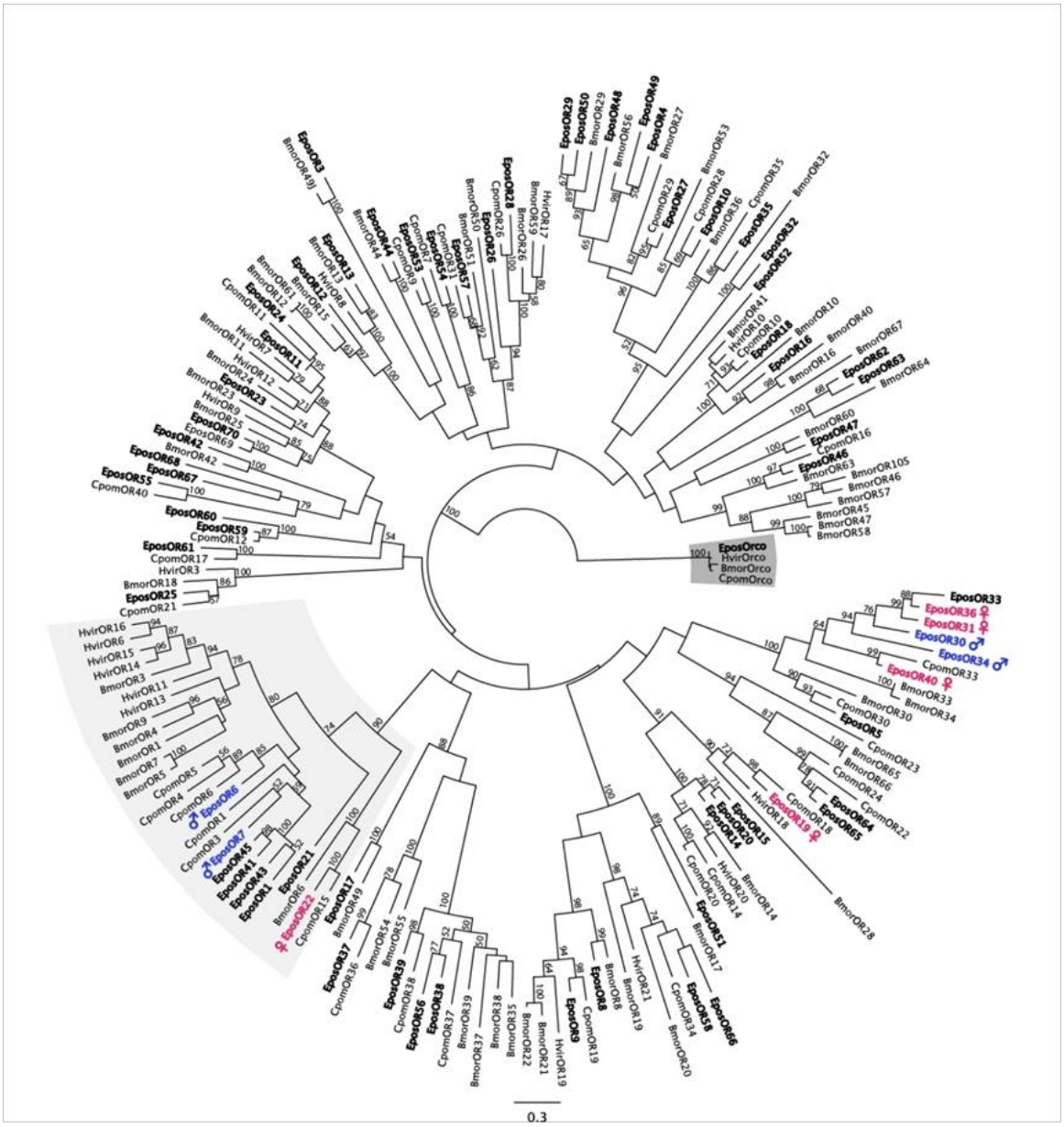

Figure 1. Phylogenetic tree of olfactory receptors from *E. postvittana, C. pomonella, H. virescens* and *B. mori*. The lepidopteran 'pheromone receptor' clade is shaded. *E. postvittana* ORs in bold font, male biased *E. postvittana* ORs in blue font and female biased *E. postvittana* ORs in pink font. Node support was assessed using Bootstrap replicates of 1000.

Gene expression analyses

Of the 173 non-OR olfactory related genes examined in this study, RNAseq count data revealed normalized transcript counts ranging from 0 to 10^6 in moth antennae (Table 1). Many of these genes display sex-biased antennal expression patterns; however the statistical significance of the biased expression could not be assessed because the RNAseq data was conducted on single male and female antennal transcriptomes.

Table 1. IR, GR, ABPX, OBP, PBP, SNMP, CSP, GST, CXE and CYP transcript counts in antennal tissue from male and female *E. postvittana* as determined by RNAseq. Data represent transcript counts from a single transcriptome made from male or female antennal mRNA.

Gene	RNASeq Data (FPKM)		Gene	RNASeq Data (FPKM)		Gene	RNASeq Data (FPKM)	
	female antennae	male antennae		female antennae	male antennae		female antennae	male antennae
EposABPX01	207197	359151	**EposCYP16**	130	26	**EposGST12**	429	277
EposABPX02	110396	123337	**EposCYP17**	389	213	**EposGST13**	1134	477
EposABPX03	209842	349917	**EposCYP18**	13144	8585	**EposGST14**	546	743
EposABPX04	32000	29399	**EposCYP19**	299	57	**EposGST15**	175	160
EposABPX05	774	526	**EposCYP20**	5912	3127	**EposGST16**	645	348
EposABPX06	77347	69661	**EposCYP21**	129	86	**EposGST17**	1669	1286
EposCSP01	56630	60959	**EposCYP22**	448	259	**EposGST18**	397	313
EposCSP02	107403	39694	**EposCYP23**	1464	528	**EposGST19**	1506	750
EposCSP03	18851	12635	**EposCYP24**	723	239	**EposGST20**	744	401
EposCSP04	188642	185436	**EposCYP25**	128	123	**EposGST21**	70	31
EposCSP05	97360	14303	**EposCYP26**	206	74	**EposGST22**	121	115
EposCSP06	173	417	**EposCYP27**	167	86	**EposIgluR**	83	42
EposCSP07	142154	72392	**EposCYP28**	206	59	**EposIR01**	60	37
EposCSP08	117	32	**EposCYP29**	46	28	**EposIR03**	199	144
EposCSP09	600	115	**EposCYP30**	526	282	**EposIR04**	117	44
EposCSP10	429	54	**EposCYP31**	482	226	**EposIR07d**	37	21
EposCSP11	185	118	**EposCYP32**	246	137	**EposIR08a**	1534	1086
EposCSP12	100	28	**EposCYP33**	69	30	**EposIR21a**	169	89
EposCSP13	69	12	**EposCYP34**	31	35	**EposIR25a**	503	351
EposCXE01	28	26	**EposCYP35**	88	32	**EposIR41a**	181	118
EposCXE02	2	1	**EposCYP36**	2009	1086	**EposIR68**	146	99
EposCXE03	5	7	**EposCYP37**	47	32	**EposIR75a**	84	46
EposCXE04	247	179	**EposCYP38**	130	761	**EposIR75b**	132	56
EposCXE05	49	31	**EposCYP39**	236	114	**EposIR75c**	642	521
EposCXE06	30	14	**EposCYP40**	26	21	**EposIR75d**	133	95
EposCXE07	262	197	**EposCYP41**	441	193	**EposIR75e**	27	23
EposCXE08	2	0	**EposCYP42**	240	107	**EposIR75f**	66	24
EposCXE09	0	0	**EposCYP43**	420	564	**EposIR76b**	1064	931
EposCXE10	1	0	**EposCYP44**	477	290	**EposIR87a**	298	174
EposCXE11	2	0	**EposCYP45**	213	113	**EposIR93a**	46	24
EposCXE12	0	0	**EposCYP46**	163	460	**EposOBP01**	3106	1202
EposCXE13	0	0	**EposCYP47**	135	34	**EposOBP02a**	376	190
EposCXE14	220	332	**EposCYP48**	383	1728	**EposOBP02b**	347	173
EposCXE15	210	237	**EposCYP49**	1091	369	**EposOBP03**	8153	1779
EposCXE16	470	522	**EposCYP50**	53	13	**EposOBP04**	956	513
EposCXE17	905	426	**EposCYP51**	132	116	**EposOBP05**	5392	1443
EposCXE18	175	159	**EposGOBP01**	85834	48015	**EposOBP06**	286028	104121
EposCXE19	6	0	**EposGOBP02**	425890	326245	**EposOBP07**	286536	75329
EposCXE20	6	3	**EposGR01**	20	3	**EposOBP08**	125	36
EposCXE21	441	274	**EposGR02**	28	17	**EposOBP09**	482	236
EposCXE22	326	178	**EposGR03**	11	12	**EposOBP10**	187	73
EposCXE23	107	36	**EposGR04**	131	56	**EposOBP11**	404	19
EposCXE24	49	21	**EposGR05**	97	87	**EposOBP12**	214	55
EposCYP01	583	343	**EposGR06**	91	49	**EposOBP13a**	10626	84
EposCYP02	9	3	**EposGR07**	148	69	**EposOBP13b**	11403	92
EposCYP03	252	90	**EposGR08**	139	72	**EposOBP14**	355	1489
EposCYP04	1129	828	**EposGR09**	65	6	**EposOBP15**	10305	5676
EposCYP05	187	119	**EposGST01**	67	32	**EposOBP16**	482	319
EposCYP06	5716	4096	**EposGST02**	560	364	**EposOBP17**	24	44
EposCYP07	443	169	**EposGST03**	406	206	**EposOBP18**	901465	239296
EposCYP08	445	249	**EposGST04**	14241	14685	**EposOBP19**	10622	8108
EposCYP09	986	331	**EposGST05**	863	663	**EposOBP20**	8030	5565
EposCYP10	1466	528	**EposGST06**	195	103	**EposPBP01**	120672	1196560
EposCYP11	36	35	**EposGST07**	2064	3719	**EposPBP02**	130951	32283
EposCYP12	829	439	**EposGST08**	2050	898	**EposPBP03**	34956	134567
EposCYP13	13	23	**EposGST09**	8668	5555	**EposSNMP01**	2371	927
EposCYP14	24340	13737	**EposGST10**	676	439	**EposSNMP02**	3069	2852
EposCYP15	392	255	**EposGST11**	55536	59452			

Of the 70 *E. postvittana* OR genes identified in the antennal transcriptomes or genome, 65 had normalized RNAseq counts greater than one (Table 2). Transcripts for the EposOR genes 8,

11, 13, 69 and 70 were not detected in the male or female antennal transcriptomes by RNAseq analysis. Interestingly, EposOR8 and EposOR13 were detectable by PCR using the same RNA starting material from which the transcriptomes were made, suggesting rare transcripts may be lost in transcriptome generation and assembly. RNAseq data has been deposited into a Sequence Read Archive (SRA) database online (http://www.ncbi.nlm.nih.gov/) under the accession number SRP036070.

Table 2. OR mRNA expression in antennal and body tissue of female and male *E. postvittana* as determined by qPCR and OR transcript counts in antennal tissue as determined by RNAseq. qPCR data represent the mean fold change to reference genes from three biological replicates. RNAseq data represent transcript counts from a single transcriptome made from male or female antennal mRNA. BLD = Below Limit of Detection.

	qPCR data (fold change to reference genes)				RNASeq Data (FPKM)	
Gene	**female antennae**	**female body**	**male antennae**	**male body**	**female antennae**	**male antennae**
EposOR1	0.03263	0.00024	0.07130	BLD	**669**	**358**
EposOrco	2.62100	BLD	3.55188	BLD	**4252**	**2442**
EposOR3	0.00768	BLD	0.00663	BLD	**216**	**143**
EposOR4	0.03408	BLD	0.03337	0.00011	**246**	**219**
EposOR5	0.01009	0.00002	0.00138	BLD	**843**	**84**
EposOR6	0.00073	BLD	0.04183	0.00600	**3**	**24**
EposOR7	0.00315	0.00010	0.01561	0.00138	**0**	**215**
EposOR8	0.00053	BLD	0.00006	0.00002	**0**	**0**
EposOR9	0.00145	BLD	0.00127	BLD	**46**	**16**
EposOR10	0.00791	0.00007	0.00624	0.00115	**96**	**30**
EposOR11	BLD	BLD	BLD	BLD	**0**	**0**
EposOR12	0.00143	BLD	0.00192	0.00001	**262**	**204**
EposOR13	0.00028	0.00004	0.00071	0.00059	**0**	**0**
EposOR14	0.01350	0.00003	0.00650	0.00028	**188**	**50**
EposOR15	0.03509	0.00502	0.02606	0.02465	**211**	**96**
EposOR16	0.00265	BLD	0.00726	0.00023	**8**	**4**
EposOR17	0.00560	0.00493	0.01428	0.00325	**2**	**2**
EposOR18	0.03153	0.00029	0.02091	BLD	**107**	**58**
EposOR19	0.07996	0.00007	0.03561	BLD	**544**	**107**
EposOR20	0.01524	0.00139	0.01714	0.00381	**136**	**46**
EposOR21	0.00729	0.00002	0.00099	0.00003	**150**	**12**
EposOR22	0.07837	0.00007	0.02709	BLD	**295**	**129**
EposOR23	0.00058	0.00025	0.00145	0.00108	**1**	**1**
EposOR24	0.01978	BLD	0.02527	BLD	**41**	**23**
EposOR25	0.03330	0.00034	0.03440	0.00260	**289**	**78**
EposOR26	0.05425	0.01337	0.11274	0.01719	**40**	**29**
EposOR27	0.05022	BLD	0.04528	0.00128	**301**	**181**
EposOR28	0.01670	BLD	0.00903	0.00009	**40**	**29**
EposOR29	0.04849	0.00020	0.02772	0.00027	**248**	**155**
EposOR30	0.02020	0.00398	0.69629	0.02740	**2**	**1480**
EposOR31	0.00616	BLD	0.00003	BLD	**386**	**0**
EposOR32	0.04552	BLD	0.03881	BLD	**83**	**29**
EposOR33	0.00640	BLD	0.00047	0.00004	**222**	**0**
EposOR34	0.01131	0.00043	0.32708	0.00233	**1**	**2239**
EposOR35	0.00408	BLD	0.00543	BLD	**59**	**32**
EposOR36	0.02528	BLD	0.00014	0.00004	**248**	**0**
EposOR37	0.00393	0.00008	0.01092	0.00008	**51**	**45**
EposOR38	0.04631	0.00006	0.11024	0.00197	**225**	**123**
EposOR39	0.09887	BLD	0.19689	BLD	**200**	**121**
EposOR40	0.32466	0.00007	0.00507	0.00135	**2040**	**2**
EposOR41	0.00446	0.00019	0.00911	BLD	**174**	**109**
EposOR42	0.05713	BLD	0.09239	BLD	**236**	**148**
EposOR43	0.09436	0.00068	0.04906	0.00181	**155**	**111**
EposOR44	0.00199	BLD	0.00317	BLD	**114**	**67**
EposOR45	0.01907	0.00015	0.02347	0.00011	**480**	**234**
EposOR46	0.00932	0.00015	0.01168	0.00079	**78**	**45**
EposOR47	0.08845	0.00096	0.07746	0.00430	**184**	**101**
EposOR48	0.06465	0.00007	0.07733	0.00034	**302**	**196**
EposOR49	0.00271	0.00022	0.00522	0.00008	**18**	**11**
EposOR50	0.00054	0.00008	0.00144	0.00010	**7**	**3**

EposOR51	0.05788	0.00038	0.06611	0.00031	**402**	**219**
EposOR52	0.00986	BLD	0.00955	BLD	**100**	**42**
EposOR53	0.01144	0.00003	0.01077	0.00011	**184**	**131**
EposOR54	0.02487	0.00003	0.02158	0.00011	**153**	**99**
EposOR55	0.00098	0.00001	0.00569	0.00026	**149**	**93**
EposOR56	0.00100	BLD	0.00071	BLD	**20**	**10**
EposOR57	0.00114	BLD	0.00610	0.00001	**22**	**30**
EposOR58	0.00405	0.00001	0.00339	0.00003	**201**	**95**
EposOR59	0.01240	0.00061	0.00962	0.00044	**56**	**20**
EposOR60	0.00464	0.00010	0.01035	BLD	**103**	**96**
EposOR61	0.01409	0.00006	0.01719	BLD	**271**	**96**
EposOR62	0.00589	BLD	0.00451	BLD	**104**	**51**
EposOR63	0.00017	BLD	0.00011	BLD	**33**	**14**
EposOR64	0.05393	BLD	0.01088	BLD	**591**	**136**
EposOR65	0.01115	0.00005	0.00184	0.00004	**415**	**76**
EposOR66	0.00506	0.00020	0.00296	BLD	**31**	**11**
EposOR67	0.02124	0.00001	0.01068	0.00007	**433**	**157**
EposOR68	0.02752	0.00027	0.02945	BLD	**172**	**96**
EposOR69	BLD	BLD	BLD	BLD	**0**	**0**
EposOR70	BLD	BLD	BLD	BLD	**0**	**0**

The 70 OR genes were tested by qPCR expression analysis and 67 were detected in male or female antennae or bodies. Transcripts for the EposOR genes 11, 69 and 70 were not detected in any tissues despite attempts with multiple primer pairs. Of the 67 OR transcripts that were detected in *E. postvittana*, 64 genes showed enriched expression in antennae compared to bodies, while 30 were detected exclusively in male and/or female antennae and not detected in male or female bodies. Four genes, EposORs 6, 7, 30 and 34 were found to have significantly higher mRNA expression in male antennae compared to female antennae ($p=0.046$, $p=0.009$, $p=0.004$ and $p=0.002$, respectively) (Figure 2A). Five receptors, EposORs 19, 22, 31, 36 and 40 were found to have significantly higher mRNA expression in female antennae compared to male antennae ($p=0.023$, $p=0.032$, $p=0.031$, $p=0.012$ and $p=0.018$, respectively) (Figure 2B).

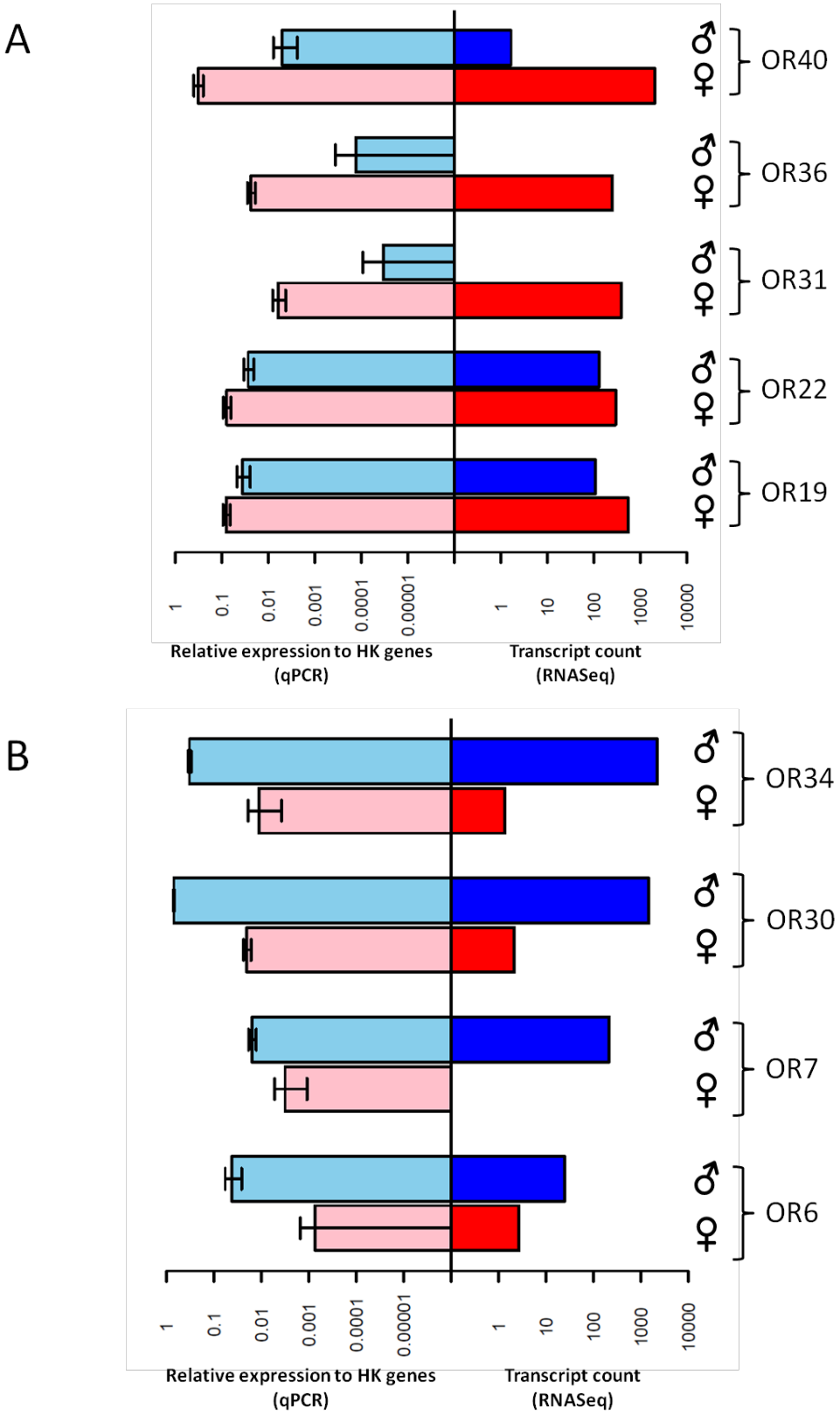

Figure 2. Female-biased (A) and male-biased (B) antennal OR mRNA expression in *E. postvittana*. Comparison of OR mRNA expression in male (♂) and female (♀) antennae as determined by quantitative real-time PCR (left panel) and RNASeq (right panel). For qPCR data, bars represent MEAN (+/- SEM) OR mRNA expression relative to reference genes from three biological replicates. For RNAseq data, bars represent transcript counts from a single transcriptome made from male or female antennal mRNA.

Discussion

Transcriptome sequencing has become a popular approach to identifying genes involved in tissue specific functions. Here we have used this approach to identify genes involved in the peripheral events of odorant and pheromone perception in the invasive lepidopteran pest *Epiphyas postvittana*. In total, 266,710 and 270,708 putative transcripts were produced from male and female antennae, respectively. From these we inferred the presence of 234 predicted proteins that are potentially involved in odorant and pheromone binding, reception and degradation. The average transcript length of our male (N50 =1,277 bp) and female (N50 = 1,319 bp) antennal transcriptomes made it relatively straight forward to determine the full length coding sequence of most of these genes through examination of untranslated regions and open reading frames of single contigs. To date we have verified the full-length sequence of thirty ORs, three OBPs, two SNMPs and one CXE by PCR.

Several olfactory-related genes have been previously identified in *E. postvittana*. In 2002, through microsequencing of purified protein and degenerate PCR Newcomb et al. were able to identify EposPBP1, EposPBP2, EposGOBP1 and EposGOBP2. In 2008 Jordan et al. generated EST libraries from male antennae which enabled them to identify sequences of 12 more binding proteins, three ORs, two SNMPs and 25 degrading enzymes. In the current study we have expanded the number of identified olfactory-related genes from *E. postvittana* to include: 33 odorant binding proteins, 13 chemosensory proteins, 70 odorant receptors, 19 ionotropic-like receptors, two SNMPs, 24 carboxylesterases, 22 glutathione-S-transferases and 51 cytochrome p450s. In addition, we identified nine *E. postvittana* GRs from antennal transcriptomes, including orthologs of the putative CO2 and sugar receptors identified in other moths (Krieger et al., 2002, Wanner and Robertson, 2008, Groβe-Wilde et al., 2011, Bengtsson et al., 2012, Poivet et al., 2013). The 70 ORs that we have identified from *E. postvittana* antennae correlates well with the number of glomeruli (50 – 70) observed in the antennal lobe of other tortricid moths (Masante-Roca et al., 2005, Varela et al., 2009). Overall, based on comparison with other moth antennal transcriptomes and genomes, we believe this represents the vast majority of the predicted proteins involved in peripheral signal transduction and as such is a valuable resource for understanding odorant and pheromone reception in this species as well as to aid in the identification of olfactory-related genes from other insects.

We first looked at ORs that were either part of the so-called pheromone receptor clade or showed male-biased expression in adult antennae as candidates for being involved in pheromone reception. Of the 70 ORs, eight from *E. postvittana* were identified as being part of the phylogenetic clade associated with a role in pheromone reception in other moths. Seven of the

eight EposORs that fall within this 'PR clade' contain the characteristic 'PWE' amino acid motif within the final transmembrane domain that is seen in members of this group. One receptor, EposOR21 does not contain this motif and phylogenetic analysis suggests this receptor is basal to other ORs in the clade. EposORs 1, 41, 43 and 45 share relatively high (~61%) identity at the protein level and form their own group within the clade and may have arisen by recent gene duplication events. The two male-biased EposORs within the PR clade, EposOR6 and EposOR7, are most closely related to ORs from *Cydia pomonella*, CpomOR6 and CpomOR3, respectively. The final receptor that falls in the pheromone receptor clade, EposOR22 is female-biased and is closely related to CpomOR15 (Bengtsson et al., 2012).

In terms of male-biased expression, EposOR6 and 7 from within the pheromone receptor clade and EposOR30 and 34 from a more distant part of the tree all showed significantly greater levels of expression in male compared with female antennae. This is the first case we are aware of where receptors from outside the pheromone receptor clade display male-biased expression. These two groups of receptors (EposOR6 and 7; EposOR30 and 34) form clusters of genes physically within the genome (unpublished draft genome and BAC analysis). In addition, we identified five receptors that showed female-biased expression, including two receptors (EposORs 31 and 36) that are closely related to the two male-biased ORs that are not part of the PR clade. None of the female-biased receptors (EposOR19, 22, 31, 36, 40) were closely related to those found to be female-biased in their expression in *B. mori* (BmorOR19, 30, 45, 46, 47) (Wanner et al., 2007).

In this study we have identified a large set of olfactory-related genes from the horticultural pest *E. postvittana* which provides a valuable tool for investigators to use to identify olfactory-related genes from other moths. The use of next generation sequencing has proven to be an extremely efficient and effective tool for full-length gene identification and expression analyses. In particular, EposOR genes that showed significant sex-biased mRNA expression by qPCR also showed biased expression patterns by RNAseq. While we could not conduct statistical analyses on RNAseq data, it is worth pointing out that biased expression patterns were consistent with our qPCR data which could be statistically analyzed. Since our interest in conducting these OR gene expression analyses was to identify sex-biased genes for functional testing, we would have identified the same genes through non-statistical analysis of RNAseq data as we did from our qPCR experiment.

Now that we have identified genes with putative roles in sex-pheromone reception we can begin functional characterization, in HEK293 cell-based assays (Corcoran et al., 2014), of ORs of interest as well as to address fundamental questions regarding the roles of PBPs in pheromone delivery, SNMP1 and 2 in pheromone reception and ODEs in pheromone degradation.

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III

A method to study lepidopteran olfactory receptors using HEK293 cells

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Key words:
Lepidoptera, olfactory receptor (OR), HEK293, high-throughput

Abstract

The development of rapid and reliable assays to characterize insect odorant receptors (ORs) and pheromone receptors (PRs) remains a challenge for the field. Typically insect ORs and PRs are functionally characterized either *in vivo* in transgenic *Drosophila* or *in vitro* through expression in *Xenopus* oocytes. While these approaches have succeeded, they are not well suited for high-throughput screening campaigns. The development of a practical, robust and consistent assay for functional studies on ORs and PRs would allow for high-throughput screening for ligands, as well as for compounds that could be used as novel olfactory-based pest management tools. Here we demonstrate the use of human embryonic kidney cells (HEK293) transfected with inducible receptor constructs for the functional characterization of ORs in 96-well plates using a fluorescent spectrophotometer. Using EposOrco and EposOR3 from the pest moth *Epiphyas postvittana* as an exemplar, we generated HEK293 cell lines with robust and consistent responses to ligands in functional assays. Single-cell sorting of cell lines by FACS facilitated the selection of isogenic cell lines with maximal responses, and the addition of epitope tags on the N-termini allowed the detection of recombinant proteins in homogenates by western blot and in cells by immunocytochemistry. Here we thoroughly describe the methods used to generate these OR-expressing cell lines, demonstrating that they have all the necessary features required for use in high-throughput screening platforms.

Introduction

Moths rely upon their ability to detect volatile chemical compounds, using olfactory receptors (ORs) housed in their antennae, to find host plants and conspecific mates for reproduction. Olfactory receptors have been studied by investigators for decades in a wide variety of insects in an effort to discern basic fundamental questions such as: How many ORs do insects have and what volatile compounds do they respond to? Are these receptors specific for individual ligands or are they broadly 'tuned' to classes of volatile compounds? Much progress has been made over the years in answering these types of questions (Benton, 2006, Leal, 2013) and technological advances have facilitated the process. Next generation sequencing techniques have made it relatively easy to identify ORs and other olfactory-related genes from an insect of interest (International Silkworm Genome, 2008, Groβe-Wilde et al., 2011, Zhan et al., 2011, Bengtsson et al., 2012, Heliconius Genome Consortium, 2012, Poivet et al., 2013, You et al., 2013, Corcoran et al., 2014), and experimental systems have been developed in which ORs can be expressed and tested for responsiveness to various ligands. In flies there is an *in vivo* system which requires the generation of transgenic *Drosophila* expressing the OR in a specific 'empty' neuron for subsequent electrophysiological characterization (Dobritsa et al., 2003, Hallem et al., 2004). While this system has proven to work well for functionally characterizing some ORs, it is not available outside *Drosophila* and non-dipteran ORs may not function properly in this system (Syed et al., 2006). Alternatively, *in vitro* systems have been developed through which insect ORs have been deorphaned including *Xenopus* oocytes, Sf9 cells and HEK293 cells.

The order Lepidoptera contains many of the world's pests of horticultural crops. Historically, control strategies have relied upon the use of insecticides, natural enemies and, more recently, mating disruption to manage pest populations. While these control strategies have proven to be efficacious, there is increasing incentive to develop more modern, targeted control tools with lower costs, less health and safety concerns and less environmental risks than existing tools. The insect olfactory system is the new target for modern pest control tools; once ORs are identified and functionally characterized from a pest moth, the opportunity exists to identify compounds that have some sort of effect on volatile detection at the receptor level. However, the key requirement for the identification of compounds that affect OR-ligand interactions is a practical, robust and consistent *high-throughput* OR assay system.

Sf9 cells are a cell line derived from ovarian tissue from the moth *Spodoptera frugiperda*. Investigators have used this cell line to express ORs from several species for functional characterization (Kiely et al., 2007, Anderson et al., 2009, Jordan et al., 2009). Because Sf9 cells have been shown to express an endogenous Orco orthologue (Kiely, 2008, Smart et al., 2008),

investigators simply transfect Sf9 cells with a plasmid containing the OR of interest, load the cells with a calcium-sensitive fluorophore and measure responses to compounds in individual cells using a fluorescent microscope. The '*Xenopus*' system utilizes frog eggs from the African clawed frog, *Xenopus laevis*, to express insect ORs for functional characterization (Sakurai et al., 2004, Nakagawa et al., 2005, Mitsuno et al., 2008, Miura et al., 2009, Miura et al., 2010, Wanner et al., 2010, Wang et al., 2011, Leary et al., 2012, Xu et al., 2012, Liu et al., 2013, Zhang and Löfstedt, 2013). In this system, oocytes are co-injected with cRNA encoding the OR co-receptor Orco and the OR of interest and later monitored electrophysiologically for ligand-induced depolarization. While the Sf9 cell and *Xenopus* oocyte assays have proven valuable for de-orphaning lepidopteran ORs, they have characteristics that do not make them amenable for high-throughput screening. For example, Sf9 cells transfected with OR genes typically have low response rates in functional assays which precludes their use in plate reader-based formats. The *Xenopus* system relies upon electrophysiological recordings from individual frog eggs which is too time-intensive to be considered a true high-throughput screening assay, despite recent technological advances in experimental setup (Papke and Smith-Maxwell, 2009). In addition, both systems yield relatively high intra and inter-assay variation of response profiles of ORs to ligands, presumably due to the nature of their transient and heterogeneous expression of exogenous proteins.

Human Embryonic Kidney 293 (HEK293) cells are an immortalized mammalian cell line (Graham et al., 1977) commonly used in the biotechnology and pharmaceutical industries to study receptor-ligand interactions and to identify molecules that may affect these interactions (Miller et al., 2011). HEK293 cells can be easily genetically modified to stably express heterologous recombinant proteins in an isogenic and regulated fashion (Jones et al., 2005, Abu-Hamad et al., 2006). The ability of HEK293 cells to stably and isogenically express recombinant proteins and their ability to be frozen and thawed creates an assay system with relatively low intra and inter-assay variation. These characteristics, as well as other cell properties (i.e., growth rate, adhesion to surfaces) make HEK293 cells an optimal high-throughput option for characterizing lepidopteran ORs and for identifying novel molecules that may affect OR-ligand interactions.

Human embryonic kidney cells were first used to characterize insect ORs in 2006 when investigators transfected PR genes from *Bombyx mori* into an HEK293 cell line expressing a Gα15 gene which allowed them to monitor OR activation using fluorescent calcium-sensitive dyes and fluorescent microscopes (Groβe-Wilde et al., 2006, Groβe-Wilde et al., 2007, Forstner et al., 2009). More recently, a group of researchers has modified a commercially available expression vector and used it to generate HEK293 cell lines with stable and inducible expression of insect Orco and ORs. The modified pcDNA5FRTO expression vector allows the expression of two genes

from the same plasmid, which theoretically creates an isogenic cell line with equal expression of both genes (Bohbot et al., 2011, Jones et al., 2011, Pask et al., 2011, Pask et al., 2013b, Kumar et al., 2013). Using this system these investigators have used 384-well plate formats to identify compounds that affect or inhibit Orco responses to agonistic compounds with high-throughput fluorescent spectrophotometers (Jones et al., 2012, Rinker et al., 2012, Pask et al., 2013a). While these studies have clearly demonstrated that HEK293 cells can be used to functionally characterize insect ORs in a high-throughput assay, a critical component of their system, the modified expression vector, is not commercially available so the methods used to generate their OR-expressing HEK293 cells lines are difficult to replicate.

The focus of this article is to demonstrate the utility and capabilities of using HEK293 cells to study lepidopteran ORs and to thoroughly describe the methods used to generate high-throughput compatible cell lines. Using a previously characterized OR from the horticultural pest *Epiphyas postvittana* (Jordan et al., 2009), we examined the ability of HEK293 cells to express the *E. postvittana* Orco and EposOR3 by western blot and confocal microscopy. We then tested HEK293 cell lines expressing EposOrco and EposOR3 for their ability to respond to the insect Orco agonist VUAA1 (Jones et al., 2011) and previously identified EposOR3 agonists (Jordan et al., 2009) using a calcium-sensitive fluorophore in a plate reader-based format.

Methods

EposOR Cloning

The cDNA used in this experiment to amplify full length EposOR DNA is the same as that used to evaluate EposOR mRNA expression in Corcoran et al (2014), therefore the insects used, RNA extraction and cDNA synthesis methods used in this experiment are identical.

Primers designed to amplify full length EposOrco and EposOR3 were designed manually using the sequences reported in Corcoran et al (2014) and the Primer 3 application within Geneious v6.0.5 software (Biomatters, http://www.geneious.com/). Two sets of primers were designed for each gene: a first set designed to amplify the full length gene from cDNA and a second set designed to add NotI and ApaI restriction sites and an N-terminal c-Myc (myc, MEQKLISEEDL) or V5 (GKPIPNPLLGLDST) epitope tag to full length EposOrco or EposOR3, respectively.

Using the first set of primers, full length EposOrco and EposOR3 were amplified from cDNA prepared from male *E. postvittana* antennae using Platinum Taq DNA Polymerase (pTaq,

Life Technologies) using the following PCR reaction conditions: an initial two minute incubation at 94°C followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 90 seconds, followed by a final seven minute incubation at 72°C. PCR reactions were run on 0.7% TAE agarose gels and bands of the expected size (1425 and 1233 bp for EposOrco and EposOR3, respectively) were extracted and purified using a QIAquick Gel Extraction Kit (Qiagen).

Purified, full length DNA for each gene was ligated into pCR8/GW/TOPO-TA (Life Technologies) and used to transform One-Shot Top-10 cells (Life Technologies) following the manufacturer's protocol. Colonies were tested for successful transformation with pCR8/EposOR'X' by PCR using pTaq and full length, gene-specific primers using the following PCR reaction conditions: an initial ten minute incubation at 94°C followed by 25 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 90 seconds. Colonies that tested positive were grown in LB broth overnight and plasmids were isolated using a NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel). Purified plasmids were sequenced and pCR8 plasmids containing the correct EposOR DNA sequence were used for further cloning efforts.

Five prime and three prime restriction sites and N-terminal epitope tags were added to EposOrco and EposOR3 by PCR using the second set of primers and pCR8 plasmids with verified DNA sequences as template. PCR reaction products and pcDNA4-TO and pcDNA5-TO (Life Technologies) expression vectors were then double-digested using NotI and ApaI (New England BioLabs) according to the manufacturer's protocol. NotI/ApaI-digested products were run on 0.7% TAE agarose gels and bands of the approximate expected size (1487, 1307, 5056 and 5645 bps for EposOrco, EposOR3, pcDNA4-TO and pcDNA5-TO, respectively) were extracted and purified using a QIAquick Gel Extraction Kit (Qiagen).

Purified, NotI/ApaI-digested, epitope-tagged full length EposOR DNA was then ligated into NotI/ApaI-digested pcDNA4/TO (EposOrco) or pcDNA5/TO (EposOR3) using T4 DNA ligase (New England Biolabs) and used to transform One-Shot Top-10 cells (Life Technologies) following the manufacturer's protocol. Colonies were tested for successful transformation with pcDNA4-TO/myc-EposOrco (pcDNA4/EposOrco) or pcDNA5-TO/V5-EposOR3 (pcDNA5/EposOR3) by PCR using pTaq and full length, gene-specific primers using the PCR reaction conditions described above. Colonies that tested positive were grown in LB broth overnight and large quantities of high-quality pcDNA4/EposOrco and pcDNA5/EposOR3 plasmids were produced using a PureLink HiPure Plasmid Midiprep Kit (Life Technologies) following the manufacturer's protocol.

Cell culture and cell line generation

HEK293 cells were grown in T-25 or T-75 cell culture flasks in a cell culture incubator at 37°C with 5% CO_2. Cells were cultured using DMEM (high glucose, Life Technologies) mammalian cell culture media supplemented with 10% heat-inactivated fetal bovine serum (FBS). Stably transfected cell lines were grown for up to six months with no obvious changes in growth rate, morphology or transfected gene expression (see results). Cells stably expressing ORs were aliquoted and frozen at -80°C and transferred to liquid nitrogen to create cell line 'banks' for future use. Cell lines were cultured for a maximum of six months and then discarded and cells from the original 'bank' were used for additional culture and functional testing.

Prior to transfection into wild type HEK293 cells, pcDNA6/TR was linearized using BstZ171 (New England Biolabs), run on a 0.7% TAE agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Five micrograms of linearized pcDNA6/TR plasmid and 15 µL of Lipofectamine2000 (Life Technologies) transfection reagent were each diluted into 500 µL of Optimem medium (Life Technologies) and incubated at room temperature (RT) for 10 minutes, after which they were mixed together and incubated for an additional 60 minutes at RT. The pcDNA6/Lipofectamine2000 mixture was then added to a T-25 cell culture flask containing wild type HEK293 cells at approximately 70% confluency and incubated overnight (37° C, 5% CO_2). The following morning, the medium was removed from the flask and replaced with fresh cell culture medium containing 20 µg/mL blasticidin (Gold Biotech, USA). Cells were cultured for approximately two weeks in the presence of the blasticidin until an antibiotic-resistant cell line was established, then the blasticidin concentration was reduced to 10 µg/mL. The resulting tetracycline repressor expressing (TREx) cell line was passaged three times and frozen prior to further use.

Isogenic TREx/HEK293 cell lines were generated by fluorescence-activated cell sorting (FACS) using a BD ARIA II machine (University of Auckland, New Zealand). Cells were lifted from a confluent culture, pelleted and resuspended in DPBS (Life Technologies) containing 5% fetal bovine serum. Individual cells were deposited into each well of five 96-well tissue culture plates containing normal cell culture medium containing 10 µg/mL blasticidin. Isogenic TREx HEK293 cell lines were grown for approximately three weeks in the presence of 10 µg/mL blasticidin during which time they were scaled-up into 48, 24, 12 and 6-well dishes.

Isogenic TREx/HEK293 cell lines were tested for Tet-Repressor (TR) expression by evaluating their ability to repress expression of N-terminally c-myc-tagged cyano fluorescent protein (CFP) from pcDNA5-TO. Briefly, a sub culture of each clonal cell line was transfected with pcDNA5-TO/CFP, then induced with 1 µg/mL doxycycline (Sigma), then evaluated for CFP

expression by western blot (see below, western blot methods) using an anti-c-myc antibody (Life Technologies). The isogenic TREx/HEK293 cell line showing the lowest non-induced CFP expression as well as the greatest fold-change between non-induced and induced CFP expression was chosen for further use. The chosen isogenic TREx HEK293 cell line was passaged three times, frozen at -80^{o}C and thawed prior to transfection with EposOrco.

Prior to transfection into isogenic TREx/HEK293 cells, pcDNA4/EposOrco was linearized using PciI (New England Biolabs), run on a 0.7% TAE agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Transfection conditions of the isogenic TREx HEK293 cell line with pcDNA4/EposOrco were identical to that described above, except that stably transformed cells were selected using zeocin antibiotic selection reagent (Life Technologies). Cells stably expressing pcDNA4/EposOrco were selected using 400 μg/mL zeocin for approximately two weeks, after which the zeocin concentration was reduced to 200 μg/mL and blasticidin (10 μg/mL) was added to the cell culture medium. The resulting TREx/HEK293 EposOrco (TEO) cell line was passaged three times, frozen at -80^{o}C and thawed prior to further use.

The expression level of EposOrco in the TEO cell line was evaluated by western blot using an anti-c-myc antibody in non-induced and induced cells. EposOrco function was evaluated using the agonist VUAA1 and Fluo4-AM (Life Technologies), a calcium-sensitive fluorescent indicator, on an Omega FluoStar plate reader (see below, functional assay methods). After confirmation of inducible EposOrco expression and function, the TEO cell line was single-cell sorted and cultured as described above, except that isogenic cell lines were grown in the presence of 200 μg/mL zeocin and 10 μg/mL blasticidin.

Isogenic TEO cell lines were tested for EposOrco function as described above. The isogenic TEO cell line showing the most favorable response profile to the Orco agonist VUAA1 and growth characteristics (i.e., cell morphology and growth rate) was chosen for further transfection with EposOR3. The chosen isogenic TEO cell line was frozen at -80^{o}C and thawed prior to further use.

The isogenic TEO cell line was transfected with pcDNA5TO/EposOR3. Transfection conditions were identical to those described above except for the following: for transient expression the plasmids were not linearized prior to transfection and the cells were not put under antibiotic selection. For stable expression, cells were cultured in the presence of 200 μg/mL hygromycin (Gold Biotech, USA) for approximately two weeks, after which the concentration was reduced to 100 μg/mL and zeocin (200 μg/mL) and blasticidin (10 μg/mL) were added to the cell culture medium. The resulting TEO/EposOR3 (TEO/OR3) cell line with stable gene expression was passaged three times, frozen at -80^{o}C and thawed prior to further use.

Isogenic TEO/OR3 cell lines were generated by single-cell sorting as described above, except that isogenic cell lines were grown in the presence of 100 μg/mL hygromycin, 200 μg/mL

zeocin and 10 μg/mL blasticidin. Isogenic TEO/OR3 cell lines were frozen at -80°C and thawed prior to functional testing.

Functional assay

On day one cells were lifted from a confluent culture and 25,000 viable cells were plated into each well of a poly-d-lysine-coated, black-walled 96-well cell culture plate (Becton Dickinson). Cells were grown overnight in a cell culture incubator at 37°C with 5% CO_2. On day two the cell culture medium was removed from the plates, fresh medium was added to top four rows, and fresh medium containing 1 μg/mL doxycycline induction reagent was added to the bottom four rows. Cells were grown at 37°C with 5% CO_2 for sixteen to twenty hours prior to testing for ligand-induced receptor activation using the calcium-sensitive indicator reagent Fluo4-AM (Life Technologies).

For functional testing, the cell culture medium was removed from the plates, cells were rinsed once with assay buffer (DPBS containing 1 mM probenicid, pH 7.1) and 50 μL of loading buffer (assay buffer containing 1 μM Fluor4-AM and 0.2% pluronic acid) was added to each well. Cells were incubated with the loading buffer for 30 minutes at RT in the dark after which the plates were rinsed twice with assay buffer. Ninety-nine microliters of assay buffer was then added to each well and plates were incubated again for 30 minutes at RT in the dark.

Fluo4-AM loaded cells were transferred to an Omega FluoStar plate reader system and fluorescence was measured by exciting wells at 485 nM and reading the emission at 535 nM. Baseline fluorescence was determined for each well prior to treatment with compound and the resulting response was expressed as a percent increase in fluorescence relative to baseline. For all experiments, 11 μL of 10X compound was added to each of three wells containing non-induced cells and to each of three wells containing induced cells. Cells were only tested once with a compound then discarded, and the outer rows and columns of each plate were not used to reduce edge effects. For screening experiments test compounds were used at 30 μM (1X). Citral, geraniol, geranial, geranyl acetate, 1, 4 cineol, ethyl hexanoate, hexanol and hexanal (Sigma-Aldrich) were diluted to 6 mM in 100% DMSO prior to a 1:20 dilution to 10X concentration in assay buffer. Cells were also tested for response to a negative vehicle control (0.5% DMSO in assay buffer) or positive control (50 μM VUAA1 in 0.5% DMSO). Resulting fluorescence was measured for a period starting approximately ten seconds after application of compound for up to 60 seconds. For time-course experiments, fluorescence was measured in wells for five seconds prior to and three minutes after treatment with vehicle, VUAA1 (50 μM in 0.5% DMSO) or geranyl acetate (5 μM in 0.5% DMSO) using the Omega FluoStar plate reader

injection system. For dose response experiments, ½ serial dilutions of compound were performed in 100% DMSO prior to a 1:20 dilution to 10X concentrations in assay buffer. Resulting fluorescence was measured for a period starting approximately ten seconds after application of compound for up to 60 seconds. Data used for dose response analyses was taken from the maximal response obtained, approximately 25 seconds after compound application. The mean response (+/- SEM) from three non-induced or three induced wells receiving the same treatment were used to create dose-response curves using the non-linear regression function and EC_{50} values were statistically analyzed using the sum of squares F-test function in the GraphPad Prism data analysis software (GraphPad, Inc.). Cells were tested for response to compounds in at least three independent experiments.

OR detection in HEK293 cells by western blot and immunocytochemistry

TEO/OR3 (heterogenic) cells were lifted from a confluent culture and 2 x 10^6 cells were plated into each of two T-25 cell culture flasks and incubated overnight at 37°C with 5% CO_2. Doxycycline (1 µg/mL) was added to one of the T-25 flasks and cells were allowed to grow for sixteen hours at 37°C with 5% CO_2. The cell culture medium was removed from each flask, cells were rinsed twice with PBS and lifted using TrypLE (Life Technologies) cell dissociation reagent. Cells were pelleted at 300 x *g* for 5 minutes.

Cell pellets were incubated for thirty minutes on a rotator at room temperature with lysis buffer containing; 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1X protease inhibitor cocktail (Roche), Benzonase 1 U/mL (Sigma-Aldrich), and 1% Zwittergent 3-16 (Calbiochem). The samples were centrifuged at 21,000 x *g* for thirty minutes at room temperature and the supernatant was collected. Total protein concentration was analyzed using a BioRad DC Protein Assay kit, and 10 µg of total protein was placed in an equal volume of loading solution containing; 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM DTT, 3 M urea, and incubated at 37°C for thirty minutes to ensure denaturation prior to loading onto 4-12% SDS PAGE gels (Life Technologies). Western blot analysis was carried out by transferring the proteins onto a nitrocellulose membrane using an iBlot (Life Technologies), then blocked with 5% non-fat milk powder in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5), and incubated with the relevant primary antibodies (myc-tagged EposOrco was detected using a rabbit-anti-myc antibody (Sigma Aldrich), V5-tagged EpOR3 was detected using a goat anti-V5 antibody (Pierce, USA)). Epitope-containing bands were visualized using alkaline phosphatase conjugated secondary antibodies (either anti-goat or anti-mouse (Sigma-Aldrich)) with 5 mL of NBT/BCIP solution (Pierce, USA).

For immunocytochemistry analyses, 2 x 10^5 TEO/OR3 (heterogenic) cells were plated into 6-well dishes containing poly-d-lysine coated coverslips and cells were cultured overnight at 37°C with 5% CO_2. On day two the cell culture medium was removed from the plates and replaced with fresh medium with or without 1 µg/mL doxycycline induction reagent (3 wells each) and cultured overnight at 37°C with 5% CO_2. On day 3, the cell culture medium was removed from the wells and the cells were fixed by incubation with 4% paraformaldehyde for ten minutes at room temperature. Cell membranes were then permeabilized by treatment with 0.3% Triton X-100 for ten minutes at room temperature.

Non-induced and induced cells were incubated with 1 µg/mL each of rabbit anti-c-myc and mouse anti-V5 antibodies (Sigma-Aldrich). As an antibody control, additional induced cells were incubated with 1ug/mL each of purified rabbit and mouse IgG control antibodies (Sigma-Aldrich). All cells were incubated with primary antibodies for thirty minutes at room temperature after which they were washed three times with PBS containing 5% FBS. All cells were then incubated with 1 µg/mL each of anti-mouse IgG-Alexa488 (Life Technologies), anti-rabbit IgG-Alexa594 (Life Technologies) and Hoechst (Sigma-Aldrich) nuclear stain for thirty minutes at RT in the dark. Cells were washed three times with PBS containing 5% FBS then rinsed twice with PBS to remove residual FBS from cells. Cover slips were then dried and mounted on slides using ProLong Gold anti-fade mounting reagent (Life Technologies).

EposOrco, EposOR3 and cell nuclei were visualized in TEO/OR3 cells using a Zeiss LSM 510 META confocal microscope (ImaGene-iT, Lund, Sweden). Images of Alexa488, Alexa594 and Hoechst were captured from cells using identical microscope and camera settings (i.e., excitation strength, detector gain, exposure).

Results

HEK293 cells were transfected with pcDNA6/TR and a heterogenic cell line with stable, constitutive tetracycline-repressor expression was produced by antibiotic selection. This TREx/HEK293 cell line was subcloned and variable repressor expression was evident among clonal TREx cell lines indicative of different integration events and/or occurrences (Figure 1).

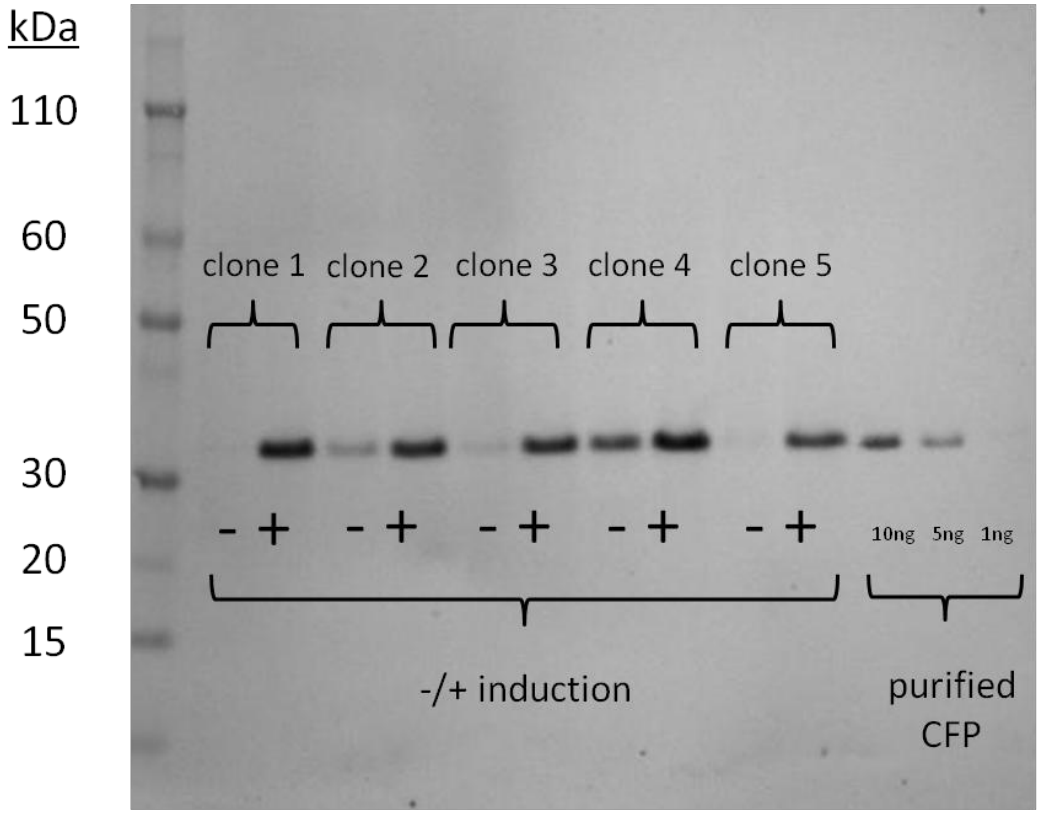

Figure 1. Variable tetracycline repressor expression levels in isogenic TREx/HEK293 cell lines. Cell lines stably expressing TR were transfected with pcDNA5/CFP and resulting CFP expression levels were detected by western blot in non-induced and induced cells.

EposOrco was transfected into a clonal TREx cell line and the cells were placed under antibiotic selection to create a cell line with stable, inducible EposOrco expression. The resulting heterogenic TREx/HEK293 EposOrco (TEO) cell line was subcloned to generate cell lines with isogenic and consistent EposOrco expression. EposOR3 was then transfected into an isogenic TEO cell line and cells were placed under antibiotic selection to generate a cell line with stable, inducible EposOR3 expression (TEO/OR3 cells). Heterogenic TEO/OR3 cells with stable EposOR3 expression were subcloned to generate cell lines with isogenic and consistent EposOrco and EposOR3 expression.

In order to demonstrate that OR genes can be detected at the protein level in HEK293 cells, EposOrco and EposOR3 were semi-purified from TEO/OR3 cells with stable, heterogenic OR expression and detected by western blot. EposOrco and EposOR3 protein was only detected in

extracts from induced cells (Figure 2). Doublet bands were seen for both EposOrco and EposOR3, which likely represent variations in the degree of glycosylation of the proteins. For EposOR3 a low molecular weight band was observed (~17 kDa) which is likely a truncated or degraded form of the protein. Verification of the entity of these bands could be achieved through protein sequencing, however this technique was not available for our use for these experiments.

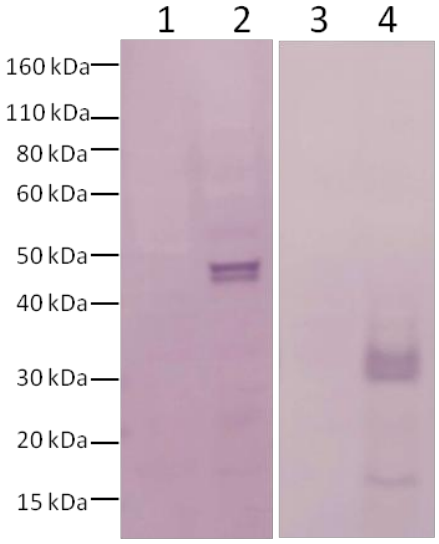

Figure 2. Western blot detection of myc-EposOrco (A) and V5-EposOR3 (B) in lysates prepared from a heterogenic TEO/OR3 cell line. Lanes: 1) c-myc-EposOrco detection in non-induced cells, 2) c-myc-EposOrco detection in induced cells, 3) V5-EposOR3 detection in non-induced cells, 4) V5-EposOR3 detection in induced cells.

Immunocytochemistry experiments conducted on stably expressing, heterogenic TEO/OR3 cells showed expression of EposOrco and EposOR3 in induced cells but not in non-induced cells or in induced cells using control antibodies. EposOrco and EposOR3 appeared to be co-localized in the cell membranes of TEO/OR3 cells (Figure 3), however, because immunocytochemistry experiments were conducted on permeabilized cells using antibodies that recognize intracellular (N-terminal) epitope tags, it is impossible to differentiate intracellular OR protein from that present in the cell membrane. However, because this cell line responded to non-membrane-permeable compounds in functional assays, it is evident that at least some EposOrco and EposOR3 are present in the cell membrane.

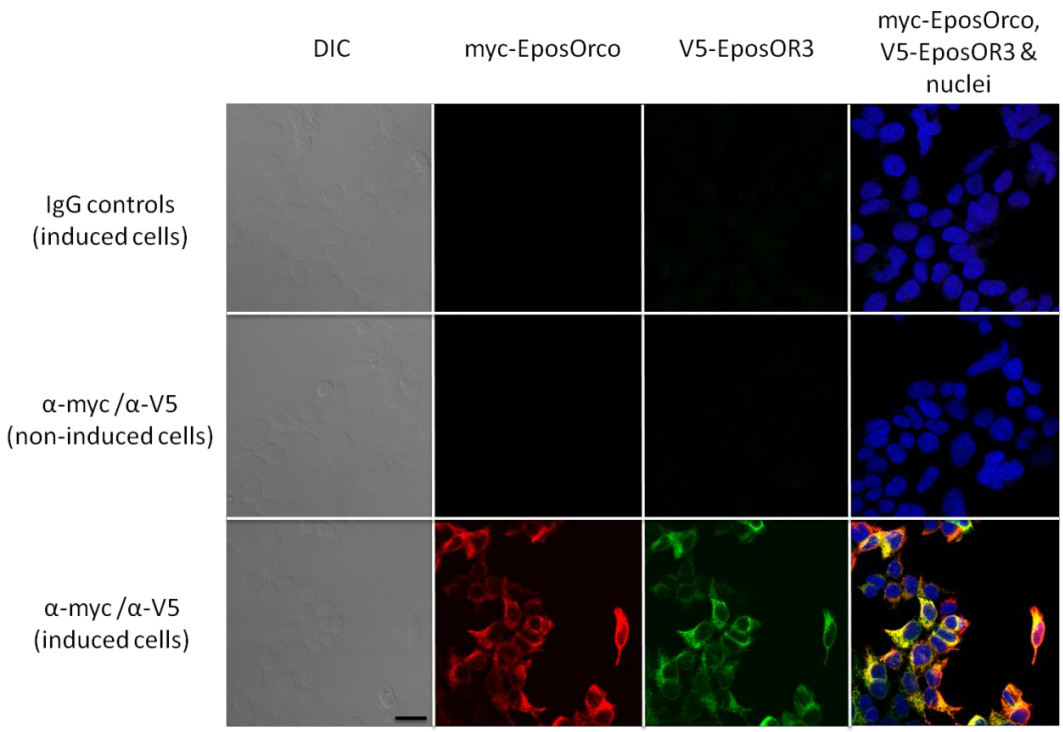

Figure 3. Immunocytochemistry conducted on TEO/OR3 cells. Images taken on a Zeiss LSM 510 META confocal microscope. Top panel: cells stained with mouse and rabbit IgG control primary antibodies. Bottom panels: non-induced (middle panel) and induced cells (bottom panel) stained with mouse anti-V5 (EposOR3) and rabbit anti-c-myc (EposOrco) antibodies. All cells were stained with Hoechst nuclear stain, anti-mouse IgG-Alexa488 and anti-rabbit IgG-Alexa594. Scale bar = 20 μm.

Isogenic TEO cell lines displayed distinct levels of EposOrco expression (data not shown) and variable response to the Orco agonist VUAA1 (Figure 4). Expression levels of EposOR3 varied between cell lines with transient and stable expression, as well as between the stably expressed isogenic EposOR3 cell lines (data not shown). In screening experiments, cells expressing EposOrco and EposOR3 responded only to VUAA1 and geranyl acetate. Responses of the TEO/OR3 cell line to geranyl acetate varied between cell lines with transient and stable EposOR3 expression and between isogenic cell lines. Response magnitudes of TEO/OR3 cells with stable EposOR3 expression were approximately twice that of cells with transient expression, and the response magnitude of some isogenic TEO/OR3 cell lines were 25 percent higher than the heterogenic cell lines from which they were derived (Figure 5).

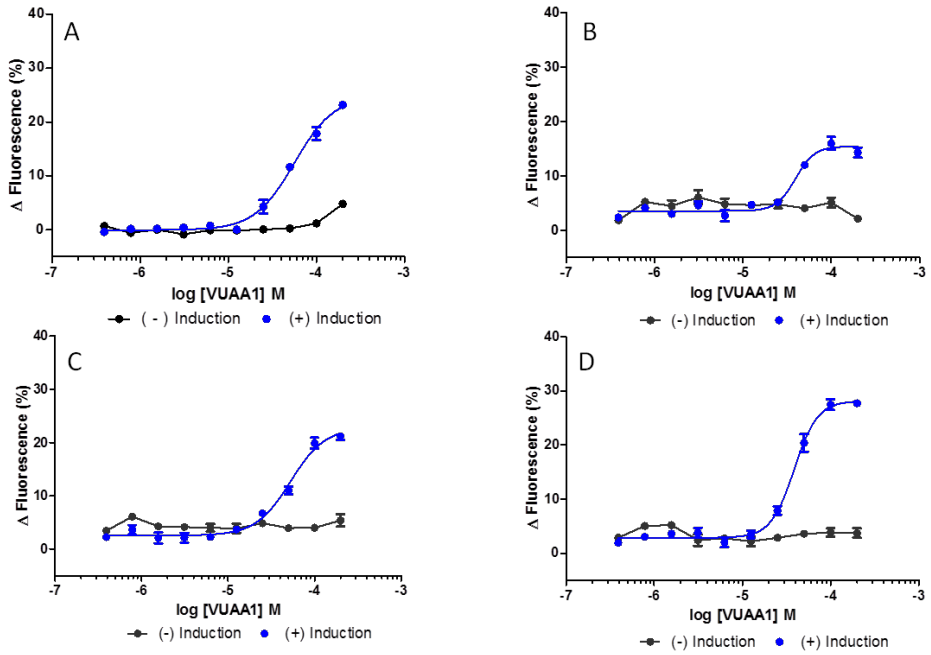

Figure 4. Response of TEO cell lines with stable EposOrco expression to various doses of the Orco agonist VUAA1. Response to compound was measured in heterogenic (A) and isogenic cell lines (B-D). Data represent the mean (+/- SEM) response from three wells per treatment.

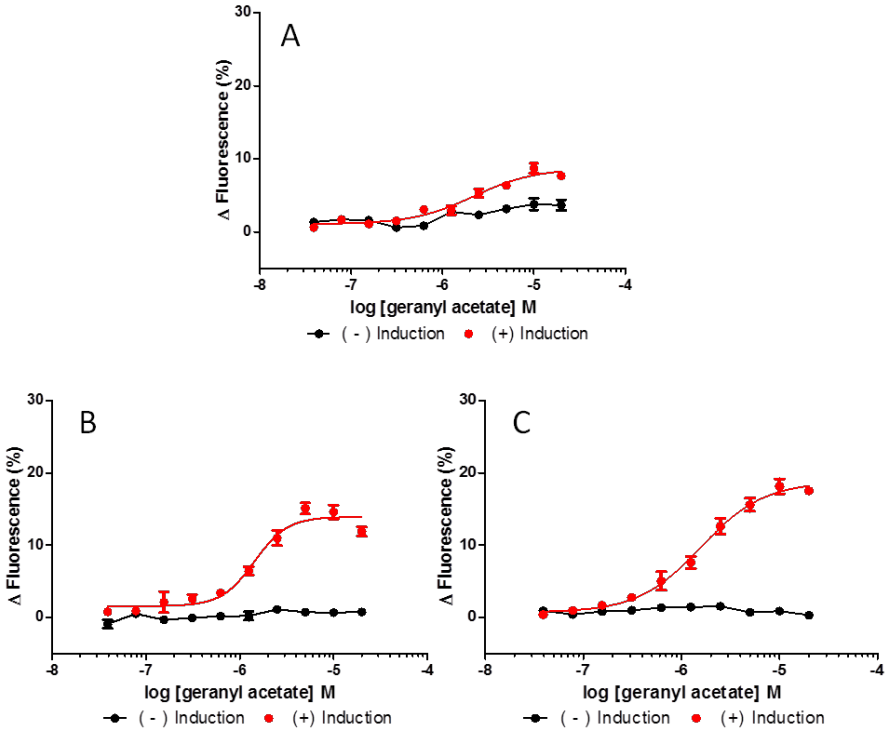

Figure 5. Response of TEO/EposOR3 cells to geranyl acetate. Cells with transient (A), stable, heterogenic (B) or stable, isogenic (C) EposOR3 expression. Data represent the mean (+/- SEM) response from three wells per treatment.

The temporal response kinetics of TEO/OR3 cells were measured for approximately three minutes following application of compound. Response of TEO/OR3 cells to VUAA1 and geranyl acetate peaked approximately 25 seconds after the application of the compound (Figure 6). Once

the peak of the response to compound had been identified the assay could be converted to an 'endpoint' assay, wherein cells were treated with compound or dose and measured for response 25 seconds later.

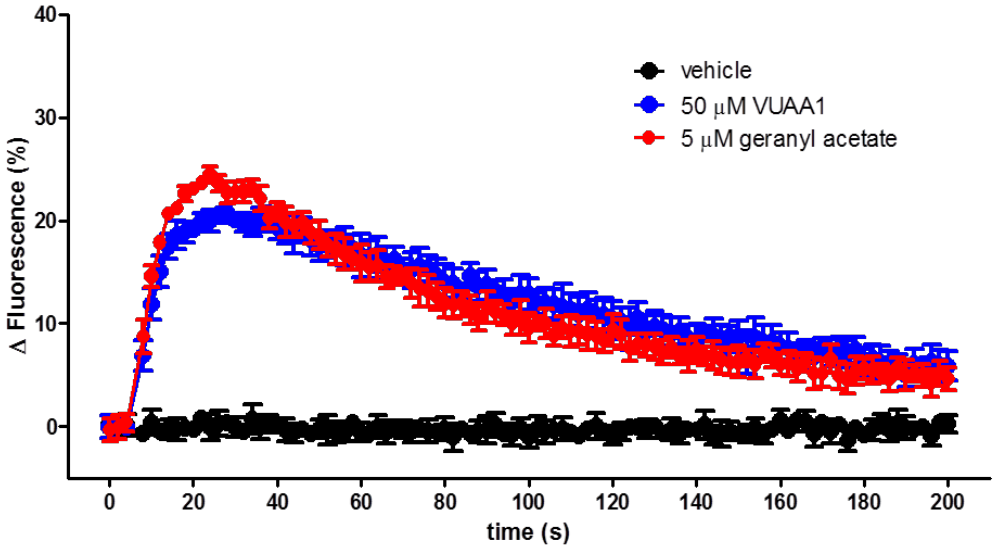

Figure 6. Kinetics of response of isogenic TEO/EposOR3 cells to treatment with vehicle, VUAA1 or geranyl acetate. Baseline fluorescence was measured for five seconds prior to and 200 seconds after treatment with a single dose of compound using the Omega FluoStar plate reader injection system. Data represent the mean (+/- SEM) response from three wells per treatment.

Isogenic TEO/OR3 cells were kept in culture for three months and no obvious deleterious effects to cell health (morphology, growth rate) were apparent. Cell lines were tested for response to ligands every four weeks to evaluate variation in response profiles. Cell lines gave consistent responses to geranyl acetate over the three month period (Figure 7). EC_{50} values of 1.03, 1.58 and 1.42 μM for cells tested at 4, 8 and 12 weeks, respectively, were not significantly different ($F_{2,78} = 2.756$, $P < 0.0697$).

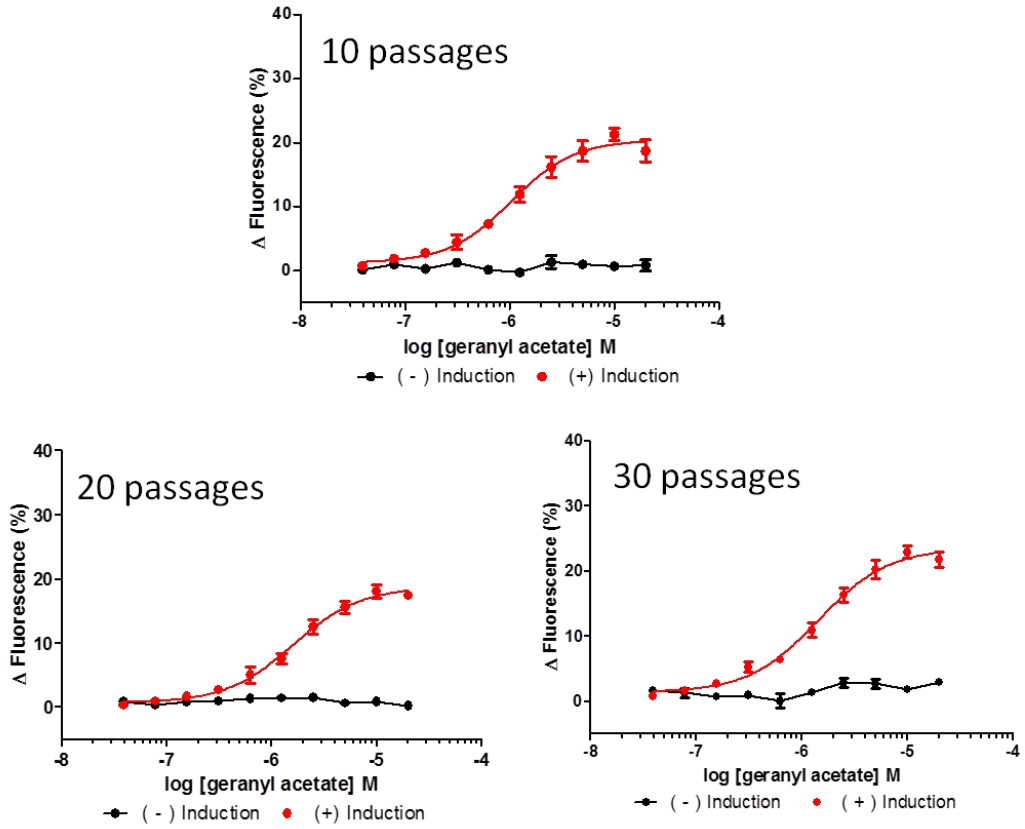

Figure 7. Consistency in response of isogenic TEO/EposOR3 cells to geranyl acetate over time. Non-induced and induced cells were tested for response after 10, 20 and 30 passages (4, 8 and 12 weeks) of culture. Data represent the mean (+/- SEM) response of cells to compounds from three wells per treatment.

Discussion

We have generated TREx/HEK293 cell lines that express EposOrco and EposOR3 under the regulation of the 'Tet-Repressor' which constitutively represses expression of both genes until an induction agent is added to the culture medium. The ability to repress the expression of EposOrco appears to be critical for the health and survival of HEK293 cells. Based on our earlier attempts at establishing the HEK293 system, constitutive expression of Orco can have deleterious effects on cell health (Law, 2013). Cell lines with stable, constitutive Orco expression died after several weeks of cell culture, whereas cell lines with stable, inducible Orco expression were maintained for up to six months with no signs of changes to cell health, growth rate or response profiles in functional assays. In addition to protecting cells from the effects of constitutive - possibly toxic - protein expression, the TREx system also serves as an endogenous control for functional assays; cell lines transfected with OR genes can be tested for response to compound with or without the specific genes being expressed. Using 96-well plates, a plate reader and the calcium-sensitive indicator Fluo4-AM we examined the responsiveness of EposOrco to VUAA1 in TREx/HEK293

cells. The small molecule VUAA1 has been previously shown to agonize Orco from a number of insects including *Anopheles gambiae*, *Drosophila melanogaster*, *Harpegnathos saltator* and the noctuid moth *Heliothis virescens* (Jones et al., 2011). We have found that VUAA1 is capable of activating EposOrco and we used this to indirectly evaluate the expression levels of Orco in subcloned cell lines. A TREx/HEK293 cell line with isogenic EposOrco expression that responded maximally to VUAA1 was chosen for further transfection with EposOR3.

Previously, using the Sf9 cell-based assay, Jordan et al. (2009) tested EposOR3 against a panel of odorants that were known to generate electrophysiological responses in *E. postvittana* antennae. Using this system they found that EposOR3 responded to 15 odorants at high concentrations and dose-dependently to citral, geranial, geraniol and geranyl acetate. In the current study, using HEK293 cells, we tested EposOR3 against odorants that gave dose-dependent responses in Sf9 cells however we only found a consistent response to geranyl acetate. One possible explanation for differences in responses observed between the two cell lines is that the EposOR3 gene we used in this study contained four amino acid differences compared to the EposOR3 gene used in Sf9 cells (Corcoran et al., 2014). It is possible that changes to these four residues affect ligand specificity, however we did not address this question in the present study. Another possible explanation is that for some unknown reason ORs show different response profiles when expressed in different assay systems. A more thorough comparison of the response profiles of multiple ORs to multiple ligands in both systems may help to address this question. In Sf9 cells EposOR3 responded to geranyl acetate dose-dependently with an EC_{50} of 14 nM and in HEK293 cells EposOR3 responded dose-dependently with an EC_{50} of 1 μM. The different EC_{50} values obtained in the two systems could be due to the differences in EposOR3 sequence used, or could be due to different sensitivities between the equipment used to measure ligand-induced receptor activation. Despite the different response profiles of EposOR3 observed in the current study and Jordan et al. (2009), we found robust and consistent responses of EposOR3 to geranyl acetate in HEK293 cells.

With the purpose of minimizing intra and inter-assay variation of OR response to ligand, we compared the responses of EposOR3 to geranyl acetate when transiently and stably expressed in a TREx/HEK293/EposOrco cell line. We found that transient expression of EposOR3 in HEK293 cells did lead to the production of functional EposOR3, however the responses to ligand were extremely variable within and between transfections (data not shown). When the TREx/HEK293/EposOrco cell line was transfected with EposOR3 and placed under antibiotic selection, the magnitude of the response to compound increased substantially. In addition, as the cells were placed under selective pressure to retain the plasmids, the inter-assay variation in response to compound became negligible. Finally, in an attempt to reduce intra-assay variation in

OR response to compound we single-cell sorted our cell lines, generating cell lines with stable and isogenic expression of transfected genes. Evaluation of protein expression and function in isogenic derivative cell lines indicated variation in expression levels between cells from the same original transfection. This single-cell sorting not only produced an isogenic background for the subsequent transfection of other genes but also allowed for the selection of cell lines with maximal signal to noise (i.e., response) profiles. We have found that transient and stable, heterogenic OR expression gave dose-dependent responses to ligand which may be sufficient for deorphaning ORs in HEK293 cells. However, we also found that the response magnitude of an OR to ligand can be increased by subcloning heterogenic cell lines which would be optimal for high-throughput screening efforts.

When first screening ORs for response to various ligands it is critical to know that the transfected proteins are expressed in the cells. If responses are obtained from induced cells but not from non-induced cells then it is clear that the protein is being expressed. However, when no response is obtained from induced cells it can be unclear as to why there was no response. For this reason, we modified our OR genes to contain c-myc (Orco) or V5 (OR3) epitope tags on their N-termini. The incorporation of these epitope tags allowed us to evaluate OR protein expression by western blot and confocal microscopy using commercially available anti-epitope antibodies. In addition to generating confidence in negative response profiles of ORs to ligands, having epitope tags on functional OR proteins allows for the exploration of other fundamental questions surrounding insect OR biology such as interactions between Orco and ligand-binding ORs (Carraher et al., 2013).

In this study we generated an isogenic TREx/HEK293 cell line and used it for further transfection with EposOrco. We subcloned this cell line by FACS and chose an isogenic cell line, with high EposOrco expression and response to VUAA1, for further work with EposORs. Alternatively, several mammalian TREx cell lines are commercially available, including one that utilizes the so-called Flip-In system (Life Technologies). The Flip-In system is designed to facilitate the generation of an isogenic cell line during the transfection process through directed incorporation of a specific plasmid (pcDNA5FR-TO, Life Technologies) into a single, defined locus in the cell genome. While this cell line has proven to work for functionally characterizing insect ORs, we have purposely chosen not to use it. When pcDN5FR-TO is incorporated into the genome of Flip-In TREx/HEK293 cells the cell line should lose resistance to zeocin and gain resistance to hygromycin. When we transfected Flip-In TREx/HEK293 cells with EposOrco in pcDNA5FR-TO the cell line gained resistance to hygromycin but did not lose its resistance to zeocin, implying there was more than one Flip-In site present in the cell line. There are currently three commercially available TR-sensitive plasmids for use in TREx mammalian cells (Life

Technologies). pcDNA4-TO, pcDNA5-TO and pTREx-DEST30-TO (and their variants) have three different antibiotic resistance genes (zeocin, hygromycin and G418, respectively) allowing for the generation of cell lines with stable and inducible expression of three exogenous genes. Because the Flip-In cell line retained resistance to zeocin, we could not confidently use this cell line for further transfection with pcDNA4TO. Without a modified vector capable of expressing two genes, we could not use the Flip-In TREx/HEK293 cell line to stably express EposOrco, EposORs and a third gene of interest (i.e., EposSNMP1). For this reason, we have developed the alternative method presented here for the generation of TREx/HEK293 cell lines with isogenic OR expression.

While the generation of HEK293 cell lines stably expressing EposORs was a considerable (approximately four months) time investment initially (Figure 8), we now have a frozen 'cell bank' from which we can thaw a particular cell line and test in functional assays in one week's time. The robust and consistent responses in functional assays suggest these cell lines are optimally suited for high-throughput screening platforms, however to date we have only tested these cell lines using 96-well plates and an Omega FluoStar plate reader. The next step would be to test these cell lines on a 384-well plate-compatible, high content fluorescent imaging plate reader system. Nevertheless, the methods described above have allowed us to generate cell lines that express functional ORs which will allow us to begin deorphaning the recently identified (Corcoran et al., 2014) OR repertoire from the pest moth *E. postvittana*.

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IV

Identification of pheromone receptors from a horticultural pest, the lightbrown apple moth *Epiphyas postvittana*

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Epiphyas postvittana, pheromone receptor, HEK293 cells, *Xenopus* oocyte

Abstract

Pheromone receptors (PRs) have been identified from several moth species and functionally characterized *in vitro* through expression in *Xenopus laevis* oocytes and Human Embryonic Kidney (HEK) 293 cells. These studies have shown that some PRs are relatively specific for certain compounds while others are more broadly tuned to several pheromone compounds, including both behavioral agonists and antagonists. Here, we express previously identified candidate PRs from the pest moth *Epiphyas postvittana* in HEK293 cells along with EposOrco and EposSNMP1 and test for responsiveness to a panel of 62 pheromone-related compounds. We found that EposOR1, EposOR6 and EposOR45 all respond to *E. postvittana* pheromone components and to pheromone components used by other moths, some of which have known function as behavioral antagonists in this species. None of the EposORs tested in these experiments showed responses to *E. postvittana* pheromone components alone. Responses obtained for EposOR1 and EposOR6 to pheromones in HEK293 cells were confirmed through expression and functional testing in *Xenopus* oocytes. Finally, we show that activation of EposOR6 with the major pheromone component, E11-14:OAc, or the behavioral antagonist, Z11-14:OAc, prevents subsequent re-activation by either compound in this cell-based system, implying that a pheromone degrading enzyme may be required to reset the system. These results lead us to propose that in *E. postvittana* behavioral agonism and antagonism may be mediated through a peripheral molecular mechanism.

Introduction

The first moth olfactory receptors (ORs) were identified from *Heliothis virescens* through BLAST searching of an *H. virescens* genomic database using sequences of known ORs from *Drosophila* (Krieger et al., 2002). Soon thereafter, using similar bioinformatic techniques, additional OR genes were identified from *H. virescens* that displayed male-biased antennal expression patterns, making them candidate receptors for female-produced pheromone compounds (Krieger et al., 2004). Around the same time a male-biased OR was identified in the domesticated silkworm moth, *Bombyx mori,* and through heterologous expression in *Xenopus* oocytes this OR was found to respond to a *B. mori* pheromone, bombykol (Sakurai et al., 2004). Additional ORs were identified in *B. mori* (Krieger et al., 2005), and one was found to respond to a second *B. mori* pheromone when expressed in *Xenopus* oocytes (Nakagawa et al., 2005) and HEK293 cells (Groβe-Wilde et al., 2006). In 2007, Groβe-Wilde et al. were able to test the male-biased HvirORs in functional assays and found that three of them responded to components of the *H. virescens* pheromone blend (Groβe-Wilde et al., 2007). Since these pioneering experiments, pheromone receptors have been identified in additional moth species including *Mythimna separata, Plutella xylostella, Diaphania indica, Antheraea polyphemus, Ostrinia spp., Amyelois transitella, Spodoptera spp.* and *Agrotis segetum* (Mitsuno et al., 2008, Forstner et al., 2009, Miura et al., 2009, Miura et al., 2010, Wanner et al., 2010, Xu et al., 2012, Montagne et al., 2012, Liu et al., 2013, Zhang and Löfstedt, 2013).

Moth PRs have been de-orphaned *in vitro* through heterologous expression in *Xenopus* oocytes and HEK293 cells. The *Xenopus* oocyte system was used by Sakurai et al. (2004) to characterize the first moth PR from *B. mori* and has since become the method of choice *in vitro* assay for the functional characterization of lepidopteran PRs. In this system, cRNA of the PR of interest and the OR co-receptor Orco (Benton et al., 2006) are injected into oocytes and later tested for response to compounds through electrophysiological monitoring of currents across the oocyte membrane (Luetje et al., 2013). HEK293 cells were used by Groβe-Wilde et al (2006, 2007) to characterize PRs from *B. mori* and *H. virescens* but have only been used to study moth PRs on one occasion since (Forstner et al., 2009). The majority of current research on insect ORs using HEK293 cells has focused on characterizing dipteran ORs (Bohbot et al., 2011), identifying pest control tools that target Orco (Jones et al., 2011) and evaluating signaling pathways of OR-Orco receptor complexes (Wicher et al., 2008). HEK293 cells can be modified to have stable, inducible expression of insect ORs (Corcoran et al., 2014b) and can be tested for ligand-induced OR activation by whole cell patch-clamping or through the use of calcium-sensitive fluorescent indicators.

To date, all known lepidopteran PRs group together phylogenetically and all but HvirOR6 (Krieger et al., 2004, Wang et al., 2011) are expressed more highly in male antennae than in female antennae. Of these lepidopteran PRs, some seem to be highly tuned to one or two pheromone components while others seem to be more broadly tuned to several components (Wanner et al., 2010, Zhang and Löfstedt, 2013). Interestingly, some of these broadly tuned receptors have been found to respond to pheromone compounds produced by conspecific and heterospecific females. However, in most *in vitro* functional studies pheromone binding proteins (PBPs) were not used to deliver pheromone components. While the exact role of PBPs in pheromone-induced receptor activation is not fully understood (Leal, 2013), studies that used PBPs in *in vitro* assays have observed increased PR sensitivity and specificity to ligands (Groβe-Wilde et al., 2006, Groβe-Wilde et al., 2007, Forstner et al., 2009). Furthermore, most *in vitro* studies on moth PRs test receptors against a small panel of ligands, usually being restricted to pheromone compounds known to be used by that particular species. Because of this, the response profiles of moth PRs that have been characterized could be found to be broader or more specific upon further testing with wider panels of pheromone compounds in conjunction with the incorporation of PBP-mediated compound delivery.

Epiphyas postvittana, the lightbrown apple moth, is an important horticultural pest native to Australia. Several pheromone compounds have been found to be present in the moth's pheromone gland, however only four of these, (*E*)-11-tetradecenyl acetate (E11-14:OAc), (*E,E*)-9,11-tetradecadienyl acetate ((E,E)9,11-14:OAc), (*E*)-11-tetradecen-1-ol (E11-14:OH) and (*E*)-11-hexadecenyl acetate (E11-16:OAc), have been shown to elicit electrophysiological responses in male *E. postvittana* antennae (El-Sayed et al., 2011). *E. postvittana* lives in sympatry with other moth species that use closely related pheromone compounds, some of which have been shown to elicit electrophysiological responses in moth antennae and serve as behavioral antagonists in wind tunnels (Rumbo et al., 1993) and field experiments (Stephens et al., 2008, Kye-Chung Park, pers. comm.). Over the last few decades the moth has spread to New Zealand, North America and parts of Europe (Danthanarayana, 1975, Tooman et al., 2011, He et al., 2012). Traditionally, the pest was controlled through the use of pesticides and natural predators. More recently, control tactics have shifted towards the use of the moth's own pheromone compounds to trap the pest and to interfere with its ability to reproduce (Suckling and Brockerhoff, 2010). While mating disruption strategies have proven effective in controlling this insect pest, a better understanding of pheromone reception could facilitate the identification and development of more efficient tools that target molecular entities in the moth's peripheral olfactory system. The first step in developing a better understanding of pheromone reception in *E. postvittana* would be to identify the moth's PRs. Based on electrophysiological data and field experiments, we would expect *E.*

postvittana to have PRs that respond to E11-14:OAc, (E,E)9,11-14:OAc, E11-14:OH, E11-16:OAc, (Z)-11-tetradecenyl acetate (Z11-14:OAc) and (Z,E)-9,11-tetradecenyl acetate ((Z,E)9,11-14:OAc).

Here, using previously identified ORs from *E. postvittana*, we screen ten putative PRs (Corcoran et al., 2014a) for their responsiveness to a panel of 62 pheromone compounds, including the known *E. postvittana* pheromone components and behavioral antagonists (El-Sayed, 2012). Sensory Neuron Membrane Protein-1 (SNMP1) is a CD36-like membrane protein that has been identified in a wide variety of insects and has been shown to be present in the neurons containing PRs by *in situ* hybridization experiments (Rogers et al., 2001). SNMP1 has been shown to be necessary for pheromone detection in *Drosophila* (Benton et al., 2007), however whether or not SNMP1 is necessary in moths remains unclear. Moth PRs have been shown to be functional *in vitro* when co-expressed with only Orco (Nakagawa et al., 2005, Mitsuno et al., 2008), however when moth PRs are expressed in *Drosophila* sensilla they do not function without SNMP1 (Syed et al., 2010a). Because the role of SNMP1 in pheromone reception in moths is unclear, we use HEK293 cell lines with stable, regulated expression of EposOrco and EposSNMP1 to express candidate EposPRs for functional testing. EposPRs that responded to pheromone components were then expressed in *Xenopus* oocytes to validate results obtained using HEK293 cells. Finally, using two pheromone compounds that were found to activate EposOR6 we demonstrate that treatment with either compound inhibits the subsequent re-activation of the receptor with either compound in cell-based assays.

Methods

Putative PR gene identification and cloning for expression in HEK293 cells

Putative EposPR genes used in these experiments were identified based on sexually dimorphic gene expression in antennae and phylogenetic analyses (Corcoran et al., 2014a). Ten genes, EposORs 1, 6, 7, 21, 22, 30, 34, 41, 43 and 45 were amplified from antennal cDNA and cloned into the mammalian expression vector pcDNA5TO (Life Technologies) as previously described (Corcoran et al., 2014b). Briefly, DNA for each gene was amplified using primers designed to amplify full length genes and cloned into the entry vector pcR8 (Life Technologies). Plasmids containing each EposOR gene were sequenced and plasmids containing EposOR DNA with verified sequences were used as templates for a subsequent PCR in which V5 epitope tags and 5' and 3' restrictions sites were added to the DNA with a second set of primers. Purified DNA for each gene was then digested using NotI/ApaI (EposORs 1, 6, 7, 22, 34, 41, 43 and 45), KpnI/NotI

(EposOR21) or NotI/XhoI (EposOR30) restriction enzymes and cloned into pcDNA5TO. Plasmids containing each EposOR gene were purified and sequenced and plasmids containing the correct EposOR DNA sequence were used for transfection into HEK293 cells. Large quantities of high-quality pcDNA5/EposOR'X' plasmids were produced using a PureLink HiPure Plasmid Midiprep Kit (Life Technologies) following the manufacturer's protocol.

Cell line generation

EposSNMP1 was amplified using gene-specific primers as described above except that V5-tagged EposSNMP1 was cloned into the expression vector pTREx-DEST30. Prior to transfection into a 'TEO' cell line with isogenic and inducible EposOrco expression (Corcoran et al., 2014b), pTREx-DEST30/EposSNMP1 was linearized using PciI (New England Biolabs), run on a 0.7% TAE agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Five micrograms of linearized plasmid and 15 µL of Lipofectamine2000 (Life Technologies) transfection reagent were each diluted into 500 µL of Optimem medium (Life Technologies) and incubated at room temperature (RT) for ten minutes, after which they were mixed together and incubated for an additional 60 minutes at RT. The plasmid/Lipofectamine2000 mixture was then added to a T-25 cell culture flask containing TEO cells at approximately 70% confluency and incubated overnight (37°C, 5% CO_2). After 16 hours the medium was removed from the flask and replaced with fresh cell culture medium containing 500 µg/mL G418 (Gold Biotech, USA). Cells were cultured for approximately two weeks in the presence of the G418 until an antibiotic-resistant cell line was established, then the G418 concentration was reduced to 250 µg/mL and zeocin (200 µg/mL) and blasticidin (10 µg/mL) were added to the cell culture medium. The resulting TEO/EposSNMP1 (TEOS) cell line was passaged three times and frozen prior to further use. Isogenic TEOS cell lines were generated by single-cell sorting as described in Corcoran et al (2014b), except that isogenic cell lines were grown in the presence of 250 µg/mL G418, 200 µg/mL zeocin and 10 µg/mL blasticidin. Isogenic TEOS cell lines were frozen at -80°C and thawed prior to further use. EposSNMP1 expression was verified in isogenic TEOS cell lines by RT-PCR prior to further use. An isogenic TEOS cell line with inducible expression of EposSNMP1 was used for transfection with EposORs.

Prior to transfection into an isogenic TEOS cell line, pcDNA5TO containing EposORs 1, 6, 7, 21, 22, 30, 34, 41, 43, and 45 were linearized with FspI (EposORs 1, 6, 7 30, 41, 43 and 45), BstZ171 (EposORs 21 & 22) or PciI (EposOR34), run on a 0.7% TAE agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Each linearized pcDNA5TO/EposOR plasmid was transfected into the same isogenic TEOS cell line using the methods described above, except that

cells were cultured for approximately two weeks in the presence of 200 μg/mL hygromycin. Once an antibiotic-resistant cell line was established, the hygromycin concentration was reduced to 100 μg/mL and zeocin (200 μg/mL), blasticidin (10 μg/mL) and G418 (250 μg/mL) were added to the cell culture medium. The resulting TEOS/EposOR'X' cell lines were passaged three times, frozen at -80°C and thawed prior to functional testing.

Confirmation of gene expression by western blot and RT-PCR

Western blot detection of EposOR protein was conducted as previously described (Corcoran et al., 2014b) with the following exception. The anti-V5 antibody used to detect EposOR3 in Corcoran et al. (2014b) has been discontinued by the supplier (Thermo Scientific, PA1-27080). Hence, in this study, two different commercially available anti-V5 antibodies (Sigma, V8012 and V8137) were tested for their ability to detect EposORs in cell lines.

Each TEOS/EposOR'X' cell line was plated into two T-25 cell culture flasks and allowed to grow overnight. Doxycycline (1 μg/mL) was then added to one of the flasks for each cell line to induce expression of EposOrco, EposSNMP1 and EposOR'X'. Cells were incubated overnight at 37°C with 5% CO_2. Cells were then collected from each flask and RNA was extracted using TRIzol RNA extraction reagent (Life Technologies) according to the manufacturer's protocol. RNA extracted from each sample was treated with DNAseI (Life Technologies) and converted to cDNA using iScript cDNA Synthesis Kit (BioRad) with random hexamers and oligo dT primers following the manufacturer's protocol. cDNA was synthesized from each sample with and without reverse transcriptase to allow for detection of genomic DNA contamination by PCR. Full length EposOrco, EposSNMP1 and EposOR'X' was amplified from each cDNA sample by PCR using Platinum Taq DNA polymerase and gene-specific primers using the following reaction conditions: an initial two minute incubation at 94°C followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 90 seconds, followed by a final seven minute incubation at 72°C.

Pheromone compounds

A panel of pheromone compounds was chosen based on the chemical space (i.e., chain length, functional group and double bond position) of *E. postvittana* pheromone components and pheromones used by sympatric and closely related species (Table 1). Pheromone compounds were purchased from Bedoukian Research or Pherobank BV and were all isometrically pure, with the exception of Z-7-tetradecen-2-one (Z7-14:Kt) which was a sum of isomers of unknown

proportion. Chemical purity of compounds ranged from 90 to 99%. Each pheromone compound was diluted to 100 mM in DMSO and kept at 4°C until use in experiments.

Table 1. Pheromone compounds used in screening experiments. Compounds are listed using shorthand notation. Full chemical names can be found at http://www.pherobase.com/.

alcohols	acetates	aldehydes & other
E9-12:OH	E9-12:OAc	E11-14:Ald
Z9-12:OH	E9-12:OAc	Z11-14:Ald
E10-12:OH	E9-12:OAc	16:Ald
Z10-12:OH	E9-12:OAc	Z9-16:Ald
E7-14:OH	E7-14:OAc	E11-16:Ald
Z7-14:OH	E8-14:OAc	Z11-16:Ald
E9-14:OH	Z9-14:OAc	Z7-14:Kt
Z9-14:OH	E10-14:OAc	(*E*)-β-farnesene
E10-14:OH	Z10-14:OAc	
Z10-14:OH	E11-14:OAc	
E11-14:OH	Z11-14:OAc	
Z11-14:OH	E12-14:OAc	
E12-14:OH	Z12-14:OAc	
Z12-14:OH	16:OAc	
E9-16:OH	E9-16:OAc	
E10-16:OH	E10-16:OAc	
Z10-16:OH	Z10-16:OAc	
E11-16:OH	E11-16:OAc	
Z11-16:OH	Z11-16:OAc	
(E,E)7,9-12:OH	(E,E)7,9-12:OAc	
(E,Z)7,9-12:OH	(E,Z)7,9-12:OAc	
(E,E)8,10-12:OH	(E,E)8,10-12:OAc	
(E,E)9,11-14:OH	(E,E)9,11-14:OAc	
(Z,E)9,11-14:OH	(Z,E)9,11-14:OAc	
(Z,E)9,12-14:OH	(Z,E)9,12-14:OAc	
(E,E)10,12-14:OH	(E,E)10,12-14:OAc	
(Z,E)7,11-16:OH	(Z,E)7,11-16:OAc	

HEK293 cell-based assays

The materials and methods used to test TEOS/EposOR'X' cell lines for response to pheromone components are identical to those previously described (Corcoran et al., 2014b). Briefly, each cell line was lifted from culture flasks and 25,000 cells were plated into each well of black-walled, poly-d-lysine coated 96-well plates. Cells were incubated overnight at 37°C with 5% CO_2. On day two, the cell culture medium was removed from the plates, fresh medium was added to the top four rows, and fresh medium containing 1 μg/mL doxycycline induction reagent was added to the bottom four rows. Cells were incubated again overnight at 37°C with 5% CO_2. The following morning, cells were rinsed and loaded with the calcium sensitive indicator Fluo4-AM. After

incubation and rinsing, cells were tested for response to pheromone compounds using an Omega FluoStar plate reader (BMG Labtech). Following baseline fluorescence determination, wells were treated with different compounds or doses and monitored for changes in fluorescence for 60 seconds. Response to compound was expressed as the mean percent increase in fluorescence relative to baseline for three non-induced and three induced wells per treatment. Experiments were repeated at least three times to confirm results.

For screening experiments, three non-induced and three doxycycline-induced wells of cells were tested for a response to a single dose (30 μM) of each of the 62 pheromone components. Test compounds were diluted to a 200X concentration (6 mM) in 100% DMSO, then diluted 1:20 to 10X (300 μM) in assay buffer. Compounds were diluted to 1X (30 μM) by adding 11 μL of compound to 99 μL of assay buffer in wells. In addition, three wells of non-induced and three wells of induced cells on each plate were treated with a negative (vehicle, 0.5% DMSO in assay buffer) and positive (50 μM VUAA1) control. VUAA1 directly agonizes Orco (Jones et al., 2011) and its use provides confidence that EposOrco was present and functional in each cell line and that the reporter assay was operating properly. Each well of cells was treated with only one compound during screening experiments.

Compounds that elicited a response in cell lines during screening experiments were tested further in dose response studies. Serial dilutions of each compound were performed in 100% DMSO starting at 200X, followed by 1:20 dilutions to 10X in assay buffer. Compounds were then diluted 1:10 to 1X in wells during the experiment by adding 11 μL of compound to 99 μL of assay buffer in wells. Each dose of each compound was tested in three non-induced and three induced wells of cells. Starting concentrations of dose titrations were between 3 – 300 μM depending on the compound. Dose response curves and EC_{50} values were generated using the non-linear regression function of GraphPad Prism software. Each well of cells was treated with only one compound during dose response experiments. Experiments were repeated at least three times to confirm results.

In a proof of principle experiment we evaluated the ability of HEK293 cells expressing EposORs to be re-activated after an initial treatment with activating ligand. Using a previously described HEK293 cell line (TEO/OR3) expressing a receptor tuned to geranyl acetate (GA) (Corcoran et al., 2014b), we treated cells with vehicle or ~EC_{70} concentrations of VUAA1 (50 μM) or GA (5 μM) and measured their response using the methods described above. After treatment, cells were rinsed and incubated at RT for 30 minutes prior to re-treatment. Cells that were treated the first time with vehicle, VUAA1 or GA were each treated with vehicle, VUAA1 or GA the second time to cover all possible treatment combinations. In addition, at the time of application

of the second treatment, non-treated cells were stimulated with vehicle, VUAA1 or GA as an assay control.

Using a similar experimental design, we then tested our TEOS/EposOR6 cell line for its ability to be re-activated following treatment with an ~EC_{70} concentration of VUAA1 (50 μM), E11-14:OAc (10 μM) or Z11-14:OAc (100 nM). Cells that were treated with each compound were rinsed and incubated at RT for 30 minutes prior to re-treatment. Cells that were treated the first time with VUAA1, E11-14:OAc or Z11-14:OAc were each treated with VUAA1, E11-14:OAc or Z11-14:OAc the second time. In addition, at the time of application of the second treatment, non-treated cells were dosed with VUAA1, E11-14:OAc or Z11-14:OAc as an assay control.

We then took our TEOS/OR6 cell line and evaluated the effects of pre-treatment with E11-14:OAc or Z11-14:OAc on the subsequent response to dose titrations of Z11-14:OAc or E11-14:OAc, respectively. Using the same methods described above, cells expressing EposOR6 were treated with 10 μM E11-14:OAc or a negative control (10 μM (*Z*)-12-tetradecenyl acetate (Z12-14:OAc)), rinsed and incubated at RT for 30 minutes after which they were treated a second time with a dose titration of Z11-14:OAc. Similarly, cells expressing EposOR6 were treated with 100 nM Z11-14:OAc or a negative control (100 nM Z12-14:OAc), rinsed and incubated at RT for 30 minutes after which they were treated a second time with a dose titration of E11-14:OAc. Dose response curves and EC_{50} values were generated using the non-linear regression function of GraphPad Prism software.

Xenopus oocyte assays

EposOR1 and EposOR6 were screened for responses to selected pheromone components using the *Xenopus* oocyte assay system at the Department of Biology, Lund University, Lund, Sweden.

Full length EposOrco, EposOR1 and EposOR6 DNA was modified by PCR to contain a BamHI restriction site followed by the Kozak sequence 'GCCACC' immediately preceding the start methionine on the 5' end and a XhoI restriction site immediately following the stop codon on the 3' end. Modified DNA for each gene was double digested using BamHI and XhoI restriction enzymes, ligated into pcDNA5TO and transformed into One Shot Top10 chemical competent *E. coli* cells. Colonies were tested for the presence of pcDNA5TO/EposOrco, pcDNA5TO/EposOR1 or pcDNA5TO/EposOR6 by colony PCR using full-length, gene-specific primers. For each gene, plasmids were purified from colonies testing positive for insertion using a NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel). Plasmids were sequenced and those containing the correct EposOR DNA sequences were used for further cloning efforts. Full-length EposOR DNA was double digested from plasmids using BamHI and XhoI restriction enzymes (New England Bio),

purified by gel electrophoresis and ligated into BamHI and XhoI-digested pCS2+ vector using T4 DNA ligase (New England Bio) according to the manufacturer's protocol.

The materials and methods used to produce and inject cRNA and test oocytes for responses to compounds are identical to those previously described (Zhang and Löfstedt, 2013). Briefly, cRNA was synthesized and purified using the mMESSAGE mMACHINE kit (Ambion) and used for injection into oocytes surgically removed from *X. laevis* frogs. Oocytes were micro-injected with 100 ng of cRNA encoding EposOrco and either EposOR1 or EposOR6 and allowed to incubate for three to five days prior to functional testing. Responses to compounds were detected by monitoring changes in whole-cell inward currents in oocytes using the two-electrode voltage clamp technique. Oocytes expressing EposOrco and EposOR1 or EposOR6 were perfused with 100 μM solutions of E11-14:OAc, (E,E)9,11-14:OAc, E11-14:OH, E11-16:OAc, Z11-14:OAc or (Z,E)9,11-14:OAc in assay buffer containing 0.1% DMSO. Initial screening experiments were conducted to determine the response magnitude of an individual oocyte to each compound, after which compounds were delivered to oocytes in order of increasing response magnitude in an attempt to eliminate the so-called de-sensitization affect of oocytes responding to compounds. Once the optimal compound delivery order was established for EposOR1 and EposOR6, five to ten oocytes were screened for responses to pheromone components.

Results

HEK293 cell lines were generated with stable and inducible expression of EposOrco, EposSNMP1 and EposOR1, 6, 7, 21, 22, 30, 34, 41, 43 or 45. Unfortunately, despite multiple attempts with multiple antibodies, we were unable to detect EposOR protein by western blot in any of our cell lines, including cell lines in which we've previously detected OR protein and seen responses to odorants (Corcoran et al. 2014b). Instead, RT-PCR was used to detect full length mRNA for each gene in non-induced and induced cells for each cell line, confirming that genes were being expressed at the transcriptional level (Figure 1). A small amount of mRNA was detected in non-induced cells, however substantially more mRNA was detected in induced cells suggesting the majority of expression was repressed in non-induced cells. Interestingly, two bands (~1200 and 700 bps) were detected in the TEOS/EposOR34 cell line using primers designed to amplify full length EposOR34. These PCR products were purified and sequenced and it was found that the smaller band was missing 532 bp perfectly aligned with 'GT'/'AG' residues present in the middle of the coding sequence. EposOR34 DNA was re-amplified by PCR, ligated into pcDNA5TO and

transfected into TEOS cells and the same results were observed, suggesting HEK293 cells were somehow splicing the EposOR34 transcript.

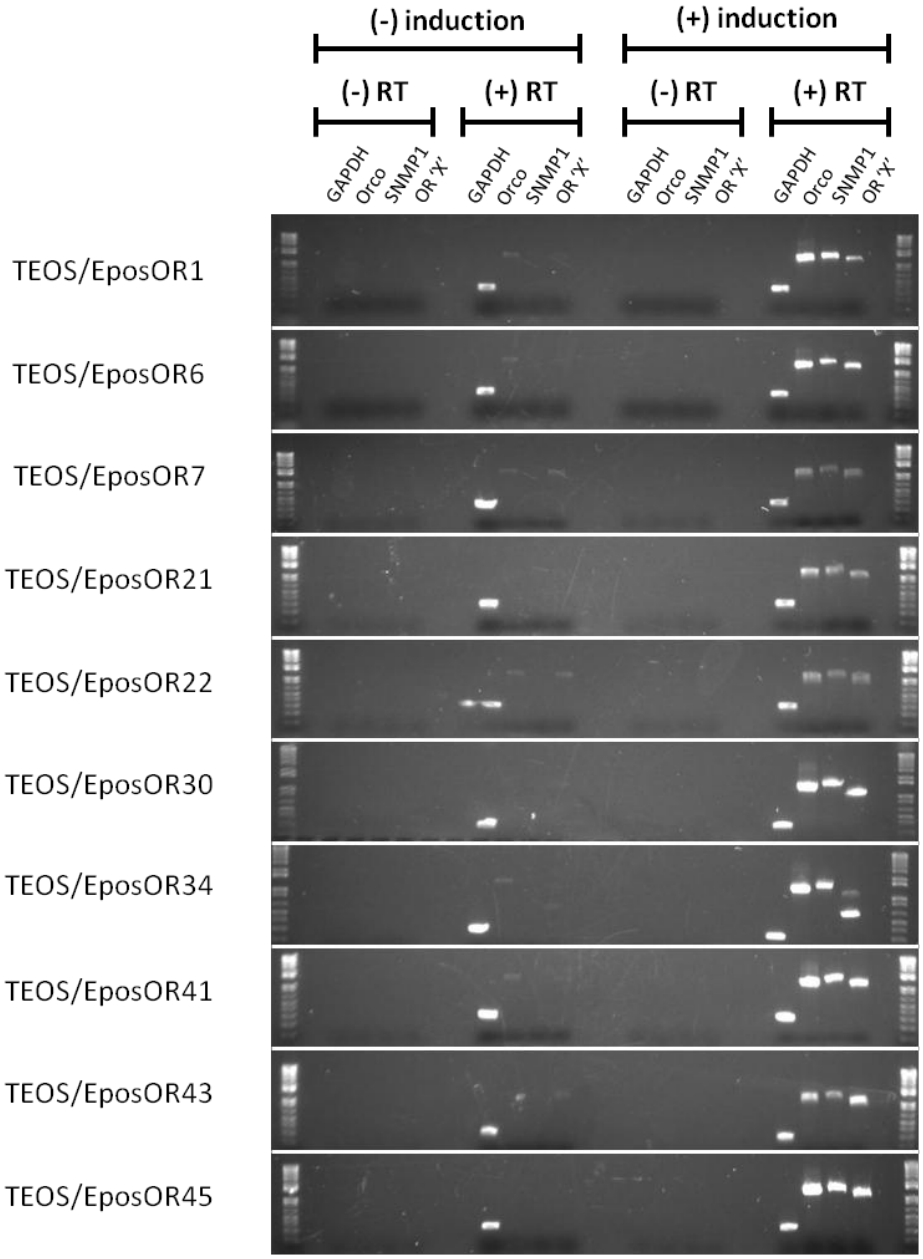

Figure 1. PCR of full length GAPDH, EposOrco, EposSNMP1 and EposOR'X' from mRNA purified from TEOS cell lines. RT-PCR conducted on non-induced and induced cells for each cell line with ((+) RT) and without ((-) RT) reverse transcriptase.

Of the ten EposORs tested in this study, three receptors, EposOR1, EposOR6 and EposOR45 responded to pheromone components when expressed in HEK293 cells. In initial screening experiments using 30 μM doses of the 62 pheromone compounds, cells expressing EposOR1 responded to (E,E)9,11-14:OAc, (Z,E)9,11-14:OAc and (*Z,E*)-9,12-tetradecadienyl acetate ((Z,E)9,12-14:OAc), cells expressing EposOR6 responded to E11-14:OAc, (E,E)9,11-14:OAc, Z11-14:OAc, (Z,E)9,11-14:Ac and (Z,E)9,12-14:OAc and cells expressing EposOR45

responded to (*E*)-9-tetradecen-1-ol (E9-14:OH), (*E*)-10-tetradecen-1-ol (E10-14:OH) and E11-14:OH (Figure 2). Non-induced cells did not respond to any of the 62 compounds for all cell lines. Cells expressing EposORs 7, 21, 22, 30, 41 and 43 did not respond to any of the 62 pheromone components tested (data not shown). Each cell line did not show a response to vehicle control in induced or non-induced cells and did show a response to VUAA1 in induced cells but not in non-induced cells. These results confirm that EposOrco was present and functional in each cell line and that the assay was operating properly during screening experiments of pheromone compounds. Cells expressing EposOR34 did not respond to pheromone components either, however, this is most likely due to the majority of transcript being truncated in cells and therefore not coding for full length EposOR34 protein.

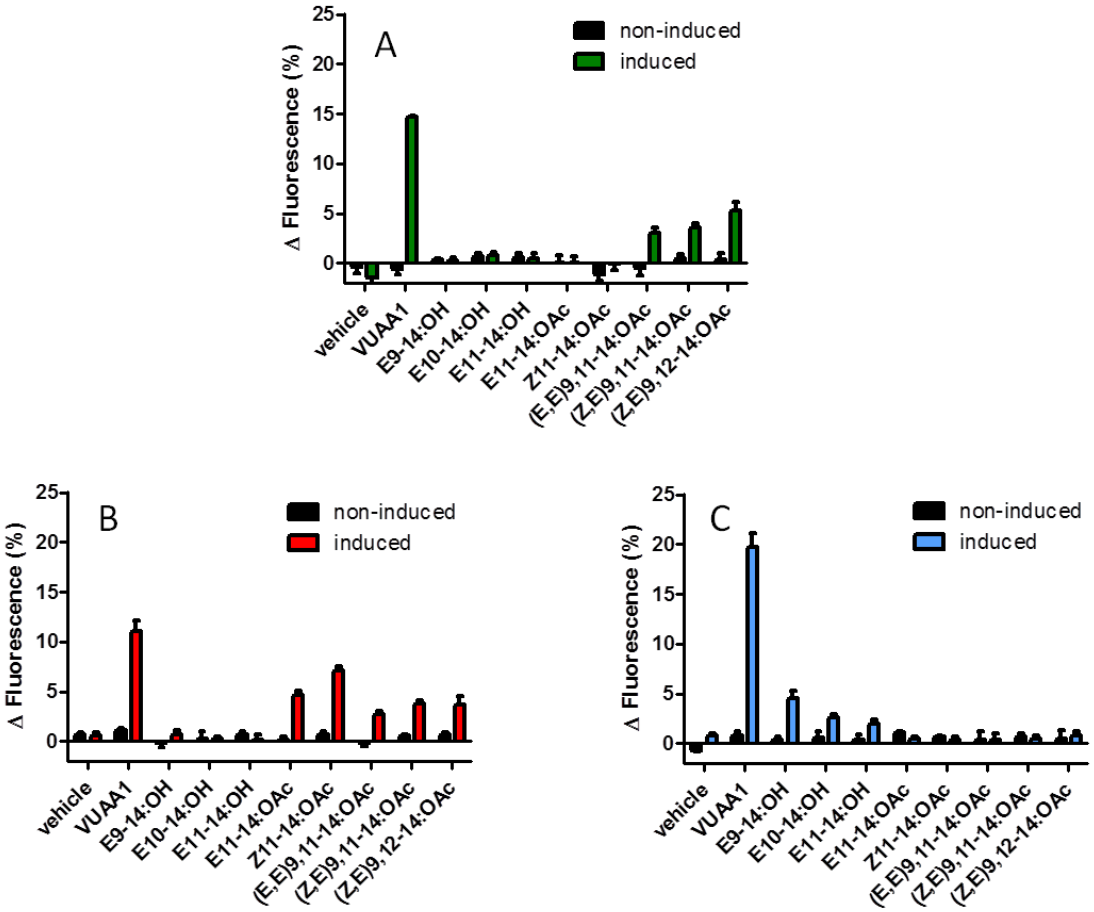

Figure 2. Response of non-induced and induced TEOS cells expressing EposOR1 **(A)**, EposOR6 **(B)** and EposOR45 **(C)** to vehicle, VUAA1 (50 μM) or pheromone components (30 μM) that elicited responses in screening experiments. Data represent the mean response (+/- SEM) from three wells per treatment.

Compounds that elicited responses in cell lines expressing EposOR1, EposOR6 or EposOR45 were then tested further in dose response experiments. In all cases non-induced cells did not show a response to test compounds. TEOS/EposOR1 cells consistently responded dose-

dependently to (E,E)9,11-14:OAc, (Z,E) 9,11-14:OAc and (Z,E)9,12-14:OAc. Similar response magnitude and sensitivity was observed for the three compounds and EC_{50} values ranged from 1.2 to 3.4 µM between experiments. TEOS/EposOR6 cells consistently responded dose-dependently to E11-14:OAc, (E,E)9,11-14:OAc, Z11-14:OAc and (Z,E)9,11-14:Ac and (Z,E)9,12-14:OAc. EposOR6 was more sensitive to Z11-14:OAc than all other activating compounds, and EC_{50} values ranged from 20 to 40 nM between experiments. The response magnitude of EposOR6 to Z11-14:OAc was consistently twice as high as other activating compounds. Similar response magnitudes and sensitivities were observed for the four other compounds and EC_{50} values ranged from 2 to 5 µM between experiments. TEOS/EposOR45 cells consistently responded dose-dependently to E9-14:OH, E10-14:OH and E11-14:OH, and EC_{50} values ranged from 600 to 700 nM, 1 to 3 µM and 800 to 900 nM between experiments for each compound, respectively. The response magnitude of EposOR45 to the three compounds varied with E9-14:OH giving the greatest response, E10-14:OH giving a response approximately 75% that of E9-14:OH and E11-14:OH giving a response of approximately 40% that of E9-14:OH (Figure 3).

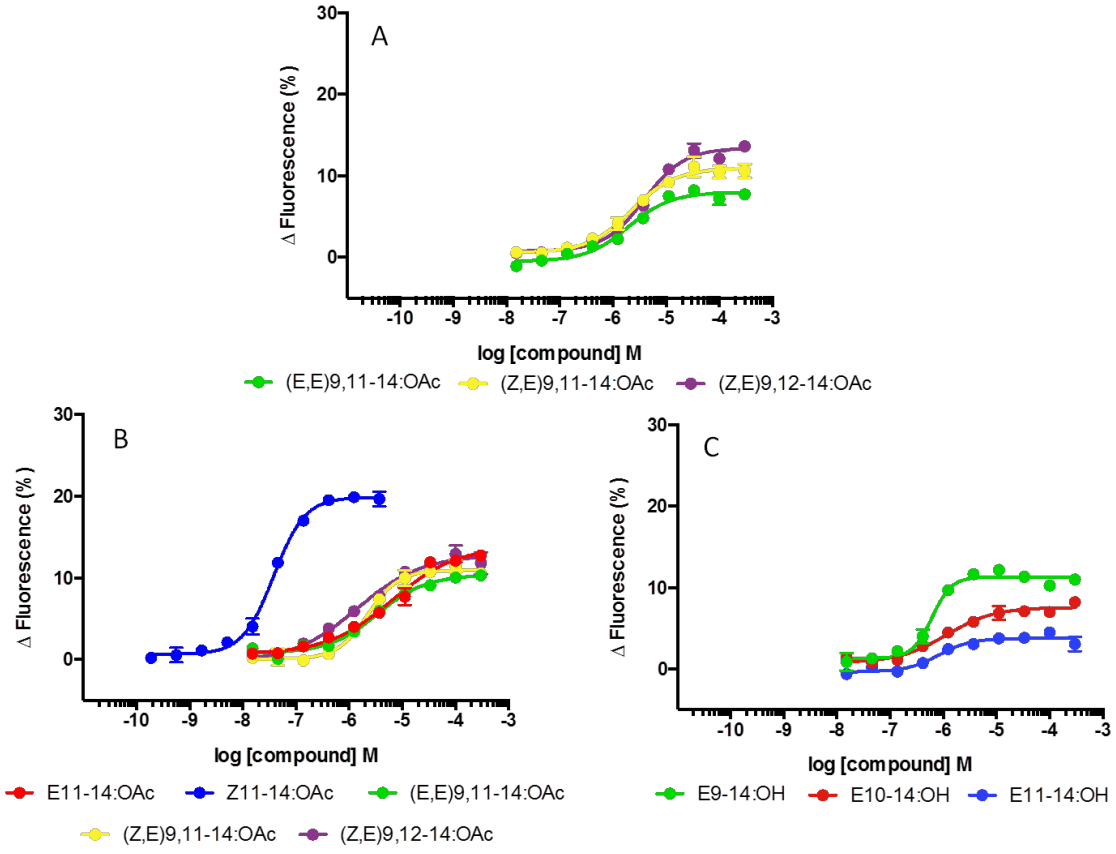

Figure 3. Response of TEOS cells expressing EposOR1 **(A)**, EposOR6 **(B)** or EposOR45 **(C)** to dose titrations of pheromone components identified in screening experiments. Data represent the mean response (+/-SEM) from three wells per treatment.

To validate responses obtained in HEK293 cell lines in a second system, EposOR1 and EposOR6 were tested for response to selected pheromone compounds when expressed in

Xenopus oocytes. In screening experiments, EposOR1 had consistent, above background responses to (E,E)9,11-14:Ac and (Z,E)9,11-14:Ac. Occasional responses were observed to E11-14:OAc and E11-16:OAc, however these responses were not consistent between individual oocytes. EposOR6 had consistent, above background responses to E11-14:Ac, (E,E)9,11-14:OAc, Z11-14:OAc and (Z,E)9,11-14:OAc. Neither EposOR1 nor EposOR6 were tested against (Z,E)9,12-14:OAc in *Xenopus* oocytes. Oocytes not expressing an EposOR did not respond to any of the pheromone compounds tested (Figure 4).

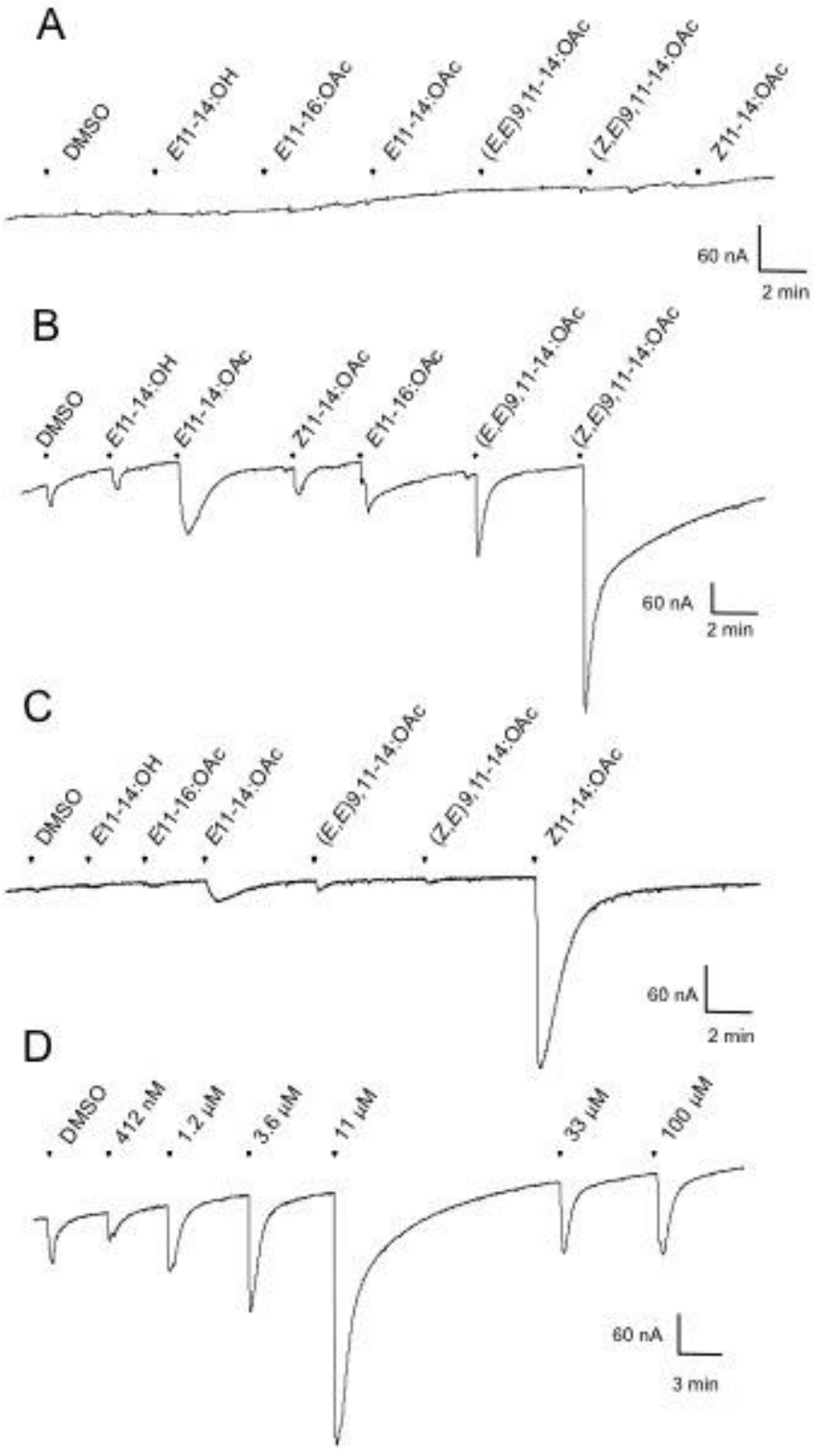

Figure 4. Response of 'empty' *Xenopus* oocytes **(A)** or oocytes expressing EposOR1 **(B)** or EposOR6 **(C)** to select pheromone components. Response of EposOR1 to a dose titration of (E,E)9,11-14:Ac **(D)**.

HEK293 cells expressing EposOrco and EposOR3 responded to VUAA1 and geranyl acetate and cells pre-treated with either compound were able to be re-activated by both compounds after cells were rinsed (Figure 5A). Cells expressing EposOrco and EposOR6 responded to

VUAA1, E11-14:OAc and Z11-14:OAc. Cells that responded to VUAA1 were able to be re-activated by VUAA1, E11-14:OAc and Z11-14:OAc. Cells that responded to E11-14:OAc or Z11-14:OAc were able to be re-activated by VUAA1 but were not re-activated by E11-14:OAc or Z11-14:OAc (Figure 5B). The response of TEOS/EposOR6 cells to dose titrations of E11-14:OAc and Z11-14:OAc were significantly decreased, in terms of magnitude and sensitivity, following treatment with Z11-14:OAc or E11-14:OAc, respectively (Figure 6).

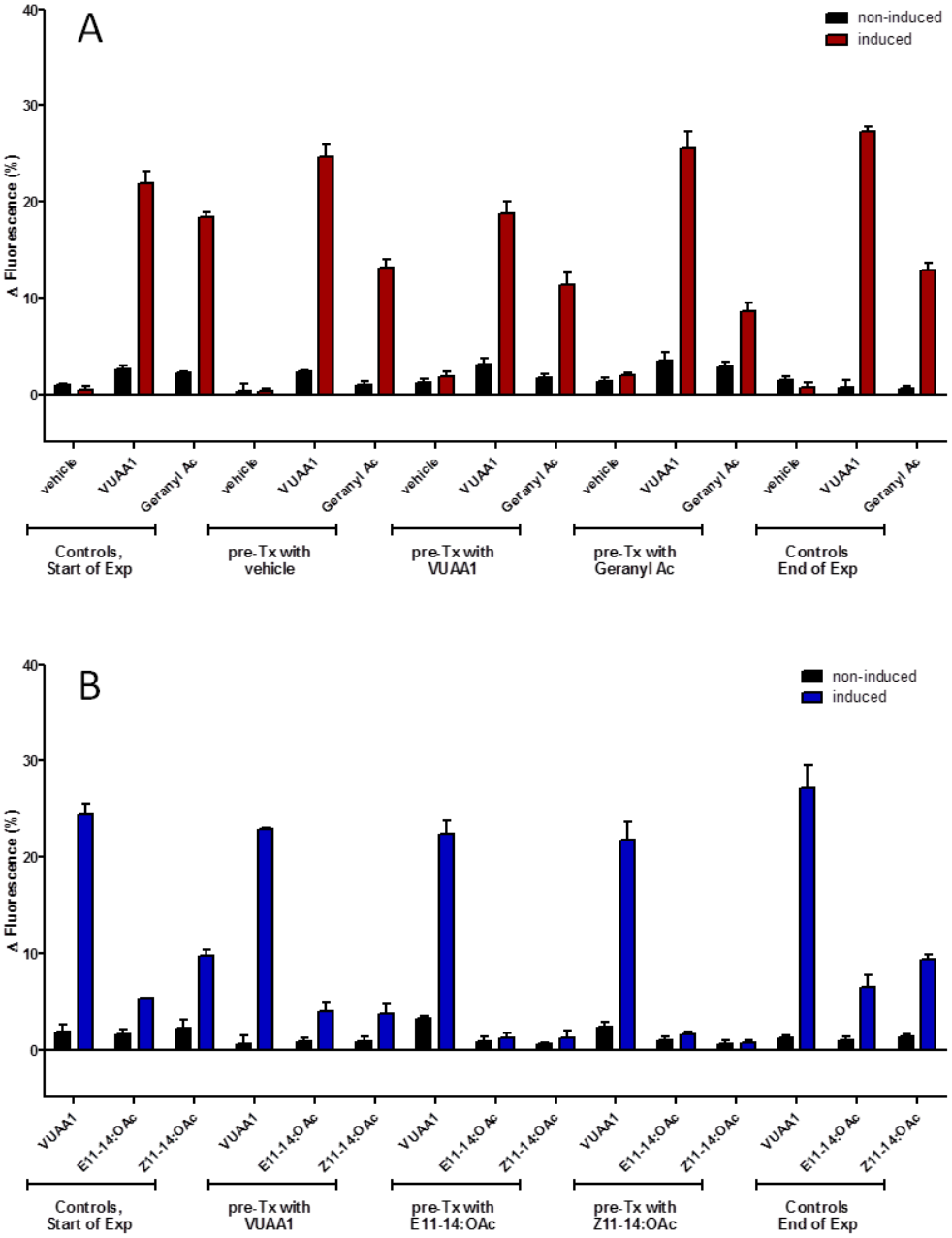

Figure 5. Repeated treatment of 'TEOS' cells expressing EposOR3 **(A)** or EposOR6 **(B)** with activating ligands or controls. Cells were treated once at the beginning of experiment (pre-Tx), rinsed three times and allowed to sit for 30 minutes prior to re-treatment with activating ligand or control. Data represent mean response (+/-SEM) from three wells per treatment.

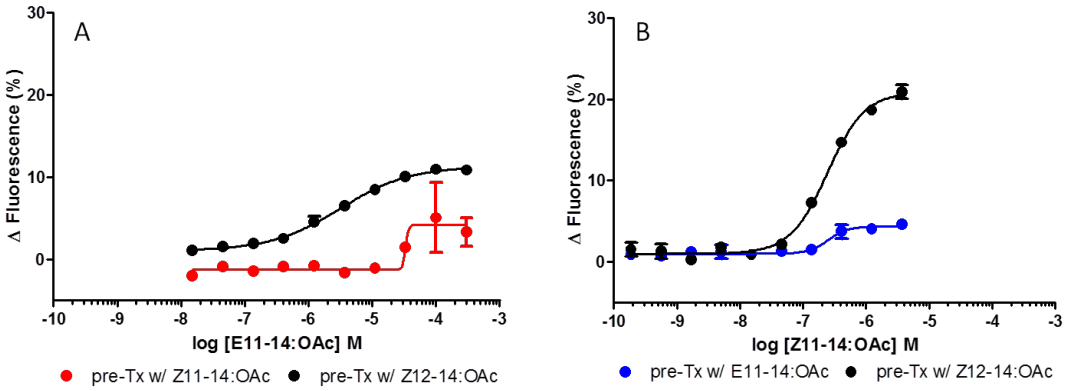

Figure 6. Inhibition of response of 'TEOS' cells expressing EposOR6 to various doses of **(A)** E11-14:OAc following pre-treatment with Z11-14:OAc or control (Z12-14:OAc) or **(B)** Z11-14:OAc following pre-treatment with E11-14:OAc or control (Z12-14:OAc). Data represent mean (+/- SEM) responses from three wells per treatment.

Discussion

Ten *E. postvittana* ORs that were either members of the 'PR clade' or showed male-biased expression were assessed for their ability to respond to sex pheromone components in HEK293 cells expressing EposOrco and EposSNMP1. Three ORs responded to sex pheromone components used by *E. postvittana* or other moths and these results concur with electrophysiological data conducted previously (Rumbo, 1983, Rumbo et al., 1993, Stephens et al., 2008, El-Sayed et al., 2011). EposOR6 responded to the major sex pheromone component, E11-14:OAc, to a minor component, (E,E)9,11-14:OAc, to two known behavioral antagonists, Z11-14:OAc and (Z,E)9,11-14:OAc and to (Z,E)9,12-14:OAc. Interestingly, EposOR6 was more sensitive to the behavioral antagonist Z11-14:OAc than it was to the attractive compounds. EposOR1 responded to a minor component, (E,E)9,11-14:OAc, and to the behavioral antagonist, (Z,E)9,11-14:OAc, as well as to a structurally related compound, (Z,E)9,12-14:OAc. Preliminary results from oocytes agreed with responses observed in HEK293 cells. In both heterologous expression systems receptors responded to the same pheromone components with EposOR6 and EposOR1 having greater response magnitudes to Z11-14:OAc and (Z,E)9,11-14:OAc, respectively. The third PR we identified, EposOR45, responded to a second minor pheromone component, E11-14:OH, as well as to two closely related alcohols, E9-14:OH and E10-14:OH in HEK293 cells but has not yet been tested in the *Xenopus* system to confirm results. It is not known if (Z,E)9,12-14:OAc, E9-14:OH or E10-14:OH elicit electrophysiological responses in moth antennae, however they are pheromone components of other moths (El-Sayed, 2012) and could possibly act as behavioral antagonists in *E. postvittana*.

Previously, EposOR1 did not respond to the *E. postvittana* pheromone components in an Sf9 cell-based assay (Jordan et al., 2009) with methyl salicylate found to be its best ligand using this insect cell-based assay system (Kiely et al., 2007). We tested our TEOS/EposOR1 cell line against the odorants that gave responses in Jordan et al. (2009) and did not see any responses to these compounds (data not shown). Responsiveness of EposOR1 to the *E. postvittana* pheromone components was observed using the oocyte system, providing confidence in the results obtained using the HEK system. One possible explanation as to why EposOR1 responds to pheromone components in HEK cells and *Xenopus* oocytes but not in Sf9 cells is that there exist inherent differences between the systems that in some way affect the receptor's ability to respond to ligands. For example, it is theoretically possible that the different cell types express and secrete carrier proteins that could promote or inhibit ligand interaction with the heterologously expressed proteins, leading to differences in the results observed between the systems. Because EposOR1 responds in two out of three assay systems we are confident that its responses to *E. postvittana* pheromone components are real.

Cell lines expressing EposORs 7, 21, 22, 30, 34, 41 and 43 did not respond to any of the 62 pheromone components tested in cell-based assays. We found that the mRNA for one receptor, EposOR34, was being spliced in HEK293 cells at specific nucleotides (GT/AG) normally associated with intron/exon boundaries. For whatever reason, this suggests that HEK293 cells may not be suitable for the expression of some OR genes. Full length mRNA was detected in cell lines for all other EposORs, indicating that these genes were being expressed at least at the transcriptional level. The EposORs all have epitope tags allowing for detection at the protein level as described in Corcoran et al. (2014b), however the critical reagent, the anti-V5 antibody, has since been discontinued by the supplier. Attempts to detect our OR genes with other commercially available α-V5 antibodies have not been successful, even in cell lines in which OR protein has been detected previously (e.g., EposOR3) (Corcoran et al., 2014b). Until EposOR protein expression is confirmed in cell lines we cannot confidently conclude that these receptors were available at the cell membrane surface to respond to any of the pheromones tested.

Only one of the three EposPRs we identified, EposOR6, displayed male-biased expression patterns by qPCR; EposOR1 and EposOR45 showed equal expression in male and female antennae (Corcoran et al., 2014a). Of the moth PRs previously described from other species, only one has been shown to be equally expressed in male and female antennae (Krieger et al., 2004, Nakagawa et al., 2005) with all others showing male-biased expression. To date all characterized moth PRs, including those we've identified in this study, are members of the so-called pheromone receptor clade, further reinforcing the importance of this clade and its members in generating the diversity of receptors involved in mate recognition in moths. However, this clade also contains

ORs that display female-biased (Bengtsson et al., 2012, Liu et al., 2013, Corcoran et al., 2014a) or non-biased expression patterns, and several male-biased members of this clade have been identified that did not respond to pheromone components in functional assays (Wang et al., 2011, Liu et al., 2013). It is possible that the other EposORs within and the male-biased EposORs outside the PR clade may respond to compounds that were not tested in these experiments. De-orphaning of these EposORs may require testing with a broader panel of test pheromones and odorants.

The three *E. postvittana* PRs we identified are all relatively specific, with each being activated by three to five pheromone components. Interestingly, each receptor responded to compounds that are structurally similar: EposOR1 responded to three 14-carbon diene acetates, EposOR6 responded to five 14-carbon acetates with one or two double bonds and EposOR45 responded to three 14-carbon alcohols. These results suggest that EposPRs may be tuned to pheromone compound classes instead of specific pheromone compounds; however, in these experiments the organic solvent DMSO was used to deliver pheromone compounds which may not reflect what occurs *in vivo*. Several studies have shown that PBPs can increase the specificity of moth PRs to pheromone compounds in *in vitro* assays (Groβe-Wilde et al., 2006, Groβe-Wilde et al., 2007, Forstner et al., 2009). It is possible that the responses of EposPRs may become more specific to certain compounds if pheromone compounds are delivered to receptors using EposPBPs. Furthermore, in flies it has been shown that the response of PRs to pheromone compounds is affected by the presence of SNMP1 (Syed et al., 2006, Benton et al., 2007, Syed et al., 2010b), and because of this we co-expressed candidate EposPRs with EposOrco and EposSNMP1 in HEK293 cells. In preliminary experiments, comparisons between cells expressing EposPRs with and without EposSNMP1 have not revealed any differences in receptor specificity to ligands (data not shown). Future work on characterizing EposPR specificity should focus on characterizing EposPBP expression in antennae and the ability of EposPBPs to bind and deliver pheromones to EposPRs in cell lines with and without EposSNMP1 present.

EposOR1 and EposOR6 both respond to components of the *E. postvittana* pheromone blend (behavioral agonists) as well as to pheromones produced by other moths that inhibit the attractiveness of the moth's pheromone blend (behavioral antagonists). Olfactory receptor neurons (ORNs) each typically express a single ligand-binding OR gene (Vosshall et al., 2000, Ray et al., 2008). Neuroanatomical studies have shown that these ORNs project to different regions of the macroglomerular complex in the moth brain (Hansson et al., 1992, Lee et al., 2006), and it is thought that input from these ORNs is translated by higher structures of the brain into a behavioral response (reviewed in Nawrot, 2012). If, for example, the ORN that responds to the behavioral agonist 'fires', the moth will be attracted to the source, but if the ORN that responds to

the behavioral antagonist 'fires' as well, the moth will not be attracted to the source. According to this model, behavioral antagonists inhibit the attractiveness of behavioral agonists by means of a central (i.e., brain) and not peripheral (i.e., receptor) mechanism through which one stimulatory signal overrides another. This model makes sense as long as there are distinct ORNs that respond to behavioral agonists or antagonists, but not both. To date, several moth PRs have been found to respond to behavioral agonists and antagonists, however in these species there is always an additional PR or PRs that respond specifically to the agonist or antagonist (Wanner et al., 2010, Zhang and Löfstedt, 2013). This is not the case with *E. postvittana*; based on our results, the only receptors that responded to behavioral agonists also responded to behavioral antagonists. Because *E. postvittana* may not have PRs specifically tuned to behavioral agonists or antagonists, and because both E11-14:OAc and Z11-14:OAc have been found to stimulate ORN firing in *E. postvittana* (Stephens et al., 2008), we examined the possibility that behavioral antagonists inhibit the attractiveness of behavioral agonists through some sort of peripheral mechanism.

When conducting screening experiments on PRs using the oocyte system compounds should be delivered in order of increasing response magnitude as an inhibitory effect is commonly observed after stimulation of the PR following treatment with certain pheromone compounds (Zhang and Löfstedt, 2013). This inhibition has traditionally been ascribed to some sort of de-sensitization effect of either the PR or the oocyte itself through an unknown mechanism following pheromone-induced receptor activation. Indeed, this phenomenon was observed in our own experiments with *Xenopus* oocytes; the responses of EposOR1 and EposOR6 to pheromone compounds were affected when preceded by stimulation with pheromone compound (Figure 4C, 4D). Unfortunately, when these experiments were conducted, due to our limited access to this assay system, a thorough examination into the inhibitory effects of pheromone treatment on the response of PRs to subsequent pheromone treatment, particularly that of E11-14:Ac and Z11-14:Ac on the response of EposOR6, could not be conducted. However, our unlimited access to the HEK293 cell based system offered us to examine this interaction more closely.

Using HEK293 cells expressing EposOrco and EposOR3, a receptor tuned to geranyl acetate, we demonstrated that ORs can be re-activated by ligands in cell-based assays. This allowed us to examine whether EposOR6 was capable of being re-activated by Z11-14:OAc (behavioral antagonist) following treatment with E11-14:OAc (behavioral agonist) and vice versa. Contrary to what we found with cells expressing EposOR3, EposOR6 could not be re-activated by either pheromone component following treatment with either compound, even when cells were rinsed several times, suggesting that there is a difference in binding affinities of odorants for their ORs and pheromones for their PRs in this *in vitro* system. In our experiments

geranyl acetate could be washed off of EposOR3 but neither pheromone could be washed off of EposOR6. These results are similar to what was observed in the oocyte system, except that in the oocyte system the receptors were able to respond to subsequent pheromone treatment more quickly. This difference is likely due to the fact that in the oocyte system the receptors are continuously washed (4 mL/min) with buffer solution whereas in the HEK system the cells (and receptors) are rinsed several times with 100 μL of assay buffer.

One potential alternative interpretation of these results and/or explanation as to why EposOR6 won't respond to pheromone following initial treatment with pheromone is because it simply can't be reactivated in this assay system. While we cannot dismiss this possibility, it would require that PRs behave fundamentally differently than general odorant receptors. Using the exact same system and methods, we found that EposOR3 was capable of responding to geranyl acetate repeatedly, suggesting that insect olfactory receptors do not get internalized, de-coupled from Orco, or undergo an inherent period of de-sensitization following ligand-induced receptor activation. Based on the assumption that the only fundamental difference that exists between EposOR3 and EposOR6 is the ligand that activates the receptor and the dynamics of this interaction, the parsimonious explanation for the observed difference in their ability to be reactivated in our assay system has to do with the ligand or the ligand/receptor interaction.

Olfactory receptors reside in ORNs and are surrounded by lymph that contains various types of enzymes that degrade odorants and pheromones (Vogt, 2005). While the exact mechanism is not totally clear, it is thought that pheromone degrading enzymes (PDEs) are responsible for terminating the pheromone-induced stimulation of ORNs, possibly by directly removing pheromones from PRs (reviewed in Leal, 2013). Our data, as well as experiments conducted on *B. mori* PRs in *Drosophila* (Syed et al., 2006), support the model that PDEs reset the system by removing pheromones from PRs. In our cell-based assays there are no PDEs present to degrade the pheromones, which may explain why we could not re-activate EposOR6 after treatment with E11-14:OAc or Z11-14:OAc.

If PDEs are responsible for removing pheromones from PRs, then the rate at which an ORN can send signals to the insect brain will be determined by the speed at which the pheromone is degraded by the PDE. If two pheromone components that stimulated the same PR were degraded at different rates then the 'firing' rates of the ORN would differ depending on which pheromone activated the PR. Indeed, studies conducted on the Japanese scarab beetle, *Popillia japonica*, have shown that a single PDE degrades the sex pheromone, R-japonilure, and its behaviorally antagonistic isomer, S-japonilure, at different rates (Ishida and Leal, 2008). Based on these data, we speculate that differential rates of degradation of E11-14:OAc and Z11-14:OAc

by PDEs may serve as the peripheral mechanism through which behavioral agonism and antagonism is mediated in *E. postvittana* (Figure 7).

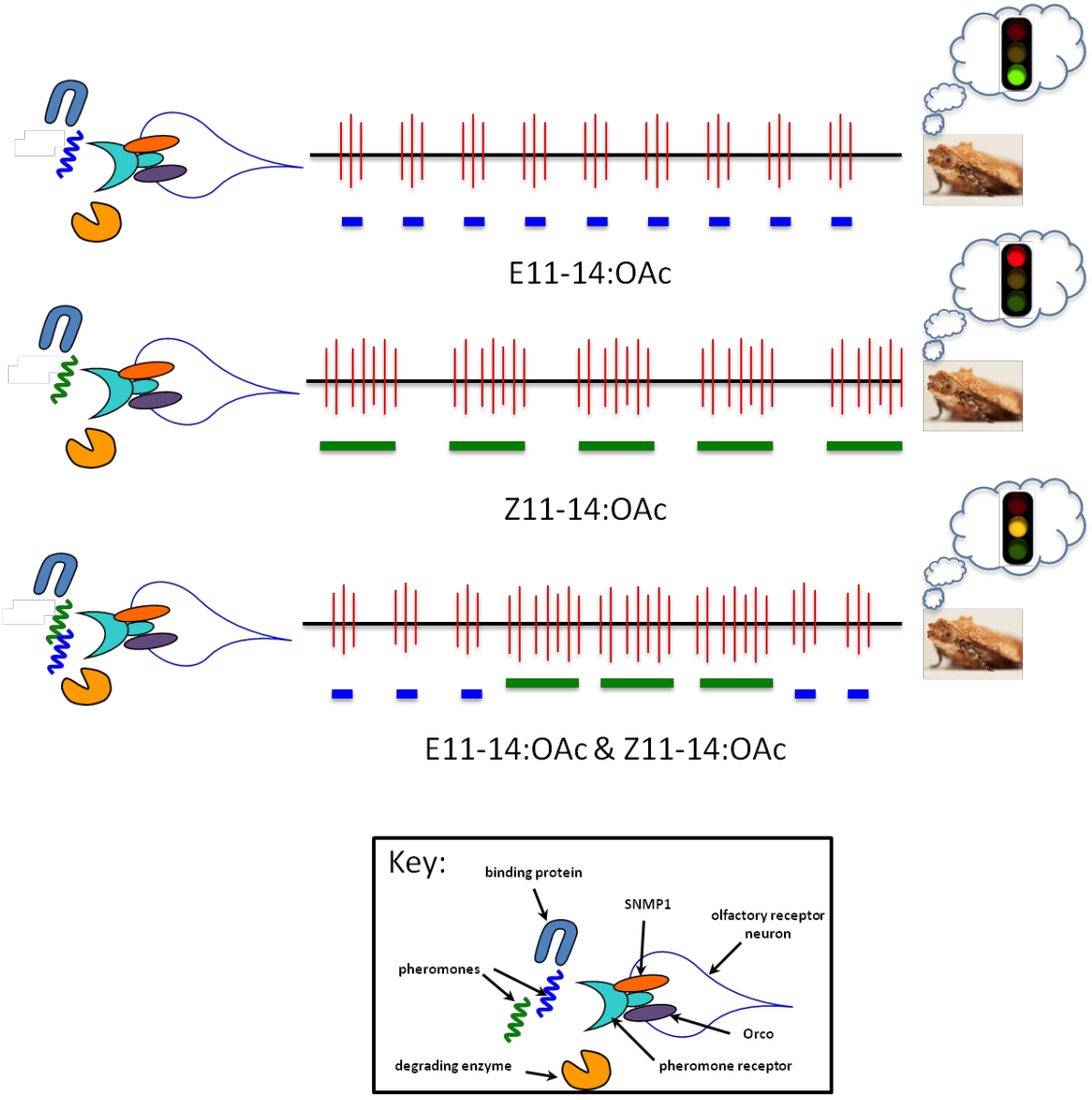

Figure 7. Hypothetical model depicting proposed mechanism for differences in behavioral response following activation of a single ORN by two different compounds. Different rates of pheromone degradation by PDEs lead to different firing rates of ORNs which lead to different behavioral responses. ORN firing rate caused by E11-14:OAc causes attraction (behavioral agonism). ORN firing rate caused by Z11-14:OAc or E11-14:OAc and Z11-14:OAc inhibits attraction (behavioral antagonism). Red vertical lines represent ORN firing. Blue dashes represent E11-14:OAc activation of EposOR6. Green dashes represent Z11-14:OAc activation of EposOR6.

In our theoretical model, the behavioral antagonist, Z11-14:OAc, is degraded more slowly by a PDE than the behavioral agonist, E11-14:OAc, and the ORN does not 'fire' at the 'attractive' rate in the presence of both compounds. In our model we exemplify disturbed firing rates of ORNs, but it could also be that degradation of the behavioral antagonist is so slow that the response of the ORN to the behavioral agonist is simply attenuated. Electroantennogram and single-sensillum recordings should be conducted on *E. postvittana* antennae using repeated exposure to E11-14:OAc and Z11-14:OAc to examine ORN firing rates following treatments with these compounds.

Recently we have identified a suite of olfactory-related proteins, including odorant degrading enzymes (ODEs), from the antennae of *E. postvittana* (Corcoran et al., 2014a). Phylogenetic comparisons of EposODEs to a PDE from the moth *Antheraea polyphemus* (Ishida and Leal, 2005) have allowed us to identify a candidate EposPDE, and future research efforts should evaluate the kinetics of the degradation of *E. postvittana* pheromone components and behavioral antagonists, as well as the ability of EposPBPs to deliver these compounds to PRs.

In summary, we have identified three PRs from the pest moth *E. postvittana* that together respond to three of the four known pheromone compounds used by this species (El-Sayed et al., 2011), as well as to biologically relevant behaviorally antagonistic pheromone compounds (Rumbo et al., 1993, Stephens et al., 2008, Kye-Chung Park, pers. comm.). In these experiments we did not identify a receptor that responds to the fourth pheromone compound, E11-16:OAc. Because we have not yet been able to verify EposOR protein expression in the cell lines we tested, we cannot rule out one of these OR genes as being a receptor for E11-16:OAc. Interestingly, at least two of the three PRs we identified respond to *E. postvittana* pheromone components and to behaviorally antagonistic pheromones. Cell-based assays using two of these compounds have shown that treatment of EposOR6 with these compounds inhibits further receptor activation and that a PDE may be required to remove them from the receptor. These results serve as proof of principle that ligand-induced receptor activation can be inhibited through blockage of the pheromone binding site, and that molecular entities such as PDEs may be appropriate targets for inhibitory compounds targeting the insect olfactory system. The recombinant HEK293 cell lines used in these experiments provide the opportunity to screen for inhibitors or constitutive activators of pheromone reception for use in the management of this and potentially related tortricid pests.

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V

Conclusion

The aim of this PhD research project was to identify and functionally characterize pheromone receptors from the horticultural pest, *Epiphyas postvittana*. During the course of this research several significant achievements were made which not only allowed attainment of this goal, but also extended our understanding of the olfactory biology of the moth, contributed a wide body of information to the field of insect olfaction in general, and provided insight and new tools which may aid in the development of novel pest control technologies. While the goals set forth in this PhD were ultimately achieved, this work has undoubtedly opened the door to many more research questions, and work on the *E. postvittana* olfactory system should be considered far from complete.

Summary of key results

Using standard bioinformatic and molecular biology techniques 243 olfactory-related genes, including 70 olfactory receptors (ORs), were identified from the antennae of *E. postvittana*. Through qPCR and RNAseq gene expression analyses it was found that several of the OR genes displayed sex-biased expression patterns, making them candidates for being the receptors that respond to female produced pheromones (male-biased ORs) or the receptors that respond to oviposition-related olfactory cues (female-biased ORs). The same OR genes were identified as being sex-biased using both qPCR and RNAseq, which suggests that RNAseq may be a sufficient technique for use in future studies focused on identifying differentially expressed genes. The generation of high quality, blast-searchable, male and female antennal transcriptomes allowed the identification of the full-length sequences of the majority of these genes, providing the critical information required to clone the genes of interest for functional testing in a heterologous expression system.

A novel heterologous expression system was developed for testing the *E. postvittana* candidate pheromone receptor genes. Human Embryonic Kidney (HEK293) cells were genetically modified to express *E. postvittana* ORs under the control of a tetracycline-inducible regulatory system, and the regulated expression of ORs proved to be critical for the generation of cell lines with stable, isogenic gene expression. The ability to generate HEK293 cell lines with stable and isogenic OR gene expression allowed the development of a robust, consistent, and relatively high-throughput plate reader-based assay system.

Using the HEK293 assay system, candidate *E. postvittana* pheromone receptor (PR) genes were tested for responsiveness to a wide panel of pheromone compounds. Out of ten candidate pheromone receptor genes tested, only three responded to pheromone components. Surprisingly, only one of these three receptors displayed male-biased mRNA expression patterns, which raises speculation about this characteristic being appropriate for its use in identifying candidate PR genes. However, all three of these genes grouped phylogenetically with all other known lepidopteran PRs, strengthening the importance of this group of ORs in pheromone reception. Two genes, EposOR30 and EposOR34 displayed the highest degree of male-biased expression but did not group phylogenetically with other known PRs and did not respond to pheromone compounds in our HEK293 screening experiments.

Together these three receptors responded to three of the four known pheromone compounds used by *E. postvittana*; a receptor for the fourth compound, E11-16:Ac, was not identified in these studies. Interestingly, EposOR6 responded to the main component of the *E. postvittana* pheromone blend as well as to the behavioral antagonist Z11-14:Ac. In fact, all three pheromone receptors identified responded equally well, if not better, to compounds that were not part of the *E. postvittana* pheromone blend as they did to components that were. The response profiles obtained for two of these receptors using the HEK293 assay system were confirmed using the *Xenopus* oocyte assay system. In our experiments behavioral agonists and antagonists both stimulated the same pheromone receptors, which led us to investigate interactions between these compounds and the pheromone receptors they activated. Using EposOR6, it was found that pre-treatment with pheromone compounds inhibited the subsequent reactivation of the receptor. The inhibitory effects that were observed in this *in vitro* system led to the formulation of the hypothesis that pheromone degrading enzymes may be required to remove pheromones from the receptors they activate, and that in *E. postvittana* behavioral agonist and antagonist-induced receptor activation may be mitigated by the pheromone degrading enzymes that are present in the moth's antennae. Further experimentation is required to test the validity of this hypothesis.

Extending our understanding of Epiphyas postvittana olfactory biology

As noted throughout this thesis, *E. postvittana* is a horticultural pest that has gained the attention of agriculturists, biologists and politicians for decades. Over time, considerable effort has been made to improve our understanding of the biology and phenology of the moth, as well as our ability to protect agricultural economies by controlling its horticultural impact. In recent years,

the focus of research on the moth has been to better understand the molecular entities involved in olfactory perception, with the primary driving force being to identify molecular targets for novel pest control technologies. Indeed, this was the underlying motivation for trying to identify the *E. postvittana* PRs in this thesis. In pursuit of this goal a lot was learned about the molecular entities involved in olfactory perception in *E. postvittana*.

The genes that were identified in these studies are likely to represent the vast majority, if not the entire compliment of odorant receptors, odorant binding proteins (OBPs) and odorant degrading enzymes (ODEs) that are used by *E. postvittana* to sense its environment through olfactory pathways. Admittedly, for the IR, GR, and possibly for the OBP and ODE families, it is likely that there are more genes to be identified that were not present in the antennal transcriptomes; it's possible that other members of these families may be expressed in different tissues or developmental stages and not in male or female adult antennae. For the OR gene family however, because genes were identified through exhaustive searching of transcriptomic and genomic libraries, it is likely that all of the *E. postvittana* olfactory receptors have been identified. The identification of the entire OR repertoire of *E. postvittana* serves as the first step towards our comprehension of how the moth perceives its olfactory environment, and because the majority of these OR sequences are full-length it will be relatively easy to experimentally determine the response profiles of each in functional assays. Once these ORs are functionally characterized, investigators will be able to know exactly what odorant molecules the moth is capable of detecting. An understanding of this "palette" will one day create an exciting story when linked back to the ecology of the animal.

Phylogenetic analysis of the *E. postvittana* ORs provides insight into the relatedness of these genes with ORs from other moths. Through these analyses we can now conclude that some of the *E. postvittana* OR genes are orthologous to those found in other moth species, whereas some have relatively low homology to ORs in other species. For example, while most *E. postvittana* ORs show high sequence similarity to ORs from *C. pomonella, H. virescens* and *B. mori*, EposORs 30, 31, 33, 34 and 36 are all more closely related to each other than they are to ORs from these other moths (Chapter 2, Figure 1). These paralogous *E. postvittana* ORs are likely to have functions that are highly relevant and specific to the moth, relative to ORs that are conserved amongst various moth species. In the case of this particular group of *E. postvittana* ORs, the fact that they are some of the most highly expressed ORs, and that most show male or female-biased expression patterns makes them even more attractive for functional characterization. In the current study, EposORs 30 and 34 were identified as having highly male-biased expression patterns suggesting they may be receptors for the female-produced pheromone compounds, however, in functional experiments these ORs did not respond to any of

the pheromone compounds tested, including all those used by *E. postvittana*. It is unclear why these ORs display such biased expression patterns if they are not receptors for the female pheromone compounds, given what is known about *E. postvittana* foraging ecology and reproductive biology. These data suggest that there may be some uncharacterized environmental or reproductive olfactory cues that exist that are used more by males than females in this species.

All three of the *E. postvittana* pheromone receptors that were identified in functional assays responded to multiple, structurally related pheromone compounds. The moth uses four different compounds in its pheromone blend: a 14-carbon monounsaturated acetate (E11-14:Ac), a 14-carbon bi-unsaturated acetate ((E,E)9,11-14:Ac), a 14-carbon monounsaturated alcohol (E11-14:OH) and a 16-carbon monounsaturated acetate (E11-16:Ac). Interestingly, despite some overlap of the receptivity profiles, EposOR1 responded best to 14-carbon bi-unsaturated acetates, including (E,E)9,11-14:Ac, EposOR6 responded best to 14-carbon monounsaturated acetates, including E11-14:Ac, and EposOR45 responded best to 14-carbon monounsaturated alcohols, including E11-14:OH. In these studies, no receptor was found that responded well to the fourth pheromone component, E11-16:Ac.

The response profiles of the three PRs suggest that pheromone *perception* in *E. postvittana* may not simply be a function of its receptors' ability to be activated by a given pheromone compound; the observation that these PRs respond to behaviorally agonistic *and* behaviorally antagonistic pheromone compounds suggests a more complicated system is at work. If there were *E. postvittana* PRs that responded to behavioral agonists or to behavioral antagonists, but not both, then this observation would not be so perplexing; the signals derived from the more specific PRs could simply override the other less-specific PRs and initiate the appropriate behavioral response. Based on these experiments, this does not seem to be the case with *E. postvittana*.

There are several, not necessarily mutually exclusive explanations and/or theories as to how this system may operate, based on the observations that the *E. postvittana* PRs respond to agonistic and antagonistic compounds: One is that the results observed in these studies may simply be artifacts of *in vitro* experimentation and do not accurately reflect what occurs *in vivo*. As noted previously, it is well known that the sensillum lymph, where PRs and ligands interact, is full of proteins that bind and transport pheromones as well as proteins that degrade them. These proteins may serve as a sort of molecular filter whereby relevant pheromones are protected from enzymatic degradation in the antennae through binding to carrier proteins. If some of the pheromones that are functional in *in vitro* assays are not recognized and carried by these binding proteins in the antennae, then it's possible they would be degraded before they ever reached the PRs, despite their ability to activate the receptors. Alternatively, another possible explanation for

these observations is that, in *E. postvittana*, the activation of these PRs by behavioral agonists and antagonists leads to neuronal firing that differs depending on which ligand activated the receptors. For example, it's possible that subtle differences in neuronal firing rate or spike amplitude could be significant enough to lead to different signal interpretations by the moth brain, leading to different behavioral responses. Because our experiments were performed in heterologous expression systems it was impossible to observe any differences in behavioral agonist or antagonist-induced receptor activation; in our experiments, both simply activate the same receptor. In chapter four a theory was introduced that incorporates both of these explanations, proposing that a degrading enzyme present in the sensillum lymph affects the neuronal firing rate, and thus behavioral response, because of differing degradation kinetics of its interactions with behavioral agonists and antagonists. Nevertheless, regardless of the explanation, our results suggest that in *E. postvittana* olfactory perception may not correlate directly with pheromone reception. In these studies we identified several *E. postvittana* PRs, and their responsiveness to ecologically relevant pheromone compounds *in vitro* suggests that a complicated pre- or post-receptor-activation "filter" is affecting these interactions or their effects, *in vivo*.

Impacts on the wider field of insect olfaction

The insight gained during the course of this research not only has increased our understanding of *E. postvittana* olfactory biology, but also that of the wider field of insect olfaction. Some of the findings of this work reinforce or add to what is currently understood about insect olfaction, or provide tools that may aid other investigators in their studies of the olfactory systems of other insects, and yet others may provide new information to or slightly change our current understanding.

The 243 olfactory-related genes that were identified in *E. postvittana* add to our current understanding of insect olfaction through comparison to the genes that have been identified in other insects. For example, 70 OR genes were identified which is very similar to the number identified in other moths, and a closer look at the OR genes themselves reveal similarities in sequence, length and hypothetical structure, further reinforcing our current models of insect OR evolution. Similarly, comparisons of other genes, such as IRs and GRs, between *E. postvittana* and more distantly related species such as mosquitos and flies, provide evolutionary perspectives of gene evolution. The ability to identify these genes and to obtain the full-length sequence of the majority of them through the generation of transcriptomic libraries will undoubtedly reinforce the value of this technique in insect olfactory gene identification.

The gene sequences identified in these studies represent one of the largest datasets of full-length, olfactory-related genes from an insect outside of *Drosophila melanogaster*. The easiest way to identify genes from an organism is through the use of the sequence of related genes from other organisms (i.e., homology-based techniques). Indeed, the first *E. postvittana* genes were identified by searching through antennal transcriptomes with the sequences of ORs, OBPs, ODEs, etc., that were identified from other organisms. While the genes from organisms as distantly related as flies can be used to identify related genes in moths, the more closely related the organisms are, the more similar the genes will be and thus more amenable to homology-based gene identification. This dataset of lepidopteran olfactory genes will serve as an invaluable tool to those seeking to identify similar genes in other moths.

Sex-biased ORs were identified from male and female *E. postvittana* antennae using qPCR and RNAseq gene expression analysis techniques. In these experiments, extensive and elaborate qPCR experiments were conducted initially through which four male-biased and five female-biased OR genes were identified. After completing the qPCR experiments the antennal transcriptomes were generated which allowed for RNAseq expression analysis. As OR gene expression analyses had already been conducted using qPCR, there was not a need to conduct extensive (e.g., replicated) RNAseq analyses. The same sex-biased OR genes were identified using both techniques, suggesting that in future studies RNAseq may be a sufficient technique for identifying candidate genes for further study, depending on statistical requirements.

As discussed in Chapter 2, excluding *E. postvittana* ORs, all known male-biased lepidopteran ORs fall within the so-called "male-biased" or "pheromone receptor" clade of olfactory receptors. Two of the male-biased *E. postvittana* OR genes, EposOR30 and EposOR34, do not group with those in the "male-biased" or "pheromone receptor" clade, representing the first known male-biased OR genes that are not related to this group. This finding not only challenges the use of the term "male-biased" as a descriptor of this group, but also should draw focus away from OR genes within this group as the putative receptors for odorant compounds that may have male-biased ecological importance. Furthermore, only one of the PRs identified in this study displayed male-biased antennal expression, while two were equally expressed between the sexes. These results suggest that the current belief that receptors for female-produced pheromone components should show male-biased expression patterns might not always be true and caution should be exercised when using this characteristic to identify candidate PR genes.

The development of the HEK293 cell-based functional assay will offer the field a new method for conducting functional studies on insect olfactory receptors. As discussed in chapter three, currently available *in vitro* heterologous expression systems have inherent characteristics

that impose limits on their efficiency and effectiveness. The HEK293 system, while not perfect, provides the opportunity to functionally characterize receptors in a small scale, relatively high-throughput manner, which opens the door to previously impractical experimentation from both fundamental and applied science perspectives. For example, while it is currently thought that OBPs and ODEs have a role in olfactory reception due to their ability to bind or degrade odorants in *in vitro* systems, their exact role remains speculative due to our inability to recapitulate the complete system in functional assays. While certainly not as complete as the moth antenna, HEK293 cells offer the ability to have stable, regulated expression of multiple genes, providing the opportunity to incorporate other molecular entities, such as ODEs, into functional assays with ORs. Cells can easily be made which stably express Orco, an OR and a secreted ODE. Functional studies could then be conducted in which odorant-induced OR activation in the presence of ODEs was analyzed with and without OBP-mediated odorant delivery. Similarly, the potential role of sensory neuron membrane proteins (SNMPs) could be examined by generating cell lines that stably express Orco, SNMPs and PRs and using them to evaluate differences in selectivity and sensitivity of PR responses following OBP-mediated pheromone delivery. To date, these types of studies have never been reported, most likely because other *in vitro* functional assay systems are not capable of performing these experiments in an efficient and effective manner.

Finally, as discussed in Chapter 4, two of the PRs that were identified responded to components of the *E. postvittana* pheromone blend as well as to compounds that act as behavioral antagonists. The third receptor responds to compounds that are not part of the moth's pheromone blend as well, but the effects of these compounds on moth behavior have not yet been tested. Because no PRs were identified that specifically responded to behavioral agonists or behavioral antagonists alone, it is unclear how behavioral responses to PR activation are mitigated in *E. postvittana*. While further work needs to be done to validate these results, these data suggest that a previously undescribed mechanism for mitigating behavioral responses to ligand-induced receptor activation may exist in certain moths.

Towards the development of olfactory-based pest control technologies

The primary goal of this PhD research was to identify pheromone receptors from the horticultural pest moth, *Epiphyas postvittana,* so they could be evaluated as potential targets for olfactory-based pest control technologies. The ultimate goal of the long-term project is to identify compounds that somehow interfere with the interaction between the moth's pheromones and pheromone receptors; theoretically, by interfering with the male moth's ability to detect the

pheromone plume of conspecific females, reproduction rates would be affected and population numbers of the pest could be controlled. A secondary goal of this research was to develop a heterologous expression system for functional characterization of insect olfactory receptors that was compatible with modern high-throughput screening platforms. Succeeding in achieving both of these goals has not only opened the door for the development of olfactory-based pest control technologies for the control of *E. postvittana*, but also provided a tool that should help anyone interested in conducting high-throughput screening of insect olfactory receptors.

In these studies, three different pheromone-responsive ORs were identified from *E. postvittana*. Using the HEK293 expression system, these receptors were screened against large panels of pheromones in order to identify agonistic compounds, demonstrating that the HEK293 expression system is suitable for the expression and functional testing of lepidopteran ORs in a relatively high-throughput fashion. Furthermore, investigations into E11-14:Ac and Z11-14:Ac inhibition of EposOR6 activation (Chapter 4) provide proof of principle that the responsiveness of PRs can be inhibited *in vitro*, laying the foundation for high-throughput screening campaigns designed to identify inhibitory compounds that target *E. postvittana* PRs.

The HEK293 assay system will aid in the development of novel, olfactory-based pest control technologies in ways beyond identifying compounds that target ORs. As mentioned above, the HEK293 system allows for the practical incorporation of various other olfactory-related molecular entities, such as SNMPs, OBPs and ODEs, into an *in vitro* functional assay. This feature provides the opportunity to screen for compounds that affect pheromone reception by targeting these other molecules. For example, it is theoretically feasible to produce HEK293 cells that stably express the insect OR co-receptor, a pheromone receptor and a secreted odorant degrading enzyme and to use these cells in functional assays to screen for compounds that interfered with the enzyme's ability to degrade the pheromone compound. Similarly, this system could be used in a high-throughput format to identify compounds that inhibited the ability of OBPs to carry pheromones or other odorants through the sensillum lymph. While the exact roles of OBPs and ODEs in pheromone reception are not clear, they are known to be involved (see Chapter 4), and interfering with their roles in this capacity could possibly affect pheromone reception enough to affect the moth's pheromone perception. This HEK293 expression system will offer investigators the opportunity to pursue these previously impractical research questions.

Future work on E. postvittana

During the course of this PhD research a tremendous amount of information was produced regarding the molecular entities involved in olfaction in *E. postvittana*. From this information, several candidate pheromone receptors were identified, some of which were found to respond to components of the moth's pheromone blend when tested in functional assays. While this research helped to answer several questions regarding pheromone reception in *E. postvittana*, it raised many more during the process. However, a foundation has now been laid which will allow the next investigators to pursue these research questions, as well as to explore previously un-addressable research questions relating to insect olfaction.

In HEK293 experiments, no receptors were identified that responded to the fourth component of the *E. postvittana* pheromone blend, E11-16:Ac. It's possible that the receptor for this pheromone compound was indeed one of the receptors that were tested, yet for some reason it did not respond in these experiments. Candidate PR gene expression was only verified in HEK293 cells at the mRNA level, not the protein level, and it's possible that certain genes are not expressed well in this system (see Chapter 4). Further work is needed to verify that candidate PR protein is expressed at sufficient levels in HEK293 cell membranes to gain confidence in negative results obtained using this system. If certain genes are not found to express well in HEK293 cells, these genes should be tested for response to pheromone compounds using an alternative *in vitro* expression system.

In these studies, the solubility of the compounds that were tested was completely ignored and it's possible that a receptor for E11-16:Ac was not identified in these experiments because of inherent differences in solubility of this compound compared to other pheromone components. In theory, the HEK293 cells expressing the receptors were exposed to the exact same concentration of each of the compounds in screening experiments, however it is possible, and perhaps likely, that certain compounds were not tested at the theoretical concentrations. While various structural groups of compounds (i.e., saturation, functional group) displayed activity in these experiments, and thus proved to be in solution, future work should include measurements of true compound concentration in the buffers used in this assay system.

All three of the PRs that were identified in these studies responded to structurally related pheromone compounds in the HEK293 assay system. Two of these receptors, EposOR1 and EposOR6, were also tested in *Xenopus* oocytes, however a thorough comparison of the response profiles of the receptors between the two systems was not possible; only preliminary experiments could be conducted due to very limited access to the oocyte assay system. In these experiments oocytes expressing each receptor were tested for a response to compounds

identified in HEK293 assays, however one compound, (Z,E)9,12-14:OAc, was not available for use in these experiments. Furthermore, screening experiments were not conducted on the third *E. postvittana* PR, EposOR45, in the oocyte system. More work is required on testing the EposPRs in the oocyte system to complete the screening experiments for all receptors and compound combinations, as well to conduct concentration-response studies on all compounds that elicit responses in screening experiments. Upon completion, PR response profiles will be able to be compared between the two systems. In addition to validating the results obtained for the receptors using HEK293 cells, it will be interesting to see if there are any discrepancies between the two systems, such as differences in receptor selectivity or sensitivity to ligands.

Using HEK293 cells expressing EposOR6 it was found that receptor activation with either E11-14:Ac or Z11-14:Ac prevented further stimulation by either compound, which prompted further experimentation designed to investigate the inhibitory effect of pheromone treatment on PRs. This effect was also observed in *Xenopus* oocytes, however, only retrospectively, because at the time that oocyte experiments were being conducted this phenomenon had not yet been observed in HEK293 cells. In experiments using EposOR1 and EposOR6 it was necessary to deliver compounds to oocytes in order of increasing magnitude of response. Stimulation of a PR-expressing oocyte with a pheromone decreased the magnitude of or completely inhibited the response of the same oocyte to subsequent stimulation by the same dose of a different compound or a higher dose (once past a certain threshold) of the same compound. Further experiments should be done in which EposOR6 is re-tested in the oocyte system in experiments designed to evaluate the inhibitory effect of stimulation of the receptor by activating ligands, in a way similar to that done in the HEK293 system. Furthermore, using both assay systems, additional experiments should be designed to test whether this phenomenon occurs with other PRs.

The responses of PRs obtained using the HEK293 and oocyte assay systems should be related back to what is observed *in vivo*. Theoretically, using modern electrophysiological techniques such as single-sensillum recordings, neurons can be identified that respond to each of the *E. postvittana* pheromone components. Based on the HEK293 assay results, there should be a neuron that responds to (E,E)9,11-14:Ac, a neuron that responds to E11-14:Ac and (E,E)9,11-14:Ac, and a neuron that responds to E11-14:OH. Once these neurons were identified, they could be tested for activation by the other compounds that were found to stimulate the PRs in *in vitro* assays, either confirming or refuting the results obtained in the HEK293 and *Xenopus* oocyte assay systems.

In order to address the hypothesis that was put forth in Chapter 4, two different types of experiments should be conducted: One, a set of *in vivo* electrophysiological experiments in which neurons that respond to E11-14:Ac and/or Z11-14:Ac are identified and repeatedly stimulated by

the two pheromone components. Any inhibitory effects of pheromone stimulation upon the ability of the receptor to subsequently be re-activated should be detectable through careful examination of neuronal firing rate and spike amplitude. And two, the *E. postvittana* pheromone degrading enzymes need to be identified and evaluated for their ability to degrade E11-14:Ac and Z11-14:Ac. *In vitro* enzyme-kinetics experiments could then be conducted in which the degradation rates of pheromone compounds by these enzymes could be compared, and *in situ* expression analyses could be conducted to determine the localization of these enzymes in conjunction with E11-14:Ac and Z11-14:Ac responsive receptors.

All of the functional work that was conducted during these studies was focused on identifying and characterizing the *E. postvittana* pheromone receptors. In total, only 10 of the 70 OR genes that were identified were tested in functional assays, and of those 10, only three were found to respond to pheromone compounds. The full-length sequences are known for the vast majority of the remaining 67 'orphan' receptors, and a proven method for their expression and functional testing is now in place. While it would require a significant amount of work to clone and test all of these genes, functionally characterizing the moth's entire olfactory repertoire would provide an invaluable resource to the field. It would be incredibly useful to understand the spectrum of odorants that are detectable, from the more molecular-biological perspective of an individual OR through to the more ecological perspective of the entire organism. Similarly, 173 other proteins, including IRs, GRs, carrier proteins and degrading enzymes, were identified from the antennal transcriptomes, and, as the full-length sequence of most of these genes are known, they too can begin to be functionally characterized in an effort to unravel the complex olfactory system of *E. postvittana*.