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Functional characterization of pheromone receptors in the tobacco budworm *Heliothis virescens*

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Abstract

Functional analyses of candidate *Heliothis virescens* pheromone odorant receptors (HvORs) were conducted using heterologous expression in *Xenopus* oocytes. HvOR6 was found to be highly tuned to *Z*9-14:Ald, while HvOR13, HvOR14 and HvOR16 showed specificity for *Z*11-16:Ald, *Z*11-16:OAc and *Z*11-16:OH, respectively. HvOR15, which had been considered a candidate receptor for *Z*9-14:Ald did not respond to any of the pheromone compounds tested, nor to 50 other general odorants. Thus, while HvOR15 is specifically expressed in *H. virescens* male antennae, its role in pheromone reception remains unknown. Based on our results and previous research we can now assign pheromone receptors in *H. virescens* males to each of the critical *H. virescens* agonistic pheromone compounds and two antagonistic compounds produced by heterospecific females.

Keywords: odorant receptors, pheromone receptors, *Heliothis virescens*, *Xenopus oocytes*.

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Introduction

The ability of male moths to perceive and respond to extremely low concentrations of volatile chemicals in the sex pheromone emissions of conspecific females has been studied for a considerable period of time (Karlson & Butenandt, 1959; Roelofs, 1995). Indeed, this high sensitivity has made moth sexual communication a model system for understanding the mechanisms of animal sensory perception at the molecular level (Rutzler & Zwiebel, 2005; Benton *et al.*, 2006; Touhara & Vosshall, 2009; Kaupp, 2010).

Major findings in a number of insect chemosensory systems over the past decade have added greatly to our understanding of pheromone processing by male moths (Rutzler & Zwiebel, 2005; Sato *et al.*, 2008; Wicher *et al.*, 2008). Arguably, the most advanced work in this area has been focused on the silkworm moth *Bombyx mori*, which has a relatively simple pheromone composed mostly of *E*10*Z*12-16:OH (bombykol) and about 10% *E*10*Z*12-16:Ald (bombykal) (Kaissling & Kasang, 1978). Pheromone-responsive sensilla in the male moth antennae house two olfactory receptor neurons (ORNs) per sensillum, one of which expresses the seven-transmembrane *B. mori* odorant receptor (BmOR), BmOR1, that reversibly binds to, and is activated by bombykol, resulting in male attraction (Sakurai *et al.*, 2004). A distinct receptor, BmOR3, is expressed in the second ORN and binds bombykal which inhibits male behavioural response (Nakagawa *et al.*, 2005). Additional studies of *B. mori* have examined other molecular aspects of pheromone reception such as the roles of specific odorant-binding proteins (Grater *et al.*, 2006; Zhou *et al.*, 2010), sensory neuron membrane proteins (SNMPs, Rogers *et al.*, 1997), and an atypical, evolutionarily conserved receptor-like transmembrane protein, BmOR2, which is coexpressed with odorant receptors (ORs) and acts as a co-receptor to enhance OR function (Nakagawa *et al.*, 2005). The existence of a high-quality genome sequence of *B. mori* has facilitated these efforts (Xia *et al.*, 2008).

A second moth species for which there is considerable information regarding pheromone reception and response

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is *Heliothis virescens*, the tobacco budworm. This species has a more complex sex pheromone blend and fewer genomic tools than *B. mori*. Nevertheless, a series of researchers has succeeded in providing insight into neuronal activation by the diverse pheromonal compounds (Berg *et al*., 1998; Vickers & Christensen, 2003; Baker *et al*., 2004) and identified and characterized several pheromone-binding proteins (PBPs) and receptors for some of the compounds (Krieger *et al*., 1993, 2002, 2005, 2009; Rogers *et al*., 2001; Grosse-Wilde *et al*., 2007; Kurtovic *et al*., 2007).

Sex pheromone glands of female *H. virescens* produce at least seven compounds, not all of which have been found to affect male behaviour (Groot *et al*., 2009). The pheromone compound with the highest titer is *Z*11-16:Ald (see full names in Experimental Procedures), which together with *Z*9-14:Ald must be present to elicit oriented male flight behaviour (Vetter & Baker, 1983). *Z*11-16:OH is produced by the pheromone gland of *H. virescens* and by its congener *Heliothis subflexa.* This compound inhibits the response of males when they are exposed to relatively high concentrations in a pheromone plume (Vetter & Baker, 1983). However, lower amounts of this alcohol in combination with *Z*11-16:Ald and *Z*9-14:Ald enhance attraction of *H. virescens* males (Ramaswamy *et al*., 1985). Lastly, *Z*11-16:OAc which is produced by *H. subflexa* and not by *H. virescens* females (Groot *et al*., 2009), inhibits responses of male *H. virescens* to an otherwise attractive pheromone blend (Vetter & Baker, 1983). Field studies have shown that female *H. subflexa* genetically manipulated to produce smaller than normal amounts of this acetate attract more interfering *H. virescens* males than typical *H. subflexa* females (Groot *et al*., 2006).

Electrophysiological recordings from single male sensilla have demonstrated that there are three basic types of pheromone-responsive sensilla in male antennae (Baker *et al*., 2004; Gould *et al*., 2010). One of the two ORNs in an A-type sensillum is activated by *Z*11-16:Ald. B-type sensilla are most sensitive to *Z*9-14:Ald. One neuron in the C-type sensilla is activated by *Z*11-16:OAc, and a second neuron in the C-type sensilla is activated by *Z*11-16:OH.

At a molecular level, genes encoding some *H. virescens* ORs (e.g. HvOR11, HvOR13, HvOR14, HvOR15 and HvOR16) are preferentially expressed in *H. virescens* male antennae (Krieger *et al*., 2004, Vasquez *et al*., 2010). Furthermore, *in situ* hybridizations (Grosse-Wilde *et al*., 2007; Krieger *et al*., 2009) demonstrate that *HvOR11* and *HvOR13* gene expression is associated with A-type sensilla, while *HvOR14* and *HvOR16* expression is apparently co-localized to C-type sensilla (Grosse-Wilde *et al*., 2007; Baker, 2009). Heterologous expression experiments with these genes in a modified HEK293 cell line (Grosse-Wilde *et al*., 2007) indicate that the primary ligands of HvOR13, HvOR14 and HvOR16 are *Z*11-16:Ald, *Z*11-16:OAc and *Z*11-16:OH, respectively. Heterologous expression of HvOR13 in *Drosophila* further confirmed that *Z*11-16:Ald is a ligand of this receptor (Kurtovic *et al*., 2007). Taken together, these findings provide functional information on the bulk of the pheromone-sensing HvORs with the exception of the receptor(s) associated with B-type sensilla that are involved in the response to the critical *H. virescens* pheromone compound, *Z*9-14:Ald.

A recent study demonstrating that the differential response of *H. virescens* and *H. subflexa* to *Z*9-14:Ald genetically maps to a chromosomal location that includes both *HvOR6* and *HvOR15* (Gould *et al*., 2010) predicts that *Z*9-14:Ald is the ligand of at least one of these two HvOR proteins. Data from Grosse-Wilde *et al*. (2007) and a broader analysis by Baker (2009) suggested that the best candidate receptor for *Z*9-14:Ald would be HvOR15 because it is preferentially expressed in male antennae and is not co-localized with any of the other pheromone receptors. HvOR6, however, was considered a less likely candidate receptor for *Z*9-14:Ald based on its expression levels in antennae of both sexes as well as expression in both the abdomen and proboscis (Krieger *et al*., 2004; Baker, 2009). The relevance of non-antennal expression is somewhat questionable in light of studies detailing ovipositor expression of HvOR13 (Widmayer *et al*., 2009) and labellum-based chemosensory activity in both *Manduca sexta* (Kent *et al*., 1986) and *Anopheles gambiae* (Kwon *et al*., 2006).

The goal of the current study was to identify the receptor for *Z*9-14:Ald and to further characterize the functional properties of HvOR11, HvOR13, HvOR14, and HvOR16. To achieve this, we cloned full-length *H. virescens* cDNAs encoding candidate pheromone receptors and the highly conserved, non-conventional DmOR83b-like HvOR2 directly from *H. virescens* male antennae. We then functionally characterized these receptors *in vitro* using heterologous expression in *Xenopus* oocytes and two-electrode, voltage-clamp physiological recordings. This system has been successfully employed in functional studies of numerous insect ORs as well as several putative pheromone receptors (Nakagawa *et al*., 2005; Mitsuno *et al*., 2008; Miura *et al*., 2009; Wang *et al*., 2010), providing a basis for its use here to characterize pheromone receptors in *H. virescens*.

Results

HvOR6 is a specific receptor for the critical pheromone compound, Z9-14:Ald

Based on the analyses of Grosse-Wilde *et al*. (2007) and Baker (2009), HvOR6 and HvOR15 were considered as

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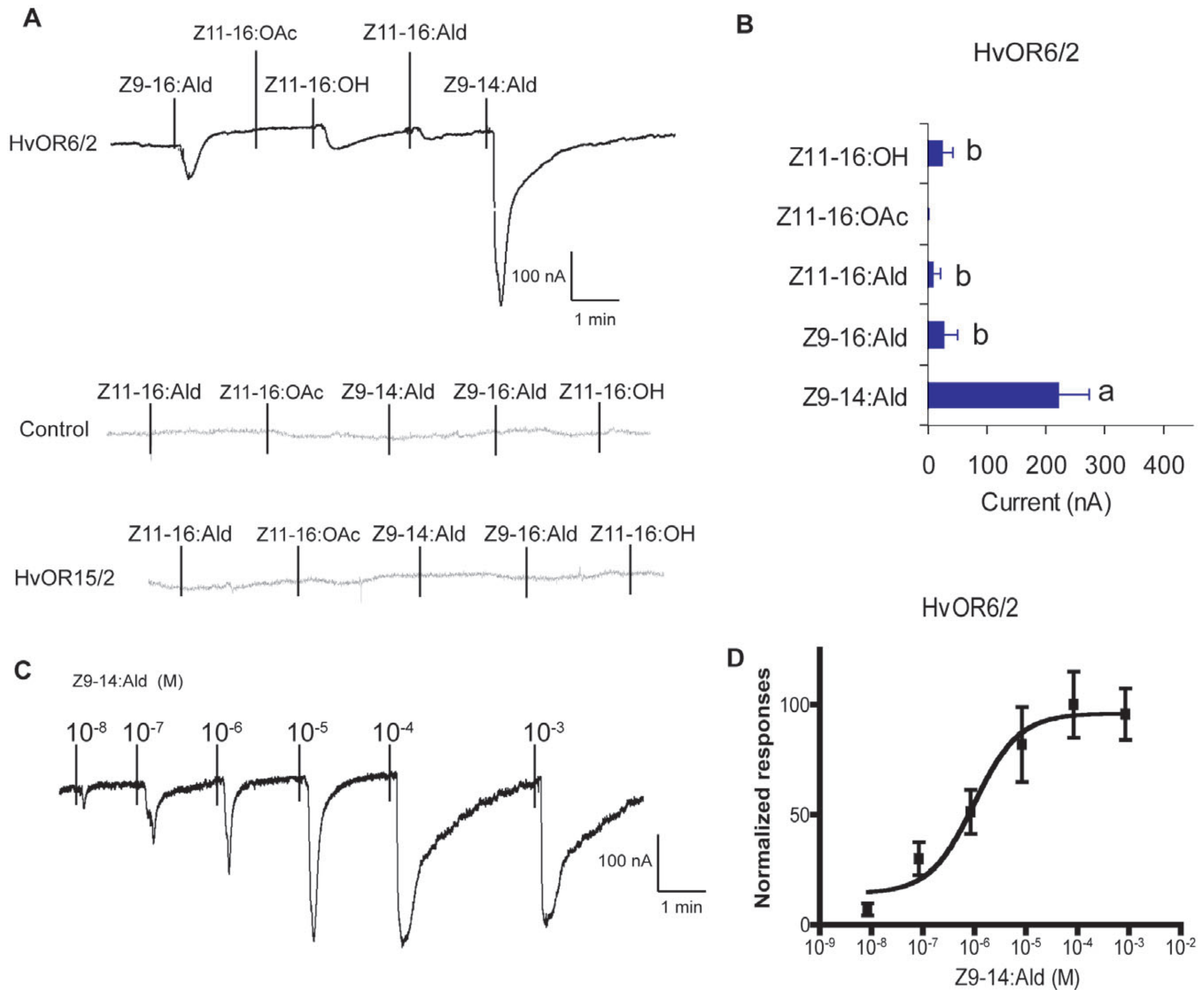

Figure 1. Responses of *Xenopus* oocytes with co-expressed HvOR6/HvOR2 or HvOR15/HvOR2 to stimulation with pheromone compounds. (A) (*Upper*) Inward current responses of HvOR6/HvOR2 *Xenopus* oocytes in response to 10^{-4} M solution of pheromone compounds. (*Middle*) Buffer-injected *Xenopus* oocytes fail to respond to any of the pheromone stimuli. (*Lower*) HvOR15/HvOR2 *Xenopus* oocytes fail to respond to any of the tested pheromone stimuli. (B) Response profile of HvOR6/HvOR2 *Xenopus* oocytes. Error bars indicate SEM (n = 7). (C) HvOR6/HvOR *Xenopus* oocytes stimulated with a range of *Z*9-14:Ald concentrations. (D) Dose–response curve of HvOR6/HvOR2 *Xenopus* oocytes to *Z*9-14:Ald. Responses are normalized by defining the maximal response as 100. $EC_{50} = 9.79 \times 10^{-7}$ M. Error bars indicate SEM (n = 6).

candidate receptors for *Z*9-14:Ald. HvOR15 and HvOR2 (hereafter HvOR15/2), when co-expressed in *Xenopus* oocytes, did not facilitate cellular responses to any of the five candidate pheromone components at a concentration of 10^{-4} M (Fig. 1A, lower). Furthermore, no responses were elicited in similar tests with 14:Ald, 16:Ald, 16:OAc, 16:OH, Z7-16:Ald, Z7-16:OAc, Z9-16:OAc, Z9-14:OH, Z9-16:OH (data not shown), nor to 50 other general odorants across a range of chemical classes (Supplementary Table S1).

In contrast, oocytes co-expressing HvOR6/2 robustly responded to 10^{-4} M *Z*9-14:Ald and had minimal response to the other four candidate pheromone compounds (Fig. 1A upper, B) and Z9-16:OH (~30 nA, data not shown). In dose–response studies, even the lowest concentration of *Z*9-14:Ald, 10^{-8} M, elicited measurable responses from oocytes with co-expressed HvOR6/2; the EC_{50} of HvOR6/2 responses to *Z*9-14:Ald was 9.79×10^{-7} M (Fig. 1C, D). No responses were elicited by concentrations of 10^{-4} M of 14:Ald, 16:Ald, 16:OAc, 16:OH, Z7-16:Ald, Z7-16:OAc, Z9-16:OAc, Z9-14:OH (data not shown).

HvOR13 is tuned to the major pheromone component, Z11-16:Ald

Xenopus oocytes co-expressing HvOR13 and HvOR2 (hereafter referred to as HvOR13/2) responded robustly

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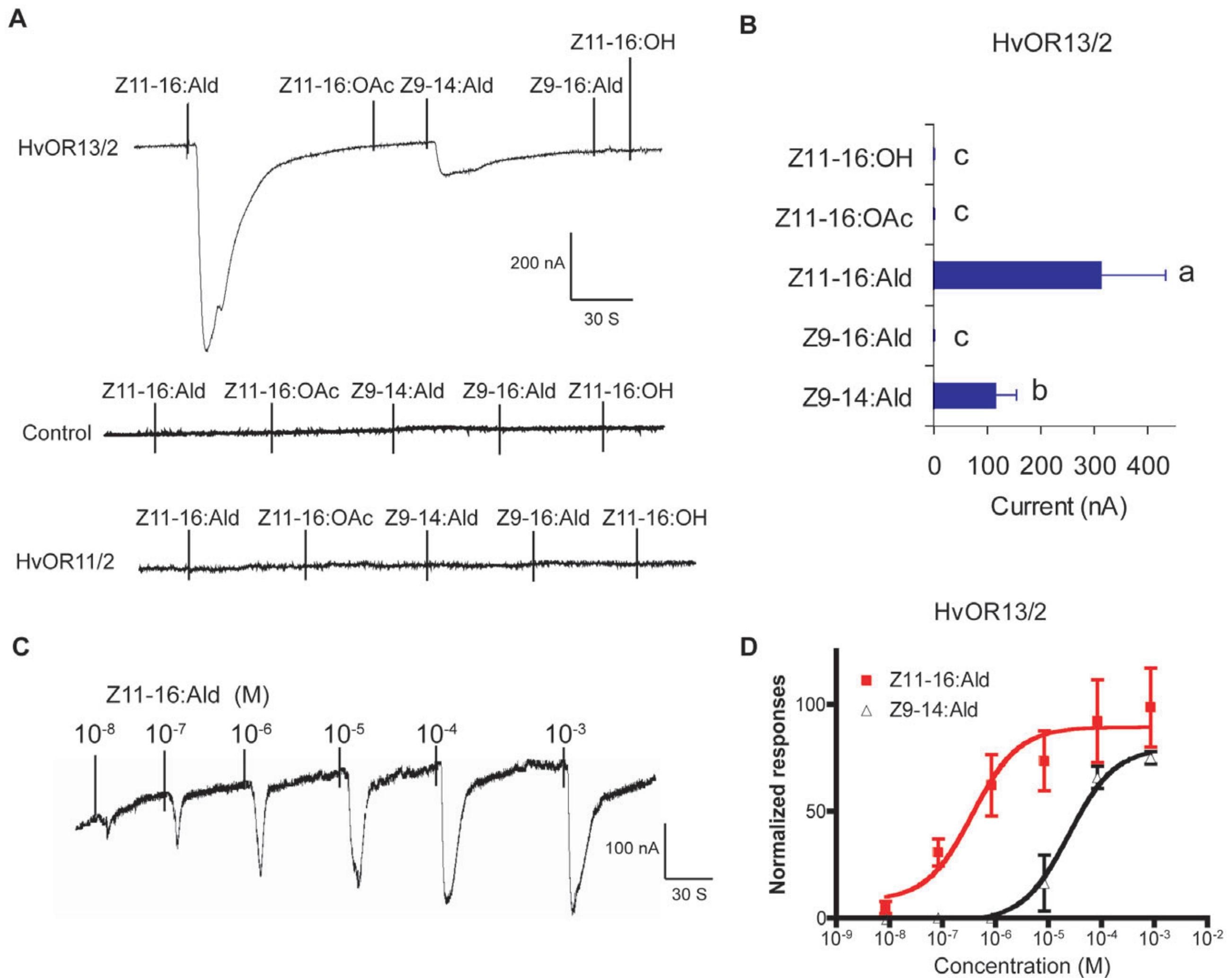

Figure 2. Responses of *Xenopus* oocytes with co-expressed HvOR13/HvOR2 or HvOR11/HvOR2 to stimulation with pheromone compounds. (A) (*Upper*) Inward current responses of HvOR13/HvOR2 *Xenopus* oocytes in response to 10^{-4} M of pheromone compounds. (*Middle*) Buffer-injected oocytes fail to respond to any of the five pheromone stimuli. (*Lower*) HvOR11/HvOR2 oocytes fail to respond to any of the tested pheromone compounds. (B) Response profile of HvOR13/HvOR2 *Xenopus* oocytes. Error bars indicate SEM (n = 6). (C) HvOR13/HvOR2 *Xenopus* oocytes stimulated with a range of *Z*11-16:Ald concentrations. (D) Dose–response curve of HvOR13/HvOR2 *Xenopus* oocytes to the two pheromone components *Z*11-16:Ald and *Z*9-14:Ald. Responses are normalized by defining the maximal response as 100. *Z*11-16:Ald $EC_{50} = 3.67 \times 10^{-7}$ (n = 7) and *Z*9-14:Ald $EC_{50} = 2.40 \times 10^{-5}$ (n = 4). Error bar indicates SEM.

to *Z*11-16:Ald and to a much lesser extent to *Z*9-14:Ald at a concentration of 10^{-4} M (Fig. 2A upper). In these studies, responses evoked from *Z*11-16:Ald had a mean amplitude of 311 nA in HvOR13/2-expressing oocytes compared with only 114 nA for *Z*9-14:Ald (Fig. 2B). Control oocytes injected with buffer alone were unresponsive to any of the five compounds (Fig. 2A middle). To further delineate the differential responses of HvOR13/2 to these two ligands, we conducted dose–response studies with *Xenopus* oocytes expressing HvOR13/2. Oocytes responded to *Z*11-16:Ald even at 10^{-8} M, the lowest concentration tested, and the EC_{50} to *Z*11-16:Ald was 3.67×10^{-7} M (Fig. 2C, D). In contrast, the response to *Z*9-14:Ald was two orders of magnitude lower than to *Z*11-16:Ald, with an EC_{50} of 2.40×10^{-5} M (Fig. 2D).

In as much as HvOR11 and HvOR13 share a high degree of amino acid similarity and are expressed in the same sensilla (Krieger *et al.*, 2004, 2009), HvOR11 had been considered a candidate pheromone receptor. However, in agreement with results of experiments using HEK293 cells (Krieger *et al.*, 2009), when HvOR11 and HvOR2 were co-expressed in *Xenopus* oocytes they failed to respond to any of the five candidate pheromone compounds (Fig. 2A, lower). Similar tests with 14:Ald, 16:Ald, 16:OAc, 16:OH, *Z*7-16:Ald, *Z*7-16:OAc, *Z*9-16:OAc, *Z*9-14:OH and *Z*9-16:OH also failed to elicit any response (data not shown).

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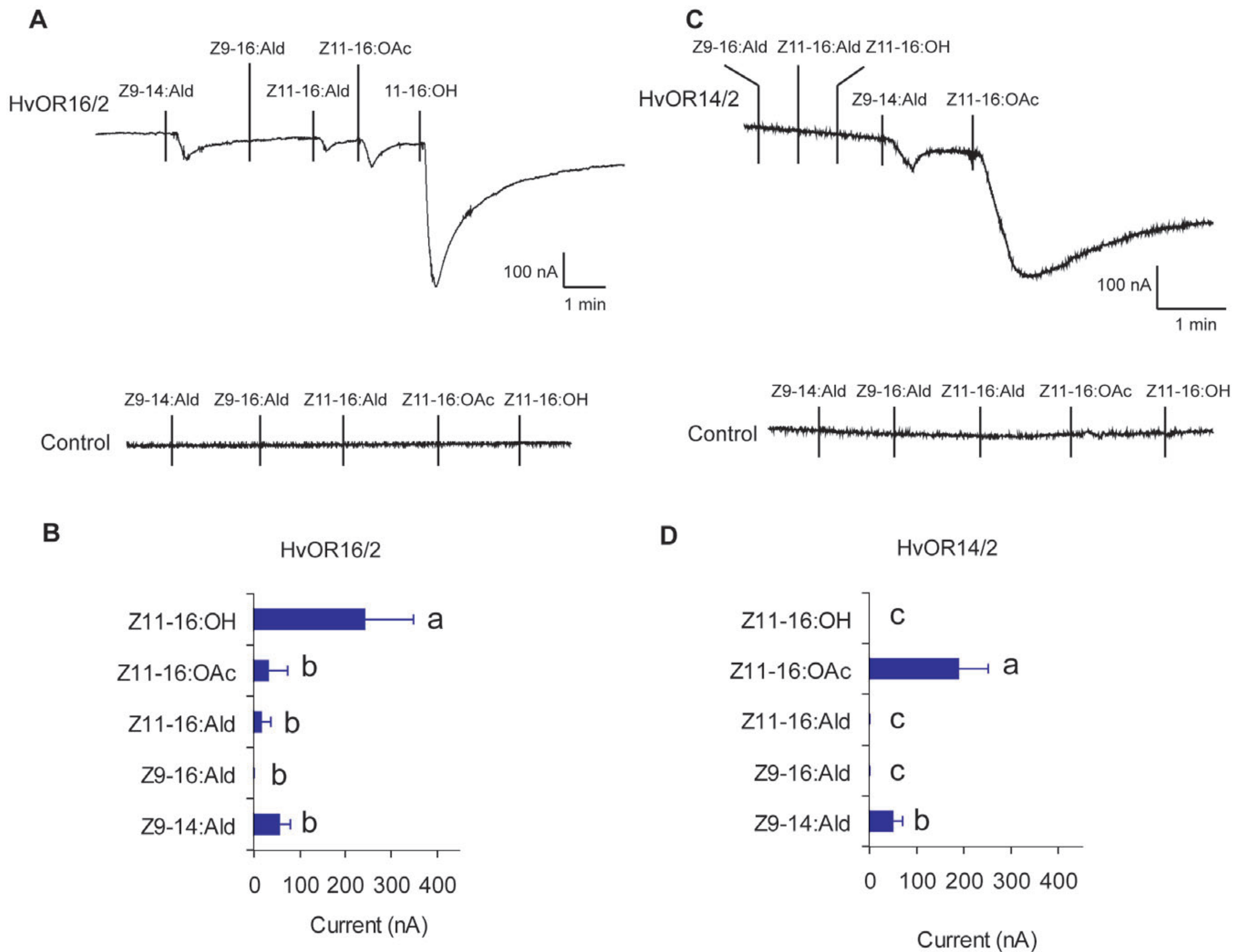

Figure 3. Responses of *Xenopus* oocytes with Co-expressed HvOR14/HvOR2 or HvOR16/HvOR2 to stimulation with pheromone compounds. (A) (*Upper*) Inward current responses of HvOR16/HvOR2 *Xenopus* oocytes in response to 10^{-4} M of pheromone compounds. (*Lower*) Buffer-injected oocytes fail to respond to any of the pheromone compounds. (B) Response profile of HvOR16/HvOR2 *Xenopus* oocytes. Error bars indicate SEM ($n = 9$). (C) (*Upper*) Inward current responses of HvOR14/HvOR2 *Xenopus* oocytes in response to 10^{-4} M of pheromone compounds. (*Lower*) Buffer-injected oocytes fail to respond to any of the five *Heliothis* pheromone compounds. (D) Response profile of HvOR14/HvOR2 *Xenopus* oocytes. Error bars indicate SEM ($n = 7$).

HvOR14 and HvOR16 are, respectively, tuned to Z11-16:OAc and Z11-16:OH

Heterologous expression of HvOR14 and HvOR16 in a modified HEK293 cell line indicated that these proteins could act as receptors for *Z*11:16:OAc and *Z*11-16:OH, respectively (Grosse-Wilde *et al*., 2007). As shown in Fig. 3A, B, co-expression of HvOR16/2 in *Xenopus* oocytes lead to robust responses to *Z*11-16:OH and only modest responses to *Z*9-14:Ald, *Z*11-16:Ald and *Z*11-16:OAc at a concentration of 10^{-4} M. There was notably no response in HvOR16/2-expressing oocytes when exposed to this high concentration of *Z*9-16:Ald.

HvOR14/2-co-expressing oocytes responded strongly to *Z*11-16:OAc at a concentration of 10^{-4} M while displaying a much smaller response to *Z*9-14:Ald and no response to any of the other three pheromone compounds tested (Fig. 3C, D).

Discussion

The present data indicate that HvOR6, and not HvOR15, acts as a pheromone receptor for *Z*9-14:Ald. This is somewhat surprising because past studies have shown that HvOR15 has specific expression in *H. virescens* male antennae, while HvOR6 has been found by reverse transcriptase (RT)-PCR to be expressed in male and female antennae, and is also expressed in the proboscis and abdomen of adults (Krieger *et al*., 2004). Moreover, the observation that in the *Xenopus*-based functional studies reported in the present study, HvOR15 does not appear to

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act as a receptor for any of the active *Heliothis* pheromone compounds or, for that matter, any of 50 other general odorants, leads us to question the role this protein plays in pheromone reception in *H. virescens*.

HvOR13, the receptor for the main compound in the *H. virescens* pheromone blend (*Z*11-16:Ald), is temporally and spatially co-expressed with HvOR11 in cells beneath the same sensilla, but no ligand for HvOR11 has been identified (Krieger *et al*., 2009). Baker (2009) predicted that the ligand for HvOR11 might be (*Z*)-11-hexadecenoic acid (*Z*11-16:COOH), the presumed breakdown product of *Z*11-16:Ald. In single sensillum recording experiments with *H. virescens* antennae, however, *Z*11-16:COOH evoked no neuronal response (Lee, 2006), but it is possible that this acid may have been too hydrophilic to enter the sensilla when it was tested in this manner (Baker, 2009). Following the same logic, (*Z*)-9-tetradecenoic acid (*Z*9-14:COOH) could be the ligand of HvOR15, but this has not yet been tested.

Grosse-Wilde *et al*. (2007) conducted a series of heterologous expression experiments with HvOR13, HvOR14 and HvOR16 that were individually expressed in transformed HEK293 cells. In their experiments, HvOR13-producing cells showed responses but very little specificity for any of the pheromone compounds when the stimuli had been dissolved in 0.1% DMSO. However, when HvOR13-producing HEK293 cells were exposed to *Z*11-16:Ald in the presence of the *H. virescens* PBP, PBP2, the sensitivity for *Z*11-16:Ald significantly increased and the specificity of HvOR13 for *Z*11-16:Ald became apparent. In the absence of PBP2, the EC_{50} value of HvOR13 for *Z*11-16:Ald was approximately 1.2 nM. When the experiment was performed with PBP2 augmentation, the EC50 value of HvOR13 was almost four orders of magnitude lower, at 0.0002 nM. Although this one result in Grosse-Wilde *et al*. (2007) supports the importance of PBPs in male response specificity, it should be noted that PBP1 was not found to enhance receptor specificity and that PBPs did not enhance specificity or sensitivity of other *H. virescens* ORs.

In our experiments with *Xenopus* oocytes, PBPs were not used. However, HvOR2, the *Heliothis* homologue of the non-conventional and obligate *Drosophila* DmOR83b receptor (Larsson *et al*., 2004), was co-injected with the HvORs based on our preliminary finding that HvOR13 did not respond to its ligand, Z11-16:Ald, when HvOR2 was not co-injected. Furthermore, findings of a number of studies indicate that this highly conserved, atypical receptor type enhances the response of other moth pheromone receptors (Sakurai *et al*., 2004; Nakagawa *et al*., 2005) and is required for the functional heterologous expression of a large number of anopheline ORs (Wang *et al*., 2010). In our experiments, oocytes co-expressing HvOR13/HvOR2 responded solely to *Z*11-16:Ald when stimulated with low pheromone concentrations. Even at high concentrations, the oocytes had only a low response to just one additional pheromone compound, *Z*9-14:Ald. This suggests that HvOR13, when co-expressed with HvOR2, can manifest odorant specificity in the absence of PBP2.

Our results do not prove that PBPs are not important in enhancing specificity and/or sensitivity of some *H. virescens* pheromone receptors *in vivo*. Further testing of *H. virescens* pheromones with and without PBPs is certainly needed. It is likely that the relative importance of PBPs versus the ORs themselves as determinants of specificity will vary depending on the specific pheromonal compound and the species tested. Clearly, in the case of *Drosophila melanogaster* the PBP named ‘lush’ is critical for reception of one of its pheromonal compounds, *Z*11-18:OAc, also known as 11-*cis* vaccenyl acetate (Xu *et al*., 2005). Recently, Wanner *et al*. (2010) used the *Xenopus* oocyte system to functionally characterize pheromone receptors in the moth, *Ostrinia nubilalis*. They included the DmOR83b homologue in each of their experiments but did not include PBPs. They found that some pheromone receptors had high specificity while others did not. Perhaps this is a case in which specificity for some, but not all, pheromonal compounds is determined by PBP.

Importantly, our data are consistent with *in vivo* single sensillum recordings from *H. virescens* antennae exposed to pheromone compounds (Baker *et al*., 2004) and with *in situ* hybridization studies on spatial localization of HvOR14 and HvOR16 within the male antennae (Grosse-Wilde *et al*., 2007). The single sensillum recordings found that one ORN in the C-type trichoid sensilla primarily responded to *Z*11-16:OAc, while a second C-type sensillum ORN responded most strongly to *Z*11-16:OH. The *in situ* hybridization studies found that HvOR14 and HvOR16 were expressed in adjacent cells co-localized within the same sensillum. Taken together with the data of Baker *et al*. (2004) and Grosse-Wilde *et al*. (2007), the results reported here support the model that HvOR14 and HvOR16 are the functional pheromone receptors for *Z*11-16:OAc and *Z*11-16:OH, respectively, and are expressed in discrete ORNs that are associated with *H. virescens* C-type sensilla.

To date there have been no published *in situ* hybridization studies carried out to test whether HvOR6 and HvOR15 expression is co-localized. However, the functional data reported in the present study, as well as previously reported single sensillum electrophysiological recordings (Baker *et al*., 2004), suggest that HvOR6 is expressed in B-type sensilla ORNs which respond to *Z*9-14:Ald. Similarly, it is possible that HvOR15 is expressed in the adjacent ORN within the B-type sensillum. The model presented in Fig. 4 summarizes the information that we have generated and how it fits into our

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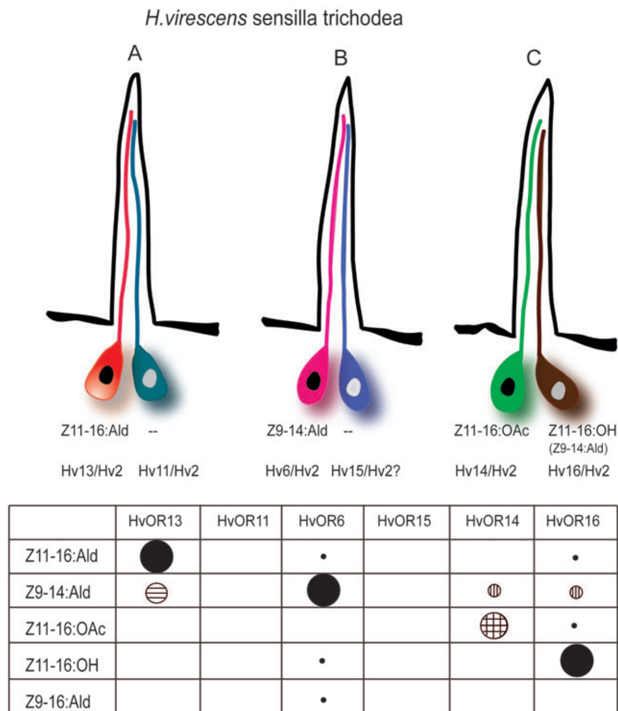

Figure 4. A model depicting receptor–neuron functional associations for *Heliothis virescens* sensilla trichodea. A, B and C indicate three types of sensilla trichodea that are tuned to specific *Heliothis* pheromone components, based on previous single-sensillum recording results (Baker *et al*., 2004); '-' indicates that no ligand has been identified for a specific neuron by single-sensillum recording. (*Bottom*) the response spectra of HvORs to five tested pheromone components (summarized from Figs 1–3, and Grosse-Wilde *et al*., 2007; Baker, 2009; Krieger *et al*., 2004, 2009). The size of circles represents the magnitude of the response of HvORs to candidate pheromone components at a concentration of 10^{-4} M. Filled circles represent a response of '>200 nA', Checkered circles represent '150–200 nA', Horizontally striped circles represent '100–150 nA', Vertially striped circles represent '50–100 nA' and small filled circles represent '0–50 nA'.

understanding of peripheral sensory perception of *Heliothis* pheromones in *H. virescens* males.

Bombyx mori, with its sequenced genome and relatively simple pheromonal system, has served as an excellent model for increasing our understanding of the mechanistic basis for moth sexual communication. The experiments reported in the present study, as well as many previous innovative studies of the *Heliothis* pheromone system, are now moving us ahead in understanding a moth species with a much more complex sexual signalling system than previously thought.

Experimental procedures

Insects

Heliothis virescens larvae were obtained from a laboratory colony (Gould *et al*., 2010). The larvae were reared on an artificial diet (Sheck & Gould, 1995). Pupae were selected by sex and placed in separate test tubes. Male antennae were excised at the base, 3–5 days after eclosion, and were immediately put in RNAlater® RNA stabilization solution (Ambion Inc., Austin, TX, USA) and stored at –80°C until use.

Pheromone components

(*Z*)-11-hexadecenal (*Z*11-16:Ald), (*Z*)-9-tetradecenal (*Z*9-14:Ald), (*Z*)-9-hexadecenal (*Z*9-16:Ald), (*Z*)-11-hexadecenyl acetate (*Z*9-16:OAc), and (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH) (all 93–95% minimum purity) were purchased from Bedoukian (Danbury, CT, USA). Stock solutions (1 M) were prepared in dimethyl sulfoxide (DMSO) and stored at –20°C. Before experiments, the stock solution was diluted in Ringer's buffer (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$ and 5 mM HEPES pH 7.6). 1X Ringer's buffer containing 0.1% DMSO was used as a negative control.

RNA isolation and cDNA synthesis

Total RNA, prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, was used for oligo dT-primed cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen) for the generation of templates for subsequent PCR reactions. Negative control samples with no reverse transcriptase were included in each cDNA synthesis and subsequent PCR analysis.

PCR amplification

Full-length coding sequences of candidate pheromone ORs of *Heliothis virescens* were PCR-amplified from pools of total cDNA prepared from male *H. virescens* antennae using *PfuUltra* High-Fidelity DNA polymerase (Stratagene/Agilent, Cedar Creek, TX, USA). Amplification reactions (25 μl) included 0.25 μl *Pfu* DNA polymerase, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 0.2 mM dNTPs (N = C,G,A,T) and 100 pmol of each primer. All amplification reactions were carried out using a DNA Engine Dyad (MJ Research, Waltham, MA, USA) under the following conditions: 94°C for 2 min; 34 cycles of 94°C for 30 s, 54–58°C for 30 s, 72°C for 1.5 min; and 72°C for 10 min. PCR amplification products were run on a 1.0% agarose gel and verified by DNA sequencing. PCR primers (Supplementary Table S2) were designed based on the nucleotide sequences reported by Krieger *et al.* (2004).

Receptor expression in Xenopus *oocytes and two-electrode, voltage-clamp electrophysiological recordings*

Previously reported protocols (Lu *et al*., 2007; Wang *et al*., 2010) were used for assaying and analysing odorant-induced whole-cell currents recorded from HvOR- and control-injected *Xenopus* oocytes. In brief, full-length coding sequences of HvOR cDNAs amplified from *H. virescens* antenna by RT-PCR were first cloned into pENTR/D-TOPO (Invitrogen) and then subcloned into pSP64DV by means of the Gateway LR reaction. The pSP64DV vector was a Gateway-compatible destination vector converted from pSP64T-Oligo with the Gateway Vector Conversion System (Invitrogen). cRNAs were synthesized from linearized vectors with mMESSAGE mMACHINE SP6 (Ambion). Human Gα15 (a gift from H. Hatt) and *An. gambiae* Gαq RNA was transcribed from pSGEM-Gα15 and pSP64T-Gαq. Mature healthy oocytes

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(stage V–VII) were treated with 2 mg/ml collagenase S-1 in washing buffer (96 mM NaCl, 2 mM KCl, 5 Mm $MgCl_2$, and 5 mM HEPES [pH 7.6]) for 1–2 h at room temperature. Oocytes were later microinjected with 27.6 ng HvORs cRNA and 27.6 ng HvOR2 cRNA. Preliminary tests demonstrated that there was no cellular response to any of the candidate pheromones when microinjecting cRNAs of HvOR13 alone or HvOR13 plus $G\alpha 15$/$G\alpha q$, so HvOR2 cRNA was always co-injected with the candidate pheromone receptor cRNA. After injection, oocytes were incubated for 4–7 days at 18°C in 1X Ringer's solution (96 mM NaCl, 2 m MKCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 5 mM HEPES [pH 7.6]) supplemented with 5% dialysed horse serum, 50 mg/ml tetracycline, 100 mg/ml streptomycin and 550 mg/ml sodium pyruvate. Whole-cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Odorant-induced currents were recorded with an OC-725C oocyte clamp (Warner Instruments, Hamden, CT, USA) at a holding potential of −80 mV. Oocytes were exposed to compounds in ascending order of concentration with an interval between exposures that allowed the current to return to baseline. Data acquisition and analysis were carried out with Digidata 1322A and Pclamp8.2 software (Axon Instruments Inc., Union City, CA, USA). Statistical comparison of responses of oocytes to the candidate ligands was assessed using a single classification ANOVA procedure in JMP 4.0.4. Dose–response data were analysed using GraphPad Prism 4.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2583.2010.01045.x

Table S1. Odor lists used in this study.

Table S2. Primers used to clone full-length HvOR cDNAs.

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Odor Number	Name	CAS #
1	2-acetylthiazole	24295-03-2
2	2-acetylpyridine	1122-62-9
3	2-acetylthiophene	88-15-3
4	acetophenone	98-86-2
5	2-methylphenol (o-cresol)	95-48-7
6	3-methylphenol (m-cresol)	108-39-4
7	4-ethylphenol	123-07-9
8	benzaldehyde	100-52-7
9	3-methyl-2-cyclohexen-1-ol	21378-21-2
10	4-methylcyclohexanol	589-91-3
11	heptanal	111-71-7
12	4-methylphenol (p-cresol)	106-44-5
13	n-valeric acid	109-52-4
14	hexanoic acid	142-62-1
15	acetic acid	64-19-7
16	butyric acid	107-92-6
17	heptanoic acid	111-14-8
18	isovaleric acid	503-74-2
19	nonanoic acid	112-05-0
20	2-ethylphenol	90-00-6
21	2-ethoxythiazole	15679-19-3
22	2-isobutylthiazole	18640-74-9
23	2,4,5-trimethylthiazole	13623-11-5
24	4-methylthiazole	693-95-8
25	2,4-dimethylthiazole	541-58-2
26	4-propyl benzaldehyde	28785-06-0
27	methylbenzoate	93-58-3
28	methyl-2-methylbenzoate	89-71-4
29	ethyl acetate	141-78-6
30	ethyl butyrate	105-54-4
31	ethyl caproate	123-66-0
32	ethyl formate	109-94-4
33	ethyl propinoate	105-37-3

34	isoamylacetate	123-92-2
35	octyl acetate	112-14-1
36	phenethyl acetate	103-45-7
37	2-nonanone	821-55-6
38	3-octanone	106-68-3
39	acetone	67-64-1
40	cyclohexanone	108-94-1
41	1-hepten-3-ol	4938-52-7
42	1-octen-3-ol	3391-86-4
43	6-methyl-5-hepten-2-one	110-93-0
44	methyl salicylate	119-36-8
45	decanal	112-31-2
46	hexanal	66-25-1
47	1-octanol	72-69-5
48	heptane	142-82-5
49	amyl acetate	628-63-7
50	1-hexen-3-ol	4798-44-1

Primer Name	Primer Sequence (5'-3')
HvOR2F	CACCATGATGACCAAAGTGAAGGCCC
HvOR2R	TTACTTGAGTTGTACCAACACCATG
HvOR6F	CACCATGAACTTACGAAAATTCTTATTC
HvOR6R	TCATTCTTCCTTTGTCTGCG
HvOR11F	CACCATGCATCTTGCAGGCAATGC
HvOR11R	TTAAAACGTGCGTAGAAAAGCG
HvOR13F	CACCATGAAAATCCTATCGGACGGT
HvOR13R	TTATTCTTCTTCTGCAACTGTTTTC
HvOR14F	CACCATGACAGGCATACGTGACTT
HvOR14R	TCACTTACTGCGTAGAAAGGTG
HvOR15F	CACCATGACTGGTTTTCGTGATTTCG
HvOR15R	TCACATGCTGCGTAGAAAAGC
HvOR16F	CACCATGGGTCTCCGTCAATTTCT
HvOR16R	ATGGCCACATTCACATACTTC