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HR11 and HR13 Receptor-Expressing Neurons Are Housed Together in Pheromone-Responsive Sensilla Trichodea of Male *Heliothis virescens*

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Abstract

The highly specific recognition of female-released sex pheromones in insects by sensory neurons of the male antenna requires specific receptors. Recently, a small family of related candidate pheromone receptors has been identified for a few moth species. In this study, the candidate pheromone receptor HR11 from *Heliothis virescens* has been characterized. HR11 was found to be expressed in numerous cells located in short and long sensilla trichodea on the male antenna. The HR11 cells are stereotypically arranged in a paired pattern together with HR13 cells, which respond to the major component of the sex pheromone blend. Triple in situ hybridization approaches revealed that each pair of an HR11 cell and an HR13 cell was ensheathed by supporting cells, which express pheromone-binding proteins, thus constituting a structural unit. The paired pattern of HR11/HR13 cells is reminiscent of the pattern described for BmOR-1- and BmOR-3-expressing cells in the antenna of *Bombyx mori*, which respond to bombykol and bombykal, respectively. These results suggest that the ligand for HR11 may be related to the HR13 ligand and furthermore imply that an arrangement of cells expressing related receptor types in the same sensillum may be a general principle in moth pheromone detection systems.

Key words: expression, insect, olfaction, pheromone, receptor

Introduction

To ensure reproduction and survival, many insects employ sophisticated pheromone signaling systems in mate finding behavior. In moths, females release a distinct blend of several pheromone components to attract the males, which possess specialized olfactory neurons on their antenna to detect the signal molecules (Blomquist and Vogt 2003; de Bruyne and Baker 2008). These pheromone-responsive neurons are housed in particularly long hair-like cuticle structures called sensilla trichodea (Steinbrecht and Gnatzy 1984), but also shorter trichoid hairs on the same antenna contain sensory neurons responding to pheromones (Baker et al. 2004; Hillier and Vickers 2007). In several moth species, including *Bombyx mori* (Kaissling et al. 1978), *Antheraea polyphemus* (Meng et al. 1989), and *Manduca sexta* (Kaissling et al. 1989), electrophysiological recordings from sensilla trichodea of male antennae have found pairs of sensory neurons within a single hair each responding to a distinct component of the female sex pheromone blend. Moreover, recent studies have identified pheromone receptors in *B. mori* and demonstrated expression of the bombykol receptor BmOR-1 and the bombykal receptor BmOR-3 in neighboring cells of long sensilla trichodea (Krieger et al. 2005; Nakagawa et al. 2005). Together, these findings support the notion that moth neurons, which respond to a pheromone component, are housed together in special sensilla types and are not associated with neurons responding to general odorants.

On the antenna of male *Heliothis virescens*, different types of long trichoid sensilla (A, B, and C) have been classified according to the responsiveness of sensory cells to different pheromonal compounds (Almaas and Mustaparta 1991; Baker et al. 2004; Hillier and Vickers 2007). The majority of hairs (80%) represent A-type sensilla trichodea housing a neuron that detects the major compound of the female sex pheromone blend, Z-11-hexadecadienal (Z11-16:AL) (Baker et al. 2004). Recently, we have identified genes encoding a small family of candidate pheromone receptors in *H. virescens* that are specifically expressed in cells located in long sensilla trichodea on the male antenna (Krieger et al. 2004; Grosse-Wilde et al. 2007). Analysis of their topographic expression revealed that HR13-expressing cells

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matched the number and location of sensory cells, which respond to the major sex pheromone component in A-type sensilla. Furthermore, functional analysis of the heterologously expressed receptors demonstrated that the HR13 receptor is specifically activated by Z11-16:AL (Grosse-Wilde et al. 2007; Kurtovic et al. 2007). Thus, this receptor type most likely renders a neuron in sensilla trichodea type A responsive to the major sex pheromone component, Z11-16:AL. So far, single sensillum recordings have detected only the Z11-16:AL-responsive neuron in sensilla trichodea type A, although backfill experiments have shown that there is a second sensory neuron in this hair type (Almaas and Mustaparta 1991; Hansson et al. 1995; Berg et al. 1998). To address the question if the second cell in A-type sensilla is a pheromone-responsive cell, we set out to assess if any of the candidate pheromone receptors is expressed in this cell. We have found that the cells that are colocalized with HR13 cells in sensilla trichodea express the candidate pheromone receptor HR11. Thus, our results indicate that A-type sensilla trichodea contain 2 sensory cells, each of which expresses a distinct pheromone receptor type. Although the ligand for this receptor type is still elusive, the results indicate that the combined arrangement of neurons expressing pheromone receptors in the same trichoid sensillum as described for the bombykol and bombykal cells in *B. mori* (Kaissling et al. 1978; Steinbrecht and Gnatzy 1984) may be a basic principle in the moth pheromone detection system.

Materials and methods

Animal rearing and tissue preparation

Heliothis virescens (Lepidoptera, Noctuidae) eggs and pupae were kindly provided by Bayer CropScience, Monheim, Germany. Pupae were sexed and allowed to develop to adults at room temperature. Antennae and thorax tissue were dissected from cold anesthetized animals. To follow the distribution of HR receptors, antennae were cut into fragments. The filamentous antennae of *Heliothis* consist of around 80 flagellar segments (annuli) (Almaas and Mustaparta 1991). Starting from the base, which is connected directly to the head and proceeding to the tip of the antenna, 8 fragments (F1–F8), each consisting of 10 segments, were obtained (Figure 4A). Due to a slight variability in the antennal length, the F8 fragment consisted of 9–12 segments. Fragments F1–F8 were collected separately in reaction tubes cooled by liquid N_2. All tissues were stored at –70 °C until RNA isolation.

To study the time course of HR expression in the late preadult phase, we staged *H. virescens* pupae as described earlier (Picimbon et al. 2001). Briefly, criteria for developmental staging were adapted from morphological characteristics originally described for *M. sexta* and *Lymantria dispar* (Schwartz and Truman 1983; Vogt et al. 1989). Changing pigmentation patterns in wings, proboscis, antennae, and legs were used to stage the pupae relative to the day of adult eclosion (E0) and allowed to distinguish pupal stages corresponding to 5 (E-5), 4 (E-4), 3 (E-3), 2 (E-2), or 1 day (E-1) before eclosion. Antennae from newly eclosed adults (E0) and from the different pupal stages (E-1 to E-5) were dissected, immediately frozen at liquid N_2 temperature and then stored at –70 °C until RNA isolation.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA isolation and cDNA synthesis

Total RNA from male antennae, female antennae, thoraces (from both sexes), male antennal fragments (F1–F8), and male antennae of different developmental stages (E-5 to E0) were isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany) following the supplier's protocol. Poly $(A)^+$ RNA was isolated with oligo $(dT)_{25}$ magnetic dynabeads (Invitrogen) according to the specifications of the manufacturer from 100 μg total RNA of antennae and thoraces or 10 μg of each of the different pupal total RNAs. Final elution of poly$(A)^+$ RNAs was in 30 μL water. For male antennal fragments, total RNAs were directly used to transcribe mRNAs into cDNAs.

The mRNAs from the various tissues were transcribed into cDNAs in a reaction (total volume 35 μL) containing 10 μL poly$(A)^+$ RNA solution or 10 μL of total RNA solution (1 μg RNA) from antennal fragments, 0.6 μL 20 mM deoxynucleotide triphosphate mixture, 1 μL 50 mM $MgCl_2$, 1.5 μL first strand synthesis buffer (250 mM Tris pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 2 μL 100 mM dithiotreitol, 0.3 μL Rnasin (Promega, Mannheim, Germany), 1 μL oligo-dT18 primer (3 pmol), and 0.5 μL Superscript II reverse transcriptase (Invitrogen). The cDNA synthesis was performed for 50 min at 42 °C followed by 15 min at 65 °C.

PCR conditions

One or 2 μL from the cDNA synthesis reactions was employed in 50 μL standard PCRs. The presence of distinct receptor mRNAs in the different tissues was approached using receptor-specific primer pairs (Krieger et al. 2004). Primer pairs designed for HR11 and HR13 spanned at least 1 predicted intron region; this allowed to distinguish PCR products resulting from possible genomic contaminations in the cDNA preparation.

For testing the integrity of the cDNA templates, we applied a primer pair (Krieger et al. 2004) directed against the rl31 gene (Acc. No. AJ298149), which encodes the *H. virescens* homologue of the ribosomal L31 protein (Tanaka et al. 1987). The expected sizes for the PCR products were 331 bp for RL31, 651 bp for HR11, 745 bp for HR13, 499 bp for HR14, 343 bp for HR15, and 475 bp for HR16.

PCR reactions with cDNAs from antennal fragments or from pupal antennae were performed for 1 min 40 s at 94 °C, then 19 cycles with 94 °C for 1 min, 55 °C for 40 s and 72 °C for 1 min, with a decrease of the annealing temperature

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by 0.5 °C per cycle. Subsequently, 19 further cycles at the condition of the last cycling step (45 °C annealing temperature) were run, followed by incubation for 7 min at 72 °C. Using HR15 and RL31 primers, the annealing temperature in the first 19 cycles was decreased from 50 to 40 °C, followed by 19 cycles with 40 °C. PCR products were run on 1.2% agarose gels and visualized by ethidium bromide staining.

In situ hybridization

Antennae were dissected from 1- to 2-day-old moths, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen at –22 °C on the object holder. Cryosections (12 μm) of antennae were thaw mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and air dried at room temperature. Single- or 2-color double in situ hybridizations were performed following protocols reported previously (Krieger et al. 2002, 2004). In 2-color in situ hybridizations, 2 different either digoxigenin (DIG)-labeled or biotin-labeled antisense RNA probes were employed. For visualization of biotin-labeled probes, the TSA kit (Perkin Elmer, Boston, MA), including a streptavidin HRP conjugate and FITC-tyramides as substrate was used. DIG-labeled probes were detected by an anti-DIG alkaline phosphatase-conjugated antibody in combination with HNPP/Fast Red (Fluorescent detection Set, Roche). Sections were mounted using glycerol/PBS 3:1.

Biotin-labeled or DIG-labeled antisense RNAs were generated following recommended protocols using the T3/T7 RNA transcription system (Roche) and linearized recombinant Bluescript plasmids carrying the cDNA for pheromone-binding protein 1 (PBP1) (Krieger et al. 1993), HR11, and HR13 (Krieger et al. 2004). Hybridized sections were analyzed on a Zeiss LSM510 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). Powerpoint (Microsoft) and Photoshop (Adobe systems, San Jose, CA) were used to arrange figures; images were only altered to adjust the brightness or contrast for uniform tone within a single figure.

Results

Expression of HR11 in the male antenna

RT-PCR experiments with cDNAs from male and female antennae of *H. virescens* have demonstrated a male-specific or male-predominant expression for the candidate pheromone receptor types HR11, HR13, HR14, HR15, and HR16, indicative for a specific function in males (Krieger et al. 2004). Moreover, in situ hybridization experiments employing probes for the male-specific receptors have shown that HR14, HR15, and HR16 are expressed in only a very low number of cells on the male antenna and that none of these receptor types is expressed in the cell that is colocalized with HR13 neurons in the same sensillum (Krieger et al. 2004; Grosse-Wilde et al. 2007). Therefore, in search for a receptor that might be expressed in a neuron that neighbors the HR13-expressing cell in long sensilla trichodea type A, the HR11 receptor type was an assessed candidate. First, we set out to visualize and to localize the cells that express HR11 on longitudinal sections through male antennae. The *H. virescens* antennae consist of about 80 segments, carrying the sensory hairs on the leading edge, the surface that is directed forward during flight. The very long pheromone-sensitive sensilla trichodea are only found on the 50 proximal segments of the male antennae; shorter sensilla trichodea are found on all segments (Almaas and Mustaparta 1991). Using labeled HR11 antisense RNA for in situ hybridization experiments on 12 μm longitudinal sections from the male antenna, between 3 and 6 labeled cells were visualized within the area confined by a segment (Figure 1A,B). All labeled cells were located below the surface carrying short (Figure 1A) and long (Figure 1B) sensilla trichodea but were never detected below the opposing scaled side (Figure 1A). Strikingly, the number of HR11-expressing cells and their location under the long pheromone-responsive sensilla trichodea type resembled very much the distribution pattern of cells expressing HR13 (Figure 1C) (Krieger et al. 2004; Gohl and Krieger 2006).

HR11 and HR13 are expressed in neighboring cells in the same sensillum hair

To determine if the HR11 probe may label the HR13 cells or if the cells are colocalized within the same sensillum hair, double in situ hybridization experiments were performed. Employing biotin-labeled HR11 and DIG-labeled HR13 antisense RNA should allow to visualize HR11- and HR13-expressing cells on the same section. As documented in Figure 1D, HR11 and HR13 probes labeled distinct populations of cells. However, the green-labeled HR11 cells were localized in very close proximity to red-labeled HR13 cells; pairs of green- and red-labeled cells appeared in each segment (Figure 1D). The pairing and the close association suggest that HR11 and HR13 cells are located in the same sensillum hair.

This notion obtained further support from double in situ hybridization experiments employing an antisense RNA probe for PBP1 (Krieger et al. 1993). Within a sensillum hair the sensory neurons are surrounded by supporting cells expressing PBPs (Steinbrecht and Gnatzy 1984). Recently, we demonstrated that HR13-expressing neurons are surrounded by supporting cells that express PBP1 and PBP2 (Grosse-Wilde et al. 2007). Probing longitudinal sections of male antennae with PBP1- and HR11-specific probes gave a very similar picture (Figure 2); HR11-expressing cells were accompanied by cells expressing PBP1 (Figure 2A). At higher magnification and analyzing consecutive confocal planes (Figure 2B–E), it is obvious that green-labeled PBP1 cells form a kind of cave around red-labeled HR11 cells. In addition the green-labeled cells encircle an unlabeled space, most likely representing the position of a HR13 cell. In order to scrutinize this notion, triple in situ hybridization experiments were performed applying a biotin-labeled

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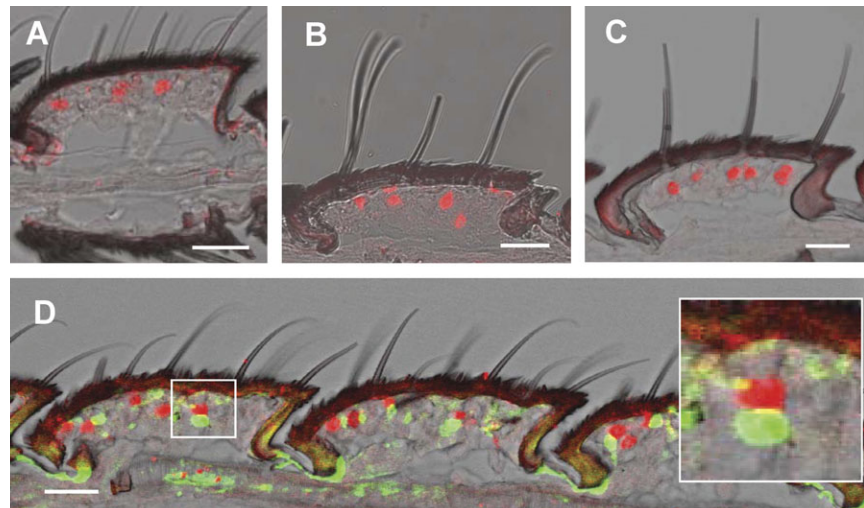

Figure 1 Expression of HR receptor types in the male antenna. **(A–C)** In situ hybridization on longitudinal sections through the antenna using DIG-labeled probes specific for HR11 (A–B) or HR13 (C). Receptor-expressing cells are visualized by red fluorescence. The HR11 probe labeled cells below short (A) and long sensilla trichodea (B). The HR13-specific probe (C) labeled a similar number of cells. **(D)** Double in situ hybridization with a combination of DIG-labeled HR13 and biotin-labeled HR11 antisense RNAs on a longitudinal section of a male antenna. The HR11 and HR13 probes labeled different, but closely associated subpopulations of cells. Several pairs of green (HR11) and red (HR13) cells are located below the antennal surface, which carries sensilla trichodea. The inset shows a higher magnification of the boxed area with 2 adjacent cells expressing HR11 or HR13, respectively. Pictures represent optical sections selected from a stack of confocal images; the red and green fluorescence channels have been overlaid with the transmitted-light channel. Scale bars: 20 μm.

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PBP1 probe and DIG-labeled antisense probes for HR11 and HR13 simultaneously. These experiments visualized neighboring red-labeled receptor-expressing cells, which were surrounded by green-labeled PBP1-expressing cells (Figure 2F–I). Together these results strongly suggest that HR11 and HR13 cells are housed together in the same sensillum hair and are surrounded by supporting cells, which express PBP1.

Expression of HR11 in the female antenna

The RT-PCR analyses of the HR11 and HR13 expression in the antennae of male and female *H. virescens* have revealed, besides very strong bands with cDNAs from males, a weak band with female cDNAs for the HR 11 receptor type (Figure 3A), suggesting that HR11 is also expressed in female antennae. In order to find out whether the observed sex difference may reflect differences in the number of receptor-expressing cells or may result from lower transcript levels in single receptor cells of females, in situ hybridization experiments were performed with antennal sections. These experiments revealed in general only 1 labeled cell per antennal segment (Figure 3B), which was located below short sensilla trichodea; occasionally, 2 or no labeled cells were observed. These results indicate that HR11 is expressed only in a small number of cells in female antennae and explain the faint RT-PCR band from female antennae (Figure 3A).

In accordance with the finding that HR13-expressing cells cannot be detected on the female antenna (Gohl and Krieger 2006), double in situ hybridization experiments with DIG-labeled HR13 and biotin-labeled HR11 antisense probes revealed only green-labeled HR11 cells on female antennal sections (Figure 3C), thus confirming the negative RT-PCR-result for HR13 (Figure 3A).

Receptor expression along the antenna

The expression of HR11 and HR13 receptors in distinct but neighboring subpopulations of olfactory neurons can only be demonstrated by in situ hybridization. Such histochemical studies are limited to a few segments of the male antennae. In order to evaluate if the 2 receptor types are only expressed in certain segments or along the entire length of the antenna, RT-PCR experiments were performed using HR receptor–specific primer pairs and cDNAs prepared from consecutive antennal fragments. The flagellar antenna of *H. virescens*, which consists of ~80 segments (Almaas and Mustaparta 1991), was divided into 8 fragments (F1 = head to F8 = tip) (Figure 4A). Each fragment comprised 10 segments, the last fragment (F8) varied slightly in length (9–12 segments). RT-PCR experiments with cDNAs from the 8 fragments and HR11-specific primers gave a PCR band of the predicted size from F1 t-o F8 indicating the presence of HR11-expressing cells from the base to the tip of the antenna (Figure 4B). Experiments with HR13-specific primers gave a very similar result. The intensities of the PCR bands for the control gene rl31 showed no differences between fractions F1 and F8. The lower intensities of PCR bands for HR13 in F3 and F7 might be explained by slight differences in the efficiency of reverse transcription of HR13 mRNA in these fractions. To verify that HR11- and HR13-expressing

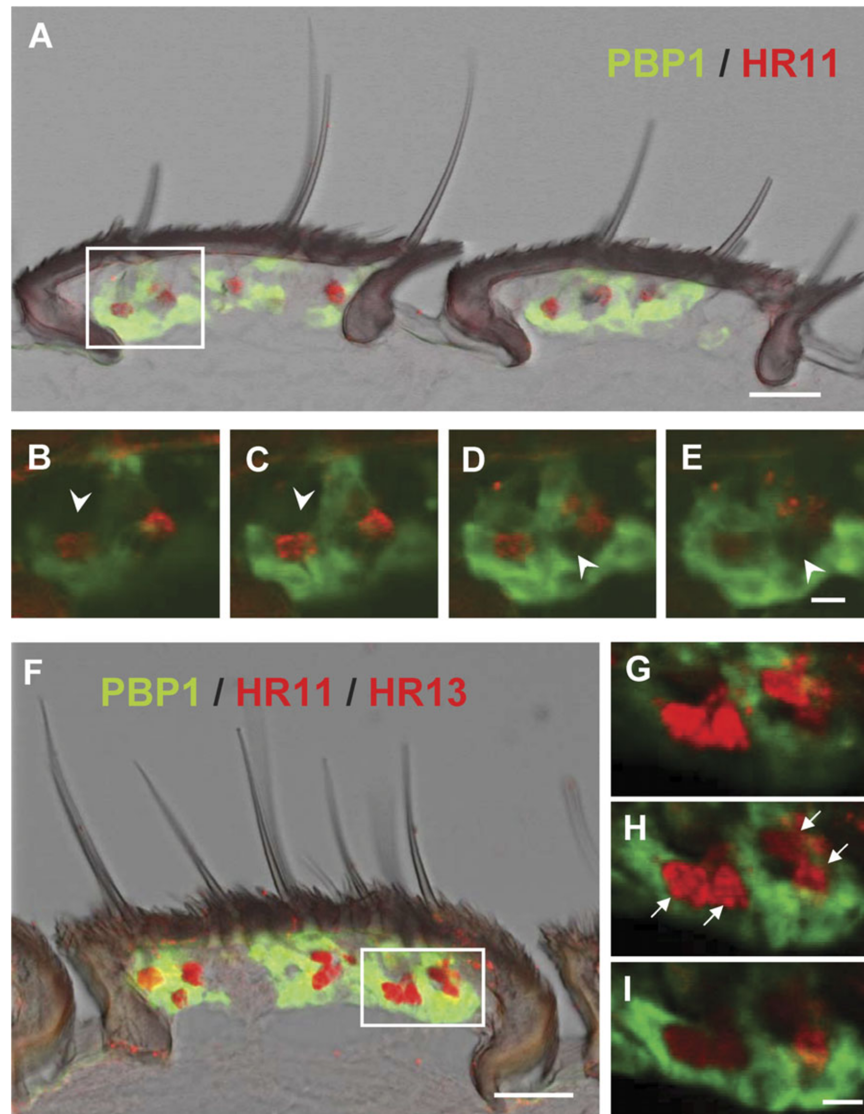

Figure 2 Localization of HR11-, HR13-, and PBP1-expressing cells on the antenna. **(A)** Double in situ hybridization employing DIG-labeled HR11 and biotin-labeled PBP1 antisense RNAs on a section through the male antenna visualized by red (DIG-label) or green (biotin-label) fluorescence. A single optical plane from a stack of confocal images is shown; the red and green fluorescence channels have been overlaid with the transmitted-light channel. Red-labeled HR11 cells are surrounded by green-labeled PBP1 cells. Consecutive optical planes from the boxed area are shown in **(B–E)** at higher magnification; only the red and green fluorescence channels are displayed. Green PBP1 cells encircle red HR11 cells and an unlabeled area (arrowheads). **(F)** Triple in situ hybridization using a biotin-labeled PBP1 probe and DIG-labeled antisense RNA probes for HR11 and HR13 on the same section. **(G–I)** Different consecutive optical planes of the area boxed in F. The appearance of 2 red-labeled cells (arrows), which are closely surrounded by green cells expressing PBP1, strongly suggests a location of HR11-, HR13-, and PBP1-expressing cells in the same sensillum. Scale bars: 20 μm in (A and F); 10 μm in (E and I).

cells are located side by side along the entire length of the antenna, double in situ hybridizations were performed on sections through proximal (segments 1–50; characterized by long sensilla trichodea) and distal (segments 51–80 carrying only short sensilla trichodea) parts of the antenna. As exemplarily shown in Figure 4C, the red and green in situ hybridization signals are located in close proximity, both in the proximal as well as in the distal part of the antenna. These results indicate that HR11/HR13 pairing is maintained along the entire antenna, from the base to the tip.

Onset of receptor expression during pupal development

To examine if during development the onset of expression of the 2 ‘‘paired’’ receptor types HR11 and HR13 may coincide, RT-PCR experiments were performed using cDNAs from antennae of males at different developmental stages (Figure 5). Pupae staged as 1–5 days before eclosion (E-1 to E-5) and newly eclosed animals (E0) were assessed. For comparison, transcript levels for related pheromone receptor types, HR14, HR15, and HR16, were determined. Primers specific for the constitutively expressed rl31 gene (Tanaka

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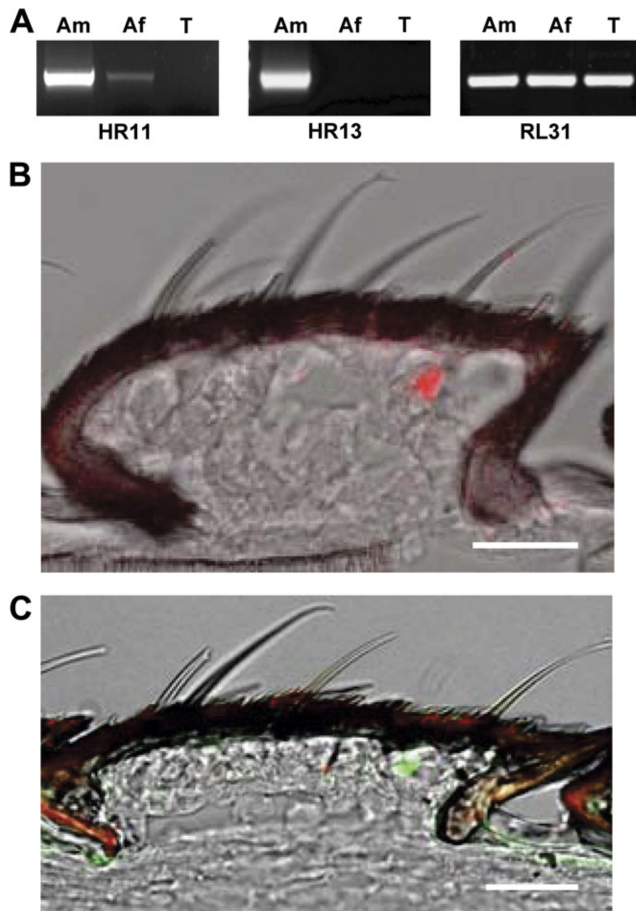

Figure 3 Expression of HR11 in the female antenna. **(A)** Detection of receptor transcripts. RT-PCRs were performed with cDNAs from different *Heliothis* tissues and primer pairs specific for HR11, HR13, and RL31. Am, antenna male; Af, antenna female; and T, thorax. Strong PCR bands of the expected size were obtained for both receptor types with cDNAs from male antennae. In addition, HR11-specific primers led to the amplification of a weak band with female antennal cDNAs. No bands were obtained with thorax cDNAs. Primers specific to the RL31 control gave bands of similar intensities in all tissues tested. **(B** and **C)** In situ hybridization on longitudinal sections of female antennae. The transmitted light and fluorescence channels have been overlaid. (B) In situ hybridization using a DIG-labeled HR11 probe. Regularly only 1 receptor-expressing cell was labeled per section (red fluorescence). (C) Double in situ hybridization using a biotin-labeled HR11 probe and a DIG-labeled HR13 probe. Only a single green-labeled HR11-expesing cell was detected; no labeling was found for HR13. Scale bars in (A and B): 20 μm.

et al. 1987) were used to control the integrity of the cDNA templates. PCR bands with rl31 primers had the correct size and similar intensity for all cDNA preparations (E-5 to E0). Bands for the different receptor types also corresponded to the size expected from the primer design. Comparing the appearance and intensity of PCR bands for different receptor types during pupal developmental stages revealed significant differences. For HR14, HR15, and HR16, gene transcripts were not found before stages E-2 and E-1. In contrast for HR11 as well as for HR13, the first faint bands appeared at stages E-5 to E-4; the intensity increased in older stages from E-3 to E0 (Figure 5). These results indicate that the HR11 and HR13 receptor gene expression begins up to 5 days prior to eclosion and implies an onset of expression of these receptors several days before expression of the related receptor types.

Discussion

In this study, we have found that the majority of long sensillar hairs on the antenna of male *H. virescens* contain 2 closely associated sensory cells. Stereotypically, 1 cell expresses the pheromone receptor HR13, and the other cell expresses the candidate pheromone receptor HR11 (Figure 1). This arrangement and the observation that each HR11/HR13 cell pair is ensheathed by supporting cells expressing pheromone-binding protein (Figure 2) strongly suggest that both cells are located within the same sensillum. The findings that the dendrite of 1 of the cells of pheromone-responsive long sensilla trichodea is equipped with HR13 protein (Gohl and Krieger 2006) and that HR13 responds to Z11-16:AL (Grosse-Wilde et al. 2007; Kurtovic et al. 2007) strongly suggest that HR13 is the receptor type, which mediates the response of type A sensilla to Z11-16:AL previously observed in electrophysiological experiments (Almaas and Mustaparta 1990; Baker et al. 2004). These studies have also demonstrated that only 1 neuron in A-type sensilla was responsive. The results of previous backfill experiments however suggested that a second neuron may exist in this hair type (Almaas and Mustaparta 1991; Hansson et al. 1995; Berg et al. 1998). This notion is confirmed by results of the present study, providing strong evidence, that sensilla trichodea type A indeed contain a second neuron. Furthermore, the expression of the candidate pheromone receptor HR11 suggests that these neurons may also respond to a pheromone. The ligand for the receptor type HR11 is still elusive, but analyses of heterologously expressed receptors may provide some insight, although this approach is hampered by the fact that the electrophysiological studies have not provided any evidence for a pheromone component, which may activate the second neurons of the A-type sensilla (Berg et al. 1995; Baker et al. 2004; Hillier and Vickers 2007). However, the sequence similarity of HR11 with the HR13 receptor and other functionally characterized pheromone receptors of several moth species (Nakagawa et al. 2005; Grosse-Wilde et al. 2006; Mitsuno et al. 2008), including the bombykol receptor BmOR-1 and the bombykal receptor BmOR-3 from *B. mori*, suggests a role of HR11 in pheromone detection.

The finding that the 2 cells, which express the related receptor types HR11 and HR13, respectively, are housed within a single sensillum is strikingly similar to the organization of the pheromone-responsive sensilla from *B. mori*, where the 2 neighboring neurons housed in long sensilla trichodea express the related receptor types BmOR-1 and BmOR-3 (Krieger et al. 2005; Nakagawa et al. 2005).

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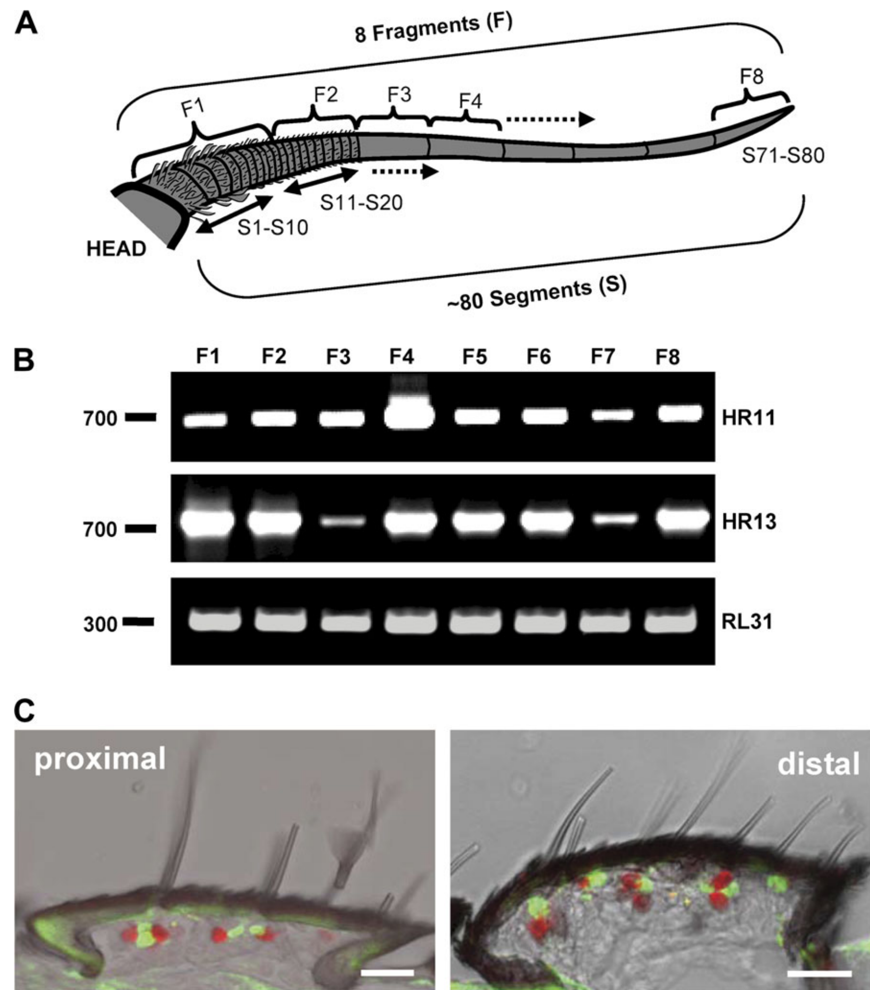

Figure 4 Expression of HR11 and HR13 along the male antenna. **(A)** Schematic drawing indicating the segmental organization of the *Heliothis virescens* antenna and the division made to dissect male antennal fragments. **(B)** RT-PCRs using cDNA preparations from antennal fragments F1–F8 and receptor-specific primers. PCR bands of the expected size based on the primer design were obtained for HR11 and HR13 in all fragments from the base to the tip of the male antenna, indicating receptor expression along the complete length of the antenna. The position of molecular weight markers (in bp) is indicated. **(C)** HR11 and HR13 double in situ hybridization on 1 of the 50 proximal (characterized by long sensilla trichodea) and 1 of the 30 distal segments of a male antenna showing that pairing of HR11- (green) and HR13-expressing cells (red) is maintained along the antenna. Images represent 1 optical plane from a stack of confocal images. Scale bars: 20 μm.

Moreover, the related candidate pheromone receptors HR14 and HR16 are also expressed in adjacent cells (Grosse-Wilde et al. 2007). Thus, the paired arrangement of cells expressing related receptor types in the same sensillum hair may be a general principle in pheromone detection systems of moths. This notion is supported by the results of electrophysiological recordings from antennal sensilla from several moth species, demonstrating 2 chemosensory neurons that are located in male sensilla trichodea, each responding to a distinct but structurally related pheromonal compound (Kaissling et al. 1989; Meng et al. 1989; Todd and Baker 1999).

Such a paired arrangement of sensory neurons in an individual structural unit may have several advantages over a separated location of individual neurons in different sensilla. First, the dendrites of the paired cells are bathed in the same sensillum lymph, which contains PBPs (Maida et al. 2000; Vogt 2003; Grosse-Wilde et al. 2007). Because PBP subtypes seem to interact specifically with distinct pheromones (Plettner et al. 2000; Bette et al. 2002; Mohl et al. 2002; Maida et al. 2003), the PBP subtypes in a sensillum lymph may contribute to the specificity of the system (Grosse-Wilde et al. 2006). In addition to PBP subtypes interacting with specific ligands, PBPs also contribute to specificity by interacting with specific receptors (Pophof 2002, 2004; Laughlin et al. 2008). In line with this view, recent studies have shown that the HR13 cells and the cells housed in the same sensillum are surrounded by supporting cells, which express the 2 PBP subtypes PBP1 and PBP2 (Grosse-Wilde et al. 2007). In addition, PBP2 but not PBP1 mediated a response to Z11-16:AL, the ligand for HR13. So, it is conceivable that the PBP1 may interact with the unknown ligand for HR11. Secondly, because the sex pheromone blend of moths

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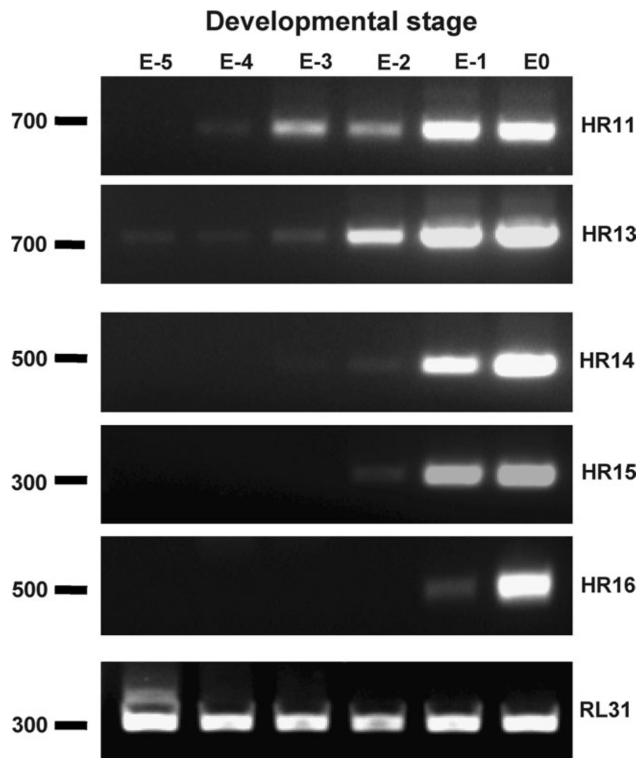

Figure 5 Expression of *Heliothis virescence* pheromone receptor types during development. E-1, E-2, E-3, E-4, and E-5 denote days before emergence (E0). RT-PCRs were performed using receptor- and RL31-specific primer pairs and cDNAs prepared from male antennae of the indicated pupal stages. All PCR products were of the size expected from the primer design. The position of molecular weight markers (in bp) is marked. The results indicate an early onset of HR11 and HR13 gene expression in development. In comparison, HR14, HR15, and HR16 gene expression appeared to be turned on later. Very similar band intensities in the different developmental stages were obtained for the RL31 control indicating the integrity of the cDNA preparations and similar transcript levels in the developmental stages E-5 to E0.

is composed of several components and distinct concentration ratios of the compounds are critical for a proper behavioral response (Linn et al. 1986; de Bruyne and Baker 2008), it has been suggested that colocalization of sensory neurons in the same sensillum may provide the basis for a higher fidelity to register and report the relative ratios of the pheromone components to which the paired neurons are tuned to (Todd and Baker 1999). In this way, cocompartmentalization might increase the animals' ability to discern relative ratios of components in single or different intertwined pheromone strands in the air. This may also contribute to distinguish if a pheromone signal originated from a single source or from separate sources.

The results of the in situ hybridization studies have shown that on female antennae, HR11 is expressed only in a low number of cells (Figure 4) and that there are no HR13 expressing cells. Thus, the stereotypic pairing of HR11 and HR13 cells is restricted to the antenna of males. This implies that a different mechanism of receptor gene choice is active in male and female sensilla and that in male trichoid hairs the choice of the HR11 gene in 1 cell is coordinated with the choice of the HR13 gene in the neighboring cell. Although the mechanisms underlying gene choice of olfactory receptors in moths are poorly understood, recent studies of the *Drosophila* olfactory system have hinted at a possible mechanism for a coordinated receptor expression. In the fruitfly, stereotyped pairing of olfactory cells expressing distinct receptors has been documented for sensilla on the maxillary palp and the antenna (Hallem et al. 2004; Couto et al. 2005; Fishilevich and Vosshall 2005). From analyzing expression of receptor genes in neighboring olfactory neurons of sensilla basiconica on the maxillary palp, evidence has been provided suggesting that receptor choices are coordinated by asymmetric segregation of regulatory proteins from the common progenitor cell (Ray et al. 2007). Future studies will assess if similar mechanisms may govern the coordinated receptor expression in *H. virescens*.

Although there is convincing evidence that HR13-expressing cells in male *H. virescens* respond to the main component of the female-released sex pheromone blend and thus play a crucial role in triggering specific mating behavior, the functional relevance of HR11-expressing cells needs to be clarified. However, not only the characteristic features of the sequence render HR11 a candidate pheromone receptor but also the high number of HR11-expressing cells on the male antenna as well as the stereotyped colocalization with HR13 cells in the long sensilla hairs implies a role of HR11 receptor in the detection of a behaviorally highly relevant chemical signal.

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