

Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*)

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The remarkable responsiveness of male moths to female released pheromones is based on the extremely sensitive and selective reaction of highly specialized sensory cells in the male antennae. These cells are supposed to be equipped with male-specific receptors for pheromonal compounds, however, the nature of these receptors is still elusive. By using a combination of genomic sequence analysis and cDNA-library screening, we have cloned various cDNAs of the tobacco budworm *Heliothis virescens* encoding candidate olfactory receptors. A comparison of all identified receptor types not only highlighted their overall high degree of sequence diversity but also led to the identification of a small group of receptors sharing >40% identity. In RT-PCR analysis it was found that distinct members of this group were expressed exclusively in the antennae of male moths. *In situ* hybridization experiments revealed that the male-specific expression of these receptor types was confined to antennal cells located beneath sensillar hair structures (sensilla trichoidea), which have been shown to contain pheromone-sensitive neurons. Moreover, two-color double *in situ* hybridization approaches uncovered that cells expressing one of these receptor types were surrounded by cells expressing pheromone-binding proteins, as expected for a pheromone-sensitive sensillum. These findings suggest that receptors like *Heliothis* receptor 14–16 (HR14–HR16) may render antennal cells responsive to pheromones.

The olfactory system of insects, most notably of moths, is renowned for its remarkable sensitivity and selectivity; it has been an invaluable model system for studying fundamental aspects of olfaction (1). Insects detect volatile chemostimulants by means of chemosensory neurons housing in multiporous cuticular hairs, comprising olfactory sensilla (2). These specialized cells generate electrical signals upon interaction with appropriate chemical compounds. Experimental evidence indicating that the underlying chemolectrical transduction process is mediated by means of odor-activated G protein–second-messenger cascades, a mechanism used by most chemosensory cells (3), supports the notion that receptors for odorous compounds in insects should be members of the G protein-coupled receptor superfamily; however, it was only with the aid of sequenced genomes that genes encoding candidate odorant receptors from insects were identified recently in fly (4–8), mosquito (9–11), and moth (12) models. Despite the progress in identification and characterization of insect olfactory receptors, receptors for insect pheromones are still elusive. This status is more noteworthy given that the pioneering work in identifying signaling molecules for communication between individuals was performed on insects; in fact, the first known pheromone (bombykol) was isolated from the silkworm *Bombyx mori* (13, 14). Pheromones, originally defined as chemical compounds that are produced and secreted by individuals and received by other members of the same species in which they release a definite reaction (15), are considered to be a unique class of environmental signals, which may require a unique detector system. In fact, the olfactory system of many insect species is divided into two subsystems: one that is specialized to accomplish the task of processing pheromonal signals and one for nonpheromonal

odorant processing (16). The notion that cellular mechanisms of pheromone detection may also be unique was not confirmed by recent studies, which have indicated that mechanisms involved in transducing and processing pheromone and nonpheromone chemical signals appear to be quite similar and also appear to be modifications of overall common processes (3, 17). Moreover, it appears that insect pheromones arose from coopting molecules that had other purposes for signal functions, which were shaped by selective forces so that the signal might be discriminated more easily (18). Based on these observations, it seems to be conceivable that receptors for pheromones might be specialized members of the insect olfactory receptor family. One could imagine some identifying features that might distinguish pheromone receptors among general olfactory receptors. A characteristic trait of insect olfactory receptor types is the extreme diversity of their sequences (9, 12, 19). Because pheromone detection is probably under different kinds of selective pressure than detection of food odors or host odors, one might speculate whether receptors for pheromones are more conserved than other olfactory receptor types. Furthermore, candidate receptors for sex pheromones released by female moths are expected to be expressed only (or preferentially) in antennae of male moths, and more specifically, in cells that can be assigned to male-specific pheromone-sensitive sensory hairs (20, 21). Therefore, putative olfactory receptors from the moth *Heliothis virescens* were assessed for sequence similarities as well as for their tissue- and cell-specific expression patterns in an attempt to identify candidate pheromone receptors.

Materials and Methods

Animals and Tissue Preparation. *H. virescens* pupae (provided by Bayer CropScience) were sexed and allowed to develop into adults at room temperature. Antennae and other body parts were dissected from cold-anesthetized animals. Tissue for RNA isolation were frozen immediately at liquid-nitrogen temperature and stored at -70°C . The experiments complied with the National Institutes of Health *Principles of Animal Care* and the German laws for animal protection.

Identification of Receptor Sequences. To find new genes encoding candidate chemoreceptors, we extended the strategy that was used successfully to detect the first candidate olfactory receptors in *H. virescens* (12) by using the sequences of *Heliothis* receptor 1–9 (HR1–HR9) and of newly identified HRs, as well as the sequences of candidate olfactory receptors from *Drosophila melanogaster* (4–8) and *Anopheles gambiae* (9–11) in BLAST analyses (22, 23) of the nonpublic *Heliothis* genomic database.

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Abbreviations: DIG, digoxigenin; HR, *Heliothis* receptor; PBP, pheromone-binding protein.

Data deposition: The sequences reported in this paper have been deposited in the EMBL database [accession nos. AJ748325 (HR10), AJ748326 (HR11), AJ748327 (HR12), AJ748328 (HR13), AJ748329 (HR14), AJ748330 (HR15), AJ748331 (HR16), AJ748332 (HR17), AJ748333 (HR18), AJ748334 (HR19), AJ748335 (HR20), and AJ748336 (HR21)].

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Several short (175–690 bp) genomic sequences were detected, showing partial sequence similarity to amino acid strings of HRs or *Drosophila* and *Anopheles* chemosensory receptors.

Sense and antisense primers matching these candidate receptor exon sequences within the *Heliothis* genome were used in standard PCRs with antennal cDNA or genomic DNA. PCR conditions were as follows: 1 min and 40 s at 94°C; and 19 cycles at 94°C for 1 min, 55°C for 40 s, and 72°C for 1 min, with a decrease of the annealing temperature by 0.5°C per cycle. Subsequently, 19 further cycles at the condition of the last cycling step were performed, followed by incubation for 7 min at 72°C. PCR products of the expected size were cloned by using the pGem-T vector system (Promega) and verified by sequencing. Digoxigenin (DIG)-labeled probes for library screening were obtained by standard PCR using the DIG PCR labeling mixture (Roche), the cloned genomic or cDNA fragment as template, and the corresponding primer pair. Labeled PCR products were gel-purified by using Gene Clean and diluted in hybridization solution [30% formamide/5× SSC/0.1% laurylsarcosine/0.02% SDS/2% blocking reagent (Roche)/100 µg/ml denatured herring-sperm DNA].

Screening of the Antennal cDNA Library. DIG-labeled PCR fragments encoding short putative receptor regions were used for the detection of corresponding full-length cDNAs in a library made from the antennae of male *H. virescens* moths (24). In addition, DIG-labeled partial cDNAs discovered with the genomic fragments were used as screening probe. Screening of the cDNA library was performed as described (12). Filters were hybridized to DIG-labeled probes at 37°C. Posthybridization washes were performed twice for 5 min in 2× SSC/0.1% SDS at room temperature, followed by three washes for 20 min each at 37°C or 60°C in 0.1× SSC/0.1% SDS. Hybridized probes were detected by using an anti-DIG alkaline phosphatase-conjugated antibody (Roche) and disodium 3-{4-methoxy-spiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo(3.3.1.1^{3,7})decan]-4-yl} phenylphosphate (CSPD) substrate (Applied Biosystems). cDNA inserts from positive phage were subcloned into the Bluescript II SK(+) vector and sequenced. Phage-carrying cDNAs encoding candidate olfactory receptors were present at a frequency ranging from 1:45,000 to 1:360,000 in the library.

To obtain the complete 5' end of HR16 cDNA, RACE PCR was performed with the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. Analysis of the RACE products allowed us to supplement the coding region of HR16 by adding a sequence for the first 52 aa, which was flanked by an upstream in-frame stop codon.

Sequencing and Sequence Analysis. Sequencing was performed on an ABI310 sequencing system by using vector- and cDNA-derived primers and the BIG dye cycle-sequencing kit (Applied Biosystems).

Database searches and sequence analyses were made by using the EMBL Heidelberg UNIX sequence-analysis resources (<https://genius.embnet.dkfz-heidelberg.de>). Sequence comparisons were performed with the DNA for the EMBL/GenBank and SwissProt databases. Based on a CLUSTAL (25) alignment of the HR1–HR21 protein sequences, an unrooted neighbor-joining tree (26) was calculated with the MEGA program (27). In cases in which the start methionine in the ORFs was not verified by the presence of an upstream in-frame stop codon or by RACE experiments, also amino acids upstream of the first methionine in the sequence were integrated into the alignment.

RT-PCR. Total RNA from antennae and different body parts were isolated by using TRIzol reagent (Invitrogen). For antennae, RNAs were isolated separately from males and females; for all other tissues, a mixture of male and female tissue was used.

Poly(A)⁺ RNA from the various tissues was isolated from total RNA with oligo(dT)₂₅ magnetic dynabeads (Dyna, Oslo), transcribed into cDNA and used in PCR as described (12) with the following specific primer pairs: HR6, s5'-AGGAAATACCG-TAAAGGGG-3' and as5'-GAAAGGTGAAATACGA-CAACG-3'; HR11, s5'-CAGGCATTACGGGTCCAA-TGG-3' and as5'-GGTCAAACATCGACACGCTGC-3'; HR13, s5'-CGGTCTACTTACTCGGCTTGG-3' and as5'-CTGTGCGACTGTCTGAGCATC-3'; HR14, s5'-GTTCA-CACTGTACCTCACTGG-3' and as5'-GAACAACATTGGC-CCGAATAC-3'; HR15, s5'-CAACTACCAACCTAAAG-CCGG-3' and as5'-GGCTACTCTGTACTCGTTC-3'; and HR16, s5'-CGAGACCAAGTTCCAAAGTGG-3' and as5'-GAGGTCTTCAAATCGCAGCCA-3'.

The primer pairs designed for HR11 and HR13 spanned at least one predicted intron region, which allowed us to check the cDNA preparations for possible genomic contaminations. Primers (s5'-CAACGAAGTTGTAACCTCGTG-3' and as5'-TTCT-TGGCTAGCGTCCACAT-3') directed against the *H. virescens* rl31 gene (GenBank accession no. AJ298149) were used to test the integrity of the cDNAs. Based on the primer design, the expected sizes for the PCR products were as follows: 331 bp for RL31, 746 bp for HR6, 651 bp for HR11, 745 bp for HR13, 499 bp for HR14, 343 bp for HR15, and 475 bp for HR16. PCR conditions were as described for the preparation of DIG-labeled probes. By using the RL31 primers, the annealing temperature in the first 19 cycles was decreased from 50°C to 40°C, followed by 19 cycles with 40°C. PCR products were analyzed on agarose gels and visualized by ethidium bromide staining.

In Situ Hybridization. Antennae of 1- to 2-day-old moths were embedded in Jung tissue-freezing medium (Leica, Nussloch, Germany) supplemented with 1/3 of 20% glucose and frozen at –22°C on the object holder. Cryosections (12 µm) of antennae were thaw-mounted on slides coated with Vectabond (Vector Laboratories) and air-dried at room temperature for at least 30 min.

Preparation of DIG-labeled or biotin-labeled antisense riboprobes and *in situ* hybridization was performed as described in ref. 12. Hybridizations of DIG-labeled probes were visualized by the anti-DIG alkaline phosphatase-conjugated antibody in combination with HNPP/Fast Red fluorescence-detection set (Roche). For biotin-labeled probes, the tyramide-signal amplification (TSA) kit (PerkinElmer), including an anti-biotin-streptavidin horseradish peroxidase conjugate and fluorescein-labeled tyramides, was used as substrate. In two-color double *in situ* hybridization experiments, tissue sections were hybridized to two (either DIG- or biotin-labeled) antisense RNA probes. Visualization of hybridization signals was performed by incubating the sections first with HNPP/Fast Red, followed by the TSA kit development. Sections were mounted by using the SlowFade Light Antifade kit with DAPI (Molecular Probes). Analyses of the sections were performed on a LSM510 Meta laser-scanning microscope (Zeiss). Images were arranged in WORD (Microsoft) and PHOTOSHOP (Adobe Systems, San Jose, CA) and were not altered except to adjust the brightness or contrast for uniform tone within a single figure.

Results

Cloning of Candidate Chemosensory Receptors. Data mining of a partial genomic database from *H. virescens* in combination with cDNA-library screening (12) led to the identification of numerous cDNA clones containing long ORFs. BLAST comparisons of the sequences to entries in the EMBL/GenBank and SwissProt databases revealed that several of the newly isolated cDNAs appear to be related to some of the candidate olfactory receptors from *Heliothis* (12), *Drosophila* (4–8), or *Anopheles* (9–11), thus suggesting that the isolated cDNA clones encode novel members

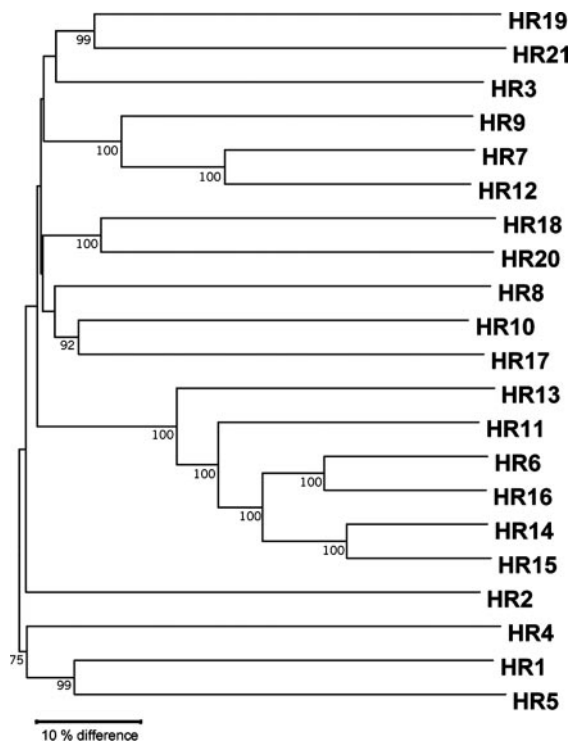


Fig. 1. Neighbor-joining tree showing the sequence relatedness between putative chemosensory receptors in *H. virescens*. The distance tree was calculated by using the MEGA program and is based on a CLUSTAL alignment of HR1–HR9 (12) and HR10–HR21 (this study). Branch lengths are proportional to the percentage of sequence difference. (Scale bar, 10% difference.) Bootstrap-support values (only >75% shown) are based on 1,000 replicates. The tree is rooted at the midpoint.

of the olfactory receptor family. Each of the clones HR11, HR14, HR17, and HR19 comprised a full-length receptor sequence, as indicated by stop codons upstream to an ATG start codon and at the end of the sequence. The cDNA clones HR12, HR13, HR15, HR18, and HR21 contain no in-frame stop codon upstream of a predicted start-methionine, but based on distinct motifs and the length of the sequences (which matches that of full-length receptors), it is likely that they comprise the complete coding region. The HR10, HR16, and HR20 cDNAs did not include the entire receptor sequence, but RACE experiments allowed completion of the coding region of HR16.

Sequence Comparisons. Comparison of the sequences of all identified olfactory receptors of *H. virescens* revealed a very high degree of diversity, resulting in overall sequence identities of 8–15%; nevertheless, based on several common sequence motifs, they could be affiliated to the family of putative olfactory receptors. Despite a very high degree of sequence diversity within the family, analysis of the sequence relatedness allowed us to assign some receptors to groups sharing at least 40% identity. As shown by the neighbor-joining tree in Fig. 1, one group is formed by HR7 and HR12, sharing 52% amino acid identity, and a second group comprises six HRs (HR6, HR11, and HR13–HR16), which have at least 40% of their amino acids in common. Within this group, HR6 and HR16, as well as HR14 and HR15, form distinct subgroups, based on amino acid identities of 69% and 73%, respectively. The second group of receptors was considered for further analysis.

Tissue Specificity of Expression. To explore whether these related receptor types share a common and characteristic expression

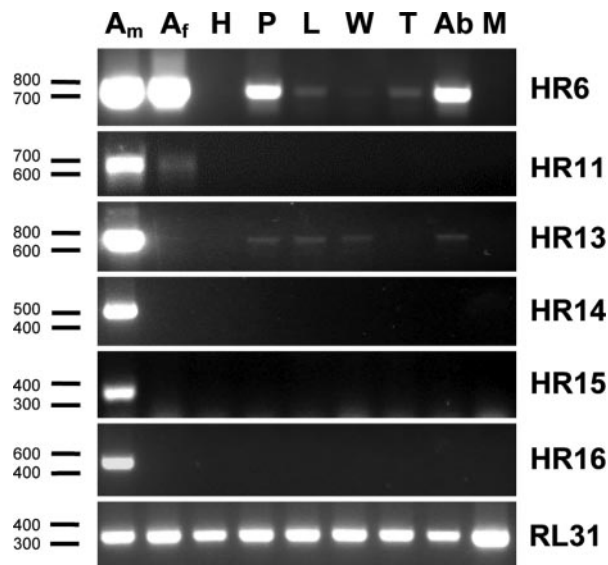


Fig. 2. Tissue specificity of chemosensory receptor expression. RT-PCRs were performed with specific primer pairs and cDNAs prepared from different *Heliothis* tissues. Reaction products were visualized by ethidium bromide staining and UV illumination. Bands were of the expected size based on the primer design. HR14–HR16 expression was detected only in the antennae of males. The results for HR11 indicate differences in the expression levels between sexes. HR13 was predominantly found in male antennae, but RNA for this receptor was detected also in other tissues. For HR6, very high levels of RNA were detected in antennae; lower levels were found in several other tissues. Bands of similar intensities obtained with primers specific to the RL31 control indicate the integrity of the various cDNA preparations. Am, antenna male; Af, antenna female; H, head without appendices; P, proboscis; L, leg; W, wing; T, thorax; Ab, abdomen; M, muscle. The position of marker bands (bp) is indicated on the left.

pattern, RT-PCR experiments were performed by using cDNA preparations from various tissues of *H. virescens* and specific primer pairs for each of the subtypes (Fig. 2). All PCR bands obtained were of the expected size, based on the primer design. Comparison of the results obtained by means of the receptor-specific primers indicate a very strong expression for the HR6 type in male and female antennae. A significantly lower expression level was found in the proboscis and abdomen; also, weak bands were obtained with cDNAs from leg, wing, and thorax, confirming previous observations (12). For HR11, a PCR band was obtained only with cDNA from antennae, with a striking difference of the expression levels in the two sexes; a much stronger band was obtained with cDNA from male antennae, compared with female antennae. For HR13, a very strong PCR band was obtained only with cDNA from male antennae. In addition, faint bands were detected with cDNAs from the proboscis, leg, wing, and abdomen; occasionally, a very faint band was also obtained with cDNA from female antennae (not shown in Fig. 2). For the receptor types HR14–HR16, PCR bands were seen only with cDNA from male antennae, suggesting that these receptor subtypes are male-specific and expressed exclusively in the antennae of male moths.

Topographic Expression of Male-Specific Receptors in the Antenna.

The antennae of *H. virescens* exhibit characteristic differences in the two sexes, consistent with the sensitive detection of female-released sex pheromones by the males. Only the male antenna contains long, curved sensory hairs, which are characterized as sensilla trichoidea type 1, housing pheromone-responsive neurons. These sensory hairs are arranged in three to four rows on one side of the 50 proximal segments of the filamentous antenna;

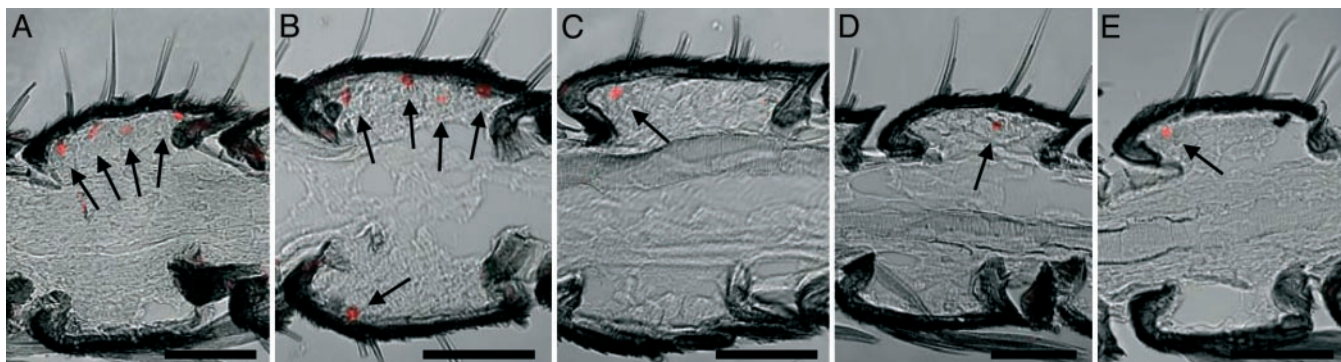


Fig. 3. Expression of specific receptor types in the antenna of *H. virescens*. DIG-labeled antisense RNA probes for HR13 (A and B), HR14 (C), HR15 (D), and HR16 (E) were hybridized to longitudinal cryosections through the antennae of male moths and visualized by fluorescence detection using HNPP/Fast Red. A and C–E show sagittal planes; B displays a horizontal plane. Pictures represent optical sections selected from a stack of confocal images generated by a laser-scanning microscope. The fluorescence channel is overlaid with the transmitted-light channel. Arrows indicate labeled cells. (Scale bars, 50 μm .)

the opposite side comprises no sensilla, but rather, scales (21). Additional pheromone-responsive neurons have been found in a type of shorter sensory hairs, which are classified as sensilla trichoidea type 2; this type of sensilla is located on the leading edge of all ≈ 80 segments of the male antenna (20, 21). To explore whether the characteristic receptor types may be expressed in neurons located in sensilla trichoidea, *in situ* hybridization experiments were performed to visualize cells expressing HR13–HR16 in cryosections of the proximal segments from male antenna. Although the delicate tip of the long sensilla trichoidea type 1 broke off frequently during sectioning of the antennae, it was possible to unequivocally assign receptor-expressing cells to long sensillar hair structures (Fig. 3). Fig. 3A shows a sagittal plane of a longitudinal section through a proximal segment of the male-moth antenna, which was assessed with a HR13 antisense probe and analyzed by means of a confocal laser-scanning microscope; the picture represents an overlay of a transmission image and fluorescence image. Labeled cells are visible only in a distinct area of the sections just beneath the long sensilla hairs; no labeled cells are seen on the opposite side, where the antenna carries scales rather than sensilla. To demonstrate that the labeled cells are not restricted to a particular topographic region of the antennae, which happened to be met by the section plane, but rather are indeed located beneath the sensory hairs, longitudinal sections representing a horizontal plane were probed. The section shown in Fig. 3B represents an almost-medial plane of the tubular structure of an antennal segment. Long hairs are visible on both sides, with all labeled cells located beneath these sensilla. The results of experiments assessing longitudinal sections of antennae with probes specific for HR14–HR16 are shown in Fig. 3C–E, respectively. In all sections, labeled cells were found only beneath the long hairs. The considerably smaller number of labeled cells per segment is notable, as compared with HR13; only one (as shown in Fig. 3C–E) or very few labeled cells per segment were observed. Cells labeled by the HR13–HR16 receptor probes were also found under short sensory hairs, which were most likely sensilla trichoidea type 2, on proximal and distal antennal segments (data not shown). Together, the results indicate that the receptor types HR13–HR16 are expressed in cells, which can be assigned to long sensory hairs and, thus, could be pheromone-responsive cells.

Association of Pheromone-Binding Protein (PBP)- and Receptor-Expressing Cells. In a trichoid sensillum of male-moth antennae, pheromone-sensitive neurons are generally accompanied by supporting cells that express PBP; PBP is not expressed in the olfactory neurons (28). Therefore, attempts were made to assess

whether cells expressing HR14, HR15, or HR16 may be positioned in close association with cells expressing PBP. In a first approach, longitudinal sections through the male antennae were probed with a biotin-labeled antisense RNA for the specific PBP of *H. virescens* (24). The resulting hybridization signals could clearly be assigned to rows of long sensory hairs (sensilla trichoidea type 1) (Fig. 4). Two-color double *in situ* hybridization experiments were performed with DIG-labeled antisense RNAs for HR14, HR15, or HR16, each in combination with biotin-labeled RNA for PBP to visualize the cells that express a distinct receptor type as well as the cells that express the PBP on the same tissue section. The hybridized tissue sections were analyzed by means of laser-scanning confocal microscopy, generating stacks of confocal images. The results shown in Fig. 5 were obtained with probes for HR15 and PBP; a series of confocal images taken at different planes of a longitudinal section through two segments of the proximal antennae is shown. Cells with HR15 RNA are shown in red, and cells with PBP RNA are shown in green. In both segments in A, green PBP cells are visible

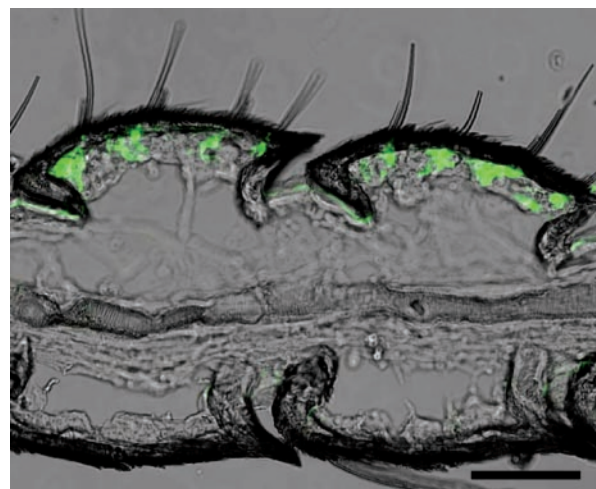


Fig. 4. Expression of PBP in the antenna of a male *H. virescens*. *In situ* hybridization was performed on a longitudinal cryosection with biotin-labeled antisense RNA. Hybridization signals were visualized by a detection system, indicating PBP-positive cells by green fluorescence. The image represents an optical section selected from a stack of confocal images that were generated by a laser-scanning microscope. Two segments of the filamentous antenna are shown. Hybridization signals can be assigned to cells lying under rows of long sensilla trichoidea; no labeling was detected on the scaled side. (Scale bar, 50 μm .)

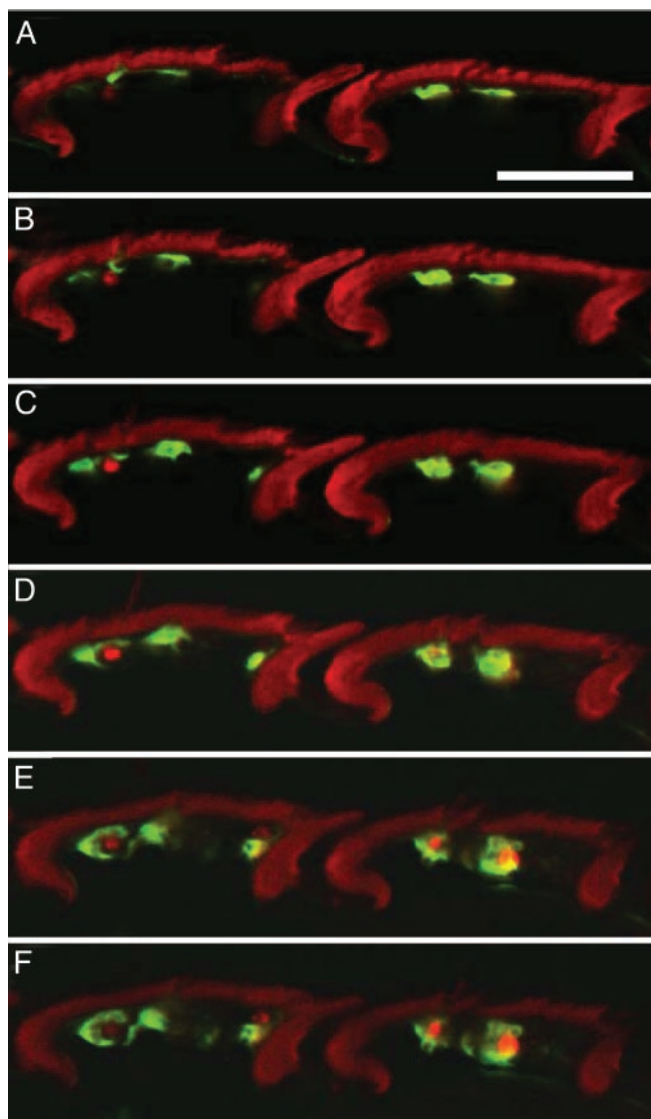


Fig. 5. Gene expression of HR15 and PBP in the antenna. Two-color *in situ* hybridization was performed on a section through the male antenna by using a combination of DIG-labeled HR15 and biotin-labeled PBP antisense RNAs. Hybridization signals were visualized by detection systems, indicating cells bearing HR15 transcripts by red fluorescence and PBP-positive cells in green. The side of two antennal segments carrying the sensilla hairs is shown. Images represent selected confocal images from a stack of confocal images that were taken with a laser-scanning microscope. Cells expressing HR15 were found to be accompanied by cells expressing PBP. (Scale bar, 50 μm .)

beneath the cuticle. At deeper optical layers (left segments in *B* and *C*; right segment in *D* and *E*) a red HR15 cell emerges, accompanied by green-labeled PBP cells. It is notable that in the left segment, the red cell is hardly visible in the deepest optical layer but the green cells are still there (*F*). It is also notable that, in the right segment, the yellow color in *D* and *E* may indicate a coverage of the red cell by green cells at this level. Similar results were obtained for combinations of HR14 and HR16 with PBP. The notion that receptor-expressing cells may be wrapped up by PBP-expressing cells was studied in more detail by optical sectioning of hybridized tissue sections at higher magnification. The result of such an analysis employing a combination of HR16 and PBP probes is shown in Fig. 6. At the top (*A* and *B*), only green-labeled PBP cells are visible; in *C* the red HR16 cell emerges. This cell is clearly visible in *D–F* and is surrounded by

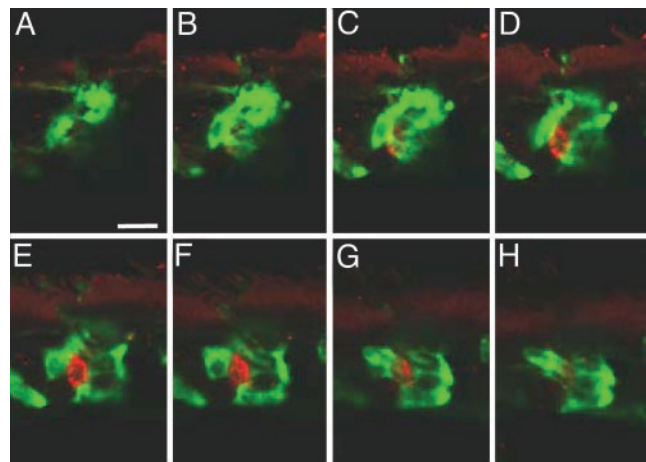


Fig. 6. Colocalization of cells expressing the HR16 receptor type and PBP. Two-color *in situ* hybridization performed with DIG-labeled HR16 and biotin-labeled PBP antisense RNAs on a section through the male antenna. Hybridization signals were visualized by detection systems indicating cells bearing HR16 transcripts by red fluorescence and PBP-positive cells in green. All images show different optical planes of the section in an area just below the surface carrying the sensilla hairs with cells expressing HR16 or PBP. Images represent selected confocal images from a stack taken with a laser-scanning microscope. By following the sequence of images, it is obvious that cells expressing PBP surround a HR16-expressing cell. (Scale bar, 10 μm .)

green PBP cells. In *G* and *H*, the red HR16 cell disappears and the whole area is covered by the green PBP cells. These observations support the view that cells expressing the male-specific receptor types HR14–HR16 are surrounded closely by cells expressing PBP.

Discussion

Based on the assumption that candidate pheromone receptors might be members of the olfactory receptor family but might be more conserved than the highly diverse general olfactory receptors, in this study a small set of putative olfactory receptors was identified from the moth *H. virescens*, which share an array of characteristic features consistent with those predicted for pheromone receptors of moths. These receptors were found to be expressed in a sex-specific manner only in antennae of male moths. In many moth species, the antennae are sexually divergent; usually, only male antennae contain sex-pheromone-responsive cells, which may comprise as many as 50% of all chemosensory antennal cells (29). However, in *H. virescens* also, females might possess a few sensory neurons responding to pheromones (20). Interestingly, HR13 was found to be highly expressed in male antennae (Fig. 2), but gave only a very faint band in PCR experiments with preparations from female antennae. Electrophysiological recordings from individual olfactory cells in male antennae of *Heliothis* have demonstrated that components of the female sex pheromone are perceived by means of specific sensory cells in sensilla trichodea types 1 and 2, whereas host-plant odors are perceived by means of sensory cells of other sensilla types (20, 21). The observation that the novel receptor types HR13–HR16 were expressed in cells that could be confined to long sensilla trichodea supports the view that they are candidate pheromone receptors. The different number of cells expressing the various receptor types may be of particular interest in view of previous electrophysiological findings indicating that a rather high number of sensilla trichodea type 1, and also of type 2, contain neurons tuned to (*Z*)-11-hexadecenal, one of the major pheromone components, whereas the secondary pheromone component, (*Z*)-9-tetradecenal, activates significantly fewer cells (20, 21, 30). For other minor

components of the complex female pheromone blend (31, 32), how many cells are activated is unknown.

The possible role of the novel receptor types as pheromone receptors is further strengthened by the observation that the receptor-expressing cells are surrounded by cells expressing PBP (Figs. 5 and 6). By means of immunohistochemistry and electron microscopy, it has been shown that the PBPs are associated with pheromone-sensitive sensilla, primarily the specialized long trichoid sensilla in male antennae of noctuids (28, 33), and almost all sensilla trichoidea were found to be labeled with a PBP antibody in male *H. virescens* (34). Although the presence of PBPs may not be an ultimate proof for pheromone responsiveness of the sensilla, the finding that cells expressing receptor types HR14–HR16 are regularly wrapped by PBP-expressing cells further substantiates the evidence that these male-specific receptors represent candidate pheromone receptors. This view is not impaired by the fact that, based on sequence identities, they are grouped with receptor types (HR6, HR11, and HR13) that

are to some extent also expressed in other tissues and female antennae (Fig. 2). It is possible that these subtypes are not specialized for pheromones but may interact with structurally similar compounds. Alternatively, it is conceivable that at least HR11 and HR13, which show very low expression levels in female antennae, may be involved in pheromone detection because autodetection of pheromonal compounds by females has been demonstrated for several moths species, including noctuids (35).

The characteristic features of the identified male-specific receptor types render them candidate pheromone receptors, which may open avenues for exploring molecular aspects of chemical communication in insects. Furthermore, designing compounds affecting these receptors may allow novel strategies of pest control in agriculture and public health.

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