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MINI REVIEW

Review of pheromone receptors in heliothine species: expression, function, and evolution

Ke Yang[1,2] & Chen-Zhu Wang[1,2]*

[1]State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China, and [2]CAS Center for Excellence in Biotic Interactions, University of Chinese Academy of Sciences, Beijing China

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Abstract

Heliothine species (Lepidoptera: Noctuidae, Heliothinae) include some of the world's most devastating pest species, such as *Heliothis virescens* (Fabricius) in the Americas and *Helicoverpa armigera* (Hübner) in the Old World. Sex pheromone communication of these agricultural pest species has been investigated from pheromone receptors (PRs) to brains and behavior in detail. In recent years, great progress has been made in the mechanistic dissection of pheromone reception, demonstrating that PRs play a key role in determining the response characteristics of pheromone-sensitive olfactory receptor neurons. In this review, we focus on the functional characterization of PRs in heliothine species, and summarize recent progress in the identification of receptors tuned to principal sex pheromone components including Z11-16:Ald, Z9-16:Ald, Z9-14:Ald, and other related alcohols and acetates. Evolution of PRs in ligand selectivity is also discussed. The efficiency and faithfulness of three frequently used heterologous expression systems – *Xenopus laevis* Daudin oocytes, modified HEK 293 cells, and *Drosophila* 'empty neuron' mutants – are compared, and the research strategies, skills, and perspectives in the studies are envisioned. Finally, we propose future research topics on PRs in heliothine species.

Introduction

Sex pheromones play significant roles in intraspecific sexual communication and interspecific reproductive isolation, especially for moth species. Mate finding is largely dependent on males detecting and responding to the conspecific sex pheromones released by females in almost all moth lineages. Male moths evolved remarkably sensitive and selective olfactory systems to precisely respond to multicomponent mixtures of female-emitted sex pheromone components. Numerous odorant receptor neurons (ORNs) are housed in thousands of sensilla sensitive to pheromone components in the antennae. When the pheromone molecules enter the antennal hemolymph through the pores, it is supposed to be bound by pheromone-binding proteins (PBPs) and transported to pheromone receptors (PRs) (Vogt et al., 2002; Krieger et al., 2004; Touhara & Vosshall, 2009; Fleischer & Krieger, 2018; Venthur & Zhou, 2018). PRs are a subset of odorant receptors (ORs), which are seven transmembrane receptors but unrelated to the vertebrate G-protein coupled chemoreceptors. PRs can be directly stimulated by pheromone-related signals by forming a unique class of heteromeric ligand-gated ion channels with the co-receptor Orco (Sato et al., 2008; Wicher et al., 2008). Current data suggest that sensory neuron membrane proteins (SNMPs) could act in concert with PRs to capture cognate pheromone molecules delivered by PBPs on the surface of ORN dendrites (Benton et al., 2007; Gomez-Diaz et al., 2016). After being transduced, pheromone signals are relayed to the macroglomerular complex in the antennal lobes (Hansson et al., 1991; Rogers et al., 2001; Namiki et al., 2014). The signals from multiple sex pheromone blends are integrated in the antennal lobe, and finally pheromone information is relayed to the protocerebrum (Wong et al., 2002). In the

*Correspondence: Chen-Zhu Wang, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China. E-mail: czwang@ioz.ac.cn

whole process, PRs largely determine the selectivity and sensitivity of corresponding ORNs to pheromones, and play a key role in the olfactory coding of sex pheromones in moths.

Heliothinae is a cosmopolitan subfamily of noctuid moths, containing about 400 species. The larvae primarily attack flowers and fruits of herbaceous plants. Three globally distributed pest species – *Helicoverpa armigera* (Hübner), *Helicoverpa zea* (Boddie), and *Heliothis virescens* (Fabricius) – pose major threats to agricultural systems, particularly to high-value crops such as cotton, maize, sunflower, soybean, and tomato (Cunningham & Zalucki, 2014). Successive and economically significant outbreaks of *H. armigera* have recovered again in China since 2010 (Guan et al., 2016). More recently *H. armigera* has invaded South America and cause great damage to agriculture and horticulture in many areas (Jones et al., 2019).

Heliothine species are excellent model systems for examining pheromone production, detection, and evolution in closely related species. Multicomponent pheromones have been described in major pest species of heliothine species and their relatives. Studies on pheromone function have been corroborated by studies on pheromone gland composition, behavior, electrophysiology, and field trap attraction (Hillier & Baker, 2016), which have laid an excellent foundation to further analyze the PR's function and evolution in heliothine species. Most of the studies on sex pheromone identification and male moth behavior to sex pheromones, as well as the functional analysis of underlying PRs, have focused on two *Heliothis* species [*H. virescens* and *Heliothis subflexa* (Guenée)] and three *Helicoverpa* species [*H. armigera*, *H. zea*, and *Helicoverpa assulta* (Guenée)]. Moreover, *H. virescens* and *H. subflexa* (Laster, 1972), *H. zea* and *H. armigera* (Laster et al., 1985), and *H. armigera* and *H. assulta* (Wang & Dong, 2001) can be hybridized interspecifically in the laboratory, which provided valuable material for the study of genetics and evolution of pheromone communication in moth species. Therefore, this review is limited to these five heliothine species and focuses on the expression, function, and evolution of their PRs.

Sex pheromone blends and responding sensilla in heliothine species

The sex pheromone components of *H. armigera*, *H. assulta*, *H. zea*, *H. virescens*, and *H. subflexa* are listed in Table 1. All species use Z11-16:Ald as the major pheromone component except for *H. assulta* that use Z9-16:Ald as the major component and Z11-16:Ald as the second component (Cork et al., 1992). Z9-16:Ald serves as the second pheromone component of *H. armigera* (Nesbitt et al., 1979), *H. subflexa* (Teal et al., 1981), and *H. zea* (Pope et al., 1984). However, Z9-16:Ald is not a pheromone component of *H. virescens* that uses Z9-14:Ald as its second pheromone component (Roelofs et al., 1974; Vickers et al., 1991) (Table 1). The pheromone-sensitive sensilla have been characterized by single sensillum recording in male antennae of *H. virescens* (Almaas & Mustaparta, 1991; Berg et al., 1995), *H. subflexa* (Baker et al., 2004), *H. zea* (Grant et al., 1989; Cossé et al., 1998), *H. armigera*

Table 1 Sex pheromone components of the best-studied heliothine species

Species	Distribution	Hybridization	Sex pheromone component (percentage)
Heliothis virescens	North and South America[1]	With *H. subflexa*[2] ♀ *H. subflexa* × ♂ *H. virescens* (F1 males: sterile; F1 females: fertile)	Z11-16:Ald (100); Z9-14:Ald (0.4-6)[3,4,5]
Heliothis subflexa	North and South America[1]	With *H. virescens*[2]	Z11-16:Ald (100); Z9-16:Ald (42.9); Z11-16:OH (5.8)[6,7]
Helicoverpa zea	North and South America[1]	With *H. armigera*[8] ♀ *H. armigera* × ♂ *H. zea* (mating incidence was extremely low)	Z11-16:Ald (100); Z9-16:Ald (1.8)[9]
Helicoverpa armigera	Asia, Southeast Asia, Australia, Africa, southern Europe, eastern Pacific[1]	With *H. zea*[8]; *H. assulta*[10] ♀ *H. armigera* × ♂ *H. assulta* (only fertile males)	Z11-16:Ald (100); Z9-16:Ald (2.5)[11]
Helicoverpa assulta	Asia, Southeast Asia, Australia[1]	With *H. armigera*[10]	Z9-16:Ald (100); Z11-16:Ald (6.5)[12]

References: [1]Hillier & Baker (2016); [2]Laster (1972); [3]Roelofs et al. (1974);[4]Klun et al. (1980); [5]Vickers et al. (1991); [6]Teal et al. (1981); [7]Vickers (2002); [8]Laster et al. (1985); [9]Pope et al. (1984); [10]Wang & Dong (2001); [11]Nesbitt et al. (1979); [12]Cork et al. (1992).

(Wu et al., 2013, 2015, Chang et al., 2016; Xu et al., 2016), and *H. assulta* (Berg & Mustaparta, 1995; Berg et al., 2005; Wu et al., 2013, 2015; Chang et al., 2016; Xu et al., 2016). Based on the tuning specificity, the pheromone-sensitive sensilla are classified into three functional types, A, B, and C: type A specifically responds to Z11-16:Ald, type B responds to Z9-14:Ald, and type C to Z9-16:Ald, Z9-14:Ald, and some other structurally related compounds (Berg & Mustaparta, 1995; Cossé et al., 1998; Baker et al., 2004; Wu et al., 2015; Xu et al., 2016, 2017). B- and C-type sensilla are further classified into subtypes according to their response spectra in *H. armigera* and *H. assulta* (Xu et al., 2016). Against this background, these heliothine species provide a good model to study the mechanism of pheromone communication during speciation.

Identification, diversity, and phylogenetic characteristics of PRs in heliothine species

Insect ORs were first identified in *Drosophila melanogaster* Meigen by analyzing the database of *Drosophila* genomic DNA sequences (Clyne et al., 1999; Gao & Chess, 1999; Vosshall et al., 1999). Getting the full-length sequence of heliothine PRs was the first step toward their functional analysis. Using known *Drosophila* OR genes data, together with analyzing *H. virescens* genomic sequence data and antennal cDNA-library screening, Krieger et al. (2002) first identified a series of OR sequences in *H. virescens*. In 2004, the same group identified six candidate PRs (HvirOr6, 11, 13, 14, 15, and 16) by further screening of antennal cDNA and genomic sequences with probes against ORs of *H. virescens* and ORs sequences from other insects (Krieger et al., 2004) (Figure 1). HvirOr13, 14, 15, and 16 were found to be exclusively expressed in pheromone-responsive sensilla trichoidea of male antennae, and shared more than 40% amino acid identity. This was the first report about the identification of candidate PR sequences in lepidopteran species. In the same year, Sakurai et al. (2004) identified the gene encoding the receptor BmOR1 from *Bombyx mori* (L.) and functionally characterized the protein in the *Xenopus* oocyte expressing system as receptor for the major sex pheromone component bombykol of the silk moth; this was the first study describing the specific function of an insect PR. In 2011, five PR sequences (Or6, Or13, Or14, Or15, and Or16) homologous to the *H. virescens* PR sequences were reported in *H. subflexa* (Vásquez et al., 2011) (Figure 1). Zhang et al. (2010) cloned the full-length cDNAs of three PRs from *H. armigera* (HarmOr11, HarmOr13, and HarmOr16) and three PRs from *H. assulta* (HassOr11, HassOr13, and HassOr16) by RACE PCR based on the nucleotide sequences of PRs in *H. virescens*. Later, by mining antennal transcriptome data of *H. armigera*, the identified sequences of PRs in *H. armigera* were increased to six (HarmOr11, HarmOr13, HarmOr16, HarmOr14, HarmOr15, and HarmOr6) (Liu et al., 2012). Furthermore, the full-length sequences of two PRs from *H. assulta* (HassOr14b and HassOr6) and one PR from *H. armigera* (HarmOr14b) were identified by RACE PCR using primers designed on the corresponding ORs of *H. virescens* (Jiang et al., 2014). In 2015, Xu et al. (2015) identified HassOr15 by analysis of the *H. assulta* antennal transcriptome. Together with HassOr14 (the sequence of which is submitted into GenBank AHI44516.1), seven PRs (Or6, 11, 13, 14, 14b, 15, and 16) were identified in both *H. armigera* and *H. assulta* (Figure 1).

The phylogenetic analysis of the identified PRs reveals seven clusters, comprising Or13, Or11, Or16, Or6, Or14b, Or14, and Or15 in heliothine species (Figure 1). The most neighbored PR cluster with the outgroup cluster of Orco is Or13. The Or6 and Or16 clades are clustered in the same branch. Similarly, the Or14 and Or14b clades fall in the same branch (Figure 1). Up to now, the Or14b clade has only been identified from *H. armigera* and *H. assulta* in heliothine species (Figure 1).

The expression pattern of PRs in heliothine species

The relative expression patterns of PRs in the male antennae have been investigated by using quantitative real-time PCR in four heliothine species, *H. virescens*, *H. subflexa*, *H. armigera*, and *H. assulta* (Vásquez et al., 2011; Yang et al., 2017). We normalized the PRs' expression levels by transferring the expression data with highest expression PR as '1', and calculated the relative expression level of the other PRs from the same species (Figure 2). Or13 has a high expression level in *H. armigera*, *H. virescens*, and *H. subflexa*, but a lower level in *H. assulta* (Figure 2). For *H. assulta*, HassOr14b has the highest expression level, nearly five- to six-fold higher than that of HassOr13 (Figure 2).

The in situ hybridization analysis proved that Or13 is adjacently localized with Or11 in type A sensilla in *H. virescens* (Baker, 2009; Krieger et al., 2009), *H. armigera* and *H. assulta* (Chang et al., 2016), and Or14b is localized with Or6 or Or16 in type C sensilla in *H. assulta* (Yang et al., 2017). As the A-type sensilla responding to Z11-16:Ald are the most abundant type in the male antennae of *H. armigera* (Xu et al., 2016), *H. virescens* (Almaas & Mustaparta, 1991), and *H. subflexa* (Baker et al., 2004), whereas C-type sensilla responding to Z9-16:Ald are the most abundant in the male antennae of *H. assulta*, we suggest that the PR expression levels are roughly correlated with the number of sensilla containing the respective PR-expressing neurons on the male antenna in heliothine species.

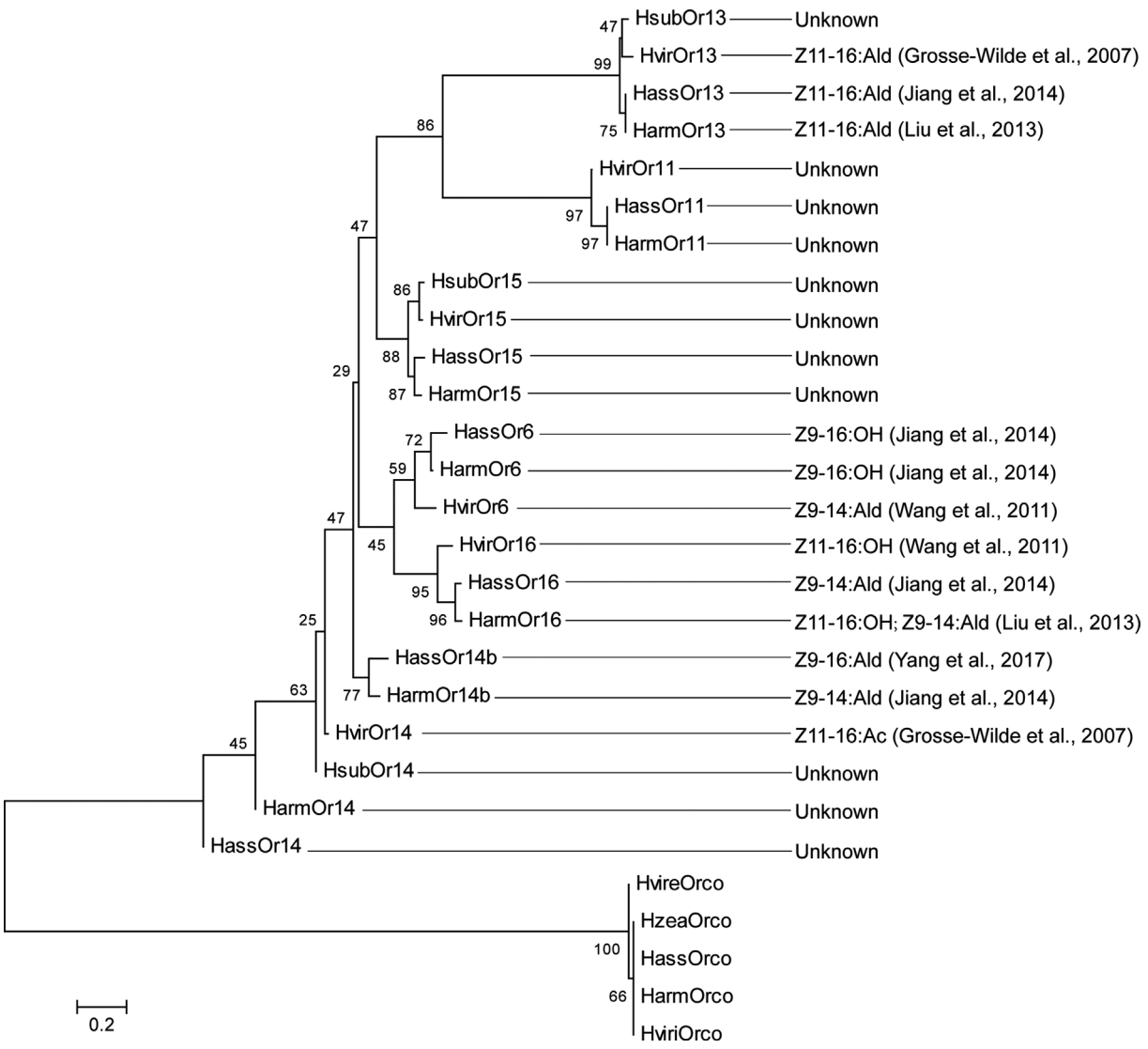

Figure 1 Phylogenetic relation and determined ligand specificity of pheromone receptors (PRs) in heliothine species. The Orco lineage is defined as an outgroup. Bootstrap values are based on 1 000 replicates, and the values are shown at corresponding nodes. The bar indicates the phylogenetic distance value. Some partial PR sequences were not included. Abbreviations: Harm, *Helicoverpa armigera*; Hass, *Helicoverpa assulta*; Hvir, *Heliothis virescens*; Hsub, *Heliothis subflexa*; Hviri, *Heliothis viriplaca* (Hufnagel); Hzea, *Helicoverpa zea*. Tuning specificity is based on previous PR functional analysis in *H. armigera* (Liu et al., 2013; Jiang et al., 2014), *H. assulta* (Jiang et al., 2014; Yang et al., 2017), and *H. virescens* (Grosse-Wilde et al., 2007; Wang et al., 2011).

Function of PRs in heliothine species

Heterologous expression systems, including *Xenopus laevis* Daudin oocytes, modified HEK293 cells, and *D. melanogaster delta-halo* mutant with an empty ab3A neuron and *Or67d-GAL4* mutant have been used successfully for the functional characterization of candidate heliothine PRs in vivo. This allowed to assign distinct components of the heliothine pheromone blend to distinct PR types.

The receptor for Z11-16:Ald

Z11-16:Ald is the major pheromone component of heliothine species except for *H. assulta* (Hillier & Baker, 2016). However, *H. assulta* also use Z11-16:Ald as the secondary pheromone component (Wang et al., 2005). Gohl & Krieger (2006) combined antibody staining with in situ hybridization to examine the localization of PRs in specific sensilla, and found that the HvirOr13 gene was expressed in ORNs beneath sensilla trichodea. Moreover, the protein

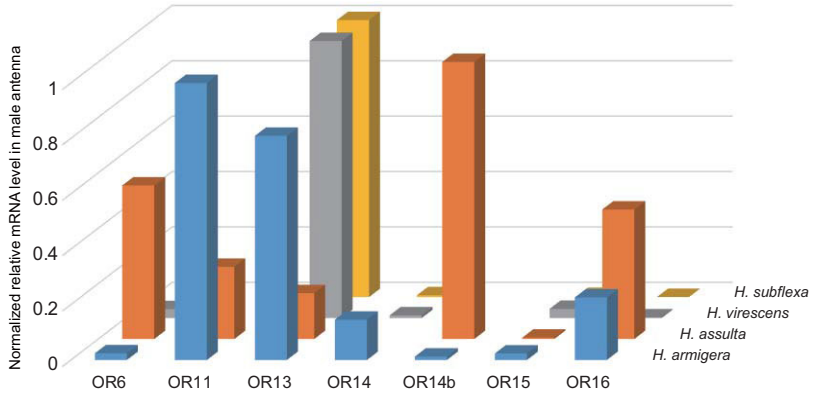

Figure 2 Normalized relative expression pattern of the heliothine pheromone receptors (PRs) in male antenna. In order to compare the PR's relative expression pattern among species, the PR with the highest expression level was normalized to '1' in each species, and the relative expression of the other PRs were calculated accordingly. The expression data shown were based on previous PR expression analysis in male antenna of *Helicoverpa armigera*, *Helicoverpa assulta* (both Yang et al., 2017), *Heliothis virescens*, and *Heliothis subflexa* (both Vásquez et al., 2011).

was located to the dendrites of these ORNs projecting into the sensillum shaft (Gohl & Krieger, 2006). By using a modified HEK293 cell line, Grosse-Wilde et al. (2007) discovered that HvirOr13 is specifically tuned to Z11-16:Ald, which was further proved using the *X. laevis* oocyte expression system (Wang et al., 2011) (Figure 1) and the *Drosophila* empty neuron system (Kurtovic et al., 2007). Later on, the functional analyses of orthologs of HvirOr13 in other heliothine species were carried out by using the *Xenopus* oocytes expression system. HarmOr13 and HassOr13 were also specifically tuned to Z11-16:Ald in *H. armigera* and *H. assulta*, respectively (Liu et al., 2013; Jiang et al., 2014) (Figure 1). Together with the fact that Or13 is highly expressed in ORNs located in the type A sensilla in male antennae of *H. armigera* and *H. virescens*, and relatively lower expressed in male antennae of *H. assulta*, this strongly supports the notion that Or13 is the receptor to Z11-16:Ald, the principal pheromone component in heliothine species. Remarkably, its function and expression pattern are very conservative across heliothine species.

Receptors for Z9-16:Ald

Z9-16:Ald is the major pheromone component of *H. assulta* and the secondary pheromone component of *H. armigera*, *H. zea,* and *H. subflexa* (Table 1). HarmOr6 was considered to be the receptor of Z9-16:Ald in *H. armigera* that use Z9-16:Ald as the secondary pheromone components (Liu et al., 2013). However, a series of functional studies in the *Xenopus* oocyte system found the best ligand of HarmOr6 or HassOr6 is Z9-16:OH but not Z9-16:Ald (Jiang et al., 2014; Chang et al., 2016; Yang et al., 2017) (Figure 1). Xu et al. (2016) demonstrates that HassOr6 has a relatively lower expression level in male antennae of *H. assulta*, which is not consistent to the high abundance of the C-type sensilla trichodea responding to Z9-16:Ald in male antennae of *H. assulta* (Xu et al., 2016).

In *H. assulta*, the PR tuned to Z9-16:Ald is expected to be highly expressed in male antennae because the C-type sensilla trichodea responding to Z9-16:Ald are predominant in male antennae, a situation reminiscent to *H. armigera* where numerous A-type sensilla contain an ORN expressing Or13 (Wu et al., 2013; Xu et al., 2016). When the functions of HarmOr14b and HassOr14b were characterized for the first time by using the *Xenopus* system, the oocytes expressing HarmOr14b responded to Z9-14:Ald, whereas HassOr14b failed to respond to any chemicals (Jiang et al., 2014). In a later study, Yang et al. (2017) validated the sequences and expression patterns of all seven PRs in *H. assulta*, and found that HassOr14b is the most highly expressed receptor among them in the male antennae of *H. assulta* (Figure 2). Proving that the oocytes expressing HarmOr14b are specifically tuned to Z9-14:Ald, the functional study discovers that HassOr14b is specifically tuned to Z9-16:Ald (Figure 1) (Yang et al., 2017). Together with the fact that HassOr14b is highly expressed and the C-type sensilla are predominant in male antennae of *H. assulta*, we confirm that HassOr14b is the receptor to Z9-16:Ald, and the ORNs expressing Or14b are located in the type C sensilla in *H. assulta*.

Z9-16:Ald is the secondary pheromone blend component for *H. armigera* (Table 1). However, oocytes expressing HarmOr14b are specifically tuned to Z9-14:Ald (Jiang et al., 2014; Yang et al., 2017). Liu et al. (2013) suggest that HarmOr6 could be the receptor of Z9-16:Ald because the

Xenopus oocytes expressing HarmOr6 are moderately responsive to Z9-16:Ald (Liu et al., 2013) although the best ligand of HarmOr6 is Z9-16:OH (Figure 1). Therefore, the receptors tuned to Z9-16:Ald in *H. armigera*, *H. zea*, and *H. subflexa* are still elusive.

Receptors for Z9-14:Ald

Z9-14:Ald is the second component in the sex pheromone blend of *H. virescens*, but acts as an antagonist in the pheromone communication of *H. assulta* (Boo et al., 1995; Wu et al., 2015) (Table 1). In the pheromone communication of *H. armigera*, Z9-14:Ald acts as an agonist in small amounts (0.3%) (Rothschild, 1978; Zhang et al., 2012; Wu et al., 2015) but an antagonist in higher amounts (1% and above) (Gothilf et al., 1978; Kehat & Dunkelblum, 1990; Wu et al., 2015). However, SSR results demonstrate that the sensilla thrichodea responding to this chemical are quite common in *H. assulta*, *H. virescens*, *H. armigera*, and *H. zea*. Wang et al. (2011) found that HvirOr6 from *H. virescens* is specifically tuned to Z9-14:Ald by using the *Xenopus* oocytes expression system (Figure 1). However, in the profile of the test chemicals, Z9-16:OH was not included (Wang et al., 2011). The receptors specifically tuned to Z9-14:Ald in *H. armigera* and *H. assulta* are HarmOr14b and HassOr16, respectively (Jiang et al., 2014). HarmOr6 and HassOr6 are also responsive to Z9-14:Ald, but the most effective ligand for these PRs is Z9-16:OH (Jiang et al., 2014) (Figure 1). HarmOr16 is tuned to both Z9-14:Ald and Z11-16:OH, and the best ligand for HarmOr16 is Z11-16:OH (Liu et al., 2013). As HarmOr14b has a relatively low expression level in male antenna, we suggest that HarmOr14b is not the major receptor to Z9-14:Ald in *H. armigera*. HassOr16 has the second highest expression level in *H. assulta*, we suggest that HassOr16 plays an important role in detecting Z9-14:Ald in *H. assulta*. Whether HarmOr6, HassOr6, or HarmOr16 also take part in the detection of Z9-14:Ald must await further analysis.

Receptors for Z11-16:OH and Z9-16:OH

Z11-16:OH is one of the sex pheromones for *H. subflexa*, but is a behavioral antagonist to *H. virescens* (Vickers & Baker, 1997), *H. zea* (Quero & Baker, 1999), and *H. armigera* (Kehat & Dunkelblum, 1990). HvirOr16 from *H. virescens* and HarmOr16 from *H. armigera* are tuned to Z11-16:OH (Wang et al., 2011; Liu et al., 2013). Moreover, Chang et al. (2017) confirm that HarmOr16 is required for the detection of Z11-16:OH in *H. armigera* by mutagenesis through the CRISPR-Cas 9 technique. Z9-16:OH is found in the pheromone gland extracts of *H. armigera* and *H. assulta* (Cork et al., 1992; Wang et al., 2005), and acts as behavioral antagonist for *H. assulta*. HarmOr6 and HassOr6 are sensitively tuned to Z9-16:OH (Jiang et al., 2014) (Figure 1). We suggest that Or6 is the receptor of Z9-16:OH in *H. armigera* and *H. assulta*.

Receptors for Z11-16:Ac and Z9-16:Ac

Z11-16:Ac is a behavioral antagonist to *H. virescens* (Vickers & Baker, 1997), *H. zea* (Quero et al., 2001), and *H. armigera* (Xu et al., 2016), but is an agonist to *H. assulta* (Xu et al., 2016). As shown by functional analysis in modified HEK293 cells (Grosse-Wilde et al., 2007) and the *Xenopus* oocytes expression system (Wang et al., 2011) HvirOr14 is tuned to Z11-16:Ac (Figure 1). However, the PR tuned to the Z11-16:Ac in other heliothine species is still unknown. Z9-16:Ac is one of the components of the pheromone gland extracts of *H. assulta* (Cork et al., 1992). Interestingly, this compound is an antagonist for Chinese *H. assulta* colonies but an agonist for Korean colonies when mixed with Z11-16:Ac (Cork et al., 1992). Receptors for Z9-16:Ac are still unknown in the heliothine species. We expect that the functional analysis of not yet analyzed PRs such as receptors from the Or15 clade may finally close this gap in knowledge.

Evolution of PRs in heliothine species

The sex pheromones released from female moths play a critical role in species-specific communication. According to the asymmetric tracking model, male preference varies more frequently than the composition of the female pheromone does, thus the male olfactory system may track the variation in the female pheromone (Phelan, 1992; Groot et al., 2016). How male moths change their olfactory responses and how this eventually affects the evolution of behavior remains unclear. With regard to illuminating adaptive processes in the male olfactory system, heliothine species are good models to study the diversity of PRs and the evolution of PR's functions. There are at least three aspects that need consideration in the context of changes in the pheromone response phenotype of male moths caused by adaptive changes in PRs.

Birth and death of PR genes

Most multigene families are subject to 'birth-and-death' evolution (Wyatt, 2014). The OR gene family has expanded from a small number of ancestral OR genes, predominantly through a series of uneven crossing over and tandem duplication events (Kratz et al., 2002). However, OR gene losses also occur by deletion or from pseudogenization by nonsense or frameshift mutations (Nei et al., 2008; Andersson et al., 2015). PR sequence clades in lepidopteran species may be formed in both directions. Based on the phylogenetic tree of PRs in these insects, it seems that *Or13* could be more ancestral than other PR genes. It

is still a mystery that the *Or14b* gene identified from *H. armigera* and *H. assulta* was absent in other heliothine species. When an identical copy of an existing gene is produced, it may later get separated within the genome, but needs much time to accumulate enough mutations for functional divergence (Heckel, 2010). Gene expansion by uneven crossing over may immediately create a novel phenotype (Heckel, 2010). Heliothine species are evolutionarily close enough, and some species could be interspecifically hybridized and produce fertile hybrids in laboratory, providing a good opportunity to study the early stages of divergence and the genetic basis of their phenotypes. For example, differential male responses to three female-produced chemicals that maintain sexual isolation in *H. subflexa* and *H. virescens* are all controlled by a single quantitative trait locus in the genome (Gould et al., 2010). F1 male analysis proved that the mutation with one copy of some PRs would suffice to react to the pheromone components, and could broaden the tuning of its ORN (Gould et al., 2010). From the hybridization analysis from *H. armigera* and *H. assulta*, it is demonstrated that the population ratio of the two male-specific types of olfactory sensory neurons responding to two sex pheromone components is controlled by a major gene, and that the allele of *H. armigera* is dominant (Xu et al., 2017). Moreover, two new subtypes with broader response spectra emerged in the hybrids, implying introgression related to PR genes might occur from *H. assulta* into *H. armigera* through repeated backcrossing (Xu et al., 2017). The evolutionary mechanism of PRs diversification needs to be analyzed further, especially from the hybrids.

Changes in gene expression patterns

Adaptive changes in the pheromone response of moths could also be due to changes in the number of PR-expressing cells on the antennae or the expression level of PRs in single ORNs. The closely related heliothine species *H. armigera* and *H. assulta* share two pheromone components, Z11-16:Ald and Z9-16:Ald, but in reverse ratios, 100:2.5 and 6.5:100, respectively. HarmOr13 and HassOr13 are both specifically tuned to Z11-16:Ald, but have different Or13 expressing cell numbers in males of the two species possibly reflecting adaptions in cell number to the amount of pheromone in the female-released pheromone blend (Liu et al., 2013; Jiang et al., 2014; Yang et al., 2017). How such adaptations in cell numbers have evolved in heliothine moths is unclear. One possibility is that changes in genes regulating neurogenesis have affected the PR's expression and localization. Such regulatory genes have been identified in *Drosophila* where *acj6*, *E93*, *Fer1*, *onecut*, *sim*, *xbp1*, and *zf30c* control the expression patterns of more than 30 ORs (Jafari et al., 2012).

Functional shift of PR genes

It is easy to understand that PRs keep the stability in expression and function under strong selective pressure. Genetic mapping by using interspecific hybridizations indicates that some PR genes form a tightly linked cluster of duplicated genes in the chromosomes (Gould et al., 2010). However, it remains an unsolved mystery how a PR shifts from one function to another under stabilizing selection. One possibility is that the PRs could change their tuning specificity by amino acid mutation(s). In heliothine moths such evolutionary processes appear to be represented in HarmOr14b and HassOr14b that are 91% identical in the amino acid sequences (Yang et al., 2017), but have a different ligand selectivity. HarmOr14b is specifically tuned to Z9-14:Ald (Jiang et al., 2014), whereas HassOr14b is specifically tuned to Z9-16:Ald (Yang et al., 2017). Also in the Or16 clade, functional expansions and shifts in PRs are represented. The amino acid identity of HarmOr16 with HassOr16 and HvirOr16 is 94.1 and 88.2%, respectively, and that between HassOr16 and HvirOr16 is 87.4%. Whereas HvirOr16 is specifically tuned to Z11-16:OH, and has very weak response to Z9-14:Ald (Wang et al., 2011), HarmOr16 is sensitively tuned to both Z11-16:OH and Z9-14:Ald (Liu et al., 2013). In addition, HassOr16 is specifically tuned to Z9-14:Ald, and shows only very weak response to Z11-16:OH (Jiang et al., 2014; Yang et al., 2017). Compared with HvirOr16, this PR represents a complete PR's functional shift.

Leary et al. (2012) reported that a single amino acid mutation could change the ligand selectivity of a PR in *Ostrinia furnacalis* (Guenée) and *Ostrinia nubilalis* (Hübner). Yang et al. (2017) found two single-point mutations, T355I and F232I, responsible for the difference in ligand selectivity of a PR between two *H. armigera* and *H. assulta*. This evidence suggests that for the sibling species or the different geographical populations of the same species, the functional shift of their PRs could be achieved by 'one-step' and that a single amino acid change could induce the functional change just like in *O. furnacalis* and *O. nubilalis* (Leary et al., 2012). For the closely related species such as *H. armigera* and *H. assulta* (Yang et al., 2017), it would be more common that the PR's functional change entails at least 'two steps': the first step might be the functional extension, which could adapt to the extension in the sex pheromone, whereas the second step might be the functional shift that could adapt the evolved sex pheromone released by the female. Each step would require one or few single-point(s) mutation(s) (Figure 3).

Generally, the transmembrane domains (TMDs) and extracellular loops (ECLs) constitute the ligand binding domain in ORs (Guo & Kim, 2010; Yuvaraj et al., 2020). In

2018, Butterwick et al. (2018) solve the cryo-EM structure of the insect odorant receptor Orco, suggesting that the loose packing of helices S1–S6 form the extracellular pocket, and the residues lining this pocket are required in defining odorant specificity both in Orco and in ORs. Interestingly, the single amino acid mutation that could change the ligand selectivity of a PR in *O. furnacalis* and *O. nubilalis* is located in the predicted third TMD (Leary et al., 2012). The two single-point mutations, T355I and F232I, which determine the ligand selectivity of a PR between *H. armigera* and *H. assulta*, are both located in the predicted TMDs (Yang et al., 2017; Butterwick et al., 2018).

Critical points and new approaches in functional identification of pheromone receptors

Although significant progress has been made in the functional analyses of PRs in lepidopteran species, some challenges remain: (1) genetic manipulation tools are very limited compared to *D. melanogaster*; (2) gene silencing through RNA interference is difficult to achieve particularly in lepidopteran species (Terenius et al., 2011); and (3) in some cases the results of functional analyses of PRs by using different methods are inconsistent (Wang et al., 2018). We summarize our experiences and put forward some perspectives about the PR's study in five aspects.

Using multiple heterologous expression systems

Heterologous expression systems, including *X. laevis* oocytes (Wetzel et al., 2001), modified HEK293 cells (Grosse-Wilde et al., 2006), the Sf9 cell line expression system (Kiely et al., 2007), and *D. melanogaster* 'empty neuron' systems (Dobritsa et al., 2003) have been used successfully for the functional characterization of candidate moth PRs in vivo. For identifying the function of moth PRs the *Xenopus* oocytes expression system is most widely used (Zhang & Löfstedt, 2015). In this system, both the complementary RNAs (cRNAs) of a candidate *PR* and the corresponding *Orco* are concomitantly injected into

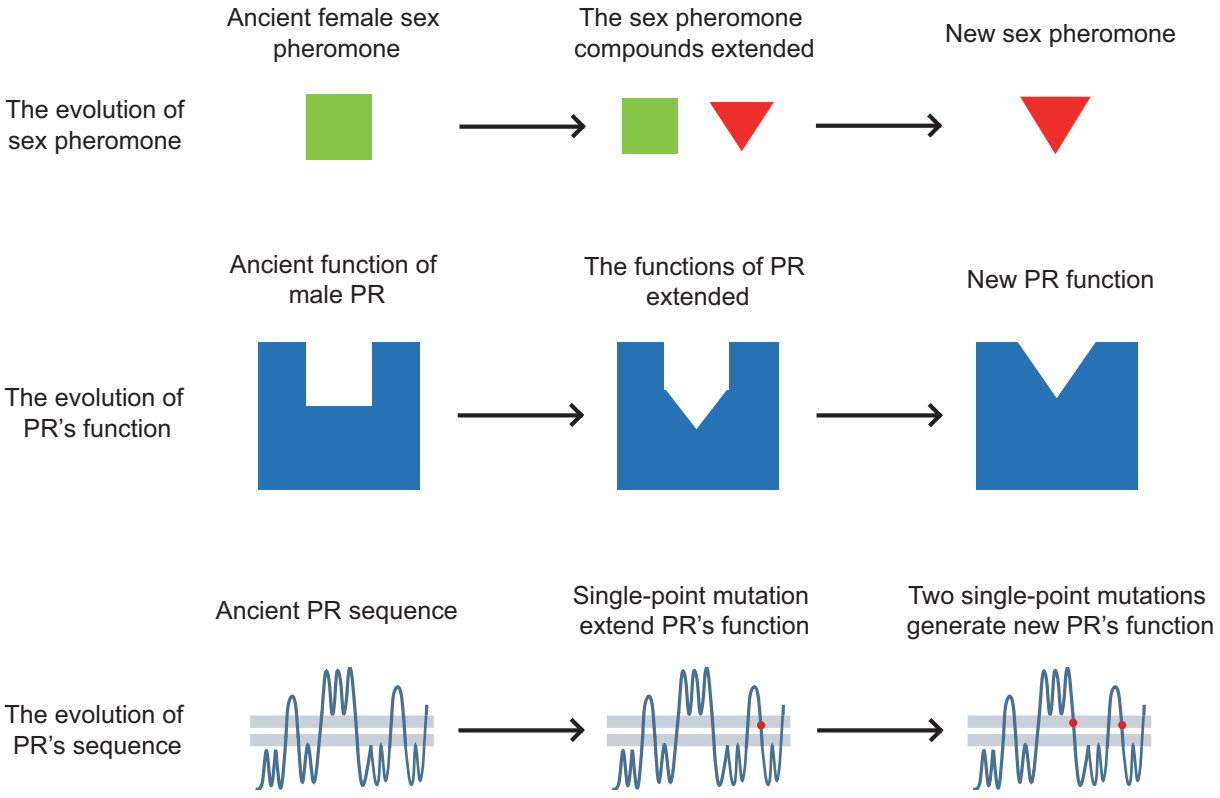

Figure 3 Schematic view of one of the possible processes in the evolution of sex pheromone and pheromone receptor (PR) function. For the evolution of sex pheromone, the green square represents the ancient female sex pheromone, the red triangle represents the newly emerged sex pheromone. For the evolution of PR function, the blue form with the square-shaped cavity represents the ancient function of male PR, the blue form with a triangle and square notch represents the extension of the PR's function, and the blue form with a triangle notch represents the new PR's function. The predicted topology of HassOr14b (Yang et al., 2017) at the bottom represents the evolution of PR's sequence, the mutation sites are highlighted by dots (●).

oocytes of *X. laevis* for PR and Orco protein expression. In general, 3–5 days post-injection *Xenopus* oocytes are tested for their response to a panel of pheromones and pheromone-related compounds solubilized in buffers flowing over the oocyte using the two-electrode voltage-clamp system (de Fouchier et al., 2014). For analyzing PRs in the HEK293/Gα protein cell line expression system, vectors containing PR sequences have been transfected to generate stable PR-expressing cell lines. When simulated by pheromones and related compounds, coupling of these exogenous proteins elicits an increase in the level of intracellular Ca^{2+}, and the signals could be measured via calcium imaging systems with the help of calcium-sensitive dye responding to calcium release by the cells (Grosse-Wilde et al., 2007; Krieger et al., 2009; Corcoran et al., 2014). For the Sf9 cell line expression system, which already expresses the *Spodoptera frugiperda* (JE Smith) Orco, the strategy for PR expression and analysis is quite similar to that used for HEK293 cells (de Fouchier et al., 2014). The vectors containing PR sequences were transfected to the Sf9 cells and the PR-expressing cells were finally tested by the calcium imaging system.

The *Drosophila* empty neuron expression systems are to some extent a faithful mimicry of olfactory systems of moths. By replacing an endogenous OR gene with a candidate moth PR gene, the PRs are expressed in corresponding *Drosophila* ORNs in the antenna. Then the ORNs in the *Drosophila* antenna are stimulated by moth pheromone-related compounds, and the responses of the PR-expressing ORNs are monitored by the single sensillum recording system (Dobritsa et al., 2003; Hallem et al., 2004; Hallem & Carlson, 2004, 2006). There are mainly two *Drosophila* 'empty neuron' expression systems. One is the ab3A neuron in which the endogenous *Drosophila DmelOr22a* gene has been mutated (Syed et al., 2006), and the other is the T1 neuron wherein the coding sequence of a PR (Or67d) is replaced (Kurtovic et al., 2007; Syed et al., 2010; Montagné et al., 2012). As the T1 neuron expresses SNMP1, a protein necessary for proper detection of pheromones (Jin et al., 2008), it is widely used for the functional analysis of moth PRs (Benton et al., 2007; Kurtovic et al., 2007; Syed et al., 2010; Montagné et al., 2012).

These heterologous expression systems have advantages and drawbacks in PR functional analysis. For example, the *Xenopus* oocytes expression system is more practical in high throughput screening the function of ORs (Di et al., 2017). However, the heterologous expression systems lack other olfactory elements such as PBPs and SNMPs, which may affect the pheromone response of some PRs. The *Drosophila* T1 empty neuron system expresses *Drosophila* PBPs and SNMPs, which may increase the accuracy in characterizing some PRs' function (Wang et al., 2016), but the PR genes cannot always be expressed in transgenic fly lines (de Fouchier et al., 2017). As each system has its pros and cons (Wang et al., 2016, 2018; Hou et al., 2019), it would be recommended to use multiple methods in identifying the function of PRs.

Combining the data from multiple level approaches in the real moth system

The identification of the sex pheromones in various heliothine species has paved the way to further study the chemoreception mechanism underlying pheromone detection in the moths. The behavioral and electrophysiological responses to pheromones of the moths as well as the expression patterns of PRs in antennae always reflect the functional characteristics of the real moth system. In this regard, we should keep in mind that using heterologous expression systems is crucially important for functional characterization of PRs in heliothine moths, but that these systems are far away from the natural situation after all. Therefore, for a comprehensive understanding of the specific function of a certain PR for the detection of a distinct pheromone compound, we should connect the results from its functional analysis with respective data from behavioral and electrophysiological analyses.

The responding profiles of pheromone-sensitive sensilla largely depend on the ORNs expressing specific PRs, whereas the expression pattern of PRs could largely reflect the abundance of ORNs and related pheromone-sensitive sensilla. Based on the single sensillum recording results, which include the specificity and abundance information of ORNs, we could support the function of heliothine PRs by combining this information with the expression pattern of the PRs. The A-type sensilla specifically tuned to Z11-16:Ald are the most abundant pheromone-sensitive sensilla in *H. virescens* and *H. armigera* (Berg et al., 1998; Wu et al., 2013; Xu et al., 2016). Consequently, the expression level of the receptor tuned to this chemical should be the highest among all the PRs in *H. virescens* and *H. armigera* (Vásquez et al., 2011; Liu et al., 2013; Jiang et al., 2014; Yang et al., 2017). In this respect, the best matched PR in *H. virescens* and *H. armigera* is Or13, which specifically responds to Z11-16:Ald in heterologous expression systems (Figure 4). Similarly, the C-type sensilla responding to Z9-16:Ald are the most abundant pheromone-sensitive sensilla in *H. assulta* (Xu et al., 2016), and Or14b is the most highly expressed among all PRs in *H. assulta*. Finally, the functional analysis proves that HassOr14b is specifically tuned to Z9-16:Ald (Yang et al., 2017) (Figure 4).

Concluding function from amino acid sequence relatedness of PRs

The amino acid sequences and functions of PRs are relatively conserved in closely related species. As more and

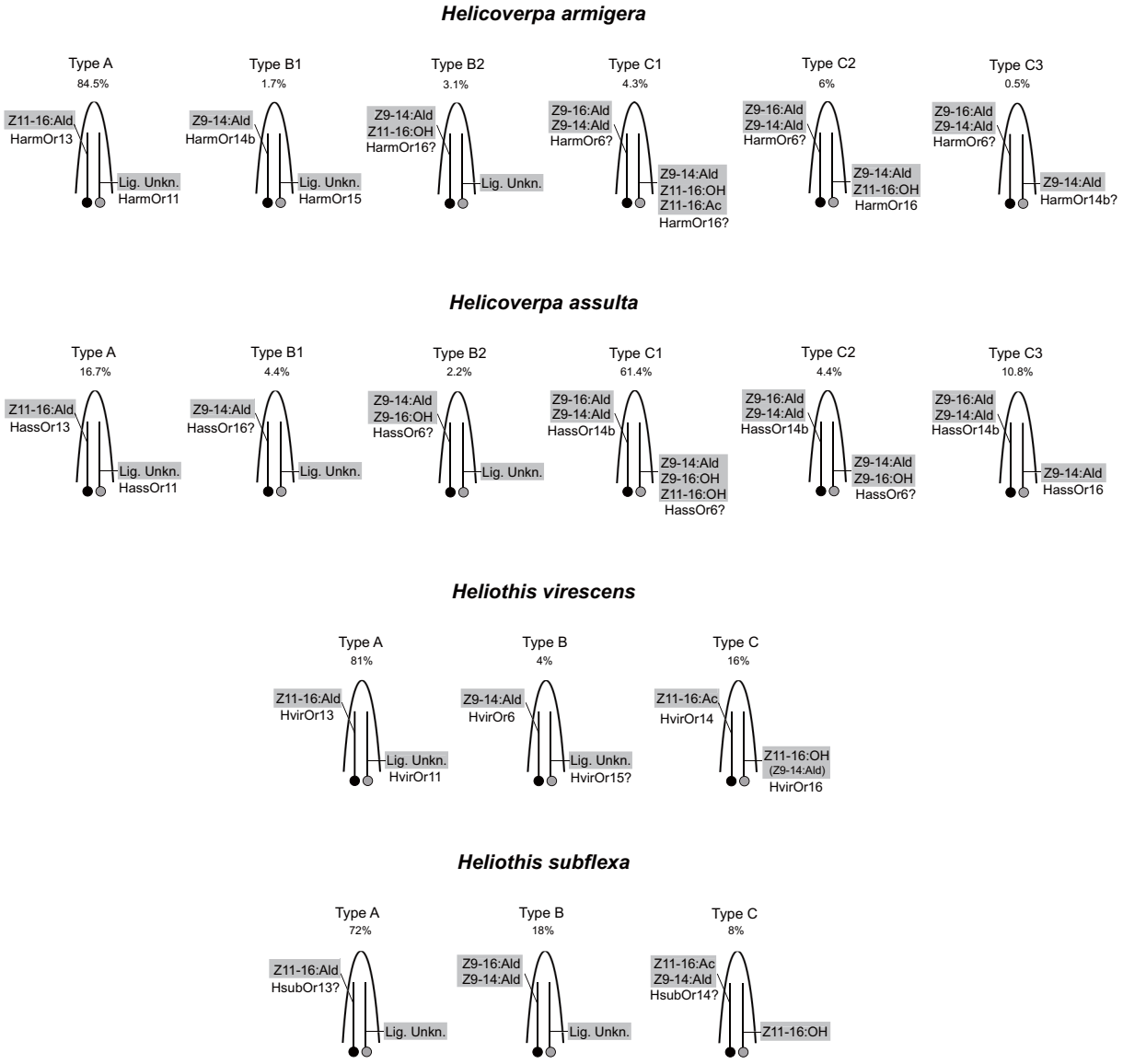

Figure 4 Model of sensillar compartmentalization arrangements in type A, B, and C trichoid sensilla and the putative locations of pheromone receptors (PRs) in the olfactory receptor neurons (ORNs) in male antennae of *Helicoverpa armigera*, *Helicoverpa assulta*, *Heliothis virescens*, and *Heliothis subflexa*. The different sensillum types and the percentages of each type are based on the results of indicated compounds to which various ORNs respond by single sensillum recordings (Baker et al., 2004; Berg et al., 2005; Wu et al., 2013, 2015; Xu et al., 2016). The putative localization of PRs in ORNs are based on matching of PR tuning profiles and ORNs responding profiles (Grosse-Wilde et al., 2007; Wang et al., 2011; Liu et al., 2013; Jiang et al., 2014; Yang et al., 2017) and/or have been characterized via in situ hybridization studies as being expressed on particular ORNs (Krieger et al., 2009; Chang et al., 2016; Xu et al., 2016; Yang et al., 2017). 'Lig. Unkn.', ORNs have no known ligand; '?' indicates uncertain function or localization of PR.

more PRs' functions have been characterized in different species, one may be tempted to assume that the function of, for example, ligand specificity of a very related but yet uncharacterized PR could be predicted from its sequence identity to functionally identified PRs. However, this assumption is not always true because the functional change can result from only one or a few amino acid mutations. For the same reason, we suggest that the accuracy and integrity of the amino acid sequence is crucial to the identification of a PR's function, no matter of which expression system is used. Moreover, the selection of the appropriate expression vector could also be important at

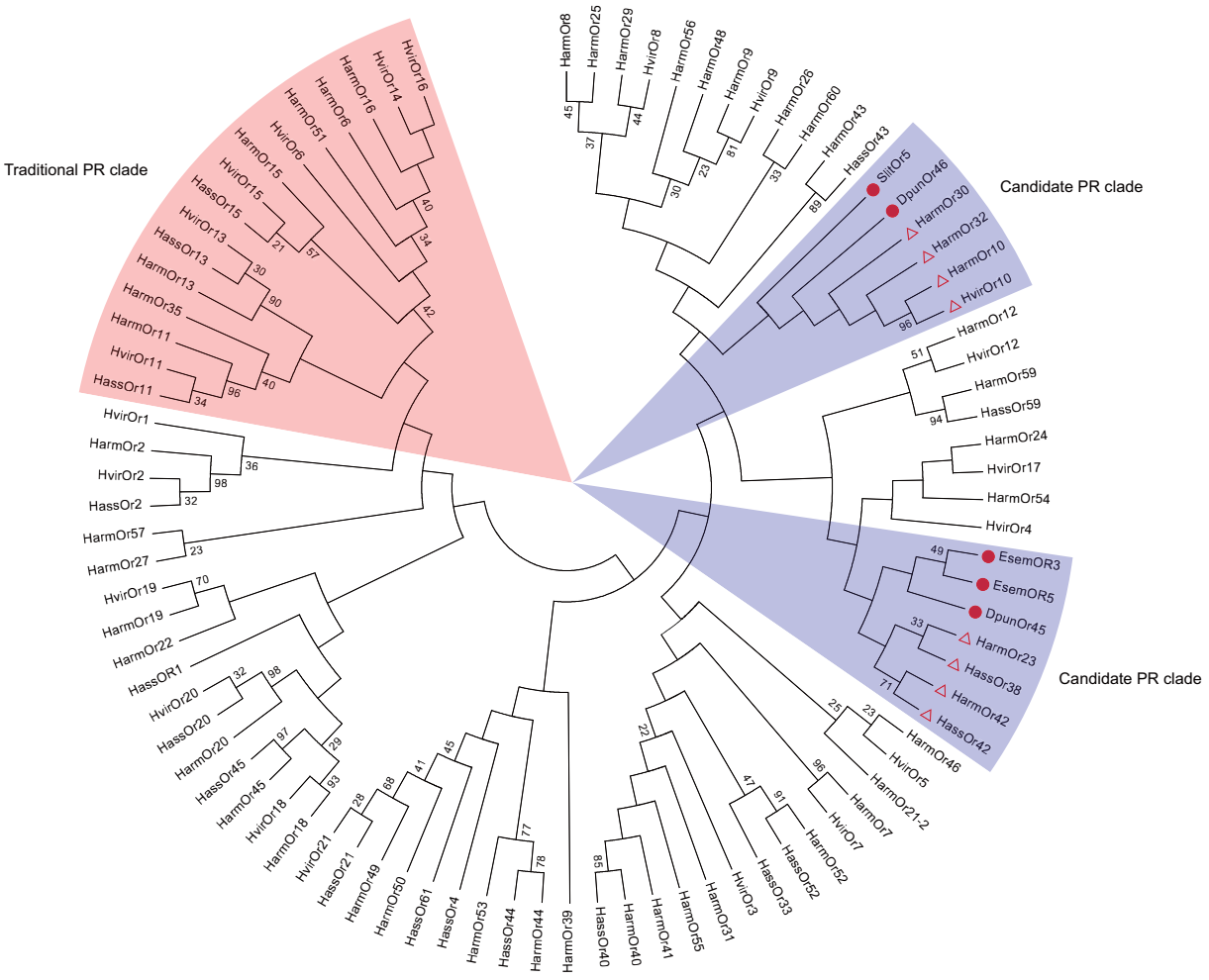

Figure 5 Phylogenetic analysis of odorant receptors (ORs) in heliothine species and novel lineage of candidate pheromone receptors (PRs) in other lepidopteran species. Bootstrap values are based on 1 000 replicates, and the values are shown at corresponding nodes. Some partial OR sequences were not included. Abbreviations: Harm, *Helicoverpa armigera*; Hass, *Helicoverpa assulta*; Hvir, *Heliothis virescens*; Slit, *Spodoptera littoralis*; Dpun, *Dendrolimus punctatus*; Esem, *Eriocrania semipurpurella*. The OR sequences in heliothine species are from *H. armigera* (Liu et al., 2012, 2014), *H. assulta* (Xu et al., 2015), and *H. virescens* (Krieger et al., 2002). The traditional PR clade is marked in red, the candidate novel PR clades are marked in blue. In the candidate novel PR clades, the reported PRs are marked by dots (●) – based on recent studies in *E. semipurpurella* (Yuvaraj et al., 2017), *S. littoralis* (Bastin-Héline et al., 2019), and *D. punctatus* (Shen et al., 2020) – and the ORs in heliothine species are marked by triangles (△).

least for the *Xenopus* oocytes expression system (Yang et al., 2017). In general, the PR's functions reported by studies using different expression systems are well coincided (Grosse-Wilde et al., 2007; Wang et al., 2011; Wang et al., 2018).

Developing in vivo functional identification methods

Recently, genetical manipulation of gene expression in non-model systems has become reality by using new biotechnical techniques such as CRISPR/Cas9 (Yan et al., 2017). These methods have been used in studies of the function of PRs in moths, including heliothine species (Chang et al., 2017). Upon the knock out (by CRISPR) of a distinct PR, the changed moth behavior could be observed or tested, which reflected the function of the PRs. When HarmOr16 in *H. armigera* is knocked out by the CRISPR/Cas9 system, the electrophysiological and behavioral responses of the mutant males to Z11-16:OH are abolished (Chang et al., 2017). As the combined analysis of a PR in heterologous expression systems and through a

gene knock out generated by the CRISPR/Cas9 systems is a convincing way to characterize the function of PRs, it is expected to be widely used in the PR's functional analysis in the future (Chang et al., 2017).

Searching the receptors beyond the PR clade

The phylogenetic analysis clearly shows that nearly all PR sequences in moth species clustered in one clade, separate from other odorant receptor clades (Zhang & Löfstedt, 2015). The various PRs would presumably allow the olfactory system to expand the array of compounds that it can detect, which lays the basis for male moths to detect the unstable sex pheromones. For heliothine species, the male moth expresses 6-7 PRs in the traditional PR clade (Jiang et al., 2014; Zhang & Löfstedt, 2015), and there are 2–4 sex pheromones in a heliothine female (Hillier & Baker, 2016). From the previous studies of PR functional analysis, we suggest that most receptors tuning to the sex pheromones in heliothine species could be found in the PR sequences clade (Figure 1). Anyway, exceptions have been found in other moths. In *Eriocrania semipurpurella* (Stephens) and *Dendrolimus punctatus* (Walker), the ORs tuning to the pheromone-related compounds are not clustered in the traditional PR clade (Yuvaraj et al., 2017; Shen et al., 2020). Moreover, it seems that there is more than one PR clade in *Spodoptera littoralis* (Boisduval) (Bastin-Héline et al., 2019). Therefore, it is possible that some PRs in heliothine species may not cluster in the traditional PR clade. Based on these newly reported PR sequences, eight ORs in heliothines are clustered in two candidate new PR clades, which await further functional analysis (Figure 5). However, the expression patterns of these ORs seem not sex-biased (Liu et al., 2012; Xu et al., 2015).

Future perspectives

Big strides have been made in characterization of PRs and in understanding their function in heliothine pheromone detection. However, there are still several aspects and open questions that remain to be explored to get a more complete picture of their function in heliothine moths. These include:

1. Further verification of the function of known PRs. For example, whether Or6 clades in *H. armigera* and *H. assulta* take part in the detection of Z9-16:Ald or Z9-14:Ald is unknown.
2. Functional characterization of more heliothine PRs. Although the PRs tuned to the major pheromone component have been identified in some heliothine species, the functions of the whole set of PRs are still incomplete. Even for the most-studied species such as *H. armigera*, *H. virescens*, or *H. assulta*, the function of some PRs such as Or11 or Or15 is still unknown. Moreover, it would be interesting to explore the function of ORs that are not clustered in the traditional PR clades, in order to test whether they also take part in pheromone detection.
3. Analysis of the function of PRs highly expressed in female antennae (Vásquez et al., 2011; Liu et al., 2013; Yang et al., 2017).
4. Characterization of male sex pheromones and their tuning PRs. More studies should be implemented in identifying male pheromones and their PRs in heliothine species. Female sex pheromones in the five heliothine species have been characterized, but male courtship pheromones have been identified only in *H. virescens* (Teal & Tumlinson, 1989) and *H. armigera* (Huang et al., 1996).
5. Inheritance analysis of PRs in closely related species. More studies should focus on the PR's function in the hybrids of closely related species, as hybridization could give a broader perspective on the functional evolution of PRs.
6. Analysis of the regulatory mechanism of PR expression. The factors that determine the number and localization of the PR-expressing cells in a given species are largely unexplored (Koutroumpa et al., 2016). Thus, more efforts should be taken to reveal such regulatory elements which could also give interesting new insight into how differences in the expression of distinct PRs have evolved across heliothine species.

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