

RNAi-Induced Electrophysiological and Behavioral Changes Reveal Two Pheromone Binding Proteins of *Helicoverpa armigera* Involved in the Perception of the Main Sex Pheromone Component Z11–16:Ald

Kun Dong^{1,2} · Liang Sun³ · Jing-Tao Liu² · Shao-Hua Gu² · Jing-Jiang Zhou⁴ · Ruo-Nan Yang² · Khalid Hussain Dhilloo^{2,5} · Xi-Wu Gao¹ · Yu-Yuan Guo² · Yong-Jun Zhang²

Received: 18 October 2016 / Revised: 16 December 2016 / Accepted: 21 December 2016 / Published online: 9 January 2017
© Springer Science+Business Media New York 2017

Abstract Pheromone binding proteins (PBPs) are thought to play key roles in insect sex pheromone recognition; however, there is little *in vivo* evidence to support this viewpoint in comparison to abundant biochemical data *in vitro*. In the present study, two noctuid PBP genes *HarmPBP1* and *HarmPBP2* of the serious agricultural pest, *Helicoverpa armigera* were selected to be knocked down by RNA interference, and then the changes in electrophysiological and behavioral responses of male mutants to their major sex pheromone component (Z)-11-hexadecenal (Z11–16:Ald) were recorded. There were no significant electrophysiological or behavioral changes of tested male moths in response to Z11–16:Ald when either single PBP gene was knocked down. However, decreased sensitivity of

male moths in response to Z11–16:Ald was observed when both *HarmPBP1* and *HarmPBP2* genes were silenced. These results reveal that both *HarmPBP1* and *HarmPBP2* are required for the recognition of the main sex pheromone component Z11–16:Ald in *H. armigera*. Furthermore, these findings may help clarify physiological roles of moth PBPs in the sex pheromone recognition pathway, which in turn could facilitate pest control by exploring sex pheromone blocking agents.

Keywords *Helicoverpa armigera* · Pheromone binding proteins · RNA interference · Sex pheromone detection

Kun Dong and Liang Sun contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10886-016-0816-6) contains supplementary material, which is available to authorized users.

✉ Yong-Jun Zhang
yjzhang39@hotmail.com

- ¹ Department of Entomology, China Agricultural University, Beijing 100193, China
- ² State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China
- ³ Key Laboratory of Tea Biology and Resources Utilization, Ministry of Agriculture, Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou 310008, China
- ⁴ Department of Biological Chemistry and Crop Protection, Rothamsted Research, Harpenden AL5 2JQ, UK
- ⁵ Department of Entomology, Faculty of Crop Protection, Sindh Agriculture University Tandojam, Tandojam, Pakistan

Introduction

Semiochemicals such as sex pheromones and host volatiles are vital for insects' foraging and copulation (Karg and Suckling 1999). For moth species, sex pheromones are comprised of multiple components at precise ratios and are normally emitted by females and recognized specifically and sensitively by their conspecific males (Kaissling 1971, 1986; Wang et al. 2005). This accurate and specific perception benefits from male moths' sophisticated olfactory system as numerous antennae sensilla especially the sensilla trichodea which are highly sensitive to different female produced sex pheromone components are located on their antennae (Field et al. 2000; Takanashi et al. 2006). Species-specific pheromone molecules enter the lymph of pheromone-sensitive sensilla trichodea via multipores (Steinbrecht 1997; Sun et al. 2014) and it is commonly accepted that several different groups of olfactory proteins such as soluble carrier proteins pheromone-binding proteins (PBPs), chemosensory proteins (CSPs) and membrane-bound

odorant receptors (ORs), ionotropic receptors (IRs) and sensory neuron membrane proteins (SNMPs) are responsible for the detection of sex pheromones (Leal 2013; Zhang and Löfstedt 2015; Zhang et al. 2015).

Pheromone Binding Proteins are small (15–17 kDa) water-soluble proteins with six conserved cysteines (Leal et al. 1999; Pelosi et al. 2006, 2014) and are presumed to be synthesized by non-neuronal auxiliary cells (trichogen and tormogen cells) of the odorant sensory neurons (OSNs) and secreted into the sensillum lymph at extremely high concentrations (up to 10 mM) (de Santis et al. 2006; Steinbrecht et al. 1992; Zhang et al. 2001). There, they are thought to solubilize, bind and transport hydrophobic sex pheromone molecules across the aqueous sensillar lymph to the specific pheromone receptors (PRs) (Chang et al. 2015; Gu et al. 2013; Jin et al. 2014; Sun et al. 2013a). The first PBP gene was identified in the silkworm, *Antheraea polyphemus* (Vogt and Riddiford 1981) and since then their orthologs have been identified in many species of moth (Vogt et al. 2015). Commonly, there are three PBP genes in a moth, with each displaying high levels of sequence identity across species to form three conserved sub-groups (Vogt et al. 2015). Given that the female produced sex pheromone generally contains multiple components, it has been hypothesized that different PBPs might specifically recognize distinct sex pheromone components. For example, two PBPs of the gypsy moth, *Lymantria dispar* have different preferential binding abilities to enantiomer sex pheromone components (Plettner et al. 2000). In *A. polyphemus*, specific conformational changes are associated with different PBPs binding specific sex pheromone components (Mohl et al. 2002). In *Bombyx mori*, both bombykol and bombykal can activate the BmorOR1-expressing cells, but these cells can be made specifically sensitive to bombykol in the presence of PBP (Grosse-Wilde et al. 2006). However, studies on other species have revealed PBPs that show no discrimination among different pheromone components, and different PBPs that can bind the same sex pheromone component (Campanacci et al. 2001; Gu et al. 2013; Guo et al. 2012). The co-expression of different PBP genes in the same sensilla is consistent with a shared binding ability (Forstner et al. 2006; Maida et al. 2001).

The cotton bollworm, *Helicoverpa armigera*, is one of the most serious agriculture pests worldwide and represents a major threat to cotton and other crops (Guo 1997). The sex pheromone of *H. armigera* contains two major components, (*Z*)-11-hexadecenal (Z11–16:Ald) and (*Z*)-9-hexadecenal (Z9–16:Ald), with a ratio of 97: 3 showing the strongest attraction toward *H. armigera* males in the field (Kehat and Dunkelblum 1990; Wang et al. 2005; Wu et al. 1997). Notably, Z11–16:Ald accounts for a much larger proportion of the sex pheromone blend in comparison to Z9–16:Ald and is considered the most important main component of the *H. armigera* sex pheromone. Zhao et al. (2006)

proposed that male moths of *H. armigera* responded strongly to Z11–16:Ald at the electrophysiological level, but not to Z9–16:Ald. Furthermore, Wu et al. (2013) suggested that Z11–16:Ald alone (a ratio of Z11–16:Ald: Z9–16:Ald of 100:0) could successfully evoke upwind flight and subsequent behaviors.

Previously, three PBP genes, *HarmPBP1–3* have been identified from *H. armigera* (Zhang et al. 2011). A fluorophore displacement assay suggests that HarmPBP1 and HarmPBP2 interact more with Z11–16:Ald than with the other pheromone component, and that Z11–16:Ald interacts more with HarmPBP1 and HarmPBP2 than with HarmPBP3 (Guo et al. 2012). These results imply that both HarmPBP1 and HarmPBP2 rather than HarmPBP3 are more likely associated with Z11–16:Ald perception. However, there is still no *in vivo* evidence to support this hypothesis. In the present study, we choose to knockdown both the *HarmPBP1* and *HarmPBP2* genes by RNA interference, and then determine the electrophysiological and behavioral responses of males to Z11–16:Ald. Our results revealed that both PBPs are involved in Z11–16:Ald recognition.

Methods and Materials

Insect Materials *Helicoverpa armigera* were collected from the crop fields of Xiajin county, Shandong Province, China. A colony was established and maintained in the laboratory at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Larvae were reared on an artificial diet under the conditions 26 ± 1 °C, $60\% \pm 5\%$ RH, and L14 h: D10 h. Male and female moths were allowed to emerge in separate cages. Virgin males were collected for the RNA interference 24 hr after eclosion. All the adult moths were fed with 10% honey solution.

Synthesis and Injection of dsRNA Total RNA was isolated from the antennae of two-day-old males using the SV Total RNA Isolation System (Promega, Madison, WI) following the manufacturer's protocol. First strand cDNA was synthesized using the SuperScript™ III Reverse Transcriptase System (Invitrogen, Carlsbad, CA). The full sequences of *HarmPBP1* and *HarmPBP2* were amplified from antennal cDNA by PCR. A fragment of the green fluorescent protein (GFP) gene was used as control. These three genes were sub-cloned into pGEM-T vector and used as templates for the target sequence amplification. The target sequences of three genes were amplified by reverse transcription PCR (RT-PCR) using specific primers containing the T7 promoter at the 5' end (Table 1). The PCR products, 551 bp of *HarmPBP1*, 536 bp of *HarmPBP2* and 711 bp of *GFP* (containing the T7 promoter), were electrophoresed on a 1% agarose gel and then purified as templates for dsRNA synthesis using a T7 Quick High Yield

Table 1 Primers used in this study

Primer name	Sequence (5' - 3')
<i>For dsRNA Synthesis</i>	
HarmPBP1- forward	<u>TAATACGACTCACTATAGGATGGAGTTCATCGATCG</u> ^a
HarmPBP1- reverse	<u>TAATACGACTCACTATAGGTTAGACTTCGGCCAAGAC</u> ^a
HarmPBP2- forward	<u>TAATACGACTCACTATAGGATGGCCGACTCAAGATGG</u> ^a
HarmPBP2- reverse	<u>TAATACGACTCACTATAGGCTAGGCGGCAGTCATG</u> ^a
GFP- forward	<u>GAATTAATACGACTCACTATAGGGAGAAAGGGCGAGGAGCTGTTACCCG</u> ^a
GFP- reverse	<u>GAATTAATACGACTCACTATAGGGAGACAGCAGGACCATGTGATCGCGC</u> ^a
<i>For qRT-PCR</i>	
HarmPBP1- forward	GGAAGGCTACGAGTTCACGAA
HarmPBP1- reverse	ATCGTCAGCGCCATGTTTCT
HarmPBP1- probe	FAM-CGCCATCCTCTGCCTCTCCTCCA-TAMRA
HarmPBP2- forward	GGTTTGATTACGGCTGTGA
HarmPBP2- reverse	CTTGAGCTCGTGATCTTGGT
HarmPBP2- probe	FAM-CCAAGCCATCGAGGACCACTGCA-TAMRA
HarmPBP3- forward	GACGCCAACGGCAGGAT
HarmPBP3- reverse	CGTCACCGTCTTGTTTCATGCT
HarmPBP3- probe	FAM-CGCCGCCGAGTTCGCCAA-TAMRA
Harm-actin- forward	TCTCTCCAGCCCTCATTCTTG
Harm-actin- reverse	TTCTGCATACGGTCAGCGATAC
Harm-actin- probe	FAM-CCAACGGCATCCACGAGACCACCT-TAMRA

^a“_” represented the T7 RNA polymerase promote

RNA Synthesis Kit (New England Biolabs, Ipswich, MA). In the process of synthesis, the incubation time was extended for 4 hr to increase yield. The dsRNA was isopropanol precipitated, re-suspended in nuclease-free water and then stored at -80°C until use. Both yield and quality were evaluated by 1% agarose gel electrophoresis, and amount quantified by a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE).

Twenty-four-hour-old male moths were anesthetized under CO_2 , and then 2 μl nuclease-free water, or 2 μl of each of the three single dsRNA solutions (contain 10 μg dsPBP1, dsPBP2 or dsGFP), or a 4 μl mixture of both dsPBP1 and dsPBP2 (1:1) was injected into the thorax of the moth using a micro-syringe (Hamilton, Bonaduz, Switzerland), separately. Subsequently, moths of all the treatments including non-dsRNA injected, water-injected, dsGFP-injected, dsPBP1-injected, dsPBP2-injected, and dsPBP1-dsPBP2-mixture injected were reared under normal conditions. Antennal samples were collected after treated moths were recovered for 24, 48 or 72 hr, respectively.

Quantitative Real-Time PCR (qRT-PCR) Measurement

Total RNA was isolated from male antennae of all treatments. The cDNAs were synthesized according to the above description. Thirty individuals from each treatment were selected and each treatment was performed with three biological replicates. The β -actin gene (GenBank accession No.EU527017) of

H. armigera was used as reference gene to normalize target gene expression. The primers of the target and reference genes used in the qRT-PCR were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA) (Table 1). Amplification reactions for qRT-PCRs were performed on an ABI Prism 7500 Fast Detection System (Applied Biosystems). Each reaction contains 10 μl of Premix *ExTaq* (TaKaRa, Kyoto, Japan), 0.4 μl of each primer (10 μM), 0.8 μl of the probe (10 μM), 0.4 μl of Rox Reference Dye II, 1 μl of cDNA template and 6.0 μl of sterilized H_2O . PCRs were conducted with 40 cycles as follows: 95°C for 15 sec and 60°C for 34 sec. The comparative $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001) was conducted for data analysis.

Electroantennogram Analysis Since the results of qRT-PCR showed that the expression levels of target genes had a significant reduction compared to controls at 72 hr after injection, this time point was chosen for electroantennogram (EAG) recording. Each tested moth was anaesthetized using CO_2 . The antennae were carefully removed at the base, and a few terminal segments at the distal end were excised (Sun et al. 2014). Then single antenna was connected between two electrode holders using conductive gel (Spectra 360 Electrode Gel) immediately. The compound Z11-16:Ald was dissolved in liquid paraffin (Fluka, Buchs, Switzerland) at 1 mM (approximately 200 ng/ μl) to examine antennal sensitivity in different treatments. Liquid paraffin was also set up

as a blank control. Before EAG recording of Z11–16:Ald, the EAG responses of different treated males to (*E*)-2-hexenal were examined. The results showed that (*E*)-2-hexenal could elicit stable recordings on male antennae, and there was no significant difference between different treated groups (data not shown). Hence, (*E*)-2-hexenal was selected as the reference compound to normalize all the EAG recordings. Filter paper strips (4 mm × 30 mm) were loaded with 10 µl of test chemical solution and then inserted into a glass Pasteur pipette. The tip of the pipette was inserted approximately 3 mm into a small hole in the wall of a metal tube (9 mm diameter × 12 cm long) directed at the antennal preparation. An air stimulus controller (ModelCS-55, Syntech, Hilversum, Netherlands) was used for air and odor delivery. A constant flow (300 ml/min) of activated carbon-filtered air passed over the antenna through the open end of the metal tube positioned 5 mm from the antenna. During odor stimulation, 30 ml/min of air was applied through the Pasteur pipette into the main air flow for 0.2 sec. Signals were recorded for 5 sec with each compound at 30 sec intervals. EAG recordings were made using an IDAC-2 recording unit with amplifier and computer board (Syntech). The EAG values were calculated by subtracting the mean response of the blank control and then converted to a percentage value of the mV response to the accompanying standard (Kendra et al. 2005). Each treatment was tested for at least 15 individuals, and each antenna repeated three times.

Wind Tunnel Bioassay To evaluate the males' behavioral responses of all treated groups to Z11–16:Ald, wind tunnel assays were performed in a plexiglass wind tunnel (2.5 m long, 1 m wide, 1 m high) under the conditions of 27 ± 1 °C, 70% relative humidity, 0.3 lux of red light and air speed of 20 cm/s. A 20 µg/µl solution of Z11–16:Ald was used in taxis tests. Moths were tested during their scotophase at the third day after injection. Prior to experiments, moths were placed in mesh cages and then moved into the tunnel room to acclimate for 20 mins. Twenty microliters of pheromone solution was loaded in the rubber septa as a lure. The rubber septa were fixed on a 50 cm high, 30 cm long-wide organic glass holder close to the upwind end of the tunnel. Approximately 84–92 male moths were tested in each treatment. Moths were released into the tunnel from mesh cages two meters downwind of the lure and 30 cm above the tunnel floor. Assembling responses of moths to Z11–16:Ald were tested during a 5-min bioassay period and scored for the following behaviors: (1) taking flight; (2) upwind flight, zig-zag flight towards the lure; (3) continuously flew or landing within 30 cm of the lure; (4) contacted the lure.

Data Analysis Data from qRT-PCR, EAG and wind tunnel tests were analyzed using SAS 9.0 for Windows software (SAS 9.0 system for Windows, 2002, SAS Institute Inc.,

Cary, NC). ANOVA and Duncan's new multiple range test ($P = 0.05$) were used to determine whether differences in *HarmPBPs* mRNA levels or EAG values among different treatment groups were significant. In wind tunnel assays, the percentage of male responses to the lure was calculated for each of the four behavioral phases as the number of males exhibiting a given behavior phase divided by the number of tested males. Differences in the males behavioral responses of non-injected, water-injected, dsGFP-injected, and dsPBP1&2-injected were analyzed using Chi-square 2 × 2 tests for each behavioral phase with the threshold of significance set at $P = 0.05$.

Results

Effect of dsRNA Treatment on HarmPBP1 and HarmPBP2 Transcript Levels To investigate the RNAi efficiency, qRT-PCR assays were performed. The injection of dsRNA decreased expression levels of target genes. Briefly, injection of dsPBP1 significantly decreased *HarmPBP1* mRNA levels after 48 hr compared to the non-injected, dsGFP-injected and water-injected groups. At 72 hr post injection *HarmPBP1* transcript levels were reduced up to 70% compared to control groups (Fig. 1a). Injection of dsPBP2 gave similar results in decreasing of *HarmPBP2* transcript levels (Fig. 1b). In addition, the transcript levels of both *HarmPBP1* and *HarmPBP2* was significantly reduced after 72 hr when a mixture of dsPBP1 and dsPBP2 was injected (Fig. 2).

There was no significant influence on levels of *HarmPBP1* mRNA when dsPBP2 was injected (Fig. 2a), and similarly, injection of dsPBP1 did not affected the expression of *HarmPBP2* (Fig. 2b). Furthermore, we also assessed the mRNA levels of *HarmPBP3* between the controls and dsRNA-treated groups. Injection of PBP1 and PBP2 dsRNA had no significant effect on *HarmPBP3* mRNA levels (Figure S1). These results suggest that specific dsRNA could induce a significant reduction of a specific target gene, with no off-target effects.

Effect of RNAi on Electrophysiological Responses to Sex Pheromone of Z11–16:Ald In order to dissect the physiological function of HarmPBP1 and HarmPBP2 in the perception of Z11–16:Ald, the electrophysiological responses of male moths to this compound were recorded in EAG experiments. There was no significant decrease in response in dsPBP-treated groups in comparison to non-dsRNA treated or dsGFP-treated groups when either dsPBP1 or dsPBP2 was injected into male moths (Fig. 3). However, EAG activity of males in response to Z11–16:Ald was reduced (approximate 50%) when both *HarmPBP1* and *HarmPBP2* were silenced by injection of a mixture of dsPBP1 and dsPBP2.

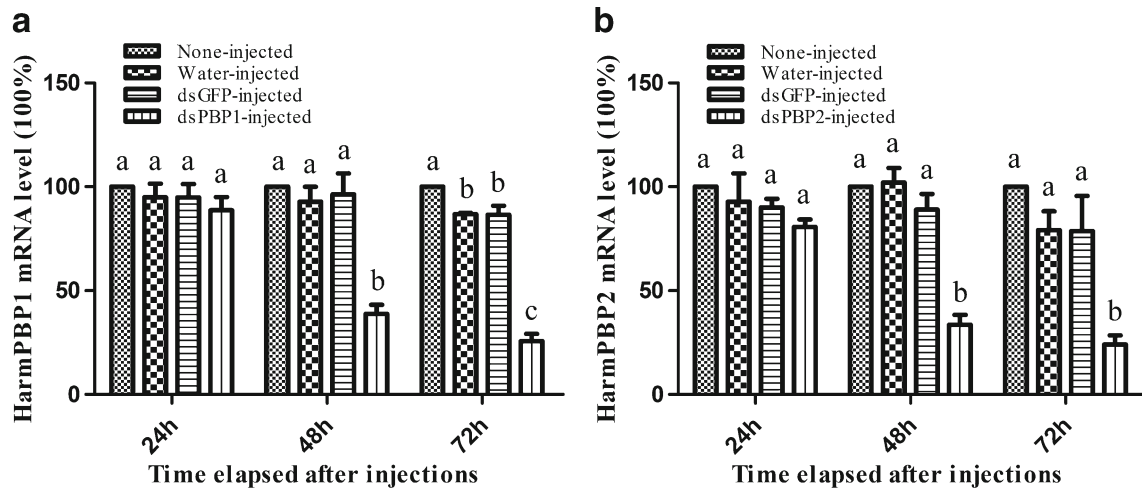


Fig. 1 qRT-PCR measurements of *HarmPBP1* (a) and *HarmPBP2* (b) transcripts in non-injected, water-injected, dsGFP-injected, and single dsPBP1-injected *Helicoverpa armigera* male antennae. The expression of *HarmPBP*s was normalized using β -actin, and the mRNA transcript

levels of *HarmPBP*s were examined at 24, 48 and 72 hr after injection. Data represents the mean values \pm S.E.M of three independent replicates. Different letters within the same figure indicate that the values were significantly different ($P < 0.05$)

Effect of RNAi on Behavioral Responses to Z11–16:Ald

Based on the results of the EAG experiments described above, wind tunnel assays were conducted to investigate the behavioral responses of dsPBP1&2-treated moths and three control groups to Z11–16:Ald. The results showed that 74% of non-injected male moths initiated flight from the starting position, 58% undertook zig-zag flight, 26% flew up to within 30 cm of the lure, and approximately 15% finally reached the lure. There was no significant difference in response to the lure between the non-injected group and the water or dsGFP-injected groups (water-injected group: $\chi^2_{\text{Taking flight}} = 0.87$,

$P > 0.05$; $\chi^2_{\text{Upwind flight}} = 0.25$, $P > 0.05$; $\chi^2_{\text{Close to the lure}} = 0.09$, $P > 0.05$; $\chi^2_{\text{Landing the lure}} = 0.11$, $P > 0.05$; dsGFP-injected group: $\chi^2_{\text{Taking flight}} = 2.3$, $P > 0.05$; $\chi^2_{\text{Upwind flight}} = 0.07$, $P > 0.05$; $\chi^2_{\text{Close to the lure}} = 0.12$, $P > 0.05$; $\chi^2_{\text{Landing the lure}} = 0.78$, $P > 0.05$). A reduction in these behaviors was observed in males that had been injected with a mixture of dsPBP1 and dsPBP2. In these males 56% initiated flight, 37% flew upwind, 11% came close to the lure, and 7% reached the lure (the mixture of dsPBP1 and dsPBP2 injected group: $\chi^2_{\text{Taking flight}} = 5.27$, $P = 0.022$; $\chi^2_{\text{Upwind flight}} = 8.01$, $P = 0.005$; $\chi^2_{\text{Close to the lure}} = 6.72$, $P = 0.01$; $\chi^2_{\text{Landing the lure}} = 3.4$, $P > 0.05$) (Fig. 4).

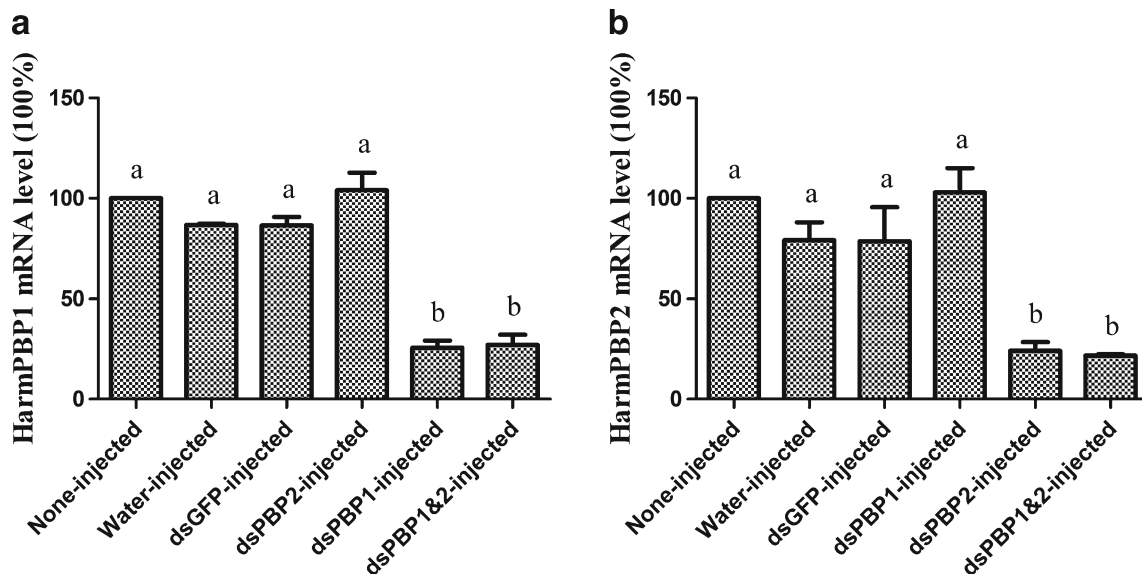


Fig. 2 qRT-PCR analysis of *HarmPBP1* (a) and *HarmPBP2* (b) transcripts in non-injected, water-injected, dsGFP-injected, single dsPBP1-injected and a mixture of dsPBP1 and dsPBP1-injected *Helicoverpa armigera* male antennae. The expression of *HarmPBP*s was normalized

using β -actin, and the mRNA transcript levels of *HarmPBP*s were examined at 72 hr after injection. Data represents the mean values \pm S.E.M of three independent replicates. Different letters within the same figure indicate that the values were significantly different ($P < 0.05$)

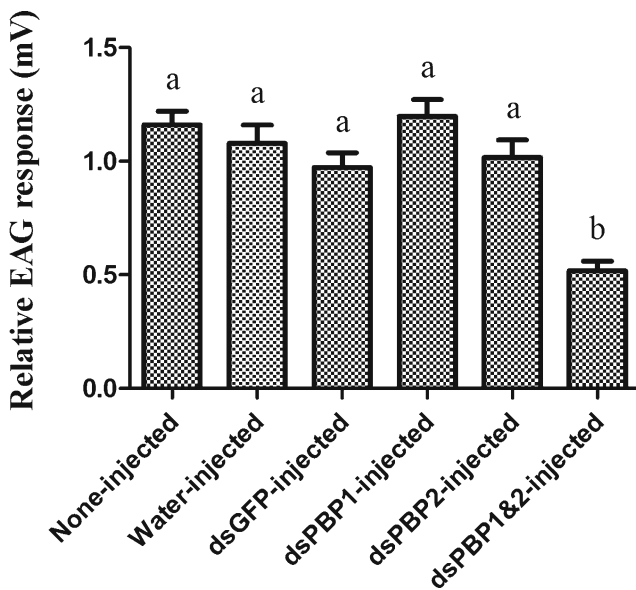


Fig. 3 Electrophysiological responses of dsRNA-treated and control *Helicoverpa armigera* males to Z11–16:Ald. Data represents the mean values \pm S.E.M of three independent replicates. Different letters within the same figure indicate that the values were significantly different ($P < 0.05$)

Discussion

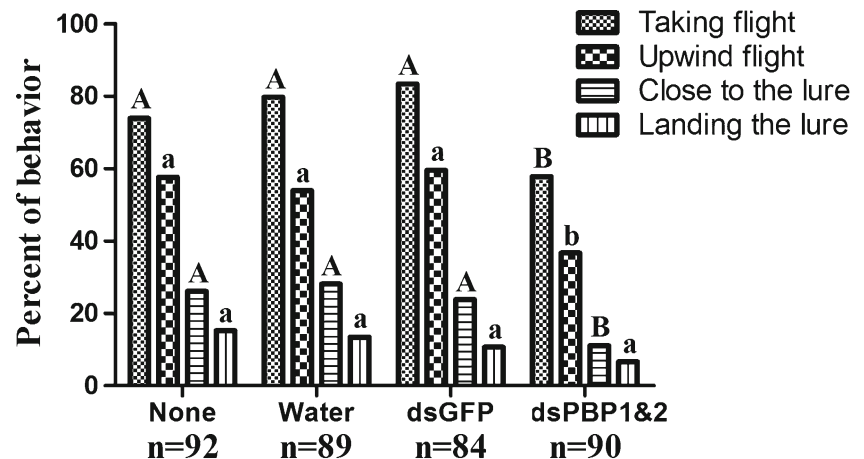
Pheromone Binding Proteins are highly expressed in the pheromone-sensitive sensilla trichodea, and are thought to bind and transport sex pheromone components across the sensillum lymph to pheromone receptors (PRs) (Pelosi et al. 2006, 2014; Steinbrecht et al. 1992; Vogt et al. 2015; Vogt and Riddiford 1981). Although some results from heterologous systems suggest that sex pheromones can be detected by ORs alone (van der Goes van Naters and Carlson 2007), the viewpoint that the sensitivity of PRs in response to sex pheromones can be enhanced by expressed PBPs is well accepted (Chang et al. 2015; Forstner et al. 2009; Große-Wilde et al. 2007; Van den Berg and Ziegelberger 1991). In addition, some fluorophore displacement assays suggest that PBPs had

binding specificity to different sex pheromone components, and each subgroup of PBP is tuned to a specific component of the sex pheromone blend (Grosse-Wilde et al. 2006; Gu et al. 2013; Mohl et al. 2002; Plettner et al. 2000). However, in *H. armigera*, three recombinant PBPs did not show selective binding abilities. In particular, both HarmPBP1 and HarmPBP2 showed strong interaction with the same sex pheromone component Z11–16:Ald, implying that these two PBPs could be associated with Z11–16:Ald recognition (Guo et al. 2012). As a result, we employed the RNAi method to investigate whether both HarmPBP1 and HarmPBP2 play roles in Z11–16:Ald recognition of male *H. armigera*.

Direct injection of dsRNA is invasive and may cause high mortality (Jaubert-Possamai et al. 2007). In this study, a low mortality (less than 10%) was observed in both non-injected and injected groups at 72 hr after injection, indicating that dsRNA injection was suitable for target gene interference in *H. armigera*. Additionally, it has been reported that injected dsRNA can cause off-target silencing which means the unintended genes may be reduced in expression by RNAi (Jackson et al. 2003). In the current work, qRT-PCR analysis showed that *HarmPBP1* mRNA levels were not affected by dsPBP2 injection (Fig. 2a), similarly, injection of dsPBP1 had no significant effect on the expression of *HarmPBP2* (Fig. 2b). Furthermore, supplementary results also showed that the injection of PBP1 and PBP2 dsRNA had no significant effect on *HarmPBP3* mRNA levels (Figure S1). Such qRT-PCR results revealed that specific dsRNA injection could reduce specific target gene expression in the RNAi assays undertaken in this study.

A significant reduction in the transcript levels of *HarmPBP1* or *HarmPBP2* (up to approximately 70%) was found 72 hr after injection of dsPBP1 or dsPBP2, respectively, indicating that dsRNA injection is responsible for the observed reduction in the expression levels of these two PBP genes. However, there was no significant difference in the electrophysiological responses (EAG recording) of male moths to Z11–16:Ald between the single PBP dsRNA injected

Fig. 4 Behavioral responses of *Helicoverpa armigera* males to Z11–16:Ald. Symbols in the same behavioral category with different letters were significantly different (Chi-square test, $P < 0.05$)



moths and control groups (Fig. 3). This phenomenon has two potential explanations. First, *HarmPBP1* and *HarmPBP2* showed high expression levels in the Z11–16:Ald-sensitive sensilla trichodea, and the 70% reduction of mRNA level of *HarmPBP1* or *HarmPBP2* may not be sufficient for a significant decrease in the response of males to Z11–16:Ald. Second, both of these two PBPs are involved in the perception of Z11–16:Ald, and knockdown of any single PBP gene (*HarmPBP1* or *HarmPBP2*) cannot on their own affect the male's recognition of Z11–16:Ald. It has been reported that a 50% reduction in transcripts levels of an OBP is sufficient to generate reduced antennal responses to several semiochemicals in the mosquito, *Culex quinquefasciatus* (Pelletier et al. 2010), suggesting that the first explanation seems unreasonable. No difference in response to Z11–16:Ald between single dsPBP-treated moths and control groups could be due to the combinatorial contribution of *HarmPBP1* and *HarmPBP2*. Thus, we performed a combinatorial RNA silenced strategy with a 1:1 mixture of dsPBP1 and dsPBP2 injection to confirm this hypothesis. Interestingly, the EAG activity of males in response to Z11–16:Ald had a substantial reduction (approximately 50%) when both *HarmPBP1* and *HarmPBP2* were silenced together. In subsequent behavioral trials, a significant reduction in behaviors was observed in the number of dsPBP1 and dsPBP2 mixture treated males responding to the lure compared to the controls. These results suggest that both *HarmPBP1* and *HarmPBP2* are associated with the recognition of major sex pheromone component, Z11–16:Ald.

Recent studies have revealed that GOBPs and CSPs may be also involved in pheromone detection in some lepidopteran species (Zhang et al. 2014; Zhou et al. 2009). In *H. armigera*, an antenna-specific expressed classic OBP (*HarmOBP7*) showed high affinity for Z11–16:Ald (Sun et al. 2013b), which suggests that this protein may participate in the detection of Z11–16:Ald. However, in the current work, it seems that the presence of *HarmOBP7* cannot compensate for the reduction of the two *HarmPBPs*. This phenomenon may be due to the distribution or functional differentiation of OBPs. First, besides Z11–16:Ald, *HarmOBP7* also shows high affinities to some aromatic compounds. Such broad binding abilities indicate that *HarmOBP7* may act as a GOBP to play a diversity of roles. Second, *HarmOBP7* may be located in different sensilla from that of *HarmPBP1* and *HarmPBP2*. Third, the expression levels of *HarmOBP7* may be not sufficient to compensate for the knocked down PBPs. Moreover, recent reports have revealed that OBPs undergo specific conformational changes upon binding their ligands and only in some cases such changes could enable OBPs to interact with the olfactory receptor and then generate a physiological response (Laughlin et al. 2008). Therefore, GOBPs without suitable conformational change may be not able to trigger the subsequent physiological response.

In summary, we have successfully silenced two PBP genes of *H. armigera* using the RNAi method. The EAG and wind tunnel assays provided *in vivo* evidence which supports the previous conclusions from ligand binding assays that both *HarmPBP1* and *HarmPBP2* were responsible for the recognition of the major sex pheromone component, Z11–16:Ald. This finding suggests a redundant recognition by two PBPs of a single sex pheromone component, and may serve as a foundation for better understanding of sex pheromone recognition in moths.

Acknowledgements This work was supported by the Chinese National Basic Research Program (2012CB114104), the National Natural Science Foundation of China (31272048, 31471778, 31672038, and 31621064) and the Beijing Nova Program (Z151100003150118). This manuscript has been edited by the native English-speaking experts of Elsevier Language Editing Services.

References

- Campanacci V, Krieger J, Bette S, Sturgis JN, Lartigue A, Cambillau C, Breer H, Tegoni M (2001) Revisiting the specificity of *Mamestra brassicae* and *Antheraea polyphemus* pheromone-binding proteins with a fluorescence binding assay. *J Biol Chem* 276:20078–20084
- Chang H, Liu Y, Yang T, Pelosi P, Dong S, Wang G (2015) Pheromone binding proteins enhance the sensitivity of olfactory receptors to sex pheromones in *Chilo suppressalis*. *Sci Rep* 5:13093
- De Santis F, Francois MC, Merlin C, Pelletier J, Maibèche-Coisne M, Conti E, Jacquín-Joly E (2006) Molecular cloning and *in situ* expression patterns of two new pheromone-binding proteins from the corn stemborer *Sesamia nonagrioides*. *J Chem Ecol* 32:1703–1717
- Field LM, Pickett JA, Wadhams LJ (2000) Molecular studies in insect olfaction. *Insect Mol Biol* 9:545–551
- Forstner M, Gohl T, Breer H, Krieger J (2006) Candidate pheromone binding proteins of the silkworm *Bombyx mori*. *Invertebr Neurosci* 6:177–187
- Forstner M, Breer H, Krieger J (2009) A receptor and binding protein interplay in the detection of a distinct pheromone component in the silkworm *Antheraea polyphemus*. *Int J Biol Sci* 5:745–757
- Grosse-Wilde E, Svatos A, Krieger J (2006) A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor *in vitro*. *Chem Senses* 31:547–555
- Große-Wilde E, Gohl T, Bouché E, Breer H, Krieger J (2007) Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. *Eur J Neurosci* 25:2364–2373
- Gu SH, Zhou JJ, Wang GR, Zhang YJ, Guo YY (2013) Sex pheromone recognition and immunolocalization of three pheromone binding proteins in the black cutworm moth *Agrotis ipsilon*. *Insect Biochem Mol Biol* 43:237–251
- Guo YY (1997) Progress in the researches on migration regulatory of cotton bollworm and relationships between the pest and its host plants. *Acta Entomol Sin* 40:1–6
- Guo H, Huang LQ, Pelosi P, Wang CZ (2012) Three pheromone-binding proteins help segregation between two *Helicoverpa* species utilizing the same pheromone components. *Insect Biochem Mol Biol* 42:708–716

- Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 21:635–637
- Jaubert-Possamai S, Le TG, Bonhomme J, Christophides GK, Rispé C, Tagu D (2007) Gene knockdown by RNAi in the pea aphid *Acyrtosiphon pisum*. *BMC Biotechnol* 7:1–8
- Jin JY, Li ZQ, Zhang YN, Liu NY, Dong SL (2014) Different roles suggested by sex-biased expression and pheromone binding affinity among three pheromone binding proteins in the pink rice borer, *Sesamia inferens* (Walker) (Lepidoptera: Noctuidae). *J Insect Physiol* 66:71–79
- Kaissling KE (1971) Handbook of sensory physiology. In: Beidler LM (ed) *Insect olfaction*. Springer, Berlin, pp 351–431
- Kaissling KE (1986) Chemo-electrical transduction in insect olfactory receptors. *Annu Rev Neurosci* 9:121–145
- Karg G, Suckling M (1999) Applied aspects of insect olfaction. In: Hansson BS (ed) *Insect olfaction*. Springer, Berlin Heidelberg, Berlin, pp 351–377
- Kehat M, Dunkelblum E (1990) Behavioral response of male *Heliothis armigera* (Lepidoptera: Noctuidae) moths in a flight tunnel to combinations of components identified from female sex pheromone glands. *J Insect Behav* 3:75–83
- Kendra PE, Montgomery WS, Mateo DM, Puche H, Epsky ND, Heath RR (2005) Effect of age on EAG response and attraction of female *Anastrepha suspensa* (Diptera: Tephritidae) to ammonia and carbon dioxide. *Environ Entomol* 34:584–590
- Laughlin JD, Ha TS, Jones DNM, Smith DP (2008) Activation of pheromone sensitive neurons is mediated by conformational activation of pheromone binding protein. *Cell* 133:1255–1265
- Leal WS (2013) Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu Rev Entomol* 58:373–391
- Leal WS, Nikonova L, Peng G (1999) Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. *FEBS Lett* 464:85–90
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408
- Maida R, Krieger J, Gebauer T, Lange U, Ziegelberger G (2001) Three pheromone-binding proteins in olfactory sensilla of the two silkworm species *Antheraea polyphemus* and *Antheraea pernyi*. *Eur J Biochem* 267:2899–2908
- Mohl C, Breer H, Krieger J (2002) Species-specific pheromonal compounds induce distinct conformational changes of pheromone binding protein subtypes from *Antheraea polyphemus*. *Invertebr Neurosci* 4:165–174
- Pelletier J, Guidolin A, Syed Z, Comel AJ, Leal WS (2010) Knockdown of a mosquito odorant-binding protein involved in the sensitive detection of oviposition attractants. *J Chem Ecol* 36:245–248
- Pelosi P, Zhou J, Ban L, Calvello M (2006) Soluble proteins in insect chemical communication. *Cell Mol Life Sci* 63:1658–1676
- Pelosi P, Mastrogiacomo R, Iovinella I, Tuccori E, Persaud KC (2014) Structure and biotechnological applications of odorant-binding proteins. *Appl Microbiol Biotechnol* 98:61–70
- Plettner E, Lazar J, Prestwich EG, Prestwich GD (2000) Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth *Lymantria dispar*. *Biochemist* 39:8953–8962
- Steinbrecht RA (1997) Pore structures in insect olfactory sensilla: a review of data and concepts. *Int J Insect Morphol Embryol* 26:229–245
- Steinbrecht RA, Ozaki M, Ziegelberger G (1992) Immunocytochemical localization of pheromone-binding protein in moth antennae. *Cell Tissue Res* 270:287–302
- Sun M, Liu Y, Wang G (2013a) Expression patterns and binding properties of three pheromone binding proteins in the diamondback moth, *Plutella xylostella*. *J Insect Physiol* 59:46–55
- Sun YL, Huang LQ, Pelosi P, Wang CZ (2013b) A lysine at the C-terminus of an odorant-binding protein is involved in binding aldehyde pheromone components in two *Helicoverpa* species. *PLoS One* 8:e55132
- Sun L, Xiao HJ, Gu SH, Guo YY, Liu ZW, Zhang YJ (2014) Perception of potential sex pheromones and host-associated volatiles in the cotton plant bug, *Adelphocoris fasciaticollis* (Hemiptera: Miridae): morphology and electrophysiology. *Appl Entomol Zool* 49:43–57
- Takanashi T, Ishikawa Y, Anderson P, Huang Y, Löfstedt C, Tatsuki S, Hansson BS (2006) Unusual response characteristics of pheromone-specific olfactory receptor neurons in the Asian corn borer moth, *Ostrinia furnacalis*. *J Exp Biol* 209:4946–4956
- Van den Berg MJ, Ziegelberger G (1991) On the function of the pheromone binding protein in the olfactory hairs of *Antheraea polyphemus*. *J Insect Physiol* 37:79–85
- van der Goes van Naters W, Carlson JR (2007) Receptors and neurons for fly odors in *Drosophila*. *Curr Biol* 17:606–612
- Vogt RG, Riddiford LM (1981) Pheromone binding and inactivation by moth antennae. *Nature* 293:161–163
- Vogt RG, Große-Wilde E, Zhou JJ (2015) The Lepidoptera odorant binding protein gene family: gene gain and loss within the GOBP/PBP complex of moths and butterflies. *Insect Biochem Mol Biol* 62:142–153
- Wang HL, Zhao CH, Wang CZ (2005) Comparative study of sex pheromone composition and biosynthesis in *Helicoverpa armigera*, *H. assulta* and their hybrid. *Insect Biochem Mol Biol* 35:575–583
- Wu DM, Yan YH, Cui JR (1997) Sex pheromone components of *Helicoverpa armigera*: chemical analysis and field tests. *Entomol Sin* 4:350–356
- Wu H, Hou C, Huang LQ, Yan FS, Wang CZ (2013) Peripheral coding of sex pheromone blends with reverse ratios in two *Helicoverpa* species. *PLoS One* 8:e70078
- Zhang DD, Löfstedt C (2015) Moth pheromone receptors: gene sequences, function, and evolution. *Front Ecol Evol* 3:105
- Zhang S, Maida R, Steinbrecht RA (2001) Immunolocalization of odorant-binding proteins in noctuid moths (Insecta, Lepidoptera). *Chem Senses* 26:885–896
- Zhang T, Gu S, Wu K, Zhang Y, Guo Y (2011) Construction and analysis of cDNA libraries from the antennae of male and female cotton bollworms *Helicoverpa armigera* (Hübner) and expression analysis of putative odorant-binding protein genes. *Biochem Biophys Res Commun* 407:393–399
- Zhang YN, Ye ZF, Yang K, Dong SL (2014) Antenna-predominant and male-biased CSP19 of *Sesamia inferens* is able to bind the female sex pheromones and host plant volatiles. *Gene* 536:279–286
- Zhang J, Walker WB, Wang G (2015) Pheromone reception in moths: from molecules to behaviors. *Prog Mol Biol Transl Sci* 130:109–128
- Zhao XC, Yan YH, Wang CZ (2006) Behavioral and electrophysiological responses of *Helicoverpa assulta*, *H. armigera* (Lepidoptera: Noctuidae), their F1 hybrids and backcross progenies to sex pheromone component blends. *Journal Comp Physiol A* 192:1037–1047
- Zhou JJ, Robertson G, He X, Dufour S, Hooper AM, Pickett JA, Keep NH, Field LM (2009) Characterisation of *Bombyx mori* odorant-binding proteins reveals that a general odorant-binding protein discriminates between sex pheromone components. *J Mol Biol* 389:529–545