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CRISPR/Cas9 mediated gene knockout reveals a more important role of PBP1 than PBP2 in the perception of female sex pheromone components in *Spodoptera litura*

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ABSTRACT

Three different pheromone binding proteins (PBPs) can typically be found in the sensilla lymph of noctuid moth antennae, but their relative contributions in perception of the sex pheromone is rarely verified *in vivo*. Previously, we demonstrated that *SlitPBP3* plays a minor role in the sex pheromone detection in *Spodoptera litura* using the CRISPR/Cas9 system. In the present study, the roles of two other *SlitPBPs* (*SlitPBP1* and *SlitPBP2*) are further verified using the same system. First, by co-injection of Cas9 mRNA/sgRNA into newly laid eggs, a high rate of target mutagenesis was induced, 51.5% for *SlitPBP1* and 46.8% for *SlitPBP2* as determined by restriction enzyme assay. Then, the homozygous *SlitPBP1* and *SlitPBP2* knockout lines were obtained by cross-breeding. Finally, using homozygous knockout male moths, we performed electrophysiological (EAG recording) and behavioral analyses. Results showed that knockout of either *SlitPBP1* or *SlitPBP2* in males decreased EAG response to each of the 3 sex pheromone components (Z9,E11-14:Ac, Z9,E12-14:Ac and Z9-14:Ac) by 53%, 60% and 63% (for *SlitPBP*1 knockout) and 40%, 43% and 46% (for *SlitPBP2* knockout), respectively. These decreases in EAG responses were similar among 3 pheromone components, but were more pronounced in *SlitPBP1* knockout males than in *SlitPBP2* knockout males. Consistently, behavioral assays with the major component (Z9,E11-14:Ac) showed that *SlitPBP1* knockout males responded in much lower percentages than *SlitPBP2* knockout males in terms of orientation to the pheromone, along with reduction in close range behaviors such as hairpencil display and mating attempt. Taken together, this study provides direct functional evidence for the roles of *SlitPBP1* and *SlitPBP2,* as well as their relative importance (*SlitPBP1* > *SlitPBP2*) in the sex pheromone perception. This information is valuable in understanding mechanisms of sex pheromone perception and may facilitate the development of PBP-targeted pest control techniques.

1. Introduction

The pheromone binding proteins (PBPs), a group of odorant binding proteins (OBPs), play important roles in sex pheromone perception of lepidopteran insects by binding and transporting hydrophobic pheromones across the aqueous sensilla lymph to the cognate pheromone receptors (PRs) on the dendritic membrane of olfactory neurons (Krieger et al., 2004, 2009; Zhang et al., 2016). PBPs are small (15–17 kDa) water-soluble proteins with 6 conserved cysteines that are important in maintaining the tertiary structure by forming 3 disulfide bonds (Wojtasek and Leal, 1990; Leal et al., 1999). The first insect PBP, also the first insect OBP, was identified in *Antheraea polyphemus* using a radio labeled pheromone (Vogt and Riddiford, 1981). Currently, PBP genes have been identified in many lepidopteran species, using PCR-based and transcriptome or genome sequencing-based strategies (Jin et al., 2014; Gu et al., 2013; Forstner et al., 2006; He et al., 2017; Pesenti et al., 2008). Phylogenetic analyses show that PBPs and general odorant binding proteins (GOBPs) (another OBP group) from Lepidoptera form a unique subfamily, suggesting that PBP/GOBPs have evolved independently within the order of Lepidoptera (Liu et al.,

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2015a; Zhang et al., 2013; Yasukochi et al., 2018; Vogt et al., 2015).

PBPs primarily function to bind and transport hydrophobic pheromone molecules across the aqueous sensilla lymph to the receptors on dendritic membranes of the olfactory sensory neurons (Vogt and Riddiford, 1981; Pelosi et al., 2018). Multiple PBPs within a single species have been found in numerous lepidopteran species (Allen and Wanner, 2011; Gong et al., 2009; Zhang et al., 2013; Xiu and Dong, 2007; Xiu et al., 2008; Venthur and Zhou, 2018), thus, the functional differentiation (ligand selectivity and sensitivity) of PBPs in sex pheromone perception is proposed. *In vitro* assays have shown binding selectivity of pheromone components in some species, such as *Agrotis ipsilon* (Gu et al., 2013), *A. polyphemus* and *Antheraea pernyi* (Maida et al., 2003), *Lymantria dispar* (Plettner et al., 2000) and *Mamestra brassicae* (Maïbèche-Coisné et al., 1997), while no such obvious selectivity has been observed in other species, such as *B. mori* (Grater et al., 2006), *Plutella xyllotella* (Sun et al., 2013), *Helicoverpa armigera*, *H. assulta* (Guo et al., 2012), and *Spodoptera exigua* (Liu et al., 2015a). For *S. litura*, we previously identified 3 PBP genes (Xiu et al., 2008; Yang et al., 2010), and demonstrated that the 3 genes differed in sex biased expression and *in vitro* binding ability for sex pheromones, but each of 3 PBPs showed no obvious preference among pheromone components (Liu et al., 2012, 2013). To date, the functional differentiation among PBPs remains controversial, and *in vivo* functional studies will provide more convincing evidence.

Previously, by using the newly developed gene editing technique CRISPR/Cas9 (Cong et al., 2013; Jinek et al., 2012), the PBP3 gene in *S. litura* was *in vivo* demonstrated to play a minor role in sex pheromone perception (Zhu et al., 2016a). In the present study, two other PBP genes (*SlitPBP1* and *SlitPBP2)* were further evaluated for their selectivity and sensitivity to different pheromone components, using the same CRISPR/Cas9 technique along with electrophysiological and behavioral assays. The results provide new insights into the functional role of PBPs, as well as an important reference for developing PBP-based behavioral interference control strategies for *S. litura*.

2. Materials and methods

2.1. Insect

The *S. litura* used in this study were reared in the laboratory on an artificial diet (Huang et al., 2002) at 26 ± 1 °C with a photoperiod of 14 h light: 10 h dark, and 65 ± 5% relative humidity (RH). Pupae were sexed and kept in separate containers. After emergence, moths were fed on 10% honey water.

2.2. In vitro synthesis sgRNA and Cas9-coding mRNA

The sgRNA target site was determined by ZiFiTi (http://zifit.partners.org/ZiFiT/CSquare9Nuclease.aspx) (Fig. 1 A). The sgRNA template was generated by long unique oligonudeotides (Table S1) according to our previously described method (Zhu et al., 2016a). The sgRNA was transcribed *in vitro* with MAXIscript T7 Kit (Ambion, Austin, TX, USA) following the manufacturer's instruction. The Cas9-coding mRNA was transcribed *in vitro* by a pre-linearized pTD1-T7-Cas9 vector using mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. Purified Cas9 mRNA and sgRNA were stored at – 80 °C.

2.3. Microinjection and mutation analysis

The microinjection was performed as previously described (Zhu et al., 2016a). Twenty-four hours after the injection of sgRNA and Cas9-coding mRNA, 20 injected eggs were randomly selected and pooled to extract the genomic DNA for PCR amplification with the primers (Table S1). Portions of the resulted PCR products were subjected to the restriction enzyme digestion (RED) assay described by Zhu et al. (2016a). The restriction enzyme cutting sites for *Hpy*CH4 III and *Bse*R I (Fig. 1 A) adjacent to PAM sequence of *SlitPBP1* and *SlitPBP2,* respectively, were used for the RED assay. The mutation frequencies were calculated using the methods described by Guschin et al. (2010).

The uncleaved bands of RED assay were recovered and cloned into *pEASY*-T3 cloning vector (TransGen, Beijing, China) following manufacturer's instructions, for TA clone sequencing to determine the mutations. Portions of the PCR products from the injected eggs were directly sequenced with the primer PBP1–F-3 and PBP2–F-3 (Table S1) to check the occurrence of mutation.

2.4. Screening of homozygous mutation line

To obtain the homozygous mutation line, generation 0 (G0) moths were sibling crossed with each other or with wild-type moths in single pairs. After oviposition, genotype of the paired G0 moths were individually analyzed by RED assay. The offspring of the G0 single pairs were held back for production of G1 moths, if their male and/or female parent was tested as the chimeric by RED assay. Similarly, the G1 moths were sibling crossed with each other in single pairs to produce G2 progeny, and after laying eggs, the genotype of the G1 parent were analyzed by RED assay and further by TA clone sequencing. At this stage, the mutations were evaluated according to the sequencing results, and single pair lines with mutations that would result in the complete loss of PBP function were used for further screening of the homozygous mutation lines. The mutation was checked by RED assay in each generation of the screening, and was confirmed in the G3 when the homozygous mutation was achieved.

2.5. Off target analysis

The potential off target sites were predicted by CasOT (Xiao et al., 2014) from a *S. litura* transcriptome (GenBank accession: PRJNA232395). The fragments containing the potential off target sites were amplified with the specific primers (Table S1) and sequenced to analyze the off target effect.

2.6. Electrophysiological assay

The antennae of virgin male *S. litura* moths of 3 days old (3d-old) were excised at the base, and a few terminal segments of the antennae were removed to facilitate the electrical contact. The ends of an isolated antenna were connected by electroconductive gel (SPECTRA 360, Fairfield, NJ, USA) to the two electrodes, respectively. Three sex pheromone components were dissolved in hexane and tested at 5 concentrations (0.1, 1, 10, 50, 100 ng/μL). A filter paper strip (2.5 × 0.9 cm) was loaded with 10 μL test solution and the solvent was allowed to evaporate for 3 min. A 10 μL hexane on the filter paper was used as the control. The EAG signals were recorded with EAG software (Syntech, The Netherlands) according to the previously study (Yang et al., 2009). The sex pheromone compounds were tested from low to high dosage. For *SlitPBP1*$^{-/-}$, 6 wild type and 9 mutant male moths were tested and for *SlitPBP2*$^{-/-}$, 8 wild type and 15 mutant male moths were tested to perform the EAG recording. Three technical repeats were conducted for every dosage with the interval for 30 s.

2.7. Behavioral assay

The male *S. litura* moths were placed individually in plastic cups on the first day of eclosion and fed with 10% honey water. The moths were starved for 12 h prior to the tests at the second or third day. Assays were performed in an environmentally controlled darkroom (26 ± 1 °C and 65 ± 5% RH) fitted with faint red light. To avoid exposure to sex pheromone components prior to the test, the male moths were kept separate from female moths in another room maintained under the same conditions.

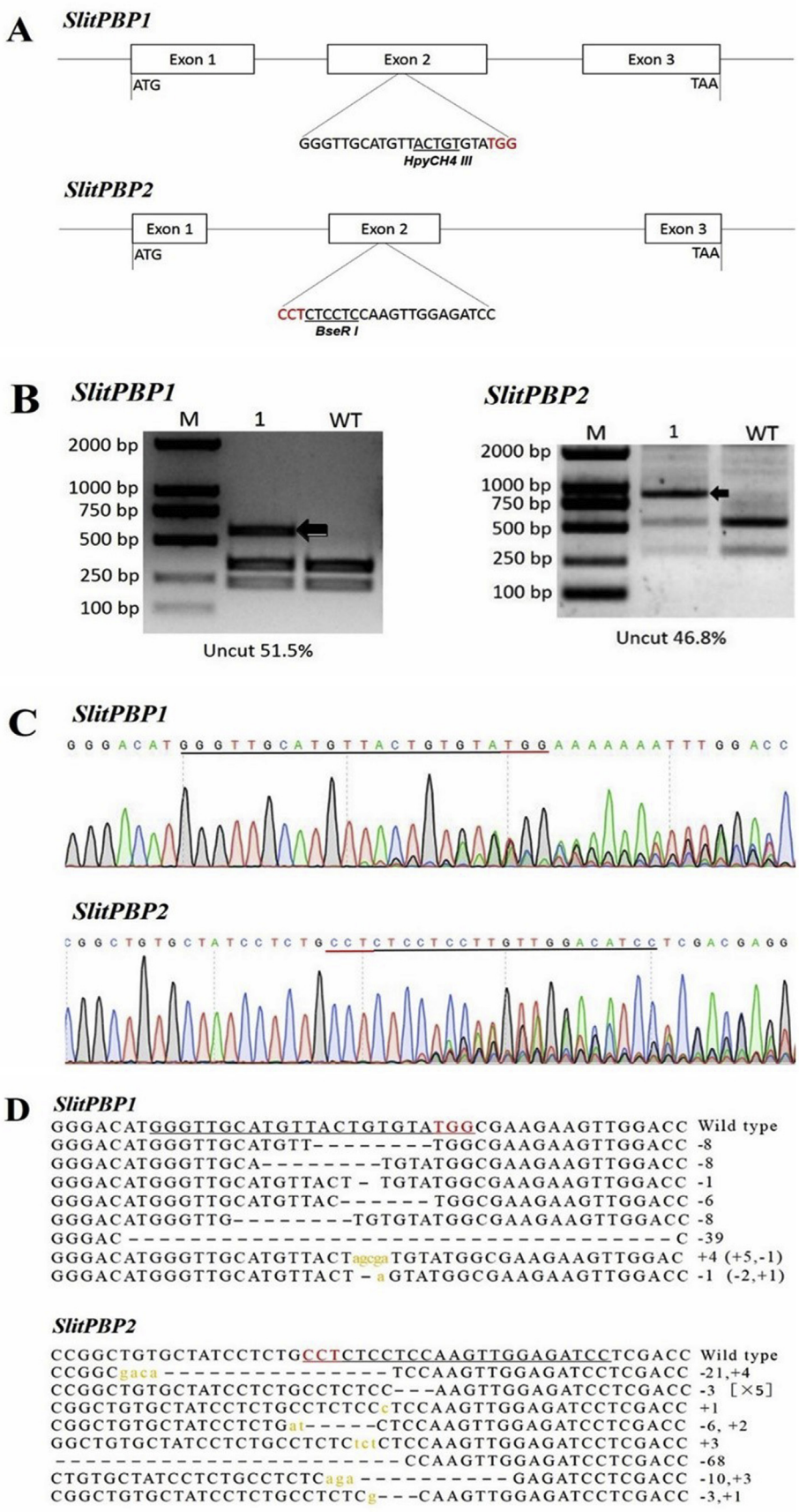

Fig. 1. Targeted mutations of *SlitPBP1* and *SlitPBP2* induced by CRISPR/Cas9. A: Diagram of *SlitPBP1* and *SlitPBP2* genes and sgRNA targeting site. The sgRNA targeting site is at the exon 2, containing a protospacer and a protospacer adjacent motif (PAM) sequence (in red text). The restriction enzyme (*Hpy*CH4 III and *Bse*R I) cutting site is underlined. **B:** The mutation efficiency of pooled sample, determined by the RED assay. M, marker; 1, RED treated PCR products of the *SlitPBP1* or *SlitPBP2* genes from the genomic DNA of 20 injected eggs; WT, control group, with PCR products from un-injected eggs. The arrow indicates the uncleaved bands by *Hpy*CH4 III *or Bse*R I. This two bands designate the occurrence of the mutations at the target site of the *SlitPBP1* and *SlitPBP2* genes. The percentages of the uncleaved bands (51.1% and 46.8%) were calculated by the relative intensity of the uncleaved band to the total bands in lane 1. **C:** Representative sequencing chromatogram of PCR product from the injected eggs. The top sequence indicates the WT sequence, with the target site underlined; and the bottom chromatogram shows multiple peaks, indicating the occurrence of the mutations. **D:** Mutant sequences determined by TA clone sequencing from the uncleaved bands in Fig. 1 **B**. The WT sequence is shown at the top with the target site underlined and PAM underlined in red. In mutant sequences, deletions are shown as dashes and insertions as yellow lower case letters. The net change in length is marked at the right of each sequence (+, insertion; −, deletion). The number of times each mutant allele was isolated is shown in brackets. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

To perform the assay, glass Y-tube olfactometers (3.0 cm inner diameter, 25 cm stem length, 20 cm arm length, and 75° angle between the arms) were used. Two filter paper strips containing either 1 μg test compound (Z9,E11-14:Ac) and 1 μg hexane were placed at each end of the two arms of an olfactometer. A constant airflow (0.2 L/min), which was filtered through activated charcoal and humidified with distilled water, was introduced into each chamber by an independent air sampling pump. The male moths in 4–8 h of scotophase were used for Y-tube assay (Li et al., 2017).

A male moth was placed at the downwind end of the Y-tube, and allowed for 5 min to choose between the two arms. The males that walked more than 5 cm into one arm was regarded as responding to the odor source in that arm. The behaviors of male moths after it selected the arm with test compound (hair pencil displaying and mating attempt) were also counted. Each moth was tested only for one time. The placement of the pheromone and solvent was altered in the Y-tube after two moths were tested to avoid a directional or any other bias, and the filter paper containing the test compound was replaced with a new one for each test. After two moths were tested in an olfactometer, a different clean Y-tube was used. Twelve individuals were tested for each mutant line and wild type moths. To minimize the experimental error by the testing order, mutant moths and wild type moths were alternated in the assay.

3. Results

3.1. Targeted mutations induced by CRISPR/Cas9 system

According to the results of RED assay, the indel frequencies of *SlitPBP1* and *SlitPBP2* were 51.5% and 46.8% respectively (Fig. 1B). On the representative sequencing chromatogram of PCR products, multiple peaks appeared at one base behind the cutting site of CRISPR/Cas9, indicating the occurrence and target specificity of mutations (Fig. 1C). To confirm the indel sequences, 15 clones of *SlitPBP1* and *SlitPBP2* were randomly selected for sequencing. Eight clones of *SlitPBP1* were successfully sequenced, displaying mutation types of deletion (6 clones), insert (one clone) and indel (one clone) (Fig. 1D). All 15 *SlitPBP2* clones were successfully sequenced, and 12 ones displayed different mutations including 6 deletions, 2 inserts and 4 indels (Fig. 1D).

The remaining injected eggs were kept until hatching and were raised to adults. The moths from injected eggs were designated as injection resulted moths (IJ moths), and the moths from un-injection wild type eggs were named WT moths. In total, 26 *SlitPBP1* IJ moths (14 females and 12 males) and 38 *SlitPBP2* IJ moths (16 females and 22 males) were obtained. These moths were sibling crossed with each other or paired with wild type isomerism moths, resulting in 14 single pairs of *SlitPBP1* and 22 single pairs of *SlitPBP2*. Finally, 10 single pairs of *SlitPBP1* (Fig. S1A) and 10 single pairs of *SlitPBP2* (Fig. S2A) laid fertilized eggs. After laying eggs, each IJ moths was checked for the genome typing by RED assay. It showed that 12 *SlitPBP1* IJ moths and 17 *SlitPBP2* IJ moths were RED positive, respectively (Fig. S1A and Fig. S2A). The numbers of injected eggs and resulted larvae, pupae and moths are listed in Table 1.

The detailed mutant sequences of RED positive *SlitPBP1* IJ moths were further obtained by TA clone sequencing. About 10 (8–12) clones of each RED positive moths were sequenced, and in total of 119 sequenced clones, 76 clones contained deletions, 19 clones contained inserts, and 8 clones contained indels (Table S2 and Fig. S3).

3.2. Establishment of homozygous lines with SlitPBP1 or SlitPBP2 knockout

Based on results of RED assay and TA clone sequencing of G0 moths, G1 offspring from the G0 single pair #4 and #6 were mixed together and used to screen the *SlitPBP1* homozygous mutant lines. As a result, 2 G1 single pairs successfully mated and laid fertilized eggs (Fig. S1B), and TA clone sequencing showed that male and female moths in the G1 single pair #1 had the same mutant genotype of a GC insertion at the target site (Fig. 2A and Fig. S4A). This insertion resulted in the premature termination in protein translation, and thus a highly truncated protein of 113 amino acids (164 amino acids in the WT protein), leading to loss of the protein function. This mutant genotype was further screened. In G2, 10 single pairs laid fertilized eggs, and female and male moths of single pair #1, #2 and #9 were homozygotes (Fig. S1C).

Similarly, G1 offspring from all the RED positive G0 single pairs were mixed and used to screen *SlitPBP2* homozygous mutant lines, finally, 11 *SlitPBP2* G1 single pairs successfully mated and laid fertilized eggs (Fig. S2B). Fortunately, the male and female moths of single pair #9 had the same mutant genotype with a net change of 13 bp insertion (Fig. S4B). This mutation resulted in formation of a stop codon, leading to the premature termination in protein translation, and thus a highly truncated protein of 114 amino acids (171 amino acids in the WT protein). In G2, 5 single pairs laid fertilized eggs, among which the female and male moths of single pair #5 were homozygotes (Fig. 2B and Fig. S2C). The homozygous mutant lines of *SlitPBP*1 (*SlitPBP1*$^{-/-}$) and *SlitPBP2* (*SlitPBP2*$^{-/-}$) were used for further physiological and behavioral assays.

To confirm that the mutant PBP proteins have lost the sex pheromone binding ability, the mutant *SlitPBP1* selected for functional verification. *SlitPBP1* was expressed in the bacterial system, and the recombinant protein was used to perform the binding experiment with the 3 sex pheromone components, with the method as previous report (Liu et al., 2012). The result was exactly same as expected, showing no binding between the truncated PBP1 and the pheromone components (Fig. S5). Therefore, the function of *SlitPBP1* or *SlitPBP2* would be completely lost in the homologous knockout moths used in the present study.

To determine the efficiency of mutation transmission to the progeny, 99 G1 moths (11 moths per brood) of *SlitPBP1* were tested by RED assay. As a result, 62.6% (62 of 99) individuals were recognized as the mutants (Table 2). However, G1 moths from different single pair strains (broods) showed different mutant frequencies, ranging from 100% (11/11) for strain 4# to 18.2% (2/11) for strain 7# (Table 2). Of the 62 RED positive moths, 11 moths from 2 single pair broods were further tested by TA clone sequencing. It was found that 2 moths were heterozygous mutants, and 9 moths were compound heterozygous mutants (Fig. S6).

3.3. Detection of off target effect in the G0 chimera of SlitPBP1 and SlitPBP2

By using CasOT, 88 and 99 potential off target sequences were predicted for *SlitPBP1* and *SlitPBP2,* respectively, from the *S. litura* transcriptome data, and the top 12 ones for *SlitPBP1* and 8 ones for *SlitPBP2* (Table S3) were selected for off target detection by a PCR based method. Results showed no off target mutation occurred in the G0 chimera of *SlitPBP1* (Fig. S7) and *SlitPBP2* (Fig. S8).

Table 1
Mutagensis of *SlitPBP1* and *SlitPBP2* gene induced by gRNA/Cas9.

Gene	sgRNA/Cas9 mRNA concentration (ng/μL)	No. of injected eggs	No. of hatched eggs	No. of pupae	No. of moths
SlitPBP1	150/300	336	56	41 (19♀/22♂)	26 (14♀/12♂)
SlitPBP2	150/300	328	88	53 (25♀/28♂)	38 (16♀/22♂)

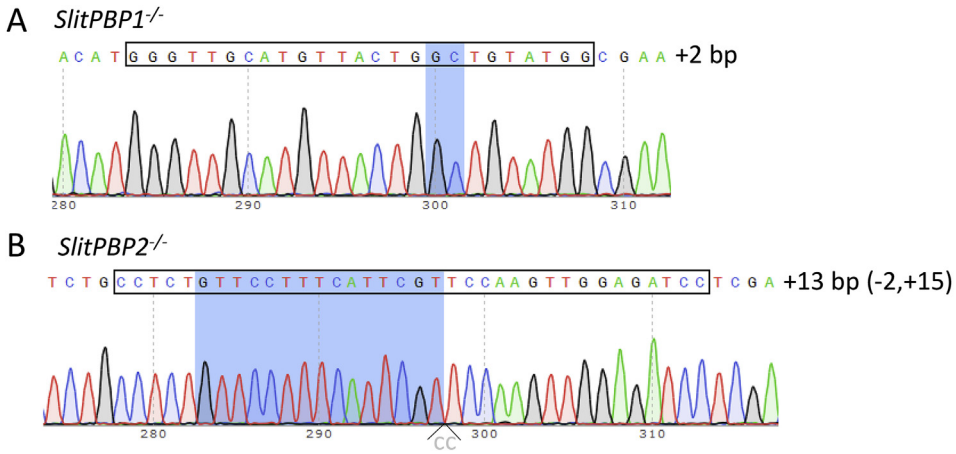

Fig. 2. The genotypes of *SlitPBP1* (A) and *SlitPBP2* (B) homozygous mutants. The target sites are indicated by boxes. The insertions are indicated in blue shadows, and the lower case letters 'cc' under the panel B indicates the deletion. The net change in length is marked at the right of each sequence (+, insertion; −, deletion). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Mutation frequencies determined by the RED assay.

Single pairs	G0 ♀ chimeric frequency (%)	G0 ♂ chimeric frequency (%)	G1 mutant frequency (%)
#1	48.3	33.5	27.3 (3/11)
#2	0	45.5	90.9 (10/11)
#3	0	51.6	45.5 (5/11)
#4	59.8	64.7	100 (11/11)
#6	48.5	57.1	63.6 (7/11)
#7	46.2	0	18.2 (2/11)
#8	58.2	0	72.7 (8/11)
#9	63.4	0	63.6 (7/11)
#10	57.7	0	81.8 (9/11)

3.4. Electrophysiological and behavioral assay of SlitPBP1 and SlitPBP2 function

The electroantennogram (EAG) responses of *SlitPBP1*$^{-/-}$ and *SlitPBP2*$^{-/-}$ mutant males to the 3 sex pheromone components were respectively measured in G4 generation. Compared with WT male moths, *SlitPBP1*$^{-/-}$ moths showed consistently lower EAG responses to all 3 components at all dosages tested, and the difference were significant to the major pheromone Z9,E11-14:Ac at all dosages of 10–1000 ng, to the minor component Z9,E12-14:Ac at 500 ng and Z9 -14:Ac at 500–1000 ng (Fig. 3A). Similarly, male *SlitPBP2*$^{-/-}$ showed consistently lower EAG responses, significantly to the major component Z9,E11-14:Ac at the dosage 10–1000 ng, the minor component Z9,E12-14:Ac at 1–1000 ng, and the third component Z9 -14:Ac at 1000 ng (Fig. 3B).

To compare the reduction extents of EAG responses among 3 pheromone components and between two PBP knockout insect lines, the averaged EAG reductions (%) to a component of different dosages were calculated. It showed that EAG reductions among 3 pheromone components (Z9,E11-14:Ac, Z9,E12-14:Ac and Z9-14:Ac) were similar, being 53, 60 and 63% respectively for *SlitPBP1*$^{-/-}$ males, and 40, 43 and 46% respectively for *SlitPBP2*$^{-/-}$ males. However, EAG reductions in *SlitPBP1*$^{-/-}$ males were significantly higher than that in *SlitPBP2*$^{-/-}$ males for all 3 pheromone components and *SlitPBP2*$^{-/-}$ were significantly higher than that in *SlitPBP3*$^{-/-}$ (Fig. 4A) while all of three *SlitPBP*s displayed no significant preference among the 3 pheromone components (Fig. 4B).

Behavioral responses to the major component (Z9,E11-14:Ac) were further tested at 4–8h of scotophase using a Y-tube olfactometer (Fig. 5). The homozygous mutants of *SlitPBP1* and *SlitPBP2* in G5 generation were used for behavioral assay. Compared with WT moths, *SlitPBP1*$^{-/-}$ and *SlitPBP2*$^{-/-}$ male moths displayed lower response percentages, and in comparison, *SlitPBP1*$^{-/-}$ males were lower than *SlitPBP2*$^{-/-}$ males in the response percentages. WT moths showed 83, 75 and 33% in pheromone selection, hair pencil displaying and mating attempt respectively; while the percentages were 50, 33, 33% for *SlitPBP2*$^{-/-}$ males and 33, 8, 0% for *SlitPBP1*$^{-/-}$ males, respectively.

4. Discussion

The CRISPR/Cas9 system has been developing rapidly and offers a wide breadth of applications in insects. Indeed, it has been used in functional studies of olfactory genes in several insects, such as *S. litura* (Zhu et al., 2016a, 2017), *Helicoverpa armigera* (Chang et al., 2017; Ye et al., 2017), *Locusta migratoria manilensis* (Li et al., 2016), *S. littoralis* (Koutroumpa et al., 2016), *B. mori* (Liu et al., 2017; Shiota et al., 2018), *Chilo suppressalis* (Dong et al., 2019), *Harpegnathos saltator* (Yan et al., 2017) and *Ooceraea biroi* (Trible et al., 2017). For insect PBP genes, we used this system, and demonstrated in a novel manner that *SlitPBP3* in *S. litura* plays a minor role in the sex pheromone perception (Zhu et al., 2016a). To further elucidate the relative contribution and component preference of the three PBP genes in perception of the female sex pheromones, the present study expanded this system to the other two *S. litura* PBP genes, *SlitPBP1* and *SlitPBP2*.

In noctuid moths, up to 3 PBP genes have been identified in antennae of each single species (Liu et al., 2015; Forstner et al., 2006; Leal et al., 2013), suggesting functional differentiation of the PBP genes in the sex pheromone perception. Furthermore, these PBPs displays different expression levels in male antennae and different male/female ratios in expression in the antennae (Allen et al., 2005; Abraham and Wanner, 2011; Newcomb et al., 2002). In *S. litura*, the sex expression profile by real-time quantitative PCR (qPCR) demonstrated that 3 *SlitPBPs* (PBP1, PBP2 and PBP3) were differentially expressed in male antennae (10.00: 3.33: 1.04) and between males and females (male/female, 2.70: 1.38: 0.71) (Liu et al., 2013). This strongly suggests that 3 *SlitPBPs* are of different importance in sex pheromone perception (PBP1 > PBP2 > PBP3), considering that the female sex pheromone is primarily perceived by male antennae. Further *in vitro* ligand binding assays revealed the binding affinities were consistent with the expression results. *SlitPBP1* had the highest affinities for the 3 sex pheromone components (K_i = 0.57 − 1.07 μM), *SlitPBP2* had middle affinities (K_i = 2.20– 4.46 μM), while *SlitPBP3* had lowest affinities (K_i = 4.77–21.75 μM) (Liu et al., 2012, 2013). The present *in vivo* functional study and our previous study with PBP3 (Zhu et al., 2016a) confirm this different importance of PBP1 > PBP2 > PBP3 in the sex pheromone perception in *S. litura*.

While this relative importance is consistently supported, some discrepancy is noted among results obtained by *in vivo*and *in vitro* functional assays, and in tissue-sex profiling studies. For example, the relative contribution of PBP1 among 3 PBPs based on *in vitro* ligand binding affinities and sex expression profiles appears to be skewed when compared to the EAG assay of the PBP knockout moths, similar to

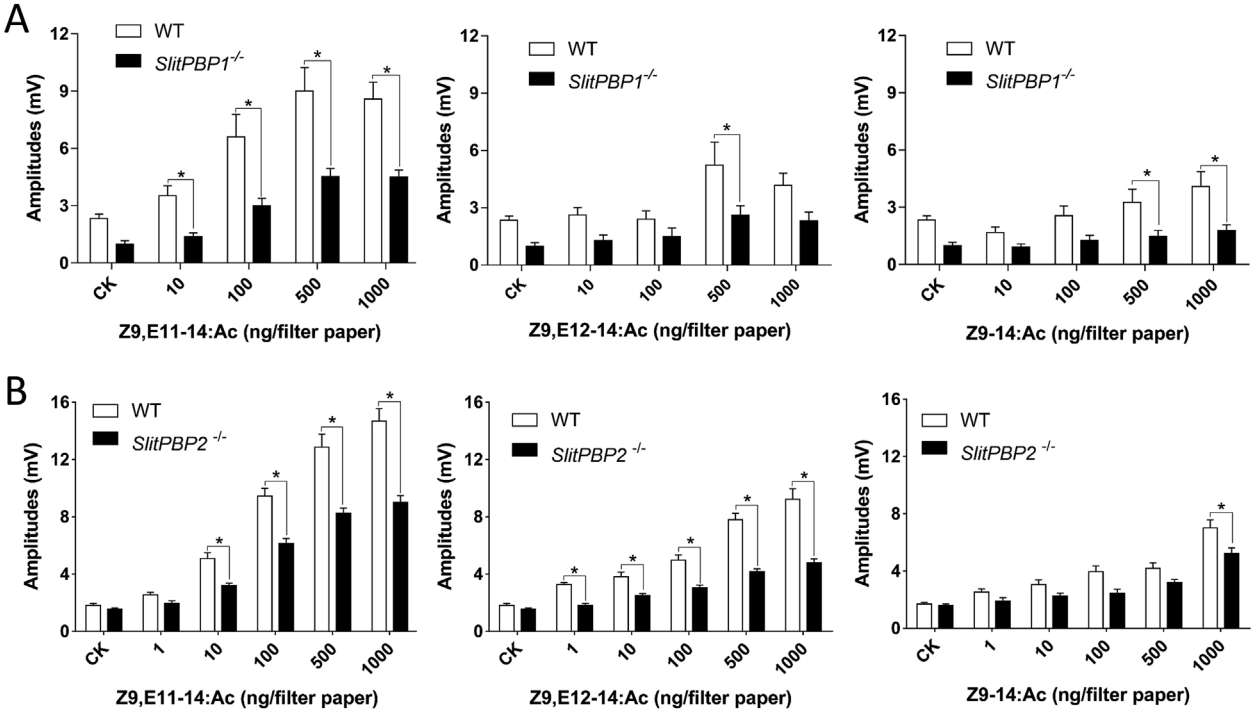

Fig. 3. EAG responses of WT and *SlitPBP1* knockout (A) and *SlitPBP2* knockout (B) males to the sex pheromone components (Z9,E11–14:Ac, Z9,E12–14:Ac, Z9-14:Ac) at the 3rd day after eclosion. The statistical significance between WT and the mutant line was analyzed with unpaired Student's t-test. Error bars represent SEM. The asterisks indicate significant differences between the two groups ($p < 0.05$).

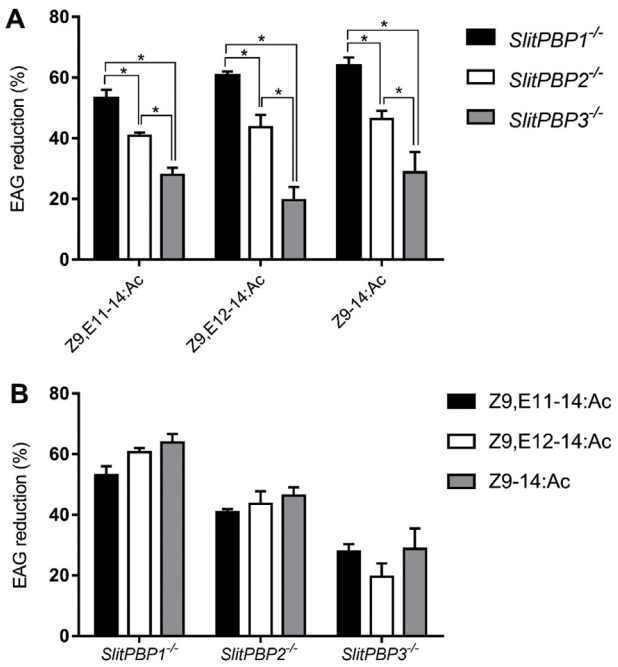

Fig. 4. Reduction in EAG response (%) of male moths with a knockout gene of *SlitPBP1*, *SlitPBP2* or *SlitPBP3*. **A**: Reduction in EAG response of same sex pheromoone between different mutant lines. **B**: Reduction in EAG response of same mutant line between different sex pheromone compounts. The perecntags of reduction was calcuted based on response of WT moths. The original data for *SlitPBP3* was from our previous study. The statistical significance between different groups were analyzed with unpaired Student's t-test. Error bars represent SEM. The asterisks indicate significant differences between two groups ($p < 0.05$).

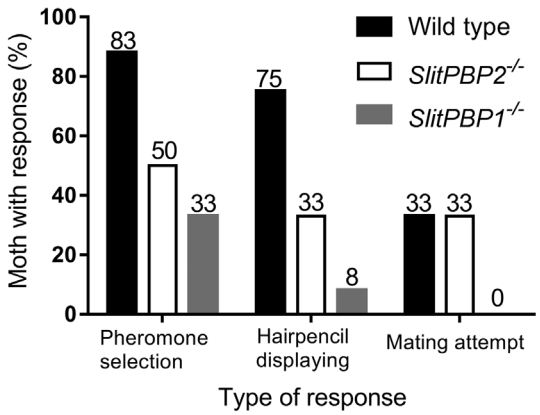

Fig. 5. Behavioral response (%) of WT and *SlitPBP* knockout male moths in Y-type olfactometer assay. The major sex pheromone component Z9,E11-14:Ac (1000 ng) was used, and hexane served as control.

that of *C. suppressalis* (Dong et al., 2019). This discrepancy implies that, in addition to three PBPs, some other genes may also contribute to the binding and transportation of the sex pheromones. It has been reported that GOBPs (especially GOBP2) have high binding affinities for sex pheromone components in *S. litura* (Liu et al., 2015b) and even in other lepidopteran insects, such as *S. exigua* (Liu et al., 2015a), *Amyelois transitella* (Liu et al., 2010), *C. suppressalis* (Sajjad et al., 2017) and *Loxostege sticticalis* (Yin et al., 2012). This is not necessarily unexpected, as most of the main contributors to the ligand-binding pocket are conserved between GOBPs and PBPs, as indicated in *B. mori* (Zhou et al., 2009) and *P. xyllostella* (Zhu et al., 2016b). In addition to GOBPs, CSP19 in *Sesamia inferens* (Zhang et al., 2014) also displays high binding affinities for sex pheromone components. Alternatively, a compensation effect may exist among PBPs and other related genes, although qPCR quantification in the present study with PBP1 knockout moths showed that 4 of the most probable OBP genes (PBP2, PBP3 and

two GOBPs) were not different in expression levels between PBP1 knockout and wild type males (data not shown). Thirdly, the interaction among PBPs and even between PBP and OR may also contribute to this discrepancy. It is reported that a binary OBP mixture exhibited an enhanced ligand affinity compared to a single OBP in *Anopheles gambiae* (Qiao et al., 2011) and *Holotrichia oblita* (Wang et al., 2013), and that PBPs enhanced the sensitivity of ORs to sex pheromones in *P. xyllostella* (Sun et al., 2013) and *C. suppressalis* (Chang et al., 2015). Further, an interesting result is that mating attempts were completely prevented by silencing PBP1, while weak effect (53% in reduction) was observed on the EAG with the absence of this protein. This provides strong evidence to the fact that behavior is the result of an integrated detection of multiple elements of information, and simultaneously, supports a crucial role of PBPs and other OBPs in olfaction.

Another question our present study addressed is whether each PBP is specifically tuned to a single component of the pheromone blend, as results obtained by *in vitro* studies were not consistent among moth species and PBPs in a same species, with some studies supporting it (Gu et al., 2013; Maida et al., 2003; Plettner et al., 2000 and Maïbèche-Coisné et al., 1997) and others did not (Grater et al., 2006; Sun et al., 2013; Guo et al., 2012; Liu et al., 2012, 2015b). By using the *in vivo* functional study, our present study and a previous study (Zhu et al., 2016a) demonstrate that all 3 PBPs in *S. litura* display no obvious selectivity among 3 pheromone components, based on percentages of EAG reduction in PBP knockout males in relation to the wild type males. This is consistent with the results of *in vitro* binding assays (Liu et al., 2012, 2013), and similar to the results of recent *in vivo* functional studies with PBP1 in *H. armigera* (Ye et al., 2017), PBP1 in *B. mori* (Shiota et al., 2018) and PBP1 and PBP3 in *C. suppressalis* (Dong et al., 2019). However, the selectivity of PBPs for pheromone components may vary between insect species, which requires further verification in additional lepidopterans.

In conclusion, by electrophysiological and behavioral assays using CRISR/Cas9 induced PBP knockout moths, it is demonstrated that both *SlitPBP1* and *SlitPBP2* play important roles in the female sex pheromone perception in *S. litura*. Combined with our earlier study with *SlitPBP3*, we conclude that the relative importance is PBP1 > PBP2 > PBP3 in the sex pheromone perception, while each PBP is not specifically tuned to a single pheromone component. Our study provides new insights into the functional role of PBPs, as well as an important reference for developing PBP-based behavioral interference techniques in the pest control.

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Appendix A. Supplementary data

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