In vivo functional characterisation of pheromone binding protein-1 in the silkmoth, Bombyx mori

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Male moths detect sex pheromones emitted by conspecific females with high sensitivity and specificity by the olfactory sensilla on their antennae. Pheromone binding proteins (PBPs) are highly enriched in the sensillum lymph of pheromone sensitive olfactory sensilla and are supposed to contribute to the sensitivity and selectivity of pheromone detection in moths. However, the functional role of PBPs in moth sex pheromone detection in vivo remains obscure. In the silkmoth, Bombyx mori, female moths emit bombykol as a single attractive sex pheromone component along with a small amount of bombykal that negatively modulates the behavioural responses to bombykol. A pair of olfactory receptor neurons, specifically tuned to bombykol or bombykal, co-localise in the trichodeum sensilla, the sensillum lymph of which contains a single PBP, namely, BmPBP1. We analysed the roles of BmPBP1 using BmPBP1-knockout silkmoth lines generated by transcription activator-like effector nuclease-mediated gene targeting. Electroantennogram analysis revealed that the peak response amplitudes of BmPBP1-knockout male antennae to bombykol and bombykal were significantly reduced by a similar percentage when compared with those of the wild-type males. Our results indicate that BmPBP1 plays a crucial role in enhancing the sensitivity, but not the selectivity, of sex pheromone detection in silkmoths.

Male moths utilize sex pheromones emitted by conspecific females to identify and locate their mates¹,². To detect the minute amounts of sex pheromones that are diluted in the air, male moths have evolved a sophisticated olfactory system that can detect conspecific pheromones with extreme sensitivity and specificity. Molecular mechanisms underlying the detection and discrimination of sex pheromone components by male moths have been one of the major topics of research in the field of insect olfaction.

Sex pheromones emitted by female moths are detected by sex pheromone receptor proteins, which are expressed on the dendritic membrane of pheromone-specific olfactory receptor neurons (ORNs) in the sensilla trichodea located on the antennae of male moths³⁴. These ORNs are bathed in an aqueous solution referred to as sensillum lymph. Because most pheromone molecules are highly hydrophobic, they are believed to be solubilised into the sensillum lymph and are transported to pheromone receptors after they bind with small soluble proteins (about 15kDa) named pheromone binding proteins (PBPs) that are highly enriched in the sensillum lymph⁴⁶. PBPs, which belong to the odorant binding protein (OBP) family of insects⁵, are expressed in accessory cells surrounding the ORNs and are secreted into the sensillum lymph of pheromone-sensitive olfactory sensilla⁶. In addition to the solubilisation of pheromones, PBPs have been proposed to participate in the discrimination of sex pheromone components based on the fact that each moth species possesses multiple PBPs that exhibit different binding affinities to different sex pheromone components, as has been demonstrated in in vitro binding assays using several moth species⁹⁻¹¹.
Figure 1. Generation of BmPBP1-knockout silkmoths. (a) Schematic representation of the genomic structure of BmPBP1 (top) and target sequences of transcription activator-like effector nucleases (TALENs; bottom). Exons are indicated by blue boxes and the start/stop codon locations are shown. TALENs were constructed to target sequences in the second exon. The sequences of TALEN recognition sites are shown at the bottom of the genomic structure. (b) TALEN-induced mutant alleles generated in this study. The wild-type sequence is aligned with the deletion mutant sequences of BmPBP1. The deletions are indicated by a dashed line. The red box in the 4-bp deletion sequence indicates the position of a frame shift. Right and left TALEN recognition sequences are highlighted in red and blue characters, respectively. The black arrow under the sequences indicates the 3′ primer site used for genotyping by using genomic PCR. (c) A representative genomic PCR analysis of the 4-bp deletion allele is shown. The PCR products obtained using genomic DNA isolated from the wings of a mutation-homozygous individual (4del/4del), or a mutation-heterozygous individual (WT/4del), and a wild-type individual were separated by electrophoresis. The PCR primers corresponding to sequences flanking
The silkmoth Bombyx mori is one of the model insects used in sex pheromone communication research. Peripheral pheromone detection system of this species, including relationships among the sensillum types, ORNs, pheromone receptors, and PBPs, is well characterised. Female silkmoths emit bombykol ([E,Z]-10,12-hexadecadien-1-ol) and bombykal ([E,Z]-10,12-hexadecadienial) from their sex pheromone gland at a typical ratio of 11:13. Of these two compounds, only the major component, bombykol, is sufficient to induce pheromone source orientation behaviour in male moths, whereas the minor component, bombykal, negatively modulates the initiation of this behaviour. Male silkmoths detect these pheromones using long sensillum trichodea on their antennae. This type of sensilla contain a pair of bombykol- and bombykal-sensitive ORNs that express sex pheromone receptors specific to bombykol (BmOR1) and bombykal (BmOR3), respectively. In the silkmoth, one PBP gene (BmPBP1) and two PBP-like genes (BmPBP2, 3) have been reported. However, only BmPBP1 has been shown to be expressed in the sensillum lymph of pheromone sensitive sensilla and its associated accessory cells at mRNA and protein levels, whereas BmPBP2 and BmPBP3 are expressed in accessory cells that are not associated with the pheromone-sensitive sensilla. In addition, immunohistochemical analysis has shown that other OBPs that have been examined (GOBP1, GOBP2, and ABPX) are rarely or not expressed in the long sensilla trichodea of male antennae. These findings suggest that BmPBP1 is involved in the detection of bombykol and bombykal whereas BmPBP2 and BmPBP3 play roles in the detection of compounds other than sex pheromones. However, the possible functions of BmPBP1 have yet to be conclusively established, particularly with respect to whether BmPBP1 plays a crucial role in the discrimination between bombykol and bombykal.

To characterise the functional role of PBPs in the detection of sex pheromones in vivo, we generated BmPBP1−/-knockout silkmoth lines by using transcription activator-like effector nuclease (TALEN)-mediated gene targeting in order to elucidate the in vivo functions of BmPBP1 in the sex pheromone detection of male silkmoths. By electroantennogram (EAG) recordings, we show that the response of BmPBP1−/-knockout male antennae to both bombykol and bombykal was significantly reduced compared with those in the wild-type moths. We also established that the sensitivity of the initiation of pheromone source orientation behaviour is reduced in BmPBP1−/-knockout male moths. On the basis of our results, we discuss whether BmPBP1 contributes to the sensitive and selective detection of bombykol and bombykal in male silkmoths.

**Results**

**Establishment of BmPBP1−/-knockout silkmoths.** To characterise the functional role of PBPs in the detection of sex pheromones in vivo, we generated BmPBP1−/-knockout silkmoth lines using TALENs that were designed to target the second exon of the BmPBP1 gene, which is located on the 19th chromosome (Fig. 1a). By genomic PCR screening of G1 individuals, followed by sequence analyses, we obtained two genetic lines with 4- and 12-bp deletions in the target region of the TALENs, respectively (Figs 1b and S1). From these two genetic lines, we selected a line that had the 4-base deletion. This deletion caused a frame shift at the 55th amino acid residue of the BmPBP1 protein and the introduction of a stop codon at the 73rd amino acid residue. Given that the first 22 amino acids of this protein constitute a signal peptide, this premature translational termination would result in a truncated protein of only 51 amino acids, compared with the 142 amino acids of the wild-type protein. The mutant BmPBP1 protein contained only three out of the nine residues that form the bombykol binding pocket and only one out of the six cysteine residues required for proper folding, resulting in the synthesis of a loss-of-function BmPBP1 protein (Fig. 1c).

To confirm the 4-bp deletion at the transcript level, we amplified full-length coding sequences of BmPBP1 from the antennal cDNA of homozygous mutant (BmPBP1−/-) male moths using RT-PCR. After cloning the PCR products into a sequence plasmid vector, we sequenced 11 clones and confirmed that the DNA insert in all the clones had the same deletion as that in the genomic sequence, further supporting our observation that this allele encodes a substantially truncated BmPBP1 protein. We noted that both BmPBP1−/- female and male moths were fertile, and that their offspring grew normally to adulthood.

**EAG analysis of BmPBP1−/-knockout male antennae.** To determine the effects of the loss of function of BmPBP1 on the olfactory response in male moths, we recorded the EAG responses of male antennae to sex pheromone components. The peak EAG amplitudes of the antennae of BmPBP1−/-knockout male moths were significantly reduced compared with those of the antennae of wild-type males (Fig. 2a,b). Notably, the degree of reduction in EAG amplitude was similar between the responses to bombykol and bombykal (Table 1), suggesting that BmPBP1 contributes to the detection of these two components to a similar extent, and thus is not likely to have selectivity for either of the two compounds. Although the peak amplitudes were significantly lower than those of the wild-type males, antennae from the BmPBP1−/-knockout males showed clear dose-dependent responses to bombykol and bombykal (Fig. 2a,b), suggesting that male moths can detect bombykol and bombykal, albeit with low sensitivity, in the absence of functional BmPBP1.
To exclude the possibility that the loss of BmPBP1 affected the olfactory response of antennae as a whole, we analysed the EAG response of male antennae to general odorants, linalool and citral. The peak EAG amplitudes for the response to both linalool and citral were not significantly different between the antennae of the BmPBP1-knockout and wild-type males (Figs 2c and S2). Although linalool reportedly inhibits most of bombykol receptor neurons and activates some of them, our results that BmPBP1-knockout did not affect EAG responses to general odorants indicate the lowered response of BmPBP1-knockout male moths to pheromones was not due to a general effect on olfactory detection.

Behavioural analysis of BmPBP1-knockout male moths. Finally, using a closed-box assay, we investigated whether the reduced response of antennae to bombykol caused by BmPBP1 mutation affected the behavioural responsiveness to bombykol. In this assay, we used wing flapping behaviour as a criterion for initiation of the pheromone source orientation behaviour. Dose-response analysis revealed that the percentage of BmPBP1-knockout males that initiated the pheromone source orientation behaviour was lower than that of the wild-type males (Fig. 3a). As in wild-type males, BmPBP1-knockout males did not show any behavioural
Figure 3. Behavioural response of BmPBP1-knockout males to pheromones. The behavioural response percentages of BmPBP1−/−/BmPBP1−/− males to different doses of bombykol (a) or bombykal (b) are plotted. The asterisk indicates significant differences between the groups (*p < 0.05), as determined using Fisher’s exact probability test for comparing pairs of data.

responses to bombykal even at the highest dose tested (100 ng; Fig. 3b). These results indicate that the behavioural responsiveness to bombykol was reduced in BmPBP1−/− males.

Discussion
In this study, we demonstrated that the loss of functional BmPBP1 led to the lowering of EAG responses to both bombykol and bombykal to a similar extent. These observations indicate two important aspects of BmPBP1 function: (1) BmPBP1 is necessary for the sensitive detection of bombykol and bombykal and (2) BmPBP1 is not involved in the discrimination between bombykol and bombykal. Our results are consistent with those of a recent study by Ye et al., wherein the authors observed significant reductions in the EAG responses of the antennae of PBPI-knockout Helicoverpa armigera males to three sex pheromone components in this species.

Previous studies suggested two different molecular mechanisms underlying the specific response of male silkmoths to bombykol. On the basis of an in vivo opened sensillum tip analysis, Pophof reported that BmPBP1 mediates the response of BmOR1 to bombykol but not to bombykal, and thus proposed that response selectivity to pheromones is intermediated by PBPs specialized in the recognition of particular ligands. Using HEK293T cell expression system, Große-Wilde et al. showed that BmOR1 was able to respond to both bombykol and bombykal when these compounds were solubilised by dimethyl sulfoxide (DMSO), but responded only to bombykol when BmPBP1 was used as a solubiliser instead of DMSO21, suggesting that BmPBP1 selectively solubilised bombykol and that the interplay between bombykol and BmPBP1 is important for the selective response of BmOR1 to bombykol. Accordingly, these authors proposed that other PBPs that bind to bombykol and mediate the bombykol response should be present in the sensilla trichodea of male moths. In contrast, it has been demonstrated that Xenopus oocytes or Sf21 cells, co-expressing BmOR1 and the co-receptor BmOrco, responded specifically to bombykol that was dissolved in DMSO, suggesting that specific interaction between bombykol and BmOR1 defines the specificity of the response18,32. Our results, demonstrating that BmPBP1 mediates the response to both bombykol and bombykal in vivo, are consistent with the latter mechanism because a BmOR1-expressing bombykol-sensitive ORN and a BmOR3-expressing bombykal-sensitive ORN co-localise in the same sensilla in the silkmoth, and are thus bathed in the same sensillum lymph containing BmPBP1.22 Further, our results are consistent with the docking simulation and in vitro binding assay reported by Gräter et al., which showed that BmPBP1 bound to both bombykol and bombykal with nearly the same affinity33.

Although the responsiveness of the antennae of BmPBP1-knockout males to sex pheromones was considerably lower than that of the antennae of the wild-type males, BmPBP1-knockout male antennae still showed dose-dependent EAG responses to bombykol and bombykal. Therefore, even in the absence of a functional BmPBP1, subsets of pheromone molecules can reach the dendritic membranes of ORNs and activate pheromone receptor proteins expressed on the membranes. The residual EAG response of the antennae of BmPBP1-knockout males to bombykol and bombykal is dose-dependent, indicating that bombykol and bombykal are detected by BmOR1 and BmOR3, respectively. In this regard, it has been reported that at least some of the olfactory tubules in the sensillum trichodea come into contact with the dendritic membrane of ORNs in the giant silkmoth Antheraea polyphemus, thereby raising the possibility of a direct pathway from the olfactory pores to the dendritic membranes. Further studies are accordingly required to clarify how pheromone molecules reach the pheromone receptors in the absence of solubilisation by BmPBP1.

We also demonstrated the effects of BmPBP1-knockout at the behavioural level, showing that the percentage of male moths that initiate pheromone source orientation behaviour was significantly reduced in BmPBP1-knockout males. To gain a more precise understanding of the functional role of BmPBP1 in the sex
pheromone communication system, it would be informative to examine not only the sensitivity of behavioural response initiation but also the efficiency of male orientation to female moths and successful copulation.

In summary, by using BmPBP1-knockout moths, we showed that BmPBP1 contributes to the sensitivity of pheromone detection, but does not play a significant role in the discrimination of bombykol and bombykal. Apart from the solubilisation and transportation of pheromones, PBPs have also been proposed to play additional roles\(^\text{36}\), including the protection of pheromone molecule from enzymatic degradation\(^\text{37}\) and rapid inactivation of pheromones\(^\text{38}\). Detailed physiological analyses of BmPBP1-knockout moths will shed further light on the underlying mechanisms and modes of action of BmPBP1 in pheromone detection in vivo.

**Methods**

**Animals and chemicals.** The wi-pnd strain of Bombyx mori, which is non-diapausing and characterised by non-pigmented eggs and eyes, was used for the generation of BmPBP1-knockout moths. Larvae were reared on an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a 16:8 (light/dark) photoperiod. The purity (>99.5%) of synthetic bombykol and bombykal were verified by gas chromatography under previously described conditions\(^\text{25,40}\).

**Construction of TALEN vectors and synthesis of RNA for injection.** TALEN expression vectors were constructed as described previously\(^\text{41,42}\). The RNA used for injection was prepared as described previously\(^\text{28}\) using a Qiagen Hispeed plasmid midi kit (Qiagen, Hilden, Germany) and an mMMESSAGE mMACHINE T7 Ultra Transcription kit (Ambion, Austin, USA). Following EAG measurements, the data were analysed using a custom-written programme (MATLAB; Mathworks, Natick, MA, USA). The data for wild-type and BmPBP1−/− males were obtained by crossing BmPBP1+/BmPBP1+ females with BmPBP1−/− males.

**Screening of mutagenised moths.** G1 eggs were obtained by the sibling mating of G0 adults. Genomic DNA of G1 eggs from different broods was extracted separately using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The region surrounding the target site was PCR amplified using genomic DNA as the template and specific primers (sense: 5′-CGACCTTCGCAAGGTATGAT-3′ and antisense: 5′-AGGCACATTATAGGCGCATCC-3′). The PCR products were sequenced directly using an ABI3700 DNA analyser (Applied Biosystems, Foster City, CA, USA). G1 broods that showed overlapping sequencing patterns with the target sequence were reared to the adult stage. After the G1 moths had been crossed with wild-type adults, their genomic DNA was extracted, PCR amplified, and sequenced, as described above, to identify the mutagenised individuals. BmPBP1+/BmPBP1− males were obtained by crossing BmPBP1+/BmPBP1+ females with BmPBP1−/− males.

**Reverse-transcription-PCR.** Total RNA was extracted from male moth antennae using TRizol reagent (Invitrogen, Carlsbad, CA, USA) as described previously\(^\text{29}\). The extracted RNA was reverse transcribed using an oligo(dT) adaptor primer (Takara-Bio, Otsu, Japan) and AMV reverse transcriptase (Takara-Bio, Otsu, Japan) at 42 °C for 30 min. The cDNA of BmPBP1 was amplified using Ex Taq DNA polymerase (Takara-Bio, Otsu, Japan) and a primer pair for BmPBP1 (5′-ATGCTCAGATCAGGACGATG-3′ and 5′-TCAAACTTCAAGCTAAAATTTCTCCC-3′), with thermal cycling at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis on 2.0% agarose gels. The cDNA was cloned into a PGEM-T Easy vector (Promega, Madison, USA), and the PCR products were sequenced using an ABI3700 DNA analyser (Applied Biosystems, Foster City, CA, USA).

**Electroantennogram (EAG) recordings.** The antennae of male moths were excised at the base, and a few segments at the tip were cut off. The antennae were then mounted on the EAG probe using electrode gel (SPECTRA 360; Parker Laboratories, Fairfield, NJ, USA). A glass cartridge (inner diameter, 5 mm) was prepared for stimulation by inserting a filter paper (1.5 × 1.5 cm), and 5 μL of pheromone solution in n-hexane or neat n-hexane (control) was administered. For general odorant stimulation, 5 μL of 10% (v/v) linalool or citral (Wako, Osaka, Japan) in paraffin oil (Sigma-Aldrich, St. Louis, USA) was used as the stimulant. A charcoal-purified airstream (1 L/min) was passed through a glass pipette and directed onto the antenna. The EAG responses were amplified using a custom-made amplifier (Minegishi and Kanzaki, unpublished), low-pass filtered at 300 Hz, and digitised at 1 kHz (USB-6210; National Instruments, Austin, USA). The data were analysed using a custom-written programme (MATLAB; Mathworks, Natick, MA, USA). Following EAG measurements, the genotype of all the males was confirmed by PCR as described in the section ‘Screening of mutagenised moths’. The percentage reduction in peak EAG amplitude (Table 1) was calculated by dividing the peak EAG amplitude of BmPBP1+/BmPBP1− by that of wild-type males.

**Behavioural experiments.** The behavioural responses to bombykol were examined as described previously\(^\text{36}\), with the exception that the male silkmoths were used within 1 to 5 days after eclosion. The moths were exposed to increasing doses of bombykol or bombykal (0.01, 0.1, 1, and 10 ng) at 1-min intervals. Wing flapping within 10 s of the stimulation that lasted for more than 10 s was counted as a response. Subsequent to these behavioural experiments, the genotype of all the males was determined by PCR.

**Statistical analysis.** To assess the statistical significance of differences in the EAG and behavioural response data for wild-type and BmPBP1-knockout moths, we used Student’s t-test and Fisher’s exact probability test for comparing pairs of data, respectively, by using Microsoft Excel 2010 and a commercial macro programme (Statcel version 3; Seiun-sya, Japan). The error bars shown in figures represent SEMs.
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Author Contributions
Y.S., T.S., T.D., Y.I., and R.K. designed the research; Y.S., T.S., H.M., and T.F. performed the research; T.D., S.M., and H.S. contributed new reagents/analytic tools; Y.S. and T.S. analysed the data; and Y.S. and T.S. wrote the paper.

Additional Information
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