

Candidate pheromone binding proteins of the silkworm *Bombyx mori*

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Abstract Pheromone reception is thought to be mediated by pheromone binding proteins (PBPs) in the aqueous lymph of the antennal sensilla. Recent studies have shown that the only known PBP of *Bombyx mori* (BmorPBP1) appears to be specifically tuned to bombykol but not to bombykal, raising the question of whether additional subtypes may exist. We have identified two novel genes, which encode candidate PBPs (BmorPBP2, BmorPBP3). Comparison with PBPs from various moth species have revealed a high degree of sequence identity and the three BmorPBP-subtypes can be assigned to distinct groups within the moth PBP family. In situ hybridization revealed that BmorPBP2 and BmorPBP3 are expressed only in relatively few cells compared to the number of cells expressing BmorPBP1. Double-labeling experiments have shown that the two novel BmorPBPs are expressed in the same cells but are not co-expressed with BmorPBP1. Furthermore, unlike BmorPBP1, cells expressing the newly identified PBPs did not surround neurons containing the BmOR-1 receptor. The results indicate that BmorPBP2 and BmorPBP3 are located in sensilla types, which are different from the long sensilla trichodea.

Keywords Insect · *Bombyx mori* · Olfaction · Pheromone detection · Pheromone binding protein

Data deposition: The sequences reported in this paper have been deposited in the EMBL database under accession nos. AM403100 (BmorPBP2) and AM403101 (BmorPBP3).

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Abbreviations

BmorPBP *Bombyx mori* pheromone binding protein
BmOR *Bombyx mori* olfactory receptor
DIG Digoxigenin

Introduction

Throughout the animal kingdom pheromone-induced behaviour plays a critical role in survival and reproduction. In particular, nocturnal insects rely strongly on distinct chemical cues for intraspecific communication (Hansson 1995; Blomquist and Vogt 2003; Howard and Blomquist 2005); sex-pheromone signaling of moths represents one of the most remarkable examples (Schneider 1992). Female moths release a blend of sex-pheromones to attract males over long distances and males detect the released pheromones with extreme sensitivity and selectivity (Kaisling 1987; Baker et al. 2004). In male moths, pheromone detection is mediated by specialized sensory neurons in trichoid sensilla on the antennae. Soluble pheromone binding proteins (PBPs) in the sensillum lymph surrounding the dendrites of the pheromone-sensitive cells are thought to transfer the usually hydrophobic pheromone molecules to the dendritic membrane of the sensory neurons. PBPs have been identified in various moth species and in some species several subtypes were found (reviewed in Vogt 2003; Pelosi et al. 2006). The identification of multiple subtypes within one species (Krieger et al. 1991; Maida et al. 2000; Abraham et al. 2005) has led to the concept that a distinct PBP-subtype may be tuned to bind and transport only a defined pheromone component. Indeed, recent work indicated that a

PBP-subtype interacts differentially with various pheromone components and displays distinct binding specificities for different pheromone components (Feixas et al. 1995; Plettner et al. 2000; Mohl et al. 2002; Bette et al. 2002; Maida et al. 2003). In the silkworm, *Bombyx mori*, only one PBP (BmorPBP) has been identified to date (Krieger et al. 1996); a finding which appeared to coincide with the apparently very limited complexity of the sex-pheromone blend (bombykol, bombykal). The expression of BmorPBP was confined to the supporting cells surrounding the two pheromone-responsive neurons in long sensilla trichodea (Steinbrecht 1999), which make up the majority of sensilla hairs on male antennae. Each of the two sensory neurons in this sensillum type appears to mediate the response to either bombykol or bombykal respectively (Kaissling et al. 1978). Furthermore, it has been demonstrated that one cell expresses the receptor-type BmOR-1 and the second cell the receptor-type BmOR-3 (Nakagawa et al. 2005; Krieger et al. 2005). Recent functional analysis employing a reconstituted system revealed that the PBP plays an important role in solubilizing the pheromone component in an aqueous medium and, furthermore, that the BmorPBP only mediates a specific action of bombykol, but not that of bombykal (Grosse-Wilde et al. 2006). These results not only demonstrated the role and specificity of PBPs in pheromone reception but also suggest that, in *B. mori* at least, one additional binding protein should exist, mediating the response to bombykal. In a search for further PBPs in *B. mori* we set out to analyse the partial *B. mori* genome database and to screen an antennal cDNA library for genes, which may encode BmorPBP-related pro-

teins. Here we describe the identification of two novel candidate PBP genes and the exploration of their expression pattern in the antennae of *B. mori*.

Materials and methods

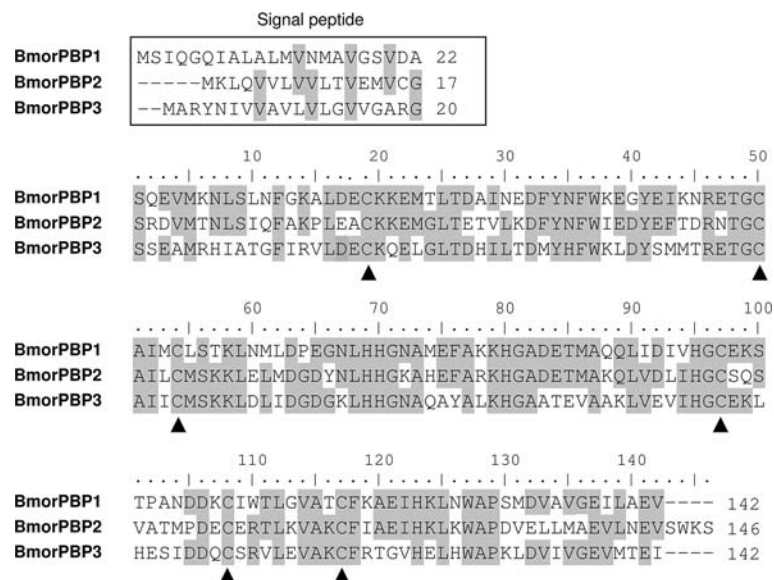
Animals and tissue preparation

Bombyx mori were obtained from Worldwide Butterflies (Dorset, UK) and Seritech (Stratford upon Avon, UK). Cocoons were kept at room temperature until emergence of the animals; after hatching males and females were separated and kept at 8°C. Antennae and heads were dissected from 1 to 4-days old, cold-anaesthetized animals. Tissues for RNA isolation were immediately frozen at liquid nitrogen temperature and stored at -70°C.

Identification of new PBP sequences

To find genes encoding new pheromone binding proteins in *B. mori*, BLAST searches of the *Bombyx* genome database at NCBI were performed with the coding region of the known pheromone binding protein of *B. mori* (Krieger et al. 1996), renamed here as BmorPBP1. This led to the identification of several genomic fragments, which encoded amino acid strings closely related to BmorPBP1. One genomic fragment of 11254 bp (Contig 010432) contained the exons for BmorPBP1 and putative exon regions for two new *B. mori* pheromone binding proteins (Fig. 1). Specific sense and antisense primers to these putative binding protein exons were used to amplify corresponding

Fig. 1 Comparison of *B. mori* PBPs. Alignment of amino acid sequences deduced for BmorPBP2 and BmorPBP3 (this study) with BmorPBP1 (Krieger et al. 1996). The signal peptides found in the PBP precursors are depicted separately above the sequences of the native proteins. Amino acids identical in at least two sequences are shaded grey. The length of the native proteins is indicated at the C-terminal end. Six conserved cysteines characteristic for PBPs are marked by an arrowhead



regions from cDNA of male *B. mori* antennae. PCR conditions were: 1 min 40 s at 94°C, then 19 cycles with 94°C for 30 s, 55°C for 40 s and 72°C for 1 min, with a decrease of the annealing temperature by 0.5°C per cycle. Subsequently, 19 further cycles at the condition of the last cycling step were performed followed by incubation for 7 min at 72°C. PCR products were gel purified by GeneClean™ (Q BIOgene, Irvine, CA, USA) and cloned using the pGEM-T vector system (Promega, Madison, WI, USA). After verification by sequencing the amplified sequences were used to prepare Digoxigenin (DIG)-labeled probes for screening an antennal cDNA library of *B. mori*. Labeled probes were obtained by standard PCR including specific sense and antisense primers, the DIG PCR labeling mixture (Roche, Mannheim, Germany) and plasmids carrying the corresponding cDNAs. PCR conditions were as described above. PCR products were gel purified by GeneClean and diluted in hybridization solution (30% formamide, 5× SSC, 0.1% lauroylsarcosine, 0.02% SDS, 2% blocking reagent [Roche], 100 µg/ml denatured herring sperm DNA). Screening of the cDNA library phage DNA was performed as described (Krieger et al. 2002, 2005). Posthybridization membranes were washed twice for 5 min in 2× SSC, 0.1% SDS at room temperature, followed by three washes in 0.1× SSC, 0.1% SDS for 20 min each at 60°C. Hybridized probes were detected using an anti-DIG AP-conjugated antibody (Roche) and CSPD (Applied Biosystems, Foster City, CA, USA). cDNA inserts from positive phage were subcloned into the Bluescript II SK+ vector and sequenced.

Sequence analysis

Sequencing was performed on an ABI310 sequencing system using vector and cDNA derived primers and the BIG dye cycle sequencing kit (Applied Biosystems). Sequence analyses were made using HUSAR (Heidelberg unix sequence analysis resources; <http://www.genius.embnet.dkfz-heidelberg.de>). The unrooted neighbor joining tree was calculated with the MEGA program (Kumar et al. 2001) based on a ClustAL alignment (Thompson et al. 1994) including protein sequences for moth pheromone binding proteins taken from the EMBL/GenBank DNA Data Bank.

Reverse transcription (RT)-PCR

Total RNAs from the antennae of males and females were isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany). Poly (A)⁺ RNA was isolated from

total RNA with oligo (dT)₂₅ magnetic dynabeads (Dyna, Oslo, Norway), transcribed into cDNA as previously described and used in PCR with the following specific primer pairs.

BmorPBP1: 5′-tct caa gaa gtc atg aag aac-3′, as5′-tea aac ttc agc taa aat ttc-3′; BmorPBP2: 5′-gtg gat ccc gag atg tga tga c-3′, as5′-cac ctc att taa gac ctc tgc c-3′; BmorPBP3: 5′-cgg tct cac gga cca cat cct g-3′, as5′-ctt ctc cta caa tga cgt cga g-3′. Based on a comparison of the BmorPBP1, BmorPBP2 and BmorPBP3 cDNA sequences to the genomic DNA the primer pairs were designed to span intron regions, which allowed to check the cDNA preparations for possible genomic contaminations. To test the integrity of the different cDNAs, primers (5′-cat aaa cga agt tgt tac ccg tg-3′, as5′-ggc tgg cat caa cat ttt ctg tc-3′) directed against the gene encoding the *B. mori* homolog of the ubiquitous ribosomal L31 protein (Tanaka et al. 1987) were used (NIAS KaikocDNA database, clone ID brP-0946). PCR conditions were: 1 min 40 s at 94°C, followed by 20 cycles with 94°C for 30 s, 47°C for 40 s and 72°C for 1 min 30 s. Finally, an incubation for 8 min at 72°C was performed. Based on the primer design the expected sizes for PCR-products originating from cDNA were 329 bp for RL31, 439 bp for BmorPBP1, 431 bp for BmorPBP2 and 344 bp for BmorPBP3. PCR products were analyzed on agarose gels and visualized by ethidium bromide staining.

In situ hybridization

Hybridization to cryosections

Antennae of 1 to 2-days-old moths were embedded in Tissue-Tek O.C.T.™ compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen at -22°C on the object holder. Cryosections (12 µm) of antennae were thaw mounted on slides and air dried at room temperature for at least 30 min. If not used directly slides were stored at -70°C. In situ hybridization with single antisense RNA probes or two color double in situ hybridizations with two different either DIG- or biotin-labeled probes, as well as visualization of hybridization was performed as reported previously (Krieger et al. 2002). DIG-labeled probes were detected by an anti-DIG AP-conjugated antibody in combination with HNPP/Fast Red (Fluorescent detection Set; Roche); for biotin-labeled probes the TSA kit (Perkin Elmer, Boston, MA, USA), including an anti-biotin streptavidin HRP-conjugate and FITC-tyramides as substrate was used. Sections were mounted in glycerol/PBS 3:1.

Preparation of hybridization probes

Biotin-labeled or DIG-labeled antisense riboprobes for BmorPBP1 to BmorPBP3 were generated using a T3/T7 RNA transcription system (Roche) and linearized recombinant Bluescript plasmids following recommended protocols.

Data analysis

Sections and antennal fragments were analyzed on a Zeiss LSM510 Meta laser scanning microscope (Zeiss, Oberkochen, Germany). Figures were arranged in Powerpoint (Microsoft) and Photoshop (Adobe systems, San Jose, CA, USA); images were not altered except to adjust the brightness or contrast for uniform tone within a single figure.

Results

Identification of cDNA sequences encoding novel *B. mori* PBPs

BLAST analysis of the partial *Bombyx* genome database (Mita et al. 2004; Wang et al. 2005) led to the identification of two sequences on a 11,254 bp long contig highly related to the BmorPBP1. Based on the genomic sequence information, PCR approaches were used trying to amplify corresponding fragments from cDNA of *B. mori* male antennae. The PCR-products were verified by sequencing, labeled and applied to screen an antennal cDNA library. This search led to the identification of two cDNA clones, which corresponded to the identified genomic sequences; they were named BmorPBP2 and BmorPBP3.

The isolated cDNA clone encoding BmorPBP2 was 792 bp long and contained an open reading frame for a polypeptide of 152 amino acids, flanked by a stop codon; a start methionine was missing. Comparison of the BmorPBP2 sequence with the corresponding genome regions and the sequence of BmorPBP1 indicated that the BmorPBP2 precursor starts with a signal peptide of 17 amino acids, followed by 146 amino acids representing the native protein (Fig. 1). Thus, the first 11 amino acids were not encoded by the cloned BmorPBP2 cDNA. The isolated cDNA clone encoding BmorPBP3 (518 bp) comprised the complete PBP precursor consisting of a 20 amino acids long signal peptide followed by 142 amino acids of the native protein (Fig. 1). A sequence comparison revealed that the three BmorPBPs share a high number of amino acid residues, including the six conserved cysteines

(Fig. 1), a hallmark for PBPs as well as odorant binding proteins of insects (Vogt 2003). The highest degree of sequence identity is found between BmorPBP1 and BmorPBP2 (57.7%), the lowest for the pair BmorPBP2/BmorPBP3 (47.2%). BmorPBP1 and BmorPBP3 have an identity of 49.3%.

Comparison of BmorPBPs to other pheromone binding proteins

To assess the relatedness of the three PBPs from *B. mori* to PBPs from other lepidopteran species, an identity dendrogram was calculated based on a ClustAL alignment including 42 PBP sequences. The resulting neighbor joining tree (Fig. 2) suggests that based on their amino acid identity Lepidopteran PBPs divide into three main groups each comprising PBPs from various species. Furthermore, it clearly indicates the relatedness of the three BmorPBPs to PBPs of other moths. Moreover, the tree also revealed that the three BmorPBPs are clearly separated from each other and are assigned to different branches. This indicates that each BmorPBP-subtype is more identical to PBPs from other species than to the other BmorPBP-subtypes. BmorPBP1 and BmorPBP3 are most closely related to their counterparts in *Manduca sexta*; whereas BmorPBP2 appeared to be most related to PBP2 of *Sesamia nonagrioides*.

Structure of the BmorPBP genes

Comparing the cDNA sequences with genomic sequences allowed the exact location of each gene on Contig 010432 to be determined and exon and intron regions to be assigned. It was found that the BmorPBP3 gene is positioned around 2,400 bp downstream of BmorPBP2 and the BmorPBP1 gene about 700 bp upstream of BmorPBP2 (Fig. 3). Not only are all three BmorPBP genes located in close proximity, they also share the same exon/intron structure; three exons are separated by two introns. The first exon encodes the signal peptide and amino acids 1–22 of the native protein. The second exon comprises amino acids 23–82 and the third exon covers the C-terminal end of the protein beginning from amino acid 83. The three BmorPBP genes share highly conserved intron/exon boundaries but they differ in the length of their introns. Whereas intron 1 has about the same length in BmorPBP1 and BmorPBP2 it is significantly shorter in BmorPBP3. In contrast intron 2 is longer in BmorPBP3 compared to the other two BmorPBP genes (Fig. 3).

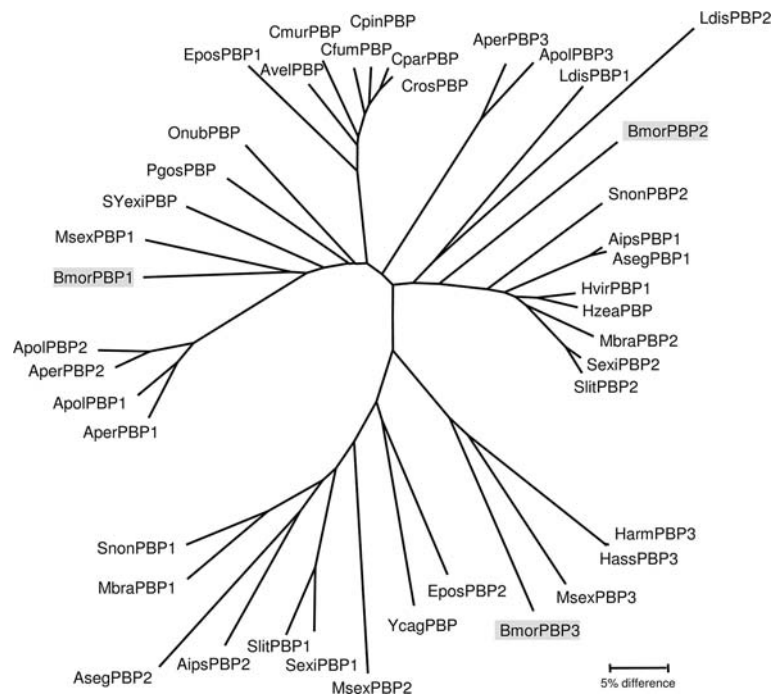


Fig. 2 Sequence relatedness of pheromone binding proteins. Neighbor joining tree based on the identity between pheromone binding proteins from different species. The distance tree was calculated using the MEGA program and is based on a ClustAL alignment of the sequences indicated. Branch lengths are proportional to percentage sequence difference; scale bar 5% difference. The following sequences have been used (name, accession number): AipsPBP1, AY301985; AipsPBP2, AY301986; AperPBP1, X96773; AperPBP2, X96860; AperPBP3, AJ277265; ApolPBP1, X17559; ApolPBP2, AJ277266; ApolPBP3, AJ277267; AsegPBP1, AF134292; AsegPBP2, AY301987; AvelPBP, AF177641; BmorPBP1, X94987; BmorPBP2 and

BmorPBP3, this study; CfumPBP, AF177642; CmurPBP, AF177647; CparPBP, AF177648; CrosPBP, AF177655; CspinPBP, AF177650; EposPBP1, AF416588; EposPBP2, AF411459; HarmPBP3, AAO16091; HassPBP3, ABB91374; HvirPBP1, X96861; HzeaPBP, AF090191; LdisPBP1, AF007867; LdisPBP2, AF007868; MbraPBP1, AF051143; MbraPBP2, AF051142; MsexPBP1, AF323972; MsexPBP2, AF117588; MsexPBP3, AF117580; OnubPBP, AF133643; PgosPBP, AF177656; SexiPBP1, AAU95536; SexiPBP2, AAU95537; SlitPBP1, AAY21255; SlitPBP2, AAZ22339; SnonPBP1, AAS49922; SnonPBP2, AAS49923; SyexiPBP, AF177657; YcagPBP, AF177661

Tissue specificity of expression

To address the functional roles of the novel putative PBPs their patterns of expression in the antennae and head tissues of *B. mori* were assessed by RT-PCR experiments (Fig. 4). PCR was performed using cDNA prepared from tissues of male or female moths using primer pairs specific for each BmorPBP. As an intrinsic control for the cDNA preparations primers specific for RL31 were applied leading to a PCR band of the correct size with all cDNA preparations.

In RT-PCR experiments, employing primers for the three BmorPBPs PCR resulted in bands of the predicted size only when antennal cDNA was used as template. Primers specific for BmorPBP1 gave a strong band for male antennae, and only a weak band for female antennae. This result confirms previous data obtained by Northern blot analysis indicating BmorPBP1 is predominantly expressed in male antennae and only at low expression levels in female

antennae (Krieger et al. 1996). Using BmorPBP2 primers, a slightly stronger band was obtained with cDNA from male antennae compared to female antenna (Fig. 4). No differences between the sexes were seen using BmorPBP3 primer.

Expression of BmorPBPs in the male antenna

The pectinate *B. mori* antenna is formed by pairs of antennal side branches, which emanate from the antennal stem. The majority of sensilla hairs housing sensory neurons are located on the inner side of the antennal side branches (Schneider and Kaissling 1957; Krieger et al. 2005). To determine which antennal cells may express the different BmorPBP-types, in situ hybridization experiments were performed on cryosections of side branches from male antennae. On sections through an antennal side branch the BmorPBP1 probe labeled a high number of cells distributed beneath the antennal surface carrying

Fig. 3 Location on genome and gene organisation of BmorPBPs genes. The *upper drawing* indicates the position of the three PBPs on Contig 010432. The organisation of the BmorPBP1, BmorPBP2 and BmorPBP3 genes and of the identified cDNAs are shown below; the length of the genes is depicted in half the scale of the corresponding cDNA. Regions in *dark grey* (a, b, c) indicate the protein coding regions. The region of the signal peptide is shown in *light grey*. The striped region in BmorPBP2 indicates part of the coding region not present in the identified cDNA. 5' and 3' non-coding regions are shown in *white*. Intron regions are marked by *shading*. All three PBPs exhibit the same three exons two introns structure

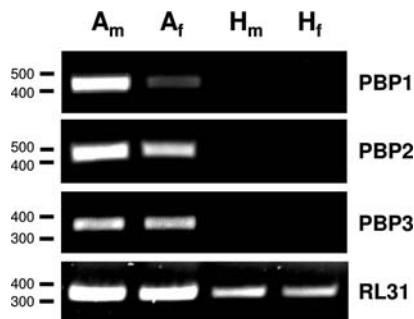
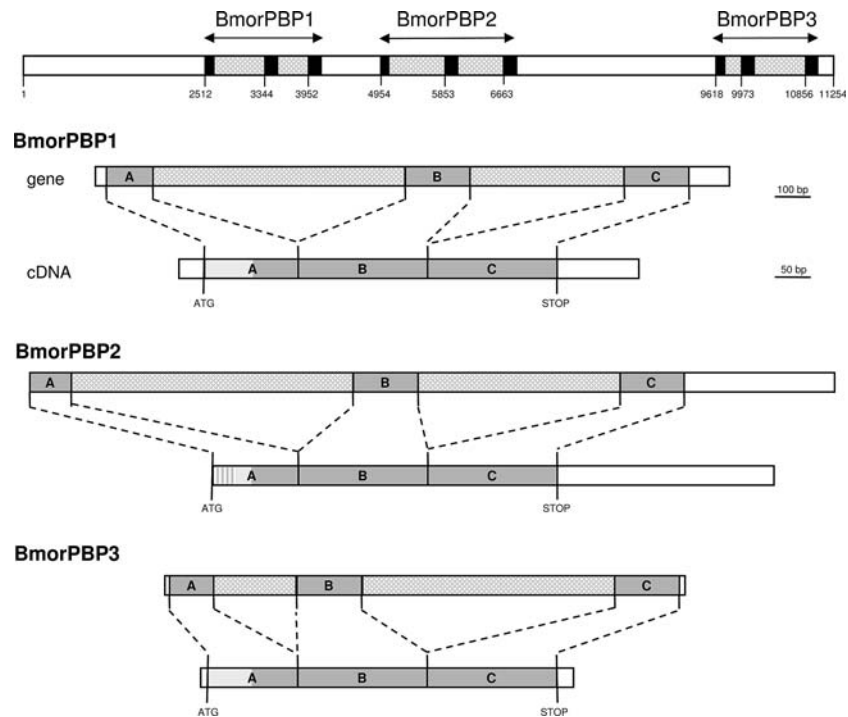


Fig. 4 Expression of BmorPBP-types in different tissues. RT-PCRs were performed with primer pairs specific for the three BmorPBP-types or RL31 and cDNAs prepared from antennae and heads of male and female *B. mori*. Reaction products were visualized by ethidium bromide staining and UV-illumination. A_m antenna male, A_f antenna female, H_m and H_f male and female heads, respectively, without appendices. The position of marker bands (bp) is indicated left. BmorPBP transcripts were detected only in antennae

the sensilla hairs (Fig. 5a), thus confirming previous observations (Krieger et al. 2005). The probes for BmorPBP2 or BmorPBP3 gave a quite different labeling pattern (Fig. 5b, c). On most of the sections made from different regions of the antenna and antennal side branches no labeled cells were found and positive sections comprised only one or two labeled cells. In general, they were localized near the midline of the antennal side branch. These results indicate that the number and the topographic location of cells

expressing BmorPBP2 or BmorPBP3 differs significantly from BmorPBP1; they are expressed in a much lower number of cells which appear to be dispersed over the entire length of the antenna. To test whether PBP-subtypes may be co-expressed in the same cell, two-colour double in situ hybridisation experiments were performed. Figure 6a shows a cross section through an antennal side branch, which has been probed with BmorPBP1 and BmorPBP2. A high number of red labeled BmorPBP1 cells and one green labeled BmorPBP2 cell are visible; there is no overlap of the two colors. A similar result was obtained when the combination of BmorPBP1 and BmorPBP3 was employed (Fig. 6b). Together these results indicate that BmorPBP1 is not co-expressed with BmorPBP2 or BmorPBP3. In contrast, probing a section with a BmorPBP2 and a BmorPBP3 probe (Fig. 6c) resulted in cells which were not only labeled by the BmorPBP2 probe (green fluorescence), but which were also positive for BmorPBP3 (red fluorescence), indicating that both PBPs are expressed in the same cell.

Association of PBP- and receptor-expressing cells

In moths, cells expressing pheromone-binding proteins appear to be closely associated with pheromone-sensitive neurons (Steinbrecht 1999; Vogt 2003). For *B. mori* it has been shown that cells expressing BmorPBP1 are associated with neurons in the pher-

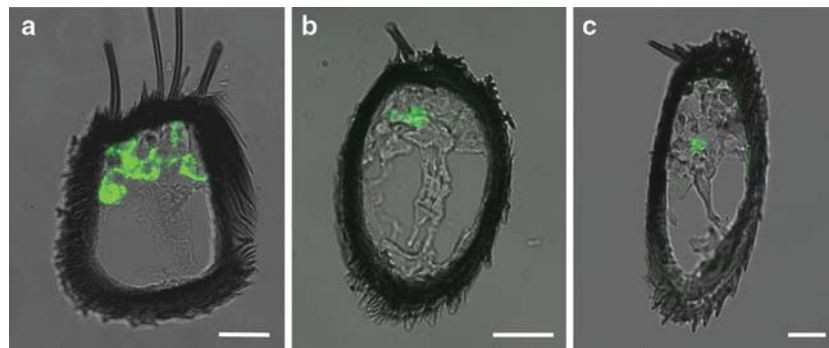


Fig. 5 Antennal localisation of cells expressing different BmorPBP-types. In situ hybridization with biotin-labeled anti-sense RNA probes for the three BmorPBP-types were performed on cryosections through the male antenna. Hybridization signals were visualized by detection systems indicating PBP-positive cells by *green fluorescence*. Images are representative for several independent in situ hybridization experiments performed with each probe on antennal sections from at least eight

individuals. **a** BmorPBP1-labeled cells were found regularly below sensilla hairs and are distributed through the complete width of the antenna. On sections carrying cells positive for BmorPBP2 (**b**) and BmorPBP3 (**c**) only one or two cells were labelled. Pictures represent optical sections selected from stacks of confocal images generated by a laser scanning microscope; the fluorescence channel is overlaid with the transmitted-light channel. *Scale bars* 20 μ m

omone-sensitive trichoid sensilla (Steinbrecht et al. 1992). Recently, it was found that the BmorPBP1-cells are closely associated with the neurons expressing the receptors BmOR-1 and BmOR-3, respectively (Nakagawa et al. 2005; Krieger et al. 2005). To determine if cells expressing the novel BmorPBP-subtypes may also be associated with receptor expressing cells, DIG-labeled antisense RNA probes for BmOR-1 were used in double in situ hybridization experiments together with biotin-labeled BmorPBP1 (Fig. 7a). The red-labeled BmOR-1 cell is surrounded by green-labeled BmorPBP1 cells. Optical sectioning at higher magnification (Fig. 7b–e) reveals that green-labeled PBP-expressing cells are surrounding the receptor-expressing cell giving the appearance of a green “cage” around the red receptor cell. Double in situ hybridizations with the BmOR-1/BmorPBP2 or BmOR-1/BmorPBP3 gave a different picture (Fig. 7f, k). Cells expressing BmorPBP2 or BmorPBP3 were clearly separated from the BmOR-1 cells. In most cases this was immediately obvious (Fig. 7f–j) or became apparent in optical sections (Fig. 7l–q). Together these results indicate that cells expressing BmorPBP2 and BmorPBP3 are localized in sensilla different from those containing the BmOR-1 neurons.

Discussion

In addition to the well-characterized pheromone binding protein from *B. mori* (Maida et al. 1993; Krieger et al. 1996; Sandler et al. 2000; Grosse-Wilde et al. 2006), we have identified in this study two novel candidate pheromone binding proteins, BmorPBP2

and BmorPBP3, from this species. Due to its apparently limited complement of pheromone components (bombykol, bombykal), it was thought that *B. mori* may only contain a single PBP-type in contrast to several moth species, including *Antheraea pernyi* and *A. polyphemus* (Krieger et al. 1991; Maida et al. 2000), *Manduca sexta* (Robertson et al. 1999), *Agrotis ipsilon* and *A. segetum* (Abraham et al. 2005), which express multiple PBP-subtypes. The coexistence of several distinct PBP-types in one species suggests a specific role of each PBP in the process of pheromone reception and led to the notion that each subtype may be tuned to a distinct component of the pheromone blend. This view was strongly supported by recent studies demonstrating that BmorPBP1 specifically mediates only a response to bombykol, but not to bombykal, in cells which express the BmOR-1 receptor (Grosse-Wilde et al. 2006), and that in *Drosophila* the response of receptor-type OR67d to a pheromonal component requires the PBP-type LUSH (Xu et al. 2005; Ha and Smith 2006).

The pheromone binding protein family of Lepidoptera represents a group of “olfactory” binding proteins, which is not present in other insect orders, suggesting that the PBP genes may have arisen within the Lepidoptera lineage (Vogt 2003). A sequence identity tree including PBPs from a large variety of species belonging to different lepidopteran families (Fig. 2) categorized nearly all PBPs into one of three distinct groups. The three BmorPBPs, although quite conserved, were found to be more related to PBPs from other species than to each other. Moreover, each of the three *B. mori* PBPs was assigned to one of the three major PBP groups; thus, each is a representative

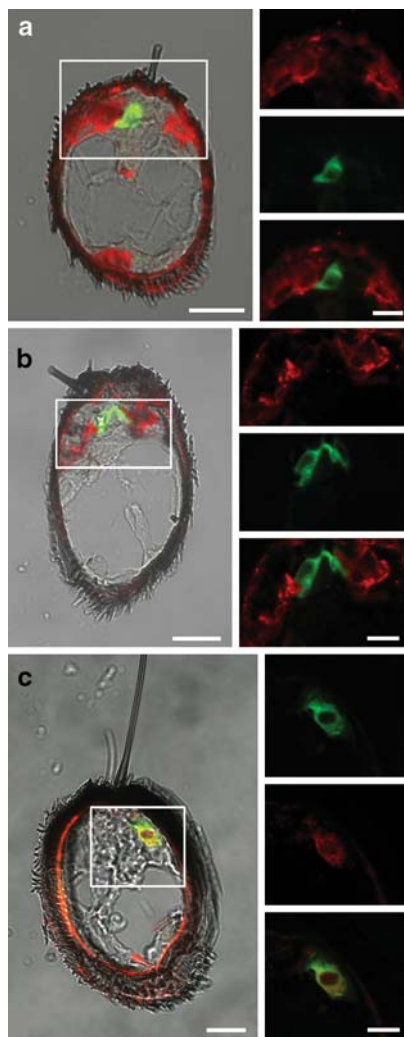


Fig. 6 Analysis of PBP colocalization by two-color in situ hybridization. Hybridization was performed on sections through the male antenna using combinations of DIG- or biotin-labeled antisense RNAs for the three BmorPBPs. Hybridization of probes was visualized by red (DIG-label) or green (biotin-label) fluorescence. In **a**, **b** and **c** images on the left represent pictures taken from a stack of optical sections; the red and green fluorescence channels have been overlaid with the transmitted-light channel. Scale bars 20 μm . Pictures to the right represent higher magnifications of the boxed areas and show the red fluorescence channel, the green fluorescence channel and the overlay of both channels, respectively. Scale bars 10 μm . **a** Hybridization with a combination of BmorPBP1 and BmorPBP2. The red-labeled BmorPBP1 expressing cells are clearly separated from a green-labeled BmorPBP2 cell. **b** Hybridization with the combination BmorPBP1/BmorPBP3 indicating expression of the two PBPs in different cells. **c** Hybridisation with the combination BmorPBP2 (green) and BmorPBP3 (red). A cell labeled by both PBP probes is shown indicating co-expression of the two proteins. Slight diffuse red colour in the periphery of the section results from autofluorescence of the cuticle

for one of the major PBP groups. This result suggests that the three genes for the *B. mori* PBPs coexist throughout lepidopteran evolution and that gene

duplication events, which probably led to the three PBPs in *B. mori*, must have occurred before radiation of the lepidopteran species. Nevertheless, the generation of the BmorPBP gene family via gene duplication is supported by the finding that the three BmorPBPs genes are located in close proximity within the genome. Furthermore, the three BmorPBP genes share the same exon/intron number and conserved exon/intron boundaries (Fig. 3), a feature also observed in PBP genes of other moth species (Vogt et al. 2002; Abraham et al. 2005), emphasising a common evolutionary history of moth PBP genes.

The binding protein BmorPBP1 is present in the long sensilla trichodea (Steinbrecht 1999; Maida et al. 2005), which represent the large majority of sensilla on male antennae. This sensillum type houses a bombykol- as well as a bombykal-responsive neuron (Kaisling et al. 1978), which are supposed to express the receptor-types BmOR-1 or BmOR-3, respectively. Both neurons are surrounded by supporting cells, which express the binding protein BmorPBP1 (Sakurai et al. 2004; Nakagawa et al. 2005; Krieger et al. 2005). The in situ hybridization analysis for BmorPBP2 and BmorPBP3 have shown that the number of cells expressing the novel PBPs as well as the location of these cells do not follow this pattern. So, they are most probably not involved in mediating pheromone responses in the long sensilla trichodea and thus are probably not a specific binding protein for bombykal. Therefore, the molecular identity of a PBP, which mediates the response to bombykal in the long sensillum type remains elusive. Blast analysis of the partial *Bombyx* genome database revealed no evidence for further PBP related genes. However, it is possible that the gene encoding a PBP for bombykal may not yet be in the database. Alternatively, it is conceivable that the homology of a PBP for bombykal is beyond the BLAST detection limit.

Both novel PBPs (BmorPBP2, BmorPBP3) were found to be expressed to about the same extend in the antenna of male and female. Expression of PBP in both sexes has been described for various moth species (Krieger et al. 1993, 1996; Callahan et al. 2000; Picimbon and Gadenne 2002). Although expression of PBPs in female antennae conflicted with the original view that female moths do not respond to their own sex-pheromone (Boeckh et al. 1965; Schweitzer et al. 1976) the discovery of female expression of PBPs has led to the concept that the corresponding sensilla may be responsive to some components of the female released sex-pheromone blend. In fact, autodetection of sex-pheromone components by females has been demonstrated for several moth species (Nesbitt et al.

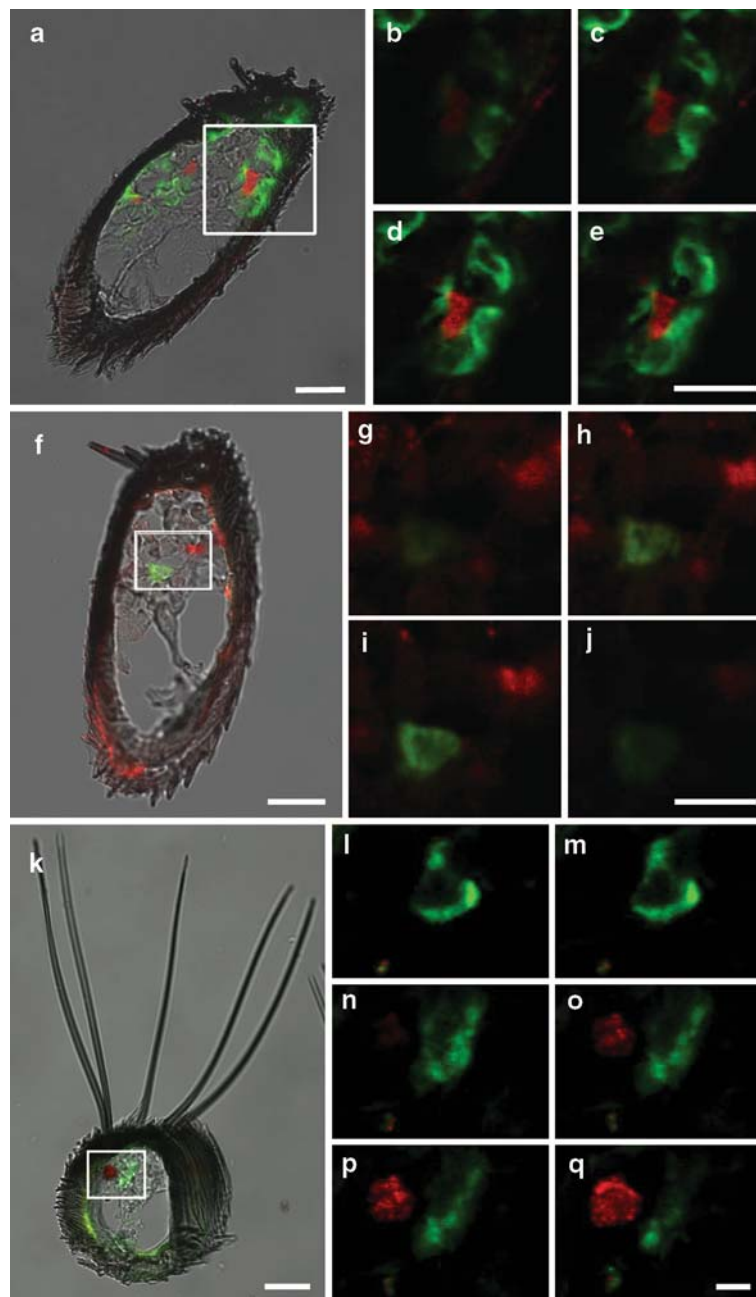


Fig. 7 Expression pattern of *B. mori* PBPs and BmOR-1 in the antenna. Two-color in situ hybridization was performed on sections through the male antenna using combinations of DIG-labeled BmOR-1 and biotin-labeled BmorPBP antisense RNAs. Hybridization signals were visualized by detection systems indicating cells bearing BmOR-1 transcripts by red and PBP-positive cells by green fluorescence. **a–e** Hybridization with a combination of BmorPBP1 and BmOR-1. Green-labeled PBP1 cells are closely associated with the red-labeled BmOR-1 expressing cell on different optical planes, strongly suggesting a location of BmOR-1- and PBP-expressing cells in the same sensillum. In contrast, cells expressing BmorPBP2 or BmorPBP3

are clearly separated from cells expressing BmOR-1. **f–j** Hybridization with a combination of BmOR-1 and BmorPBP2. **k–q** Hybridization with a combination of BmOR-1 and BmorPBP3. **a, f** and **k** Single pictures taken from a stack of optical sections; an overlay of the red and green fluorescence channels and the transmitted-light channel is shown. Higher magnifications of the areas boxed are shown on the right; these images only show the red and green fluorescence channel and represent selected optical planes taken from a stack of optical sections. Scale bars 20 μm in **a, f** and **k**; 10 μm in **b–e** and **g–j**; 5 μm in **l–q**

1973; Schneider et al. 1998; Hillier et al. 2005) and it has been proposed that autodetection of sex-pheromone components may allow the female moths to monitor their pheromone release through an antennal feedback system. Indeed, it has been found that a subset of sensilla on female antennae responds in a specific manner to at least one component of the sex-pheromone blend (Hillier et al. 2005).

Although the functional implications of the novel PBPs remain unclear, the low number of cells expressing BmorPBP2 and BmorPBP3 as well as their location closer to the midline in a cross section (Fig. 5) resembles the low number and location of the so-called medium-sized sensilla trichodea on the *Bombyx* antenna (Steinbrecht 1970, 1973); moreover the topographic expression pattern also resembles the distribution of cells expressing hitherto uncharacterized candidate pheromone receptors, such as BmOR-4 and BmOR-5 (Krieger et al. 2005). The similarity in number and distribution of the BmorPBP2 and BmorPBP3 cells to that of BmOR-4 and BmOR-5 cells suggests that they may be co-localized in the same sensillum. It is possible that they could co-operate in the reception of probably yet unknown pheromone components of *B. mori*. Although a role in detecting odors unrelated to pheromones cannot be excluded for the novel PBPs, the conservation of the PBP gene family and the uniqueness of the PBP lineage to Lepidoptera (Vogt et al. 2002), as well as the topographic expression patterns of PBPs, argue for an important and specific role in the detection of highly relevant chemical signals.

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