J Chem Ecol (2006) 32: 1703–1717
DOI 10.1007/s10886-006-9103-2

Molecular Cloning and *in Situ* Expression Patterns of Two New Pheromone-Binding Proteins from the Corn Stemborer *Sesamia nonagrioides*

Federica de Santis · Marie-Christine François · Christine Merlin · Julien Pelletier · Martine Maïbèche-Coisné · Eric Conti · Emmanuelle Jacquin-Joly

Received: 28 July 2005 / Revised: 7 December 2005 /
Accepted: 24 February 2006 / Published online: 2 August 2006

Abstract We describe the identification and characterization of two new cDNAs encoding pheromone-binding proteins (PBPs) from the male antennae of *Sesamia nonagrioides*, a species where no PBPs have been identified to date. Because PBPs are thought to participate in the first step of odor detection in a specific manner, we focused our investigation on this olfactory protein family using reverse transcription–polymerase chain reaction strategies. The deduced amino acid sequences of SnonPBP1 and SnonPBP2 revealed mature proteins of 142 and 143 amino acids, respectively, with six cysteine residues in conserved positions relative to other known PBPs. The alignment of the two mature *S. nonagrioides* PBPs with other noctuid PBPs showed high sequence identity (70–80%) with other full-length sequences from GenBank. Sequence identity between SnonPBP1 and SnonPBP2 was only 46%, suggesting that the two proteins belong to different classes of PBPs already described from the Noctuidae. Furthermore, analyses of expression patterns of SnonPBP1 and SnonPBP2 were performed by *in situ* hybridization on antennae of both sexes, and these studies revealed the expression of the two PBPs at the bases of olfactory sensilla (basiconica or trichodea) from both sexes. The possible binding properties of these two new PBPs are discussed according to their homologies with other known PBPs and *S. nonagrioides* pheromone components.

Keywords *Sesamia nonagrioides* · Noctuidae · (*Z*)-11-hexadecenyl acetate · Pheromone-binding proteins · Molecular cloning · *In situ* hybridization · RT-PCR · Stemborer

F. de Santis · M.-C. François · C. Merlin · J. Pelletier · M. Maïbèche-Coisné · E. Jacquin-Joly (✉)
UMR UPMC-INRA-INAPG «Physiologie de l'insecte: signalisation et communication»,
Centre INRA, Route de Saint-Cyr, 78026 Versailles Cedex, France
e-mail: jacquin@versailles.inra.fr

E. Conti
DSAA-Entomologia, Università degli Studi di Perugia, Borgo XX Giugno, 06121 Perugia, Italy

Present address:
F. de Santis
DSAA-Entomologia, Università degli Studi di Perugia, Borgo XX Giugno, 06121 Perugia, Italy

Introduction

In insect antennae, odorant-binding proteins (OBPs) are soluble proteins that are thought to participate in the first step of olfactory detection by specific binding with volatile molecules. Well known in insects, OBPs are proposed to be specialized in solubilization, binding, and transport of odorants across the aqueous lymph of the olfactory sensilla (Vogt and Riddiford, 1981), allowing hydrophobic molecules to reach the membrane olfactory receptors. In addition, these proteins may also participate in dendritic odorant receptor activation or odorant signal deactivation (Kaissling, 2004). They are small hydrophilic proteins synthesized by accessory cells that surround the olfactory neuron and are secreted into the sensillar lymph where they are found at high concentrations. OBPs have been identified in a variety of insect species and appear to be present throughout the Neoptera (Vogt et al., 1999; Vogt, 2003). They are divided in two groups according to their potential ligands: the pheromone-binding proteins (PBPs) are thought to bind pheromone components, whereas the general odorant-binding proteins (GOBPs) are believed to bind more "general" odorants such as those from plants. Although the designations PBP/GOBP are used to distinguish two lineages of OBPs with high sequence similarity within the Lepidoptera, this designation is also used across all insects to distinguish functional classes of OBPs based on their probable ligands.

Direct cloning and genomic analysis with insects have revealed the occurrence of multiple OBPs within the same species. For example, up to 50 OBP-related genes were identified in the fruit fly *Drosophila melanogaster* and a similar number in the mosquito *Anopheles gambiae* (reviewed in Vogt, 2003). Within OBPs, multiple PBPs are also expressed in a single species. Indeed, three PBPs are known in the silk moths, *Antheraea polyphemus* and *A. pernyi* (Saturniidae) (Raming et al., 1990; Krieger et al., 1991; Vogt et al., 1991; Maida et al., 2000) and the hawkmoth, *Manduca sexta* (Sphingidae) (Györgyi et al., 1988; Robertson et al., 1999), whereas two PBPs are known in different armyworm moths, e.g., *Mamestra brassicae* (Maïbèche-Coisné et al., 1998) and *Heliothis virescens* (Noctuidae) (Krieger et al., 1993; Abraham et al., 2003; direct GenBank submission no. AY301988). The presence of different subtypes of PBPs in the same species, together with the occurrence of different components in the sex pheromone, suggest a possible role of PBPs in specific binding/transport of pheromone components to the receptor neuron. PBPs could then contribute to the specificity of the olfactory system through an initial selective detection of odorant molecules (Leal, 2003). However, only a few studies have provided data suggesting some binding specificity between PBPs and pheromone components. For example, two different *M. brassicae* PBPs showed different binding affinities to the main pheromone component of this moth, (*Z*)-11-hexadecenyl acetate [(*Z*)-11-16:Ac] with PBP1, but not PBP2, binding the compound (Maïbèche-Coisné et al., 1997), although it may not be the only compound that PBP1 could bind (Campanacci et al., 2001). Similar results were obtained in *Lymantria dispar* (Vogt et al., 1989; Plettner et al., 2000), *A. pernyi* (Prestwich et al., 1995), and *A. polyphemus* (Du and Prestwich, 1995). In addition, *Bombyx mori* PBP showed binding to the pheromone component bombykol, but not to structurally unrelated chemicals (Wojtasek and Leal, 1999). In a competitive situation, each of the three *A. polyphemus* PBPs preferentially binds one of the pheromone components (Maida et al., 2000, 2003), and responses of the receptor neurons to pheromone appeared to depend on both the pheromone component and the type of PBPs (Pophof, 2002, 2004). Therefore, the PBPs appeared to contribute to the excitation of the receptor cells (Pophof, 2004).

The stemborer *Sesamia nonagrioides* (Lefebvre) (Lepidoptera: Noctuidae), is an important pest of maize in the Mediterranean Basin. Integrated pest management strategies

are being developed against this species, including olfactory-mediated behavioral modification centered on the sex pheromone. This pheromone consists of a blend of (*Z*)-11-16:Ac (main component), (*Z*)-11-16:OH, (*Z*)-11-16:Ald, and 12:Ac (Mazomenos, 1989). Because no OBP have been described to date in *S. nonagrioides*, we investigated its PBP repertoire by polymerase chain reaction (PCR) strategies and compared the PBPs with previously reported sequences from other noctuid moths. This homology-based approach allowed us to describe two new PBPs from antennae of male *S. nonagrioides*, which are expressed in both male and female olfactory tissues.

Methods and Materials

Insect Rearing

A culture of *S. nonagrioides* was established from pupae collected in central Italy and reared with a meridic diet (Giacometti, 1995). Larvae, sexed pupae, and adults were kept inside screened plastic containers in an environmental chamber (27 ± 1°C, 60–80% relative humidity, 16-hr light/8-hr dark photoperiod).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 200 antennae from sexually mature *S. nonagrioides* males by using an Rneasy® MiniKit (Qiagen, Hilden, Germany). Single-stranded 3′RACE-ready-cDNA and 5′RACE-ready-cDNA were synthesized from 1 μg of RNA with the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with 200 U of SuperscriptII™ (Gibco BRL, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Degenerate PCR for Isolation of PBP1 and PBP2 Gene Fragments

Degenerate primers corresponding to amino acid consensus regions of several PBPs from different species of Lepidoptera were used for PCR amplifications of *S. nonagrioides* cDNA templates: PBP1-forward (5′-GAYCAGTGYAAGAAYGAGCTTAAT-3′) and PBP1-reverse (5′-GTGAGGGATCCRTGYTTYWTNGCRAA-3′); PBP2-forward (5′-ATGAAYT TYGCYAAACCWYTA-3′) and PBP2-reverse (5′-ACTCCTGAGCCTTCCCGTGATGCA-3′). These primers have already been used successfully for the molecular cloning of PBP1 and PBP2 from *M. brassicae*, a species that also uses (*Z*)-11-16:Ac as its main pheromone component (Maïbèche-Coisné et al., 1998). PCR was carried out with 1 U of Taq polymerase (Promega, Madison, WI, USA) and consisted of 40 cycles of 30 sec at 94°C, 30 sec at 40 and 50°C for PBP1 and PBP2, respectively, and 30 sec at 72°C.

3′RACE-PCR

3′RACE amplifications were conducted on 2 μl of the *S. nonagrioides* 3′RACE-ready cDNA, with sense gene-specific primers deduced from the sequences obtained following degenerate PCR amplifications of PBP1 and PBP2 (PBP1Sn-forward: 5′-CTGGCGCGAG GAGTACGAGCTGGT-3′; PBP2Sn-forward: 5′-TGTGCCATACTCTGTCTCTCATCT-3′) and with an antisense Universal Primer Mix (UPM, Clontech). The 50-μl amplification mix was prepared according to the SMART™ RACE cDNA protocol using the Advantage2™

polymerase mix (Clontech). Touchdown PCRs were performed in the following conditions: 1 min at 94°C, followed by 5 cycles of 30 sec at 94°C and 1 min at 72°C; 5 cycles of 30 sec at 94°C, 30 sec at 70°C, and 1 min at 72°C; 30 cycles of 30 sec at 94°C, 30 sec at 68°C, and 1 min at 72°C.

5′RACE-PCR

5′RACE amplifications were conducted on 2 μl of the *S. nonagrioides* 5′RACE-ready cDNA using Universal Primer Mix (Clontech) as a sense primer and antisense gene-specific primers (PBP1Sn-reverse: 5′-GCACATCACCATGCATCCCAGGTCGCG-3′; PBP2Sn-reverse: 5′-CTTAAGGTCTGGATCGAGCAGTTCCAG-3′). Touchdown PCRs were performed as described for the 3′RACE-PCR.

Cloning and Sequencing

After gel extraction (Genelute, Sigma, St. Louis, MO, USA), the amplified cDNAs were ligated into the plasmid pCR™-II using the TOPO cloning kit from Invitrogen. Recombinant plasmids were isolated using the Plasmid Mini kit (Qiagen), and both strands were subjected to automated sequencing by Genome Express (Grenoble, France). Database searches were performed with the BLAST program (NCBI), protein analyses with MWCALC (Infobiogen) and SignalP (Nielsen et al., 1997), and sequence alignment with CLUSTALW (NPS@IBCP).

In Situ Hybridizations

Digoxigenin-labeled RNA sense and antisense probes (200 bp) were obtained for each PBP. The pCR™II-cDNA plasmids resulting from degenerate PCR amplifications of the internal gene fragments of PBP1 and PBP2 were PCR amplified by using M13 forward and reverse primers. The PCR products were used to generate digoxigenin-labeled RNA probes by *in vitro* transcription using T7 and SP6 RNA polymerase (Promega) following the manufacturer's protocol and in the presence of 1.5 U of Rnasin (Promega).

For hybridization, antennae were removed from the heads of adults of both sexes, cut into pieces, and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Fixed tissues were dehydrated in 100% methanol. The hybridization protocol was performed as described (Jacquin-Joly et al., 2000), with a hybridization temperature of 65°C, and digoxigenin was detected using alkaline–phosphatase-conjugated antidigoxigenin Ig (1:4000; Roche, Basel, Switzerland) and revealed colorimetrically with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate, toluidine salt (Roche). After sufficient staining, specimens were washed in PBS and fixed in 4% PFA, dehydrated, and embedded in Epon (Agar Scientific, Stansted, Essex, UK). Six-micrometer sections were counterstained with acridine orange. Sections were photographed, and pictures were digitized and processed with Adobe Photoshop 5.0.

Phylogenetic Analysis with Other Noctuid PBPs

Several noctuid PBP sequences were retrieved from GenBank for phylogenetic analysis. Amino acid sequences of PBPs without the leader sequence were aligned by using Multalin program (Corpet, 1988). Maximum parsimony was used to build a strict consensus tree in PAUP 4.0b10 software (Sinauer Associates, Sunderland, MA, USA). Branch support was

assessed by bootstrap analysis based on 1000 replications, and the *H. virescens* GOBP1 and 2 (GenBank accession nos. X96862 and X96863, respectively) were used as outgroups.

Results

Molecular Cloning and cDNA Sequencing of *S. nonagrioides PBPs*

By using degenerate PCR and RACE-PCR strategies, two full-length cDNAs, encoding potential PBPs, were cloned in *S. nonagrioides*. They both contained an open-reading

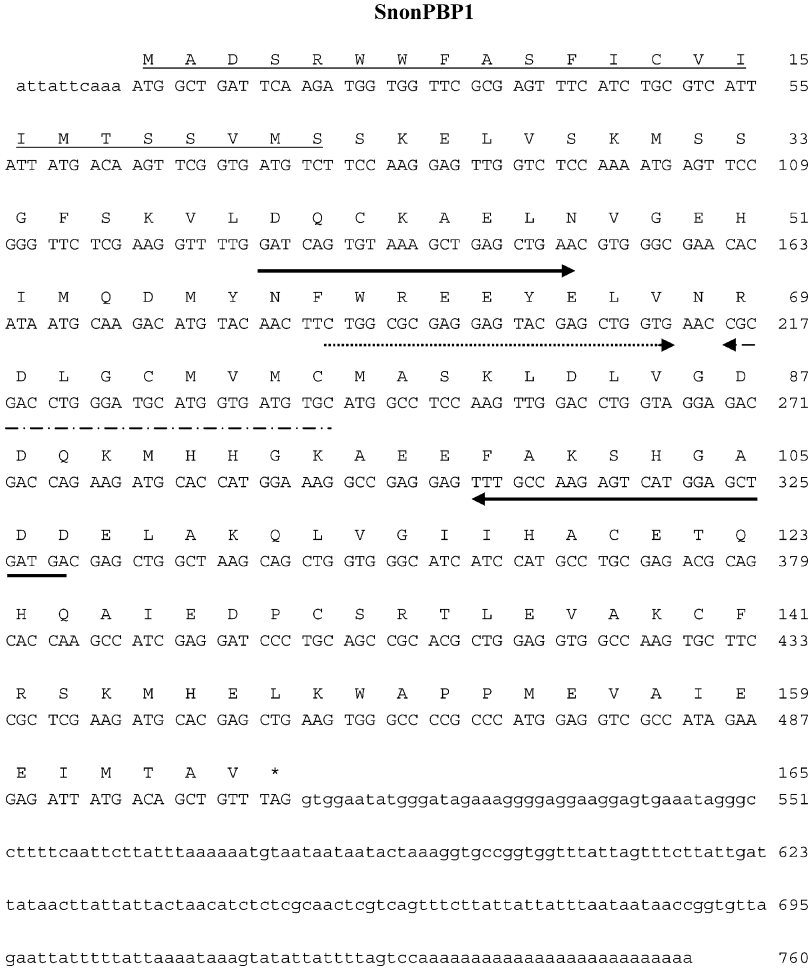

Fig. 1 Nucleotide and deduced amino acid sequences of SnonPBP1 and SnonPBP2 cDNAs from antennae of male *Sesamia nonagrioides* (GenBank accession nos. AY485219 and AY485220). The stop codons are indicated by an asterisk. The positions of the degenerate primers used in PCR for gene fragment amplifications are underlined with a bold line (PBP1-forward and -reverse; PBP2-forward and -reverse), whereas gene-specific primers for 3′RACE (PBP1Sn-forward and PBP2Sn-forward) and 5′RACE (PBP1Sn-reverse and PBP2Sn-reverse) are marked with dotted and dashed lines, respectively. Putative signal peptides at the N-termini are underlined

Snon PBP2

```
     M   A   L   H   R   S   P   I   M   S   A   R   L   A   L   V   L      17
aca ATG GCG CTG CAT CGA TCG CCC ATC ATG TCG GCA CGC TTG GCG CTG GTA CTG      54

 I   A   S   L   F   I   V   V   K   C   S   Q   E   V   M   K   N   L      35
ATC GCC AGT CTG TTC ATC GTC GTG AAA TGT TCT CAA GAA GTC ATG AAG AAT CTG     108

 T   H   H   F   S   K   P   L   E   D   C   K   K   E   M   D   L   P      53
ACC CAT CAT TTC TCT AAG CCT TTG GAA GAC TGT AAG AAG GAG ATG GAC CTC CCG     162

 D   S   V   I   T   D   F   Y   N   F   W   K   E   G   Y   E   F   T      71
GAC TCA GTG ATC ACA GAT TTC TAC AAT TTC TGG AAA GAA GGC TAC GAG TTC ACG     216

 S   R   H   T   G   C   A   I   L   C   L   S   S   K   L   E   L   L      89
AGC AGA CAT ACA GGC TGT GCC ATA CTC TGC CTC TCA TCT AAG CTG GAA CTG CTC     270

 D   P   D   L   K   L   H   H   G   K   A   Q   E   F   A   Q   K   H     107
GAT CCA GAC CTT AAG TTG CAT CAT GGA AAG GCG CAG GAG TTC GCG CAG AAA CAT     324

 G   A   D   E   A   M   A   K   Q   L   V   G   L   I   H   G   C   M     125
GGC GCT GAC GAG GCC ATG GCG AAG CAG CTG GTA GGC CTG ATC CAC GGC TGT ATG     378

 E   T   I   R   E   P   A   D   D   P   C   V   R   A   Q   N   V   V     143
GAG ACA ATC CGC GAA CCG GCC GAC GAC CCC TGC GTG AGG GCT CAG AAC GTA GTC     432

 M   C   F   K   A   K   I   H   E   L   X   W   A   P   S   L   D   L     161
ATG TGC TTC AAG GCC AAG ATA CAT GAG CTG ANC TGG GCG CCT AGC TTG GAC CTC     486

 I   V   G   E   V   L   A   E   V   *                                     171
ATC GTG GGA GAA GTC TTG GCT GAA GTC TAG  catgatgcccttggttccgtgatatacctttt   547

atcttctcttcgtcatagaaggccatcattgcatgtgatagtgatgttgttgttttgaatgcaaaacatagt   619

ttcatctttttcatttgttttgcttgagtgttttcagctagagacattacgtaaatcaaagtctttttttatc   691

aaatatcattctctgttaagaaaccaataaccagtgctcagacaacattaatgttatgtgcggttgtaatgt   763

aatgcaatgcttatgacctgcaggaataaatgcaaataagtttatatctacattacattatgtttatacatt   835

acagtacattgtgttatacaagtctgatttgtttctatctctactttaacgacaaggcttgttcaatggact   907

acagatatttctacagttagttatttgattaatatttaataattcttgtgaagagtctcctgtctcgccagt   979

tctcatcaaagggagtgggtacattgtaagttgcaagttctggatgtcatattaataaagaatacatcttta  1051

caaaaaaaaaaaaaaaaaaaaaaaaaaa                                            1080
```

Fig. 1 (continued)

frame encoding two putative proteins of 165 and 170 amino acids, named SnonPBP1 and SnonPBP2, respectively, according to their high identity with proteins from these classes in BLAST searches. The sequences for SnonPBP1 and SnonPBP2 have been deposited in GenBank under the accession numbers AY485219 and AY485220, respectively. The deduced amino acid sequences for both proteins possess a putative signal sequence of 23 (SnonPBP1) and 27 (SnonPBP2) amino acids (Fig. 1). Mature predicted SnonPBP1 protein consisted of 142 amino acids with a molecular mass of 16,117 Da and a pI of 5.49, whereas SnonPBP2 consisted of 143 amino acids with a molecular mass of 16,039 Da and a pI of 5.00. Thus, the two predicted mature proteins are small, likely soluble, likely secreted, and both have an acidic isoelectric point. Moreover, both contained six cysteines in conserved positions when aligned with other known PBPs: positions C^{19}, C^{50}, C^{54}, C^{97}, C^{108}, and C^{117} (Fig. 2).

The alignment of the two mature *S. nonagrioides* PBPs with other noctuid PBPs showed high sequence identity. *S. nonagrioides* PBP1 showed 83% identity with *M. brassicae* PBP1 (MbraPBP1), 72% with *Spodoptera exigua* PBP1 (SexiPBP1), and 88% with the partial sequence of *H. virescens* PBP2 (HvirPBP2; Fig. 3). Similarities were even higher,

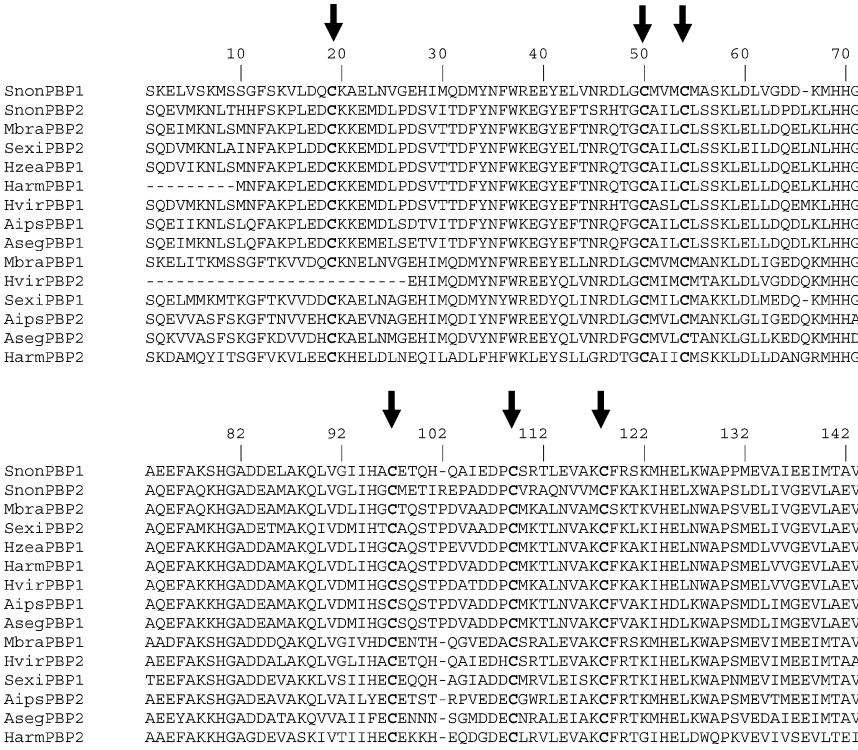

Fig. 2 Comparison of the predicted amino acid sequences of SnonPBP1 and SnonPBP2 from antennae of male *S. nonagrioides* with pheromone-binding proteins from other noctuids. The six conserved cysteines are in bold and marked by arrows. GenBank accession numbers—SnonPBP1: AY485219, SnonPBP2: AY485220 (*S. nonagrioides*); MbraPBP1: AF051143, MbraPBP2: AF051142 (*Mamestra brassicae*); SexiPBP1: AY743351, SexiPBP2: AY743352 (*Spodoptera exigua*); HzeaPBP1: AF090191 (*Helicoverpa zea*); HarmPBP1: AJ278992, HarmPBP2: AF527054 (*Heliothis armigera*); HvirPBP1: X96861, HvirPBP2: AY301988 (*Heliothis virescens*); AipsPBP1: AY301985, AipsPBP2: AY301986 (*Agrotis ipsilon*); and AsegPBP1: AF134253, AsegPBP2: AY301987 (*Agrotis segetum*)

Snon PBP1														
83	Mbra PBP1													
88	**80**	Hvir PBP2												
70	**72**	**75**	Aips PBP2											
67	**67**	**68**	**77**	Aseg PBP2										
72	**71**	**76**	**70**	**65**	Sexi PBP1									
46	44	49	42	42	46	Snon PBP2								
45	44	49	45	43	45	**80**	Mbra PBP2							
48	46	51	46	46	46	**78**	**87**	Hvir PBP1						
48	46	52	46	45	45	**79**	**88**	**92**	Hzea PBP1					
49	47	53	48	45	47	**77**	**83**	**83**	**86**	Aips PBP1				
49	47	54	48	46	49	**77**	**83**	**83**	**84**	**97**	Aseg PBP1			
50	47	53	46	46	48	**79**	**92**	**94**	**98**	**87**	**86**	Harm PBP1		
45	42	48	42	41	45	**73**	**85**	**84**	**86**	**80**	**80**	**89**	Sexi PBP2	
51	50	53	44	46	53	45	46	47	46	43	44	49	47	Harm PBP2

Fig. 3 Percentage identity of pheromone-binding proteins (PBPs) of the Noctuidae based on conserved amino acid residues. Sequences were retrieved from GenBank (accession numbers listed in Fig. 2), and signal peptides were deleted for this comparative analysis. Identities greater than 55% are in bold. Two different groups of PBPs are evident according to level of identity: Grp1 (names not bold) and Grp2 (names in bold), as defined by Picimbon and Gadenne (2002)

e.g., 95% with MbraPBP1. SnonPBP2 presented the highest identities with MbraPBP2 (80%), HzeaPBP1 (79%), and HarmPBP1 (79%). The degree of identity between SnonPBP1 and SnonPBP2 (46%) was much lower than in the interspecific comparisons within a protein (Fig. 3). Sequence similarities of noctuid PBPs were summarized in a neighbor-joining tree (Fig. 4). This analysis revealed two clusters of PBPs from various species, designated as Grp1 and Grp2, according to the two PBP groups already described in noctuids (Picimbon and Gadenne, 2002). Grp2 contained SnonPBP1, whereas Grp1 contained SnonPBP2 (Fig. 4).

PBP Expression Patterns in *S. nonagrioides*

Expression patterns of the two *S. nonagrioides* PBPs were established by *in situ* hybridization to RNA in both male and female antennae (Figs. 5 and 6). *S. nonagrioides* antennae contain about 40 flagellomeres. In males, they are comb-like (bipectinate) and 7.20 mm long and 0.70 mm wide at mid-length (Fig. 5A), whereas in females, they are filiform and 6.60 mm long and 0.15 mm wide at mid-length (Fig. 6A). In both sexes, the

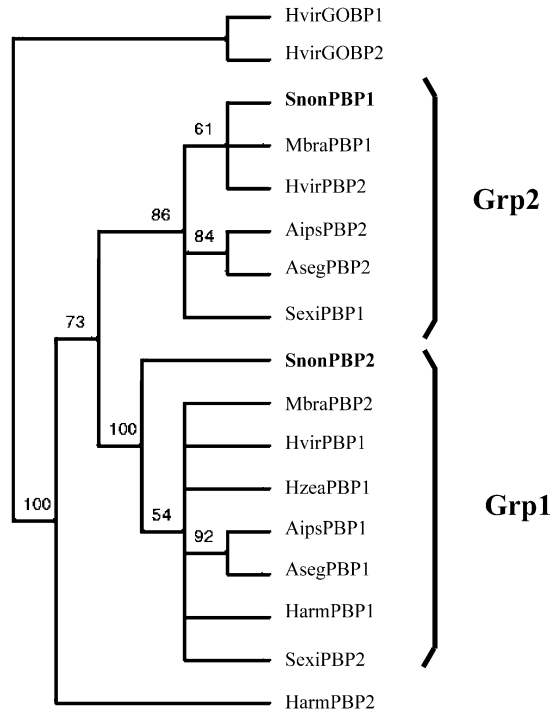

Fig. 4 Phylogenetic analysis of *S. nonagrioides* SnonPBP amino acid sequences with various noctuid PBPs. GenBank accession numbers are listed in Fig. 2. Bootstrap support values (in percent) based on 1000 replicates are indicated. Analysis indicates two groups (Grp1 and Grp2), as defined by Picimbon and Gadenne (2002)

antennae are covered dorsally with overlapping scales. The olfactory hairs, the sensilla trichodea and the sensilla basiconica, are all set on the ventral side of the flagellomere. In males, the olfactory sensilla are distributed across both portions of the lateral processes and in the medial process (Fig. 5A). In particular, the branches of the male antennae carry a great number of long sensilla trichodea, which have been characterized functionally by single cell recording (Quero et al., 2004). Although the number and the distribution of sensilla are quite different in females and males, both sexes share the same types of sensilla (Solinas and Trona, 2002).

Hybridization of male antennae with either SnonPBP1 or SnonPBP2 sense probes showed no signal (data not shown). Hybridization with either SnonPBP1 (Fig. 5B–D) or SnonPBP2 (Fig. 5E and F) antisense probes showed signals restricted to the sensillar side of the antennae (Fig. 5B), with labeling present in both lateral and medial processes (Fig. 5E). No labeling was visible on the dorsal-scaled side of the antennae. On the sensillar side, labeling was observed at the base of olfactory sensilla (Fig. 5C, D, and F), with no labeling at the bases of the sensilla chaetica (Fig. 5D), which are thought to function in gustation in other Lepidoptera (Jørgensen et al., 2006). In some sections (Figs. 5B, C, E, 6B and C), labeling could be clearly associated with numerous long sensilla trichodea. However, it was not possible to exclude expression in sensilla basiconica that differs from sensilla trichodea by their small size because a whole sensillum was rarely visible on a section. In longitudinal sections of female antennae, *in situ* hybridization revealed that both PBP1 and PBP2 are also abundantly expressed in this sex, with labeling observed at the base of the olfactory sensilla with both probes (Fig. 6B and C).

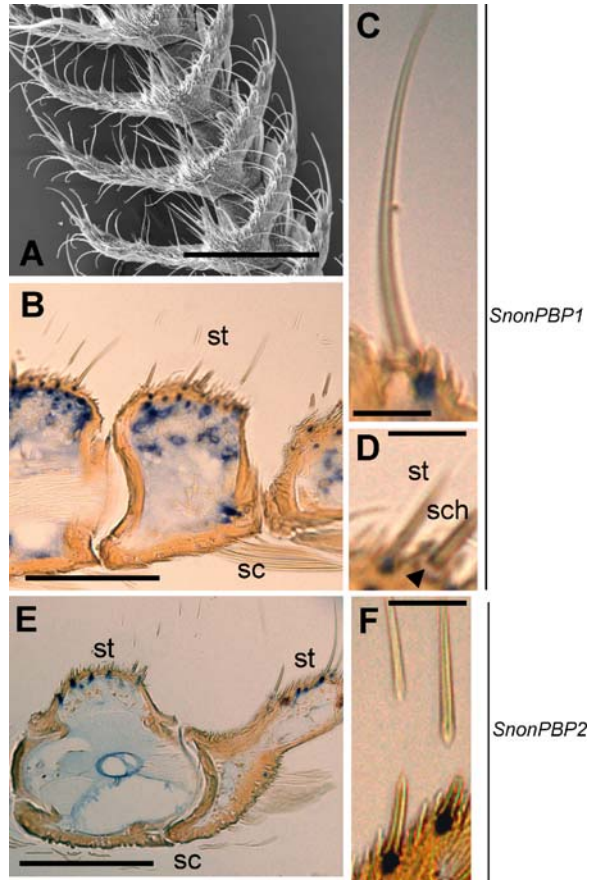

Fig. 5 Expression patterns of SnonPBP1 and SnonPBP2 revealed by *in situ* hybridization to mRNA in longitudinal and transversal sections of *S. nonagrioides* male antennae. (A) SEM of the ventral side of a male antenna. (B) Longitudinal section showing SnonPBP1 labeling on the sensilla side of the antenna. (C) SnonPBP1 labeling at the base of a sensillum trichodeum. (D) Magnification of (B) showing labeling at the base of a sensillum trichodeum with no labeling associated with a sensillum chaeticum (arrow). (E) Transversal section showing SnonPBP2 labeling on the sensilla side of the antenna. (F) SnonPBP2 expression at the base of sensilla trichodea. sc: scales; st: sensilla trichodea; sch: sensilla chaetica. Bars: 200 μm (A); 100 μm (B, E); 25 μm (C, D, F)

Discussion

Heterogeneity of PBPs Expressed in *S. nonagrioides* Antennae

In *S. nonagrioides*, no OBPs had been described prior to our study. In this species, the responses of the olfactory receptor neurons have been recently investigated, defining functional types of long sensilla trichodea distributed on the lateral branches of male antenna (Quero et al., 2004). Deciphering the PBP repertoire in this species provides the opportunity to relate PBP expression with the functional types of sensilla. Here, we have cloned two cDNAs encoding proteins that can be classified in the lepidopteran PBP family. Indeed, both of the deduced amino acid sequences present all of the typical features of OBPs: (1) they are small hydrophilic proteins with acidic isoelectric points; (2) they are destined for secretion, as demonstrated by the occurrence of a signal peptide at the N-termini; (3) they possess six cysteines in positions conserved across OBPs; and (4) their expression is associated with the olfactory sensilla, as revealed by *in situ* hybridization. The two deduced proteins are grouped in the PBP1 and PBP2 families, according to sequence homologies with noctuid PBPs and phylogenetic analyses, and thus have been named SnonPBP1 and SnonPBP2. With only 46% identity, these two PBPs clearly represent divergent proteins.

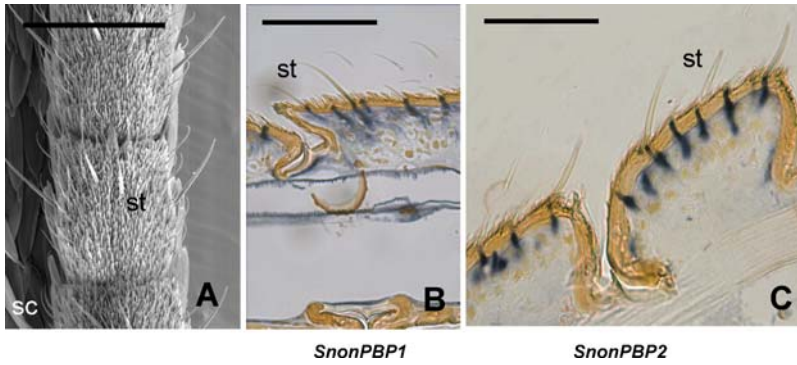

Fig. 6 Expression patterns of SnonPBP1 and SnonPBP2 revealed by *in situ* hybridization to mRNA in longitudinal sections of *S. nonagrioides* female antennae. (A) SEM of the ventral side of a female antenna. (B) Longitudinal section of female antenna with SnonPBP1 expression pattern. (C) Longitudinal section of female antenna with SnonPBP2 expression pattern. st: sensilla trichodea; sc: scales. Bars: 100 μm (A); 50 μm (B, C)

With the discovery of at least two PBPs in its antennae, *S. nonagrioides* is another example of a moth species expressing multiple PBP-like proteins (Györgyi et al., 1988; Raming et al., 1990; Krieger et al., 1991; Vogt et al., 1991; Maïbèche-Coisné et al., 1998; Robertson et al., 1999; Maida et al., 2000). In particular, noctuid species tend to have PBPs belonging to both the PBP1 and the PBP2 families. Because lepidopteran pheromone blends usually consist of several components, the occurrence of divergent PBPs within a species accounts for a possible role of PBP in specific binding. In particular, *M. brassicae* PBP1 has been shown to bind (*Z*)-11-16:Ac (Maïbèche-Coisné et al., 1997). As *S. nonagrioides* and *M. brassicae* share this main pheromone component, SnonPBP1 could be the ortholog of MbraPBP1, considering that these proteins share 83% identity. This hypothesis is supported by the establishment of the PBP1 expression pattern in correlation with the functional types of the olfactory sensilla. Indeed, PBP1 appeared to be expressed in association with numerous sensilla distributed on the lateral branches of the male antennae (Fig. 5B). These branches have been shown to carry different functional sensilla types (Quero et al., 2004), and among them, 90% containing at least one olfactory neuron responding to (*Z*)-11-16:Ac.

Because the sex pheromone of *S. nonagrioides* consists of several different components, SnonPBP2 may bind one or more of these other components. In particular, *S. nonagrioides* female pheromone also contains (*Z*)-11-16:Ald (Mazomenos, 1989), another common pheromone component between *M. brassicae* and *S. nonagrioides* (Renou and Lucas, 1994), which is detected by *S. nonagrioides* olfactory neurons housed in 48% of the sensilla trichodea located on the branches of male antennae (Quero et al., 2004). High conservation between SnonPBP2 and MbraPBP2 (80% identity), together with PBP2s from other noctuids that also use (*Z*)-11-16:Ald in their pheromone blend (*H. virescens*, *H. zea*, and *H. armigera*; see the Pherolist: http://www-pherolist.slu.se/pherolist.php), further supports the hypothesis that these PBP2s could be involved in (*Z*)-11-16:Ald binding, although no functional data are available on either SnonPBP1 or SnonPBP2. The correlation between PBP sequence identities and pheromone components has already been investigated, but there is no evidence for convergence of PBP sequences in species that use similar pheromone components (Willett, 2000a). However, when alleles of PBPs were sampled from members of the tortrix moth genus *Choristoneura* (Tortricidae) that use

pheromones with different functional groups, evidence for episodes of selection acting on PBPs was demonstrated (Willett, 2000b). However, it is not clear whether pheromone changes are causing this selection.

Through our neighbor-joining tree analysis (Fig. 4), the two newly identified SnonPBPs were easy to classify in the two previously defined groups of PBPs (Picimbon and Gadenne, 2002; Abraham et al., 2005). Indeed, SnonPBPs segregate into two subclasses. Subclass 1 (Grp1) contains PBPs reported only in noctuid species (Picimbon, 2003) and includes SnonPBP2, HzeaPBP1, HvirPBP1, AsegPBP1, and AipsPBP1. Subclass 2 (Grp2) includes SnonPBP1 together with MbraPBP1, HvirPBP2, AipsPBP2, AsegPBP2, and SexiPBP1, as well as non-noctuid PBPs (Picimbon, 2003). The classification of the two SnonPBPs in the two subgroups confirms the occurrence of two orthologous groups of PBPs in noctuids.

The Two New *S. nonagrioides* PBPs are Expressed in Male and Female Antennae

From our *in situ* hybridization results, we showed that SnonPBP1 and SnonPBP2 were abundantly expressed in both male and female antennae. Moreover, the expression is associated with the olfactory sensilla in both sexes, with no labeling associated with gustatory sensilla (Fig. 5), consistent with a role in olfaction.

Considering their role in pheromone detection, PBPs were first thought to be expressed exclusively in male antennae (Vogt and Riddiford, 1981), in contrast to GOBPs, which were present in both sexes. We now know from several examples that PBPs are expressed in antennae of both sexes (Györgyi et al., 1988; Maïbèche-Coisné et al., 1997; Callahan et al., 2000; Vogt et al., 2002), but with differences according to the insect family. In the Saturniidae, Bombycidae, and Sphingidae, PBPs are present in much higher levels in males than in females (e.g., Györgyi et al., 1988; Steinbrecht et al., 1992, 1995; Vogt et al., 2002), whereas in the Noctuidae, relatively high expression has been observed in female antennae (Maïbèche-Coisné et al., 1997; Callahan et al., 2000; Zhang et al., 2001). Our results on PBP expression in *S. nonagrioides* female antennae are consistent with these previous observations from noctuid moths. However, the function of PBPs in females remains unclear. Although many studies have failed to detect female response to sex pheromone (reviewed in Callahan et al., 2000), electrophysiological recordings from female antennae showed that *Spodoptera littoralis* as well as *M. sexta* females could respond to at least one of their pheromone components (Ljungberg et al., 1993; Kalinova et al., 2001). The behavioral significance of female PBPs and/or female detection of their own pheromone remains unknown.

The molecular characterization of SnonPBP1 and SnonPBP2 are significant not only because olfactory-mediated behavioral modification of the pest is used in integrated pest management strategies, but also because *Telenomus busseolae* Gahan (Hymenoptera: Scelionidae), an egg parasitoid, is being used effectively in crop protection against *S. nonagrioides*. Possible explanations for such efficacy is that *T. busseolae* uses the *S. nonagrioides* sex pheromone component (*Z*)-11-16:Ac as a host location kairomone and has developed morphofunctional adaptations, behavioral strategies (Conti and Bin, 2000), as well as molecular adaptations of odorant detection. Indeed, like other parasitoids, *T. busseolae* utilizes a semiochemical espionage strategy (Vinson, 1984, 1998). In behavioral laboratory tests, *T. busseolae* are attracted by the *S. nonagrioides* main pheromone component, (*Z*)-11-16:Ac (Colazza et al., 1997), as well as to contact chemicals from *S. nonagrioides* (Colazza and Rosi, 2001). The long-term goal of this work is to compare the olfactory elements involved in (*Z*)-11-16:Ac recognition in both the host and its parasitoid.

A molecular analysis of these elements should provide useful information on the coevolution of this host–parasitoid complex.

Acknowledgments This work was supported by INRA, by the Integrated Action Program GALILEO no. 06914YG (Egide, France), and by a Marie Curie fellowship to F. de Santis. We are grateful to Chiaraluce Moretti and Roberto Bonaurio (DSAA-Patologia, Università degli Studi di Perugia) for useful suggestions.

References

ABRAHAM, D., LÖFSTEDT, C., and PICIMBON, J. F. 2003. Grp2-pheromone binding protein from *Heliothis virescens*. Direct GenBank submission no. AY301988.

ABRAHAM, D., LÖFSTEDT, C., and PICIMBON, J. F. 2005. Molecular characterization and evolution of pheromone-binding protein genes in *Agrotis* moths. *Insect Biochem. Mol. Biol.* 35:1100-1111.

CALLAHAN, F. E., VOGT, R. G., TUCKER, M. L., DICKENS, J. C., and MATTOO, A. K. 2000. High level expression of "male specific" pheromone-binding proteins (PBPs) in the antennae of female noctuid moths. *Insect Biochem. Mol. Biol.* 30:507–514.

CAMPANACCI, V., KRIEGER, J., BETTE, S., STURGIS, J. N., LARTIGUE, A., CAMBILLAU, C., BREER, H., and TEGONI, M. 2001. Revisiting the specificity of *Mamestra brassicae* and *Antheraea polyphemus* pheromone-binding proteins with a fluorescence assay. *J. Biol. Chem.* 276:20078–20084.

COLAZZA, S. and ROSI, M. C. 2001. Differences in the searching behaviour of two strains of the egg parasitoid *Telenomus busseolae* (Hymenoptera: Scelionidae). *Eur. J. Entomol*. 98:47–52.

COLAZZA, S., ROSI, M. C., and CLEMENTE, A. 1997. Response of the egg parasitoid *Telenomus busseolae* to sex pheromone of *Sesamia nonagrioides*. *J. Chem. Ecol*. 23:2437–2444.

CONTI, E. and BIN, F. 2000. Parasitoids of concealed noctuid eggs and their potential in biological control of gramineae stemborers. *Redia*. 83:87–104.

CORPET, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nuc. Acids Res.* 16:10881–10890.

DU, G. and PRESTWICH, G. D. 1995. Protein structure encodes the ligand binding specificity in pheromone-binding proteins. *Biochemistry* 34:8726 – 8732.

GIACOMETTI, R. 1995. Rearing of *Sesamia nonagrioides* Lefevre on meridic diet (Lepidoptera: Noctuidae). *Redia* 78:19 – 27.

GYÖRGYI, T. K., ROBY-SHEMKOVITZ, A. J., and LERNER, M. R. 1988. Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: a tissue-specific developmentally regulated protein. *Proc. Natl. Acad. Sci. USA* 85:9851–9855.

JACQUIN-JOLY, E., BOHBOT, J., FRANCOIS, M. C., CAIN, A. H., and NAGNAN-LE MEILLOUR, P. 2000. Characterization of the general odorant-binding protein 2 in the molecular coding of odorants in *Mamestra brassicae*. *Eur. J. Biochem.* 267:6708–6714.

JØRGENSEN, K., KVELLO, P., ALMAAS, T. J., and MUSTAPARTA, H. 2006. Two closely located areas in the suboesophageal ganglion and the tritocerebrum receive projections of gustatory receptor neurons located on the antennae and the proboscis in the moth *Heliothis virescens*. *J. Comp. Neurol*. 496:121–134.

KAISSLING, K. E. 2004. Physiology of pheromone reception in insects (an example of moths). *ANIR* 6:73 – 91.

KALINOVA, B., HOSKOVEC, M., LIBLIKAS, I., UNELIUS, C. R., and HANSSON, B. S. 2001. Detection of sex pheromone components in *Manduca sexta* (L.). *Chem. Senses* 26:1175 – 1186.

KRIEGER, J., RAMING, K., and BREER, H. 1991. Cloning of genomic and complementary DNA encoding insect pheromone-binding proteins: evidence for microdiversity. *Biochim. Biophys. Acta* 1088:277–284.

KRIEGER, J., GÄNβLE, H., RAMING, K., and BREER, H. 1993. Odorant-binding proteins of *Heliothis virescens*. *Insect Biochem. Mol. Biol.* 23:449 – 456.

LEAL, W. S. 2003. Proteins that make sense, pp. 447 – 476, *in* G. J. Blomquist and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, Amsterdam.

LJUNGBERG, H., ANDERSON, P., and HANSSON, B. S. 1993. Physiology and morphology of pheromone-specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Insect Physiol.* 39:253–260.

MAÏBÈCHE-COISNÉ, M., SOBRIO, F., DELAUNAY, T., LETTERE, M., DUBROCA, J., JACQUIN-JOLY, E., and NAGNAN-LE MEILLOUR, P. 1997. Pheromone-Binding Proteins of the moth *Mamestra brassicae*: specificity of ligand binding. *Insect Biochem. Mol. Biol.* 27:213 – 221.

MAÏBÈCHE-COISNÉ, M., JACQUIN-JOLY, E., FRANÇOIS, M. C., and NAGNAN-LE MEILLOUR, P. 1998. Molecular cloning of two pheromone-binding proteins in the cabbage armyworm *Mamestra brassicae*. *Insect Biochem. Mol. Biol.* 28:815–818.

MAIDA, R., KRIEGER, J., GEBAUER, T., LANGE, U., and ZIEGELBERGER, G. 2000. Three pheromone-binding proteins in olfactory sensilla of the two silkmoth species *Antheraea polyphemus* and *Antheraea pernyi*. *Eur. J. Biochem.* 267:2899–2908.

MAIDA, R., ZIEGELBERGER, G., and KAISSLING, K. E. 2003. Ligand binding to six recombinant pheromone-binding proteins of *Antheraea polyphemus* and *Antheraea pernyi*. *J. Comp. Physiol.* 173:565–573.

MAZOMENOS, B. E. 1989. Sex pheromone components of corn stalk borer *Sesamia nonagrioides* (Lef.), Isolation, identification, and field tests. *J. Chem. Ecol.* 15:1241–1247.

NIELSEN, H., ENGELBRECHT, J., BRUNAK, S., and VON HEIJNE, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10:1–6.

PICIMBON, J.-F. and GADENNE, C. 2002. Evolution of noctuid pheromone-binding proteins: identification of PBP in the black cutworm moth, *Agrotis ipsilon*. *Insect Biochem. Mol. Biol.* 32:839–846.

PICIMBON, J.-F. 2003. Biochemistry and evolution of OBP and CSP proteins, pp. 539–566, *in* G. J. Blomquist and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, Amsterdam.

PLETTNER, E., LAZAR, J., PRESTWICH, E. G., and PRESTWICH, G. D. 2000. Discrimination of pheromone enantiomers by two pheromone-binding proteins from the gypsy moth *Lymantria dispar*. *Biochemistry* 39:8953–8962.

POPHOF, B. 2002. Moth pheromone-binding proteins contribute to the excitation of olfactory receptor cells. *Naturwissenschaften* 89:515–518.

POPHOF, B. 2004. Pheromone-binding proteins contribute to the activation of olfactory receptor neurons in the silkmoths *Antheraea polyphemus* and *Bombyx mori*. *Chem. Senses* 29:117–125.

PRESTWICH, G. D., DU, G., and LAFOREST, S. 1995. How is pheromone specificity encoded in proteins? *Chem. Senses* 20:461–469.

QUERO, C., BAU, J., GUERRERO, A., and RENOU, M. 2004. Responses of the olfactory receptor neurons of the corn stalk borer *Sesamia nonagrioides* to components of the pheromone blend and their inhibition by a trifluoromethyl ketone analogue of the main component. *Pest Manag. Sci.* 60:719–726.

RAMING, K., KRIEGER, J., and BREER, H. 1990. Primary structure of a pheromone-binding protein from *Antheraea pernyi*: homologies with other ligand-carrying proteins. *J. Comp. Physiol. B* 1160:503–509.

RENOU, M. and LUCAS, P. 1994. Sex pheromone reception in *Mamestra brassicae* L. (Lepidoptera): Responses of olfactory receptor neurons to minor components of the pheromone blend. *J. Insect Physiol.* 40:75–85.

ROBERTSON, H. M., MARTOS, R., SEARS, C. R., TODRES, E. Z., WALDEN, K. K., and NARDI, J. B. 1999. Diversity of odourant-binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol.* 8:501–518.

SOLINAS, M. and TRONA, F. 2002. Antennal sensilla of *Sesamia nonagrioides* (Lef.) (Lepidopterae: Noctuidae) Morphology and possible behavioural significance. *Boll. Zool. Agrar. Bachic. Ser. II* 34: 321–345.

STEINBRECHT, R. A., OZAKI, M., and ZIEGELBERGER, G. 1992. Immunocytochemical localization of pheromone-binding protein in moth antennae. *Cell Tissue Res.* 270:287–302.

STEINBRECHT, R. A., LAUE, M., and ZIEGELBERGER, G. 1995. Immunolocalization of pheromone-binding protein and general-odorant binding protein in olfactory sensilla of the silk moths *Antheraea* and *Bombyx*. *Cell Tissue Res.* 282:203–217.

VINSON, S. B. 1984. How parasitoids locate their hosts: a case of insect espionage, pp. 325–348, *in* T. Lewis (ed.). Insect Communication. 12th symposium of the Royal Entomological Society of London. Academic Press, London.

VINSON, S. B. 1998. The general host selection behavior of parasitoid Hymenoptera and a comparison of initial strategies utilised by larvaphagous and oophagous species. *Biol. Control.* 11:79–96.

VOGT, R. G. 2003. Biochemical diversity of odor detection: OBPs, ODEs and SNMPs, pp. 391–445, *in* G. J. Blomquist and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, Amsterdam.

VOGT, R. G. and RIDDIFORD, L. M. 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293:161–163.

VOGT, R. G., KOHNE, A. C., DUBNAU, J. T., and PRESTWICH, G. D. 1989. Expression of pheromone-binding proteins during antennal development in the gypsy moth *Lymantria dispar*. *J. Neurosci.* 9:3332–3346.

VOGT, R. G., PRESTWICH, G. D., and LERNER, M. R. 1991. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol.* 22:74–84.

VOGT, R. G., CALLAHAN, F. E., ROGERS, M. E., and DICKENS, J. C. 1999. Odorant-binding protein diversity and distribution among insect orders, as indicated by LAP, an OBP-related protein of the true bug *Lygus lineolaris* (Hemiptera, Heteroptera). *Chem. Senses* 24:481 – 495.

VOGT, R. G., ROGERS, M. E., FRANCO, M. D., and SUN, M. 2002. A comparative study of odorant binding protein genes: differential expression of the PBP1-GOBP2 gene cluster in *Manduca sexta* (Lepidoptera) and the organization of OBP genes in *Drosophila melanogaster* (Diptera). *J. Exp. Biol.* 205:719 – 744.

WILLETT, C. S. 2000a. Do pheromone-binding proteins converge in amino acid sequence when pheromones converge? *J. Mol. Evol.* 50:175 – 183.

WILLETT, C. S. 2000b. Evidence for directional selection acting on Pheromone-binding proteins in the genus *Choristoneura*. *Mol. Biol. Evol.* 17:553 – 562.

WOJTASEK, H. and LEAL, W. S. 1999. Conformational change in the pheromone-binding protein from *Bombyx mori* induced by pH and by interaction with membranes. *J. Biol. Chem.* 274:30950 – 30956.

ZHANG, S.-G., MAIDA, R., and STEINBRECHT, R. A. 2001. Immunolocalization of odorant-binding proteins in noctuid moths (Insecta, Lepidoptera). *Chem. Senses* 26:885 – 896.