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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)

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Summary

Insects discriminate odors using sensory organs called olfactory sensilla, which display a wide range of phenotypes. Sensilla express ensembles of proteins, including odorant binding proteins (OBPs), olfactory receptors (ORs) and odor degrading enzymes (ODEs); odors are thought to be transported to ORs by OBPs and subsequently degraded by ODEs. These proteins belong to multigene families. The unique combinatorial expression of specific members of each of these gene families determines, in part, the phenotype of a sensillum and what odors it can detect. Furthermore, OBPs, ORs and ODEs are expressed in different cell types, suggesting the need for cell–cell communication to coordinate their expression. This report examines the OBP gene family. In *Manduca sexta*, the genes encoding PBP1*Msex* and GOBP2*Msex* are sequenced, shown to be adjacent to one another, and characterized together with OBP gene structures of other lepidoptera and *Drosophila melanogaster*. Expression of PBP1*Msex*, GOBP1*Msex* and GOBP2*Msex* is characterized in adult male and female antenna and in larval antenna and maxilla. The genomic organization of 25 *D. melanogaster* OBPs are characterized with respect to gene locus, gene cluster, amino acid sequence similarity, exon conservation and proximity to OR loci, and their sequences are compared with 14 *M. sexta* OBPs. Sensilla serve as portals of important behavioral information, and genes supporting sensilla function are presumably under significant evolutionary selective pressures. This study provides a basis for studying the evolution of the OBP gene family, the regulatory mechanisms governing the coordinated expression of OBPs, ORs and ODEs, and the processes that determine specific sensillum phenotypes.

Key words: *Manduca sexta*, *Drosophila melanogaster*, odorant binding protein, olfactory receptor, odor degrading enzyme, gene expression, olfactory sensilla, olfaction.

Introduction

Chemosensory systems employ large families of genes whose products receive and process diverse chemical signals in manners consistent with a species' life history. Olfactory gene families provide a record of the evolution of a species' chemosensory-based behavior, and the diversity and size of a family within a species may indicate the degree to which a species utilizes chemical cues in its behavior. In insects these gene families include odorant binding proteins (OBPs), odor receptors (ORs) and odor degrading enzymes (ODEs). Moderate-sized families of homologous but divergent OBPs have been identified in many insect species (Pelosi and Maida, 1995; Krieger et al., 1997; Vogt et al., 1999; Hekmat-Scafe et al., 2000), and independently derived OBPs have also been identified in several vertebrate species (Pelosi et al., 1982; Pelosi, 1996; Pevsner et al., 1985, 1988a,b; Lee et al., 1987; Lobel et al., 1998; Tegoni, 2000). Large families of homologous but divergent ORs have been identified in the insect *Drosophila melanogaster* (Clyne et al., 1999; Gao and Chess, 1999; Scott et al., 2001; Vosshall et al., 1999, 2000), the nematode *Caenorhabditis elegans* (Troemel et al., 1995; Bargmann et al., 1998; Robertson et al., 2000, 2001), and several vertebrate species (e.g. Buck 1996; Freitag et al., 1998; Dryer, 2000; Rouquier et al., 2000). A variety of ODEs have been identified in insects (Vogt and Riddiford, 1981, 1986; Vogt et al., 1985; Rybczynski et al., 1989, 1990; Rogers et al., 1999), as well as lobster (Gleeson et al., 1992) and mammals (Ben-Arie et al., 1993). In insects, where olfactory neurons are compartmentalized within cuticular hairs (sensilla), unique combinations of ORs, OBPs and ODEs are thought to influence the odor specificities and sensitivities of the olfactory neurons (Rybczynski et al., 1990; Vogt et al., 1991a, 1999; Pelosi and Maida, 1995; Steinbrecht, 1999; Rogers et al., 1999).

Insect OBPs are small, globular, water-soluble proteins that are expressed in the support cells of olfactory sensilla and are secreted into the extracellular fluid occupying the lumen of the sensilla hairs and surrounding the ciliary dendrite projections

of olfactory receptor neurons (Vogt and Riddiford, 1981; Steinbrecht et al., 1992, 1995; Leal et al., 1999; Sandler et al., 2000). OBPs are the first gene products in the biochemical pathway detecting diverse environmental odorants, and are thought to transport odor molecules from the inner openings of pores that penetrate the sensillum cuticle to receptor proteins (ORs) located in the membranes of the olfactory receptor neurons (Vogt et al., 1985, 1999; Krieger and Breer, 1999; Wojtasek and Leal, 1999; Kaissling, 2001). The insect behaviors associated with specific odor molecules have presumably subjected the OBP gene family to selective pressures that have driven the diversification of this family. OBP homologues have been identified in numerous species of holometabolous and hemipteran insects; if they are shown also to exist in orthopteroids they would arguably be represented throughout the Neoptera, or in more than 98 % of all insect species (Vogt et al., 1999). Seven OBP sequences have been published for *Manduca sexta* (Györgyi et al., 1988; Vogt et al., 1991b; Robertson et al., 1999). Previous studies identified six OBPs in *D. melanogaster* (McKenna et al., 1994; Pikielny et al., 1994; Kim et al., 1998), and as many as 32 have been suggested to be present in the fully sequenced *D. melanogaster* genome (Kim and Smith, 2001).

OBPs are differentially expressed among diverse classes of sensilla, which have unique odor specificities. This was first suggested by the identification of three distinct OBP classes in lepidopteran species, based on N-terminal sequence analysis: the pheromone binding proteins (PBPs) and the general odorant binding proteins GOBP1 and GOBP2 (Vogt et al., 1991a). PBPs were specific to or highly enriched in male antennae, while GOBP1 and GOBP2 proteins were more equivalently expressed in antennae of both sexes. These patterns suggest that PBPs are associated with sex-pheromone-specific trichoid sensilla and GOBPs are associated with plant volatile sensitive basiconic sensilla (Vogt et al., 1991a). Differential expression of OBPs was subsequently substantiated by a series of elegant electron microscopical (EM) immunocytochemical studies in the lepidoptera *Antheraea polyphemus* and *Bombyx mori* (Laue and Steinbrecht, 1997; Maida et al., 1997, 1999; Steinbrecht, 1996, 1999; Steinbrecht et al., 1992, 1995, 1996) and in the dipteran *D. melanogaster* (Hekmat-Scafe et al., 1997; Park et al., 2000). These EM studies demonstrated both unique and combinatorial expression of different OBPs in association with morphologically and functionally distinct classes of olfactory sensilla.

The current study examines the genomic organization and patterns of expression of a subset of OBP genes of *M. sexta*: *pbp1Msex*, *gobp1Msex* and *gobp2Msex*. Previous studies suggested that these three genes are differentially expressed among distinct classes of olfactory sensilla (Györgyi et al., 1988; Vogt et al., 1991b), and as such are suitable models for elucidating genetic regulatory mechanisms underlying the determination of diverse sensillum phenotypes. The characterization of these OBP genes establishes the necessary background for investigating regulatory elements that control their spatial and temporal expression. The study concludes with an examination of the genomic organization and relationships of 25 OBP homologues in *D. melanogaster*, utilizing the completely characterized genome of this species, and a comparison between these *D. malanogaster* OBPs and 14 *M. sexta* OBPs.

Materials and methods

Animals

Manduca sexta L. were obtained as fertilized eggs (gift of Dr L. M. Riddiford, University of Washington, Seattle), and reared at 27 °C on a 16 h:18 h (L:D) light cycle. Adult tissues were taken from pharate animals within 6 h of adult emergence, anaesthetized on ice. For nucleic acid isolation, antennae were immediately frozen in dry ice and stored at –70 °C until use. For histology, antennae were dissected in Sylgard (Dupont)-lined dishes containing phosphate-buffered saline (PBS) with Tween 20 detergent (PBS-Tw; 150 mmol l^{-1} NaCl, 10 mmol l^{-1} NaH_2PO_4, pH 7.0, + 0.1 % Tween 20). For whole-mount histology, antennae were pinned (0.15 minuten pins) with their leading edge up (scales down) and bisected along their midline using a number 11 scalpel blade, somewhat like opening a clam. For paraffin-sectioned histology, antennae were pinned on their side and the scale portion of the antenna removed. To obtain larval tissue, larvae were anesthetized by submersion in water, decapitated, and heads cut open along the dorsal midline. All tissues were subsequently fixed in 2 % paraformaldehyde (PFA) in 10 mmol l^{-1} phosphate buffer (pH 7.0) overnight at 4 °C, then washed in PBS-Tw and dehydrated to 70 % methanol in H_2O and stored at –20 °C.

For the experiment examining GOBP2 expression through a larval molt cycle (Fig. 8), larvae were staged from Dr Riddiford's colony with his assistance, after the protocols of Curtis et al. (1984) and Langelan et al. (2000). Five individuals were taken and analyzed from each stage. Staging was based on morphological characteristics as follows. Spiracle apolysis (SA): an area of clear cuticle is visible surrounding the abdominal spiracles, indicating that epidermal retraction has begun (Langelan et al., 2000). Slipped head (SH): a zone of clear cuticle is visible just behind the fourth instar head capsule, revealing the underlying fifth instar head capsule. The head cap slips downward further to finally lie on top of the mandibles of the fifth instar larva. 'SH+22' and 'SH+30' were based on the appearance of the fifth instar mandibles viewed through the cuticle of the fourth instar head capsule. Approximately 22 h after SH, the head capsule is still fluid-filled and the mandibles have acquired a yellow appearance from the tanning process. Approximately 30 h after SH, the fluid within the fourth instar head capsule is reabsorbed, leaving them air-filled, and the mandibles appear dark brown.

Probes used for hybridizations

Digoxigenin-labeled antisense RNA probes were synthesized from OBP cDNA ligated into pBluescript (Genius System, Roche Biochemicals; Strategene) modified after the

methods of Byrd et al. (1996) and Rogers et al. (1997). PCR products were generated from purified plasmid (Qiagen) using the M13 forward or reverse primers and an appropriate insert gene-specific primer. RNA was synthesized from 1 μg of PCR product in the presence of 2 mmol l^{-1} each of ATP, CTP, GTP, 0.6 mmol l^{-1} UTP and 0.3 mmol l^{-1} digoxigenin-UTP using T7 or T3 RNA polymerase. The PBP1*Msex* probe encompassed base pairs (bp) 588–910 (GenBank accession number, GB-M21798) (Györgyi et al., 1988), bounded by the first internal *Eco*RI site and an *Eco*R1 site in the 3′ untranslated region (UTR). GOBP1*Msex* (GM-M73797) and GOBP2*Msex* (GB-M73798) probes encompassed full-length coding regions (Vogt et al., 1991b), and were 501 bp and 483 bp, respectively. For *in situ* hybridization studies, probes were degraded to an average size of 160 bp (Byrd et al., 1996).

Southern blot analysis

Genomic DNA (SDS-proteinase K isolation from a single *M. sexta* larva) was digested with *Eco*RV, *Cla*I, *Hin*cII, *Sca*I, *Hae*II or *Bgl*II restriction enzymes. Digested DNAs were electrophoresed overnight on a 0.8 % agarose gel (10 μg per lane), and depurinated (0.25 mol l^{-1} HCl, 25 min), denatured (0.5 mol l^{-1} NaOH, 1.5 mol l^{-1} NaCl, 45 min) and neutralized (1.0 mol l^{-1} Tris-HCl, pH 8.0, 1.5 mol l^{-1} NaCl, 45 min) on soaked Whatman paper. Digested DNAs were then transferred onto nylon membrane (Amersham; Hybond-N). A lane containing molecular mass marker was excised and stained with Methylene Blue (0.02 % in 300 mmol l^{-1} sodium acetate). The nylon membrane was prehybridized for 2.5 h at 50 °C (50 °C in 5× SSC, 0.1 % *N*-lauroylsarcosine, 0.02 % SDS, 2× Denhardt's solution, 100 μg l^{-1} herring sperm DNA) (1× SSC is 0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate) and hybridized with a digoxigenin-labeled antisense RNA probe for 16 h (20 ng l^{-1}; 50 °C in prehybridization solution containing 50 % formamide) under the same conditions in a solution containing 50 % formamide, followed by washing at room temperature in 2× SSC, 0.1 % SDS (500 ml, 5 min wash) and twice at 60 °C in 0.5× SSC, 0.1 % SDS (500 ml wash, 15 min first wash, 1 h second wash). The same membrane was hybridized three separate times with individual OBP probes (PBP1*Msex*, GOBP1*Msex* and GOBP2*Msex*) and visualized by luminous detection (Roche Biochemicals; Lumiphos-530) on X-ray film. Between hybridizations, the membrane was stripped of probe (0.2 mol l^{-1} NaOH, 0.1 % SDS, 37 °C, 30 min), equilibrated in 2× SSC (5 min), and rehybridized with a different OBP probe following a prehybridization step.

Isolation of M. sexta *OBP genomic clones*

A *M. sexta* genomic library in EMBL3 (generously provided by Dr F. Horodyski, University of Ohio) was plated at a density of 6.3×10^4 plaque-forming units (p.f.u.) per 150 mm Petri dish on a layer of *Escherichia coli* LE392 (Promega). DNA was transferred to nylon membrane (ICN), denatured (5 min) and neutralized (5 min) as above, and UV-crosslinked (in 10× SSC) on soaked Whatman paper. Membranes were prehybridized for 2.5 h at 68 °C (5× SSC, 0.1 % *N*-lauroylsarcosine, 2× Denhardt's solution, 0.02 % SDS, 100 μg l^{-1} herring sperm DNA) and hybridized with a mixture of digoxigenin-labeled PBP1, GOBP2 and GOBP1 antisense RNA probes (25 ng ml^{-1} $probe^{-1}$) under the same conditions in a solution containing 50 % formamide. Following washes (twice at 60 °C in 0.5× SSC, 0.1 % SDS), hybridized probe was visualized by luminous detection (Roche Biochemicals; Lumiphos 530) on X-ray film (Kodak, X-OMAT). Positive plaques were isolated and rescreened at low density under identical conditions. DNA from select positive clones was isolated using the Wizard Lambda Prep Kit (Promega) following recommended protocols.

Clone identities were determined by dot blot hybridization. 1 μl of each DNA sample was spotted onto dry nylon membrane (ICN) and consecutively hybridized with individual PBP1, GOBP1 and GOBP2 RNA probes following the same procedure outlined for the genomic DNA library screen (see above). After each hybridization, the membrane was stripped of probe (0.2 mol l^{-1} NaOH, 0.1 % SDS, 37 °C, 30 min), equilibrated in 2× SSC (5 min), and rehybridized with a different OBP probe following the prehybridization step. A clone that was positive for both PBP1 and GOBP2, designated M2-1S, was chosen for further analysis.

Subcloning M. sexta *genomic clone M2-1S by polymerase chain reaction*

The polymerase chain reaction (PCR) was used to generate four subclones of the M2-1S insert. Several primers were designed from published cDNA sequences for PBP1 (Györgyi et al., 1988) and GOBP2 (Vogt et al., 1991b), and the left and right arm sequences of the EMBL3 cloning vector (Stratagene). All PCR reactions were performed using the Expand Long Template PCR System (Roche Biochemicals). Each reaction (4×50 μl) used the supplied enzyme mix (1.75 U; mixture of *Taq* and *Pwo* DNA polymerases) and buffer no. 3, with 350 μmol l^{-1} dNTP, 300 nmol l^{-1} of each primer, and 20 ng M2-1S DNA. PCR was performed on a Cetus Thermocycler under oil overlay: the sequence was 3 min at 94 °C followed by 30 cycles at 94 °C (25 s), 60 °C (40 s), 68 °C (12 min for 10 cycles + a 20 s extension for each remaining cycle), and 1 cycle at 68 °C (7 min). Pooled samples were purified by phenol–chloroform extraction and precipitation (Maniatis et al., 1982). Resuspended PCR products were reamplified by PCR using primers containing either *Eco*RI or *Bam*HI sites at the 5′ end of the same gene-specific sequence. The resulting products were purified as above, digested with the appropriate restriction enzyme, and cloned into pBluescript (SK+; Stratagene).

Sequencing M2-1S subclones

All clones were fully sequenced in both directions using vector primers or primers designed to internal sequence. Sequencing was done at the University of Florida DNA Sequencing Core Laboratory (Gainesville, FL, USA) using ABI Prism Dye Terminator cycle sequencing protocols (part number 402078) developed by Applied Biosystems (Perkin Elmer Corp., Foster City, CA, USA). The fluorescently labeled

extension products were analyzed on an Applied Biosystems Model 373 Stretch DNA Sequencer (Perkin Elmer Corp.). Oligo primers were designed using OLIGO 4.0 (National BioSciences, Inc., Plymouth, MN, USA) and synthesized at the DNA Synthesis Core Laboratory (University of Florida, Gainesville, FL, USA). Nucleotide sequences were aligned and assembled using programs in the Sequencer 3.0 package (Gene Codes Corp., Ann Arbor, MI, USA).

Histological analyses

Adult tissue was prepared as described above (Animals); tissue for analysis was selected from 70 % methanol stocks. For larval tissues, heads were rehydrated to PBS, and the majority of tissue cut away from the larval antenna and maxillary palps, leaving enough head tissue for handling and orientation. For whole-mount analysis, sensory appendages (antenna, palp, galea) were cut open longitudinally by a single passage of a micro-scalpel (blade breaker, George Tiemann, Hauppauge, NY, USA) to allow probe access.

Whole-mount *in situ* hybridizations (for adult and larval tissues) were done as described by Byrd et al. (1996) and Rogers et al. (1999). Tissue was prehybridized overnight at 55 °C (in 0.6 mol l^{-1} NaCl, 10 mmol l^{-1} Tris, pH 7.5, 2 mmol l^{-1} EDTA, 1× Denhardt's, 50 μg ml^{-1} herring sperm DNA and 50 μg ml^{-1} tRNA) and hybridized for at least 24 h at 60 °C with 100 ng ml^{-1} digoxigenin-labeled probes in the pre-hybridization solution containing 50 % formamide. After washing, tissue was incubated in blocking solution alone (5 % non-fat dry milk in PBS-Tw, 2 h, 20 °C) followed by blocking solution containing alkaline phosphatase-coupled anti-digoxigenin antibody (Roche–Boehringer Mannheim; dilution 1:5000, overnight, 4 °C). Hybridized probe was visualized using Nitroblue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 20 °C following the recommended protocol (Roche–Mannheim). Tissue was photographed in whole mount under dark field illumination.

Sectioned *in situ* hybridizations were done as described by Byrd et al. (1996) and Rogers et al. (1997). Tissue was dehydrated through a graded series of ethanol and toluene (tissue stored in 70 % methanol was transferred to 70 % ethanol and carried forward), and incubated in melted paraffin (Periplast +) for 2–4 h before being embedded in plastic molds. Paraffin was additionally hardened on dry ice after trimming; sections (10 μm) were taken using razor blades mounted on top of a microtome blade, and transferred to water drops on electrostatically charged microscope slides (SuperFrost II, Fisher). After drying, slides were dewaxed by immersion in xylene, and sections were treated with Proteinase K [5 μg ml^{-1} in PBS-Tw, 15 min, room temperature (RT)]. Tissue was then treated with fix and acetic anhydride as described above. Slides were washed twice with glycine/PBS-Tw (2 mg glycine ml^{-1}, 5 min per wash) between treatments. Sections were prehybridized overnight at 42 °C (0.6 mol l^{-1} NaCl, 10 mmol l^{-1} Tris, pH 7.5, 2 mmol l^{-1} EDTA, 1× Denhardt's solution, 50 μg ml^{-1} herring sperm DNA and 50 μg ml^{-1} tRNA; 1 ml per slide) and hybridized with 100 ng ml^{-1} digoxigenin-labeled probes under the same conditions but in the presence of 50 % formamide. Following hybridization, sections were washed as described above. Tissue sections were then blocked, treated with alkaline phosphatase-coupled anti-digoxigenin antibody and stained as described above. Coverslips were placed on slides with Aquamount mounting medium (Lerner Laboratories) and samples photographed with differential interference contrast (DIC) optics. For pre-hybridization and hybridizations, slides were placed on parallel glass rods mounted on the floor of plastic Petri dish (four slides per dish) containing wet tissue and sealed with parafilm to maintain humidity; temperature-controlled incubations and washes were performed in a bacterial incubator.

Immunocytochemistry of whole-mount and sectioned material was done as described in Rogers et al. (1997) and Callahan et al. (2000). Tissues were prepared as described above for *in situ* analysis. Whole-mount tissue or dewaxed sections were blocked in 3 % non-fat dry milk (NFDM), incubated with primary antiserum (diluted 1:500, overnight, 4 °C) followed by goat IgG–horseradish peroxidase conjugate (ICN; diluted 1:100, 2 h, RT) and stained with VIP substrate (Vector) following the recommended protocols. For a negative control, sections were incubated with pre-immune serum under identical conditions. All washes and antibody treatments included 3 % NFDM in PBS-Tx (PBS containing 0.1 % Triton X-100). Permount (Fisher) was used to place coverslips on slides, which were photographed using brightfield or DIC optics. Antisera were immunohistochemically active at dilutions to 1:10,000. Primary antisera were anti-PBP*Msexta* (Györgyi et al., 1988) or anti-rGOBP2*Msexta*. rGOBP2*Msexta* was expressed from cDNA (Vogt et al., 1991b; Feng and Prestwich, 1997) and antiserum was generated in a rabbit using rGOBP2*Msexta* dissolved in 50 % Freund's Complete Adjuvant (University of South Carolina Institute for Biological Research Technology Antibody Facility).

Analysis of Drosophila *OBP genes*

Twenty five OBP homologues were identified from the *D. melanogaster* genome data base using the Blast network servers at National Center for Biotechnology Information (NCBI) and Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org/blast/) (see Table 1). The database was initially screened using six previously identified OBP sequences: OS-E, OS-F(PBPRP3), PBPRP1, PBPRP2, PBPRP5 and LUSH (McKenna et al., 1994; Pikielny et al., 1994; Kim et al., 1998), and rescreened using newly identified sequences. Criteria for selecting candidate OBPs were based on Blast *e*-values <0.05, a cutoff considered to be statistically significant (Karlin and Altschul, 1990). Data associated with the gene product accession number (AAF#) include the gene product sequence as well as a locus accession number (AE#) referencing a gene scaffold, with annotations describing the coding regions and their orientation within the scaffold sequence. Gene loci were determined using the NCBI Entrez Genome Web Server for *D. melanogaster* (www.ncbi.nlm.nih.gov/PMGifs/Genomes/7227.html) and using the gene product identifier (CG# or specific name) noted in the

Table 1. *Gene locus groups of OBP homologues in* D. melanogaster

Locus	OBP homologues
1	AAF46463 (CG12665)
2	AAF50907 (PBPRP2) AAF50909 (CG1670)
	AAF50908 (CG15457) AAF50910 (CG11748)
3	AAF52525 (PBPRP5)
4	AAF59126 (CG2297)
5	AAF58726 (CG12944)
6	AAF57515 (CG13874) AAF57520 (CG11218)
	AAF57516 (CG13873) AAF57521 (CG15129)
	AAF57519 (CG8462) AAF57522 (CG11797)
7	AAF57467 (CG13421) AAF57460 (CG13429)
8	AAF49925 (PBPRP1)
9	AAF49136 (LUSH)
10	AAF51928 (OS-E) AAF51929 (OS-F, PBPRP3)
11	AAF51918 (CG15583)
12	AAF56912 (CG18111) AAF56920 (CG15505)
	AAF56918 (CG7584) AAF56921 (CG7592)

Accession numbers (AAF#) and gene product names (in parentheses) are indicated.

Loci 10 and 11 are separated by about 100 kb and might alternatively be considered one locus.

OS-F and PBPRP3 refer to the same gene product.

sequence reference file or scaffold annotation. Introns and exons of *D. melanogaster* genes were identified by comparing translations of genomic nucleotide sequences with predicted amino acid sequences, both obtained from the gene scaffold data entries for the respective genes.

Protein sequences were aligned using ClustalX (Thompson et al., 1994); the alignment included several non-*Drosophila* proteins identified during the Blast search as having significant similarity to the *D. melanogaster* proteins. Phylogenetic analysis was performed on the ClustalX alignment matrix using Paup (Version 4.0b8 for Macintosh) (Swofford, 2000). Three analyses were performed and results displayed on a single tree (see Fig. 10A): Neighbor Joining (Saitou and Nei, 1987) (default settings, 5000 replicates) and Maximum Likelihood (Quartet Puzzling) (Strimmer and von Haeseler, 1996) (default settings, 50,000 puzzling steps), both based on Paup's mean character differences, and Maximum Parsimony (heuristic search; default settings, 5000 replicates). Algorithms ignored missing data resulting from alignment gapping.

Modifications were made to three candidate OBP genes from their predicted amino acid sequences noted in the gene scaffold annotations.

AAF50909 (gene scaffold AE003571)

Annotation suggested three exons. A truncation of the first exon was required to permit alignment using a start ATG situated mid-exon 1 (scaffold nucleotide modification: <97221..97368, 97434..97656, 98027..98227>).

AAF51918 (gene scaffold AE003600)

Annotation suggested three exons, but Blast analysis indicated that only exons 1 and 2 are OBP-related. The stop codon used was seven codons downstream from the annotated end of exon 2, adding six amino acid residues to the exon 2 domain (scaffold nucleotide modification: <70038..70376, 70440..70499>). Searching with AAF51918 identified AAF51919 as a significant homologue, but significance was only in the rejected exon 3 and AAF51519 was thus rejected as an OBP-related homologue.

AAF57521 (gene scaffold AE003795)

Annotation suggested four exons, but Blast analysis indicated that only exons 3 and 4 are OBP-related. The start ATG used was from the middle of exon 3 (scaffold nucleotide modification: complement <250658..251011, 251074..251133>).

Results

Southern blot analysis of pbp1Msex, gobp1Msex *and* gobp2Msex

High stringency Southern blots were performed on *M. sexta* genomic DNA to evaluate the genomic complexity of the three *M. sexta* OBP genes, as well as to establish hybridization conditions for library screening (Fig. 1A). Each probe generated a unique hybridization pattern indicating a lack of cross-reactivity with the respective target sequences. The PBP1*Msex* probe hybridized to only a single band in each digest, suggesting that the *pbp1Msex* gene is represented as a single copy within the genome and that there was a lack of allelic variation in the donor individual at the restriction sites generating these target sequences. The GOBP2*Msex* hybridization pattern also suggests a single copy gene. Several digests show only a single band and the other digests show more-or-less equivalent hybridization intensity in two bands. This pattern for GOBP2*Msex* suggests there may be allelic variation within several of the restriction sites generating these targets. Alternatively, there may simply be internal restriction sites within the *gobp2Msex* gene, creating multiple targets of the same gene; several such internal sites were observed in the obtained genomic sequence (see below) for each of the restriction enzymes *Eco*RV, *Hin*cII and *Sca*I, compared to only single sites for *Bgl*II and *Hae*II for PBP1. The GOBP1*Msex* hybridization pattern is consistent with the presence of an additional GOBP1 homologue. GOBP1*Msex* probe hybridized to several bands in every digest, with one band consistently more intense than the others. Multiple targets with variation in hybridization intensity suggest the presence of a second sequence similar enough to hybridize to GOBP1*Msex* probe but distinct in sequence from the *gobp1Msex* gene.

A comparison of the three blots (Fig. 1A) suggested that the probes may recognize common genomic DNA fragments. Bands labeled 2 and 4 in the PBP1*Msex* blot appeared to correspond to the equivalently numbered bands in the GOBP1*Msex* blot, and bands labeled 1 and 3 in the PBP1*Msex* blot appeared to correspond to the equivalently numbered bands in the GOBP2*Msex* blot. The *Sca*I digest yielded a single

Fig. 1. Hybridization analysis of PBP1*Msex*, GOBP1*Msex* and GOBP2*Msex*. (A) Southern blot analysis using genomic DNA isolated from a single individual; a single blot was sequentially hybridized with each probe, following stripping of the previous probe. Numbers (1–4) mark DNA fragments that appeared to hybridize with multiple OBP probes. Size markers (kb) are from *Hin*dIII-digested λ DNA. Labelled bands are discussed in the text. (B) DNA hybridization analysis of isolated genomic clones, processed under the same conditions as the Southern blot but on separate filters. Arrays of 25 clones were analyzed, and the numbers of positive clones are indicated. Arrows indicate two colonies which hybridized to both PBP1*Msex* and GOBP2*Msex* probes. Colony 2 (M2-1S) was chosen for sequence analysis.

large target for each probe (asterisks), although these do not precisely overlap; several internal *Sca*I sites were observed within the GOBP2 coding region, suggesting that *Sca*I digestion may have been incomplete. The possibility of shared targets for the different probes suggests that the three genes may be situated near each other in a single chromosome.

Isolation and characterization PBP1 *and* GOBP2 *genes*

A genomic DNA library (8×10^5 plaques) was screened with a single mixture of digoxigenin-labeled PBP1*Msex*, GOBP1*Msex* and GOBP2*Msex* antisense RNA probes. 19 positive clones were subjected to dot blot hybridization with individual probes to determine their identity (Fig. 1B): PBP1*Msex* probe hybridized to five clones; GOBP1*Msex* probe hybridized to eight clones; GOBP2*Msex* probe hybridized to five clones. Two clones were positive to both PBP1*Msex* and GOBP2*Msex* (arrows). One of these clones (no. 2, Fig. 1B) was designated M2-1S and sequenced.

A physical map of the fully sequenced M2-1S insert (9186 bp, GenBank accession number AF323972) is presented in Fig. 2A. The translational initiation and termination codons and the exon/intron boundaries of each gene were determined by alignment with published cDNA sequences for GOBP2*Msex* (Vogt et al., 1991b) and for PBP1*Msex* (Györgyi et al., 1988). *Gobp2Msex* spans 1492 bp from start codon to polyadenylation signal and *pbp1Msex* spans 1747 bp from start codon to polyadenylation signal. Both genes are oriented in the same direction, with *gobp2* upstream (5′) of *pbp1Msex*; 2741 bp separate the polyadenylation signal of *gobp2Msex* and the initiation codon of *pbp1Msex*. The coding region of each gene contains three exons, the first encoding at least part of the 5′ UTRs and the amino acid signal peptides (Vogt et al., 1991a). TATA box motifs reside 292 bp and 508 bp upstream from the respective *gobp2Msex* and *pbp1Msex* initiation codons. Also, the octamer PyCATTTPuPy, which may represent an enhancer motif (Hekmat-Scafe et al., 1997), was found 318 bp and 439 bp upstream from the respective *gobp2Msex* and *pbp1Msex* initiation codons.

The exon and intron structures of *M. sexta gobp2* and *pbp1* genes are compared in Fig. 2B along with the same structures of several other insect OBP genes, including those of several lepidopteran PBPs and six OBPs of *D. melanogaster*. The lepidopteran OBP genes show a consistent pattern of two introns, of variable length, and three exons encoding similar portions of the protein. This contrasts with a much more variable pattern among the *D. melanogaster* OBPs, where the proteins are all of similar size but the genes range from having a single coding exon (*pbprp5*) to having five coding exons (*pbprp1*). This difference in exon/intron structure between the lepidopteran and dipteran OBPs is also observed when the exon boundaries are compared within the proteins (Fig. 2C). The lepidopteran PBPs and GOBP2 have conserved boundary sites with respect to the amino acid sequences. The exon boundary sites are not conserved between the lepidopteran and *D. melanogaster* sequences and are, furthermore, variable among the *D. melanogaster* sequences.

*Expression of PBP1*Msex, *GOBP1*Msex *and GOBP2*Msex *in adult male and female antennae*

In a previous study, PBP1*Msex*, GOBP1*Msex* and GOBP2*Msex* proteins were partially sequenced directly from both male and female antennae (Vogt et al., 1991a). PBP1*Msex*

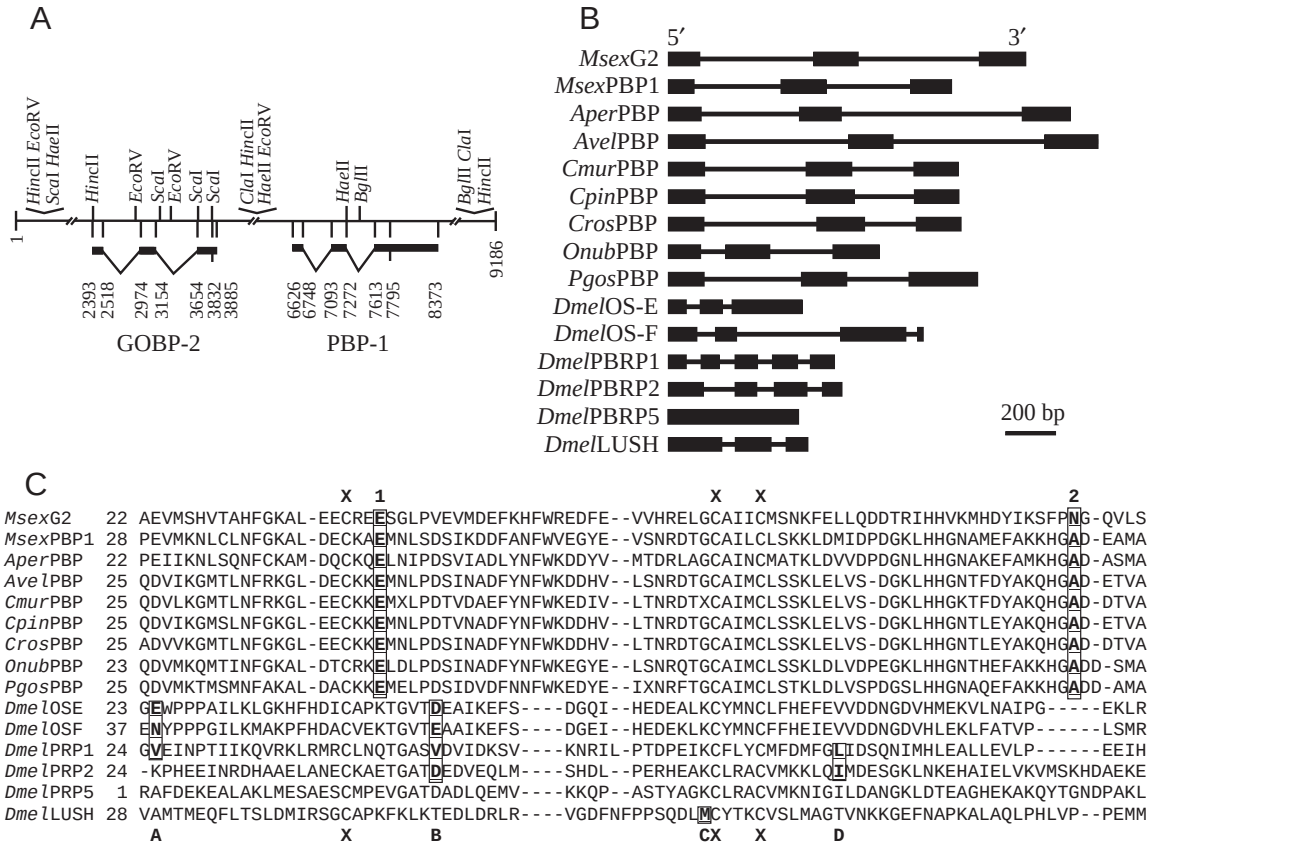

Fig. 2. Genomic organization of lepidopteran OBPs. (A) Sequence map of clone M2-1S, containing both *gobp2Msex* and *pbp1Msex*. Numbers indicate the upstream and downstream bases demarking translational initiation sites (2393, 6626), termination codons (3832, 7795), polyadenylation signals (*gobp2Msex*, AGTAAA, bp 3885; *pbp1Msex*, AATAAA, bp 8373) and boundaries between exons (heavy bars) and introns. Restriction sites relevant to Fig. 1 are indicated. The full-length sequence of M2-1S is available from GenBank (accession number AF323972). (B) Size comparison of exons and introns of OBPs. Exon/intron organization within coding regions are compared between GOBP2*Msex* and PBP1*Msex*, PBPs of several other moth species, and six OBPs of *Drosophila melanogaster*. Exon/intron boundaries were determined by comparing derived amino acid sequences with translated genomic DNA sequences. Genomic sequences are represented by large filled boxes (exons), joined by thin lines (introns); lengths are proportional to the scale bar. The 5′ ends correspond to the start ATGs and the 3′ ends correspond to the termination codons. Genes, taxa and GenBank accession numbers are: *Msex*G2 (*M. sexta* GOBP2, AF323972), Msex PBP1 (*M. sexta* PBP1, AF323972); *Aper*PBP (*Antheraea pernyi* PBP1, X57562); *Avel*PBP (*Argyrotaenia velutinana* AvelE PBP, AF177641); *Cmur*PBP (*Choristinoneura murinana* Cmur4 PBP, AF177662); *Cpin*PBP (*Choristinoneura pinus* Cpin4 PBP; AF177653); *Cros*PBP (*Choristinoneura rosaceana* CrosC PBP; AF177654); *Onub*PBP (*Ostrinia nubilalis* UZ4 PBP, AF133643). *Pgos*PBP (*Pectinophora gossypiella* PBP, AF177656) *Dmel*OSE, *Dmel*OS-F, *Dmel*PBRP1 *Dmel*PBRP2, *Dmel*PBRP5 *Dmel*LUSH – *Drosophila melanogaster* OS-E (AE003601); OS-F (PBPRP3) (AE003601); PBPRP1 (AE003541); PBPRP2 (AE003571); PBPRP5 (AE003617); LUSH (AE003516). (Krieger et al., 1991; Pikielny et al., 1994; McKenna et al., 1994; Hekmat-Scafe et al., 1997; Willett and Harrison, 1999; Willett, 2000). (C) Comparison of exon boundaries in OBP proteins. Amino acid alignments of OBP proteins in Fig. 4 are shown. The alignment is limited to regions surrounding the lepidopteran exon boundaries. Sequences were aligned using Clustal X (Thompson et al., 1994). Three of six conserved cysteine residues are marked (X). Intron/exon boundaries of the PBPs and GOBP2 are indicated by numbers (1,2); boundaries in the *Drosophila* proteins (*Dmel*) are indicated by letters (A–D). The C-terminal amino acids of exon domains are enclosed by boxes.

was more abundant in male antennae than female antennae, and was shown to associate with pheromone-sensitive long trichoid sensilla of male antennae. In females, it was not determined whether the expression of PBP1*Msex* was restricted to a subset of sensilla or occurred at low levels in the general population of sensilla. Both GOBP1*Msex* and GOBP2*Msex* were present at similar levels in male and female antennae but neither associated with pheromone-sensitive trichoid sensilla isolated from male antennae, suggesting that both GOBPs associated with sensilla involved in the detection of plant volatiles. To clarify these general observations, *in situ* hybridization and immunocytochemical studies were performed on male and female antennae.

The anatomy of male and female adult antennae is reviewed in Fig. 3. Both male and female *M. sexta* adults have flagellum-shaped antennae, which are subdivided into approximately 80 segment-like annuli (Sanes and Hildebrand, 1976; Keil, 1989; Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a,b). Fig. 3A–D shows male (A,C) and female (B,D) antennae; single annuli are represented in the inserts. Each annulus is

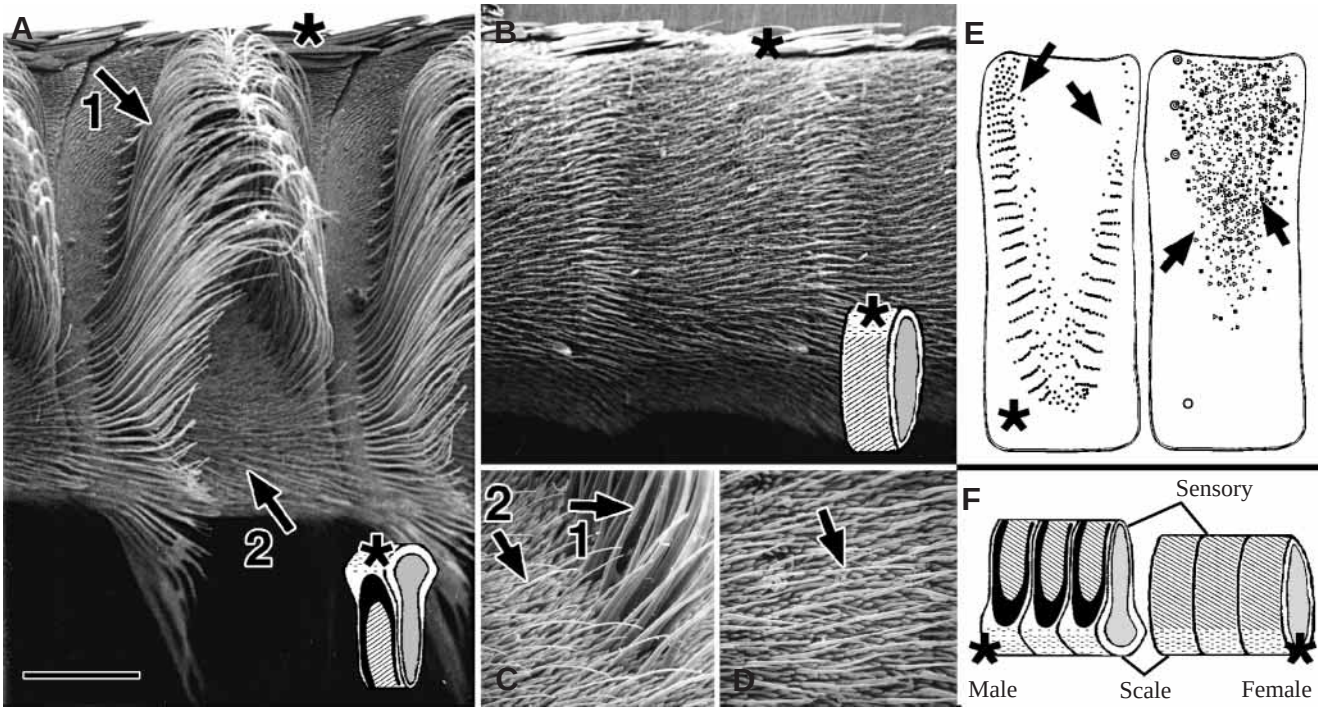

Fig. 3. Morphology of antennae and male expression of three OBPs. (A–D) Scanning electron micrographs of adult male (A,C) and female (B,D) antennae. Arrows in A point to sensilla of the peripheral (1) and mid-annular sensory (2) regions in A and C, and to a female sensillum in D. Insert diagrams in A and B indicate the structural organization of the male or female annulus; asterisks indicate scale (non-sensory) regions and hatching or solid black, sensory regions. (C) The boundary between the peripheral and mid-annular sensory regions of a male annulus; arrows identify the long trichoid sensilla (1) and the short sensilla of the mid-annular region (2). (D) A comparable region of a female antenna; the arrow points to one of many slender hair-like sensilla. Short protrusions underlying the sensilla can be seen in both C and D; these are non-sensory protrusions in the antennal cuticle. (E) Side view diagram of a male annulus, showing the distributions of olfactory sensilla (arrows) in the peripheral (left annulus) and mid-annular (right annulus) sensory regions) (Lee and Strausfeld, 1990). (F) Diagram of three male and female annuli. Sensory and scale (non-sensory, asterisk) regions are noted, as are the peripheral (black) and mid-annular (hatched) sensory regions of male and the more-or-less single homogeneous sensory region (hatched) of female. Size bar, 278 μm (A,B), 114 μm (C,D).

divided into a sensory region rich in olfactory sensilla (arrows 1 and 2 in Fig. 3A) and a largely non-sensory region (marked by asterisks) containing scales and very few sensory structures (Fig. 3F). In male antennae, the sensory region of an annulus is divided into two zones. A peripheral sensory zone (Fig. 3E, left) contains the single class of long trichoid sensilla (type I); these sensilla appear to form a horseshoe pattern when the antenna is viewed from the side as in Fig. 3E. A mid-annular sensory zone (Fig. 3E, right) contains several types of short sensilla, intermixed, including many short trichoid (type II) and basiconic (type I and II) sensilla, and a few coeloconic and styliform sensilla (Fig. 3E, right). In general, a sensillum contains 1–3 sensory neurons plus three supporting cells (thecogen, trichogen and tormogen cells). Each male antenna contains about 100,000 sensilla and 250,000 sensory neurons (Sanes and Hildebrand, 1976; Lee and Strausfeld, 1990); the long type I trichoid sensilla contain neurons that respond specifically to sex pheromone, while the mid-annular mixture of sensilla contain neurons thought to respond to plant volatiles. In female antennae, the sensory region is constructed of a single sensory zone of intermixed sensilla types, which include all those of the male antenna except for the long trichoid sensilla (Fig. 3B,D,F); a recent study identified two classes of trichoid sensilla on female antennae, suggesting that one of these classes (type A) is the equivalent of the male type I trichoid sensilla, though much shorter (Shields and Hildebrand, 2001). Several publications suggest that the total number of sensilla on female and male is similar (Sanes and Hildebrand, 1976; Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a,b); Oland and Tolbert (1988) estimated that a female antenna contained 300,000–340,000 neurons.

The distributions of PBP1*Msex* and GOBP2*Msex* are shown in whole-mount *in situ* hybridizations of adult male and female antennae in Fig. 4. In male tissue, PBP1*Msex* expression was largely restricted to the annular periphery, associating with the sex-pheromone-sensitive long trichoid sensilla. (Fig. 4A,C). A small number of scattered cells within the mid-annular region also consistently hybridized the PBP1 probe (Fig. 4D), suggesting that PBP1*Msex* is expressed within a limited number of sensilla scattered throughout this region. Expression of GOBP2*Msex* in males was restricted to the mid-annular region, corresponding to the plant-volatile-sensitive basiconic and short trichoid sensilla (Fig. 4B). In female antennae, both PBP1*Msex* (Fig. 4E) and GOBP2*Msex* (Fig. 4F) were

expressed in cells distributed throughout the sensory region, though the number of cells expressing GOBP2*Msex* was far greater than those expressing PBP1*Msex*.

The distributions of PBP1*Msex* and GOBP2*Msex* are shown by *in situ* hybridizations to sectioned adult male and female antennae (Fig. 5). For male tissue, PBP1*Msex* expression was restricted to four corners of the annulus sections (Fig. 5A), corresponding to the cells of the type I long trichoid sensilla. The sensory epithelium consists of two cell layers, with the trichogen support cells occupying the basal half and the other support cells (tormogen, thecogen) and neurons occupying the apical half (Sanes and Hildebrand, 1976). PBP1 mRNA appears to be restricted to the basal region of the epithelium, suggesting that it is primarily expressed in the trichogen cells; a single layer of negatively stained, round nuclei is clearly visible. Expression of GOBP2*Msex* in males was restricted to the mid-annular regions (Fig. 5B). Staining appears to be associated with single elongate cells (nuclei are visible by negative staining) and restricted to the basal region of the epithelium. The location of these nuclei and their appearance in a single layer suggest that GOBP2 expression may also occur predominantly in the trichogen cells of these sensilla. Unstained areas between expressing cells suggest the locations of sensilla cell clusters not expressing GOBP2.

For female tissue in section, hybridization of both PBP1*Msex* (Fig. 5C,E) and GOBP2*Msex* (Fig. 5D,F) was observed throughout the sensory epithelium, though a much larger number of cells was observed expressing GOBP2*Msex*. Cells expressing GOBP2 appeared to be located at the extreme basal region of the epithelium; negatively stained, round nuclei were seen in many of these cells. In contrast, cells expressing PBP1 appeared in the basal region but slightly above the basal border, suggesting that the cells and sensilla expressing these two genes are morphologically distinct and that these two genes are differentially expressed and not coexpressed. As in males, restriction of PBP1 and GOBP2 expression to the basal layer suggests that these genes may be expressed in only one type of sensillum support cell. A comparison of the distribution of PBP1*Msex* mRNA and protein localization is shown in female antennae in Fig. 5G,H. PBP1 mRNA (Fig. 5G) is restricted to cells in the basal region of the epithelium, while PBP1 protein (Fig. 5H) is distributed in a column extending vertically throughout the epithelium. PBP1 protein (Fig. 5H) appears to be more concentrated in the apical region, presumably within the extracellular sensillum cavity that penetrates the epithelium and extends upwards into the shaft of the sensillum hair (Keil, 1989; Laue and Steinbrechet, 1997; Steinbrecht, 1999).

Expression of GOBP1*Msex* is shown in Fig. 6. GOBP1*Msex* expression occurred in the same region as GOBP2*Msex* in both adult male (Fig. 6A–D) and female (Fig. 6D–F) antennae. In male whole mounts, cells expressing *gobp1Msex* appear to be somewhat smaller than those expressing *gobp2Msex*, suggesting that these two genes are differentially expressed within a common region. Double-labeling experiments would

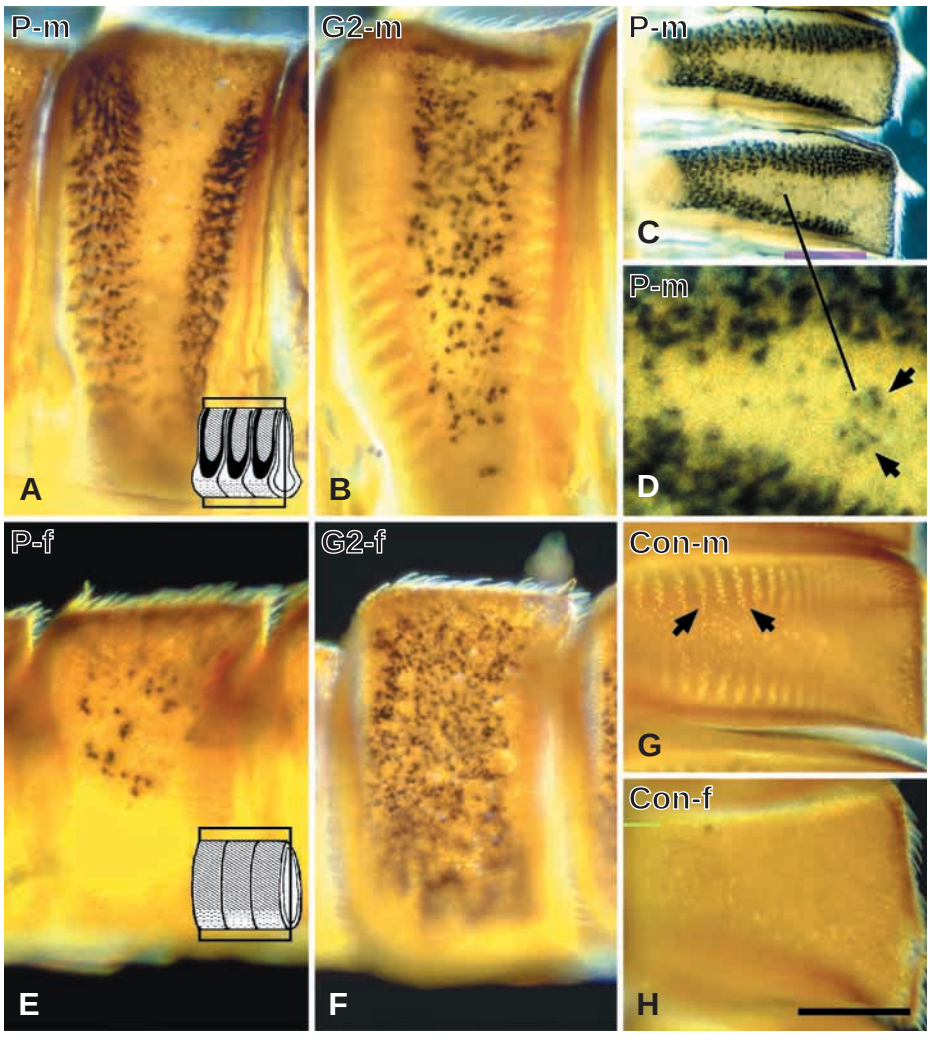

Fig. 4. Expression of PBP1 and GOBP2 in male and female antennae, in whole mount. (A–F) Bisected antennae of male (m; A–D) and female (f; E,F) adult *M. sexta* are shown probed with antisense RNA encoding PBP1 (P) or GOBP2 (G2). Insert diagrams indicate the orientation of the bisection. (C) shows details of cells of the mid-annular region expressing PBP1 (D, arrows). (G,H) Control *in situ* hybridizations (Con). Arrows in (G) indicate holes through the cuticle belonging to the long trichoid sensilla and through which olfactory dendrites pass to enter sensillum hairs. Tissue was from pharate adult animals. Size bar, 150 μm (A,B,E,F); 411 μm (C); 26 μm (D); 125 μm (G,H).

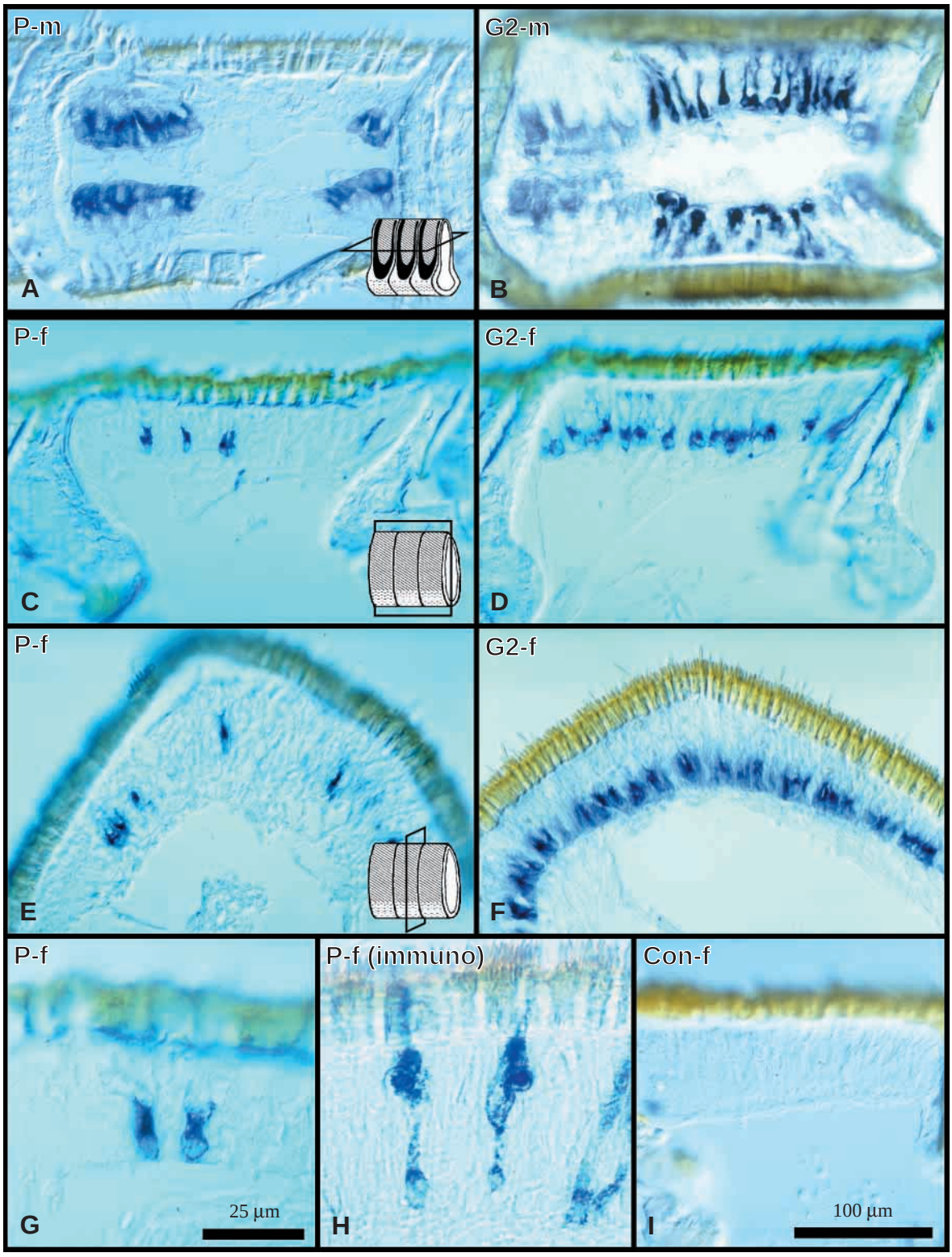

Fig. 5. Expression of PBP1 and GOBP2 in sections of male and female antennae. (A–F) Male (m; A,B) and female (f; C,F) antennae were sectioned and probed with antisense RNA encoding PBP1*Msex* (P) or GOBP2*Msex* (G2). Insert diagrams indicate the positions and orientations of sections. (G–H) Comparison of the vertical distribution of PBP1 mRNA (G, *in situ* hybridization using GOBP2*Msex* probe) and PBP1 protein (H, immunocytochemistry using PBP1*Msex* antiserum) in the female antenna. (I) Control *in situ* hybridization. Tissue was from pharate adult animals. Size bar in I, 100 µm (A–F,I); in G, 25 µm (G,H).

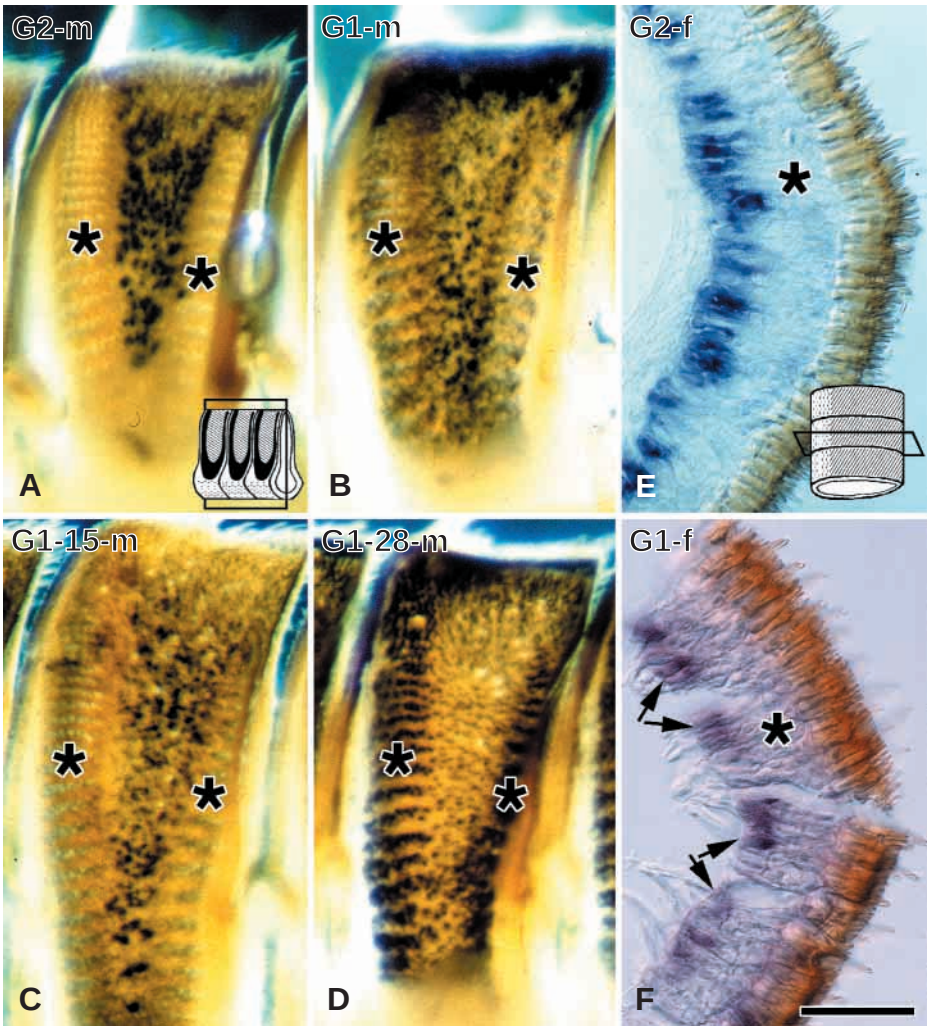

Fig. 6. Expression of GOBP1 in adult male and female antennae. (A–D) Male (m) antennae of adult *M. sexta* are shown probed in whole mount with antisense RNA encoding GOBP2 (G2), or three different clones of GOBP1 (G1). G1 probe (B) encoded the entire coding cDNA region, G1-15 probe (C) encoded the 5′ third of the coding region, and G1-28 probe (D) encoded the middle third of the coding region. G1-15 and G1-28 probes were contiguous but non-overlapping. Asterisks mark the peripheral annular regions occupied by the long trichoid sensilla. (E,F) Female (f) antennae of adult *M. sexta* are shown probed in section with antisense RNA encoding GOBP2 (G2) or GOBP1 (G1). Arrows in F indicate positive staining cells. Insert diagrams indicate the positions and orientations of cuts. Tissue was from pharate adult animals. Size bar, 188 μm (A–D) or 50 μm (E,F).

be necessary to confirm differential expression. GOBP1*Msex* probes consistently produced high background staining relative to the GOBP2*Msex* or PBP1*Msex* probes. This difference is evident in Fig. 6A and B; while full-length GOBP1*Msex* probe stained discrete cells in the mid-annular region (Fig. 6B), a more diffuse staining was also observed in the peripheral regions (asterisks, Fig. 6B) at a notably higher level than observed for the GOBP2*Msex* probe (asterisks, Fig. 6A). To improve specificity, probes were generated to specific subregions of the GOBP1*Msex* cDNA. A probe encoding the 5′ third of the coding region (G1–15, Fig. 6C) displayed reduced cross-reactivity with cells of the periphery (asterisks). In contrast, a probe encoding the middle third of the coding region (G1–28, Fig. 6D) displayed increased cross-reactivity with the periphery (asterisks).

*Larval expression patterns of GOBP2*Msex

We previously proposed that PBPs and GOBPs have different functions: PBPs process sex pheromones, which are adult- and possibly male-specific, and GOBPs process plant volatiles, which are gender- and stage-non-specific (Vogt et al., 1991a). To test this hypothesis, expression of both GOBP2*Msex* and PBP1*Msex* was investigated in larval tissue. The larval mouth of *M. sexta*, typical of any lepidopteran, is surrounded by sensory detectors that are presumably designed to assess the quality of potential food (Hanson and Dethier, 1973; Kent and Hildebrand, 1987; Glendinning et al., 1999). A pair of relatively simple antennae are situated on either side of the mandibles (Fig. 7A,B), and a pair of bilobed maxilla are situated just below the mandibles (Fig. 7A–C) (Kent and Hildebrand, 1987; Keil, 1996; Laue, 2000). At least some sensilla of the antennae and maxillary palps are presumed to detect volatile odors (Hanson and Dethier, 1973; Kent and Hildebrand, 1987). *M. sexta* larval antennae and maxillary palps contribute in the discrimination of different solanaceous plants (Hanson and Dethier, 1973). Gustatory styloconic sensilla on the maxillary galea play a commanding role in larval discrimination between solanaceous (host) and non-solanaceous (non-host) plants (del Campo et al., 2001).

Cells at the tip of the larval antennae express GOBP2 but not PBP1 (Figs 7D–I,O; 8C). Each antenna consists of three segments with basiconic sensilla on segments II and III (Fig. 7D). Whole-mount *in situ* hybridization using GOBP2 probe labeled multiple cell clusters (Figs 7E, 8Ca–k). Several preparations clearly revealed three cell clusters (Fig. 8Ca,b,h–k), with one appearing to associate with segment III (Fig. 8Ch,i). Details of these stained cell clusters are shown by the immunocytochemical analysis of sectioned antennae (Fig. 7F–I). A stained cluster in Fig. 7F associates with a large basiconic sensillum of the second antennal segment. Stained cells are shown entering segment III in Fig. 7I. No expression

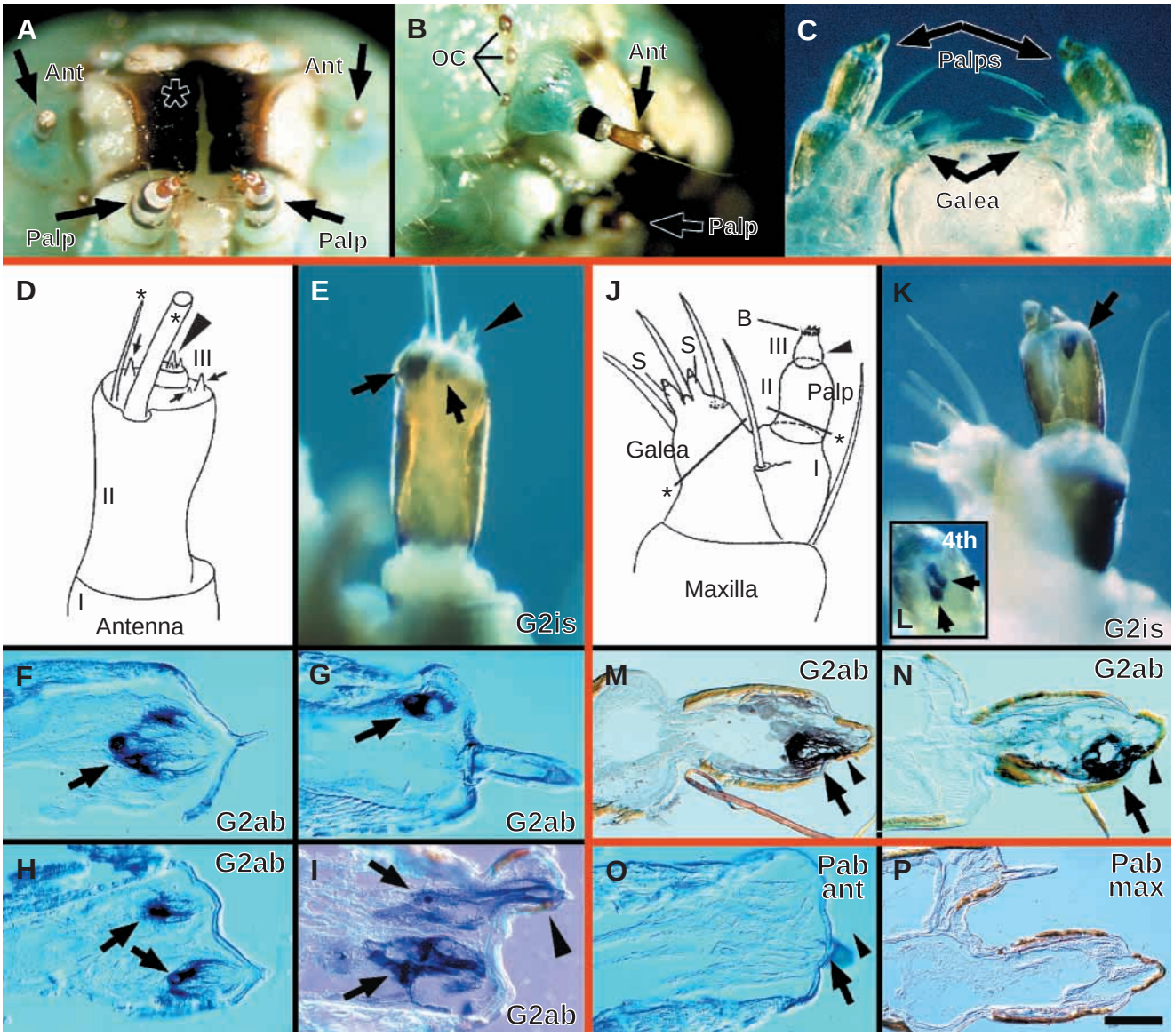

Fig. 7. GOBP2 expression in larval antennae and maxillary palps. (A–C) Mouth parts. Frontal (A), side (B) and ventral (C) views of the oral region, illustrating the position of larval antennae (Ant) and maxilla with associated palps and galea. OC, ocelli. (A,B) Fifth and (C) fourth instar larvae. (D–I) Antenna. Single larval antennae shown diagrammatically (D), in whole mount (E) and in section (F–I). (E) A whole-mount *in situ* hybridization using the antisense GOBP2*Msex* RNA probe (G2is). (F–I) Immunoreactions using GOBP2*Msex* antiserum (G2ab). (D) is modified from Kent and Hildebrand (1987), with segments I, II and III indicated. Small arrows, three basiconic sensilla on the end of the second segment; large arrowhead, three basiconic sensilla on the tip of the third segment; asterisks, mechanosensory spines. (J–N) Maxilla. Larval maxilla shown diagrammatically (J), in whole mount (K,L) and in section (M,N). (K,L) Whole-mount *in situ* hybridizations using the antisense GOBP2*Msex* RNA probe (G2is). (M,N) Immunoreactions using GOBP2*Msex* antiserum (G2ab). (J) is modified from Hanson and Dethier (1973), showing palp and galea. S, styloconic sensilla; B, basiconic-like sensilla on the tip of the maxillary palp. Arrowhead points to region on third segment of maxillary palp containing several pore-plate sensilla. Asterisks note positions of extirpation in the experiments of Hanson and Dethier (1973), which suggested differential roles of these structures in feeding decisions. (O,P) Controls. Control antennae (O) and maxilla (P) probed with PBP1*Msex* antiserum (Pab). All tissues (E-I, K-P) are from day-3 fifth instar larvae (actively feeding), except for L, which is from a fourth instar larva. Arrows over histology indicate positive staining. Arrowheads indicate third antennal segment (E,I) and point of cuticular contact of stain in third palp segment (L,M). Size bar (lower right), 542 μm (A,B), 104 μm (E), 58 μm (F), 65 μm (G), 72 μm (H), 48 μm (I), 148 μm (K), 100 μm (L), 116 μm (M,N), 66 μm (O), 116 μm (P).

of PBP was detected (Fig. 7O). These observations are consistent with those of Laue (2000), who used antisera against PBP and GOBP2 of *A. polyphemus* to immunodetect OBPs in sections of larval *Bombyx mori* and *Helicoverpa armigera* antennae at the EM level. Laue identified three basiconic sensilla (two large and one small), two chaetica and two campaniform sensilla on segment II, three basiconic sensilla (one large and two small) and one styloconic sensillum on

segment III. GOBP2 antigenicity associated with the three large basiconic sensilla; the small basiconic sensilla were not immunoreactive and no anti-PBP antigenicity was observed (Laue, 2000).

The identity of GOBP2*Msex* in antenna was confirmed using the polymerase chain reaction (RT–PCR) (data not shown). Antennal mRNA was isolated, converted to cDNA, amplified with GOBP2-specific primers and the resulting product cloned and sequenced (Rogers et al., 1999). The resulting sequence exactly matched that of the adult antennal-derived GOBP2*Msex* sequence (Vogt et al., 1991b). Similar efforts using PBP1 specific primers yielded no product, supporting the negative histology which suggests that PBP1 is not expressed in larval antennae.

Cells in the maxillary palp express GOBP2 but not PBP1 (Fig. 7K–N,P). Each maxilla consists of two lobes, the galea and palp; the palp consists of three segments with candidate volatile-sensitive sensilla on segment III (Fig. 7C,J). Whole-mount *in situ* hybridizations of fifth and fourth instar maxillary palps revealed a single cluster of at least two cells expressing GOBP2 mRNA (Fig. 7K,L). Immunocytochemical detection in sectioned tissue suggests that this cluster is located within segment II of the palp (arrows) but makes contact with the cuticle near the base of the segment III (arrowheads) (Fig. 7M,N). GOBP2*Msex* was not detected in the galea, and PBP1*Msex* expression was not detected in the maxilla (Fig. 7P).

GOBP2*Msex* expression in the maxillary palp may associate with pore plate sensilla in the side of segment III. Several reports have characterized sensory structures on the maxillary palps. Schoonhoven and Dethier (1966) described eight peg-shaped sensilla at the tip of segment III and four campaniform sensilla on the side of segment III in the region where GOBP2 immunoreactivity was observed to make cuticle contact (Fig. 7M,N). Several (2–4) of the tip sensilla were thought to be olfactory, on the basis of electrophysiological responses to plant volatiles, and the remainder were identified as gustatory or contact chemoreceptors (Schoonhoven and Dethier, 1966). Keil (1989) presented an ultrastructural analysis of the maxillary palp sensory structures in the moth *Helicoverpa armigera* and suggested that none of the tip sensilla were olfactory. Of the eight tip sensilla, five had single tip pores and three had both tip and side-wall pores; however, only the tip pores appeared not to penetrate the cuticle, suggesting that all eight sensilla were contact chemoreceptors. On the side of the palp (third segment), Keil (1989) described one singly innervated campaniform sensillum (proprioceptive), a large singly innervated digitiform organ, and two multiply innervated pore-plate sensilla. Based on structure and innervation, the digitiform organ is a candidate CO_2 detector, and the pore-plate sensilla might be olfactory detectors (Keil, 1989). The location of expressed GOBP2*Msex* suggests that it associates with one of these side-wall sensilla, possibly one or both of the multiply innervated pore-plate sensilla described by Keil. These observations further suggest that a re-evaluation of the function and identity of *M. sexta* maxillary palp sensory structures is in order.

*Downregulation of GOBP2*Msex *expression during the larval molt*

During a larval molt, the outer cuticle, including the sensillum cuticle ensheathing chemosensory neurons, is lost. OBPs are secreted into the extracellular lumen of the sensillum and are thus subject to loss during the molt; continued secretion of OBPs in the absence of sensilla cuticle would result in an energetic loss. Also, the support cells that express OBPs alter their program during a molt to extend a protrusion, which molds the new sensillum hair, and to express and secrete the cuticular proteins, which form the sensillum hair. Coexpression of OBPs at this time might strain this hair-forming process. We therefore hypothesized that OBP expression would be downregulated during the molting process. Because larval molts are regulated by ecdysteroids (Fig. 8A), and because the *M. sexta* OBPs were previously shown to be regulated by ecdysteroids in the developing adult antenna (Fig. 8B) (Vogt et al., 1993), we further hypothesized that the downregulation of larval OBP expression would correspond temporally to changes in larval ecdysteroid levels. To explore these possibilities, the larval expression of GOBP2 was examined through the molt from fourth to fifth instar, selecting animals staged relative to known ecdysteroid levels.

Expression of GOBP2*Msex* was observed to be downregulated during the larval molt, corresponding temporally to the rise and fall of larval ecdysteroids (Fig. 8A,C). Fig. 8C shows a developmental series of larval antennae, subjected to *in situ* hybridization with antisense GOBP2*Msex* probe in whole mount; the relative age of these tissues is indicated graphically in Fig. 8A. The presence of GOBP2*Msex* mRNA was detected strongly at SA 3–5 (Fig. 8Cb), weakly at SA 15-16 (Fig. 8Cc), but not detected at stages SH or SH+3 (Fig. 8Cd,e). GOBP2*Msex* mRNA was clearly visible again at SH+30 (Fig. 8Cg); under direct observation, staining was faintly apparent at SH+22 (Fig. 8Cf). This study indicates that GOBP2*Msex* expression is downregulated during a molt, turned off by SH but reinitiated by SH+22 (summarized in Fig. 8A). The temporal expression of GOBP2*Msex* correlates with the rise and fall of ecdysteroid levels as well as with expression of several other genes, which are known to be regulated by ecdysteroid levels and juvenile hormone (JH) (Fig. 8A).

Analysis of OBP gene loci in Drosophila

The full characterization of the *Drosophila* genome (Adams et al., 2000) affords the opportunity to assess the genomic organization of a large set of OBP genes within a single species. To that end, we analysed 19 potential homologues of the six previously identified *Drosophila* OBPs. Note that only the six previously identified OBPs are known from cDNAs; the coding regions of the additional OBP homologues were identified by the algorithms used by Celera Genomics (Adams et al., 2000) to characterize coding regions and intron/exon boundaries and are thus subject to the errors that may be inherent within this approach. Several of these entries were modified, as indicated in Materials and methods.

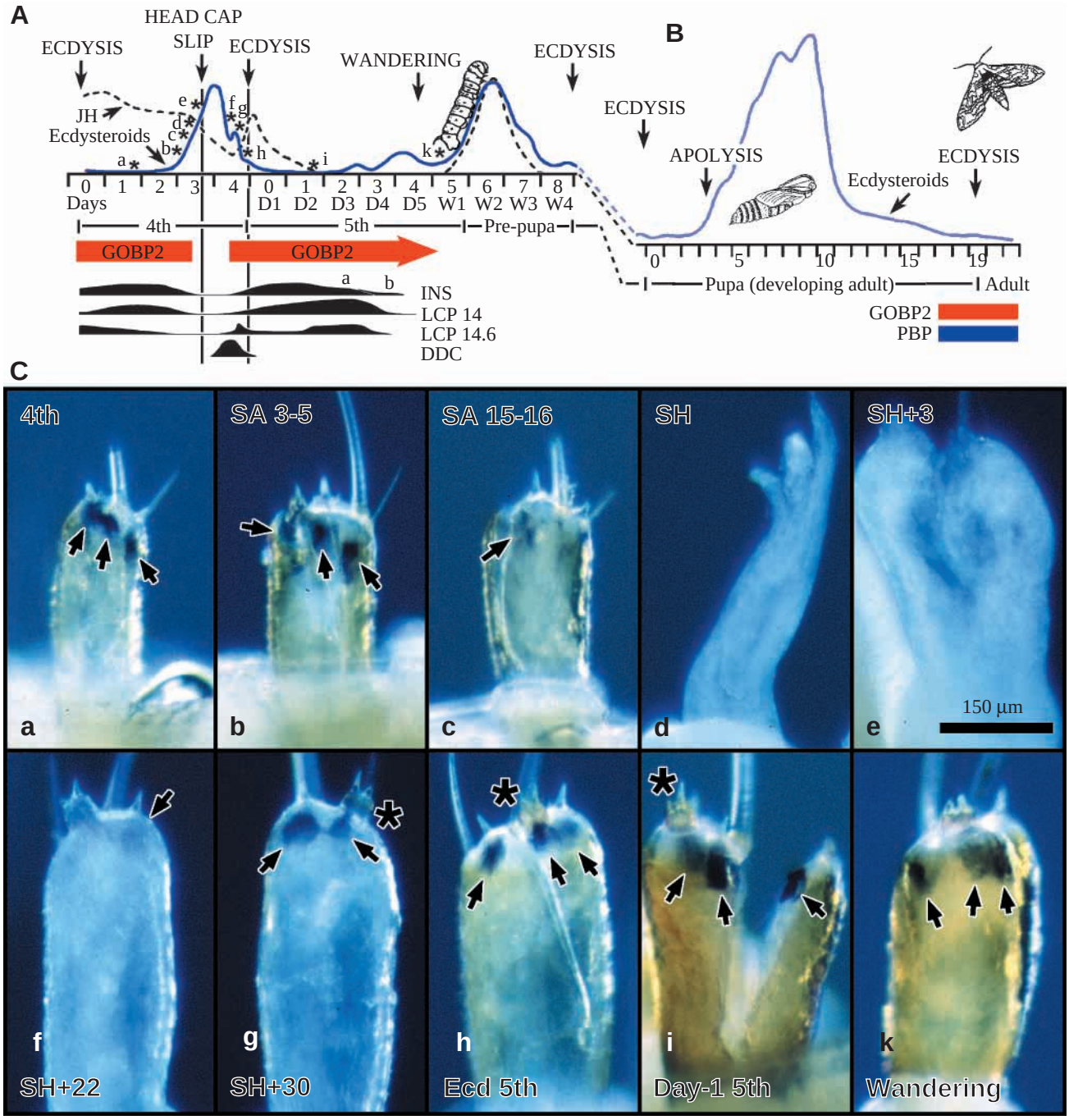

Fig. 8

All 25 *Drosophila* OBP homologues are listed in Table 1. These genes distribute among 12 loci on the three euchromatic chromosomes (Fig. 9A). Five of the 12 loci include multiple OBP genes, ranging from 2 to 6 (Fig. 9B). Many of the genes from a given multi-OBP locus are sequentially arranged; gene orientation within a multi-OBP locus appears to be arbitrary (Fig. 9B). Members of a locus tend to share significant similarity with each other based on Blast *e*-values; only the members of locus 2 shared no significant sequence similarity with other members of that locus. Further analysis might identify additional OBP homologues within the *Drosophila* genome but, for those identified here, multi-OBP loci are not the rule, but are not uncommon.

Twenty three *D. melanogaster* OBP protein sequences were aligned along with significantly similar homologues from non-drosopholid species (Fig. 10). Difference tree analyses were performed using this alignment matrix in order to quantify sequence similarities and to correlate similarities with locus (Fig. 11A). Members of a given multi-gene locus are presumably derived from another member of the same locus from gene duplications resulting from recombination

Fig. 8. GOBP2 expression in larval antenna through a fourth-fifth instar molt cycle. (A,B) The relationship between the developmental stage, levels of ecdysteroid and juvenile hormone (JH) and gene expression are indicated from fourth instar to adult, modified from Riddiford (1995). Lettered asterisks (a-k) in (A) indicate developmental stages represented by tissues shown in (C). Significant developmental events are indicated in all-capitalized text above graph. Ecdysteroid titers are from Bollenbacher et al. (1981) and Warren and Gilbert (1986); JH titers are from Fain and Riddiford (1975) and Baker et al. (1987). Expression profiles of four ecdysteroid sensitive genes are shown in black, reviewed in Riddiford (1995): INS, insecticyanin; LCP 14, larval cuticle protein; LCP 16.6, larval endocuticle protein; DDC dopa decarboxylase. Temporal expression of GOBP2*Msex* in larva is indicated in (A), from this study; expression of GOBP2Msex and PBP1Msex in pupa is indicated in (B), from Vogt et al. (1993). (C) Whole-mount *in situ* hybridizations of developmentally staged tissue, each representative of five individuals. Arrows indicate positively stained cell clusters; asterisks indicate staining entering the third antennal segment. (a-k) Tissue correlated with the time points indicated by lettered asterisks in A. 4th, feeding fourth instar larva day 1; SA, spiracle apolysis; SH, slipped head, with hours after indicated (e.g. SH+3=SH+3 h). Ecd 5th, animal within 2 h of molting; Day-1 5th is 24 h after molting. 'Wandering' is an animal on the first day of wandering (W1, in A), a non-feeding pre-pupal stage that initiates about 5 days after molting. The time between SA 15-16 and SH was approximately 8 h, and between SH+30 and Ecd 5th, approximately 5 h. Apolysis, or the detachment of the epidermis from the cuticle, occurred following stage SA15-16, and is indicated by the loss of antennal form observed at SH (d). The formation of new fifth instar larval cuticle is indicated by the structural form the antenna has reacquired by stage SH+22 (f). The fourth instar larval cuticle is not shed until ecdysis (Ecd 5th, h), after which the cuticle becomes tanned, as indicated by the brown coloration in the Day-1 5th and 'Wandering' tissues (i,k). Size bar, 150 μm.

misalignments. Thus, members of a locus might be expected to be more similar in sequence than members from different loci. The two members of locus 10 (OS-E and OS-F) share strongly supported similarity. Members of locus 12 are contained within a single branch, though with relatively weak support; this branch also includes the serum proteins of the medfly *Ceratitis capitata* (e.g. CAB64645). Members of locus 6 are distributed between two branches, one with strong support and the other with weak support. Of the multi-gene loci, only members of locus 2 show no supportable within-locus similarity, failing to group together in a single branch. The overall topology of the tree shown in Fig. 11A is consistent with an earlier analysis, which supported relationships between OS-E/OS-F and between PBPRP2/PBPRP5 but indicated considerable divergence between these two pairings, PBPRP1 and LUSH (Hekmat-Scafe et al., 2000).

Evolutionarily related genes might contain structurally conserved features such as intron/exon boundaries or exon domains. Fig. 11B shows a graphical representation of the aligned amino acid sequences (Fig. 9C). Three basic patterns were observed: OBP genes contain either a single coding exon (I), or two exons, where the first primarily encodes the N-terminal region including the signal peptide (II), or multiple exons (III). For several members of the single exon group I, it is possible that an additional 5′ exon was not identified by the Celera Genomics analysis. The N-terminal ends of CG18111 and CG13874 are particularly likely to be incomplete; both proteins align well with the other sequences and contain the six cysteines characteristic of the family but are truncated just 5′ of the most 5′ cysteine. Among the multiple exon group III, several of the exon boundaries appear to be conserved in patterns, suggesting a close evolutionary relationship of these genes. A variety of evolutionary histories might be constructed using these exon domain boundaries as significant characters and assuming different initial conditions. For example, a single boundary at position 131 (two exons) might represent the ancestral state and the additional boundary positions might have resulted from the subsequent additions or deletions of introns. The common exon boundaries observed in OS-E and OS-F, along with their sequence similarity, chromosomal pairing and coexpression, were previously noted as providing strong evidence that the two genes derived from a recent duplication event but one that predated the formation of the *D. melanogaster* species (Hekmat-Scafe et al., 2000).

The chromosomal locus positions of the *D. melanogaster* OBP genes were compared with those of 61 candidate *D. melanogaster* OR (DOR) genes (Fig. 9A). The combinatorial expression of specific OBPs and DORs may contribute to the functional phenotypes of descrete olfactory sensilla; the relative proximity of these genes might suggest a possible mechanism for such coregulation. 61 DOR genes have been identified in *D. melanogaster* (reviewed by Vosshall, 2000, 2001). In general, DOR genes are distributed throughout the genome; some reside in multi-gene loci, but most are relatively isolated from one another. Furthermore, there appears to be no consistent association between OBP and DOR loci in *D. melanogaster*, as at least 500 kbp separate most of them and there are many intervening and unrelated genes. There are closer physical associations: OBP locus 2 is about 100 kbp from DOR 19a, OBP locus 4 is about 200 kbp from DOR 43b, and OBP locus 5 is about 350 kbp from DOR 47a. OBP loci 10 and 11 are about 130 kbp apart, with OR83c situated between, about 100 kbp from locus 10 and 30 kbp from locus 11. The most striking relationship is seen in OBP locus 6 (Fig. 9B). Locus 6 encompasses eight genes including six OBP homologues and two non-OBP genes, the odor receptor OR56a (CG12501) and a gene with significant similarity to mitochondrial thioredoxin (CG8517, mtr). Excluding locus 6, the distances between DOR and OBP genes make it highly unlikely that coregulation occurs through shared regulatory sites.

A sequence comparison was made between the 25 *D. melanogaster* OBP (Table 1) and 14 *M. sexta* OBP amino acid sequences (Fig. 11C). While only six of the *D. melanogaster* genes have been shown to express in antennae (OS-E, OS-F, PBPRP1, PBPRP2, PBPRP5, LUSH), all 14 *M. sexta* genes were identified from antennal cDNA libraries of either male or female adult antennae (see Robertson et al., 1999). With

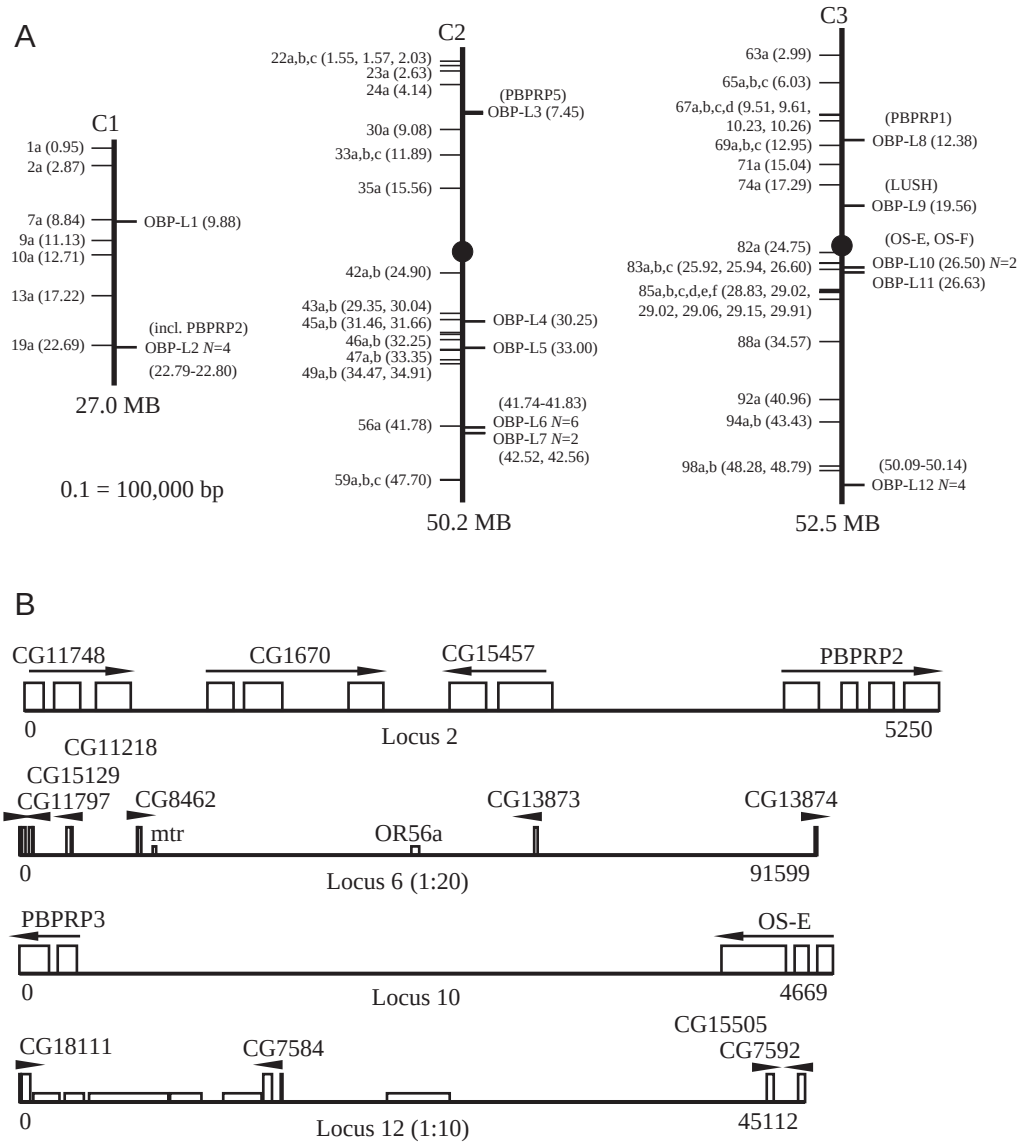

Fig. 9. *D. melanogaster* OBP homologues. (A) Physical positions of OBP (L1-L12) and DOR loci on chromosomes 1-3 (C1-C3). Numbers are in megabase units (MB); circles mark positions of centromeres. DORs are those identified by the *Drosophila* Odorant Receptor Nomenclature Committee (2000) and Leslie Voshall (67d; personal communication). Mid-point nucleotide positions of genes were determined using the NCBI Entrez genome server. (B) Spatial organization of genes at loci 2, 6, 10 and 12; based on gene scaffold annotations. Positions of all annotated genes within these regions are shown. Tall boxes are OBP genes (CG numbers) and short boxes intervening genes; exons are indicated only for OBPs, and arrows indicate orientations of OBP genes. Numbers indicate the nucleotide range of each diagram; diagrams of loci 6 and 12 are scaled (1:20, 1:10) relative to those of loci 2 and 10. Locus 7 is not illustrated; two OBP genes are separated by about 44 kbp with six unrelated annotated genes situated between. mtr, mitochondrial thioredoxin (CG8517); OR56a, olfactory receptor.

few exceptions, the OBP sequences segregate by species, consistent with the estimated divergence between the dipteran and lepidopteran lineages about 250 million years ago (see Discussion). Also, and with few exceptions, the OBPs show considerable sequence diversity, indicated by the consistently long branch lengths. Several distinct similarity groups are evident in addition to those mentioned above, notably the PBP/GOBP1/GOBP2 group of *M. sexta*, and the PBPRP2/PBPRP5/CRKBP of *D. melanogaster* and the blowfly *Phormia regina*.

Discussion

Insect OBPs comprise a highly divergent multigene family (Vogt et al., 1999). 14 OBP homologues have been identified in this work and by others from cDNAs derived from antennal mRNA of *M. sexta* (Robertson et al., 1999) and we have characterized 25 OBP homologues from *D. melanogaster*, several of which were previously identified. The three lepidopteran OBPs chosen for this study, PBP1M*sex*, GOBP1*Msex* and GOBP2*Msex*, were also previously partially characterized and shown to to be differentially associated with

Fig. 10. Alignment (ClustalX) of *D. melanogaster* OBP amino acid sequences. *Drosophila* sequences are those identified in Table 1; locus 6 genes were excluded because of significant divergence from the other OBPs. Other included proteins showed a significant relationship to the *Drosophila* proteins by Blast analysis: CAB64650, CAB64649 and CAB64645 are serum proteins of the medfly *Ceratitis capitata*, (Christophides et al., 2000), LAP (AF091118) is an OBP from the hemipteran *Lygus lineolaris* (Vogt et al., 1999) and ABPX*Msex* (AF117577) is an OBP from *M. sexta* (Robertson et al., 1999). This alignment preserved the relationships between six conserved cysteines, noted by 'X'. Exon domains are alternately in bold and underlined.

male and female antennae (Vogt et al., 1991a). In the current study, we show that *pbp1Msex* and *gobp2Msex* genes are adjacent to one another; the chromosomal position of *gobp1Msex* relative to the *pbp1/gobp2* genomic cluster was not determined. *Pbp1Msex* and *gobp2Msex* appeared to express in non-overlapping spatial domains in the adult male sensory epithelium, but in overlapping spatial domains within the adult female sensory epithelium. However, in males, *pbp1Msex* was also expressed in a small number of sensilla of the mid-annular region that intermingle with sensilla expressing *gobp2Msex*, much like the situation in the female antenna where cells expressing these two genes also intermingle. This suggests an equivalence between the mid-annular region of the male sensory epithelium and the entirety of the female sensory epithelium. GOBP1*Msex* hybridization was observed to be somewhat variable in males, consistently overlapping with *gobp2Msex* expression in males and females, but occasionally also overlapping with *pbp1Msex* expression in males. This promiscuous behavior of the GOBP1 probe in male tissue may be due to crossreactivity with an additional OBP gene that

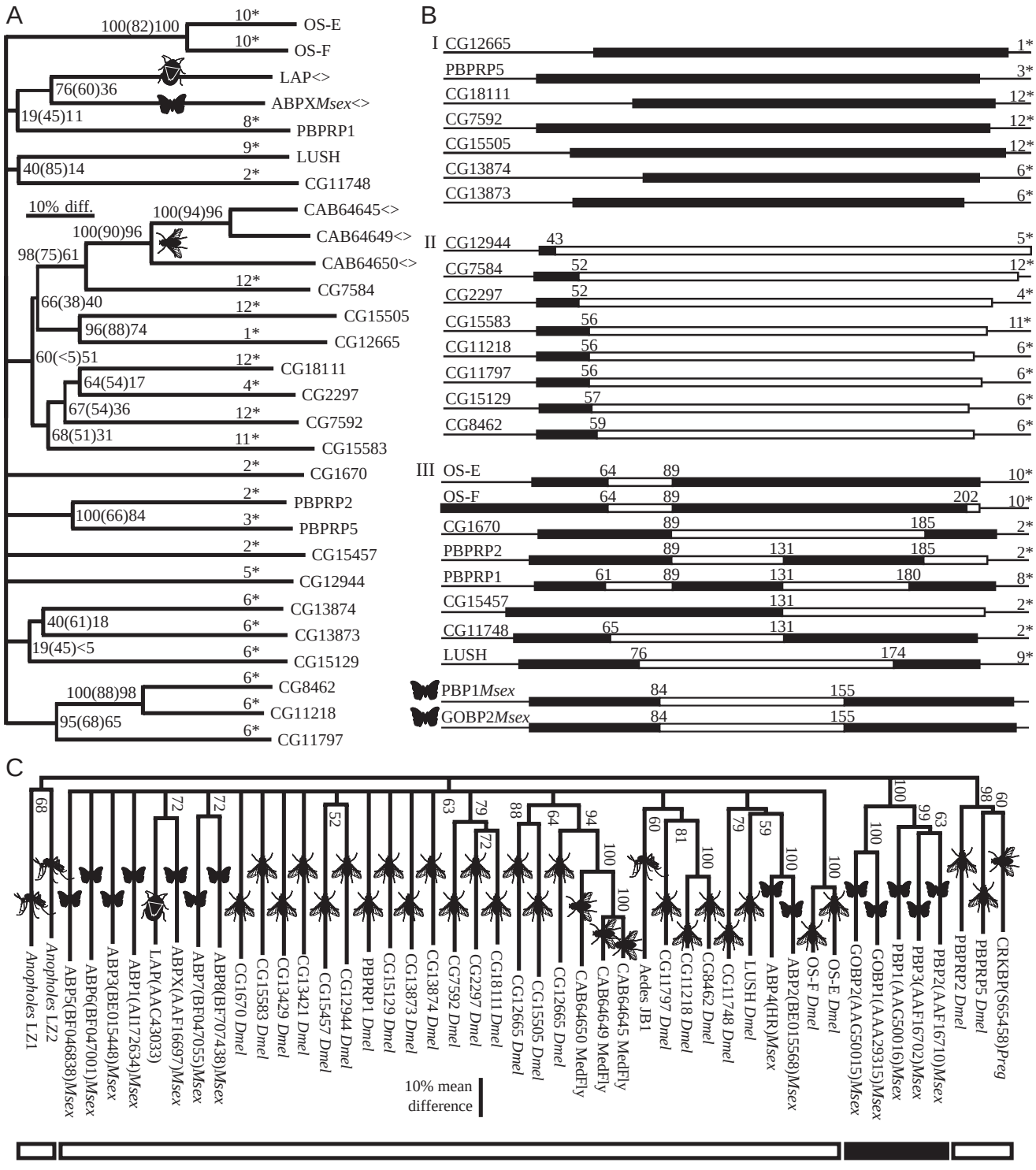

Fig. 11

shares considerable similarity with *gobp1Msex* and which coexpresses with *pbp1Msex* in the pheromone-sensitive long trichoid sensilla. This possibility is based on Southern blot analysis, which suggested that GOBP1 probe recognized multiple genes but did not crossreact with the *pbp1* target (Fig. 1A), and on the differential hybridization specificity antisense RNA probes generated from different regions of the GOBP1*Msex* sequence (Fig. 6B–D).

Adult expression of PBP and GOBP2 proteins has been previously studied in antennae of the moths *A. polyphemus* and *B. mori* by immunodetection in EM sections of identifiable olfactory sensilla (Steinbrecht et al., 1992, 1995, 1996; Steinbrecht, 1996, 1999; Laue and Steinbrecht, 1997; Maida et al., 1997, 1999). In these other studies, PBP was consistently detected in long trichoid sensilla, and GOBP2 was detected in basiconic sensilla. Both PBP and GOBP2 were detected in

Fig. 11. Comparisons of OBP sequences. (A) Amino acid sequence comparisons of 23 *D. melanogaster* OBPs. A Neighbor Joining Distance tree is shown, derived from the alignment matrix shown in Fig. 9C. Branch lengths are proportional to percentage sequence difference (scale bar represents 10% mean difference). Three methods were used for this analysis; numbers by nodes are triplets and refer in order to neighbor-joining bootstrap values (5000 replicates), maximum likelihood quartet puzzling support values (in parentheses, 50,000 puzzling steps), and maximum parsimony support values (5000 replicates). Numbers with asterisks indicate gene locus numbers identified in Table 1 and Fig. 9A. Branches are collapsed to 40% support for at least one method of analysis; all three methods yielded identical topologies at this level of support. Non-drosopohilid taxa are indicated (<>); these were identified when searching the *D. melanogaster* homologues using Blast. (B) Comparisons of exon domains of 23 *D. melanogaster* OBPs. Graphical representation of aligned amino acid sequences shown in Fig. 9C, focusing on the alignment of exon domain boundaries within the proteins, including 23 *D. melanogaster* OBPs plus PBP1*Msex* and GOBP2*Msex* (transferred from Fig. 4). Alternate exon domains are shown as filled and unfilled boxes; C-terminal amino acid numbers of exon domain boundaries are indicated, referencing their character positions in the alignment (Fig. 9C). (C) Amino acid sequence comparisons of dipteran *versus M. sexta* OBPs. A Neighbor Joining Distance tree is shown (Paup 4.0b8), derived from a ClustalX alignment (not shown). Branch lengths are proportional to percentage sequence difference (scale bar represents 10% mean difference); numbers by nodes are bootstrap values (1000 replicates). Sources of dipteran sequences are described above, except for the mosquito sequences; two sequences from *Anopheles gambiae* are from L. Zwiebel, and one sequence from *Aedes aegypti* is from J. Bohbot and R. Vogt. *M. sexta* sequences PBP1, PBP2, PBP3, GOBP1, GOBP2, ABPX and ABP1 were previously published (Györgyi et al., 1988; Vogt et al., 1991b; Robertson et al., 1999). The remaining *M. sexta* sequences were identified from ESTs submitted by H. Robertson (GenBank); this data set was downloaded and searched locally by Blast protocols using software obtained from the NCBI FTP site, and subsequently translated for alignment. Representative EST accession numbers are indicated. One sequence, ABP4, was provided by Hugh Robertson and is as yet unpublished. The broken bar at the bottom identifies major similarity groups in this analysis.

short trichoid sensilla but were never colocalized. There is as yet little information regarding the expression of GOBP1, except that its distribution among male and female antennae closely matches that of GOBP2 (Vogt et al., 1991a,b). One conference abstract report noted immunodetection of GOBP1 in at least some long trichoid sensilla of *B. mori*, suggesting that GOBP1 and PBP may occasionally coexpress (Maida et al., 1999). We also observed GOBP1 probe hybridizing to long trichoid sensilla, but suggest instead that the target being detected is a different OBP protein that is similar to GOBP1 and that coexpresses with PBPs in the pheromone-sensitive long trichoid sensilla.

Larval expression of OBPs has already been shown by northern blot in the dipteran *D. melanogaster* (PBPRP5) (Park et al., 2000), and at the immunohistological EM level in the antenna of the lepidopteran *B. mori* (GOBP2) (Laue, 2000). In the current study, GOBP2*Msex* hybridizations were positive in sensilla of the larval maxillary palp and antenna, but PBP1*Msex* hybridizations were negative; PCR-based cloning and sequencing confirmed the presence of GOBP2 transcript in antennal-derived mRNA but failed to identify any PBP1 transcript in the same mRNA sample.

Thus, this study has identified a pair of homologous OBPs that are tandemly arranged on the chromosome, but are differentially expressed between male and female adults as well as between larvae and adults. The patterns of expression of these two genes support their original naming: PBP1*Msex* is adult-specific, primarily associating with sex-pheromone sensitive neurons, whereas GOBP2*Msex* is present in a wide variety of olfactory sensilla in males, females and larvae, associating with a population of neurons that are presumed to respond to a wide range of odorants. It is worth noting that the adult sensory epithelia of female antennae and the mid-annular region of the male antennae contain a diverse and intermingling population of sensilla phenotypes, both with respect to morphology and odor responsiveness (Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a,b, 2001). These diverse sensilla provide a considerable landscape for the differential or combinatorial expression of a large number of OBP genes.

Female expression of PBPs

PBP expression in female antennae conflicts with the generalized dogma that female moths do not display any physiological or behavioral response to their own sex pheromone (Schweitzer et al., 1976; Boeckh and Boeckh, 1979; Koontz and Schneider, 1987; Hildebrand, 1996; Christensen et al., 1990; Chen et al., 1997). The first PBP was identified in *A. polyphemus*, and it appeared to be uniquely expressed in male antenna; it was isolated directly from receptor lymph of pheromone-sensitive long trichoid sensilla, and was shown to bind pheromone (Vogt and Riddiford, 1981). PBPs do in fact continue to be observed associating with sex-pheromone-sensitive sensilla of adult male antennae (e.g. Laue and Steinbrecht, 1997). However, PBP expression in female antennae has now been observed in many moth species. PBP is more abundantly expressed in male antennae than female antennae of saturniid, bombycid and sphingid families, but more equivalently expressed in male and female antennae of noctuiids (Györgyi et al., 1988; Vogt et al., 1991a; Steinbrecht et al., 1992, 1995; Laue and Steinbrecht, 1997; Nagnan-LeMeillour et al., 1996; Maïbèche-Coisné et al., 1998; Callahan et al., 2000). Female expression of PBPs has led to suggestions that female sensilla expressing these PBPs may be detecting and monitoring some component of the female-released sex pheromone or that PBPs may have broader functions than the detection of sex-pheromone odorants.

Autodetection of sex pheromone by females does in fact occur. A recent report by Schneider et al. (1998) presents data of female autodetection of sex pheromone in the tiger moth, and includes an excellent review of the literature of female autodetection. Nevertheless, in those species where

female expression of PBP has been demonstrated, pheromone detection by female olfactory sensilla has not. It might make sense for female moths to have the capability of monitoring their release through an antennal feedback system, and it may be that there are a small subset of olfactory sensilla on female antennae that respond in a specific manner to at least one component of the sex pheromone.

Assays of female response to sex pheromone have often been at the behavioral or whole antenna (electroantennogram) level. In these studies, the relevant behavior or physiological response may not have been recognized, or an electroantennogram signal may have been below the level of detection if only a small number of sensilla were involved. However, a recent study examined the odor responsiveness of 125 individual type-A trichoid sensilla from female *M. sexta* antennae to 105 different odorants (Shields and Hildebrand, 2001). Neurons from these sensilla project to a region in the female olfactory lobe which is similar to that receiving pheromonal inputs in the male olfactory lobe. Electrical responses were elicited for about 60 % of the tested odors. No responses were observed for two pheromone components that were tested, *E*10,*E*12-hexadecadienal and *E*11,*Z*13-pentadecadienal. A failure to elicit a response to pheromone may have been because no female sensilla detect pheromone, or because only one class of sensilla was tested (an annulus has about 1,100 olfactory sensilla), or because the 'wrong' pheromone components were tested. In another recent study, 200 olfactory sensilla were arbitrarily selected from either female antennae or the mid-annular region of male antennae in *M. sexta*, and tested for their responses to eight pheromone components and 24 host plant-related compounds (Kalinová et al., 2001). A small and scattered population of sensilla from the female antenna and from the mid-annular region of the male antenna responded to the pheromone component *Z*11-hexadecadienal; no other pheromone component responses were observed for these sensilla. The distribution of these *Z*11-16-aldehyde-sensitive sensilla is similar to that of the PBP-expressing sensilla of the same region (Kalinová et al., 2001).

The function of female sensilla expressing PBP remains unclear, as does the function of male sensilla which are not of the type-I long-trichoid type but which express PBP (i.e. those in the mid-annular region). It is possible that these sensilla respond to odors unrelated to pheromone. However, conservation of the PBP gene family in lepidoptera suggests that a strong and focused selective pressure has contributed to its evolution (Vogt et al., 1999). Divergent functions of sensilla expressing PBPs might be expected to steer PBP evolution in a less conserved direction. The uniqueness of the PBP lineage to lepidoptera and the patterns of PBP1 expression argue that PBPs have highly specific roles in odor detection, and that sensilla expressing PBPs, whether in males or females, play an important behavioral role for the animals.

Genomic organization of insect OBPs

The PBP and GOBP2 genes of Lepidoptera that have been characterized are highly conserved with respect to exon boundaries in their translated amino acid sequences, and are quite different from OBP genes of Diptera, both with respect to exon position and variation (Figs 2B,C, 10B). Such differences in gene structures may simply reflect the phylogenetic distance between Lepidoptera and Diptera. Alternatively, the differences may be consistent with distinct protein/gene classes. The PBPs and GOBPs comprise a single structural class of OBP within Lepidoptera, distinct from other lepidopteran OBPs as well as from OBPs identified from other insect Orders (Vogt et al., 1999; Hekmat-Scafe et al., 2000). Thus, the conserved and unique exon structure of the PBP/GOBP proteins may indicate that the gene duplications which produced this lepidopteran-specific gene lineage occurred relatively recently.

Analysis of 25 OBP homologues in *Drosophila* identified 12 OBP loci distributed across three of four chromosomes; five of these loci included clusters of two or more OBP genes (Fig. 9A,B). The remaining OBPs were individually distributed, presumably the consequence of chromosomal rearrangements which translocated these OBP genes from their sites of origin. For the multi-OBP loci, OBPs were frequently, but not always, sequentially arrayed, and orientation was about even in either direction. OBPs of a given locus tended to be more similar in sequence to other OBPs of the locus than to those outside the locus, as indicated by the grouping of OBPs of a given locus in the sequence tree (Fig. 10A). However, long branch lengths and weak support values in the tree emphasize the considerable sequence divergence that has accumulated among clustered OBP genes.

Locus 10 includes two OBP genes, OS-E and OS-F, which are oriented in the same direction (Fig. 9B), are similar in both sequence and exon structure (Fig. 10A,B), and are known to coexpress within the same sensilla and presumably the same cells (Hekmat-Scafe et al., 1997). Locus 12 contains four genes, which share similar sequence and exon structures, associating with a single branch in the sequence tree, which also includes OBPs of two single-OBP loci and the serum proteins of *C. capitata*. This association with the *C. capitata* proteins suggests a hypothesis that the locus 12 OBP homologues may be non-olfactory serum proteins. Locus 6 contains six OBPs, which also share similarities in sequence and exon structure, and has the unusual feature of being the only OBP cluster that also includes an OR gene. Locus 2 includes four OBP genes, which share common exon structures, conserving specific exon domains, but which also have highly divergent sequences.

Locus 2 OBPs demonstrate the unreliability of amino acid sequence and the value of genomic organization (locus and exon structure) in establishing the evolutionary relationships of members of a multi-gene family. The sequence divergence of the locus 2 proteins suggests that these genes are not closely related (Fig. 10A). However, the conserved exon boundary positions of the locus 2 proteins (Fig. 10B) and the close proximity of their genes (Fig. 9B) suggests the opposite, a close relationship between the locus 2 OBPs and most of the

other members of the group III genes (Fig. 10B). Indeed, the exon boundaries are potentially highly informative as characters useful for deciphering the evolutionary relationships of these genes. The sequence divergence of the locus 2 genes may indicate that these genes resulted in much earlier duplications than occurred for the genes of loci 10, 12 and 6, providing the locus 2 genes with a much longer period of time to diverge. Alternatively, the conserved exon boundaries and close physical arrangement of the genes may indicate that the locus 2 duplications were relatively recent, but that the function of the locus 2 genes, and the selective pressures acting on these genes, were such that their evolution has been more rapid than those of the other loci.

One locus 2 OBP, PBPRP2, is significantly similar in sequence to a single locus OBP, PBPRP5 (locus 3); this is especially curious because PBPRP2 is encoded by four exons while PBPRP5 is encoded by only one exon. How do two genes which differ so dramatically in genomic organization have such strongly supportable sequence similarity in an otherwise highly divergent gene family? The sequence similarity of PBPRP2 and PBPRP5 could be the consequence of convergence or homoplasy. Alternatively, PBPRP5 might represent a reinsertion of a processed mRNA of a locus 2 gene member, perhaps through some retroviral activity. However, if this occurred, then the regulatory elements for PBPRP5 would most probably be lost and the gene would either cease to express or express in a non-olfactory context, neither of which is the case (Park et al., 2000). A third possibility is that PBPRP5 is ancestral to the locus 2 cluster, that a translocated duplicate of PBPRP5 founded the locus 2 gene cluster, acquiring introns and establishing the locus 2 cluster through further duplication events. A fourth possibility is that PBPRP5 is simply a relocated locus 2 relative which lost its introns. The expression of PBPRP2 and PBPRP5 was characterized by Park et al. (2000) and Shanbhag et al. (2001). PBPRP5 was detected in sensilla of the adult antenna and in cells of the dorsal organ of the larval antenno–maxillary complex. PBPRP2 was detected in both olfactory and taste epithelium of adults, but surprisingly was not found in the receptor lymph; instead it was seen in the subcuticular spaces next to sensilla, or in a non-neuronal cavity of taste sensilla. This apparently non-sensory localization of PBPRP2 suggested that this protein does not function as an odor carrier, cautioning that OBP homologues should not be assumed to be odor carriers solely on the basis of sequence similarity (Park et al., 2000). On the other hand, sequence analyis (Fig. 11C) showed a similarity between PBPRP1 and PBPRP5 and an OBP homologue (CRKBP) isolated from taste sensilla of the blowfly, which is believed to have a role in chemodetection (Ozaki et al., 1995); thus, PBPRP2 may have a poorly understood role in processing odor-like molecules. The PBPRP5 expression patterns seem consistent with other OBPs, implying that it has retained regulatory elements that are characteristic of OBPs and arguing against an intron-free origin by retroviral reinsertion.

Regulation of OBP expression

pbp1Msex and *gobp2Msex* are coexpressed temporally, but differentially expressed spatially. In developing adult antennae, both genes were previously shown to be expressed in response to a decline in ecdysteroids (Vogt et al., 1993); in larvae, *gobp2Msex* expression ceases when ecdysteroid levels rise and resumes when levels fall (Fig. 8). In both adults and larvae, the support cells expressing and secreting OBPs have additional roles, growing out to cast the hair and expressing and secreting the proteins which form the sensillum cuticle (e.g. Sanes and Hildebrand, 1976; Keil, 1992). The support cells apparently partition their resources, temporally separating the expression and secretion of cuticle proteins from the expression and secretion of OBPs; changing levels of ecdysteroids appear to coordinate these processes.

The mechanism for regulating differential OBP expression is not known, but it must be linked to the determination and expression of sensilla phenotype. Sensilla phenotypes are characterized by many features, including morphology of the cuticular portion of the sensillum, numbers and morphologies of neurons, synaptic targets of the olfactory neurons, and the combinatorial expression of olfactory gene products including OBPs, ORs and ODEs. In *D. melanogaster*, some 30 OBP and 60 OR genes are presumably differentially expressed in specific combinations among a large number of sensilla of adult and larval chemosensory organs. Functional analysis of *D. melanogaster* antennal basiconic sensilla identified seven distinct subtypes of sensilla encapsulating 16 different types of olfactory receptor neurons (de Bruyne et al., 2001; Rogers and Firestein, 2001). These sensillum subtypes were distributed in non-overlapping spatial domains on the antennal surface, suggesting the likelihood that spatial cues have a role in the determination of phenotype. Spatial cues might also be involved in *M. sexta* antennae, influencing the phenotype of pheromone sensilla in the peripheral sensory zones of male antennae. However, the mid-annular region of male antennae and the entire sensory region of female antennae contain mixed populations of sensilla that intermingle (Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a,b; 2001); in these regions, stochastic rather than positional mechanisms may play a dominant role in determining sensilla phenotypes.

Because OBPs and ORs are expressed in different cell types, coordinated combinatorial expression of these proteins may require communication between the sensilla support cells which express OBPs and the olfactory neurons which express ORs. Such communication has been described in larval sensilla in *D. melanogaster*, where neuronal coexpression of the BarH1 and BarH2 homeodomain proteins is required for the trichogen/tormogen cells to construct a plate-like campaniform sensillum; the trichogen/tormogen cells construct a hair-like trichoid sensillum when BarH1 and BarH2 are deleted (Higashijima et al., 1992). Thus BarH1 and BarH2 must be part of a communication pathway that coordinates distinct cell types, neurons and support cells, to express a unified sensilla phenotype.

The determination of sensilla phenotype is influenced by a

series of hierarchical developmental decisions, which range from the selection of neuronally competent epithelial cells to the asymmetric differentiation of specific sensilla cells (e.g. Ghysen and Dambly-Chaudiere, 1993; Posakony, 1994; Jan and Jan 1993, 1995; Lu et al., 1998, 2000). Several studies have shown that the morphological phenotype of *D. melanogaster* olfactory sensilla (campaniform, trichoid or basiconic) is influenced by specific proneural genes that are expressed early in sensilla development (Vosshall, 2000, 2001). Expression of Atonal (bHLH) is required for the formation of campaniform sensilla (Gupta and Rodrigues, 1997), while similar expression of Amos (bHLH) is required for the formation of trichoid and basiconic sensilla (Goulding et al., 2000; Huang et al., 2000). Expression of Lozenge is required for all basiconic sensilla and some trichoid sensilla (Gupta et al., 1998). Proneural decisions might determine the final phenotype of a sensillum. For example, olfactory sensilla cells of lepidoptera have been suggested to be clonally related, deriving from a common sensory mother cell (SMC) following proneural selection of the SMC (Sanes and Hildebrand, 1976; Keil, 1992); the phenotype of these sensilla could be determined during SMC selection. However, in *D. melanogaster*, olfactory sensilla cells are suggested to be non-clonally related, and are recruited following a proliferative phase by a designated founder cell (Ray and Rodrigues, 1995; Reddy et al., 1997). In this case, determination of the mature sensilla phenotype would presumably follow recruitment.

The selection of one among many members of a gene family has been of interest regarding vertebrate ORs. In rodents, an olfactory receptor neuron selects one allele of about 1000 OR genes, and various models are being investigated for both the gene selection process and the mechanism of allelic exclusion (Chess et al., 1994; Chess, 1998; Ebrahimi et al., 2000; Mombaerts, 1999; Reed, 2000; Serizawa et al., 2000; Wang et al., 1997). One speculation is that some aspects of olfactory gene selection are cluster-dependent. In one study, neurons expressing a group of clustered OR genes all targeted adjacent glomerulae in the olfactory bulb, suggesting that OR genes residing in a cluster are subject to some level of coregulation, and further supporting a link between an olfactory neuron's selected OR gene and the neuron's synaptic target (Strotmann et al., 1999). A similar suggestion was made for two OBP genes of *D. melanogaster*. The genes encoding OS-E and OS-F (also termed PBPRP5) are adjacent to one another and are coexpressed in adult olfactory sensilla, leading to the suggestion that the clustering of these genes was linked to their coregulation. In contrast, however, we have described two lepidopteran OBP genes, *pbp1Msex* and *gobp2Msex*, which are also adjacent one another but are clearly not coexpressed.

Gene clustering is not a consequence of regulation but rather a consequence of gene duplication, the result of DNA repair following a misalignment during recombination (e.g. Freeman and Herron, 1998). The inclusion or exclusion of specific regulatory elements in the misalignment influences the relative expression of the resulting genes, translocation events distribute the genes throughout the genome, and evolutionary selection further shapes both the function and expression of the genes. Except for one very curious pairing (OR56a in locus 6), the OBP and OR genes of *D. melanogaster* are not physically linked; coregulation of specific ORs and OBPs must be accomplished in a cluster-independent manner. Presumably, the regulation and coregulation of these genes occurs at multiple levels. *pbp1Msex* and *gobp2Msex* could be temporally regulated as a cluster, but the two genes are spatially regulated in an apparently independent manner since they are differentially expressed.

Evolution of insect OBPs

Insects emerged about 400 million years ago (Mya) and include more than 800,000 named species with upper estimates ranging from 1.5–30 million species (Erwin, 1982; Kristensen, 1991). 25 of the 28 extant insect Orders belong to the division Neoptera, which emerged about 300 Mya and includes approx. 98 % of species (Kukalová-Peck, 1991; Freeman and Herron, 1998). The Neoptera include two major lineages: the orthopteroids, which include cockroachs, grasshoppers and termites, and the sister hemipteroid and holometabolous lineages, which include true bugs (hemipteroids) and moths, bees, beetles and flies (Hennig, 1981; Kristensen, 1991). OBP sequences are published for insect orders of the holometabolous and hemipteran lineages (e.g. Vogt et al., 1999), and recently have been identified in cockroaches (K. Robinson, R. Anholt, C. Schal and S. Riviere, personal communication), suggesting that this gene family is distributed throughout the Neoptera and appeared at least 300 Mya. Dipteran and lepidopteran lineages diverged before 250 Mya, initially as dipteran/mecopteran/siphonapteran and lepidopteran/trichopteran lineages; Diptera emerged by 250 Mya and Lepidoptera by 235 Mya (Whalley, 1986; Kukalová-Peck, 1991; Pashley and Ke, 1992; Friedrich and Tautz, 1997; Wiegmann et al., 2000).

In all analyses of multi-order OBPs, the PBPs and GOBPs consistently form a distinct lepidopteran subgroup (e.g. Vogt et al., 1999; Robertson et al., 1999), suggesting they form a lepidopteran specific OBP subfamily. The identity of this subfamily is supported by the current study, which suggests that *pbp1Msex* and *gobp2Msex* are related by gene duplication, their physical proximity being too close to have occurred by an arbitrary translocation event. In *D. melanogaster*, OS-E and OS-F genes also reside in close proximity; however, OS-E and OS-F are quite similar in sequence and always coexpress in olfactory sensilla (Hekmat-Scafe et al., 1997), in contrast to PBP1*Msex* and GOBP2*Msex*, which differ considerably in sequence and expression. An evolutionary analysis of OS-E and OS-F in several *Drosophila* species suggests these two genes emerged from a duplication event that occurred at least 40 Mya (Hekmat-Scafe et al., 2000). The PBPs and GOBPs diverged much earlier, at least 100 Mya, based on the identification of these genes in the lepidopteran superfamilies Bombycoidea, Sphingiodea, and Noctuoidea; the Noctuoidae are thought to have emerged as early as 100 Mya (Pashley and Ke, 1992). So far no efforts have been made to identify the

PBP/GOBP subfamily in more ancestral lepidopteran lineages. The fact that PBP1*Msex* and GOBP2*Msex* genes have retained their proximal relationship is curious, and may support their coordinated expression or indicate a unique importance in lepidopteran olfactory behaviors.

OBPs and ORs are gene products that function at the interface between the organism and its environment. No other sensory system employs such large and divergent gene families to decipher the environment. The peripheral role of gene products such as OBPs and ORs allows for a certain malleability; few other gene families are or can afford to be as volatile in their evolution. There is apparently little consistency in the known mechanisms regulating the differential expression of large gene families (Chess et al., 1994). Because of the size, diversity and differential yet combinatorial expression of the OBP and OR gene families, their genomic organizations offer not only a glimpse into the evolutionary history of chemosensory behavior, but also a potentially important model system for elucidating novel mechanisms regulating the expression of large gene families.

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Note added in proof

Since this manuscript was accepted, the identification of 34 *Drosophila* OBP homologues was published (Galindo and Smith, 2001); most were identified from the *Drosophila* genome database and named for their map locations. All sequences identified in the current manuscript were included in Galindo and Smith (2001), with the exceptions of CG15505, CG12665, CG7584 and CG2297. Combining the identifications from both manuscripts would suggest that the *Drosophila* genome contains at least 38 OBP-related homologous genes.

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