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Novel Odorant-binding Proteins Expressed in the Taste Tissue of the Fly

Masayuki Koganezawa and Ichiro Shimada

Biological Institute, Graduate School of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan

Correspondence to be sent to: Masayuki Koganezawa, Biological Institute, Graduate School of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan. e-mail: kogane@mail.cc.tohoku.ac.jp

Abstract

A taste tissue cDNA library of the fleshfly *Boettcherisca peregrina* was screened with a subtracted cDNA probe enriched with taste-receptor-tissue-specific cDNA. Seven genes were identified with sequence similarity to insect odorant-binding protein (OBP) genes. The predicted amino acid sequences of the genes contain the putative signal peptide sequence at the N-terminal and most of them conserve the six cysteines common to known insect OBPs. These genes show a high degree of sequence divergence with ~20% amino acid identity. The most striking feature was that all seven of these genes are expressed mainly in the taste tissues, such as the labellum and tarsus, unlike the known insect OBP genes expressed in olfactory tissue. The predicted amino acid sequences had the highest degree of sequence similarity to the *Drosophila melanogaster* OBPs named pheromone binding protein-related proteins (PBPRPs). These gene products are here referred to as gustatory PBP-related proteins (GPBPRPs) 1–7. Homologous GPBPRP genes were found also in *D. melanogaster* by database search and are shown to be expressed in *Drosophila* taste tissues.

Introduction

Odorant-binding proteins (OBPs) and pheromone binding proteins (PBPs) are small, water-soluble proteins identified from the chemosensory organs of vertebrates and insects (Pelosi and Maida, 1990; Pelosi, 1996). The name 'binding protein' originates from the facts that PBP of moth binds with sex pheromones (Vogt and Riddiford, 1981) and that some mammalian OBPs bind with volatile odorants (Pelosi *et al.*, 1982). There is no significant sequence similarity between insect OBPs and vertebrate OBPs, but they are presumed to play a similar role (Pelosi and Maida, 1990). The electrical response of the chemosensory receptor cell is initiated by the binding of stimulants with the receptor molecule on the cell membrane. Because the receptor cells are surrounded by a hydrophilic environment, it is considered that hydrophobic molecules such as volatile odorants or pheromones have difficulty reaching the receptor cell membrane. OBPs are thought to transport these hydrophobic molecules into the hydrophilic environment and regulate the chemosensory response. The mutation of one OBP gene of *Drosophila*, *lush*, results in abnormal chemoattractive behavior to alcohol (Kim *et al.*, 1998). The antibody to one OBP-related protein of the blowfly *Phormia regina* (chemical-sense-related lipophilic ligand-binding protein, CRLBP) blocks the response of the taste receptor cell to a stimulant containing hydrophobic molecules (Ozaki *et al.*, 1995). These functional studies support the idea that OBPs can transport hydrophobic molecules.

The taste receptor organ of the fly is the chemosensory hair, many of which are found on the labellum and tarsi. It has been one of the most intensively used preparations at the single receptor cell level for electrophysiological and pharmacological experiments because of its simple structure compared with that of vertebrates. One chemosensory hair has only four receptor cells, named the 'sugar', 'salt', 'water' and 'fourth' receptor cells, which respond specifically to sugars and amino acids, salt, water and fatty acid salts, respectively (Dethier, 1976; Hansen, 1978; Morita and Shiraishi, 1985; Morita, 1992). Despite the complexity of the vertebrate taste receptor organ, many kinds of molecular components of vertebrate taste reception have been identified, including the taste receptor molecules and the signal transduction molecules (McLaughlin *et al.*, 1992; Ugawa *et al.*, 1998; Hoon *et al.*, 1999; Adler *et al.*, 2000; Chaudhari *et al.*, 2000; Matsunami *et al.*, 2000). In contrast, the nature of taste reception at the molecular level in the fly is still unclear.

The differential screening method is often used to identify a gene specifically expressed in a certain tissue. Applying this method to the study of chemoreception, several kinds of genes have been cloned successfully, such as the olfactory receptor genes of the mammalian vomeronasal organ (Dulac and Axel, 1995), the candidate mammalian taste receptor genes (Hoon *et al.*, 1999) and the OBP genes of *Drosophila* (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). To identify genes related to taste reception in the fleshfly

Boettcherisca peregrina, we performed differential screening of a taste tissue cDNA library. This cDNA library was screened with a subtracted cDNA probe enriched with taste-receptor-tissue-specific cDNA to ensure effective screening. In this procedure, 418 cDNA clones predominantly expressed in the taste tissue were obtained, of which seven genes with sequence similarity to insect OBP genes were identified and are here referred to as gustatory pheromone binding protein-related proteins (GPBPRPs). The expression of these genes was specific to taste tissue: strikingly different from other insect OBPs, which are expressed predominantly in the antenna.

Materials and methods

Animals

Two fly species were reared in our laboratory at 25°C and used for the experiments: the fleshfly, *B. peregrina*, and the fruitfly, *D. melanogaster*. For the latter, Canton-Special (CS) was used as the wild type.

Construction of the taste tissue cDNA library

The labella of adult fleshflies were dissected out under a microscope and collected in a microcentrifuge tube cooled by dry ice–ethanol solution. The samples were stored at –80°C until use. Poly(A)$^+$ RNA was extracted using a QuickPrep mRNA purification kit (Amersham Pharmacia Biotech). The labellar cDNA library was constructed with a SuperScript Lambda System for cDNA Synthesis and λ cloning (GIBCO BRL), following the protocol provided.

To prepare taste-receptor-rich tissue (TRRT), adult fleshfly labella from which the pseudotracheal organ had been removed were collected in cold fly Ringer solution (111.2 mM NaCl, 55 mM KCl, 0.8 mM $CaCl_2$, 1.2 mM $NaHCO_3$, 0.08 mM NaH_2PO_4, 1.8 mM $MgCl_2$, 5 mM HEPES, pH 7.1). Figure 1a shows a photograph of the dissected labellum and the pseudotracheal organ. Then the dissected labella were treated with 1 mg/ml collagenase (Sigma) in Ca^{2+}-free Ringer solution (111.2 mM NaCl, 55 mM KCl, 1.2 mM $NaHCO_3$, 0.08 mM NaH_2PO_4, 2.6 mM $MgCl_2$, 5 mM HEPES, pH 7.1) for 40 min at 37°C. After the treatment, epidermal tissues were easily removed from the outer shell with microforceps. Figure 1b and c show the photograph of TRRT stained with methylene blue. TRRT have globular groups of cells connected with the outer shell (Figure 1c, arrow heads) which are presumed to contain only taste receptor cells, mechanosensory receptor cells and supporting cells (trichogen, tormogen and thecogen cells). Total RNA was extracted from TRRT using an RNeasy Mini kit (QIAGEN), then the first strand cDNA was synthesized using SuperScript II RNase H$^-$ Reverse Transcriptase (Gibco BRL). As the amount of RNA extracted from TRRT was very small, TRRT cDNA was amplified using a SMART PCR cDNA synthesis kit (Clontech), following the protocol provided.

Adaptor-ligated double-strand cDNAs of labella or TRRT were ligated into λ ZIPLox (GIBCO BRL), and then *in vitro* packaging was performed using a Gigapack III Gold packaging extract (Stratagene). Y1090(ZL) (Gibco BRL) was used as the host cell.

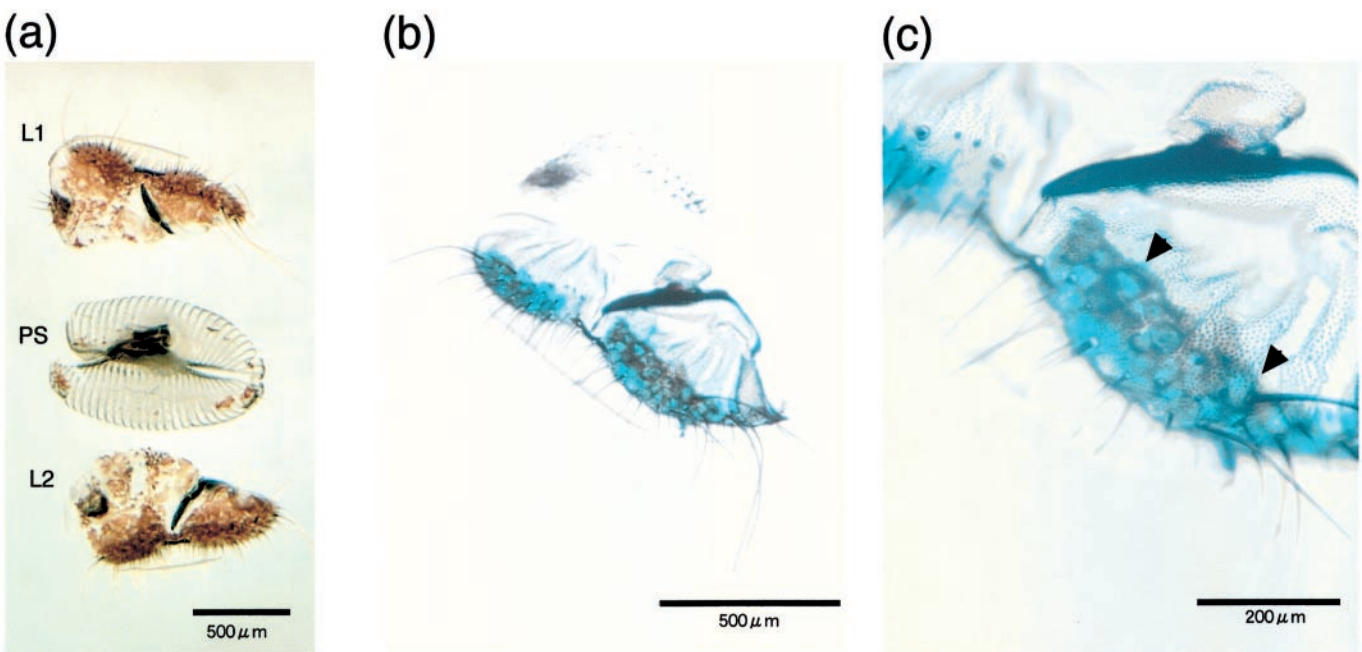


Figure 1 Preparation of taste-receptor-rich tissue (TRRT). **(a)** The dissected halves of labellum (L1 and L2) and a pseudotracheal organ (PS). **(b)** TRRT stained with methylene blue after collagenase treatment and removal of the epidermal tissues. **(c)** Globular group of cells at the base of taste hairs. The positions of the bases of the largest type taste hairs (LL type) are indicated by arrowheads.

Generation of subtracted cDNA probe

The subtraction of non-taste tissue cDNA from the taste tissue cDNA was performed using a Clontech PCR-Select cDNA subtraction kit (Clontech). TRRT and tarsus were used as the taste-receptor-cell-containing tissues. Eye, antenna and femur were used as the tissues without taste receptor cells. We generated two kinds of subtracted cDNA probe: tarsus cDNA from which femur and antenna cDNA were subtracted [T–(F+A) probe] and TRRT cDNA from which eye cDNA was subtracted (TRRT–Eye probe). The starting materials for subtraction were total RNA for the T–(F+A) probe and SMART PCR-amplified cDNA for the TRRT–Eye probe. Southern blot analysis was performed to confirm the subtraction efficiency.

Differential screening to identify genes expressed in the taste tissue

The taste-tissue cDNA libraries were screened under high stringency with the subtracted cDNA probe as the positive probe and the unsubtracted non-taste-tissue cDNA as the negative probe. Each cDNA probe was labeled with [α-^{32}P]dCTP using an RTS RadPrime DNA labeling system (Gibco BRL). The cDNA library was plated at low density to enable identification and isolation of single plaques at the first screening (2000–5000 pfu in 9 cm dish). Plaques hybridized with the positive probe, but not with the negative probe, were selected. The cDNA inserted in a λ ZIPLox vector was amplified by PCR using the vector-specific primers and Southern blot analysis was performed as the second screening. Usually, PCR was performed using AmpliTaq Gold DNA polymerase (Perkin-Elmer) and a TaKaRa PCR Thermal Cycler MP. Each PCR product was electrophoresed, transferred to a membrane (Hybond-N$^+$; Amersham Pharmacia Biotech) and hybridized with the positive probe or the negative probe under the high-stringency conditions. Again, the clone selected was that hybridized with the positive probe but not with the negative probe. The selected samples were recovered in the autonomously replicating plasmid pZL1 using an *in vivo* excision protocol provided by Gibco BRL. The cDNA inserts were sequenced using a Labstation Thermo Sequenase labeled primer cycle sequencing kit with 7-deaza-dGTP (Vistra) and a Hitachi DNA sequencer 5500, or a ThermoSequenase II cycle sequencing kit (Amersham Pharmacia Biotech) with an ABI 373A DNA sequencing system.

Analysis of cDNA sequences

After the sequences of cDNA were determined, a homology search was performed using NCBI BLAST (http://www.ncbi.nlm.nih.gov) and genes of interest were selected. After the full DNA sequence of the longest clone of each gene of interest was determined, the predicted amino acid sequence was determined. The signal peptide sequence cleavage site was predicted using the SignalP program (http://www.cbs.dtu.dk/services/SignalP). The amino acid identity was calculated from the sequence after removal of the signal peptide sequence. The Clustal X program was used for multiple sequence alignment and the PHYLIP program package (Version 3.6a for Macintosh) was used for the phylogenetic analysis.

Virtual Northern blot analysis

The term 'Virtual Northern blot' appears in the protocol of the SMART PCR cDNA synthesis kit (Clontech) and refers to the method of blotting total cDNA instead of RNA. Virtual Northern blot analysis can provide information similar to that obtained by standard Northern blot analysis. The labella, tarsi, antennae, eyes and guts of the fleshflies were collected and total RNA was extracted using an RNeasy Mini kit (QIAGEN). The total RNA sample was treated with RNase-free DNase I (Promega) to eliminate contamination with genomic DNA. The first strand cDNA was synthesized by SuperScript II RNase H$^-$ Reverse Transcriptase (Gibco BRL). Each cDNA was amplified using a SMART PCR cDNA synthesis kit (Clontech). Amplified cDNAs (1500 ng) were electrophoresed, transferred to nylon filter and hybridized with [α-^{32}P]dCTP labeled probe under high-stringency conditions. The probes were prepared from plasmid containing GPBPRP cDNA by PCR with the specific primers. The primer sequences of each GPBPRP were as follows:

GPBPRP1 forward primer,
GTTTCGAATATTCGGGCGGA;

GPBPRP1 reverse primer,
TGCCAATTCTATGGCCGCAT;

GPBPRP2 forward primer,
GCTTCGATAAAAAACAAGCCCTCG;

GPBPRP2 reverse primer,
CTTTGCAACTGCCATGCGG;

GPBPRP3 forward primer,
GCTTTAATGTCGTTTGGTGAGGAC;

GPBPRP3 reverse primer,
TCAGAGAACATGGAACCCATTAGG;

GPBPRP4 forward primer,
CATCGCTGCTGTCAGTGCC;

GPBPRP4 reverse primer,
CAATTCCTTCCATTCCTCGGGT;

GPBPRP5 forward primer,
GAGGTTGCCAAAATACGCAGC;

GPBPRP5 reverse primer,
ATCACAGCGATCAGCACCCT;

GPBPRP6 forward primer,
ATACGTAGCACTTGTCAAGCGG;

GPBPRP6 reverse primer,
CCCTATTTAAACCATTTACGCACA;

GPBPRP7 forward primer,
TTTGCTTTATTGCTGGCGCTT;

GPBPRP7 reverse primer,
CATCATCTTGTACTTGGCCGTCC.

Each primer has a similar T_m value and the annealing temperature of PCR was set at 60°C for all reactions. The sizes of PCR products are 222 bp (GPBPRP1), 260 bp (GPBPRP2), 203 bp (GPBPRP3), 271 bp (GPBPRP4), 213 bp (GPBPRP5), 305 bp (GPBPRP6) and 231 bp (GPBPRP7).

RT–PCR analysis

Labella, tarsi, antennae, heads devoid of antennae and proboscis, and guts of adult fleshflies were collected. For other experiments, the heads (including the first and second segments) of third larval stage and the labella of adult males and females were also collected. Poly(A)$^+$ RNA was extracted using a QuickPrep mRNA purification kit (Amersham Pharmacia Biotech). An RNase-free DNase I treated poly(A)$^+$ RNA sample of each tissue was used for the first strand cDNA synthesis. The amount of RNA in this first strand cDNA synthesis was standardized for each tissue. The specific primers for each GPBPRP are shown above. The number of cycles of RT–PCR was 24. The ribosomal protein 49 (RP49) gene was used as the internal control for each cDNA sample. To amplify the RP49 gene of *Boettcherisca*, we used the primers for the RP49 gene of *D. melanogaster* and performed RT–PCR at a low annealing temperature. The annealing temperature for RT–PCR of RP49 for *Drosophila* was 65°C, but that in *Boettcherisca* was 55°C. The amplified product was confirmed to have a high degree of sequence similarity with the RP49 gene of *Drosophila* (data not shown). The sequence of the primers for the RP49 gene of *Drosophila* are as follows:

RP49 forward primer,
AGATCGTGAAGAAGCGCACCAAG;

RP49 reverse primer,
CACCAGGAACTTCTTGAATCCGG.

The number of cycles of RT–PCR was determined so as not to reach the plateau of the reaction.

In the RT–PCR analysis of *Drosophila* GPBPRP gene homologs, labella, tarsi, antennae and heads devoid of proboscis and antennae were collected. One mutant, *pox-neuro*^{70} (*poxn*^{70}), was used to investigate the expression pattern of *Drosophila* GPBPRP gene homologs. The gene *poxn* encodes a possible transcriptional regulator and controls the differentiation of mechanosensory and chemosensory cells (Dambly-Chaudiere *et al.*, 1992; Nottebohm *et al.*, 1994; Awasaki and Kimura, 1997). In homozygous *poxn*^{70}/*poxn*^{70} flies, the chemosensory hairs (polyinnervated bristles) in the labellum and tarsus are transformed into mechanosensory bristles—monoinnervated bristles (Awasaki and Kimura, 1997). There are no taste receptor cells in the transformed bristle. Heterozygous *poxn*^{70}/+ flies show the normal phenotype of chemosensory hair and were used as control animals in RT–PCR analysis. Total RNA was extracted using an RNeasy Mini kit (QIAGEN). Other protocols were the same as those described above for *Boettcherisca*. The primer sequences were as follows:

CG1670 forward primer,
AATGTCATGGCTATCGCCGGTT;

CG1670 reverse primer,
TTATACTGCAACTGATCCTCGGGC;

CG11218 forward primer,
ATGAAGTTCCTGATTGTCCTCTCCG;

CG11218 reverse primer,
GGCGCGATTCTTGTAGTAGCACTC;

CG11797 forward primer,
TGAGTGCTCTTTTTGTGACTCTGGC;

CG11797 reverse primer,
GGTATCACACTTGTTCTCGCCCTTG;

CG13421 forward primer,
TCTTGACTGTCAGCGTGGTCTCC;

CG13421 reverse primer,
CTTCGGTGACCTCATCGCTCTG.

Each primer has a similar T_m value and the annealing temperature for PCR was set at 65°C for all reactions. The numbers of cycles of RT–PCR were 28 (CG11218, CG11797, CG13421 and RP49) and 42 (CG1670). The sizes of PCR products were 433 bp (CG1670), 387 bp (CG11218), 335 bp (CG11797) and 393 bp (CG13421).

Results

Isolation of seven classes of taste-tissue cDNAs with similar sequence to insect OBPs

We combined the cDNA subtraction and differential screening methods to identify the genes specifically expressed in taste receptor tissue. Two kinds of taste-tissue cDNA libraries were used: one was the labellar cDNA library and the other was the taste-receptor-rich tissue (=TRRT) cDNA library. The labellum has many taste hairs, but also contains non-taste tissue. To ensure effective screening, we prepared a TRRT and constructed the corresponding cDNA library. Then, cDNA probes were generated for differential screening by subtracting the cDNA of non-taste tissue from that of the taste tissue. This subtraction could enrich the cDNAs expressed in the taste-receptor tissue and would ensure identification of the specific genes at low expression levels.

The labellar cDNA library (20 000 pfu) and the TRRT

cDNA library (total 80 000 pfu) were screened with the T – (F + A) or TRRT – Eye probe as the positive probe and with unsubtracted, non-taste-tissue cDNA (i.e. femur + antenna cDNA or eye cDNA) as the negative probe. After multiple differential screenings, 418 cDNA clones hybridized with the positive probe but not with the negative probe were selected. After sequencing of these clones, seven classes of cDNAs with significant sequence similarity to insect OBP genes were identified by a database search with the BLAST program. Most of these clones had the highest similarity to *Drosophila* PBPRP genes (Pikielny *et al.*, 1994). Based on the taste-tissue-specific expression pattern of identified genes (see below), we named these gene products 'Gustatory PBP-related proteins (GPBPRPs) 1–7'. The size of the longest clone of each GPBPRP gene, except the poly(A) tail, was 733 bp (GPBPRP1), 627 bp (GPBPRP2), 831 bp (GPBPRP3), 907 bp (GPBPRP4), 552 bp (GPBPRP5), 555 bp (GPBPRP6) and 529 bp (GPBPRP7). The length of the predicted amino acid sequence of each gene was 144 aa (GPBPRP1), 148 aa (GPBPRP2), 148 aa (GPBPRP3), 127 aa (GPBPRP4), 132 aa (GPBPRP5), 130 aa (GPBPRP6) and 136 aa (GPBPRP7). In addition, multiple independent cDNA clones of each GPBPRP were obtained from cDNA libraries and these sequences were shown to be identical by multiple sequencing (data not shown).

Figure 2 shows the multiple sequence alignment of the predicted amino acid sequences of the identified GPBPRPs and several known fly OBPs. All GPBPRPs have the signal peptide sequence of secreted protein at the N-terminal, as shown by underlining. This suggests that GPBPRP was also a secreted protein, produced in supporting cells and secreted into the sensillum lymph, as with other known OBPs (Pelosi and Maida, 1995, Hekmat-Scafe *et al.*, 1997). All GPBPRPs, except GPBPRP4, have six cysteines: a feature in common with most insect OBPs (Figure 2a). GPBPRP4 did not contain conserved cysteines and had high degree of sequence similarity to other type of OBP, OS-D protein of *Drosophila* (McKenna *et al.*, 1994) and ejaculatory bulb protein III (PebIII) of *Drosophila (*Dyanov and Dzitoeva, 1995), which also do not have conserved cysteines (Figure 2b).

GPBPRP genes are expressed in taste tissue

Figure 3 shows the result of the virtual Northern blot analysis of GPBPRP genes. All genes were expressed in taste tissue (labellum and tarsus). Furthermore, the expression of GPBPRP genes, except for GPBPRP1 and GPBPRP4, was restricted to these taste tissues: significant expression in other tissues was not detectable. This pattern of taste-tissue specificity is novel, since most insect OBPs reported so far are expressed predominantly in antenna. There were some differences in the expression pattern of GPBPRP genes in the taste tissue. GPBPRP2, 3 and 5 genes were expressed more abundantly in tarsus than in labellum, while the expression level of GPBPRP6 and 7 was higher in labellum than in tarsus and GPBPRP1 gene was expressed in the taste tissue and antenna at equal levels, but was not detectable in eye or gut. The expression pattern of GPBPRP1 gene was similar to that of CRLBP of the blowfly (Ozaki *et al.*, 1995) and the PBPRP2 gene of *Drosophila* (Pikielny *et al.*, 1994). The GPBPRP4 gene was expressed in the taste tissue more abundantly than in other tissues, but there was significant expression in the antenna and eye also, and a trace was observed in gut.

Almost the same results were obtained by RT–PCR analysis of GPBPRP genes (Figure 4a). The expression of some GPBPRPs in antenna was observed when the number of cycles of RT–PCR was increased, as shown for GPBPRP5 and GPBPRP7 in Figure 4a, since the RT–PCR analysis is more sensitive than virtual Northern blot analysis. The expression level of most GPBPRP genes in antenna, however, was significantly lower than that in the taste tissue. The expression of GPBPRP genes is, therefore, highly localized to the taste tissues. The expression of all GPBPRP genes in TRRT was obtained by PCR using TRRT cDNA as a template (Figure 4b).

It has been shown that some PBPs of moths are expressed in the adult male antenna but not in the female antenna, so they are thought to regulate the response to pheromones (Vogt and Riddiford, 1981; Pelosi and Maida, 1990, 1995). In the GPBPRPs of the present study, sex dimorphism in expression level was not observed in the labellum (Figure 4c). Furthermore, the expression of some GPBPRPs was observed not only in adult taste tissue but also in larval tissue. GPBPRP2, 4, 5 and 7 genes were expressed in the head of the third larva (Figure 4d), which is known to contain the gustatory organs (Stocker, 1994).

GPBPRPs represent a new class of OBP

Figure 5 shows the phylogenetic tree of GPBPRPs and several known insect OBPs. The amino acid sequences (without signal peptide sequence) were aligned using the Clustal X program and the neighbor joining tree was constructed with the PHYLIP program package (V. 3.6a). The OBPs of moths are thought to be classified into at least three subfamilies: PBP; general odorant-binding protein 1 (=GOBP1); and GOBP2 (Pelosi and Maida, 1995; Vogt *et al.*, 1999). The sequence identities among OBPs in these subfamilies are ~60–80% across several species. Recently, another group of OBPs, named antennal-binding protein X (ABPX), has been identified (Krieger *et al.*, 1996; Robertson *et al.*, 1999). The amino acid identity among the ABPX group is ~60%. GPBPRPs were clearly far from such groups of OBPs of moth. Furthermore, the feature of GPBPRP was the high degree of diversity in the same species (*B. peregrina*). The amino acid sequence identity among GPBPRPs was small (typically 15–25%) and most appear to fall into the different groups. Such a high level of diversity within the same species was also reported for the PBPRPs of *Drosophila*, which are expressed mainly in antenna (Pikielny *et al.*, 1994). GPBPRP3, 5, 6 and 7 are

Figure 2 Multiple alignment of GPBPRPs and other fly OBPs. **(a)** GPBPRPs, except GPBPRP4, have six cysteines (#) commonly shared with the known insect OBPs. **(b)** GPBPRP4 has no conserved cysteines, but has high sequence similarity with OS-D protein and PebIII of *D. melanogaster*. Conserved residues are indicated by asterisks (*). Underlining indicates signal peptide sequences at the N-terminal. Abbreviations: CRLBPpreg, chemical-sense-related lipophilic ligand-binding protein of *Phormia regina* (Ozaki *et al.*, 1995); LUSH, LUSH protein of *D. melanogaster* (Kim *et al.*, 1998); OS-D, OS-D protein of *D. melanogaster* (McKenna *et al.*, 1994); PBPRP (1, 2 and 3), pheromone binding protein-related proteins *D. melanogaster* (Pikielny *et al.* 1994); PebIII, ejaculatory bulb protein III of *D. melanogaster* (Dyanov and Dzitoeva, 1995).

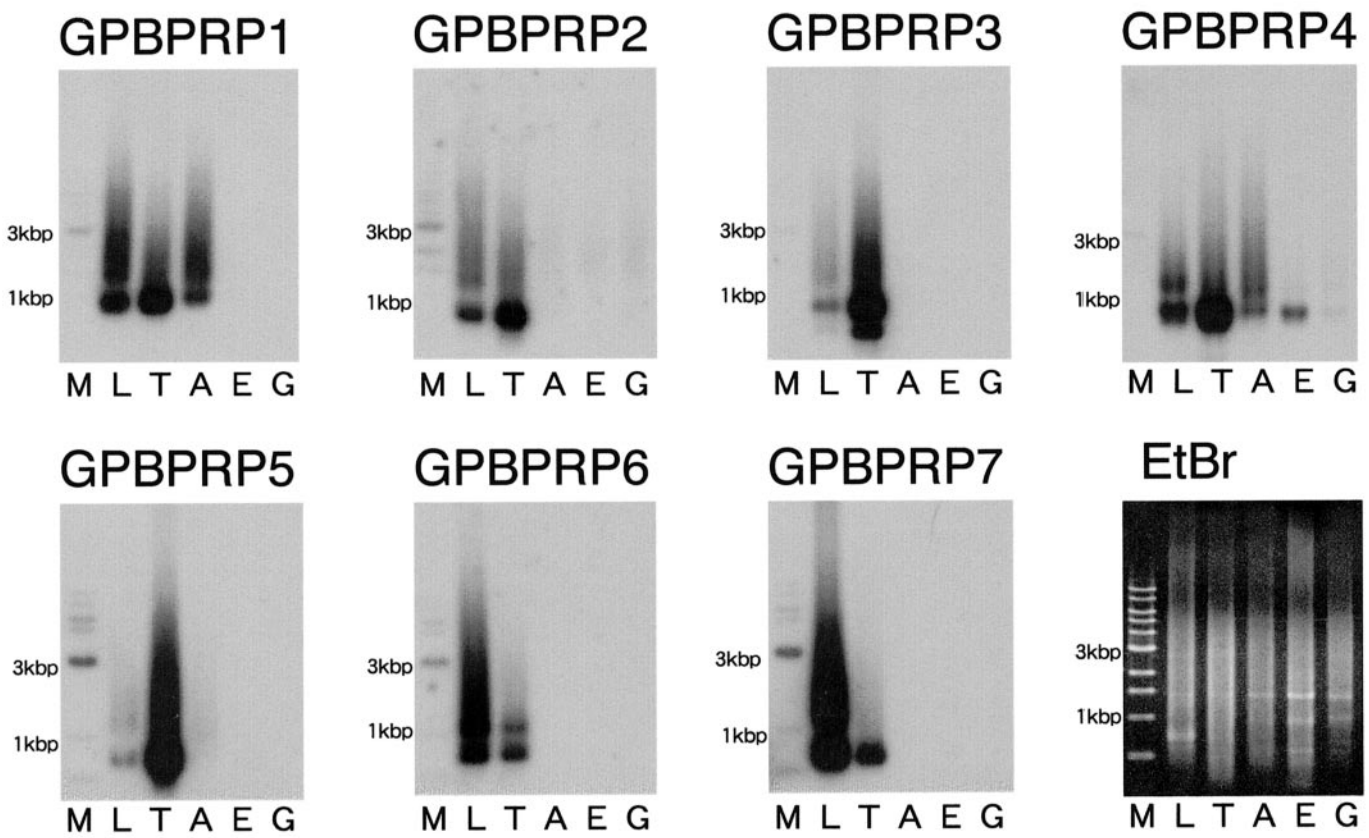


Figure 3 Virtual Northern blot analysis of GPBPRP genes. cDNA (1500 ng) amplified by using SMART PCR cDNA Synthesis Kit (Clontech) was loaded in each lane. Lanes indicated at the foot of each gel are mol. wt marker (M) and cDNAs prepared from labellum (L), tarsus (T), antenna (A), eye (E) and gut (G). The PCR fragment of each GPBPRP gene was labeled with [α-^{32}P] dCTP and hybridized under high-stringency conditions. Ethidium-bromide-stained gel (EtBr) is shown as control.

relatively far from the known *Drosophila* OBPs and may well belong to novel groups. This corresponds to the result that these four GPBPRP genes are expressed predominantly in taste tissues but little in other tissues (Figures 3 and 4a). In addition, the identity between GPBPRP5 and GPBPRP7 was relatively high (53%) and the two proteins could be classified in the same group.

GPBPRP1 has a relatively high sequence similarity to CRLBP of the blowfly, *Phormia regina* (73% identity): CRLBP is the major soluble protein in the taste sensillum of the blowfly (Ozaki *et al.*, 1995). The expression level of the GPBPRP1 gene was also the highest in the labellum among the seven GPBPRP genes (Figures 3 and 4a) and GPBPRP1 is thought to be a *Boettcherisca* homolog of CRLBP. The phylogenetic analysis placed GPBPRP1, GPBPRP2, CRLBP, PBPRP2 and PBPRP5 in the same group (bootstrap value = 57%). GPBPRP1 of *Boettcherisca*, CRLBP of *Phormia* and PBPRP2 of *Drosophila* have the same expression pattern. They are all expressed in labellum, tarsus and antenna (Pikielny *et al.*, 1994; Ozaki *et al.*, 1995).

GPBPRP4 of *Boettcherisca* has a relatively high sequence similarity with the PebIII (50% identity) and OS-D (39% identity) proteins of *Drosophila* (McKenna *et al.*, 1994; Dyanov and Dzitoeva, 1995). These proteins do not contain the six cysteines always conserved in other fly OBPs. Some proteins with significant sequence similarity to GPBPRP4 have been reported in other insects (Nomura *et al.*, 1992; Maleszka and Stange, 1997; Robertson *et al.*, 1999; Bohbot *et al.*, 1998). The similarity is high across several species in different orders, such as Diptera, Lepidoptera and Blattaria (~40% identity) and some proteins are expressed not only in antenna but also in other tissues, as with the GPBPRP4 gene (Nomura *et al.*, 1992; Maleszka and Stange, 1997).

GPBPRP-related genes in *Drosophila*

The GPBPRP sequence information obtained in the present study was used to perform a homology search with the complete *Drosophila* genome sequence (Adams *et al.*, 2000) and the GPBPRP amino acid sequences (except for GPBPRP4) were used to search the *Drosophila* protein database with the BLASTP program. Predicted gene products scoring >35 were selected. This procedure produced 13 predicted genes with GPBPRP sequence similarity: CG1670, CG7592, CG8462, CG11218, CG11748, CG11797, CG12944, CG13421, CG13873, CG13874, CG15129, CG15457 and G15883. They show a high degree

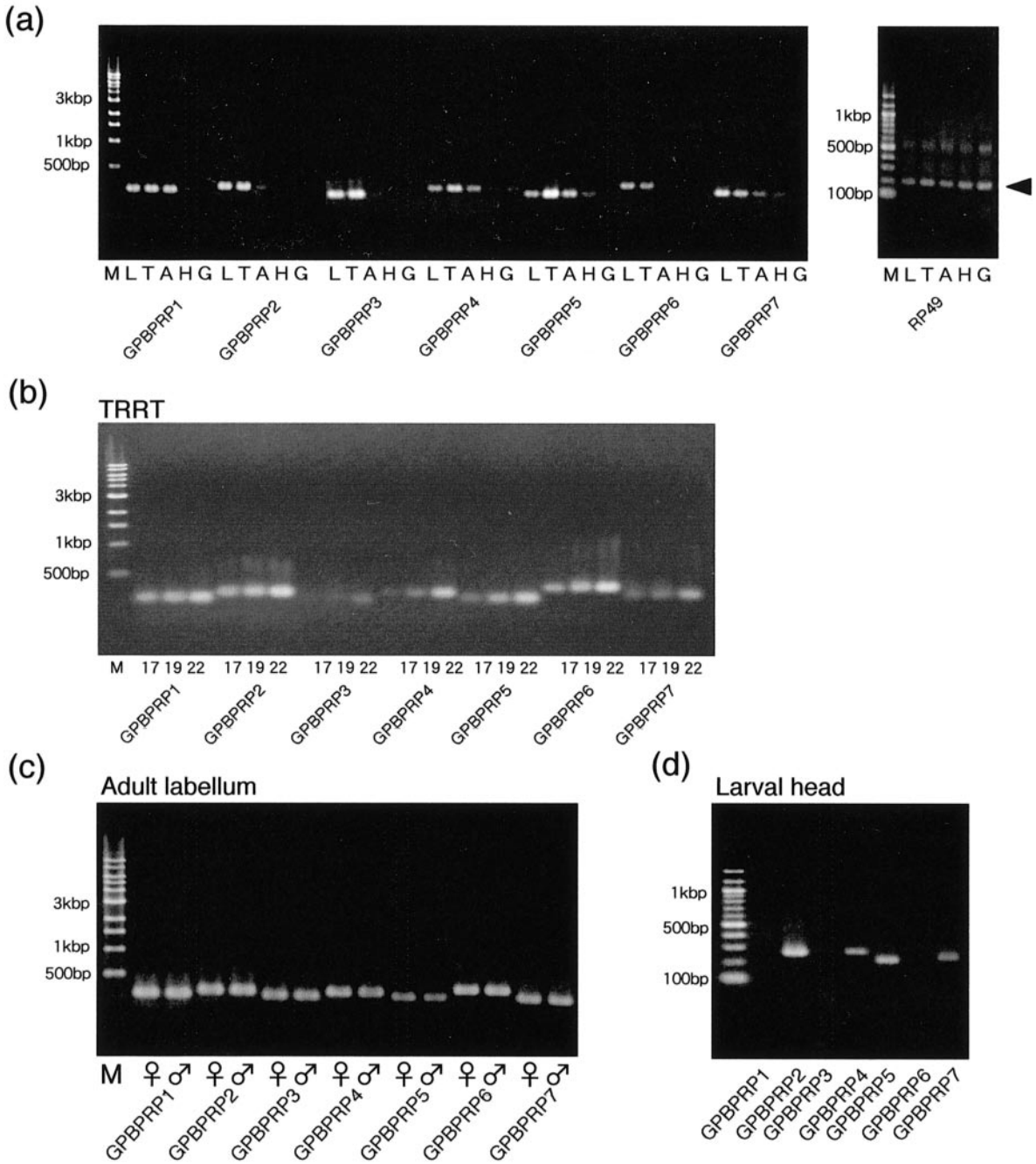


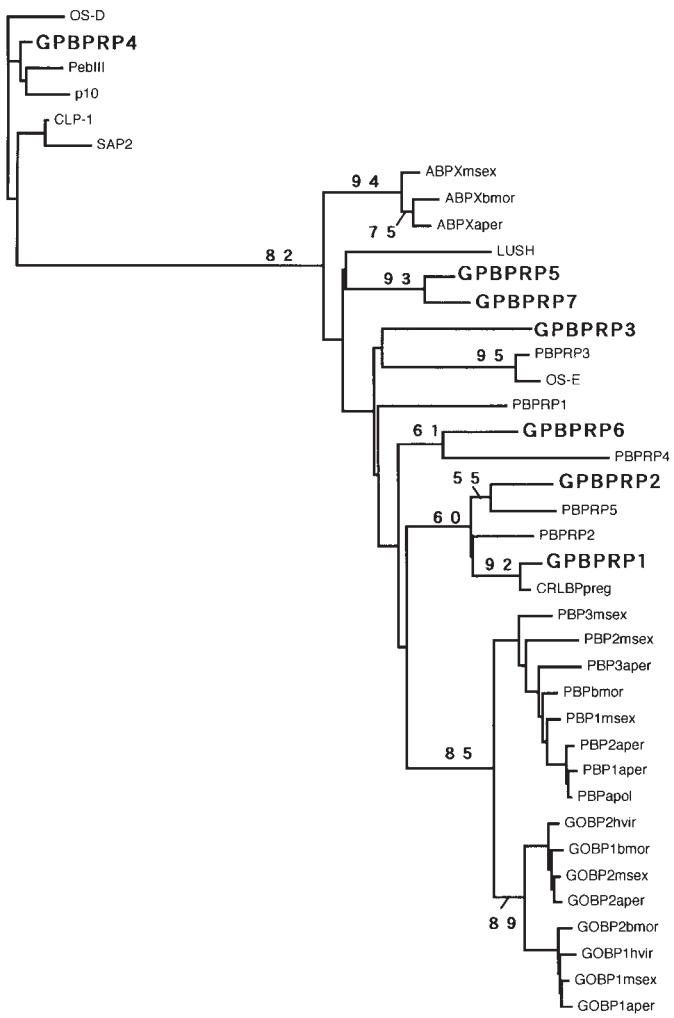
Figure 4 RT–PCR analysis of GPBPRP genes. **(a)** RT–PCR was carried out with the specific primers for each GPBPRP gene. The templates were prepared from labellum (L), tarsus (T), antenna (A), head without proboscis and antenna (H) and gut (G). The mol. wt marker is in lane M. Control RT–PCR was performed with primers of the RP49 gene of *D. melanogaster* (PCR product size is 206 bp). **(b)** PCR analysis of GPBPRP genes in TRRT. The number below each lane indicates the number of cycles of PCR. **(c)** RT–PCR analysis of GPBPRP genes in the labellum of adult males and females. **(d)** RT–PCR analysis of GPBPRP genes in the first and second segments of the third larval stage containing the chemoreceptor organs.

of sequence diversity, like the GPBPRPs of *Boettcherisca* and the PBPRPs of *Drosophila*. All of these genes had the six conserved cysteines, but in some of them the signal peptide sequence at the N-terminal could not be estimated with the SignalP program (data not shown). It is noted that many of these *Drosophila* GPBPRP gene homologs are located physically close to each other on the chromosomes. Genes on the cytogenetic map at positions 56–57 included CG8462 (56E4–5), CG11797 (56E4), CG11218 (56E4–5), CG13421 (57A6–7), CG13873 (56F1), CG13874 (56F1–2) and CG15129 (56E4). Another gene cluster involves PBPRP2 gene, CG1670, CG11748 and CG15457 mapped

to 19D2. There have been no reports of such clustering of OBP genes, other than for the very similar genes OS-F (=PBPRP3 gene) and OS-E (Hekmat-Scafe *et al.*, 1997).

Figure 6a shows the phylogenetic tree of the known OBPs of *Drosophila*, GPBPRPs of *Boettcherisca* and the GPBPRP homologs of *Drosophila* containing a recognizable putative signal peptide sequence. GPBPRP5 and GPBPRP7 of *Boettcherisca*, and the products of CG8462, CG11218 and CG11797 of *Drosophila*, have relatively high sequence similarity (33–65% identity) and appear to be in same subgroup (Figure 6a). Furthermore, the three *Drosophila* genes are cytogenetically close, at 56E4–5. We performed RT–PCR analysis to ascertain whether or not these predicted GPBPRP gene homologs are really expressed in taste tissue. Because GPBPRP3, 5, 6 and 7 of *Boettcherisca* are apparently new OBPs expressed almost exclusively in taste tissue, we selected the predicted genes of *Drosophila* that were the most similar to these four GPBPRPs. They were CG1670, CG11218, CG11797 and CG13421. Figure 6b shows the RT–PCR analysis of the expression of these four *Drosophila* GPBPRP gene homologs. As with the GPBPRP genes of *Boettcherisca*, they were expressed in labellum and tarsus. However, unlike in *Boettcherisca*, three of the four genes were also expressed in antenna at almost the same level in this preparation. No signal was observed in lane L (labellum) for CG1670 in Figure 6b, but the expression of this gene in the labellum was confirmed by RT–PCR with more cycles (data not shown).

Finally, we performed the RT–PCR analysis using the $poxn^{70}$ mutant. Figure 6c shows the RT–PCR fragments of CG11218, CG11797, CG13421 and RP49. In both labellum and tarsus, each *Drosophila* GPBPRP gene homolog was expressed almost at a same level in $poxn^{70}$ /$poxn^{70}$ fly and $poxn^{70}$/+ fly.



Discussion

Novel feature of GPBPRPs in insect OBP genes

Using differential screening, seven GPBPRP genes that have sequence similarity with known insect OBP genes were identified in the fleshfly *B. peregrina*. The special feature of these GPBPRP genes is their predominant expression in taste tissue. All GPBPRP genes are expressed in labellum and tarsus, and most of them are scarcely expressed in other (non-taste) tissues, including antenna, head and gut. Many of the known insect OBPs are expressed predominantly in the adult antenna. Some OBPs, such as PBPRP2 of *Drosophila* and CRLBP of blowfly, are reportedly expressed

Figure 5 Phylogenetic tree of GPBPRPs and other insect OBPs. Amino acid sequences (excluding the signal peptide sequence) were aligned by the Clustal X program and the phylogenetic tree was constructed by NJ methods. OS-D protein is regarded as the out group when the tree is drawn. Except for the branches in the PBP, GOBP1 and GOBP2 subfamilies of moths, bootstrap values (1000 replications) >50% are shown above branches. Abbreviations: ABPXaper, ABPXbmor and ABPXmsex, antennal binding protein X of *Antheraea pernyi* (Mameli, 1997), *Bombyx mori* (Krieger *et al.*, 1996) and *Manduca sexta* (Robertson *et al.*, 1999), respectively; CLP-1, CLP-1 protein of *Cactoblastis cactorum* (Maleszka and Stange, 1997); CRLBPpreg, chemical-sense-related lipophilic ligand-binding protein of *Phormia regina* (Ozaki *et al.*, 1995); GOBP1aper and GOBP2aper, general odorant-binding proteins 1 and 2 of *A. pernyi* (Breer *et al.*, 1990; Mameli *et al.*, 1997); GOBP1bmor and GOBP2bmor, general odorant-binding proteins 1 and 2 of *B. mori* (Krieger *et al.*, 1996); GOBP1hvir and GOBP2hvir, general odorant-binding proteins 1 and 2 of *Heliothis virescens* (Krieger *et al.*, 1993); GOBP1msex and GOBP2msex, general odorant-binding proteins 1 and 2 of *M. sexta* (Vogt *et al.*, 1991); LUSH, LUSH protein of *D. melanogaster (*Kim *et al.*, 1998); OS-D, OS-E and OS-F, OS-D, OS-E and OS-F proteins of *D. melanogaster* (McKenna *et al.*, 1994); p10, p10 protein of *Periplaneta americana* (Nomura *et al.*, 1995); PebIII, ejaculatory bulb protein III of *D. melanogaster* (Dyanov, 1994); PBP1aper, PBP2aper and PBP3aper, pheromone-binding proteins 1, 2 and 3 of *A. pernyi* (Raming *et al.*, 1990; Krieger *et al.*, 1991); PBPapol, pheromone-binding protein of *A. polyphemus* (Raming *et al.*, 1989); PBPbmor, pheromone-binding protein of *B. mori (*Krieger *et al.*, 1996); PBP1msex, PBP2msex and PBP3msex, pheromone-binding proteins 1, 2 and 3 of *M. sexta* (Gyorgyi *et al.*, 1988; Robertson *et al.*, 1999); PBPRP1-5, PBP-related proteins 1–5 of *D. melanogaster* (Pikielny *et al.*, 1994); SAP2, sensory appendage protein 2 of *M. sexta* (Robertson *et al.*, 1999).

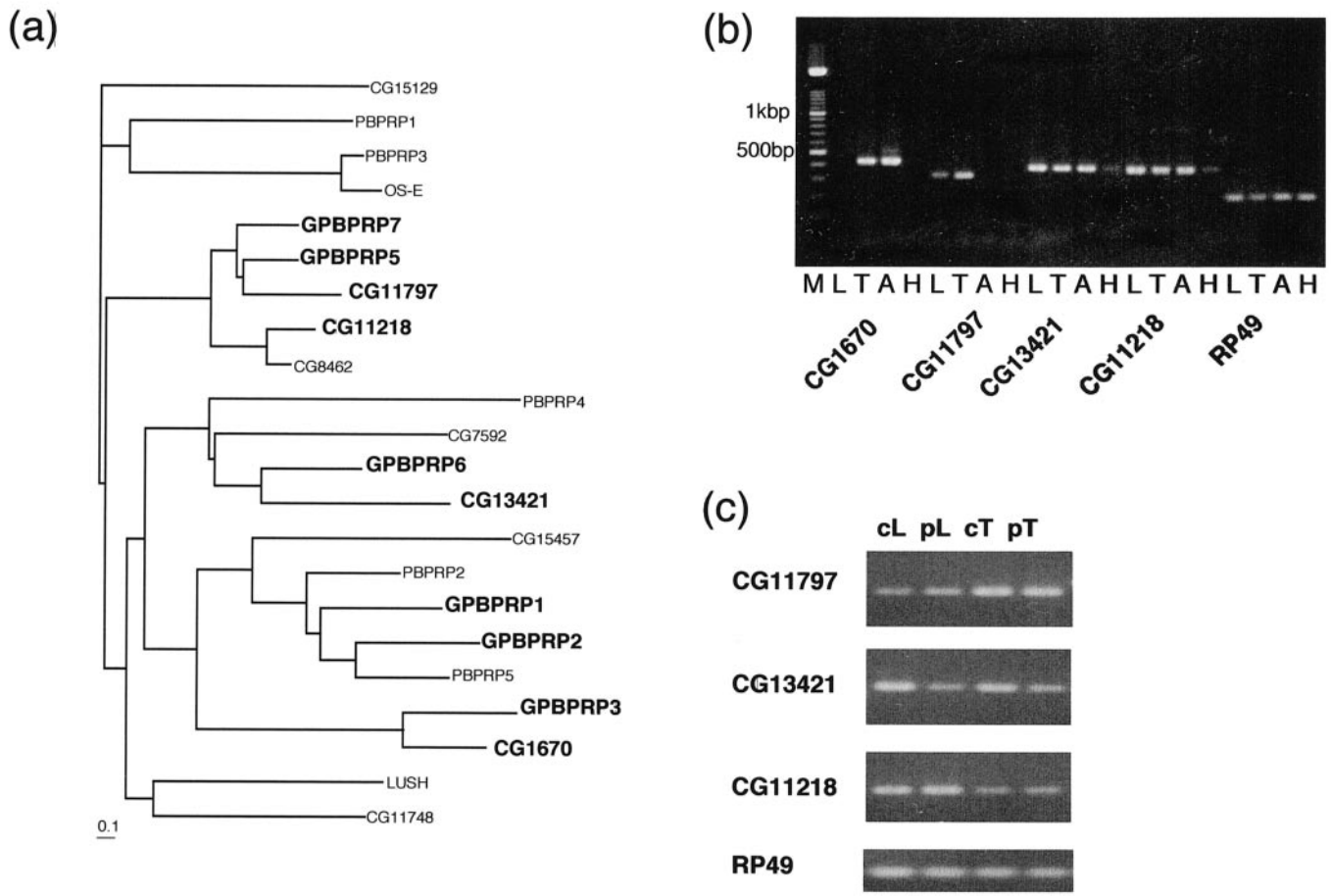


Figure 6 GPBPRP gene homologs in *Drosophila* taste tissue.**(a)** Phylogenetic tree of the known OBPs of *Drosophila*, GPBPRPs of *Boettcherisca* and the *Drosophila* homologs of GPBPRP. **(b)** RT–PCR analysis of *Drosophila* GPBPRP gene homologs. RT–PCR (28 cycles, other than 42 for CG1670) was carried out with the specific primers for each GPBPRP-related gene, designed using genomic sequence data. The templates were cDNAs prepared from labellum (L), tarsus (T), antenna (A) and head devoid of proboscis and antenna (H). (M, mol. wt marker). Control PCR was performed with RP49 gene primers. **(c)** RT–PCR analysis of *Drosophila* GPBPRP gene homologs in *poxn* mutant. The templates were cDNAs prepared from labellum of $poxn^{70}/poxn^{70}$ fly (pL) and control fly (cL) and tarsus of $poxn^{70}/poxn^{70}$ fly (pT) and control fly (cT). The numbers of cycles in RT–PCR were 29, 27, 24 and 28, for CG11797, CG13421, CG11218 and RP49, respectively.

in both olfactory and taste tissues (Pikielny *et al.*, 1994; Ozaki *et al.*, 1995). In vertebrates, one OBP-related protein, named von Ebner's gland protein (VEG protein), has been reported to be secreted in saliva (Schmale *et al.*, 1990) and tear fluid (Redl *et al.*, 1992). However, there are no insect OBPs with a taste-tissue-specific expression pattern. GPBPRPs, therefore, are considered to comprise a novel type of insect OBP that function for taste reception.

Boettcherisca GPBPRP genes were expressed in TRRT (Figure 4b). In most GPBPRP genes, the number of cycles for PCR using TRRT cDNA was lower than that for labellar cDNA at the same amplification level. This suggested that GPBPRP genes in labellum were expressed predominantly in the cells of TRRT. Because TRRT is presumed to contain only a small number of cells, including receptor and supporting cells, the expression of GPBPRP may be restricted to some of these cells. On the other hand, there were almost no differences in the expression levels of GPBPRP gene homologs between *poxn* mutant and control flies in *Drosophila* (Figure 6c). Therefore, these genes were scarcely expressed in the taste receptor cells. There are no taste receptor cells, but only the supporting cells in the transformed bristle in labellum and tarsus of $poxn^{70}/poxn^{70}$ fly. These results suggested that GPBPRP genes were expressed mainly in the supporting cells. This suggestion is supported by the observation that *Drosophila* OBPs, OS-E and OS-F proteins, are present in the supporting cells as well as in sensillum lymph (Hekmat-Scafe *et al.*, 1997).

The total numbers of chemosensory hairs on one labellum, six tarsi and two antennae of a fly are, respectively, ~300, ~1200 in the blowfly (Dethier, 1976) and ~850 in *Drosophila* (Stocker, 1994). In the fleshfly, the number of chemosensory hairs on the labellum and tarsus is about equal to that in the blowfly. However, the number on the antenna is thought to be far fewer in *Drosophila*. In our experiments, the amounts of total RNA extracted from one

labellum, six tarsi and two antennae of a fleshfly were 160, 500 and 80 ng, respectively. This suggests that the ratio of the number of supporting cells (i.e. the candidate GPBPRP producing cells) to a fixed amount of total RNA sample is at least five times larger in the antenna than in the taste tissue, based on the assumption of an equal number of supporting cells per chemosensory hair for labellum, tarsus and antenna. If the expression level of GPBPRP genes is the same for each supporting cell, the observed level of GPBPRP gene expression should therefore be five times higher in antenna than in the taste tissues in virtual Northern blot and RT–PCR analysis (since the amount of cDNA templates are equally loaded). However, the results observed clearly show that the expression level of GPBPRP genes in the taste tissue is higher than that in antenna. The true expression pattern of GPBPRP genes in the taste tissues may therefore be much more specific than that observed by virtual Northern blot analysis (Figure 3) and RT–PCR analysis (Figure 4a).

Some *Drosophila* genes with sequence similarity to GPBPRP genes were also expressed in the taste tissue (Figure 6b). However, they were less specific for taste tissue than those of *Boettcherisca*, since three of the four *Drosophila* genes were expressed significantly in antenna (Figure 6b). The true expression of the *Drosophila* GPBPRP gene homologs may still be specific to taste tissue, however, since the relative number of supporting cells to a fixed amount of total RNA sample is expected to be much larger in antenna than in the taste tissue.

Function of GPBPRPs

OBPs are thought to transport hydrophobic molecules such as pheromones or odorants into the hydrophilic environment surrounding the olfactory receptor cell and to regulate the olfactory response. Although many insect OBPs have been identified, there is little direct functional evidence for the participation of OBPs in chemoreception. Only two fly OBPs, LUSH protein in *Drosophila* and CRLBP in *Phormia*, have been investigated for their chemoreceptive function. A *lush* mutant of *Drosophila* was identified by the enhancer trapping method and the mutant flies show abnormal chemoattractive behavior, with enhanced attraction to ethanol and propanol (Kim *et al.*, 1998). CRLBP is the main soluble protein in the labellum of the blowfly and treatment of taste receptor cells with antibody to the CRLBP reduces the response to taste stimuli containing hydrophobic compounds (Ozaki *et al.*, 1995). These reports confirm that these proteins participate in insect chemoreception.

Because the expression of GPBPRPs is specific to taste tissue, GPBPRPs are expected to function only in taste reception. Tastants are generally hydrophilic, but hydrophobic molecules, such as bitter compounds, are also detected by the taste receptor cell, as shown by behavioral and electrophysiological studies (Dethier, 1976). Expression patterns that are taste-tissue-specific, lacking in sexual dimorphism and also detectable in the larval head, suggest important roles for GPBPRPs in the taste reception of various hydrophobic tastants and participation in the feeding behavior of the fly throughout the life span from larva to adult.

There were two types of GPBPRPs expression pattern in the taste tissue: GPBPRP2, 3 and 5 genes were expressed more abundantly in tarsus than in labellum; while levels of GPBPRP6 and 7 in labellum were higher than in tarsus. This difference in expression pattern between labellum and tarsus presumably reflects the different functions of these taste tissues: the tarsus perhaps being more important in detecting food and the labellum more essential in determining food intake.

Fly OBPs and mammalian OBPs

The genes with sequence similarity to GPBPRP genes of *Boettcherisca* were found in the predicted gene database of *Drosophila* (Figure 6). The number of novel GPBPRP gene homolog candidates of *Drosophila* was at least 13. Together with the known OBPs, such as the PBPRPs, the total number of OBPs in the *Drosophila* genome is large and estimated to be at least 21. The sequence similarities of these gene products were very low (~20% amino acid identity) compared with that of PBPs or GOBPs of moth (~60–80% amino acid identity in each subfamily). Furthermore, the PBPRP genes of *Drosophila* are expressed in the limited region of antenna and show different expression patterns from each other. For example, PBPRP1 gene is expressed in the anterior part of the antenna, while PBPRP5 gene is expressed in a more posterior position (Pikielny *et al.*, 1994). In the same manner as PBPRPs of *Drosophila*, each GPBPRP of *Boettcherisca* and GPBPRP homolog of *Drosophila* may be expressed differently in various types of taste hair of the labellum and tarsus. The chemosensory hairs of insects are separated from each other and the OBPs are secreted discretely in the sensillum lymph of each hair. The sequence diversity and differential expression pattern of OBPs may contribute to the specific response of each chemosensory hair, especially since it is known that some fly OBPs affect the chemosensory response (Ozaki *et al.*, 1995; Kim *et al.*, 1998).

The OBPs of rat and mouse that have been identified are fewer in number and have a higher level of sequence similarity than fly OBPs (Pevsner *et al.*, 1988; Dear *et al.*, 1991). Mammalian OBP-related proteins are uniformly secreted in the olfactory mucus (OBPs) or in the saliva (VEG protein) and are not restricted to the local fluid environment of the chemoreceptor cells. Mammalian OBPs, therefore, could not be related to specific receptor cells and their contribution to specific chemosensory responses seems rather unlikely. Furthermore, functional expression studies of the olfactory receptor genes strongly suggest that mammalian OBPs are not essential to odor reception, because the receptor gene alone expressed in a heterologous cell system

without any OBPs was able to respond to odorants (Raming *et al.*, 1993; Wellerdieck *et al.*, 1997; Krautwurst *et al.*, 1998).

The features of fly OBPs (large number, high sequence diversity and differential expression pattern) suggest that they have functional roles in both general and specialized facets of fly chemoreception, while for mammalian OBPs, their function in chemoreception seems likely to be much less direct, because of their small number, low diversity and uniform distribution compared with fly OBPs.

OBPs and chemosensory receptors

Candidate odorant receptor genes include members of several superfamilies of large G-protein-coupled receptor (GPCR) gene (Buck and Axel, 1991; Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997). The number of olfactory receptor genes expressed in the main olfactory epithelium of rodents is estimated to be ~1000 (Buck and Axel, 1991). Recently, a large GPCR gene family has been identified which is suggested to code the taste receptors for bitter taste in mammals (Adler *et al.*, 2000; Matsunami *et al.*, 2000). The number of these genes was estimated to be ~40–80 in the human genome.

In insects, novel GPCR genes of *Drosophila* have been cloned (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). These presumptive odorant receptor genes are expressed in a small subset of olfactory receptor cells. Only 41 candidate odorant receptor genes have been identified based on a genomic database analysis (Vosshall *et al.*, 2000). Recently, the taste receptor gene candidates of *Drosophila* have been reported, which have seven transmembrane domains with little sequence similarity with the known GPCRs (Clyne *et al.*, 2000; Dunipace *et al.*, 2001; Scott *et al.*, 2001). The number of these genes is estimated to be only 56. The number of chemosensory (olfactory and taste) receptors of *Drosophila* is, therefore, very small compared with that of mammals.

The number of OBP genes relative to that of chemoreceptor (olfactory and taste) genes in the fly is much larger than in mammals. This relatively large number of OBP genes and their high diversity suggest an important role of OBPs in chemoreception in the fly. With a relatively small number of chemoreceptor genes but a large number of OBP genes, both with diverse sequences, it may be that, after all, flies are almost equal to vertebrates in their ability to respond to various chemical stimulants.

This work was partly reported in abstract form (Koganezawa and Shimada, 2000). While this paper was being reviewed, a study describing the expression of a large family of *Drosophila* OBPs in gustatory and olfactory sensilla was published (Galindo and Smith, 2001).

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