Behavioral analyses of mutants for two odorant-binding protein genes, *Obp57d* and *Obp57e*, in *Drosophila melanogaster*

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(Received 5 December 2007, accepted 31 March 2008)

The odorant-binding protein (OBP) functions in chemosensation in insects. Two OBP genes, *Obp57d* and *Obp57e*, are involved in the evolution of the unique host-plant preference in *Drosophila sechellia*. Comparative analysis of the *Obp57d/e* genomic sequences in the *Drosophila melanogaster* species group has revealed that the rapid evolution of *Obp57d* and *Obp57e* has resulted in functional divergence between the two genes. Here, using *D. melanogaster* knockout strains generated by gene targeting, we examined the roles of *Obp57d* and *Obp57e* in behavioral response to a series of fatty acids. In the taste-based oviposition-site preference assay, the knockout flies showed stronger preference for acids than wild-type flies, indicating that the normal functions of *Obp57d* and *Obp57e* are associated with the suppression of positive preference for C_6-C_9 acids. Heterozygotes for each knockout haplotype also exhibited a significantly different behavioral response compared with wild-type flies, suggesting that *Obp57d* and *Obp57e* have a gene dosage effect on behavior. In contrast, the wild-type and knockout flies exhibited similar responses in the feeding assay and the odor-based free-walking assay, suggesting that the two OBPs' contribution to feeding and olfactory behaviors is small. Taken together, our results demonstrated that each of *Obp57d* and *Obp57e* in *D. melanogaster* contributes to the determination of reproductive sites, suggesting that the two OBP genes play an important role in ecological adaptation of *Drosophila*.

**Key words:** taste and odor perception, oviposition-site preference, feeding behavior

**INTRODUCTION**

The odorant-binding protein (OBP) is a water-soluble protein secreted to the extracellular lymph in insect chemosensilla, in which the OBP-containing lymph surrounds dendrites of chemosensory neurons (Pelosi et al., 2006). OBP is capable of binding odorants, which are often hydrophobic, solubilizing them into the lymph (Tsuchihara et al., 2005). With this molecular function, OBP plays an important role in insects' chemosensation, by helping hydrophobic molecules reach odorant receptors on the dendrites of chemosensory neurons and/or by removing ligands after the receptor activation (Pelosi et al., 2006). Although its name implies a function in “odorant-binding”, OBP is also shown to participate in taste sensation (Ozaki et al., 2003).

Genes encoding OBP tend to form a large family in insect genomes. Analysis of 12 *Drosophila* genomes revealed that there are more than 50 OBP genes in each genome; the number is comparable to that of odorant receptors and gustatory receptors (Vieira et al., 2007). Each OBP gene is highly diverged from others in their structure, suggesting the large diversity in their function, i.e., ligand repertoire (Graham and Davies, 2002; Hekmat-Scafe et al., 2002).

Two OBP genes, *Obp57d* and *Obp57e*, are involved in the evolution of the characteristic host-plant preference in *Drosophila sechellia*, a specialist species that exclusively reproduces on the ripe fruit of *Morinda citrifolia* (Matsuo et al., 2007). The two genes are tandemly arranged in *D. sechellia* and *D. melanogaster* genomes, and in the latter species, *Obp57d* and *Obp57e* are coexpressed in the cells associated with gustatory sensilla on each leg (Galindo and Smith, 2001; Matsuo et al., 2007). Replacement of the *Obp57d/e* genomic region with that of *D. sechellia* altered the behavioral response of *D. melanogaster* to pre-
fer hexanoic acid and octanoic acid, the toxins contained in the Morinda fruit (Matsuo et al., 2007).

Comparisons of the genomic sequences of the Obp57d/e region from 27 closely related species revealed the rapid evolution of Obp57d and Obp57e in the melanogaster species group (Matsuo, 2008). The two OBP genes arose by duplication of an ancestral OBP gene at the early stage of the melanogaster group evolution. Analysis of the amino acid sequences revealed that the two OBP genes share several conserved sites, suggesting that the two genes share common functions. At the same time, site-specific analysis of evolutionary rate indicated that the two genes have functionally diverged from each other by subfunctionalization. Furthermore, gene number variation was found in the melanogaster group. Immediately after the gene duplication and functional diversification of Obp57d and Obp57e, Obp57e was lost in the ananassae subgroup. Likewise, Obp57d was lost in the auraria-rufa lineage of the montium subgroup. Because Obp57d and Obp57e are involved in the behavioral evolution of D. sechellia, those species losing either gene are also expected to have behavioral difference. To elucidate the roles of Obp57d and Obp57e in the behavioral evolution of Drosophila, how the two OBPs are different from each other in terms of their function should be confirmed experimentally.

In addition to the inter-species variation in gene number, the intra-species polymorphism at the Obp57d/e locus has been reported (Takahashi and Takano-Shimizu, 2005). The null alleles of Obp57e were found from natural populations of D. melanogaster, at rather high frequency in some cases. One of such alleles was found worldwide, suggesting that a balancing selection by an unknown mechanism maintains the polymorphism. Examination of the phenotype caused by the loss of either gene will elucidate the mechanism underlying the balancing selection, which would play an important role in the evolution of the duplicated genes.

Here, to address these issues, we analyzed the behavioral response of the D. melanogaster Obp57d and Obp57e knockout strains. The analyses include taste- and odor-based assays against a series of fatty acids. Both of the two OBPs were shown to be involved in taste-based response to C₆-C₉ acids in oviposition behavior. Our findings provide the basis for a thorough understanding of the roles of OBP genes in behavioral evolution.

**MATERIALS AND METHODS**

**Fly strains and preparation** The knockout strains for Obp57d and Obp57e were previously generated by gene targeting (Matsuo et al., 2007). For each strain, the targeted region was replaced with a marker gene, which was then excised by Cre recombinase leaving a 34 bp loxP sequence (Fig. 1). All the strains were backcrossed to a control strain w¹¹¹b (referred to as wild-type hereafter) for five generations. Unless otherwise noted, homozygotes were used for the assays. For the oviposition-site preference assay and odor-based free walking assay, newly eclosed adults were staged for three days at 25°C with a cellulose plug (Fisher Scientific, USA) soaked with a solution containing 5% yeast extract (Difco Becton Dickinson, USA) and 5% sucrose (Wako Pure Chemical Industries, Japan). For the feeding assay, two-day-old flies staged as described above were provided with 20 mM sucrose solution overnight prior to 6 h of starvation with distilled water immediately before each assay. For the all assays, both sexes from the same strain were staged together in the same vial, so that all females were mated.

**Chemicals** All the chemicals used for the behavioral assays were purchased from Kanto Chemical (Japan), except for hexanoic acid, which were purchased from Wako Pure Chemical Industries (Japan). All the chemicals were of the highest grade available.

**Oviposition-site preference assay** The oviposition medium containing 1% yeast extract and 1% sucrose in 0.8% agar (Difco Becton Dickinson, USA) was freshly prepared immediately before the assay, in a flexible 96-well assay plate (Falcon Becton Dickinson, USA) cut into pieces with each having #2 × #4 wells that were filled with acid-containing or control medium (oviposition plate) (Fig. 2A). The acidity in the media was pH 6.7 for control medium, pH 4.5 for 20 mM hexanoic acid, pH 4.9 for 10 mM heptanoic acid, pH 5.3 for 5 mM octanoic acid, and
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pH 6.1 for 2 mM nonanoic acid. An oviposition plate was placed in an empty fly culture vial with a disc of filter paper (absorbant pads, 2.5 cm in diameter; Millipore, USA) soaked with 500 μl of distilled water. Three pairs of the staged flies (both sexes from the same strain) were introduced into the vial and allowed to lay eggs overnight at 25°C in a dark box with ventilation. The preference index (PI) was calculated for each vial as $\text{PI} = \frac{(N_{\text{acid}} - N_{\text{cont}})}{(N_{\text{acid}} + N_{\text{cont}} + N_{\text{both}})}$, where $N_{\text{acid}}$, $N_{\text{cont}}$, and $N_{\text{both}}$ are the numbers of eggs laid on acid-containing and control media, respectively. Six vials of assays were performed at the same time, and were repeated three times on different days. Mean and standard error were calculated from a total of 18 replicates for each acid concentration.

Feeding assay

Acid dissolved in 200 μl of heptan was spread over a 2.5 cm filter paper disc (absorbant pads; Millipore, USA) and air-dried. For control discs, only the solvent was applied. On the air-dried disc, 400 μl of 2 mM sucrose solution colored with brilliant blue FCF (0.25 mg/ml; Wako Pure Chemical Industries, Japan) or amaranth (0.5 mg/ml; Sigma-Aldrich, USA) was loaded, distinguishing between the acid-containing and control discs by color. Two pairs of acid-containing and control discs were placed in an assay box (Fig. 2B). Staged and starved flies were introduced into the assay box and allowed to feed for 30 min at 25°C under dark condition. The flies were immediately killed and classified into four groups according to the color of their abdomen: blue, red, purple, and white. Preference index was calculated as $\text{PI} = \frac{(N_{\text{acid}} - N_{\text{cont}})}{(N_{\text{acid}} + N_{\text{cont}} + N_{\text{both}})}$, where $N_{\text{acid}}$, $N_{\text{cont}}$, and $N_{\text{both}}$ are the numbers of flies that fed themselves with the acid-containing solution, control solution, and both solutions, respectively. More than 50 individuals were tested at the same time, and the assay was repeated three times for each acid concentration. No difference was observed between sexes, and thus the data were pooled.

Odor-based free-walking assay

The method was modified from Legal et al. (1999). An odor source consists of a filter paper disc (8 mm in diameter; Advantec, Japan) absorbing a defined amount of odorant and wrapped using a metal mesh that prevents the direct contact of the flies to the disc (Fig. 2C). For amounts of less than 1 μl, the odorants were diluted in pentadecane. Ten staged female flies were starved for 3 h with distilled water, and then introduced into a glass tube (40 cm length × 2 cm diameter) immediately after the odor source

Fig. 2. Methods for behavioral assays. (A) For the oviposition-site preference assay, the oviposition medium was prepared in a flexible 96-well assay plate, which was then cut into pieces, with each row of four wells filled with acid-containing or control medium. An oviposition plate was placed in an empty fly culture vial with a disc of filter paper humidified with 500 μl of distilled water. Three pairs of staged flies, both sexes from the same strain, were allowed to lay eggs overnight at 25°C. (B) For the feeding assay, two pairs of filter paper discs loaded with acid-containing and control sucrose solutions, distinguished by color dye, were placed in an assay box. Staged and starved flies were allowed to feed for 30 min at 25°C under dark condition. (C) For the free-walking assay, an odor source consisting of a filter paper disc absorbing a defined amount of odorant and then wrapped using a metal mesh, was placed at the one end of a glass tube. Ten staged female flies were introduced, and the number of flies in each half of the tube was recorded every 5 min for 1 h at 25°C under the illumination of fluorescent lamps. To cancel the effect of illumination, each assay tube was always paired with another that was in the opposite direction.
RESULTS

Oviposition-site preference of knockout flies for Obp57d and Obp57e  In our previous study, by using the oviposition-site preference assay, the Obp57d/e genomic region was shown to be responsible for the behavioral difference between Drosophila species in response to hexanoic acid (C₆) and octanoic acid (C₈), but not to acetic acid (C₂) and butyric acid (C₄) (Matsuo et al., 2007). In this study, we examined the oviposition-site preference in response to C₅-C₉ acids using the three Obp57d/e knockout strains, Obp57dKO, Obp57eKO, and Obp57d+eKO (Fig. 1). The dose-dependent change in the response was analyzed by the two-choice assay between acid-containing and control media (Fig. 2A). At the highest concentration, the wild-type flies avoided any of the tested acids (Fig. 3). This avoidance could be in part, but not entirely, due to the low pH in the media, because each acid gives different pH at the concentrations that induce similar behavioral responses (see Materials and Methods). At the moderate concentrations, the wild-type flies exhibited a slight preference for heptanoic acid (C₇) and octanoic acid (C₈), but not to hexanoic acid (C₆) and nonanoic acid (C₉). Similarly to the wild-type flies, the knockout flies avoided any of the tested acids at the highest concentration. However, at the moderate concentrations, the knockout flies’ preference index (PI) was higher than that of the wild-type flies, suggesting that the normal functions of OBPs57d and OBPs57e are associated with the suppression of the positive preference for the moderate concentrations of these acids. The largest difference between the knockout strains and the wild-type flies was observed for octanoic acid, while smaller but significant differences were also observed for the other acids. The Obp57dKO flies always exhibited a stronger preference for any of the tested acids than the Obp57eKO flies. This difference between the Obp57dKO and Obp57eKO flies, however, may not be explained by a simple genetic mechanism, because the double-knockout Obp57d+eKO flies’ response to the tested acids was not similar to that of the Obp57dKO.

Dominant effects of knockout haplotypes  To analyze the genetic interaction between Obp57d and Obp57e, heterozygous combinations of the Obp57dKO, Obp57eKO, and Obp57d+eKO haplotypes were examined by the oviposition-site preference assay for 5 mM of octanoic acid (Fig. 4). The Obp57d+eKO/+ flies’ response was intermediate between those observed for the wild-type and Obp57d+eKO homozygotes, indicating that Obp57d and Obp57e have a gene dosage effect. Surprisingly, the behavioral response of trans-heterozygotes Obp57dKO/Obp57eKO was not identical to that of the Obp57d+eKO/+ flies but comparable to that of the Obp57dKO homozygotes, whereas the gene dosage is identical to the former. Furthermore, the preference of Obp57dKO/+ was also similar to that of the Obp57dKO flies, suggesting that the Obp57dKO haplotype has a dominant effect leading to the behavioral shift toward the positive preference. Because the ORF of Obp57d was accurately removed by gene targeting, the dominant effect of the Obp57dKO haplotype is
hardly derived from the neomorphic action of Obp57d (Fig. 1B). Rather, it is more likely that the remaining Obp57e is involved in the dominant effect (discussed later).

In addition to the Obp57dKO haplotype, the Obp57eKO haplotype also showed a dominant effect. The behavioral response of the Obp57eKO/+ flies was significantly different from that of the wild-type flies, and was similar to that of the Obp57eKO homozygotes. Because the phenotype of the heterozygotes was within the range of those observed for the wild-type and Obp57d+eKO homozygotes, the dominant effect of the Obp57eKO haplotype appears to be caused, in this case, by a gene dosage effect of Obp57e similarly to the case observed for the Obp57d+eKO haplotype.

Feeding behavior of knockout flies Taste perception is involved in feeding behavior. We examined the roles of Obp57d and Obp57e in this behavior (Fig. 5). Unlike in the results of the oviposition-site preference assay, the wild-type flies exhibited a clear preference for moderate concentrations of C6-C8 acids in the feeding assay. For these acids, the difference in the response between the wild-type and knockout flies was smaller in the feeding assay than in the oviposition-site preference assay, suggesting that Obp57d and Obp57e are not involved in feeding behavior. The wild-type flies avoided nonanoic acid (C9) and the knockout flies exhibited a preference for it, but the differences were statistically not significant.

Odor-based behavioral response The expression of Obp57d and Obp57e is limited to gustatory organs (Galindo and Smith, 2001; Matsuo et al., 2007). However, one of the OBPs in the blowfly, which is necessary for the taste recognition of toxic substances, is able to incorporate air-borne molecule into the OBP-containing solutions, raising the possibility that OBPs expressed in gustatory organs also function in the perception of chemical stimuli without direct contact to the source, similarly to the case in olfactory organs (Ozaki et al., 2003; Tsuchihara et al., 2005). To examine the possibility that Obp57d and Obp57e are also involved in the perception of air-borne stimuli, we analyzed the flies’ behavioral response by the free-walking assay, in which flies were prevented from making direct contact to the odor source (Fig. 6). We first tested methyl hexanoate (MH), another compound involved in the D. sechellia’s behavioral adaptation to its host plant, that has been proved to be perceived by the flies as an olfactory cue (Dekker et al., 2006). The wild-type and knockout flies avoided 1 μl of MH (PI = –1), supporting the validity of the assay (data not shown). Likewise, the flies avoided C6-C8 acids as the amount of the odor source was increased, demonstrating that flies are able to sense the tested acids without direct contact. However, there was no difference between the wild-type and knockout flies, indicating that Obp57d and Obp57e are not involved in perception of air-borne stimuli from these acids.

**DISCUSSION**

In our previous study, replacement of the Obp57d1e genomic region with that of other species resulted in a significant behavioral shift in the responses to hexanoic acid and octanoic acid, whereas little effect on behavior was observed in the absence of the gene function in the knockout flies (Matsuo et al., 2007). In contrast, in this study, we observed a significant difference between the wild-type and knockout flies in the oviposition-site preference assay. In the previous study, the flies were simultaneously provided with four types of oviposition medium containing different concentrations of acid, while in this study, the flies were forced to choose between the acid-containing and control media. The two-choice assay in this study uncovered the dose-dependent shift of the flies’ response, enabling a more detailed examination of the difference between the wild-type and knockout flies. As a result, Obp57d and Obp57e were shown to be involved in the perceptions of heptanoic acid and nonanoic acid, as well as hexanoic acid and octanoic acid.

LUSH, a well-studied OBP in *D. melanogaster*, is suggested to function as an adapter for the ligand binding to the corresponding chemoreceptor (Xu et al., 2005). LUSH mutant flies have completely lost neural and behavioral responses to the ligand, cis-vaccenyl acetate.
In contrast, Obp57d*KO flies still responded to the tested acids, indicating that the two OBPs are dispensable for the perception of these acids. Nevertheless, it is still possible that OBP57d and OBP57e are adapters for particular receptors. Because the number of neurons and receptors involved in the perception of the acids tested in this study is not known and could be more than one, the loss of Obp57d and Obp57e does not necessarily mean the loss of overall response to these acids. For example, it is possible that C₆-C₉ acids are recognized by two types of receptors, one for the positive preference and the other for suppression of the positive preference. Such interaction is known between sugars and bitter compounds (Meunier et al., 2003). Bitter compounds elicit the food rejection behavior both by inhibiting sugar-activated neurons and by activating the L2 neurons that counteract the excitation of sugar-activated neurons in the other chemosensilla. It should be noted that, in the D. melanogaster legs, the bitter-activated L2 neurons are housed in a limited number of chemosensilla including 5b and 4s, where Obp57d and Obp57e are expressed (Meunier et al., 2003; Matsuo et al., 2007). OBP57d and OBP57e may function in the detection of C₆-C₉ acids by the bitter-activated neurons in those chemosensilla. Identification of the corresponding neurons and receptors is necessary to understand the molecular functions of OBP57d and OBP57e in the perception of the acids.

Results of reporter experiments have shown that Obp57d and Obp57e are expressed in the cells associated with the gustatory sensilla on the legs (Galindo and Smith, 2001; Matsuo et al., 2007). Because the chemosensory neurons on the legs send axons to the thoracic ganglion while those on the proboscis project directly to the brain, their contributions to the oviposition behavior, which is achieved by the activity of motor neurons in the thoracic ganglion, could be different (Stocker, 1994; Dahanukar et al., 2007). Indeed, difference in the wild-type flies’ response to C₆-C₉ acids was observed between the oviposition-site preference assay and the feeding assay. In the oviposition-site preference assay, the wild-type flies exhibited little preference for these acids, if any. In contrast, the wild-type flies exhibited a signifi-
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cant preference for the moderate concentration of the same acids in the feeding assay. These results are explained if the neural inputs from the legs are more important for the oviposition behavior than for feeding behavior. Obp57d and Obp57e are expressed only in the legs, and may not be involved in the feeding behavior, but in the oviposition-site determination.

The existence of a balancing selection on the polymorphism at the Obp57d/e locus has been suggested (Takahashi and Takano-Shimizu, 2005). Our results indicated that the two OBP genes are involved in the determination of larval food via the oviposition-site preference of adults. Thus, the mechanism of the balancing selection may be related to the conflict between the two developmental stages; larvae are forced to feed on a food that is pre-determined by adults, which are, on the other hand, potentially able to feed on several other foods than that which they selected as an oviposition site. Indeed, octanoic acid delays the growth of D. sechellia larvae at the concentrations higher than 1%, showing that the host chemicals hold the deleterious effect even for the larvae of such a highly adapted species (Amlou et al., 1998).

Our results also demonstrated that the flies heterozygous for the Obp57e null mutation exhibit a significantly different behavioral response compared with the wild-type flies, which is a condition favorable for balancing selection.

In the oviposition-site preference assay, a significant quantitative difference was observed between the Obp57dKO and Obp57eKO flies. However, considering the dominant effect of the Obp57dKO haplotype, all of the differences between the Obp57dKO and wild-type flies may not be attributed to the loss of Obp57d. Rather, it appears to be caused by an unexpected effect of the remaining Obp57e. By removing the Obp57d ORF, the promoter regions of Obp57d and Obp57e are tandemly arranged in the Obp57dKO haplotype (Fig. 1B). This situation may induce over-expression of Obp57e. Indeed, it was previously shown by quantitative RT-PCR analyses that the amount of Obp57e transcripts is increased 10 folds in the Obp57eKO flies (Matsuo et al., 2007). Then, how does the over-expression of Obp57e result in the enhancement of the preference for C6-C9 acids? Two explanations are possible. If OBP57e functions as transporters and/or adaptors, excess amount of OBP57e could titrate ligands, inhibiting the receptor-activation in a dominant-negative manner. Alternatively, OBP57e may have other functions than the facilitation of ligand-binding to the receptors. A proposed function of OBPs in removal of ligands after receptor-activation has not been rejected by experimental evidence (Pelosi et al., 2006). If OBP57e simultaneously functions as a transporter and a quencher, over-expression of Obp57e may disrupt the balance between the two functions and inhibit the activation of the receptors leading to the enhancement of the positive preference for C6-C9 acids. The scenario should be confirmed by further experiments. Nevertheless, the genetic alteration at the Obp57d/e region was capable of modulating the fly’s behavioral response, suggesting that the evolution at the Obp57d/e locus has a significant potential to contribute to the behavioral variation among species and intra-species populations.

Evolutionary analysis of Obp57d and Obp57e sequences suggested that the two genes have functionally diverged from each other (Matsuo, 2008). Considering the results that there was no qualitative difference between the Obp57dKO and Obp57eKO flies in response to the acids, the functional divergence between the two OBP genes may not reside in the difference in the chain length of the acid tested in this study. Instead, it is possible that the functional divergence resides in the difference of the ligand classes, such as acids vs. alcohols or esters. Although it is preferable to test highly water-insoluble compounds such as alcohols and esters by the oviposition-site preference assay, it is difficult for the current method using agar-based medium as oviposition sites. Improvement of the method is necessary to test these compounds.

This work was supported by a Grant-in-Aid for Young Scientists (B) 19770210 from the Japanese Ministry of Education, Culture, Sports, Science and Technology to T. M.

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