Conserved *cis*-regulatory elements of two odorant-binding protein genes, *Obp57d* and *Obp57e*, in *Drosophila*

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Insect odorant-binding proteins function in the sensing of odors, tastes, and pheromones. Genes encoding two odorant-binding proteins, *Obp57d* and *Obp57e*, were identified to be involved in the behavioral adaptation of *Drosophila sechellia* to its host plant. The two genes are expressed in cells associated with taste sensilla on the legs, and the expression pattern in the legs is conserved among closely related species. To identify the *cis*-regulatory elements necessary for the expression in the leg sensilla, the promoter sequences of *Obp57d* and *Obp57e* were compared among species. Two types of conserved sequence-motifs were found as candidate *cis*-regulatory elements. Functions of these conserved elements in the promoters of *D. melanogaster Obp57d* and *Obp57e* were examined by using a newly constructed vector that combines the advantages of ϕ C31 integrase-based transformation and *gypsy* transposable-element-derived insulators. By GFP-reporter assay using the new vector, it was confirmed that these conserved elements are necessary for the expression in the legs, working synergistically with each other to affect the expression level. Single-nucleotide substitutions in these elements dramatically changed the promoter activity. These results provide insight into the molecular mechanism for evolution of adaptive behavior via modulation of OBP expression levels.

Key words: gene expression, ϕ C31 integrase

INTRODUCTION

The evolution of gene expression is an important mechanism generating variation in phenotypes. Genes encoding two odorant-binding proteins, *Obp57d* and *Obp57e*, were identified to be involved in the evolution of host-plant preference in *Drosophila sechellia* (Matsuo et al., 2007; Matsuo, 2008a). Loss of the expression of these OBPs in *D. melanogaster* leads to a behavioral shift toward a preference for an otherwise avoided toxic compound, octanoic acid (Matsuo, 2012). A GFP-reporter assay showed that a small, 4-bp insertion found in the promoter of *D. sechellia Obp57e* results in the loss of expression in the *D. melanogaster* background, suggesting that the evolution of host-plant preference was caused by changes in the regulatory mechanisms of OBP expression (Matsuo et al., 2007).

In our previous study, promoters cloned from other species in the *melanogaster* species group were shown to

have diverged as well as conserved functions (Yasukawa et al., 2010). Diverged functions were observed for the expression in the mouthparts, where expression of the reporter gene in the *D. melanogaster* background, as well as mRNA levels in the original species, differed between species. On the other hand, expression in the legs was conserved among species, suggesting that the *cis*-regulatory elements for expression in the legs are conserved among the promoter sequences of these species.

In this study, to find *cis*-regulatory elements in the promoters of *Obp57d* and *Obp57e*, conserved sequence motifs were searched for in the promoter sequences. Functions of the candidate elements were tested by using a newly constructed transformation vector for precise quantification of promoter activity *in vivo*. As a result, two types of *cis*-regulatory elements that are necessary for expression in the legs were identified.

MATERIALS AND METHODS

Fly strain For ϕ C31 integrase-based transformation, an integrase-attP combined line ϕ X-86Fb (*y w*

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M{eGFP.vas-int.Dm}\ZH-2A; +; *M{RFP.attP}\ZH-86Fb*) was used (Bischof et al., 2007; provided by Dr. Konrad Basler).

Identification of conserved sequence motifs

OLIGO-ANALYSIS (van Helden et al., 1998) was used to find sequence motifs that appear specifically in the following promoter sequences: *Dmel\Obp57d* (641-bp), *Dmel\Obp57e* (441-bp), *Dsim\Obp57d* (650-bp), *Dsim\Obp57e* (467-bp), *Dyak\Obp57d* (684-bp), *Dyak\Obp57e* (445-bp), *Dana\Obp57d* (671-bp), *Drua\Obp57e* (346-bp), *Dpse\Obp57d* (680-bp) (Yasukawa et al., 2010). The *D. melanogaster* genome was used as a control.

Construction of the pGreenPelican+attB vector

A multi-cloning site of the pAttB vector (Bischof et al., 2007) was removed by digestion with *EcoRI* and *XbaI* followed by blunting using a DNA Blunting kit (TAKARA). A fragment containing gypsy-insulators, a multi-cloning site, and *eGFP* was amplified from pGreenPelican (Barolo et al., 2000) with the primers 5'-GGCTGCATCACGTAATAAG-3' and 5'-CTTACATACATACTAGAATTGATCGGC-3' by using KOD+ enzyme (TOYOBO) that produces blunt ends. The resulting fragment was ligated with the digested pAttB vector described above, to obtain the pGreenPelican+attB vector. The original pAttB vector had an additional *BamHI* site that was not described. To remove it from the pGreenPelican+attB vector, inverse PCR followed by self-ligation was performed with 5'-GGCTGCATCACGTAATAAG-3' and 5'-CCGCTAGCATAACTTCG-3' using KOD+ enzyme. The structure of the final product was confirmed by sequencing.

Cloning and modification of promoter sequences

Previously cloned promoters of *Dmel\Obp57d* and *Dmel\Obp57e* (Yasukawa et al., 2010) were used as templates for PCR amplification in this study. Primers used for *Dmel\Obp57d* promoter constructs were: 5'-GCGGC-CGCGAGCATGGAGAGATCGAAG-3' and 5'-AAAGGATC-CCAACTAGTTGAAGATATCATAGGAACT-3' (P1) for the intact 319-bp promoter, and 5'-GCGGCCGCA-CAGTTTGTATAATCAGATAGCATT-3' and P1 for the deleted 154-bp promoter. Primers used for *Dmel\Obp57e* promoter constructs were: 5'-GCGGCCG-CAAGCCGTTGTAAAGAATAAG-3' and 5'-AAAGGATC-CAGTTGCTATATTCCTAGGGAA-3' (P2) for the intact 330-bp promoter, 5'-GCGGCCGCGCATCTCCACGTATAT-TCAATTGTTTA-3' and P2 for the deleted 191-bp promoter, and 5'-GCGGCCGCGCAATTAAAAATGTGATTATCC-3' and P2 for the deleted 101-bp promoter. Recognition sites for restriction enzymes (*NotI* or *BamHI*) were added to the 5' terminal of each primer (indicated by underline). Resulting fragments were digested by *NotI* and *BamHI*

enzymes (TOYOBO), and inserted at the multi-cloning site in the pGreenPelican+attB vector.

For nucleotide substitution, inverse-PCR was performed using the intact promoter constructs as templates. Primers used for the *Dmel\Obp57d* promoter constructs were: 5'-CAGACATTCTATACCTTTTAATGAATTTATTTCTTTTGTG-3' and 5'-TATTCAATAACAACTGTATAATA-CAAAATAATTAACATAAAC-3' for disruption of the type-Ib element, and 5'-GGTTTGTATAAGTACTTCAACTAG-TTTGGGAT-3' and 5'-CAATACCAAATCGCATAAATTGAATC-3' for restoration of the type-II element. Primers for the *Dmel\Obp57e* promoter constructs were: 5'-ATGTCTATTATGATACGATGGATTCCCTA-3' and 5'-CATCTTAACCAACGAGCAATTAAATTTCAATATAATG-3' for disruption of the type-II element, 5'-GTTTATATT-ATTAATTAATCAGTCAGTTATC-3' and 5'-AATTGAA-TATACGTGGAGATG-3' for restoration of the type-Ia element, and 5'-AAAACAATCTGATTCTTTACAACG-3' and 5'-TTTTTATTTTGGATGCATATTAATAAC-3' for restoration of the type-Ib element.

Tandemly situated promoters were cloned from the *Obp57d* knockout strain (Matsuo et al., 2007) by PCR using 5'-GCGGCCGAGCCACAACTGGAGGACAG-3' and P2. The structure of all constructs was confirmed by sequencing.

Observation of GFP expression Newly eclosed homozygotes were staged for 3 days at 25°C before observation. Legs were dissected with scalpels and mounted in immersion oil (Immersol 518N, Zeiss). All six legs from an individual were examined, and the number of GFP-expressing cells was scored for each chemosensilla according to a previous study (Yasukawa et al., 2010). For each line, 10 males and 10 females were examined. In total, 80 cells were examined for 5b, 5s, and 4s sensilla that exist on both sides of each leg, and 40 cells for 4c sensilla that exist only on the single side of each leg. The expression frequency was calculated as (the number of GFP expressing cells) / (the total number of cells examined).

RESULTS

Conserved motifs in the promoter sequences of *Obp57d* and *Obp57e*

In our previous study, GFP-reporter constructs using the promoters from several species in the *melanogaster* species group reproduced the expression pattern of *Obp57d* and *Obp57e* in the legs of *D. melanogaster*, suggesting that these promoters maintain conserved *cis*-regulatory elements. To find such elements, short sequence motifs that appear specifically in these promoter sequences were searched for using OLIGO ANALYSIS, a program for the identification of conserved elements in regulatory sequences (van Helden et al., 1998). Two motifs with size of 8-bp were found (Fig. 1).

Motif-1 was found in the *Obp57d* promoters, while motif-2 was found in the *Obp57e* promoters. The direction and position of these elements relative to the translation initiation site (ATG) were conserved among species. Interestingly, promoters of *Dpse\Obp57de* and *Dana\Obp57d*

contain both motifs, each at the corresponding position. Because *Obp57d* and *Obp57e* arose via the duplication of an ancestral gene, which remains a single gene in *D. pseudoobscura* as *Dpse\Obp57de* (Matsuo, 2008b), the two motifs are likely to have originated in the ancestral gene. In the current species, either of the motifs might have experienced nucleotide substitutions. In fact, *Obp57d* and *Obp57e* are expressed in the same cells even in the current species, suggesting that the function of *cis*-regulatory elements is still conserved between *Obp57d* and *Obp57e*.

Candidate *cis*-regulatory elements in the *D. melanogaster* promoter sequences By a search allowing mismatches, additional motif-1 and motif-2 -related sequences were found in the promoter sequences of *Dmel\Obp57d* and *Dmel\Obp57e* (Fig. 2). Two motif-1-related sequences (type-Ia and -Ib) and one motif-2-related sequence (type-II) were found in both promoters, and adopted as candidate *cis*-regulatory elements. These elements were slightly diverged from the original motif-1 and motif-2 that are conserved among species, but their position was roughly maintained.

Construction of the new transformation vector for precise GFP-reporter assay In our previous study, the expression level fluctuated among independent transgenic lines even when the same promoter-reporter construct was used (Yasukawa et al., 2010). To overcome this problem, we combined the advantages of two vectors, pGreenPelican and pAttB (Barolo et al., 2000; Bischof et al., 2007). The *gypsy* transposon-derived insulators in

Fig. 1. Conserved motifs found in the promoter sequences of *Obp57d* and *Obp57e*. Two motifs were found by OLIGO ANALYSIS in the promoter sequences that were previously shown to have a conserved *cis*-regulatory function. Motif-1 is indicated as a box, and motif-2, as a circle. Arrows indicate translation initiation sites (ATG), not transcription start sites. Note that the two motifs co-exist in the *Dpse\Obp57de* and *Dana\Obp57d* promoters.

Fig. 2. Candidate *cis*-regulatory elements in the *D. melanogaster* promoters. (A) Position of type-I (indicated by boxes) and type-II (indicated by circles) elements in the promoter of *Dmel\Obp57d* and *Dmel\Obp57e*. Filled symbols indicate elements that are identical to the motifs found by OLIGO ANALYSIS (shown in Fig. 1). Arrows indicate translation initiation sites (ATG), not transcription start sites. (B) Sequences of type-I (6 bp) and type-II (20 bp) elements. Surrounding sequences are shown in gray. Boxes indicate the 8 bp motifs conserved among species (Fig. 1).

Table 1. Expression frequency at each sensillum in three independent transgenic lines using a *Dmel\Obp57d* promoter construct

line #	sex	prothoracic leg				mesothoracic leg		metathoracic leg	
		5b	5s	4s	4c	5b	4s	5b	4s
1	female	0.63	0	0.03	0.95	0.68	0.13	0	0
	male	0.58	0	0.25	1	0.7	0.15	0.13	0
2	female	0.38	0	0	0.95	0.5	0.15	0.03	0
	male	0.43	0	0	0.95	0.45	0.1	0	0.03
3	female	0.45	0	0.03	1	0.6	0.03	0.1	0
	male	0.33	0	0.05	1	0.58	0	0.1	0.05

Names of the sensillum (5b, 5s, 4s, 4c) are based on Yasukawa et al., (2010). Difference between sex was not significant except for 4s on the prothoracic leg in the line #1 by Fisher's exact test at 0.05 level. The result of line #1 is also shown in Fig. 4A.

Fig. 3. Structure of the pGreenPelican+attB vector. For a precise examination of promoter activity, site-directed transformation by ϕ C31 integrase was combined with a GFP-reporter construct equipped with *gypsy* insulators. The multi-cloning site (MCS) of the pAttB vector was replaced with an insulator - MCS - *eGFP* - insulator fragment from pGreenPelican. The entire vector sequence was integrated into the landing site in the target genome by recombination at the attB site.

the pGreenPelican vector minimize the interference from surrounding genomic sequences (enhancers). Insertion of the reporter gene at exactly the same position on the chromosome is achieved by the pAttB vector via ϕ C31 integrase-mediated transformation. The newly constructed vector (pGreenPelican+attB) has both of these functions (Fig. 3). Results from three independent strains with the same promoter construct showed an extremely stable expression level (Table 1), proving that the pGreenPelican+attB vector is a reliable tool for the precise examination of promoter activity *in vivo*. Among 24 independent comparisons of expression frequency between sex (8 sensilla positions $\times$ 3 lines), difference was significant only in one case (4s on the prothoracic leg in the line #1), suggesting that the promoter activity was same in both sexes. Hereafter, a single transgenic line was examined for each promoter construct, and the data from both sexes were pooled.

Conserved *cis*-regulatory elements necessary for expression in the leg In our previous report, use of the promoters of *Dmel\Obp57d* and *Dmel\Obp57e* (at a length of 641 bp and 447 bp, respectively) led to the

expression of GFP in cells associated with chemosensilla on the legs (Yasukawa et al., 2010). Using shorter constructs that still contain all of the three candidate elements, this expression pattern was reproduced (Figs. 4A and 5A). Promoter constructs that lack the type-Ia element, however, resulted in a reduction in the expression level (Figs. 4B and 5B). Disruption of the type-Ib element, as well as removal of both type-I elements, also abolished the expression of GFP, proving that these type-I elements are necessary for promoter function (Figs. 4C and 5C). Disruption of the type-II element in the *Dmel\Obp57e* promoter abolished GFP expression as well, suggesting that the type-II element is also necessary (Fig. 5D).

We next examined whether the activity of each element is sensitive to sequence alteration, because the sequence of type-I elements in the *Dmel\Obp57e* promoter has mismatches with the original motif-1 sequence. Single nucleotide substitutions were introduced to the type-I elements in the *Dmel\Obp57e* promoter to restore the original motif-1 sequence, resulting in a drastic increase in the expression level (Fig. 5, E and F). On the other hand, restoration of the type-II element in the *Obp57d* promoter did not result in obvious differences, suggesting that this element retains full functionality even though its sequence has diverged from the original motif-1.

Synergistic effect of promoters on the expression activity After the birth of *Obp57d* and *Obp57e* by gene duplication, the ORF of *Obp57d* disappeared in some species (Matsuo, 2008b). Such a duplication-degeneration process may leave tandemly duplicated promoters, which might affect the expression of the remaining single gene. To test the consequence of this scenario, the promoters of *Dmel\Obp57d* and *Dmel\Obp57e* were conjugated in tandem. The expression level was drastically increased from that for the *Dmel\Obp57e* promoter alone (Fig. 6 vs. Fig. 5A), and even higher than addition of those for the

Dmel\Obp57d promoter and the *Dmel\Obp57e* promoter (Fig. 6 vs. Fig. 4A + Fig. 5A) in 5b on metathoracic leg and 4s on all legs, suggesting that the *cis*-regulatory elements have a synergistic effect on promoter activity (Fig. 6).

Fig. 4. Effect of candidate *cis*-regulatory elements in the *Dmel\Obp57d* promoter. (A) Intact 319 bp construct. (B) Deletion construct of 154 bp that lacks the type-Ia element. (C) Disruption construct for the type-Ib element. (D) Restoration construct for the type-II element. The structure of the promoter construct is shown above a picture of a prothoracic leg from a typical individual (lateral view). Substituted nucleotides are shown in red. Bar graph shows expression frequency at each sensillum. All legs from 20 individuals (10 each for males and females) were examined. Each bar represents observations of 80 cells for the sensilla that exist on both sides of the legs, and 40 cells for 4c. The schematic drawing beneath the picture shows a dorsal view of the right prothoracic leg (medial side is downward). The color of the circles represents the expression frequency at each sensillum: green for above 0.5, green with hatching for between 0 and 0.5, and black for 0.

Fig. 5. Effect of candidate *cis*-regulatory elements in the *Dmel\Obp57e* promoter. (A) Intact 330 bp construct. (B) Deletion construct of 190 bp that lacks the type-Ia element. (C) Deletion construct of 101 bp that lack both type-Ia and -Ib elements. (D) Degeneration construct for the type-II element. (E) Restoration construct for the type-Ia element. (F) Restoration construct for the type-Ib element.

Fig. 6. Synergistic effect of promoters on the expression level. Full-length versions of the *Dmel\Obp57d* promoter (641 bp) and the *Dmel\Obp57e* promoter (447 bp) were tandemly conjugated. Expression frequency in 5b on metathoracic leg and 4s on all legs was significantly higher than addition of those for *Dmel\Obp57d* promoter (Fig. 4A) and *Dmel\Obp57e* promoter (Fig. 5A) by Fisher's exact test at 0.01 level.

DISCUSSION

A new transformation vector for precise evaluation of promoter activity *in vivo* The reporter assay has been widely used for the functional analysis of gene promoters in *Drosophila*. In the conventional method, a reporter-gene construct was introduced to the genome by *P*-element based transformation. Because the insertion site of the reporter gene could not be controlled by this method, each transgenic line had the reporter gene at the different position on the chromosome, resulting in the different expression pattern of the reporter gene that was influenced by the surrounding chromosomal environment such as enhancers. In fact, this “positional effect” on the expression pattern of the reporter gene was observed in our previous study using the promoters of *Obp57d* and *Obp57e* (Yasukawa et al., 2010). Even with the identical reporter-gene construct, variation of the expression level among a series of transgenic lines was so large that it was impossible to quantify and compare the promoter activity at single sensillum level.

In this study, a new transformation vector based on ϕ C31 integrase was constructed to circumvent the positional effect in the reporter assay. Because the reporter gene was integrated at the defined landing site, the surrounding environment was kept identical among independent transgenic lines, making it possible to compare the promoter activity precisely. In fact, the effect of a single nucleotide substitution was successfully detected (Fig. 5, E and F), suggesting that this vector serves as a powerful tool for the *in vivo* analysis of the promoters that have a specific expression pattern.

Tissue specificity of *cis*-regulatory elements

Defining the tissue-specificity of expression is an important function of *cis*-regulatory elements. This function is achieved by the activation or suppression of expression in a particular tissue. In this study, both type-I and type-

II elements were proven to work for activation, because deletion of the elements resulted in a loss of expression in all tissues. Although *Obp57d* and *Obp57e* are expressed in the mouthparts in some species, the modification of type-I and type-II elements changed only expression levels in the legs, not the tissue-specificity, suggesting that the expression in the mouthparts is regulated by other elements yet to be identified.

Although both type-I and type-II elements are required, it has not been proven whether they are sufficient for expression in the legs. In addition, the minimum requirements for expression, such as order and spacing among elements, are not known.

cis-regulatory elements define the expression pattern in the leg sensilla

Among the legs, it was observed that the prothoracic legs had the highest expression frequency, and the metathoracic legs had the lowest expression frequency (Figs. 4A and 5A). Increase of the promoter activity by modification of *cis*-regulatory elements increased the expression frequency in the mesothoracic and metathoracic legs (Fig. 5, E and F), suggesting that the difference among the legs is dependent to the activity of the promoter. In fact, the taste neurons on the all legs expressing Gr32a project into the identical part of the central nervous system (Koganezawa et al., 2010), suggesting that the identical neural circuit supports the taste recognition by a given type of neurons regardless of on which leg they exist. Thus, the difference among the legs in their contribution to the behavioral and physiological responses against the taste stimuli, if any, might be caused by the difference in the expression levels of receptors or OBPs.

Interestingly, the expression frequency in the 5s sensilla on the prothoracic leg was not increased even with the modified promoters (Fig. 5, E and F), suggesting that the expression level in the 5s sensilla is independent from the promoter activity. The physiological responses of the 5s sensilla to bitter compounds were different from those of 5b and 4s (Meunier et al., 2003). The low expression level of *Obp57d* and *Obp57e* in the 5s sensilla might contribute to make them functionally different from other bitter taste sensilla.

Synergistic effect of *cis*-regulatory elements

In our previous study, the level of the *Obp57e* transcript was increased 10 fold in the *Obp57d* knockout flies (Matsuo et al., 2007). Because most of the *Obp57d* ORF was replaced with a short loxP recombination target sequence, the promoter sequences of *Obp57d* and *Obp57e* were situated tandemly in the *Obp57d* knockout flies. The same promoter structure was reproduced in this study. Tandemly situated promoters induced higher expression, suggesting that the synergistic effect caused the increased transcript level of *Obp57e* in the *Obp57d* knock-

out flies.

Contribution of regulatory mechanisms for OBP expression to behavioral adaptation In this study, it was shown that a single nucleotide substitution in the *cis*-regulatory element altered expression levels drastically, suggesting that the evolution of these *cis*-regulatory elements has the potential to cause variations in OBP levels among species. On the other hand, the evolution of *trans*-regulatory factors maintains expression levels in response to the evolution of *cis*-elements (Takahashi et al., 2011). Because OBP levels affect preference behavior (Matsuo et al., 2007; Harada et al., 2008, 2012; Matsuo, 2012), fine tuning of OBP levels through the evolution of *cis*- and *trans*-regulatory factors may contribute to adaptations to chemical environments via the modulation of behavioral responses.

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