Deciphering the Genes for Taste Receptors for Fructose in *Drosophila*

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Taste sensitivity to sugars plays an essential role in the initiation of feeding behavior. In *Drosophila melanogaster*, recent studies have identified several gustatory receptor (Gr) genes required for sensing sweet compounds. However, it is as yet undetermined how these GRs function as taste receptors tuned to a wide range of sugars. Among sugars, fructose has been suggested to be detected by a distinct receptor from other sugars. While GR43A has been reported to sense fructose in the brain, it is not expressed in labellar gustatory receptor neurons that show taste response to fructose. In contrast, the Gr64a-Gr64f gene cluster was recently shown to be associated with fructose sensitivity. Here we sought to decipher the genes required for fructose response among Gr64a-Gr64f genes. Unexpectedly, the qPCR analyses for these genes show that labellar expression levels of Gr64d and Gr64e are higher in fructose low-sensitivity flies than in high-sensitivity flies. Moreover, gustatory nerve responses to fructose in labellar sensilla are higher in Gr64d and Gr64f mutant lines than in mutant flies of the other Gr64a-Gr64f genes. These data suggest the possibility that deletion of GR64D or GR64F may indirectly induce enhanced fructose sensitivity in the labellum. Finally, we conclude that response to fructose cannot be explained by a single one of the Gr64a-Gr64f genes.

**Keywords:** *Drosophila melanogaster* genetic reference panel, gustatory receptor gene, sugar receptor, taste sensitivity

INTRODUCTION

Sweet taste is an essential chemosensory modality enabling animals to detect sugars, a critical energy source for survival, and facilitate consumption of energy-rich foods. In mammals, a wide range of sugars are all recognized by a single heterodimeric taste receptor T1R2/T1R3 expressed on the surface of taste cells in the tongue (Damak et al., 2003; Li et al., 2002; Nelson et al., 2001; Zhao et al., 2003). In *Drosophila melanogaster*, sugars are detected by sugar receptors expressed in sugar-responsive gustatory receptor neurons (GRNs), which are housed in chemosensilla present on the various taste organs: labellum, legs, and pharyngeal sense organs (Montell, 2009; Stocker, 1994). Studies over the past decade have suggested that nine of the 68 gustatory receptors (GRs) serve as sweet taste receptors (Dahanukar et al., 2007; Fujii et al., 2015; Jiao et al., 2007; Miyamoto et al., 2012; Slone et al., 2007). For example, Gr5a is required for trehalose sensing and also broadly mediates responses to several other sugars (e.g. glucose, maltose, and sucrose) along with Gr64f (Dahanukar et al., 2007; Jiao et al., 2008). In contrast, Gr43a is narrowly tuned to sense fructose and sucrose (Miyamoto et al., 2012). However, we do not yet clearly understand how these GRs for sugars function as sugar receptors.

Earlier studies on natural variation in the taste sensitivity to trehalose contributed to finding the *Tre* locus, which led to
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the identification of the trehalose receptor gene Gr5a (Dahanukar et al., 2001; Tanimura et al., 1982; Ueno et al., 2001). Recently, over 200 sequenced inbred lines, called the Drosophila melanogaster Genetic Reference Panel (DGRP), have been established from a natural population, which enables the dissection of various natural phenotypic variations (Huang et al., 2014; Mackay et al., 2012). Using the DGRP lines, we previously revealed that taste sensitivities to glucose, fructose, and sucrose, as well as trehalose, are polygenic among the DGRP lines (Uchizono and Tanimura, 2017). Moreover, behavioral tests using two DGRP lines that have a different sensitivity to fructose have shown that the Gr64a-Gr64f gene cluster is involved in fructose sensitivity as well as Gr43a, the narrowly tuned fructose receptor gene. Here we examine the association of individual Gr64a-Gr64f genes with fructose sensitivity. Expression levels of Gr64d and Gr64e mRNA in labella differ between the two DGRP lines, which show high and low sensitivities to fructose, respectively. Furthermore, electrophysiological recordings of GRNs show that deletion of individual Gr64a-Gr64f genes gives rise to distinct labellar responses to fructose and glucose. Similarly, behavioral responses to fructose and glucose are different among Gr64a-Gr64f mutant flies. These analyses verify the contribution of the Gr64a-Gr64f gene cluster to taste sensitivity to fructose, as well as the several other previously suggested sugars.

MATERIALS AND METHODS

Fly stocks

Two wild-derived, inbred DGRP lines, DGRP_301 and DGRP_712, were obtained from the Bloomington Drosophila stock center (Indiana, USA) (Mackay et al., 2012). Gr64d<tm3214>, Gr64d<tm3150>, Gr64d<tm5221>, and Gr64d<tm5478> flies were donated by H. Amrein (Texas A&M Health Science Center, USA) (Fuji et al., 2015). Act5C-GAL4/CyO flies were established in Weiss et al. (2011). UAS-Gr64d; Df(2R)TM3 flies were provided by S.J. Moon (Yonsei University College of Dentistry, Korea). Flies were reared on a cornmeal-agar-yeast-wheatgerm-glucose medium at 25℃ under a 12 h light/dark cycle.

Generation of Gr64d mutant

CRISPR/Cas9 was used to generate a Gr64d mutant. Two targeting plasmids were constructed, using the following oligos, 5’-CTCTGGTTGGCATCACCCCTGC-3’ and 5’-AACCTCGTTTTGGACCTCCTCA TTG-3’ (forward), and 5’-CTAATCCGATGGCACGCTACGTC-3’ (reverse), for Gr64d: 5’-TGCTTCGCAATGAAACCTTTGC-3’ (forward) and 5’-ACCTTCGCCTGGAAACTTTAACG-3’ (reverse), for Gr64e: 5’-TGCTTCGCAATGAAACCTTTGC-3’ (forward) and 5’-ATCCGGATAGGTCCATCTGG-3’ (reverse), for Gr64f: 5’-CTGTTCAAGTGGAAACCTTTGC-3’ (forward) and 5’-CTCCGGATACGAGGGGACG-3’ (reverse), for Gapdh2: 5’-GGACACACAGTGTGAC-3’ (forward) and 5’-CCCGAATGACGACGGTAC-3’ (reverse) and 5’-ATGGAACATCGATTTGCAC-3’ (reverse) and 5’-ATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse) and 5’-GATGGAACATCGATTTGCAC-3’ (reverse). Tip recordings

Action potentials were recorded from L1, L5, and L7 sensilla using the tip-recording method, essentially as described in Uchizono and Tanimura (2017). Recordings were performed on L3, L5, and L7 sensilla with 100 mM fructose, 100 mM glucose, and 100 mM sorbitol solution. To precisely count the spikes originating from the sugar-responsive receptor neuron, the number of water spikes elicited by 100 mM fructose was subtracted from the total number of spikes elicited by 100 mM fructose or 100 mM glucose in each sensillum.

Two-choice preference test

The two-choice preference test was performed as previously described (Hiroi et al., 2004). The flies were starved (sup
plied only with water) on the basis of the time taken for 10% of the flies to die in each strain, and then given choices between 32.5 mM glucose colored with blue food dye and different concentrations of fructose (2–320 mM) colored with red food dye for 1 h in darkness. Fructose sensitivity was thus determined as relative to glucose sensitivity, as carried out previously (Uchizono and Tanimura, 2017). The preference index (PI) for fructose was calculated using the following formula: \( \frac{N^R + N^M}{2} / (N^B + N^M + N^R) \), where \( N^B \), \( N^R \) and \( N^M \) represent the number of flies colored blue, red and purple, respectively.

RESULTS AND DISCUSSION

Expression levels of Gr64d and Gr64e genes in labella differ between strains showing high- and low-sensitivity to fructose

The Gr64a–Gr64f gene locus has been implicated in the difference in fructose sensitivity between two wild-derived inbred lines, DGRP_301 and DGRP_712 (Uchizono and Tanimura, 2017). Sensitivity to fructose differs between the two lines in taste organs, labellum and tarsi; the labellar nerve response to fructose in DGRP_301 (fructose low-sensitivity line, to which we refer as LF) is notably lower than the response in DGRP_712 (fructose high-sensitivity line, to which we refer as HF). To determine which gene of the Gr64 family is involved in fructose sensitivity, we first quantified the expression levels of the Gr64a–Gr64f genes in the labella of LF and HF flies by qPCR. Unexpectedly, the expression levels of two genes, Gr64d and Gr64e, were significantly higher in LF than HF (Fig. 1). Consistent with these results, DGRP_712 (HF) has a deletion of 38 bp (3L: 4,032,908–4,032,945, relative to the reference allele in the DGRP lines) in the 3rd exon of the Gr64d gene (DGRP Freeze 2 genome browser, http://dgrp2.gnets.ncsu.edu). These results raise the possibility that down-regulation of these genes results in higher sensitivity to fructose, even though these mRNA levels might not necessarily reflect the translation levels. In addition, the expression pattern of these Gr64a–Gr64f genes are consistent with previous 5’- and 3’-RACE experiments, which suggested the bicistronic transcription of Gr64bc and Gr64de in addition to single mRNAs of Gr64a, Gr64e, and Gr64f (Dahanukar et al., 2007). The transcription levels of Gr64d and Gr64e may be partially down-regulated together in the labellum of HF flies. Furthermore, the expression level of Gr64a in labella was quite low in both lines, which is consistent with a previous expression analysis of Gr64a (Uchizono and Tanimura, 2017) using a UAS-RFP reporter (Fujii et al., 2015). Gr64f also showed surprisingly low expression levels in both lines. Given that Gr64f has been reported to be widely expressed in sugar-responsive GRNs in the labellum (Dahanukar et al., 2007; Fujii et al., 2015; Weiss et al., 2011), the translational level of Gr64f might be distinct from the mRNA level.
Labellar and behavioral responses to fructose are altered by deletion of Gr64a–Gr64f genes

In the light of the qPCR results, we wondered whether deletion of Gr64d or Gr64e gene affects labellar response to fructose. To explore the possibility, we generated a deletion allele of Gr64d (Gr64d1) using CRISPR/Cas9-mediated genome editing as described in Materials and Methods (Fig. 2). We then recorded labellar nerve responses to fructose in mutant flies of each Gr64a–Gr64f gene; Gr64aGAL4, Gr64bLEXA, Gr64cLEXA, Gr64d1, Gr64eLEXA, and Gr64fLEXA. Interestingly, Gr64d1 and Gr64fLEXA showed higher responses to 100 mM fructose than the other mutant flies, implying that loss of Gr64d and Gr64f genes may induce enhanced fructose sensitivity in the labellum (Fig. 3A). The higher response to fructose in Gr64d1 is likely to be consistent with the lower expression of Gr64d in the labellum of HF flies. Given that sugar receptors function as multimeric complexes composed of two or more subunits (Dahanukar et al., 2007; Jiao et al., 2007; 2008; Slone et al., 2007), one plausible interpretation of these results is that GR64D and GR64F competitively interact with a member of a functional fructose receptor to form another receptor, as is the case for GR64E, which was suggested to be shared by GR64A and GR64B to form distinct sugar receptors (Yavuz et al., 2014). However, we were not able to identify which gene of the Gr64 family is the member of the functional fructose receptor since these mutant lines, except for Gr64d1 and Gr64fLEXA, showed considerably lower responses to fructose, similar to the LF flies: the numbers of spikes elicted by fructose were close to zero, especially in Gr64aGAL4 and Gr64fLEXA flies.

We then further examined the behavioral responses to fructose in these mutant lines. Sensitivity curves for fructose, determined by two-choice preference tests using different concentrations of fructose against a constant concentration of glucose, successfully distinguished the fructose sensitivities in HF and LF lines (Uchizono and Tanimura, 2017). Thus, sensitivity curves were employed to compare the behavioral responses to fructose in mutant flies of each Gr64a–Gr64f gene. To this end, we first tested glucose sensitivity in each mutant line. Labellar responses to glucose were found to be diminished in Gr64cLEXA and Gr64fLEXA (Fig. 3B). The impaired glucose response in Gr64cLEXA is compatible with previous studies showing that Gr64f is required for glucose sensing (Fuji et al., 2015; Jiao et al., 2008), whereas Gr64c has not yet been implicated in the glucose response. Further work is necessary to validate whether the Gr64c gene locus deleted in Gr64cLEXA is required for the glucose response in the labellum.

Figure 4 shows the sensitivity curves of each of the Gr64a–Gr64f mutant flies. Interestingly, these six curves can be classified into three groups: (i) Gr64aGAL4 and Gr64eLEXA, (ii) Gr64dLEXA, Gr64cLEXA, and Gr64fLEXA, and (iii) Gr64d1. In contrast to the notably low fructose responses in their labellum, Gr64dLEXA and Gr64cLEXA flies preferred lower concentrations of fructose. The discrepancy may be explained by fructose responses in the other taste organs and also by impaired glucose sensitivity in the case of Gr64cLEXA. Gr64d1 flies preferred 2 mM fructose to 32.5 mM glucose, presumably due to the higher response to fructose and lower response to glucose. In addition, we tested if the expression level of Gr64d affects the behavioral response to fructose by over-expressing Gr64d using UAS-Gr64d with ubiquitous Act5C-GAL4 or Gr5a-GAL4 driver. All tested lines, including GAL4-alone and UAS-alone controls, showed similar sensitivity curves, and increased expression of Gr64d did not change fructose response (Supplementary Fig. S1). Thus, over-expression of the Gr64d does not appear to affect the formation of a functional fructose receptor, though loss of the Gr64d expression leads to higher fructose response. These results suggested that Gr64a–Gr64f gene loci are indeed associated with fructose sensitivity; nevertheless we were not able to identify the fructose receptor gene among the Gr64a–Gr64f genes. Our current analyses obviously suggest
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**Fig. 4. Behavioral responses to fructose and glucose in Gr64a–Gr64f mutant flies.** Relative sensitivity to fructose against glucose sensitivity was determined by two-choice preference tests between 32.5 mM glucose and different concentrations of fructose. PI values for fructose are shown at the following concentrations of fructose: 5, 20, 80, and 320 mM in Gr64aGAL4 (closed circle, n = 5); 2, 5, 10, and 20 mM in Gr64bLEXA (open circle, n = 5), Gr64eLEXA (closed square, n = 5), and Gr64fLEXA (open square, n = 5); 2, 5, and 10 mM in Gr64cLEXA (closed triangle, n = 5) and Gr64d1 (open triangle, n = 4). Error bars indicate SEMs.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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