Molecules and Cells

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; 2008; 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). $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64e^{LEXA}$, and $Gr64f^{LEXA}$ flies were donated by H. Amrein (Texas A&M Health Science Center, USA) (Fujii et al., 2015). *Act5C-GAL4/CyO* flies were obtained from the Bloomington Drosophila stock center. *Gr5a-GAL4/CyO* flies were established in Weiss et al. (2011). *UAS-Gr64d*; *Dr/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'-CTTCGTTGGCGTACTACCCCTGGC-3' and 5'-AAACGCCAGGGGTAGTACGCCAAC-3', and 5'-CTTCGTAACTCCTGACGCTCGCGA-3' and 5'-AAACTCGCGAGCGTCAGGAGTTAC-3', and ligating the annealed products into pU6-BbsI-chiRNA. 250 ng/μl of each plasmid was mixed together and injected into CAS-0001 ($y^2 cho^2 v^1$; *attP40{nos-Cas9}/CyO*) embryos. The emerging adult flies were crossed with a balancer stock and PCR was used to isolate mutants with deletions between the two target sequences, using the primers 5'-CCCTGCATTATACCATTGGG-3' and 5'-CAAGCCTCGACACATGAGAA-3'. The break points were verified by sequencing. We isolated several deletion alleles, and used the $Gr64d^{1}$ allele, which has a 539 bp deletion covering the 2nd and 3rd exon of Gr64d-PC, in this study.

Chemicals

D-glucose was obtained from Sigma-Aldrich Corp. (USA); D-fructose and D-sorbitol were obtained from Wako Pure Chemical Industries, Ltd. (Japan); and Food Blue No. 1 and Food Red No. 106 were obtained from Tokyo Chemical Industry Co., Ltd. (Japan).

Quantitative PCR (qPCR) assay

Total RNA was extracted using TRIzol reagent (Invitrogen) from 200 labella of 4-5-day-old flies and purified with RNeasy micro kit (QIAGEN) according to the protocol provided by the manufacturers. cDNA was synthesized from total RNA using Superscript III (Invitrogen) following the protocol provided. qPCR was carried out using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) with an Mx3000P qPCR system (Agilent Technologies). The 1 μl of synthesized cDNA in a 20 μl volume was amplified with 0.5 μM primers as follows: 10 min at 95℃, then 40 cycles of 15 s at 95℃, 30 s at 60℃, and 1 min at 72℃. A housekeeping gene *Gapdh2* was used as an internal control to normalize mRNA levels. The data finally obtained were calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). We confirmed that all primer sets used did not yield any non-specific amplification by a melting curve analysis using the products of qPCR. Triplicate reactions for each of three biological replicates were performed for each sample.

qPCR primer pairs for *Gr64a* were: 5'-GTGTGCTACCAACTGCTAAATGTC-3' (forward) and 5'-ACCTCGTTTGGACTCCTCATTG-3' (reverse), for *Gr64b*: 5'-CTATCGGTTCTACGGCGAGTAC-3' (forward) and 5'-ACTGGGTGCGCTCCATATTG-3' (reverse), for *Gr64c*: 5'-CTCAGTGATCTTCTGGGTATCTACG-3' (forward) and 5'-ATCCGCATAGTTGCCCTTGG-3' (reverse), for *Gr64d*: 5'-TGCTTCGCAATGAAACCTTTGC-3' (forward) and 5'-CTTGCATTTGCCGGAACAGC-3' (reverse), for *Gr64e*: 5'-ACCTTCGCCTGGAACTTTAACG-3' (forward) and 5'-CCTGCACTGCATCCAATAGTCC-3' (reverse), for *Gr64f*: 5'-CCGCAGTACAAGACACAGTTGAC-3' (forward) and 5'-TCCGCAAAGGACAGCATCATTC-3' (reverse), and for *Gapdh2*: 5'-CTACCTGTTCAAGTTCGATTCGAC-3' (forward) and 5'-AGTGGACTCCACGATGTATTCG-3' (reverse).

Tip recordings

Action potentials were recorded from l-type labellar chemosensilla 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 sorbitol 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: $(N^R + N^M/2) / (N^B + N^M + N^R)$, 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 $Gr64a^{GAL4}$ 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.

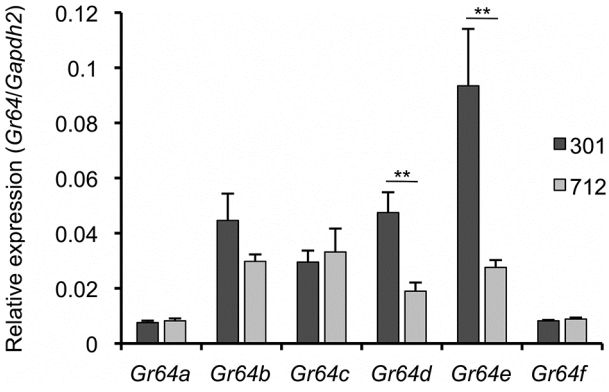

Fig. 1. Comparisons of the expression levels of *Gr64a-Gr64f* genes in the labella of strains showing high- and low-sensitivity to fructose. RNA expression levels of the *Gr64a-Gr64f* genes in labella were compared between DGRP_301 (LF) and DGRP_712 (HF) by qPCR assays. The dark and light gray bars represent the relative mRNA levels of each *Gr64* gene normalized by the level of the *Gapdh2* gene in DGRP_301 and DGRP_712, respectively (*Gr64b*, *Gr64d*, and *Gr64f*, n = 3 in triplicate; *Gr64a*, *Gr64c*, and *Gr64e*, n = 5 in triplicate). Error bars represent SEMs. The relative expression levels of each *Gr64* gene were compared between the two lines by Student's *t*-test or Welch's *t*-test (**$P < 0.01$).

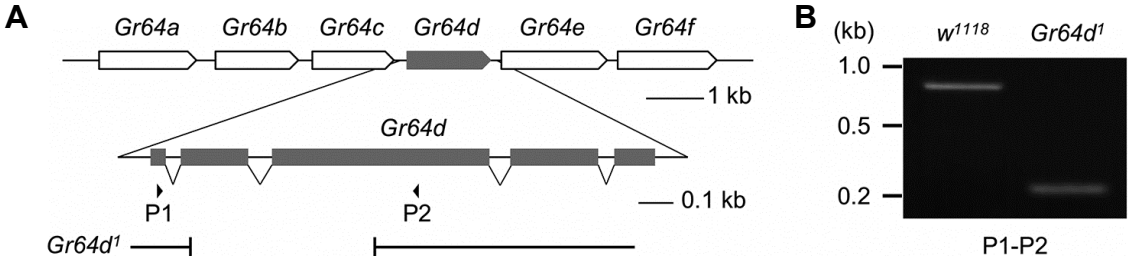

Fig. 2. Generation of the *Gr64d* mutant strain. (A) Organization of the *Gr64a-Gr64f* genomic region is shown on the top, and the exon-intron structure of the *Gr64d* is magnified below. Exons are represented as grey boxes and introns as v-shaped lines. The arrowheads show the positions of the primers used for the PCR analysis in (B). Several small deletion alleles within the *Gr64d* gene were induced by the CRISPR/Cas9 system, and one of the alleles ($Gr64d^1$) was used in this study, which contains a 539 bp deletion extending from the 2nd exon to the 3rd exon of *Gr64d* (bottom). (B) Confirmation of the presence of the deletion in $Gr64d^1$ by genomic PCR using the primer pair, P1 and P2 indicated in (A). Genomic DNA from w^{1118} was also analyzed as control.

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* (*Gr64d*^{1}) 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; *Gr64a*^{GAL4}, *Gr64b*^{LEXA}, *Gr64c*^{LEXA}, *Gr64d*^{1}, *Gr64e*^{LEXA}, and *Gr64f*^{LEXA}. Interestingly, *Gr64d*^{1} and *Gr64f*^{LEXA} 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 *Gr64d*^{1} 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 *Gr64d*^{1} and *Gr64f*^{LEXA}, showed considerably lower responses to fructose, similar to the LF flies; the numbers of spikes elicited by fructose were close to zero, especially in *Gr64a*^{GAL4} and *Gr64b*^{LEXA} flies.

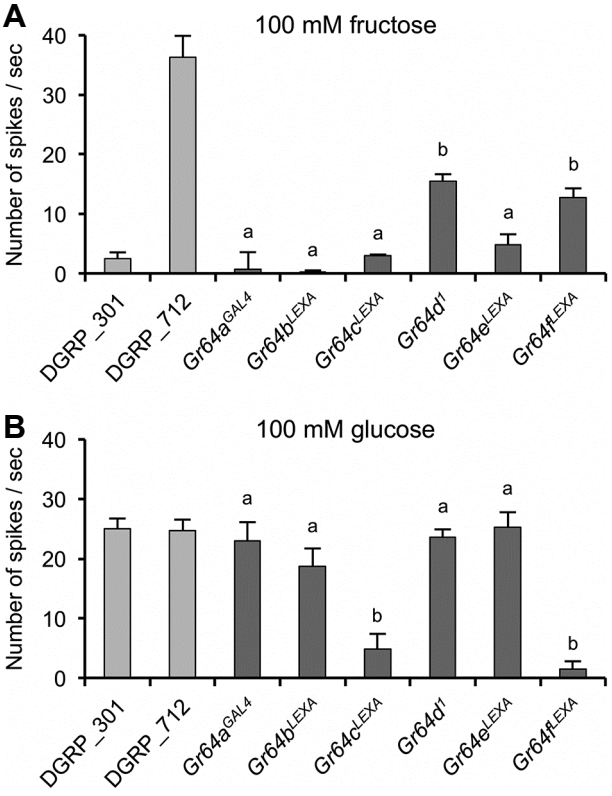

Fig. 3. Gustatory nerve responses of labellar chemosensilla to fructose and glucose in *Gr64a-Gr64f* mutant flies. Responses of l-type sensilla to 100 mM fructose (A) and 100 mM glucose (B) were recorded in *Gr64a*^{GAL4} (n = 9), *Gr64b*^{LEXA} (n = 6), *Gr64c*^{LEXA} (n = 6), *Gr64d*^{1} (n = 9), *Gr64e*^{LEXA} (n = 9), and *Gr64f*^{LEXA} (n = 6) flies. The dark gray bars represent the number of sugar spikes per second, which was calculated by subtracting water spikes elicited by 100 mM sorbitol solution from the total spikes elicited by 100 mM fructose or 100 mM glucose solution in each sensillum. Bars denoted by the same letter do not differ significantly ($P > 0.05$, one-way ANOVA with Tukey-Kramer *post hoc* tests). For comparison, the numbers of spikes elicited in DGRP_301 and DGRP_712 (Uchizono and Tanimura, 2017) are also shown by light gray bars. Error bars indicate SEMs.

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 *Gr64c*^{LEXA} and *Gr64f*^{LEXA} (Fig. 3B). The impaired glucose response in *Gr64f*^{LEXA} is compatible with previous studies showing that *Gr64f* is required for glucose sensing (Fujii 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 *Gr64c*^{LEXA} 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) *Gr64a*^{GAL4} and *Gr64e*^{LEXA}, (ii) *Gr64b*^{LEXA}, *Gr64c*^{LEXA}, and *Gr64d*^{1}, (iii) *Gr64f*^{LEXA}. In contrast to the notably low fructose responses in their labellum, *Gr64b*^{LEXA} and *Gr64c*^{LEXA} 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 *Gr64c*^{LEXA}. *Gr64f*^{LEXA} 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 overexpressing *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, overexpression 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

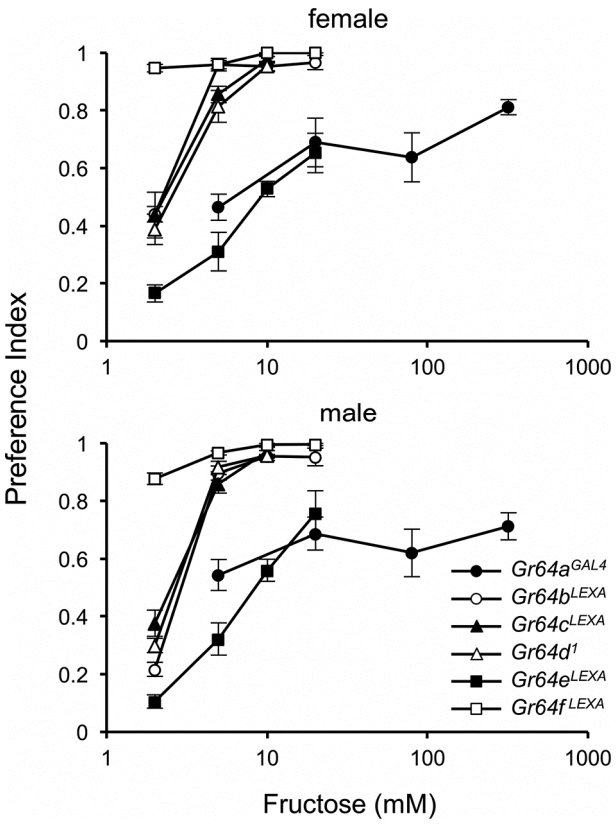

Fig. 4. Behavioral responses to fructose and glucose in *Gr64a-Gr64f* mutant flies. Relative sensitivity to fructose against glucose sensitivity was determined by two-choice preference tests between 32.5 mM glucose and different concentrations of fructose. PI values for fructose are shown at the following concentrations of fructose: 5, 20, 80, and 320 mM in $Gr64a^{GAL4}$ (closed circle, n = 5); 2, 5, 10, and 20 mM in $Gr64b^{LEXA}$ (open circle, n = 5), $Gr64e^{LEXA}$ (closed square, n = 5), and $Gr64f^{LEXA}$ (open square, n = 5); 2, 5, and 10 mM in $Gr64c^{LEXA}$ (closed triangle, n = 5) and $Gr64d^{1}$ (open triangle, n = 4). Error bars indicate SEMs.

the limitations of a study focusing on single genes from *Gr64a-Gr64f*. Further rescue experiments of these genes should thus be performed in a combinatorial manner as undertaken by Yavuz et al. (2014) to ascertain the organization of the functional fructose receptor. Our observations should be helpful for this further study.

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

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