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Development of a screening system for agents that modulate taste receptor expression with the CRISPR-Cas9 system in medaka

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ABSTRACT

Taste recognition mediated by taste receptors is critical for the survival of animals in nature and is an important determinant of nutritional status and quality of life in humans. However, many factors including aging, diabetes, zinc deficiency, infection with influenza or cold viruses, and chemotherapy can trigger dysgeusia, for which a standard treatment has not been established. We here established an engineered strain of medaka (*Oryzias latipes*) that expresses green fluorescent protein (GFP) from the endogenous taste 1 receptor 3 (T1R3) gene locus with the use of the CRISPR-Cas9 system. This T1R3-GFP knock-in (KI) strain allows direct visualization of expression from this locus by monitoring of GFP fluorescence. The pattern of GFP expression in the T1R3-GFP KI fish thus mimicked that of endogenous T1R3 gene expression. Furthermore, exposure of T1R3-GFP KI medaka to water containing monosodium glutamate or the anticancer agent 5-fluorouracil resulted in an increase or decrease, respectively, in GFP fluorescence intensity, effects that also recapitulated those on T1R3 mRNA abundance. Finally, screening for agents that affect GFP fluorescence intensity in T1R3-GFP KI medaka identified tryptophan as an amino acid that increases T1R3 gene expression. The establishment of this screening system for taste receptor expression in medaka provides a new tool for the development of potential therapeutic agents for dysgeusia.

1. Introduction

The sense of taste is essential for identification of healthful nutrients and avoidance of unhealthful compounds such as those found in spoiled food or poisonous species. The five basic taste modalities are known as sweet, sour, bitter, salty, and umami and are recognized by specific taste receptors in gustatory cells on the tongue [1–3]. Taste 1 receptors (T1Rs) are multimodal G protein–coupled receptors that are activated by sweet or umami substances [4]. The umami taste receptor is heterodimeric proteins composed of T1R1 and T1R3 and the sweet taste receptor is that of T1R2 and T1R3 subunits [5,6]. These taste receptors transduce their signals to a common effector enzyme, the β2 isoform of phospholipase C (PLC-β2) [7]. We have previously shown that changes in taste receptor gene expression in the tongue are associated with chemotherapy-induced dysgeusia in patients with head and neck cancer [8], with T1R3 gene expression being decreased after chemotherapy and T2R5 gene expression increased.

Abbreviations: PLC-β2, β2 isoform of phospholipase C; T1Rs, taste 1 receptors; T1R3, taste 1 receptor 3; T2R5, taste 2 receptor 5; MSG, monosodium glutamate; CRISPER, clustered interspaced short palindromic repeats; sgRNA, single guide RNA; qPCR, quantitative PCR; GFP, green fluorescent protein; dph, days post-hatching; KI, knock-in; WT, wild-type; GMP, guanosine 5'-monophosphate; IMP, inosine 5'-monophosphate; 5-FU, 5-fluorouracil.

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Dysgeusia has an adverse impact on quality of life and exacerbates pathological conditions such as weight loss and malnutrition. In addition to chemotherapy, it is associated with aging, diabetes, zinc deficiency, and infection with influenza or cold viruses. Its negative effect on nutritional status can prolong hospitalization of patients and impede treatment continuation [9]. Dysgeusia is one of the most frequent and serious side effects in individuals treated for cancer, resulting in loss of appetite and a reduced quality of life [10,11]. However, the causes and mechanisms of dysgeusia are complex and remain unclear, and there is no established basic method of treatment.

Monosodium glutamate (MSG) is an umami compound and a ligand for T1R3, and stimulation of the tongue with MSG upregulates expression of the T1R3 gene [12]. We recently showed that dietary supplementation with MSG increased T1R3 gene expression and maintained food intake in cancer patients undergoing chemotherapy [6], suggesting that it might prove effective for the treatment of dysgeusia by maintaining T1R3 gene expression. However, given that MSG has a harsh and bitter taste that makes it difficult to consume by itself, the identification of other active ingredients would expand food choice based on individual preference.

An animal model with a relatively simple nervous system such as a small fish may be able to compensate for some deficiencies of mammalian models used for taste research. Fish thus possess about five times as many taste buds as do rodents used in most taste studies [13,14]. We have focused on medaka (*Oryzias latipes*), whose taste buds are located on the lips, gill rakers, and pharynx [14,15] and for which many taste-related genes have been identified by bioinformatics analysis of the genome sequence, as an experimental animal [16]. Medaka are able to recognize amino acids and bitter compounds as taste stimulants, suggesting that they possess a taste recognition mechanism similar to that of humans [17,18]. Similarities between teleost and mammalian brain regions that subserve higher order sensory transduction have been proposed [19,20], and model fish species have been found to express T1R and T1R orthologs that respond to amino acids and bitter substances, respectively, as well as an ortholog of PLC-β2 [18,21]. These characteristics support the study of medaka as an animal model for research on the taste system.

We have now applied the CRISPR (clustered regularly interspaced short palindromic repeats)—Cas9 (CRISPR-associated 9) system of genetic engineering [22] to establish medaka in which taste receptor expression can be visualized directly and which therefore allow monitoring of changes in such expression in vivo.

2. Materials and methods

2.1. Fish lines and maintenance

All procedures were performed in compliance with the guidelines for proper conduct of animal experiments and related activities of the Ministry of Education, Culture, Sports, Science, and Technology of Japan as well as with the ARRIVE guidelines, and the study was approved by the animal care and use committee of Tokushima University and of National Institute for Basic Biology. The OK-Cab^lf strain of medaka was used to generate transgenic lines. All fish and embryos were maintained on a 14-h-light, 10-h-dark cycle at 22° to 26 °C. The OK-Cab (strain ID: MT830), OK-Cab^lf (strain ID: MT1335) strains and hatching enzyme were obtained from National Bio-Resource Projects Medaka (https://shigen.nig.ac.jp/medaka: Okazaki, Japan). All fish were raised in a water circulation system, with 10% of the system volume being replaced daily with dechlorinated tap water. The fish were fed brine shrimp (Miyako kagaku, Tokyo, Japan) and a powdered diet (Marubeni Nisshin Feed, Tokyo, Japan) once a day. Other fish care and breeding procedures followed the Medaka protocol book [23].

2.2. Generation of sgRNAs and Cas9 mRNA

The single guide RNA (sgRNA) expression vector pDR274 (Addgene plasmid 42250) was linearized with BsaI and isolated by electrophoresis through a 2% agarose gel. The genome-targeting sequences were 5′-GAGAAAGACTCAGGTTTAGCGGG-3′ for a T1R3 sgRNA and 5′- GATCTTCGGCCTAGACTGCGAGG-3′ for a BaitD (bait sequence) sgRNA. Template DNA was obtained by polymerase chain reaction (PCR)—mediated amplification with the use of KOD-plus-Neo (Toyobo, Osaka, Japan) and purification of the PCR product with the use of a NucleoSpin Gel and PCR Clean-Up Kit (Macherey-Nager, Landsmeer, the Netherlands). The template DNA was subjected to in vitro transcription to obtain the sgRNAs with the use of an AmpliScribe T7-flash Transcription Kit (Epicentre, Madison, WI, USA). The plasmid pCS2-hSpCas9 [24] was digested with *Not*I and was transcribed to produce Cas9 mRNA with the use of an mMESSAGE mMACHINE SP6 Kit (Life Technologies, Carlsbad, CA, USA). The sgRNAs and Cas9 mRNA were purified with an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA).

2.3. Microinjection of embryos

Purified sgRNAs and Cas9 mRNA were co-injected into medaka embryos together with purified donor DNA with the use of a micromanipulator (Narishige, Tokyo, Japan) as previously described [23]. The donor plasmid was pBS-Tbait-olhs-GFP (NBRP Medaka). Each embryo was injected with a solution (0.8 μl) containing sgRNAs for digestion of the donor plasmid and genomic DNA (10 ng/μl each), Cas9 mRNA (100 ng/μl), and donor plasmid (5 ng/μl).

2.4. RT-qPCR analysis

Medaka were deeply anesthetized with tricaine (MS-222; TCI, Tokyo, Japan) before dissection and isolation of RNA. Total RNA was extracted from various tissues of adult fish or from larvae with the use of an RNeasy Mini Kit (Qiagen) and was subjected to reverse transcription (RT) with a Primescript RT Reagent Kit (Takara, Shiga, Japan). The resulting cDNA was subjected to quantitative PCR (qPCR) analysis in a final volume of 10 μl with the use of SYBR Green and a StepOnePlus Real-Time PCR System (Life Technologies). The sequences of the qPCR primers (forward and reverse, respectively) were as follows: T1R3, 5′-CAAACTGTGGCTCTCTCCGT-3′ and 5′-GCTCTTCGGAAACTCGGTCA-3′; T2R1, 5′-TTCGTGTTCCACCCCAA-CAA-3′ and 5′-GGAGATTGAGACGCAGCACT-3′; PLC-β2, 5′-ACAA-GAGGCCACACTGGAAG-3′ and 5′-TCTGCTGGCAAAGACCTCAG-3′; and RPL7, 5′-CGCCAGATCTTCAACGGTGTAT-3′ and 5′-AGGCTCAG-CAATCCTCAGCAT-3′. Expression levels of target genes were normalized by that of the RPL7 gene.

2.5. Imaging

Medaka were anesthetized as described above before immobilization with 3% methylcellulose in a 35-mm glass-bottom dish. Three dimensional images were acquired with a Leica SP8 or Nikon A1 confocal microscope system (Leica Microsystems GmbH, Wetzlar, Germany, and Nikon, Tokyo, Japan, respectively) by 20x water immersion lenses with appropriate conditions. Wide-field fluorescent images were acquired by IX81 (Olympus, Tokyo, Japan) with sCMOS Camera ORCA FLASH4.0 (Hamamatsu Photonics,

Hamamatsu, Japan), Cyan LED of a light engine spectra-X (Lumencor, Beaverton, OR, USA) for excitation light source and FF01-520/535 emission filter and analyzed by Image J (NIH, MD, USA). For monitoring of changes in fluorescence intensity over 48 h, a Keyence BZ-X800 microscope with CFI Plan Fluor DL 10× objective lens and BZ-X filter GFP (Keyence, Osaka, Japan) was used because its simplicity of operation and short exposure time rendered it more suitable for living medaka. Fluorescence intensity was analyzed with the use of BZ-X800 Analyzer software (Keyence).

2.6. Statistical analysis

Quantitative data are presented as means ± SD or SEM. Dunnett's test was applied for multiple comparisons of gene expression, and Bonferroni's test for those of fluorescence intensity. Statistical analysis was performed with GraphPad Prism 8 (San Diego, CA, USA). BellCurve for Excel (Social Survey Research Information, Tokyo, Japan). A P value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Tissue distribution of taste-related gene expression in medaka

We first investigated the distribution of taste-related gene expression in medaka by RT-qPCR analysis of T1R3, T2R1, and PLC-β2 mRNAs in tissues isolated from 3-month-old fish of the OK-Cab strain. T1R3 (Fig. 1A) and PLC-β2 (Fig. 1B) genes were found to be expressed in tissues located around the oral cavity including lips, gill rakers, and the pharynx, whereas the T2R1 gene (Fig. 1C) was expressed substantially only in gill rakers among tissues related to the oral cavity, being also expressed in other tissues such as spleen, heart, and kidney. Transcripts encoding T1R1 or T1R2 were not detected at substantial levels (data not shown).

We next investigated the effects of exposure to MSG or the anticancer drug 5-fluorouracil (5-FU) on T1R3 gene expression in medaka. We previously showed that T1R3 gene expression was increased by MSG and reduced by 5-FU treatment both in patients with head and neck cancer and in mice [6,8]. Medaka larvae at 7 days posthatching (dph) were exposed to water containing MSG or 5-FU for 48 h before RT-qPCR analysis of T1R3, PLC-β2, and T2R1

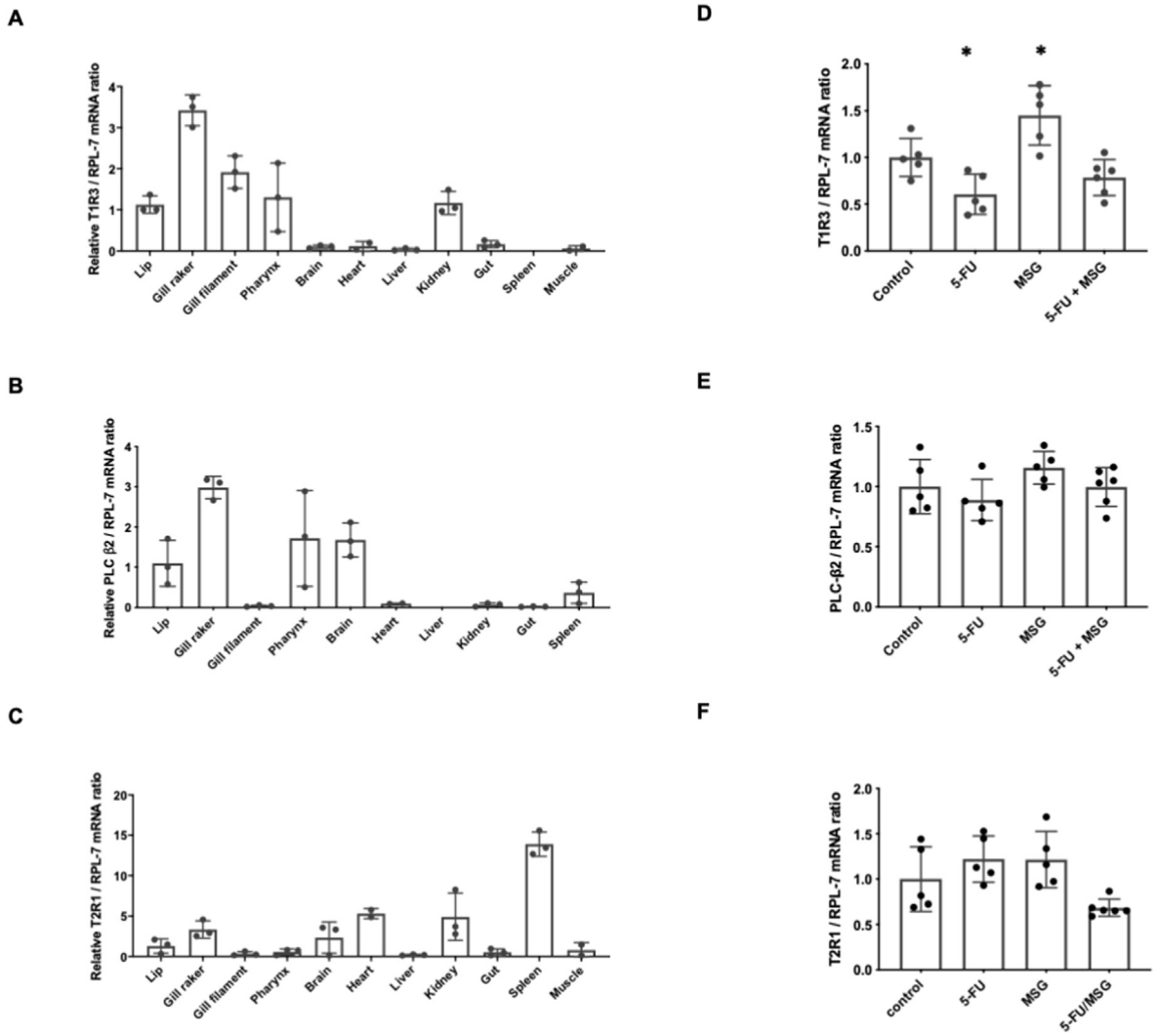

Fig. 1. Tissue distribution of and effects of MSG and 5-FU on taste-related gene expression in medaka. (A–C) RT-qPCR analysis of T1R3 (**A**), PLC-β2 (**B**), and T2R1 (**C**) mRNA abundance in various tissues of 3-month-old medaka of the OK-Cab strain. Data are expressed relative to the corresponding value for the lip and are means ± SD ($n = 3$, each). (**D–F**) RT-qPCR analysis of T1R3 (**D**), PLC-β2 (**E**), and T2R1 (**F**) mRNA abundance in 7-dph larvae of the OK-Cab strain after exposure to water containing 5-FU (100 μM) or MSG (100 μM) for 48 h. Data are expressed relative to the corresponding control value and are means ± SD ($n = 5$, each. *$P < 0.05$ versus control group, Dunnett's test).

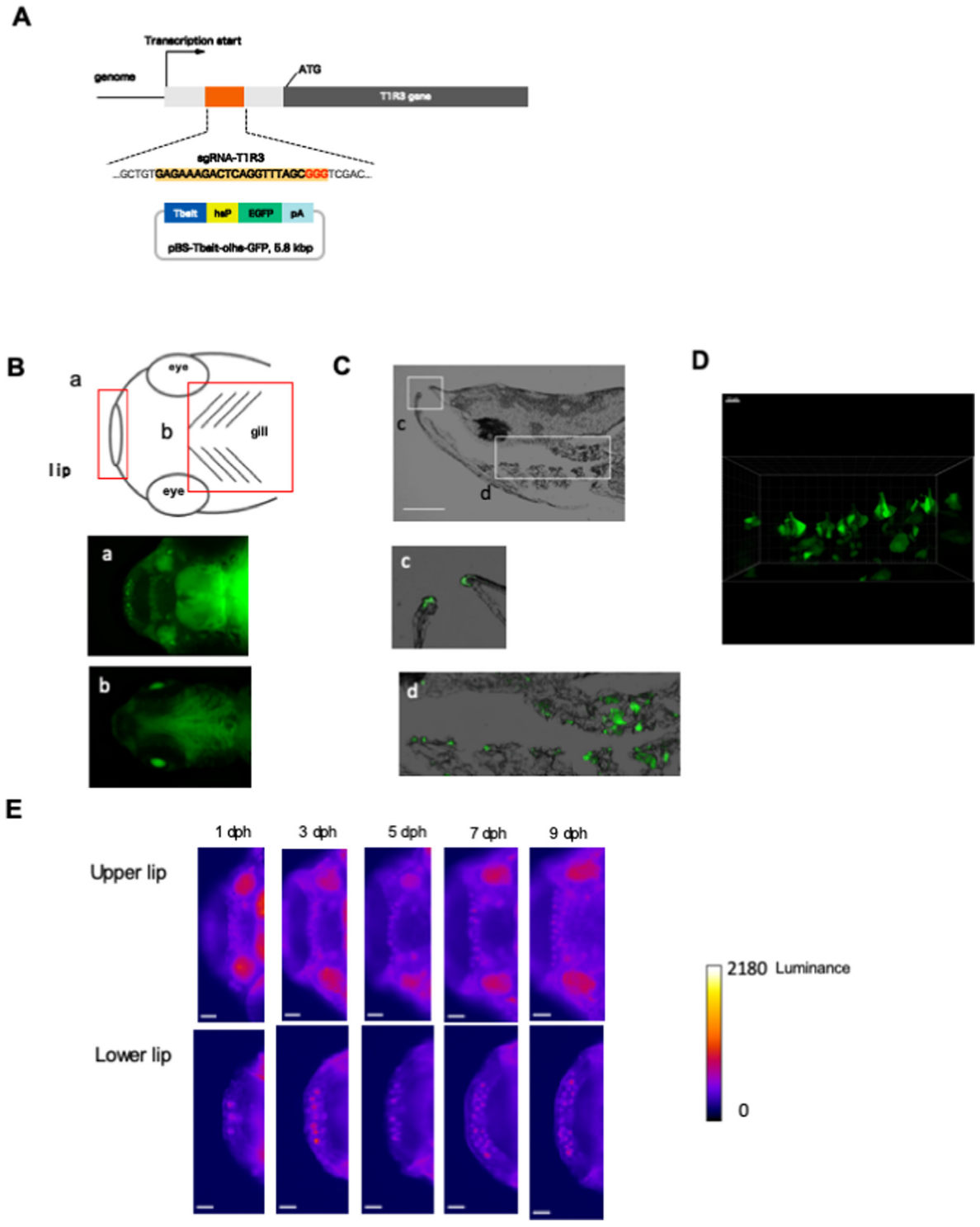

Fig. 2. Establishment of T1R3 gene-edited medaka with the CRISPR-Cas9 system. (**A**) Strategy for generation of T1R3-GFP KI medaka. The bait plasmid (pBS-Tbait-olhs-GFP) contains the Tbait sequence, the medaka Hsp70 gene promoter, the GFP coding sequence, and a poly(A) coding sequence. (**B**) GFP fluorescence images of the pharynx of T1R3-GFP KI medaka (7dph) acquired from above. Images **a** and **b** show the lip and gills. Scale bar shown 20 μm. (**C**) GFP fluorescence images of the lips, gill rakers, and pharynx acquired from the side. Images **c** and **d** show the lips as well as the gill rakers and pharynx, respectively. Scale bar, 200 μm. (**D**) Three-dimensional imaging of GFP expression in taste buds by confocal microscope. Scale bar, 10 μm. See also 3-D imaging video in supplementary data. (**E**) Confocal microscopic images of GFP expression in the upper and lower lips of T1R3-GFP KI medaka at 1 to 9 dph. Scale bars, 50 μm.

gene expression. Consistent with our previous data in mice and humans, we found that T1R3 gene expression was up-regulated by MSG and down-regulated by 5-FU in medaka larvae (Fig. 1D), with the effects of the two agents canceling each other out. In contrast, the amounts of PLC-β2 and T2R1 mRNAs were not affected by MSG or 5-FU compared with the control group (Fig. 1E and F).

3.2. Establishment of T1R3 gene-edited medaka with the CRISPR-Cas9 system

To generate medaka expressing green fluorescent protein (GFP) from the T1R3 gene locus, we injected medaka embryos of the OK-Cab^lf strain at the one-to four-cell stage with a mixture of sgRNAs for genome and plasmid digestion, a donor plasmid, and Cas9 mRNA. The target insertion site for the GFP coding sequence was located between the transcription start site and translation initiation codon of the T1R3 gene (Fig. 2A), an approach that has been shown to disrupt the function of the targeted endogenous gene [25,26]. Genomic DNA obtained from randomly selected embryos after 72 h was analyzed with a heteroduplex mobility assay in order to determine the success of gene editing (data not shown). F0 fish

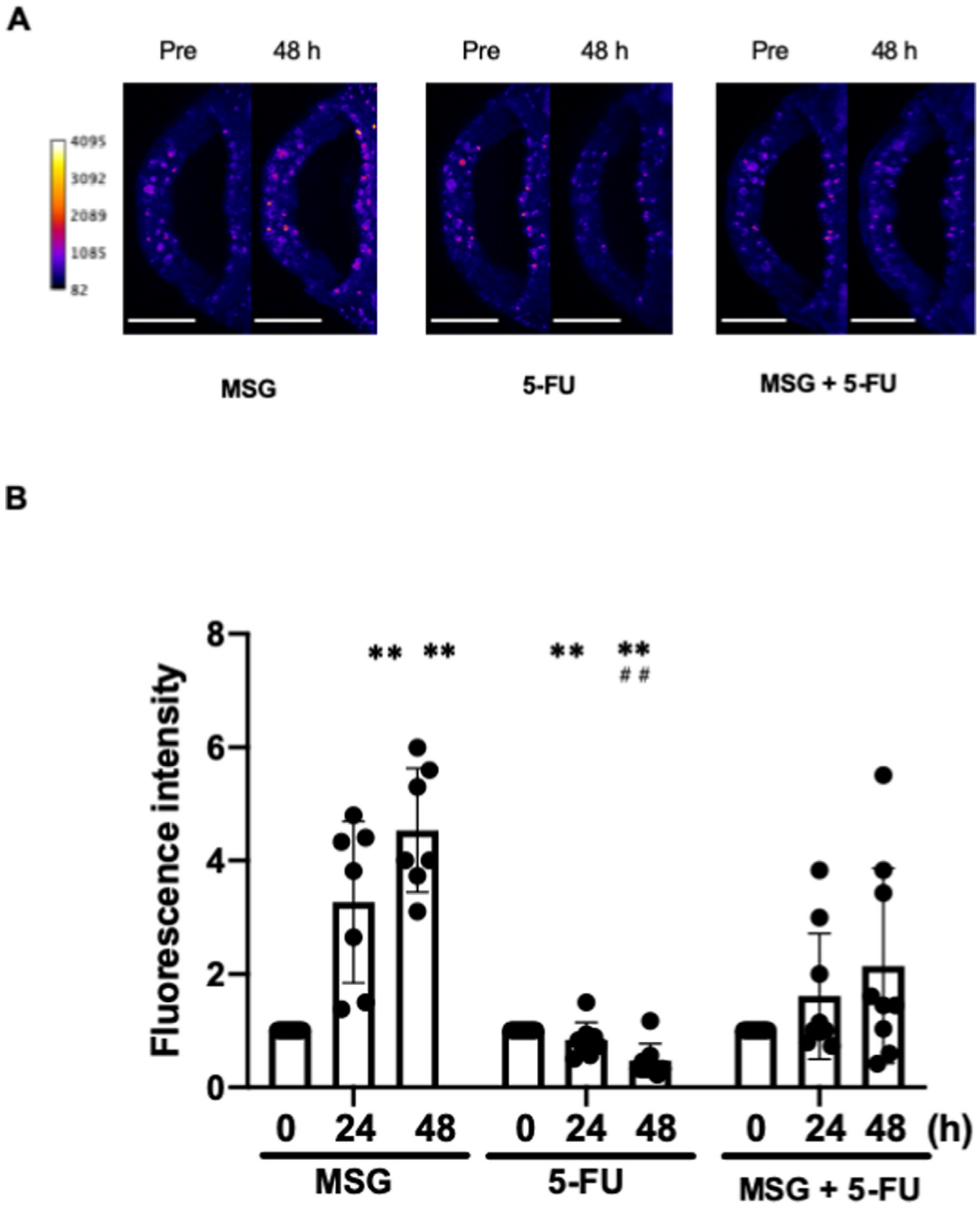

Fig. 3. Effects of MSG and 5-FU on GFP fluorescence in T1R3-GFP KI medaka. (A) Laser-scanning confocal microscopic images of GFP fluorescence in the upper lip of 7-dph T1R3-GFP KI medaka before (Pre) and after exposure to water containing 5-FU (100 μM) or MSG (100 μM) for 48 h. Scale bars, 20 μm. (**B**) Effects of exposure to MSG or 5-FU for 24 or 48 h on relative GFP fluorescence intensity in medaka treated as in (**A**). Data are means ± SEM (n = 7, 8, or 9 each group for MSG, 5-FU, or both agents, respectively). $^{**}P < 0.01$ versus time 0, $^{\#\#}P < 0.01$ versus 24 h, Bonferroni test.

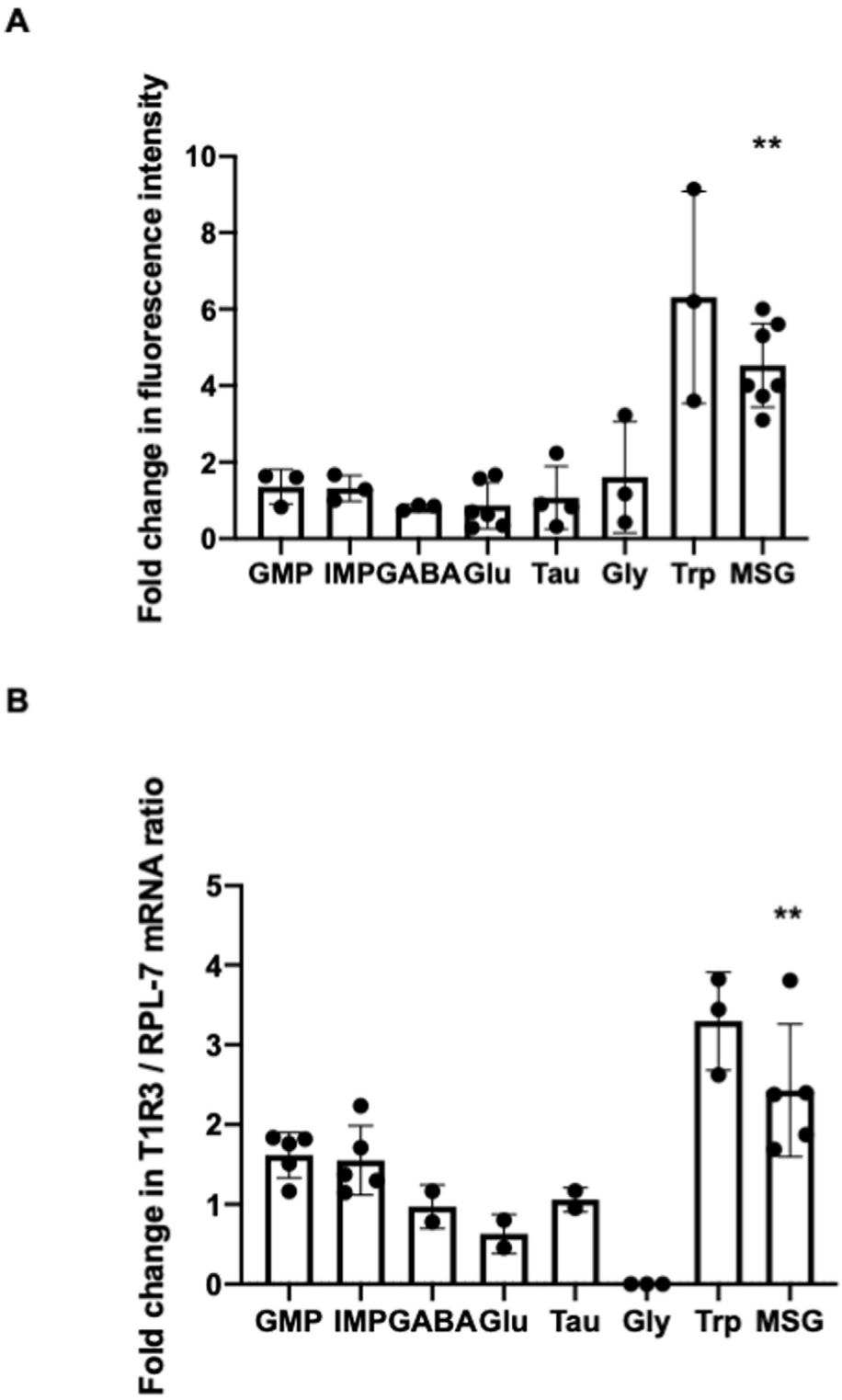

Fig. 4. Screening for agents that modulate T1R3 expression. (A) Fold change in GFP fluorescence intensity for T1R3-GFP KI medaka at 7 dph that were exposed to water containing the indicated agents at 100 μM for 48 h. Data are means ± SEM ($n = 3$ for GMP, IMP, GABA, Gly and Try, $n = 4$ for Tau, $n = 6$ for Glu, $n = 7$ for MSG) and are expressed relative to pre-exposure. (**B**) RT-qPCR analysis of the fold change in T1R3 gene expression in 7-dph WT medaka treated as in (**A**). Data are means ± SEM ($n = 3$ for GABA, Glu, Tau, and Try, $n = 5$ for GMP, IMP and MSG) and are expressed relative to control. GMP, guanosine 5′-monophosphate; IMP, inosine 5′-monophosphate; GABA, γ-aminobutyric acid; Glu, glutamate; Tau, taurine; Gly, glycine; Trp, tryptophan. ##$P < 0.01$ versus pre-exposure (**A**) or control (**B**), Dunnett's test.

with edited genomes were bred with wild-type (WT) fish of the OK-Cab strain to generate F1 fish, among which males and females harboring identical mutations were crossed to obtain heterozygous mutant lines. Fluorescence signals derived from GFP were detected in the lips and gills of T1R3-GFP knock-in (KI) fish imaged from ventral (Fig. 2B) as well as in the lips, gill rakers, and pharynx of those imaged from lateral (Fig. 2C). This pattern of GFP expression thus mimicked that of the endogenous T1R3 gene (Fig. 1A).

Confocal three-dimensional images obtained by Leica SP8 showed that the pattern of GFP expression was consistent with the shape of taste bud cells (Fig. 2D and supplementary data), supporting the notion that taste buds of the mutant fish can be directly visualized by GFP fluorescence.

To optimize imaging for comparison of expression level in T1R3-GFP KI fish, we modified the detector sensitivity and image resolution of a Nikon A1R laser-scanning confocal microscope. Comparison of image acquisition condition of HV50, HV60, and HV70 revealed that saturation occurred only at HV70, with HV50 and HV60 not exceeding a brightness value of 4,096 (data not shown). With regard to image resolution, the more detail detected at a higher resolution is offset by the negative effects of a long laser exposure time (scan speed and resolution) and we regarded to avoid the effects of repeated observations on the same individual. We therefore used a HV of 60 and a resolution of 512 by 512 pixels to examine taste buds of medaka. With these settings, we next examined the developmental time course of GFP expression (Fig. 2E). The number of taste buds and teeth increased as development of medaka progressed from 1 to 3 to 5 dph. The development of taste buds (upper lip) appeared to have stabilized at 7 dph. Images of the lower lip showed that T1R3 is also expressed around the teeth. Although not reported for medaka, fish with taste buds in the raised portion of the gums have been described [27]. We therefore decided to use medaka fish at 7 dph for screening purposes.

3.3. Effects of MSG and 5-FU on GFP expression in T1R3-GFP KI medaka

We measured the effects of exposure to 5-FU or MSG for up to 48 h on GFP fluorescence in 7-dph larvae of T1R3-GFP KI medaka. Confocal laser-scanning microscopy revealed that exposure to MSG for 48 h increased GFP expression whereas that to 5-FU reduced it, with MSG attenuating the loss of GFP expression induced by 5-FU (Fig. 3A). These effects on GFP fluorescence were thus consistent with those on T1R3 gene expression determined by RT-qPCR analysis (Fig. 1D). We also quantitated the time-dependent changes in GFP fluorescence intensity with the use of a Keyence BZ-X800 microscope. MSG was thus found to induce a time-dependent increase in fluorescence intensity that was significant at both 24 and 48 h, whereas the inhibitory effect of 5-FU was also significant at both time points and the combination of both agents had no significant effect (Fig. 3B).

3.4. Screening for agents that modulate T1R3 expression

Finally, we searched for agents that might increase T1R3 expression with the use of 7-dph T1R3-GFP KI medaka. We selected several nucleotides and amino acids as candidates, and measured their effects on the fluorescence intensity of GFP after treatment for 48 h. Umami-related nucleotides such as guanosine 5′-monophosphate (GMP) and inosine 5′-monophosphate (IMP) did not affect GFP fluorescence intensity, whereas tryptophan induced an ~6-fold increase (Fig. 4A). These data were confirmed by RT-qPCR analysis of T1R3 gene expression (Fig. 4B). Tryptophan is a bitter-tasting amino acid and induces an electrical response similar to that of the typical bitter substance quinine in mice [28], but its effects on umami and sweet receptors have been unknown. Our results now suggest that tryptophan warrants further investigation for its ability to increase T1R3 expression and activate taste signal transduction in human patients as a potential new therapeutic agent for dysgeusia. Given that tryptophan is metabolized to the neurotransmitter serotonin in vivo, possible side effects of such treatment would also need to be considered.

3.5. Conclusion

We have here developed a T1R3-GFP KI strain of medaka and established a screening system based on these fish for the identification of potential therapeutic agents for dysgeusia. We showed that the effects of MSG and 5-FU on GFP expression in the gene-edited medaka mimicked those on T1R3 gene expression in WT fish as well as in human patients [6]. In contrast to conventional studies with rodents such as mice, our medaka model allows direct visualization of changes in taste receptor expression in living animals and is therefore suitable for high-throughput screening in vivo without the need for necropsy. Our screening system based on monitoring of changes in GFP fluorescence intensity in living medaka should thus facilitate the development of new therapies for dysgeusia. The T1R3-GFP KI strain may also prove to be a useful tool for taste research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.02.082.

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