Biochemistry and Biophysics Reports 28 (2021) 101123

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Expression profiles and functional characterization of common carp (*Cyprinus carpio*) T2Rs

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ARTICLE INFO

Keywords:
Taste receptor
Bitter
In situ hybridization
Heterologous expression system

ABSTRACT

Bitter taste perception is mediated by a family of G protein-coupled receptors (T2Rs) in vertebrates. Common carp (*Cyprinus carpio*), which has experienced an additional round of whole genome duplication during the course of evolution, has a small number of *T2R* genes similar to zebrafish, a closely related cyprinid fish species, and their expression pattern at the cellular level or their cognate ligands have not been elucidated yet. Here, we showed through *in situ* hybridization experiments, that three common carp *T2R* (*ccT2R*) genes encoding ccT2R200-1, ccT2R202-1, and ccT2R202-2, were specifically expressed in the subsets of taste receptor cells in the lips and gill rakers. *ccT2R200-1* was co-expressed with genes encoding downstream signal transduction molecules, such as PLC-β2 and Gαia. Heterologous expression system revealed that each ccT2R showed narrowly, intermediately, or broadly tuned ligand specificity, as in the case of zebrafish T2Rs. However, ccT2Rs showed different ligand profiles from their orthologous zebrafish T2Rs previously reported. Finally, we identified three ccT2Rs, namely ccT2R200-1, ccT2R200-2, and ccT2R203-1, to be activated by natural bitter compounds, andrographolide and/or picrotoxinin, which elicited no response to zebrafish T2Rs, in a dose-dependent manner. These results suggest that some ccT2Rs may have evolved to function in the oral cavity as taste receptors for natural bitter compounds found in the habitats in a species-specific manner.

1. Introduction

Bitter taste perception is important for preventing animals from ingesting potentially toxic compounds [1]. Thousands of bitter compounds with diverse chemical structures are thought to be detected by a family of G protein-coupled receptors, namely T2Rs, in vertebrates [2–4]. Some human T2Rs are broadly tuned to recognize numerous compounds, whereas others are narrowly tuned to recognize a single compound [5]. T2Rs function not only as bitter taste receptors in the oral cavity but also as receptors for chemical compounds, such as acyl-homoserine lactones (produced by gram-negative bacteria) and salicin, in extra-oral tissues, such as the respiratory tract and intestine [1,6,7].

Most fish species have several T2Rs, except for coelacanth or blind cavefish [8,9], whereas there are approximately 30 different T2Rs in mammals [2–4]. Zebrafish *T2R* genes are expressed in taste receptor cells (TRCs) in the lips, gill rakers, and pharynx [10–12]. They are co-expressed with downstream signal transduction molecules, such as phospholipase C-β2 (PLC-β2) and G protein α subunit Gia [10,12]. Over the course of a decade, only one orthologous pair of T2Rs, drT2R1 (also referred to as zfT2R5) from zebrafish (*Danio rerio*) and mfT2R1 from medaka (*Oryzias latipes*), had been deorphaned with a synthetic bitter compound, denatonium benzoate, which induces an aversive response in zebrafish [11]. Recently, Behrens et al. investigated the agonist profiles of coelacanth and zebrafish T2Rs [13]. The ligand profile of the most basal coelacanth receptor lcT2R01 was found to be identical to that of drT2R1, its ortholog in zebrafish, despite >400 Myr of separate evolution. Three of the four zebrafish T2Rs investigated, namely drT2R1, drT2R2, and drT2R4, were narrowly to intermediately tuned receptors, whereas drT2R3a was an intermediately to broadly tuned receptor.

Common carp (*Cyprinus carpio*), an omnivore, is one of the most economically important breeding fish species. Common carp and zebrafish are closely related cyprinid species, and are estimated to have

Abbreviations: TRC, taste receptor cell; PLC-β2, phospholipase C-β2; WGD, whole genome duplication; ccT2R, common carp T2R; ISH, *in situ* hybridization; AUC, area under the curve; RLU, relative light units.

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https://doi.org/10.1016/j.bbrep.2021.101123
Received 30 July 2021; Received in revised form 30 August 2021; Accepted 30 August 2021
Available online 2 September 2021
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diverged 50–128 Myr ago [14–16]. Common carp has experienced an additional (4th) round of whole genome duplication (WGD) after its divergence from zebrafish 5.6–11.3 Myr ago [14,15]. The number of common carp T2R (*ccT2R*) genes was comparable to that of zebrafish T2R genes, probably due to redundancy and subsequent gene loss [13,17]. Expression patterns of five *ccT2R* genes in various tissues were studied at the tissue level by RT-PCR [17]. However, the tissue distribution of *ccT2R* gene expression remains to be elucidated by *in situ* hybridization (ISH). Moreover, to the best of our knowledge, no ligands have been identified for ccT2Rs so far.

In the present study, we investigated the expression and function of cc*T2R* genes. Through *in situ* hybridization experiments, we found that *ccT2R* genes were specifically co-expressed with *PLC-β2* and/or *Gαia* in subsets of TRCs in the lips. Furthermore, we identified natural and synthetic bitter compounds as ligands for ccT2Rs using heterologous expression system. The present study provides new insights into the function of ccT2Rs as bitter taste receptors in the oral cavity.

2. Materials and methods

2.1. Experimental animals

This study was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Both male and female common carp (*Cyprinus carpio*), ~ 3–5 cm body length, were purchased from a local commercial source. We found no difference between both sexes.

2.2. Database search, cloning, and phylogenetic analysis

The TBLASTN program was used to search for genomic sequences showing significant identity to six *ccT2R* genes, namely *ccT2R200-1*, *ccT2R200-2*, *ccT2R201*, *ccT2R202-1*, *ccT2R202-2*, and *ccT2R203-1*, which were registered in the NCBI database (https://www.ncbi.nlm.nih.gov/), in the public genome database of common carp (http://www.ensembl.org/Cyprinus_carpio/Info/Index). The entire coding regions of the six *ccT2Rs*, except *ccT2R203-2*, *ccGia*, and *ccG14*, and partial coding regions of *ccPlc-β2* (L673-G1023), which were amplified from common carp cDNA synthesized from lip tissue or genomic DNA extracted from head tissue, were used as probes for ISH or in heterologous expression systems. The complete cDNA sequences of *ccT2Rs* used in the heterologous expression systems are shown in Supplementary File 1 in the Supplementary Materials online.

The identified sequences were analyzed using the ClustalW program [18] implemented in the MEGA-X [19] available at the Molecular Evolutionary Genetics Analysis (https://www.megasoftware.net/). Phylogenetic tree was constructed based on amino acid sequence alignments using the neighbor-joining method. The stability of the tree was estimated by bootstrap analysis for 1000 bootstrap replications using the same program. Orthologous pairs of olfactory receptors, ccORA1, ccORA3, and ccORA5 from common carp and drORA1, drORA3, and drORA5 from zebrafish [20], were used as the outgroup.

2.3. Nomenclature of T2R genes

For previously known genes, we used the same gene names as the ones used in previous studies as follows: common carp T2Rs [17]; coelacanth [8]; zebrafish [9]; medaka [10]. Newly described genes were named according to the name of the closest ortholog.

2.4. In situ hybridization

In situ hybridization was performed using three individuals and more than eight sections for each gene as previously described [10]. There were no differences between individuals in the expression profiles of ccT2Rs and downstream signal transduction molecules, such as ccPlc-β2, ccGia, and ccG14. In brief, fresh-frozen sections (16-μm thick) of common carp were placed on MAS-coated glass slides (Matsunami Glass, Osaka, Japan) and fixed in 4% paraformaldehyde in phosphate-buffered saline. Prehybridization (at 58 °C for 1 h), hybridization (at 58 °C, 2 O/N), washing (0.2 × SSC at 58 °C), and development (NBT-BCIP) were performed using digoxigenin-labeled probes. Stained images were obtained using a fluorescence microscope (DM6 B, Leica, Nussloch, Germany) equipped with a cooled CCD digital camera (DFC7000 T, Leica). Double-label fluorescence ISH was performed using one individual and two sections for each combination with digoxigenin and fluorescein-labeled RNA probes. Each labeled probe was sequentially detected by incubation with a peroxidase-conjugated anti-digoxigenin antibody and a peroxidase-conjugated anti-fluorescein antibody (Roche, Indianapolis, IN, USA), followed by incubation with TSA-Alexa Fluor 555 and TSA-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) using the tyramide signal amplification method. Stained images were obtained using a confocal laser-scanning microscope (LSM 800; ZEISS, Oberkochen, Germany).

2.5. Heterologous expression system

The responses of ccT2Rs were measured using heterologous expression systems, as described previously [21]. HEK293T cells were transiently co-transfected with pEAK10 expression vectors for ccT2Rs tagged with the first 45 amino acid residues of rat somatostatin receptor 3 (sst tag) in the N-terminus to facilitate cell surface expression [22], human Gα16gust44, and mt-apoclytin-II [21], and exposed to bitter compounds. Luminescence intensity was measured using a FlexStation 3 microplate reader (Molecular Devices, San Jose, CA, USA). The response from each well was calculated based on the area under the curve (AUC) and expressed as relative light units (RLU). Bitter compounds with the highest purity were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Sigma-Aldrich (St. Louis, MO, USA), and Tokyo Chemical Industry (Tokyo, Japan). The concentrations of bitter compounds used for the screening were selected based on previous experiments [5,13]: absinthin, 100 μM; amarogentin, 1 mM; andrographolide, 333 μM; aristolochic acid, 10 μM; chloramphenicol, 1 mM; chloroquine, 10 mM; cromolyne, 1 mM; colchicine, 3 mM; denatonium benzoate, 3 mM; dimethyl-thioformamide, 300 μM; diphenylthiourea, 100 μM; methylthiourea, 300 μM; picrotoxinin, 1 mM; PROP, 1 mM; PTC, 100 μM; saccharin, 10 mM; D-(−)-salicin, 10 mM; strychinine, 30 μM; α-thujon, 300 μM. Data were collected from more than three independent experiments performed in duplicates or triplicates. Statistically significant differences were assessed using the paired t-tests.

3. Results and discussion

3.1. Phylogenetic relationships among fish T2R genes

We searched common carp *T2R* (*ccT2R*) genes in the Ensemble genome and NCBI databases, and found seven intact *ccT2R* genes. The phylogenetic tree constructed using ClustalW showed that *ccT2Rs* have their orthologs in zebrafish (Fig. 1). It was also found that two members of cc*T2R202* have recently been duplicated by the 4th round of WGD [14–16], because they are clustered in the same branch as a single ortholog in zebrafish and are located on the neighboring and duplicated 15th or 16th chromosome. In contrast, two members of cc*T2R200* were likely duplicated after the divergence of common carp and zebrafish but not by the 4th round of WGD, as they are located in the 3rd or 35th chromosome, while their orthologs in zebrafish are located in tandem within a narrow region of the same chromosome [10].

3.2. Expression of ccT2R genes in the gustatory tissues

To examine the tissue distribution of expression of genes encoding

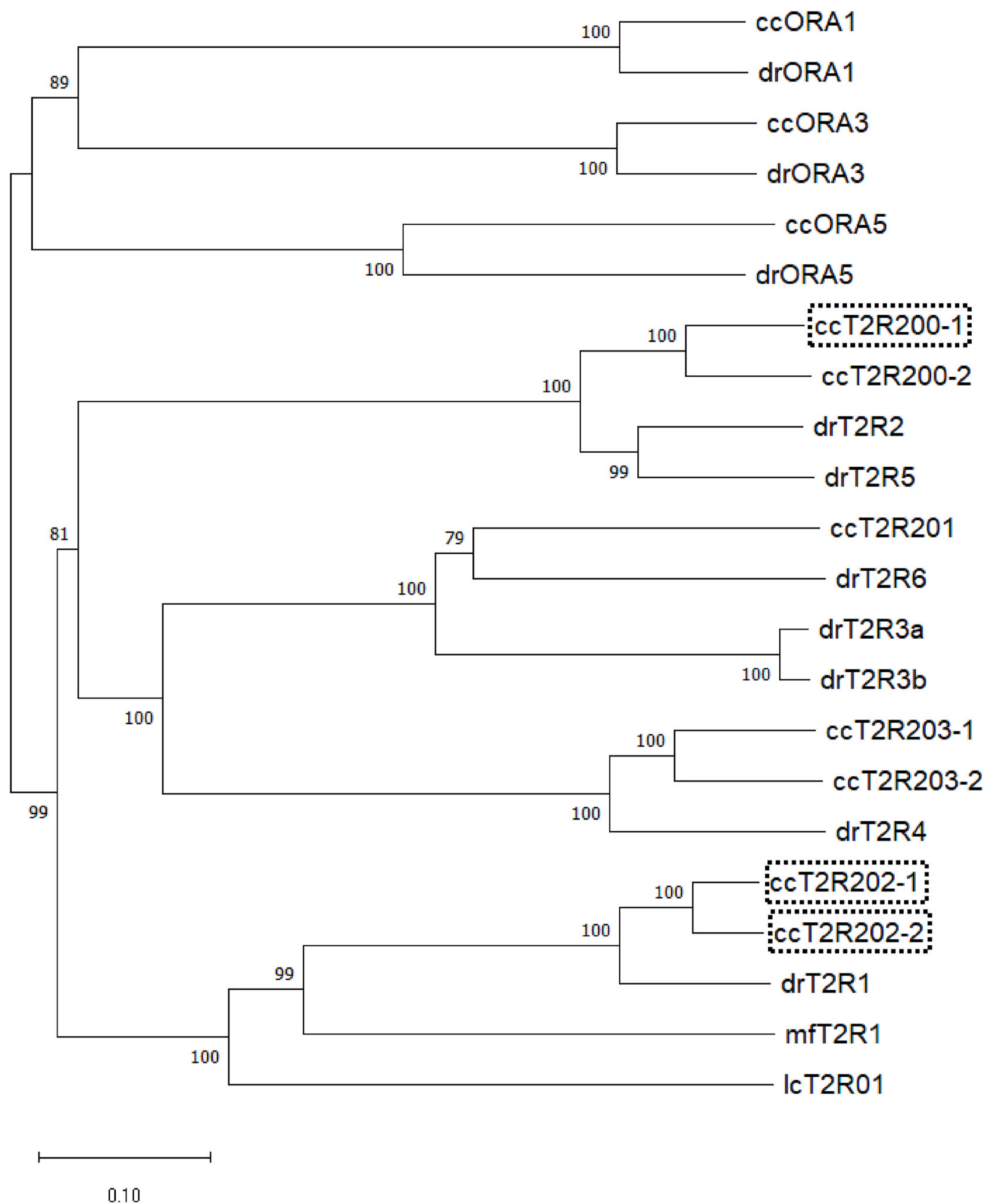

Fig. 1. Phylogenic tree showing fish T2Rs and related receptors. The tree was constructed based on amino acid sequence alignments using the neighbor-joining method. The numbers at the nodes are bootstrap support values based on 1000 bootstrap replicates. ccT2R genes surrounded by the dotted boxes show that they are expressed in the TRCs, as shown in Fig. 2. Scale bar indicates a 10% amino acid difference. cc, common carp (*Cyprinus carpio*); dr, zebrafish (*Danio rerio*); mf, medaka (*Oryzias latipes*); lc, coelacanth (*Latimeria chalumnae*).

ccT2Rs and downstream signal transduction molecules, we conducted *in situ* hybridization on sections of the lips and gill rakers. Three *ccT2R* genes, namely *ccT2R200-1*, *ccT2R202-1*, and *ccT2R202-2*, were expressed in subsets of TRCs in the lips and gill rakers, whereas no signals were detected for *ccT2R200-2*, *ccT2R201*, or *ccT2R203-1* (Fig. 2a). These results were consistent with those previously revealed by RT-PCR using TRCs-containing barbel at the tissue level [17]. Similar tissue distribution profiles between the two members of *ccT2R202* may be attributed to their well-conserved transcription regulatory regions, since they have been recently duplicated by the 4th round of WGD. However, we were unable to compare the corresponding sequences due to the absence of *ccT2R202-1* sequence in the genomic database. In contrast, the two members of *ccT2R200* showed different tissue distribution profiles, probably because they have distinct transcription regulatory regions. We previously showed that *drT2R5* and *drT2R2* (also referred to as *zfT2R1a* and *zfT2R1b*, respectively) are expressed in the TRCs of the lips, gill rakers, and pharynx [10]. *ccT2R200-2*, *ccT2R201*, and *ccT2R203-1*, which are not expressed in the gustatory tissues, were activated by some bitter compounds (see below), suggesting that they may function as extraoral T2Rs in tissues other than the gustatory tissues.

Genes encoding downstream signal transduction molecules, such as ccPlc-β2, ccGia, and ccG14, were also robustly expressed in subsets of TRCs in the lips and gill rakers (Fig. 2b). The signal frequencies for *ccPlc-β2*, *ccGia*, and *ccG14* were higher than that for *ccT2Rs*. To compare the TRCs expressing *ccT2R200-1*, which we identified the natural bitter

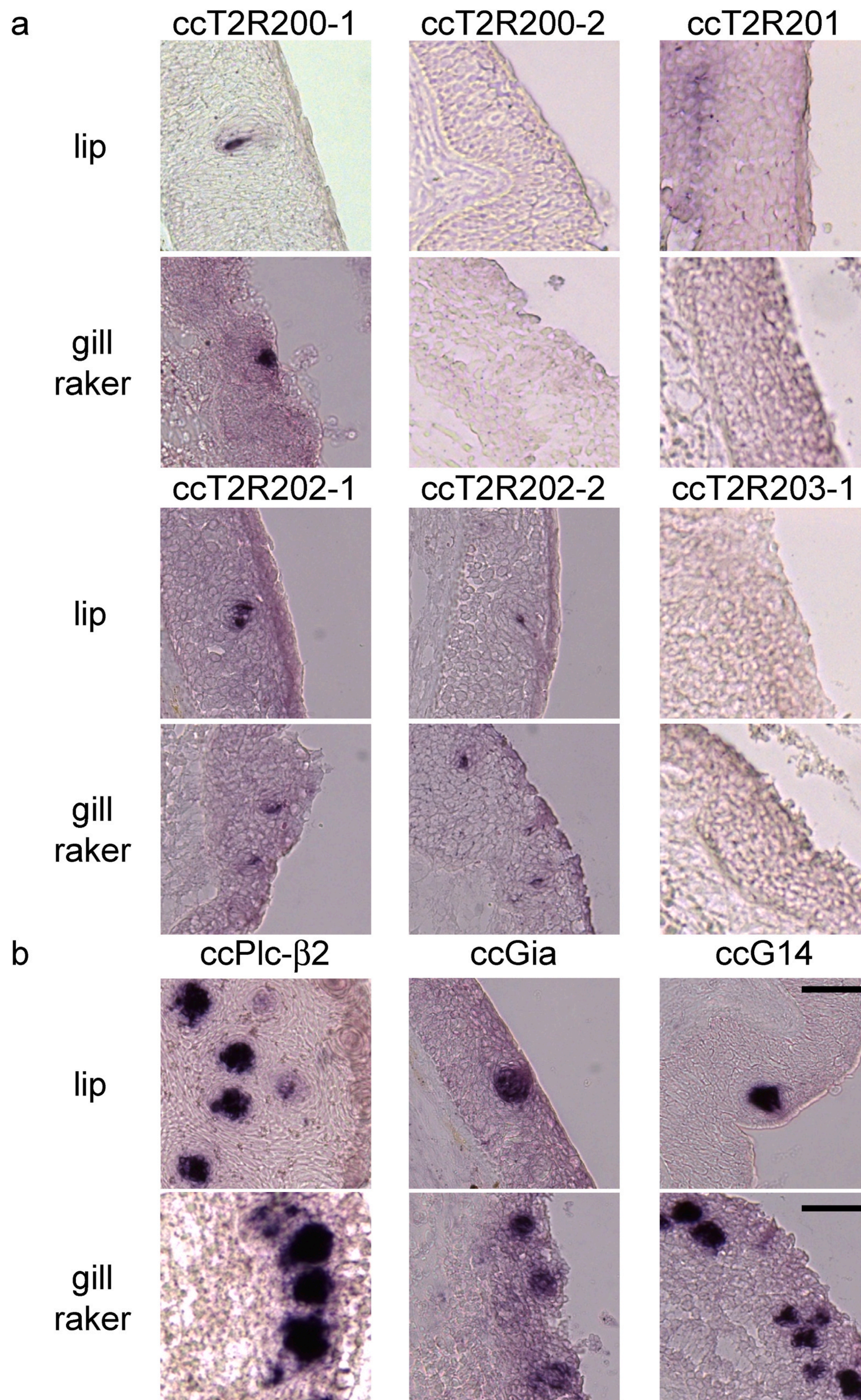

Fig. 2. Expression of genes encoding ccT2Rs and downstream signal transduction molecules in the gustatory tissues. (a) *In situ* hybridization revealed that three *ccT2R* genes, namely *ccT2R200-1*, *ccT2R202-1*, and *ccT2R202-2*, were expressed in subsets of TRCs in the lips and gill rakers. In contrast, no signals were detected for *ccT2R200-2*, *ccT2R201*, or *ccT2R203-1*. (b) Genes encoding downstream signal transduction molecules, namely ccPlc-β2, ccGia, and ccG14, were robustly expressed in subsets of TRCs in the lips and gill rakers. The frequencies of *ccPlc-β2*, *ccGia*, and *ccG14*-positive cells were higher than those of *ccT2Rs*-positive cells. Scale bars: 50 μm.

ligands for (see below), with downstream signal transduction molecules, we next performed double-label fluorescence ISH. *ccT2R200-1*-positive TRCs were also positive for *ccPLC-β2* in the lips (Fig. 3a, Supplemental Fig. 2a), suggesting that *ccT2R200-1* is involved in taste reception. Two genes encoding G protein α subunits, ccGia and ccG14, were exclusively expressed in the different subsets of the TRCs in the lips (Fig. 3b, Supplemental Fig. 2b). *ccT2R200-1*-positive TRCs were also positive for *ccGia* (Fig. 3c, Supplemental Fig. 2c) but negative for *ccG14* in the lips (Fig. 3d, Supplemental Fig. 2d). These results demonstrate that ccGia plays a pivotal role in mediating bitter taste perception via ccT2Rs in common carp, just as in case of zebrafish [12].

3.3. Characterization of ligands for common carp T2Rs

To identify ligands for ccT2Rs, we performed a luminescence-based assay using HEK293T cells transiently co-transfected with one of the

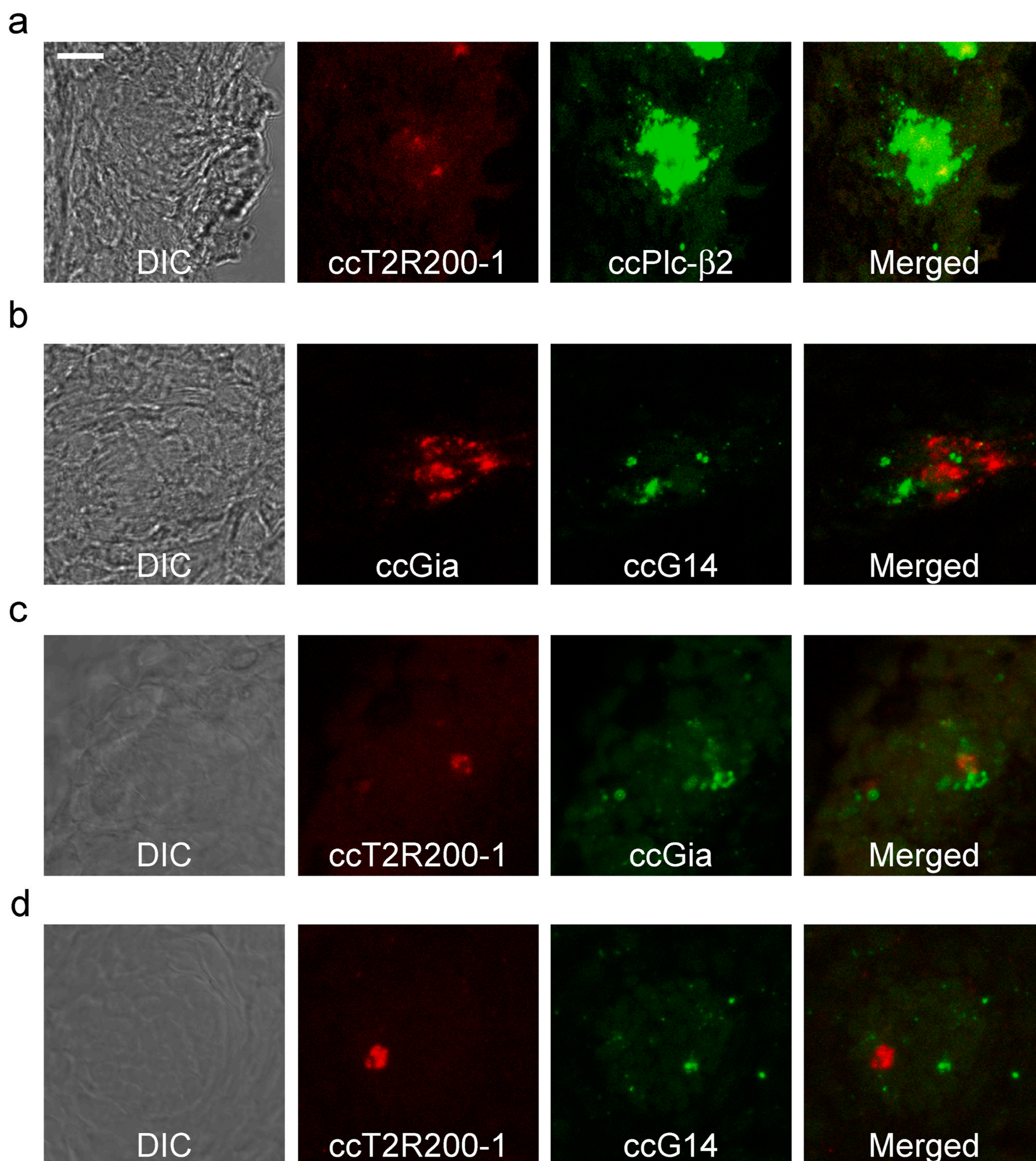

Fig. 3. The co-expression relationships among ccT2R200-1 and downstream signal transduction molecules. (a) *ccT2R200-1*-positive TRCs were also positive for *ccPLC-β2* in the lips. Scale bar: 10 μm. (b) *ccGia* and *ccG14* were exclusively expressed in different subsets of the TRCs in the lips. (c) *ccT2R200-1*-positive TRCs were positive for *ccGia* in the lips. (d) *ccT2R200-1*-positive TRCs were negative for *ccG14*.

ccT2Rs and a chimeric G protein α subunit, hGα16/gust44, as described previously [21]. We selected 11 natural and 8 synthetic bitter compounds with diverse chemical structures that were previously reported to activate human T2Rs [5] and/or used for the screening of coelacanth and zebrafish T2Rs [13].

ccT2R200-1 was strongly activated by three natural bitter compounds, amarogentin, andrographolide, and picrotoxinin, and weakly responded to absinthin, cromolyne, dimethyl-thioformamide, PROP, strychinine, and α-thujon (Supplementary Fig. 1). ccT2R200-2, which shows a high degree of identity (85%) with ccT2R200-1, was strongly activated by andrographolide, picrotoxinin, and PROP, and weakly responded to absinthin and diphenylthiourea (Supplementary Fig. 1). ccT2R201 was strongly activated by amarogentin and PROP, and weakly responded to chloramphenicol, colchicine, denatonium benzoate, and D-(−)-salicin (Supplementary Fig. 1). ccT2R202-1 and ccT2R202-2 were weakly activated by strychinine and andrographolide, respectively (Supplemental Fig. 1). ccT2R203-1 was strongly activated by picrotoxinin and PROP, and weakly responded to absinthin, andrographolide, chloramphenicol, dimethyl-thioformamide, diphenylthiourea, and methylthiourea (Supplemental Fig. 1). Hence, ccT2R200-1 and ccT2R203-1 are broadly tuned receptors, and ccT2R200-2 and ccT2R201 are intermediately tuned receptors, whereas ccT2R202-1 and ccT2R202-2 seems to be narrowly tuned receptors, although it is possible that they may respond to other bitter compounds than we tested. Having intermediately or broadly tuned ccT2Rs may account for the number of ccT2R genes comparable to that of zebrafish T2R genes, despite an additional WGD. Similarly, it was shown that chicken and turkey have only a few T2R genes, which are on average very broadly tuned [23].

For further verification of candidate agonists, we next used seven different concentrations of the putative agonists to obtain dose-response curves. We found that ccT2R200-1 was activated by the two natural bitter compounds, andrographolide (EC_{50} = 182 ± 7 μM) and picrotoxinin (EC_{50} = 510 ± 26 μM) in a dose-dependent manner, whereas ccT2R200-2 and ccT2R203-1 responded to andrographolide (EC_{50} = 335 ± 38 μM) and picrotoxinin (EC_{50} = 425 ± 99 μM), respectively (Fig. 4). To the best of our knowledge, ccT2R200-1 and ccT2R203-1 are the first fish T2Rs that were found to recognize picrotoxinin, a $GABA_A$-receptor antagonist [24]. Picrotoxinin is known to be a poisonous compound contained in fishberries (*Cocculus indicus*), the seeds of the plant *Anamirta paniculata*. In contrast, ccT2R201 responded to a synthetic bitter compound, denatonium benzoate (EC_{50} = 44 ± 5 μM)

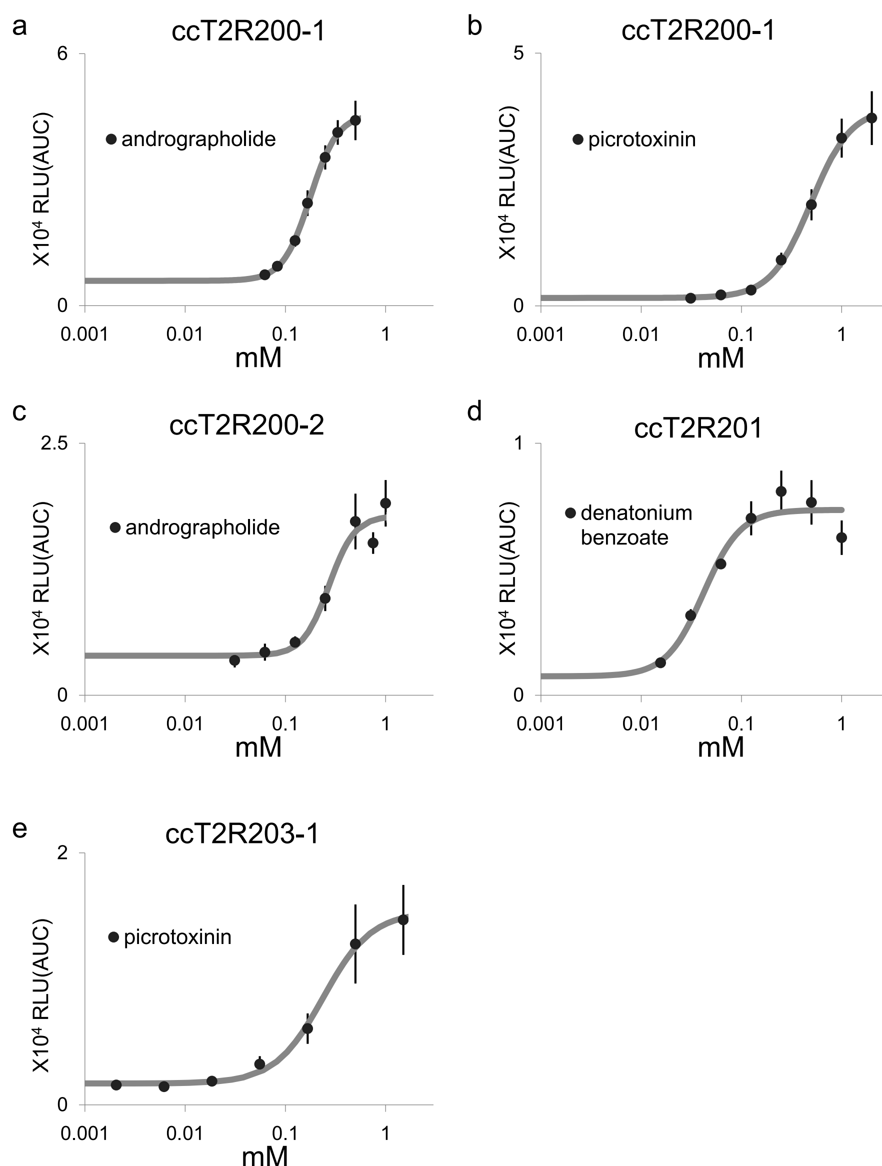

Fig. 4. ccT2Rs responded to bitter compounds in a dose-dependent manner. (a) ccT2R200-1 responded to a natural bitter compound, andrographolide (EC_{50} = 182 ± 7 μM). (b) ccT2R200-1 responded to a natural bitter compound, picrotoxinin (EC_{50} = 510 ± 26 μM). (c) ccT2R200-2 responded to andrographolide (EC_{50} = 335 ± 38 μM). (d) ccT2R201 responded to a synthetic bitter compound, denatonium benzoate (EC_{50} = 44 ± 5 μM). (e) ccT2R203-1 responded to picrotoxinin (EC_{50} = 425 ± 99 mM). Each point on the dose-response curves indicates the mean ± SEM (n = 6).

(Fig. 4).

Comparison of ligands for T2Rs between common carp and zebrafish, two closely related cyprinid fish species, indicates that drT2R2, which is orthologous and shows a high degree of identity (73%) with the two members of ccT2R200, is a narrowly tuned receptor that responds to neither andrographolide nor picrotoxinin [13]. Similarly, drT2R4, which shows the highest degree of identity (83%) with ccT2R203-1, is an intermediately tuned receptor but does not respond to any of the bitter compounds activating ccT2R203-1, including picrotoxinin [13]. Furthermore, ccT2R201, which is most closely related to drT2R3a, responds to denatonium benzoate with much higher sensitivity (EC_{50} = 44 ± 5 μM) than drT2R3a, drT2R1, mfT2R1, and lcT2R01 (EC_{50} = approximately 0.3–3 mM) [11,13]. Accordingly, the orthologous cyprinid fish T2Rs showed different ligand profiles in the present and previous studies [13]. Orthologous pairs of T2Rs between mice and humans also showed distinct ligand profiles, possibly contributing to species-specific bitter compound recognition [25]. Taken together, these results demonstrate that ccT2Rs may have evolved to function as narrowly, intermediately, or broadly tuned receptors for bitter compounds in a species-specific manner. However, we cannot exclude the possibility that drT2Rs may be activated by bitter compounds that were previously reported not to activate them in our experimental condition and vice versa. Further studies using point mutant and chimeric fish T2Rs in heterologous expression systems will be needed to elucidate the molecular mechanisms underlying the interspecies differences in the detection of bitter compounds.

4. Conclusion

Three common carp T2Rs, such as ccT2R200-1, ccT2R202-1, and ccT2R202-2, have evolved to function in the oral cavity as taste receptors for natural bitter compounds found in the habitats in a species-specific manner.

Author contributions

Y.T., and Y.I. designed the experiments. T.S., T.K., R.A., and Y.I. performed the experiments. T.S., T.K., Y.T., and Y.I. analyzed the data. Y.I. wrote the manuscript.

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.

Acknowledgments

We thank Dr. Hidenori Nishihara (Tokyo Institute of Technology) for valuable advice about the phylogenetic tree. This work was supported in part by Grants-in-Aid for Scientific Research (18KK0166 and 19H02915 to Y.I.; 18K14427 and 20H02941 to Y.T.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, by a Research Fellowship for Young Scientists (18J00315 to Y.T.) from the Japan Society for the Promotion of Science, and by the Lotte Shigemitsu Prize (to Y.T. and Y.I.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101123.

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