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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Identification and characterization of sugar receptors in the western honey bee, *Apis mellifera*

서양종꿀벌의 단맛수용체에 관한 연구

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Identification and characterization of sugar receptors in western honey bee, *Apis mellifera*

Major in Biomodulation

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ABSTRACT

The sense of taste gives animals precious information about the quality and nutritional value of food. In honey bee, taste is vital for choosing beneficial food sources, water, resins, and for nest mate identification. Although sugar detection is a crucial factor in determining the acceptability of nectar and pollen collection in the honey bee, little is known about the molecular and neural correlates underlying sugar perception in honey bees. Here we functionally identified a sugar receptor of the western honey bee, *Apis mellifera*, which were most related phylogenetically with sugar receptors of *Drosophila melanogaster* such as DmGr64a-f, Gr61a, and Gr5a. We characterized that gustatory receptor 1 of A. mellifera (AmGr1) responded to sucrose, glucose, trehalose, and maltose with a dose-dependent

manner. Notably, we firstly found that the expression patterns of AmGr1 and AmGr2 is distinct from those in Drosophila. AmGr1 alone showed fully functional, but showed different sensitivity from a heterodimer of AmGr1 and AmGr2. That is, co-expression of AmGr1 and AmGr2 demonstrated higher sensitivity to glucose, while lower sensitivity to sucrose, trehalose, and maltose, compared to AmGr1 expression alone. Expression patterns in the antenna of honey bees showed that AmGr1 and AmGr2 were co-localized in the antennal neurons or not, and especially AmGr1 were highly expressed at the distal segment of the antennae. Our study suggests that sugar receptors of the honey bee function as heterodimers (or monomer or mono-dimer), indicating that AmGr2 is required for providing honeybees with variability of sugar perceptions. This functional organization of the sugar receptor of the honey bee strongly indicates the correlation of internal and external sensing of sugars.

Keywords: Sense of taste, Gustatory receptor, Honeybee, *Drosphila melanogaster*, Sugar, Behavior

Student number: 2012-22617

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LIST OF ABBRIVIATION

Am	*Apis mellifera*
Dm	*Drosophila melanogaster*
GR	Gustatory Receptor
NCBI	National Center for Biotechnology Information
AmGr1	*Apis mellifera* Gustatory receptor 1
AmGr2	*Apis mellifera* Gustatory receptor 2
DmGr	*Drosophila melanogaster* gustatory receptor
mRNA	Messenger RNA
cDNA	Complementary DNA
qRT-PCR	quntitative Real Time-Polymerase Chain Reaction

1. Introduction

The sense of taste plays a critical role in evaluating and identifying portential food and drink by discriminating various chemical compounds in food source and in controlling energy homeostasis. it has evolved to support as a prominent regulator of feeding behavior [1-4].Tastant systems are decisive for identifying and responding to sweet, sour, salty, bitter and umami tastes. it is also able to distinguish between these various tastants to produce inborn behavioral responses. For example, animals are averse to bitter compounds, but are attracted to sweet and umami tastes [1].

In case of insect, tastant systems act a important role in multiple behaviors such as feeding, aversive avoidance, courtship, mating, and depositing eggs. Gustatory organs are broadly distributed over the whole surface of the body, enabling insects to successfuly find nonvolatile components such as latent foods or toxic compounds [2, 3].

In honey bee, Tastants especially deetected by the antennae gustatory sensilla. Gustatory sensilla on the antennae are used to evaluate food source while foraging and ingestion [4]. The number of gustatory sensilla on the antenna is highest on the last antennae segment [5], suggesting that this region is critical for detecting food source [4]. In fact, bees use the last segment of the antennae to evaluate the quality of food.

In honey bee, gustatory system act a central role in a honeybee’s life [6]. Especially the detection of sugar is a momentous in deciding whether a food source is edible, and ingesting sweet source for nutrition [7, 8]. so this ability is crucial for the survival of honey bees. Despite its crucial importance to the survival of the honey bees, little is known about the molecular and neuronal basis of sugar perception in honey bee.

Since the publication of the genome of the honeybee [9], Bioinformatic identification of gustatory receptor genes in the honeybee genome have revealed that just 10

gustatory receptor genes have been found in honeybee genome [10].

However There are no functional study to deciding the ligans of the 10 gustatory receoptors of the honey bee.

A central goal in this study is isoloation and characterization of genes encoding sweet taste receptors. The identification of sugar receptors make powerful molecular tools to examine not only the function of sweet taste receptor cells, but also logic of sweet taste perception in honeybees.

We now report the characterization and identification of *Apis mellifera* sweet taste receptors Using in situ hybridization and a heterologous expression system. We demonstrated that AmGr1 function as a sweet receptor responding to sucrose,glucose,trehalose,maltose but not fructose and AmGr2 function as a co-receptor for variability of sugar sensitivity in honeybees.

2. Materials and methods

Insect's

Honey bee species, *A. mellifera* was maintained on apiaries of Seoul National University campus surrounded by the Gwanak mountain range in Seoul, Korea.

Chemical

Sucrose, glucose, maltose, fructose were purchased from Sigma-Aldrich (St. Louis, MO); trehalose was from Fluka (St. Gallen, Switzerland)

Scanning Electron Microscopy(SEM)

Collected bees were placed in the freezer for 1 hr, after which they were decapitated and antennae were cut at their base. The antennae were then cleaned for 1 hour in PBS solution and followed by dehydration through a graded ethanol series of 25, 50, 70, 90, 100 % for 10 min each. After drying in the oven at 40^{o}C for 24 hours, the scape of the antennae was attached to double side sticky tape (3M Korea, Seoul, Korea). All samples were coated with a thin layer of gold on all sides and each antenna was fixed on a holder in the sample chamber. The samples were scanned with a SUPRA 55VP, Field-Emission Scanning Electron Microscope (Carl Zeiss, Germany). Antennae were imaged from the dorsal and ventral side. We scanned only flagellar segments 3 to 10, because there are no olfactory receptors on the first and second segments of honey bee flagellum [11]. Each segment from 3rd to 10th was scanned longitudinally at a magnification of 500 times. Two images per segment were collected. Sensilla chaetica were identified according to specific morphological characteristics as described in Frasnelli *et al.* [11]. Each type of sensilla was counted from all selected images by using image analysis tool (UTHSCSA ImageTool Version 3.0).

RNA Isolation, cDNA synthesis

Total RNA was isolated from the worker bee antennae using RNeasy mini kit(Qiagen, Valencia, CA, USA) by manufacturer's instruction, after which RQ1 RNase-free DNase Ⅰ(Promega, Madison, WⅠ, USA) was treated by manufacturer's instruction. and then RNA was used for oligo dT-primed cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen) for the generation of templates for PCR reactions.

Receptor Cloning (AmGr1,2)

Full-length coding sequences of candidate Sugar Gustatory receptors of *Apis mellifera* were PCR-amplified from pools of total cDNA prepared from worker *A. mellifera* antennae using *TaKaRa Ex-Taq* (Takara Shuzou, Kyuto, Japan). Amplification reactions (25$\mu\ell$) included 0.3$\mu\ell$ *TaKaRa Ex-Taq*, 2.5$\mu\ell$ 10Ⅹ *Ex-Taq* Buffer, 2$\mu\ell$ 2.5 mM dNTP Mixture, 2$\mu\ell$ 5 pmol of each primer 1$\mu\ell$ template cDNA, 17.2$\mu\ell$ Sterilized distilled water . All amplification reactions were carried out using a 96 Well Thermal Cycler(Applied Biosystems Inc., Foster City, Claif.) under the following conditions: 94°C for 3 min; 35 cycles of 94°C for 30 s, 58-68°C for 30 s, 72°C for 1.5 min; and 72°C for 10 min. PCR amplification products were run on a 1.0% agarose gel and verified by DNA sequencing. PCR primers (Supplementary Table S1) were designed based on the nucleotide sequences

In situ hybridization

Antennae of worker *Apis mellifera* were fixed with 4% paraformaldehyde (PFA) solution overnight, after which antennae were cut at 3-5mm long and brains were dissected out. Tissues were then washed with PBS buffer (pH 7.4), followed by dehydration and rehydration using a series of ethanol from 25%~100%. Tissues were then hybridized with a hybridization solution containing

Dig-labeled RNA probes for 20 hrs at 58°C. After several washes with PBS buffer containing 0.2% Tween 20, tissues were incubated with peroxidase (POD)-conjugated anti-DIG antibodies (Roche, Indianapolis, IN) in a blocking reagent (Roche) overnight at 4°C, followed by signal visualization process using a Tyramid signal amplification (TSA) kit by manufacturer's instruction (PerkinElmer, Waltham, MA, USA). After in situ hybridization, immunostaining using several different antibodies were performed as described previously [12]. Tissue preparations after *in situ* hybridization were subsequently incubated with rabbit anti-gustatory receptor 1 antibodies (1:200 in PBS with 0.1% Tween 20, PBSTw) at 4°C overnight. After 3-4 times washing with PBSTw, anti-rabbit secondary antibodies conjugated with cy3 fluorophore were incubated overnight at 4°C. Cell and neuronal staining was conducted with DAPI(Vector Laboratories, Inc. Burlingame, CA 94010) and anti-horseradish peroxidase (HRP) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), respectively. Stained tissue preparations were dehydrated with a series of ethanol and 100% acetone to embed into Spurr's epoxy resin [12]. Resin-embedded tissues were incubated at 60°C oven overnight and sectioned at 20μm thickness by using an automatic sliding microtome (HM 355S; Microm, Thermo Scientific). Sectioned tissues were mounted with Serva fluoromount (Crescent Chemical Co). Images were captured with a LSM 700 confocal microscope (Zeiss, Thornwood, NY) and were processed with ZEN 2009 software. Gene-specific primers for preparing RNA probes were as follows: AmGr2 (XM_397125.4): FP: 5'- CAAATTCGTGGTGGCTTAGG-3', RP: 5'-GCACGTGCGACAGATAAGAA-3'

Preperation of *Xenopus laevis* Oocyte

Matured female *Xenopus lavis* frogs were purchased from Xenopus Ⅰ(Ann Arbor, MI). Frog were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester,and oocyte were surgically removed. Oocyte were freed from the follicle cells by treatment with collagenase A for 1 h at room temperature and incubated for 24hours in modified Barth's solution [13].

In vitro transcription oocyte and microinjection

In vitro transcription of cRNA was performed by using a mMESSAGE mMACHINE SP6 Kit(Ambion) according to the manufacturer's protocol.Plasmids were linearized with EcoR1, and capped cRNA was transcribed using SP6 RNA polymerase.The cRNA were puried and re-suspended in nuclease-free water at a concentrar0ion of 1μg/1μℓ and stored at -80℃ in aliquots. RNA concentrarion were determined by UV spectrophotometry. cRNA were microinjected(27.6ng) into Xenopus laevis oocyte on stage ⅴ or ⅵ. The oocyte were then incubated at 17℃ for 3~5days in modified Barth's solution [13] sterilized by filteration.

Two-electrode voltage-clamp recording

Two-electrode voltage-clamp technique was employed to observe tastant-induced currents at holding potential of -70 mV. Signal were amplified with an OC-725C amplifier(Warner Instruments,Hamden, CT),low-pass filtered at 50 Hz and digitized at 1 kHz.Data acquisition and analysis were carried out with Digidata 1322A (Axon Instruments, Forster City,CA) and software pCLAMP 10 (Molecular Devices,LLC,Sunnyvale, CA).The data were analyzed with Graphpad 6.

Phylogenetic analysis

The amino acid sequences of the *Apis mellifera* gustatory receptors(AmGr) and *Drosophila melanogaster* gustatory receptors(DmGr) were downloaded from NCBI and previous work (Hugh et al., 2006). All amino acid sequences used for phylogenetic anlaysis were aligned with Clustal X. Phylogenetic analysis was constructed with the 16 AmGr and 55 DmGr by using MEGA5 Neigbor-Joing method. Bootstrap analysis was conducted by using 1000 Neighbor-Joining replications.

3. Results

3.1. The differences of number of sensillum chaetica per antennae segments

The results of SEM analysis are shown in Figure (1). The number of sensillum chaetica we counted were higher on distal segment of antennae. This results demonstrated that antennal distal segment is important for detecting and evaluating food source.

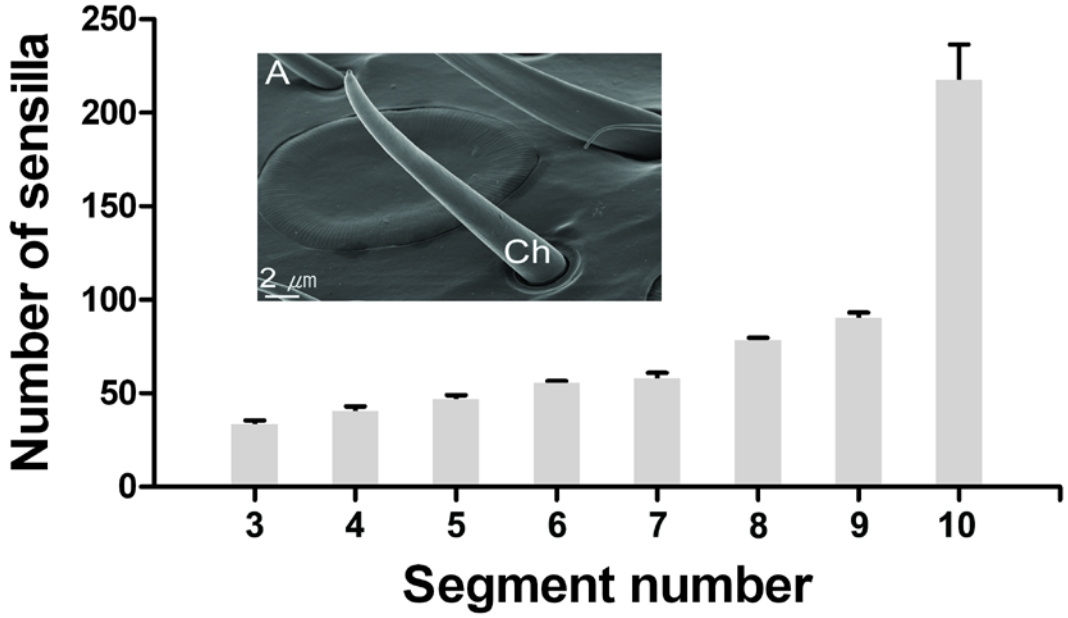

Figure 1. The mean number of sensillum chaetica of *Apis mellifera* per antenna segment. (A) Details of sensillum chaetica. Number of sensillum chaetica is higher on the distal segment of antennae. Error bars indicate SEM (n = 3).

3.2. AmGr1 and AmGr2 are highly expressed in the distal segment of antennae

The expression patterns of AmGr1 and AmGr2 genes were analyzed by qRT-PCR. The results are shown in Figure(2). The expression of gustatory receptors 1 were expressed higher in the distal segment of antennae. This results demonstrated that AmGr1 is crucial to detect chemical component in food source.

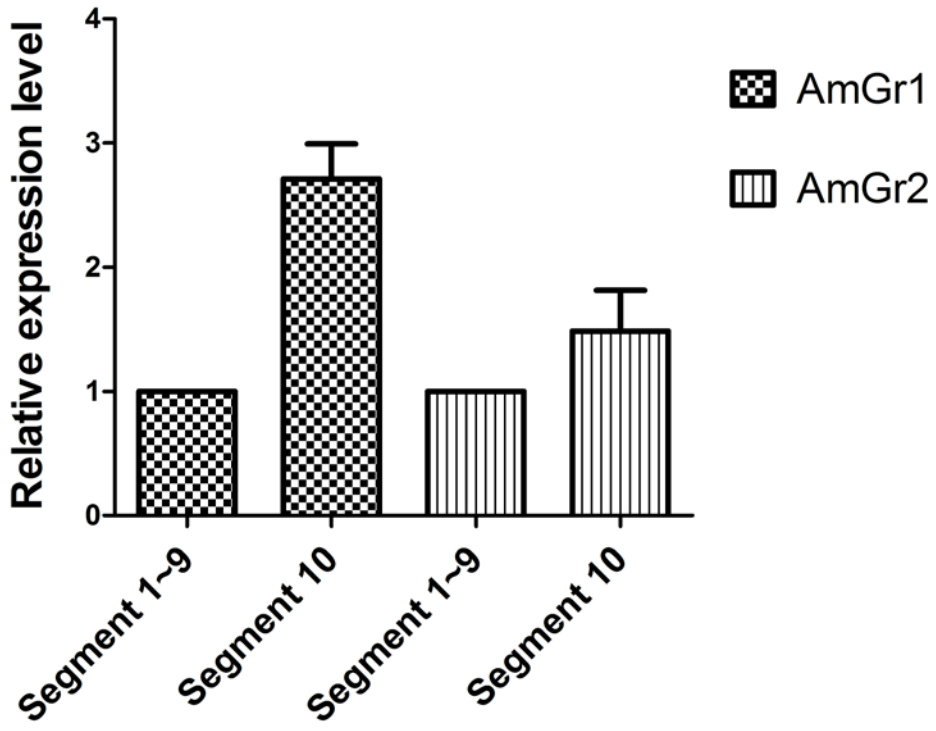

Figure 2. Expression patterns of the A*pis mellifera* sugar receptors, AmGr1 and AmGr2 in antennae segments.

AmGr1 and AmGr2 are exclusively expressed in the distal segment of the antennae.

3.3. AmGr1 and AmGr1+AmGr2 responded to sugars

In a few cases, the functional study of insect gustatory receptors using *Xenopus* oocytes and two-electrode voltage-clamp electrophysiology has been reported [14].To identify the function of candidate sugar receptors of *Apis mellifera*, we examined *Xenopus* oocyte expressing AmGr1 or AmGr2 resprectively by using Two-electrode voltage-clamp recording. *Xenopus* Oocytes injected with AmGr cRNA were stimulated with sweet tastants that have been reported to have reponsiveness in honey bees [15]. That tastants also major sugar componets in nectar [1616] and pollen [17]that provide honey bee with carbohydrates and protein respectively, which are necessary for survival.

The Oocyte expressing AmGr1 responded to sucrose, glucose, maltose, and trehalose but not fructose. The responses of oocyte injected with AmGr1 to these sugars were dose-dependent manner (Fig. 3).The Oocyte expressing AmGr2 did not showed any responses to these tastants. Actually, it has been reported that *Drosophila melanogaster* sugar receptor, DmGr64f is a co-recepotor for detection of sugar [18]. In order to identify the function of AmGr2, we injected *Xenopus* oocyte with AmGr1 cRNA and AmGr2 cRNA together. The oocyte co-expressing AmGr1 and AmGr2 showed different sensitivity to sugar tested in this study. Response of oocyte expressing AmGr1 and AmGr2 to glucose is higher, While the response to sucrose,maltose and trehalose is lower compared to oocyte expressing AmGr1 alone. The responses of oocyte injected with AmGr1 and AmGr2 to these sugars were dose-dependent manner. (Fig. 4)

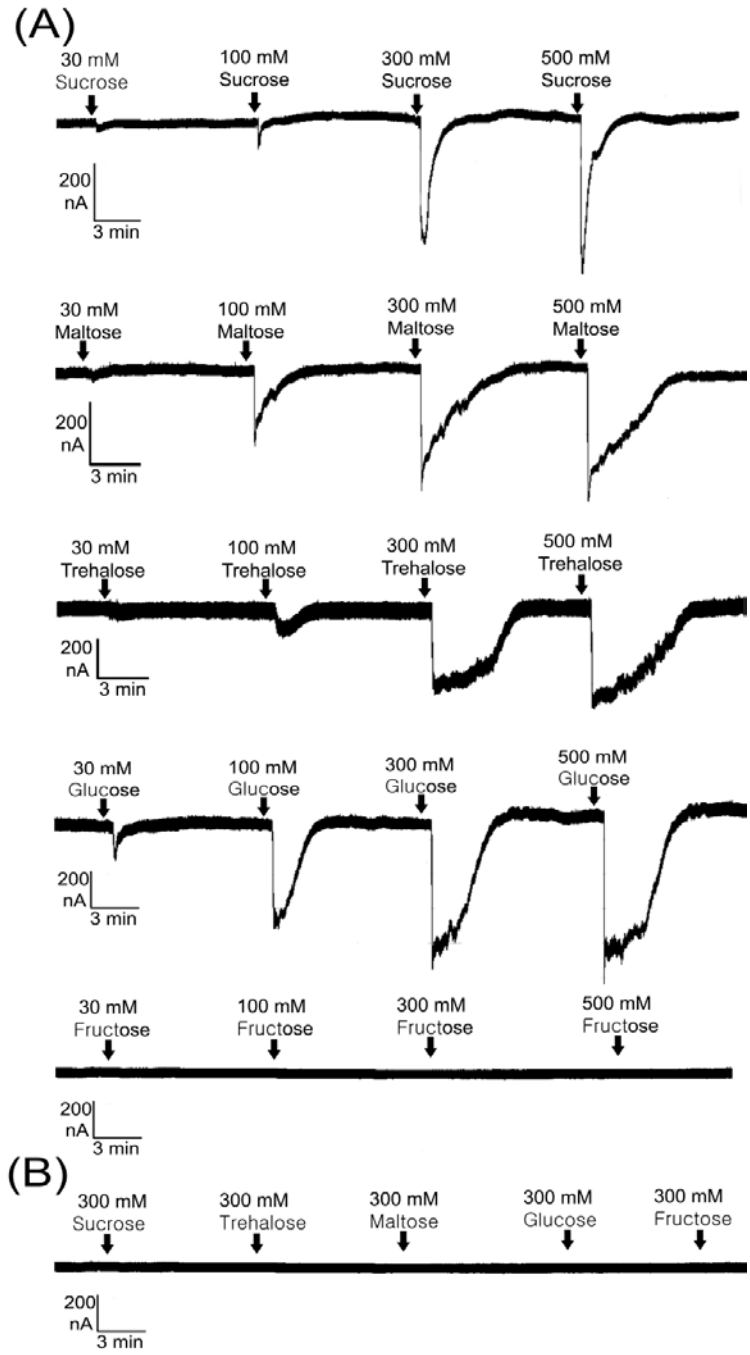

Figure 3. Current traces of *Xenopus* oocyte expressing AmGr1+AmGr2 or AmGr2 alone stimulated with a range of sugars concentrations. (A) The current of AmGr1 + AmGr2 were dependent on the dose of the sugars except for fructose. (B) The oocyte expressing AmGr2 showed no response to the sugars. The current responded to stimulations of the indicated concentrations of sugars. The oocyte was stimulated for 10 s as indicated by the black arrows

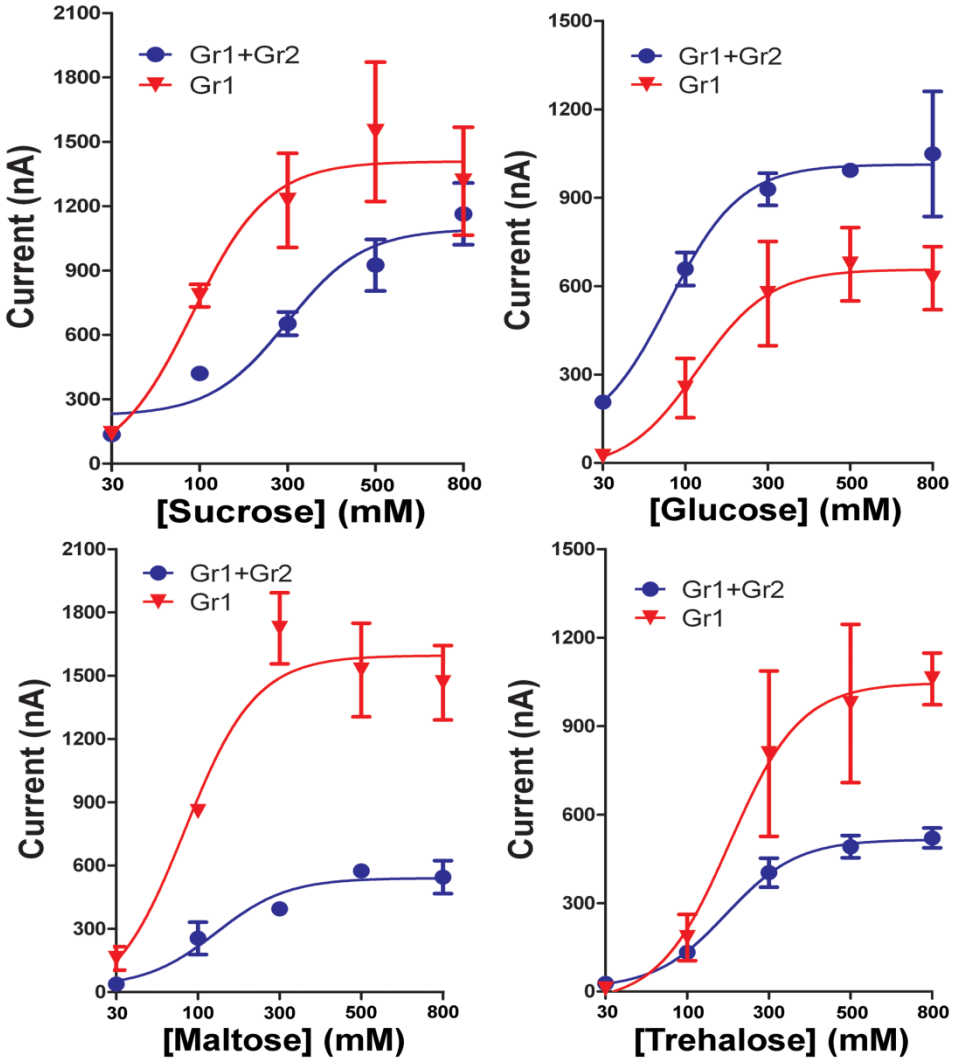

Figure 4. Two-electrode voltage-clamp recordings of *Xenopus* oocyte injected with AmGr1+AmGr2 cRNA or AmGr1 cRNA alone. (A) Dose-dependent responses of *Xenopus* oocyte expressing AmGr1+AmGr2 or AmGr1 alone to sucrose. Co-expression of AmGr1 with AmGr2 showed lower sensitivity to sucrose than expression of AmGr1 alone. (B) Dose-dependent responses of *Xenopus* oocyte expressing AmGr1+AmGr2 or AmGr1 alone to glucose. Co-expression of AmGr1 with AmGr2 showed higher sensitivity to glucose than expression of AmGr1 alone. (C) Dose-dependent responses of *Xenopus* oocyte expressing AmGr1+AmGr2 or AmGr1 alone to maltose. Co-expression of AmGr1 with AmGr2 showed lower sensitivity to maltose than expression of AmGr1 alone. (D) Dose-dependent responses of *Xenopus* oocyte expressing AmGr1+AmGr2 or AmGr1 alone to trehalose. Co-expression of AmGr1 with AmGr2 showed lower sensitivity to trehalose than expression of AmGr1 alone. Error bars indicate SEM(n =5).

3.4. Localization of sugar receotor, AmGr1 and AmGr2 in antennae

We analyzed the localization of AmGr1 and AmGr2 in antennae by double-labeling studies with *in situ* hybridization and immunostaining. As shown in Figure. , the AmGr1 is co-localized with AmGr2. Interestingly, there is also a portion of cells with non-overlapping expression of AmGr1 or AmGr2 in antennae. It also has been reported that *Drosophila melanogaster* sugar receptor, DmGr64f is coexpressed with other sugar receptors,Dm5a and Dm61a [7]. This results suggest that *Apis mellifera* sugar receptors function as heterodimer or homodimer and that AmGr2 is required as a co-receptor for dynamic range of suger taste repertoire. (Fig. 5)

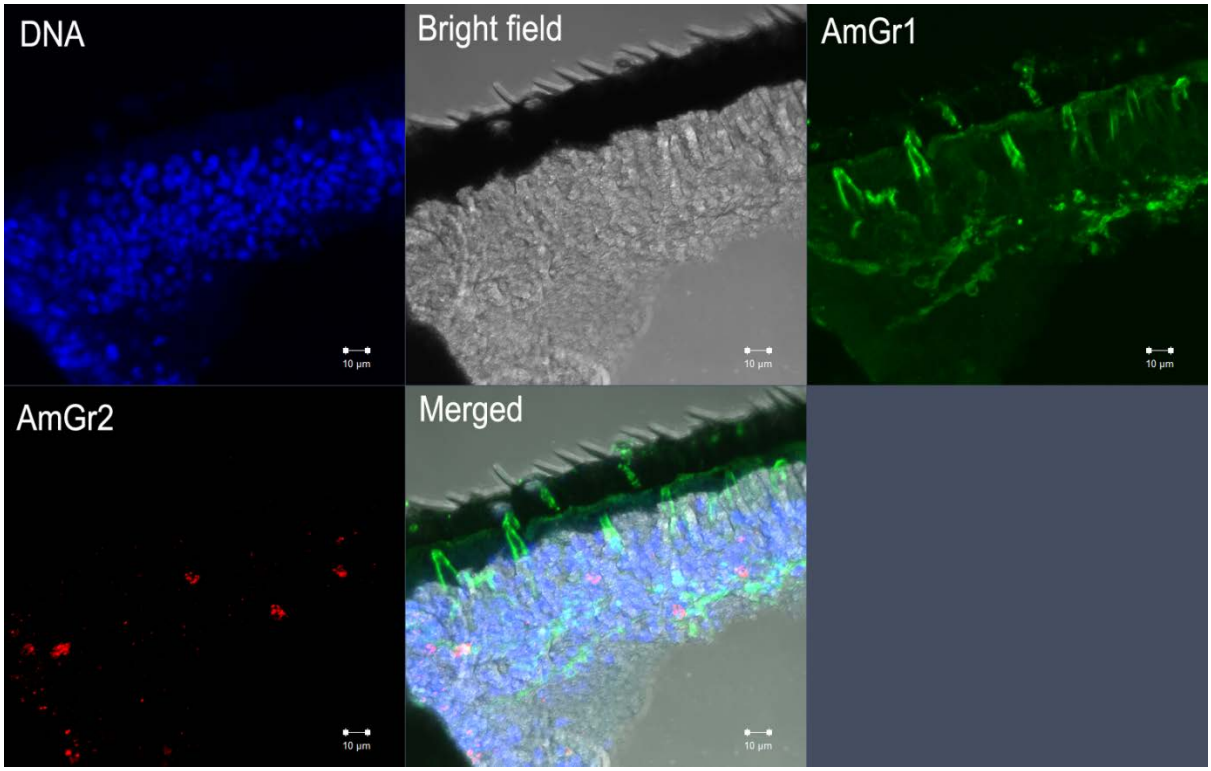

Figure 5. Localization of AmGr1 and AmGr2 in the antennae of A*pis mellifera.*

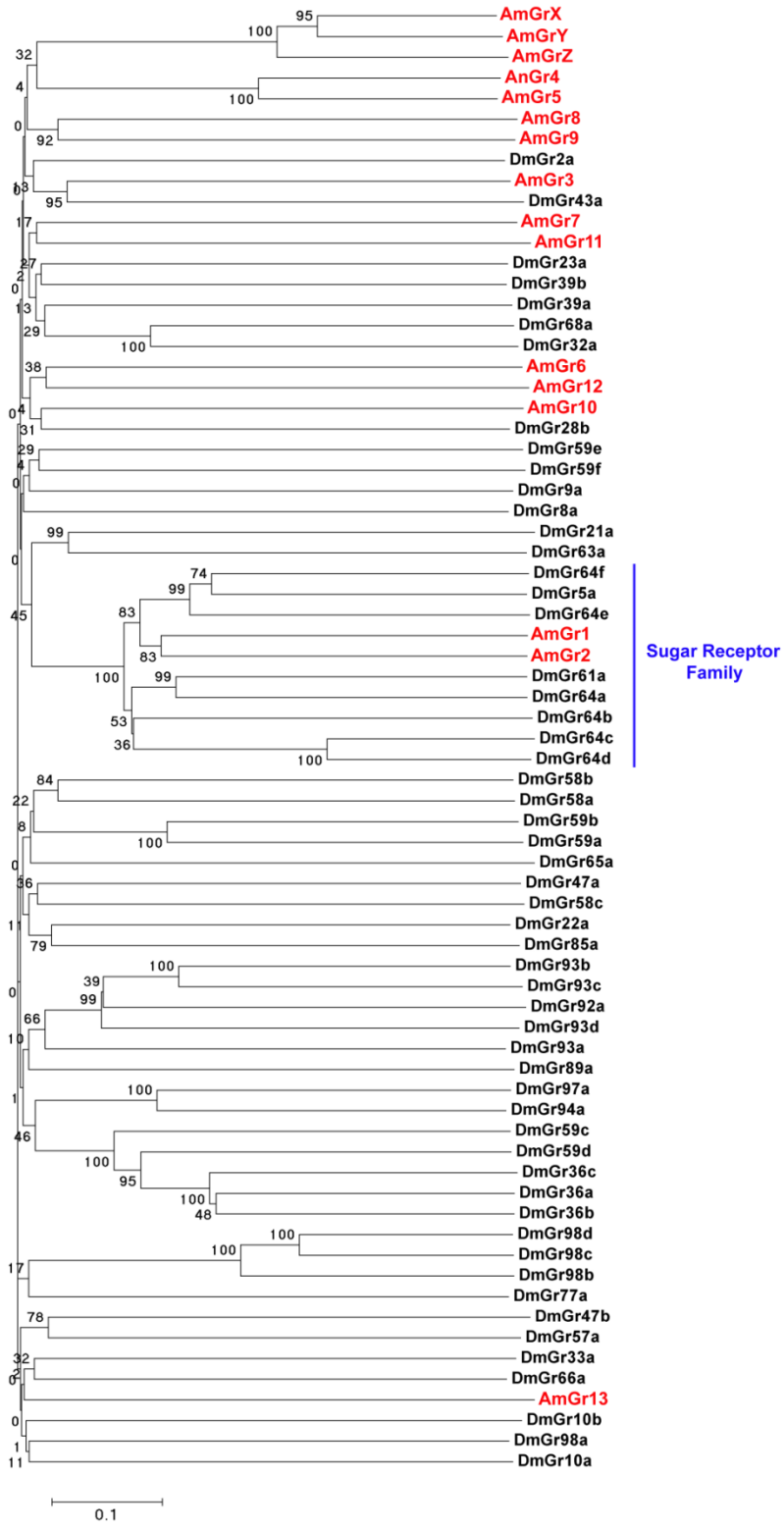

Figure 6. Phylogenetic tree of gustatory receptor proteins of *Apis mellifera* and *Drosophila melanogaster* generated in MEGA5 by using a Neighbor-Joining method. The *Apis mellefera* gustatory receotors in red. Numbers indicate bootstrap values (%) . AmGr1 and AmGr2 cluster with *Drosophila melanogaster* sugar receptors family.

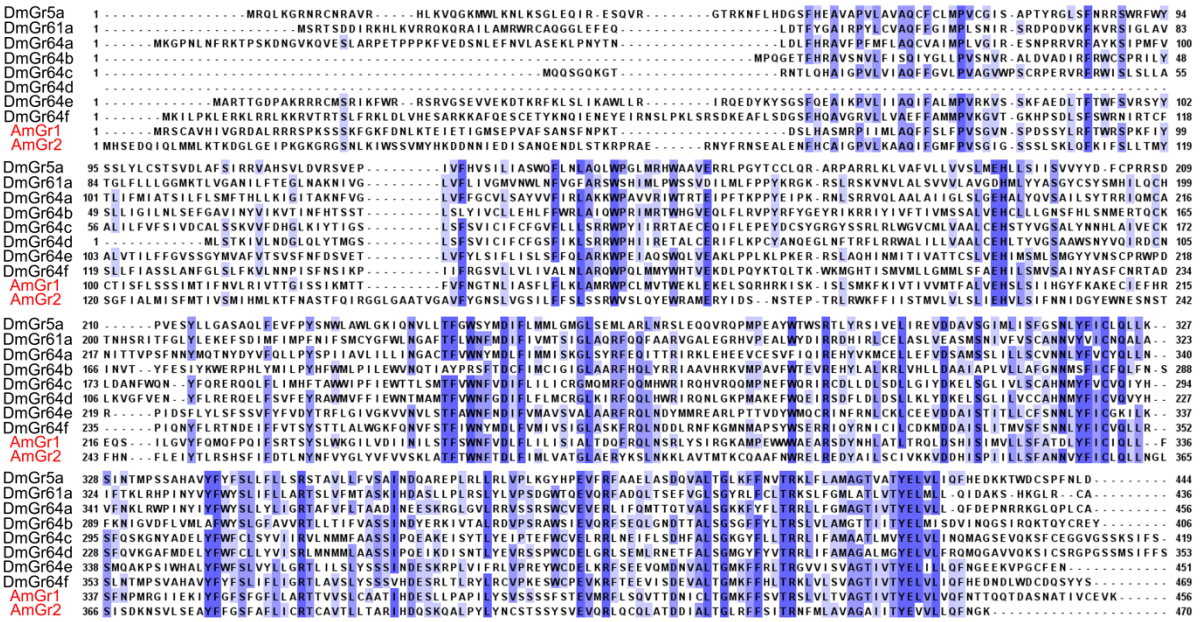

Figure 7. Multiple alignment of amino acid sequences of *Drosophila melanogaster* sugar receptors, Dm64a-f, Dm61a, and Dm5a with *Apis mellifera* sugar receptors, AmGr1-2. Alignment was generated and edited by using ClusterW and Jalview.

Table 1. Information of the primer sequences

Gene name	Accession number	Purpose		Primer sequence (5'- 3')	Size (bp)
AmGr1	XM_001123138.2	**Full coding**	**F**	ATGGAATTTGTAATTGAATTAGATAATCTAGATTCTCCTATAAGATTGTCGCCCAAGAGCAGC	**1368**
			R	TTACTTCACCTCGCATACAATTGTAGCGTTCGACGC	
		qRT-PCR	**F**	TTTCAACCCGATGCGAGGTA	**109**
			R	ATCGTGAATCGTCGCAGCAC	
AmGr2	XM_397125.5	**Full coding**	**F**	ATGCATTCGGAGGATCAAAT	**1561**
			R	TTATTTACCATTAAATTGTAAAAGTA	
		qRT-PCR	**F**	AACAGTCTGGTGGGCAGCATC	**122**
			R	CGAAGCCGTGTGGGTTCTGT	
		Probe	**F**	CAAATTCGTGGTGGCTTAGG	**732**
			R	GCACGTGCGACAGATAAGAA	
AmRP49	XM_006564316.1	**qRT-PCR**	**F**	GGGACAATATTTGATGCCCAAT	**100**
			R	CTTGACATTATGTACCAAAACTTTTCT	

4. Discussion

T1Rs family are present in the genomes of all vertebrates. However T1Rs have not been founded in any invertebtates species [19]. Interestingly, all members of the T1R family are existent in fish and they also function as heteromeric receptors [20, 21]. However, fish T1R2+3 responds to L-amino acids rather than sweet tastants [20, 21]. This demonstrate that mammalian T1R2+3 was renovated to detect sugar at certain point while the moving of vertebrates from oceans to land [19]. Insect sugar receptors have very low identity with mammalian sugar receptors. but they also can detect sweet tastants. this is reflecting the evolutional adaption to distinct ecological niches and food sources.

Honeybee sugar receptors responded to sucrose and glucose with highly sensitivity than maltose and trehalose. Usually sucrose, glucose is the dominant constituent in the nectar [22] and pollen [16]respectively. This suggest that maybe honey bees evolutionally developed sensitivity to sucrose and glucose to optimize efficiency foraging benefit cost.

In mammalian, Interestingly, T1R2 and T1R3 responsible for all responses to sugar through definite combinations of T1R2/T1R3 heterodimers and/or T1R2 and T1R3 homodimers. Actually in case of honeybee , it has been reported that responses of sucrose between unlike hair on the same antenna displayed a high grade of varialbility [4] in a similar manner with our results. In case of *Drosophila melanogaster*, it has been reported that responses of sugar among the three type of labellar gustatory sensilla are different [23, 24]. Based on localization patterns of AmGr1 and AmGr2, we suggested that combinational expression(AmGr1, AmGr2, AmGr1+2) of two sugar receptors may provide honeybees with powerful range of sugar sensing over a enormous range of concentrations in nectar or

pollen.

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서양종꿀벌 (*Apis mellifera*)의 단맛수용체에 관한 연구

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국문초록

미각은 동물들에게 먹이에 대한 질이나 영양적 가치등의 매우 중요한 정보를 제공한다. 꿀벌의 경우 미각은 유용한 먹이, 물, 수지등을 선택하고 동료들을 인지하는데 있어서 매우 중요하다. 그 중에서도 단맛에 대한 인지는 꿀벌이 수집한 화분과 꿀에 대한 용인성에 있어서 매우 중요한데, 아직까지 이에 대한 어떠한 분자적 신경학적 작용기작은 밝혀져 있지 않다.

본 논문에서는 꿀벌의 단맛수용체를 기능적으로 분석하였으며 이 단맛수용체는 기존에 밝혀진 노랑초파리의 단맛수용체와 염기서열에서의 유사성을 보였다. 서양종꿀벌 단맛수용체 1번의 경우 fructose를 제외하고 sucrose, glucose, maltose, trehalose에 농도에 따른 반응을 나타내었다. 단맛수용체 2번의 경우 어떠한 당 성분에 반응을 나타내

지 않았다. 특이하게도 단맛수용체 1번과 2번이 함께 있을 때 당 성분에 대하여 다른 민감성을 나타내었는데 단맛수용체 1번에 비하여 glucose에 대해서는 높은 민감성을 그리고 나머지 당 성분에 대해서는 낮은 민감성을 나타내었다. 실제 서양종 꿀벌의 더듬이에서의 발현위치와 발현 양상을 확인한 결과 단맛수용체 1번과 2번이 함께 위치하기도 했고 각각 위치하기도 하였으며, 특이 단맛수용체 1번이 더듬이 끝에서 매우 높게 발현하고 있었다.

본 연구의 결과 서양종꿀벌의 단맛 수용체는 기능적으로 헤토로다이머 혹은 호모다이머로 존재하며 단맛수용체 2번의 경우 서양종꿀벌에게 당 성분 인지에 있어서 인지능력의 다양성을 제공하는 것으로 생각된다.

검색어: 서양종꿀벌, 미각, 미각수용체, 당, 노랑초파리, 행동

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