The silkworm gustatory receptor BmGr63 is dedicated to the detection of isoquercetin in mulberry

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Gustatory systems in phytophagous insects are used to perceive feeding stimulants and deterrents, and are involved in insect decisions to feed on particular plants. During the process, gustatory receptors (Grs) can recognize diverse phytochemicals and provide a molecular basis for taste perception. The silkworm, as a representative Lepidoptera species, has developed a strong feeding preference for mulberry leaves. The mulberry-derived flavonoid glycoside, isoquercetin, is required to induce feeding behaviours. However, the corresponding Grs for isoquercetin and underlying molecular mechanisms remain unclear. In this study, we used molecular methods, voltage clamp recordings and feeding assays to identify silkworm BmGr63, which was tuned to isoquercetin. The use of qRT-PCR confirmed that BmGr63 was highly expressed in the mouthpart of fourth and fifth instar larvae. Functional analysis showed that oocytes expressing BmGr63 from the ‘bitter’ clade responded to mulberry extracts. Among 20 test chemicals, BmGr63 specifically recognized isoquercetin. The preference for isoquercetin was not observed in BmGr63 knock-down groups. The tuning between BmGr63 and isoquercetin has been demonstrated, which is meaningful to explain the silkworm-mulberry feeding mechanism from molecular levels and thus provides evidence for further feeding relationship studies between phytophagous insects and host plants.

1. Introduction

Relationships between phytophagous insects and their host plants are complex and have developed over long-term coevolution [1]. Plants provide food for insects, while plants with different characteristics can significantly affect insect growth, development and geographical distribution [2,3]. Phytophagous insects have developed specific feeding preferences and food adaptation strategies for different plant species [4]. In seeking food, insect chemosensory systems (including olfactory and gustatory systems) are used to detect plant-based chemical signals [5–8]. When an insect starts to feed, gustatory sensilla mainly on the mouthparts make direct contact with plant sap released by chewing [9]. Gustatory systems recognize non-volatile chemicals and are indispensable for the integrated assessment of feeding stimulants and deterrents in plants [5,10,11]. When non-volatile chemicals enter the gustatory sensilla, gustatory receptors (Grs) located on the gustatory receptor neurons will recognize and resolve the chemicals and convert chemical signals to electrical signals. These signals are then transmitted to the central nervous system and guide feeding behaviour [11,12]. Gustatory system evolution facilitates insect adaptation to different ecological niches [13,14].

The first insect Grs were identified in Drosophila melanogaster [15,16]. Additional Gr data are limited to published genome sequences of particular species including Bombyx mori [17–19], Heliconius melpomene [20], Plutella
xylostella [21–23] and Manduca sexta [24]. Insect Grs often possess seven transmembrane domains with reverse topology when compared with classical G-protein coupled receptors, with an intracellular N-terminus and an extracellular C-terminus [16,18]. Grs are diverse, with low sequence similarities of 15–25% [16], and classification into four clades including the CO2, fructose/inositol, sugar and bitter clades. Sugar Grs have been functionally characterized in several insect species. For example, Gr43a in D. melanogaster responds to fructose [25]. In P. xylostella, two spliced variants of PxyGrl3a can respond to fructose [26]. Bitter receptor function is relatively unknown and has been mainly studied in D. melanogaster. DmGr28b is necessary for saponin avoidance [27]. The subsets of DmGr66a and some other Grs in Drosophila may be responsible for detecting bitter compounds. For example, Gr33a, Gr66a and Gr93a are all required to generate a functional umbriliferone receptor [28]. Recent studies reported bitter receptor function in Lepidoptera. In Papilio xuthus, PxtGr1 is specific for the oviposition stimulant synephrine and is a key factor in host specialization [29]. PxyGrl34 from P. xylostella is responsible for the steroid plant hormone brassinolide as a feeding and oviposition deterrent [30]. PrapGr28 in Pieris rapae is tuned to sinigrin, a stimulant for larval feeding and adult oviposition [31]. However, many insect Grs have not been functionally characterized. The identification of their ligands should help elucidate insect adaption to different ecological niches and their host plant specificity.

Silkworm larvae predominantly feed on mulberry leaves. Mulberry is widely planted in the Eurasian continent and is important for domesticated silkworm culture [32]. The silkworm mulberry feeding mechanism has been studied as a representative example of interactions between a phytophagous insect and its host plants. Hamamura et al. [33–35] proposed that there were attraction, biting and swallowing factors in mulberry leaves, which stimulated silkworm feeding, and relatively few avoidance factors inhibited feeding, thereby triggering larval feeding behaviour. For instance, volatile components in mulberry such as cis-jasmonene are highly attractive to silkworm larvae and are recognized by olfactory systems [36]. Non-volatile compounds such as polysaccharides, β-sitosterol and isocoumarin act as gustatory factors to promote silkworm feeding [34,37]. Previous studies focused on the direct effects of phytochemicals on silkworm feeding behaviour, but the molecular mechanisms underlying feeding behaviour were not fully understood. With the development of genome and transcriptome sequencing, Grs in B. mori have been identified, further re-annotated and now number 76 [17,19]. In sugar clades, BmGr9 responds to fructose [38] and BmGr8 and BmGr10 to inositol [18,39]. In bitter clades, BmGr16, 18 and 53 broadly respond to the feeding deterrents coumarin and caffeine [40]. Knock-out of BmGr66 increases the plants accepted by silkworm larvae in addition to mulberry leaves, but potential BmGr66 ligands remain to be identified [41]. Mulberry-derived ligands corresponding to specific BmGrs also remain unknown. Metabolome sequencing has successfully characterized many mulberry metabolites [42]. Identification of molecular interactions between BmGrs and mulberry phytochemicals will help reveal the silkworm mulberry feeding mechanism and provide a reference for other studies of relationships between phytophagous insects and their host plants.

In the present study, multi-level experiments on the interaction between BmGrs and mulberry metabolites were conducted. BmGrs expressed in larval mouthparts, based on previous transcriptome data, were selected and full-length coding sequences were identified. Then, candidate BmGrs expression levels in larval mouthparts were determined using quantitative real-time PCR (qRT-PCR). BmGr63, with the highest expression levels, was subjected to ligand identification in voltage clamp experiments. Furthermore, RNA interference (RNAi) methods were adopted to demonstrate interactions between BmGr63 and ligand in silkworms.

2. Material and methods

(a) Animal rearing and plants

The DAZAO silkworm strain was used in all the experiments. It was maintained at the Gene Resource Bank of Domesticated Silkworms (Southwest University, Chongqing, China). Silkworm larvae were reared at 25°C under a 12:12 h (light : dark) photoperiod and fed mulberry leaves or artificial diets (purchased from Shandong Agricultural University) in feeding assays. One-day-old fourth and fifth instar larval mouthparts were collected to examine candidate BmGrs expression levels. Also, 2-day and 3-day-old fifth instar larval labrum, maxilla, labium, mandible, thoracic leg and midgut were collected to study BmGr63 expression patterns. Collected tissues were washed in phosphate buffered saline (PBS) buffer and stored at –80°C until use.

Female Xenopus laevis were purchased from Haiwei Panshi Biomedical Technology Co., Qingdao, China, and reared on frog diets at 18–20°C in our laboratory. Healthy female X. laevis individuals were anaesthetized on ice for 30 min before surgically collecting the oocytes.

The mulberry leaves of M. alba var. Shin-Ichinose (a cultivated resource) were harvested from the Mulberry Germplasm Nursery (Southwest University, Chongqing, China). To extract total active substances, freeze-dried mulberry leaf powder was soaked in 40% methanol (1:10, m/v), and vortexed three times with 10 min intervals. The solution was then placed at 4°C overnight. For oocyte experiments, the filtered extract solution was concentrated and then diluted to a working concentration in Ringer’s solution.

(b) RNA extraction and qRT-PCR analysis

Total RNA was extracted from collected tissues using an RNAiso Plus Kit (Takara Bio., Shiga, Japan). Total RNA (1 μg) was used as the template to synthesize CDNA with the PrimeScript RT Reagent Kit (Perfect Real Time) (Takara Bio., Shiga, Japan). Then, qRT-PCR assays were conducted using the QuantiTone SYBR Green PCR Kit (QIAGEN, Hilden, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a reference control for normalizing CT values. Gene-specific qRT-PCR primers were designed using Primer Premier 5 (electronic supplementary material, table S1). The 2−ΔΔCT method was used to calculate the relative fold changes in gene expression [43]. The qRT-PCR was performed in three independent technical replicates.

(c) Cloning of BmGrs and vector construction

We referred to published transcriptome data [19] to screen for Grs expressed in larval mouthparts. As these predicted BmGrs sequences were incomplete or inaccurate, specific primers were designed to obtain full-length coding sequences. PCR was conducted using Platinum SuperFi II Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) as follows: 98°C for 30 s, followed by 35–40 cycles of 98°C for 10 s, 60°C for 10 s and 72°C for 1 min, and a final extension at 72°C for 5 min. Amplified BmGrs were then cloned into a PEASY-Blunt vector (TransGen Biotech, Beijing, China) and sequenced. Grs that terminated early or were un-cloned were removed and the
rest were used as candidate Grs for follow-up experiments (electronic supplementary material, table S2).

To confirm BmGr63 localization in human embryonic kidney 293T (HEK293T) cells, an mScarlet tag with red fluorescence was fused with BmGr63 into the pcDNA3.1 vector. The full-length BmGr63 coding sequence was inserted between BgII and BcaI sites in the pT7Ts expression vector and used for cRNA synthesis.

(d) Phylogenetic analysis and transmembrane domain prediction

To investigate evolutionary relationships between BmGr63 and Gs from B. mori, and other Lepidoptera species, a phylogenetic tree based on Gr protein sequences from B. mori, M. sexta, H. melpomene, P. xuthella and P. rapae was constructed (electronic supplementary material, table S3). Amino acid sequences were aligned using MAFFT v.7.455 [44], and gap sites were removed using trimAl v.1.4 [45]. The maximum likelihood phylogenetic tree was constructed using RAxML v.8.2.12 [46] with the Jones–Taylor–Thornton amino acid substitution model with a bootstrap of 5000 replicates. The phylogenetic tree was constructed with iTOL (https://itol.embl.de). As the topology was previously reported [42].

(e) Cell culture and transfection

Cells were cultured in Dulbecco’s modified eagle medium (DMEM) (HyClone, Chicago, IL, USA) containing 10% fetal bovine serum (FBS) (Gemini, West Sacramento, CA, USA) and 1% penicillin and streptomycin (Thermo Scientific, Waltham, MA, USA) at 37°C with 5% CO2. The BmGr63 recombinant vector was transfected into HEK293T cells using liposomal polyethyleneimine (PEI) (Invitrogen, Carlsbad, CA, USA). Cells were seeded on round coverslips for 24 h until adherence. Then DNA and PEI were mixed in a ratio of 1:2.2 in 0.4 ml optimized-minimal essential medium (OPTI-MEM) for 20 min and added to wells. After 4 h, the medium was replaced by fresh OPTI-MEM medium containing 10% FBS 4 h later. At 24 h after transfection, red fluorescence was observed using a ZEISS LSM880 system equipped with a 63× oil objective (NA 1.4) and controlled by ZEN 2.1 software at an excitation wavelength of 488 nm. Acquired images were analysed using ImageJ software.

(f) Two-electrode voltage clamp recordings

The cRNA was synthesized from the linearized pT7Ts vector using BamtHI with mMESSAGE mACHINE T7 Kit (Ambion, Austin, TX, USA). Purified cRNA was diluted to 2 µg µl−1 and stored at −80°C until use. Surgically collected oocytes were treated with 2 mg ml−1 collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) in Ca2+ free buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH = 7.6) for 1–2 h at room temperature. Mature oocytes were microinjected with 25 nl BmGr63 cRNA, and RNase-free water as a negative control. Injected oocytes were incubated for 3–5 days at 18°C in Barth solution (88 mM NaCl, 1 mM KCl, 1 mM MgCl2, 0.4 mM CaCl2, 0.3 mM Ca(NO3)2, 1 mM MgSO4, 2.4 mM NaHCO3, 10 mM HEPES, pH = 7.4) supplemented with 5% dialyzed horse serum, 50 mg ml−1 penicillin, 50 mg ml−1 streptomycin, 100 mg ml−1 gentamycin and 550 mg ml−1 sodium pyruvate. The two-electrode voltage clamp technique was used to record whole-cell currents in oocytes responding to mulberry extracts and chemical compounds. Intracellular glass electrodes were filled with 3 M KCl and presented resistances of 0.3–3.0 MΩ. Signals were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT, USA) at a holding potential of −80 mV, low-pass filtered at 50 Hz and digitized at 1 kHz. When the baseline was stable, compounds for testing were perfused and current changes were recorded. Then oocytes were washed with Ringer’s solution until the current returned to a stable baseline. Test compounds were changed using a peristaltic pump (BT100-2; Longer Precision Pump Co., Ltd, Baoding, China) at a constant speed. Mulberry extracts at different concentrations were used to stimulate oocytes. Then the listed chemical compounds (electronic supplementary material, table S4) in 1.0 mM in Ringer’s solution (96 mM NaCl, 2 mM KCl, 0.1 mM CaCl2, 2.0 mM MgCl2, 5 mM HEPES, pH = 7.6) were used to record currents. When a clear current response was observed at 1 mM, compounds at gradient concentrations were tested as described.

(g) RNAi mediated by double-stranded RNA (dsRNA)

The dsRNA interfered region was designed using DRSC (https://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl). A verified BmGr63 sequence template was amplified with specific primers (shown in electronic supplementary material, table S1) containing the T7 promoter to acquire PCR products for the interfered region. PCR products were then used to yield dsRNA using a T7 High Efficiency Transcription Kit (TransGen Biotech, Beijing, China) following protocols. Green fluorescent protein (GFP) dsRNA was synthesized using the same method as the control. The obtained dsRNA was stored at −80°C until use.

For fourth instar larvae, 3 µg dsRNA was added to artificial diets and used to feed silkworms. The larvae that consumed the given diets were starved for another 12 h and then used in RNAi experiments. For fifth instar larvae, 5 µg dsRNA was brushed on mulberry leaves and used to feed silkworms. The larvae that consumed the entire leaves were starved for another 12 h, and used in RNAi experiments.

(h) Feeding assays

Artificial diet was used to examine isoquercetin effects on silkworm feeding. Freshly moulted fourth instar larvae were starved for 1 day before experiments. The dry diet weight to solution ratio was 1:1.5. Test compound solutions, in gradient concentrations, were used in experimental groups. For control groups, water was used. Larvae were placed separately into Petri dishes and the experiments were performed at 25°C. Consumed diet weight was calculated using the difference in diet wet weight before testing and at 3 h after testing.

In RNAi experiments, no choice and dual choice feeding assays were performed. Silkworms were separated into BmGr63 RNAi and control groups. In no choice feeding assays, artificial diets adding 10−3 M isoquercetin were used. In dual choice feeding assays, artificial diets with or without the addition of isoquercetin were used. The method was described above.

We investigated BmGr63 function in silkworm feeding. Freshly moulted fifth instar larvae, starved for 1 day, were used. Silkworms of uniform size were used for further assays. Three larvae were used in a group and the number of biological replicates was five groups. A similar weight of mulberry leaves was used in each group, and the weight of eaten leaves was calculated by differences in weight before and at 2 h after testing.

(i) UPLC-MS/MS system and analytical conditions

Mulberry extracts were obtained using aforementioned methods. An isoquercetin standard was diluted to different concentrations (10−3 M, 5 × 10−3 M and 10−2 M). Then the extracts and standard solutions were analysed using an LC-ESI-MS/MS system (Thermo Fisher Scientific, Waltham, MA, USA). The ultra-high performance liquid chromatography (UPLC) conditions were previously reported [42]. Relative isoquercetin content (peak area) was calculated using Thermo Scientific Xcalibur software.
domains and the N-terminus was intracellular (figure 3a). It should be pointed out that the prediction using different tools might be different (electronic supplementary material, table S2). The combination of multiple prediction methods and experimental verification should be comprehensive. Cellular localization showed that BmGr63 was localized on cell membranes, consistent with Grs functioning as membrane proteins (figure 3b).

BmGr63 expression profiles in specific mouthpart tissues (including labrum, mandible, labium and maxilla), thoracic leg and midgut were also investigated (figure 3c). The results indicated that the highest BmGr63 expression levels were observed in labrum. BmGr63 was also expressed at relatively high levels in mandible, labium, maxilla and thoracic leg, but was repressed in midgut.

(d) Identification of interaction between mulberry extracts and BmGr63 using the oocyte expression system

To determine whether BmGr63 was involved in silkworm recognition of mulberry leaves, mulberry extracts were used as stimulants for oocytes expressing BmGr63. In oocytes, currents induced by mulberry extracts were increased in a dose-dependent manner (figure 4a,b). Oocytes injected with RNase-free water were used as negative controls and no clear currents were observed at any concentration (electronic supplementary material, figure S1).

(e) Identification of BmGr63 ligand with the Xenopus oocyte expression system

Voltage clamp recordings were used to clarify which mulberry metabolite class functioned as ligands for BmGr63. Twenty metabolites, at initial concentrations of $10^{-3}$ M, included

3. Results

(a) BmgRs expression levels in silkworm larval mouthparts

The cloned sequences of BmgRs expressed in mouthparts without early termination are listed in electronic supplementary material, table S2. Although RNA-seq reflects overall trends in gene expression, qRT-PCR validation is required to verify the expression of important genes. Thus, qRT-PCR was conducted to determine the spatial-temporal expression levels of candidate BmgRs. And it showed that BmGr63 was the most highly expressed Gr in fourth and fifth instar larval mouthparts (figure 1).

(b) Phylogenetic analysis

As shown in figure 2, the phylogenetic tree was divided into CO2, fructose/inositol, sugar and bitter clades. BmGr1–3 were clustered in the CO2 clade, BmGr4–8 belonged to the sugar clade and BmGr9–10 were in the fructose/inositol clade. The remaining 66 BmgRs, were divided into bitter clades with uncharacterized functions. The BmGr63 subclade included PrapGr28, MsexGr41 and HmGr63.

(c) Bmgr63 structural and expression analysis

Full-length BmGr63 coding sequences were obtained through gene cloning and the open reading frame encoded a 417 amino acid protein (figure 3a). Using HMMPHMMTOP analysis, it was predicted that BmGr63 had seven transmembrane
sugars, flavonoids, alkaloids, amino acids, phenylpropanoids and plant hormones (electronic supplementary material, table S4). Of the three flavonoid glycosides, figure 4c,d showed that isoquercetin induced a strong response in the oocytes expressing BmGr63. The structural analogue astragalin induced a weaker response while the response to rutin was very weak. A range of isoquercetin concentrations was tested. Currents induced by isoquercetin increased from the lowest threshold concentration of $10^{-4}$ M in a dose-dependent manner (figure 4e,f). At $10^{-3}$ M and $5 \times 10^{-3}$ Mv doses, strong inward currents were observed. Water-injected oocytes as negative controls did not respond to test chemicals (electronic supplementary material, figure S1).

(f) The effects of isoquercetin on silkworm feeding

To study the stimulating or deterrent effects of isoquercetin on silkworm feeding, diet assays were conducted. Food intake increased as isoquercetin concentrations increased and this increase was significant when diets were supplemented with $10^{-3}$ M isoquercetin (figure 5a). Thus, isoquercetin functioned as a feeding stimulant for larval silkworms.

To determine if BmGr63 was involved in isoquercetin recognition in silkworms, RNAi knock-down mediated by dsRNA was used. In no choice assays, when compared with GFP dsRNA-treated larvae, BmGr63 dsRNA-treated larvae showed a reduced diet intake containing isoquercetin (figure 5b). In dual choice assays, GFP dsRNA-treated larvae preferred diets supplemented with isoquercetin, while BmGr63 dsRNA-treated larvae showed no obvious preference for diets adding isoquercetin (figure 5c). BmGr63 expression levels in larval mouthparts were determined using qRT-PCR to verify RNAi knock-down effects. As shown in figure 5d,e, BmGr63 expression levels were down-regulated by 50–70% in BmGr63 dsRNA-treated larvae when compared with GFP dsRNA-treated larvae.

(g) The influence of BmGr63 on silkworm food intake

To determine whether BmGr63 affected food intake, feeding assays were performed. When compared with GFP dsRNA-treated larvae, BmGr63 knock-down significantly decreased food consumption in silkworm larvae (figure 6a). The qRT-PCR analysis confirmed that BmGr63 levels were reduced by approximately 60% in BmGr63 dsRNA-treated larvae (figure 6b).

(h) Isoquercetin content analysis in mulberry

Isoquercetin was detected in mulberry extracts using an isoquercetin standard as reference (electronic supplementary material, figure S2). The MS/MS spectra of the standard and isoquercetin
in mulberry extracts were identical. A regression line based on the isoquercetin standard peak area was obtained. The equation was $y = 7E + 08x - 1E + 09$, $R^2 = 0.9241$. Accordingly, the isoquercetin concentration in used mulberry extracts was $1.437 \times 10^{-3}M$.

According to the mulberry leaf metabolite database, isoquercetin content was determined in 91 mulberry resources, with differences identified across resources (electronic supplementary material, figure S3). Resources with higher isoquercetin content were mainly identified in cultivated resources except for ml053. By contrast, wild resources accounted for lower isoquercetin content (electronic supplementary material, table S5).

4. Discussion

Mechanisms used by silkworms to feed on mulberry leaves are relevant to relationships between other phytophagous insects and host plants. The mulberry-derived flavonoid glycoside, isoquercetin, was previously found to trigger test biting in silkworm larvae [33–35]. Larval maxillary palps can respond to very low concentrations of isoquercetin [37]. However, the molecular mechanisms underlying host plant recognition remain unknown. From a molecular perspective, contact chemosensation with phytochemicals and ligand binding occurs via Grs [9]. We confirmed that, at the molecular level, a Gr highly expressed in silkworm larval mouthparts, BmGr63, was tuned with the feeding stimulant isoquercetin in mulberry.

(a) The biological function of BmGr63 in food selection

When insects are close to food sources, their gustatory systems detect and evaluate non-volatile feeding stimulants or deterrents and are important in the final decision to accept the food [9,47]. Lepidoptera gustatory sensilla are mainly located on maxilla and epipharynx (inner side of labrum) [48–50]. Although important roles for labellum have been demonstrated in Drosophila, gustatory perception research in Lepidoptera labrum or epipharynx is lacking [12,51,52]. The sensilla located on maxilla have received more attention. The gustatory sensilla consists of medium and lateral sensillum styloconica in the maxillary pulps and galea and are important in larval food selection [11]. The medial styloconic sensilla of the maxillary galea respond to feeding deterrents or toxic compounds such as coumarin, caffeine and nicotine [53]. Chemosensory neurons in the maxillary palps are tuned with several mulberry compounds (including β-sitosterol, CGA and quercetin glycosides) and are important in host plant selection [37]. However, the underlying molecular mechanisms were unknown. BmGr66 was reported as a determining factor for silkworm feeding preference. And it had the highest expression levels, specifically in maxilla, among mouthpart tissues [41]. It was plausible that BmGr66 on the gustatory sensilla of the maxilla, was part of a feeding deterrent recognition system. Without BmGr66, silkworms demonstrated non-specific feeding behaviours but retained to feed on mulberry leaves. This observation suggested that other Grs recognized feeding stimulants in plants. BmGr63 is highly expressed in the maxilla palps [19]. Our qRT-PCR data indicated that BmGr63 was relatively highly expressed in labrum and maxilla. Besides it, BmGr63 was specific for the feeding stimulant isoquercetin and had a positive influence on silkworm feeding. Therefore, BmGr63 appears to promote silkworm feeding on mulberry leaves and is a critical gustatory factor in feeding mechanisms.

It was also observed that BmGr63 was highly expressed in chemosensory tissues in larvae and adults [19], which further suggested BmGr63 had broader biological functions. Apart from host plant recognition in larvae, BmGr63 may also be potentially involved in mating behaviours or oviposition.

(b) Isoquercetin, a flavonoid glycoside, functions as a silkworm feeding stimulant

Mulberry leaves are rich in diverse flavonoids, with flavonoid skeletons typically modified by sugars or other groups [54,55]. The most abundant mono- and di-O-glycosylated flavonoids in mulberry leaves are isoquercetin, astragalin and rutin [56]. Silkworms have adapted to these abundant flavonoid glycosides and use them for developmental purposes. For example, dietary flavonoid glycosides are metabolized in silkworms using quercetin 5-O-glucosyltransferase to
generate quercetin 5-O-glucosides, the major constituent of cocoon flavonoids with ultraviolet shielding properties [57]. This is direct evidence for silkworms using flavonoids derived from mulberry leaves. In our study, a mulberry-derived flavonoid glycoside was shown to function as a feeding stimulant that is detected by BmGr63.

Of the three flavonoid glycosides tested, isoquercetin induced the strongest inward current in oocytes expressing BmGr63, followed by astragalin. However, rutin induced little response. In structural terms, isoquercetin and astragalin are mono-O-glycosylated flavonoids and their glycosyl ligands are glucoses. Rutin is the product of flavonoid disaccharide modification, and its glycosyl ligand is rutinose. Thus, it appears that interactions between BmGr63 and flavonoid glycosides are based on ligand structures. The current induced by undiluted mulberry extracts was 14–20 nA, and that induced by \(10^{-3}\) M isoquercetin was 16–22 nA. The isoquercetin content in mulberry extracts was determined at \(1.437 \times 10^{-3}\) M using UPLC-MS/MS. Mulberry extracts comprise multiple compounds with complex inter-relationships. However, using standards was more direct and effective. Evaluated in this manner, it is hypothesized that isoquercetin is the primary factor inducing BmGr63 responses to mulberry extracts. However, the compound types used in our assays were limited and other potential BmGr63 ligands cannot be ruled out. As receptors other than BmGr63 were not tested in oocyte and RNAi experiments, it remains to be studied whether other receptors are involved in recognizing isoquercetin.

Figure 4. Inward currents recording of oocytes expressing BmGr63. The inward current (a) and response profiles (b) of oocytes expressing BmGr63 in response to mulberry extracts at different concentrations. (c) Response profiles of oocytes expressing BmGr63 in response to different ligands at \(10^{-3}\) M. (d) Inward current responses of oocytes expressing BmGr63 to different flavonoids. (e) Inward current responses of oocytes expressing BmGr63 to isoquercetin at a range of concentrations. (f) Response profiles of oocytes expressing BmGr63 in response to isoquercetin at a range of concentrations. Data are presented as the mean ± s.e.m. (n = 5). Different letters above bars represent significant differences (p < 0.05) as determined by the ANOVA one-way Duncan’s multiple range test.
Data are presented as the mean ± s.e.m. (adding 10−3 M isoquercetin added was provided). The artificial diets made by adding 10−3 M or without isoquercetin were provided in both dsGFP and ds63 groups. Data are presented as the mean ± s.e.m. (n = 12) and analysed for statistical significance using a paired samples t-test. (c) The relationship between phytochemicals and Grs, and associated behaviours.

The majority of Grs are classified into the ‘bitter receptor’ clade with unknown functions. Bitter receptors may recognize bitter compounds that are detrimental or toxic to insects and thus elicit avoidance behaviours. For example, Gr28b is necessary for D. melanogaster to avoid saponin. PxlGr34 responds to the plant hormones BL and EBL, which are repellent to P. xylostella. However, bitter receptors could also be activated by feeding or oviposition stimulants. PxutGr1 from P. xuthus is involved in the recognition of the oviposition stimulant, synephrine. Additionally, PrapGr28 responds to sinigrin, a potent stimulant of larval feeding and adult oviposition in P. rapae. In this study, we identified the specific interaction between BmGr63 and isoquercetin, a bitter silkworm Gr and a mulberry-derived feeding stimulant.

In the constructed phylogenetic tree, relatively few orthologous relationships were observed, taking BmGr63 as an example, and it was uncommonly orthologous to PrapGr28. The sequence alignment between BmGr63 and PrapGr28 was shown in electronic supplementary material, figure S4. PrapGr28 was abundantly expressed in larval mouthparts and tuned to sinigrin, a potent stimulant of larval feeding and adult oviposition. Sinigrin is a glucosinolate, an important secondary compound in Cruciferae and its glycosyl ligand is glucose [59,60]. From this perspective, the conserved functions of BmGr63 and PrapGr28 are related to the detection of glucose-ligand glycosides. The important roles of plant-derived glycosides in Lepidoptera feeding behaviours are implied.
The ability to recognize secondary metabolites is essential for phytophagous insects. Phytochemicals may not only act as deterrents but, depending on insect ability to circumvent their detrimental effects, they could also act as stimulants and host plant indicators [47]. For insects, the stimulants are recognized during contact chemosensation and received by Grs. Then the produced stimulant signals are transferred to the brain via stimulus neurons, the electrophysiological activity of which correlates quantitatively to the strength of the behavioural response [11,61].

This study showed that BmGr63 has the highest expression levels in larval mouthparts and is specific to the mulberry-derived feeding stimulant isoquercetin. This knowledge provides a molecular basis for silkworm mulberry feeding mechanisms. In future work, comprehensive studies about Grs in Lepidoptera should be made using the combination of electrophysiology, transgenic technologies and behavioural experiments. Also, the effects of phytochemicals on insects, including interactions and transport mechanisms need further elucidation. Our interaction data between BmGrs and mulberry-derived secondary metabolites should help facilitate understanding of the specific evolutionary relationships between phytophagous insects and their host plants.

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