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A gustatory receptor tuned to D-fructose in antennal sensilla chaetica of *Helicoverpa armigera*

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ABSTRACT

Insect gustatory systems play important roles in food selection and feeding behaviors. In spite of the enormous progress in understanding gustation in *Drosophila*, for other insects one of the key elements in gustatory signaling, the gustatory receptor (GR), is still elusive. In this study, we report that fructose elicits behavioral and physiological responses in *Helicoverpa armigera* (Harm) to fructose and identify the gustatory receptor for this sugar. Using the proboscis extension reflex (PER) assays we found that females respond to fructose following stimulation of the distal part of the antenna, where we have identified contact chemosensilla tuned to fructose in tip recording experiments. We isolated three full-length cDNAs encoding candidate HarmGRs based on comparison with orthologous GR sequences in *Heliothis virescens* and functionally characterized the responses of HarmGR4 to 15 chemicals when this receptor was expressed in *Xenopus* oocytes with two-electrode voltage-clamp recording. Among the tastants tested, the oocytes dose-dependently responded only to D-fructose (EC_{50} = 0.045 M). By combining behavioral, electrophysiological and molecular approaches, these results provide basic knowledge for further research on the molecular mechanisms of gustatory reception.

1. Introduction

In insect olfaction and taste play crucial roles in detecting and discriminating chemical compounds in potential foods. The remarkable sensitivity and selectivity of the chemosensory systems depend heavily on the function of the chemosensory neurons, which in turn depend ultimately on olfactory receptors (ORs) and gustatory receptors (GRs). The first insect ORs and GRs from the fruit fly, *Drosophila melanogaster* were identified using a bioinformatics-based approach in 1999 and in 2000, respectively (Clyne et al., 2000, 1999; Vosshall et al., 1999). The discovery of ORs ushers a spectacular expansion of our understanding of the molecular basis of insect olfaction (Leal, 2013). In comparison with olfaction, molecular profiles of gustatory sensory neurons (GSNs) turn out to be complex because gustatory receptor proteins may be expressed in low amounts in the taste organs, while the affinity to gustatory ligands may be too low for affinity-based isolations (Isono and Morita, 2010). In any case, to understand the mechanism of taste sensory systems in insect, it is necessary to clarify the function of the gustatory receptors.

Similar to the OR family, the GR family includes many related members, with numbers highly variable across species. Genome analysis has revealed the presence of 68, 10, 76, 96, 62, 69, and 3 GR genes in the fruit fly *D. melanogaster*, the honeybee *Apis mellifera*, the mosquito *Anopheles gambiae*, the Argentine ant *Linepithema humile*, the red flour beetle *Tribolium castaneum*, the silkworm *Bombyx mori*, and the tobacco budworm *Heliothis virescens*, respectively (Abdel-latief, 2007; Hill et al., 2002; Krieger et al., 2002; Robertson and Wanner, 2006; Robertson et al., 2003; Smith et al., 2011; Wanner and Robertson, 2008; Zhang et al., 2011a). In other insects, only few GRs have been so far identified, most by antennal transcriptome sequencing. Among the antennal transcriptome studies of several moth species, only a single putative GR has been reported in both the codling moth *Cydia pomonella*

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and the cotton bollworm *Helicoverpa armigera* (Bengtsson et al., 2012; Liu et al., 2012), while two have been described in the tobacco hornworm *Manduca sexta* and five in the cotton leafworm *Spodoptera littoralis* (Grosse-Wilde et al., 2011; Howlett et al., 2012; Jacquin-Joly et al., 2012; Legeai et al., 2011). Some GRs are involved in the detection of sweet and bitter chemicals, as well as CO_2 in the gas state in *D. melanogaster*. DmGR5a is expressed in most sugar-sensitive GSNs and is necessary for the response to trehalose (Chyb et al., 2003; Dahanukar et al., 2001; Ueno et al., 2001). DmGR64a is essential for the detection of some other sugars, such as glucose, sucrose, maltose, turanose, maitotriose, maltitol, palatinose, stachyose, raffinose and leucrose (Dahanukar et al., 2007; Jiao et al., 2007; Slone et al., 2007). DmGR64f as a common co-receptor interacting with DmGR5a or with DmGR64a, is broadly required for the detection of most sugars, such as trehalose, sucrose, glucose and maltose (Jiao et al., 2008). Instead, DmGR43a is a specific fructose receptor (Miyamoto et al., 2012; Sato et al., 2011). DmGR66a and DmGR93a are necessary for sensing caffeine (Lee et al., 2009; Moon et al., 2006), and DmGR33a is required for detecting a wide range of bitter tasting chemicals including quinine, lobeline, denatonium and caffeine (Miyamoto et al., 2013; Moon et al., 2009). In addition, DmGR8a and DmGR66a are involved in detecting the insecticide L-canavanine (Lee et al., 2012). Coexpression of DmGR21a and DmGR63a in larvae as well as in the adults confers a CO_2 sensitivity (Kwon et al., 2007). In *B. mori*, BmGR8 and BmGR9, when expressed in heterologous systems, respond specifically to inositol and fructose, respectively (Sato et al., 2011; Zhang et al., 2011a, 2011b). Additionally, in the swallowtail butterfly *Papilio xuthus*, PxutGR1 has been reported as a gustatory receptor responding specifically to an oviposition stimulant, synephrine (Ozaki et al., 2011). Although excellent progress has been made to understand the role of the insect GR family in taste perception, most work has been focused on the model organism *D. melanogaster*. Few are the studies on the molecular mechanisms of taste perception in other insect species, particularly agricultural pests.

The highly polyphagous Old World cotton bollworm *H. armigera* (Lepidoptera: Noctuidae) is one of the most devastating agricultural pests, causing damage to crops estimated at more than 2 billion US$ every year (Sharm, 2001). Recently, *H. armigera* has successfully established at least two populations in the New World (Tay et al., 2013). Feeding is important to increase the longevity and fecundity before mating and egg-laying in this species. The adult moths usually ingest sugars (mainly sucrose, fructose, and glucose) and various free amino acids present in the floral nectar during both day and night, particularly at dusk (Zalucki et al., 1986). The larvae feed on a large number of economically important crops like cotton, peanut, tomato, wheat, soybean, tobacco, sunflower, sorghum and maize (Ambika et al., 2005; Fitt, 1989; Rajapakse and Walter, 2007). When the larvae are fed on artificial diets rich in proteins and/or carbohydrates (legumes and cereals), they produce higher larval mass and develop more rapidly than larvae fed on diets with low protein and carbohydrate content (flowers and vegetables) (Sarate et al., 2012). Both adults and larvae use the sugars as important nutrients. The characterization of the sugar receptors in *H. armigera* may contribute to the understanding of the molecular mechanisms underlying feeding behavior and the regulation of food intake, and suggest novel strategies in agricultural pest control.

In the present study, as a first step to understanding the tasting role of antennae in *H. armigera*, we studied the behavioral responses of female adults to D-fructose, and then identified the contact chemosensilla responding to D-fructose using the tip-recording technique. We also cloned three gustatory receptor candidate genes. Using a *Xenopus laevis* oocyte expression system, we obtained functional evidence that HarmGR4 specifically recognizes D-fructose, and studied its expression by using RT-PCR and *in situ* hybridization.

2. Materials and methods

2.1. Insects

H. armigera were reared at 27 ± 1 °C with a photoperiod of 16 h: 8 h, L: D in the Institute of Zoology, Chinese Academy of Sciences, Beijing. Larvae were fed on an artificial diet, mainly constituted by wheat germ, yeast and tomato paste. Pupae were sexed and males and females were put into separate cages for eclosion. 10% honey solution was used as diet for adults except for the insects used for the behavioral and electrophysiological experiments. One-to-three-day-old virgin female adults were used in all the experiments.

2.2. Chemicals

Myo-inositol and D-glucose were from Serva, New York, NY, USA. Sucrose, D-fructose, D-maltose, D-galactose, D-cellobiose, D-arabinose, D-lactose, D-trehalose, D-meleztose, D-raffinoase, D-mannose, L-rhamnose, and L-sorbose were purchased from Sigma Chemical Company (St Louis, MO, USA). All the chemicals are of analytical grade (>99.5%).

2.3. Proboscis extension reflex in response to fructose

Virgin female adult moths were used for the behavioral assay. Each individual moth was harnessed in a small plastic tube from which its head protruded, and its antennae, mandibles and proboscis were permitted to move freely. They were fed with double distilled water and left for 12 h to adapt to their new environment. Each moth was stimulated to the distal part of antennae with only one of four fructose solutions at concentrations of 0.001, 0.01, 0.1, and 1 M, a typical range of sugar concentrations in insect taste studies. Water was used as the control and solvent. The proboscis extension reflex (PER) in response to a given solution was recorded within 3 s after the antenna contacted the solution, and the PER percentage was calculated. 32 individual moths originating from the same rearing batch and in similar physiological conditions (2 days after eclosion and satiated with double distilled water until the trial) were used for each set of tests. The experiments were repeated three times with groups of insects from three different rearing batches.

2.4. Electrophysiological responses of antennal contact chemosensilla to fructose

The tip-recording technique for insect contact chemosensilla, originally described by Hodgson et al. (1955) and modified as described by Van Loon (1990) was used for electrophysiological recordings. The head together with antennae was cut and an AgCl-coated silver wire was inserted into the neck opening and connected to a copper miniconnector, which served as the recording electrode. The distal part of antenna was fixed on a small platform with double sided adhesive tape with the most distal annulus protruding. A glass capillary filled with the stimulus solution into which an AgCl-coated silver wire was inserted acted as the indifferent electrode. The detailed procedure was similar to that used in our previous work (Zhang et al., 2010, 2011a, 2011b). The moths were naive to the sugars prior to testing, and only the right antenna was used for each moth. Fructose solutions were used to stimulate individual sensilla on the tip of antenna. To avoid possible adaptation of the tested sensilla, the interval between two successive

stimulations was at least 3 min. The tip diameter of the stimulating electrode was about 50 μm, suitable for recording from a single sensillum. Action potentials (spikes) generated during the first second after stimulus onset were amplified by an amplifier (Syntech Taste Probe DTP-1, Hilversum, The Netherlands) and filtered (A/D-interface, Syntech IDAC-4, Hilversum, The Netherlands). The electrophysiological signals were recorded and analyzed with the aid of SAPID Tools software version 16.0 (Smith et al., 1990) as well as the Autospike v.3.7 software (Syntech, Hilversum, The Netherlands). Female adult *H. armigera* were used 12–48 h old after eclosion and were provided with double distilled water until the experiment. Fructose was dissolved in 2 mM KCl solution in double distilled water. A 100 mM fructose solution was used to stimulate every sensillum on the tip surface of the right antenna. The sensilla, from which electrophysiological responses to 100 mM fructose were recorded, were subsequently stimulated with a series of ascending concentrations (0.1, 1, 10 and 100 mM) of fructose to explore dose–response characteristics. Five groups of ten moths, each eclosed from a different rearing batch were tested. A solution of 2 mM KCl served as the control. Both control and test solutions were stored at 4 °C.

2.5. Cloning of the candidate gustatory receptor of H. armigera

Based on the nucleotide sequences of GRs in *H. virescens*, specific primers were designed to amplify orthologous genes in *H. armigera*. Full-length sequences were obtained through 5′ and 3′ RACE PCR. Nested PCR was used to increase the specificity and sensitivity of RACE products. All primers were designed with Primer Premier 5.0 software, UPM and NUP were used as universal primer and nested universal primer, respectively. The primer sequences are reported in Supplementary Table 1.

Total RNA was isolated from antennae collected from 30 female and 30 male moths for each species, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RACE first-strand cDNA was synthesized with SMARTScribeTM reverse transcriptase (Clontech, Mountain View, CA, USA). The 5′-RACE cDNA reaction vessel contained 1–2 μg of total RNA, 1.2 μM of the 5′ oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′), 1.2 μM of oligo-dT18 primer and 1 μl of reverse transcriptase in a final volume of 10 μL. The 3′-RACE cDNA was synthesized using 1.2 μM of oligodT-adapter primer (5′-AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}$VN). The following cycling parameters were used: 42 °C for 60 min to produce cDNA and 70 °C for 15 min to terminate the reaction. 5′-RACE PCR reactions were performed in 2 × GC Buffer and LATaq polymerase (Takara shuzo, Shiga, Japan). The downstream regions to the 3'-end of most genes were amplified by LATaq polymerase and 10 × LA PCR Buffer (Takara shuzo). The PCR program included initial denaturation at 94 °C for 2 min, followed by 5 cycles of 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C; and a final extension step of 8 min at 72 °C.

2.6. Protein expression in Xenopus laevis oocytes and electrophysiological recordings

Full-length coding sequences of HarmGR cDNAs amplified from antenna by RT-PCR were first cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and then subcloned into pCS2+ vector. cRNAs were synthesized from linearized modified pCS2+ vectors with mMESSAGE mMACHINE SP6 (Ambion, Austin, TX, USA). Mature healthy oocytes were treated with 2 mg mL^{-1} of collagenase type IA (Sigma–Aldrich) in Ca^{2+}-free saline solution (82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/5 mM HEPES, pH 7.5) for 1–2 h at room temperature. Oocytes were later microinjected with 50 ng HarmGR cRNA. Injected oocytes were incubated for 3–7 days at 18 °C in bath solution (96 mM NaCl/2 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/5 mM HEPES, pH 7.5) supplemented with 100 μg/mL gentamycin and 550 μg/mL sodium pyruvate. Whole-cell currents were recorded with a two-electrode voltage clamp. Intracellular glass electrodes were filled with 3 M KCl and presented resistances of 0.2–2.0 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. Data acquisition and analysis were carried out with Digidata 1322A and pCLAMP software (Axon Instruments Inc., Foster City, CA, USA). Dose-response data were analyzed using GraphPad Prism 6.

2.7. Tissue-specificity of HarmGR4

To illustrate and compare the expression of HarmGR4 in different tissues and organs, semi-quantitative reverse transcription PCR was carried out. Labial palpi, male antennae, female antennae, male proboscis, female proboscis, forleg tarsi, and wings of female adults, and foreguts, midguts, hindguts, maxillae, fat body of the fifth instar larvae were separately collected from 2 to 150 individuals, depending on the size of the organ, and then stored at −80 °C. Frozen tissues were transferred to a liquid nitrogen cooled mortar and ground with a pestle. The homogenate was treated with 1 mL of TriZol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA extraction was performed following the manufacturer's instructions. Total RNA was dissolved in H_2O and its integrity was verified by gel electrophoresis. RNA quantity was determined on a Nanodrop ND-1000 spectrophotometer (Nano-Drop products, Wilmington, DE, USA).

Prior to cDNA synthesis, RNA was treated with DNase I (Fermentas, Vilnius, Lithuania) to remove traces of genomic DNA. The cDNA was synthesized by First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and used as a template in PCR reactions with gene-specific primers. An actin gene fragment was used as control. Primers were designed using the Primer Premier 6 software (PREMIER Biosoft International) and their sequences are reported in Supplementary material. PCR was performed under the following conditions: 94 °C for 3 min, 39 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The number of cycles was reduced to 25 for actin. The experiment was repeated three times using three independently isolated RNA samples. PCR amplification products were run on a 1.2% agarose gel and verified by DNA sequencing.

2.8. In situ hybridization

The digixigenin-labeled probe was synthesized from linearized pGEM-T vector (Promega, Madison, WI, USA) containing 1195 bp of gene fragment of *HarmGR4* using DIG RNA labeling Kit version 12 (SP6/T7) (Roche, Mannheim, Germany). The procedure of *in situ* hybridization has been explicitly reported previously (Krieger et al., 2002; Jiang et al., 2014). Briefly, fresh female antennae are embedded in frozen medium and cut into 12 μm sections. After air drying for 10 min, they are fixed in 4% paraformaldehyde and treated in the sequence with 1 × PBS, HCl solution, 1 × PBS plus 1% Triton X-100, 1 × PBS and 50% formamide in deionized/5 × SSC. Slides are incubated overnight at 55 °C in a humidified box with 100 μL hybridization buffer containing appropriate amounts of probes. After two washings with 0.1 × SSC twice for 30 min each and rinsing shortly in TBS, each slide is treated with 1 mL of blocking reagent and incubated at room temperature for 30 min. Afterward, each slide is incubated with 100 μL anti-digoxigenin alkaline phosphatase-conjugated antibody that is diluted 1:500 in blocking reagent for 1 h at 37 °C. Following three 5 min washes

with TBS/0.05% Tween and quick rinse in DAP buffer, each slide is treated with 100 μL of NBT/BCIP substrate solution and incubated for 3 h at 37 °C. Finally, pictures were taken on a Leica DM 2500 microscope and only adjusted for contrast and brightness using Adobe Photoshop CS3.

2.9. Data analysis

The percentage of PER were first arcsine root transformed. Electrophysiological responses were quantified by counting the number of action potentials in the first second after stimulus onset, using SAPID Tools software v. 16.0 (Smith et al., 1990). The data were analyzed by One-Way ANOVA, and the differences between means were compared with the Least-significant difference multiple range test at the $P = 0.05$ level of significance. Statistical analysis was done using SPSS 17.0 software.

3. Results

3.1. Behavior experiments – proboscis extension reflex in response to fructose

We first determined the behavioral significance of fructose to the adult female using the proboscis extension reflex (PER) test. Stimulating the distal part of antennae with D-fructose at concentrations as low as 0.01 M significantly elicited PER in adult females (Fig. 1). The response increased in a dose-dependent fashion when using the fructose concentration up to 1 M (Fig. 1). This experiment clearly indicates that female *H. armigera* is equipped with a sensitive detection system for fructose in the distal part of antennae.

3.2. Electrophysiological responses of antennal contact chemosensilla to fructose

On the most distal annulus of the flagellum in *H. armigera* antenna there are many sensilla trichodea, several sensilla chaetica and one sensillum styloconicum at the tip (Fig. 2A). Only some of the sensilla chaetica evoked electrophysiological responses to fructose (Fig. 2B), with a threshold at 0.001 M of the stimulus (Figs. 2C and 3) and the firing rate of the electrophysiological responses regularly increasing with fructose concentration (Figs. 2C and 3).

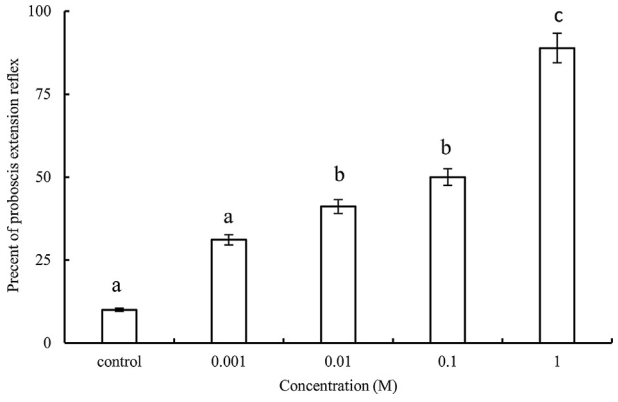

Fig. 1. Proboscis extension reflex (PER) in adult female *Helicoverpa armigera* upon antennae stimulation by 0, 0.001, 0.01, 0.1, 1 M solutions of fructose. The trial was repeated three times on three different batches of insects. Bars represent the standard errors of means (SEM). Same letters above bars represent non-significant difference between treatments, while different letters represent significant differences (LSD, ANOVA, $P < 0.05$).

3.3. Three candidate gustatory receptors identified from H. armigera

To understand the molecular mechanism of fructose detection in *H. armigera*, we then identified the candidate sugar receptors conferring sensitivity to sweet tastants. With specific primers designed from the consensus sequence of the tobacco budworm *H. virescens* gustatory receptors, HvGR1 (HR1), HvGR4 (HR4), and HvGR5 (HR5), we obtained three full-length cDNAs encoding gustatory receptors in *H. armigera*. According to the conventions of GR nomenclature and sequence of discovery, we named these gustatory receptor genes as HarmGR1, HarmGR4 and HarmGR5 (GenBank accession numbers JX982535, JX982536 and JX982537).

The full-length sequences of *HarmGR1*, *HarmGR4* and *HarmGR5* were 1466, 1659 and 1314 bp, containing open reading frames (ORFs) of 1359, 1425 and 1278 bp and encoding protein sequences of 453, 475 and 426 amino acid residues, respectively. HarmGR1 and HarmGR4, HarmGR1 and HarmGR5, and HarmGR4 and HarmGR5 shared only 23%, 26%, and 30% of identical amino acids in their sequences respectively. However, HarmGRs are very similar (more than 90% identical) to their orthologs of *H. virescens*. Notably, HarmGR4 shows 60% identity with the silkworm *B. mori* GR, BmGR9, which has been identified as the fructose receptor recently (Sato et al., 2011). Thus, we can reasonably assume that HarmGR4 is a sugar receptor in *H. armigera*.

3.4. Functional assay of HarmGR4 by using two-electrode voltage-clamp recording

We recorded electrophysiological responses of *Xenopus* oocytes expressing HarmGR4 to 15 sugars at a concentration of 0.050 M (Fig. 4A). HarmGR4 cells only showed a large response to D-fructose, a monosaccharide hexose, while the control cells without artificially expressed proteins showed no responses (data not shown). The D-fructose-induced current increased with fructose concentration from 0.005 M to 0.300 M (Fig. 4B). On the basis of the dose–response curve, the evaluated EC_{50} value of D-fructose is 0.045 M (n = 5) for HarmGR4 (Fig. 4C). Both the EC_{50} value and the threshold are close to those measured for D-fructose and receptor BmGR9 in *B. mori* (0.056 M, 0.003 M) (Sato et al., 2011), and in agreement with the sensitivity of the sensilla responding to fructose on the most distal annulus of the flagellum in *H. armigera* antennae (Fig. 3).

3.5. Tissue-specific expression of HarmGR4

The expression of *HarmGR4* in different tissues and organs was examined by RT-PCR. The results show that all PCR bands obtained were of the expected size, based on the primer design (Fig. 5). The transcript of HarmGR4 was predominantly expressed in the antennae of female moths, and foregut of the larvae. A weak band also appeared in labial palpi, male proboscis, female proboscis, male antennae, proleg tarsi and wings, as well as in maxilla and hindgut of larvae. We could not detect any expression in the midgut and in the fat body of larvae. Interestingly, the expression level in the antennae and proboscis of female adults was remarkably higher than that of male adults.

3.6. In situ hybridization

To validate the expression of HarmGR4 in the distal part of female antennae, *in situ* hybridization was performed. We indeed detected the gustatory cells labeled by HarmGR4 digoxigenin-

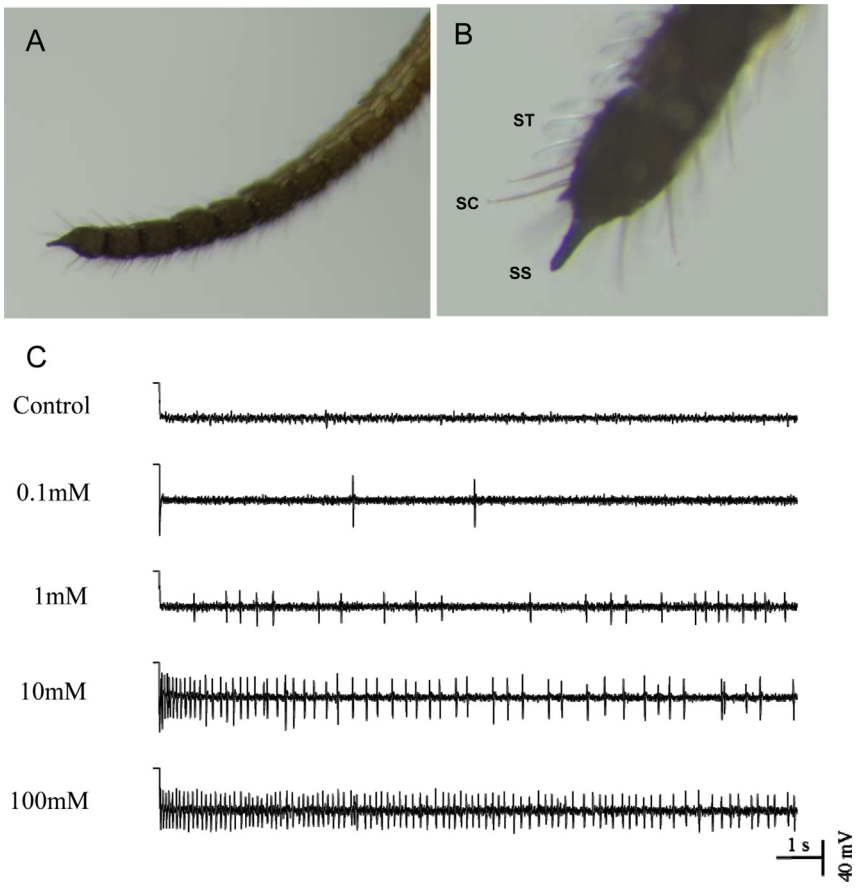

Fig. 2. Chemoreceptive sensilla on the distal part of antennae of adult female *H. armigera* and exemplary recordings of electrophysiological activity in the sensillum chaeticum. (A) The distal part of antennae. (B) The most distal annulus with several sensilla chaetica. SC: sensillum chaeticum; ST: sensillum trichodeum; SS: sensillum styloconicum. (C) Firing patterns to a series of fructose concentrations of the same sensillum chaeticum in a female adult.

labeled probes in the flagellum of female antennae (Fig. 6). HarmGR4 was virtually present in the gustatory sensory neuron of the sensilla chaetica responsive to fructose in the distal part of female antennae.

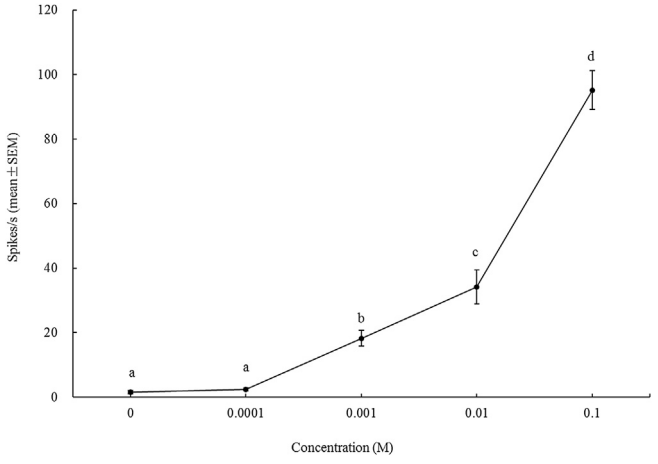

Fig. 3. Dose–response curves of electrophysiological activity in the sensillum chaeticum on the most distal annulus of adult female *H. armigera* in response to fructose at four concentrations. N = 5 for each data point. KCl at 2 mM was used as the control. Bars represent SEM. Same letters above bars represent non-significant difference between treatments, while different letters represent significant differences (LSD, ANOVA, $P < 0.05$).

4. Discussion

Detecting the sugars naturally occurring in floral nectar is crucial for lepidopteran adults, especially in females to ensure their reproductive success. The total concentration of sugars in plant nectar varies from about 118 mg mL^{-1} to 723 mg mL^{-1} of nectar, which corresponds to 0.35–2.11 M in sucrose equivalents, 0.65–4.01 M in fructose equivalents (Gottsberger et al., 1984). In adult female *H. armigera*, D-fructose stimulation of contact chemosensilla on the tarsi can elicit the proboscis extension reflex (PER) (Zhang et al., 2010). The moth of *H. armigera*, like many other insect species, moves the antennae actively not only to smell odorants, but also to detect sugar. Our behavioral assays showed that D-fructose induced the extension of the proboscis after the female antennae contacted the solution. Using tip recording technique, we further confirmed that some sensilla chaetica in the flagellum were sensitive to D-fructose. The firing rates of the sensilla were positively correlated with the PER of *H. armigera* to a series of fructose concentrations, but the electrophysiological response was much more sensitive than the behavioral response. Electrophysiological recordings from sensilla chaetica on the antennae of *H. virescens* showed responses of one mechanosensory and of several gustatory sensory neurons sensitive to sucrose and sinigrin (Jorgensen et al., 2006). Sensilla chaetica on the antenna in *S. littoralis* showed responses to fructose, and the sensilla of females responded with a higher average rate of spiking than those of males (Popescu et al., 2013). Gustatory receptors, which are expressed in the sensilla, can

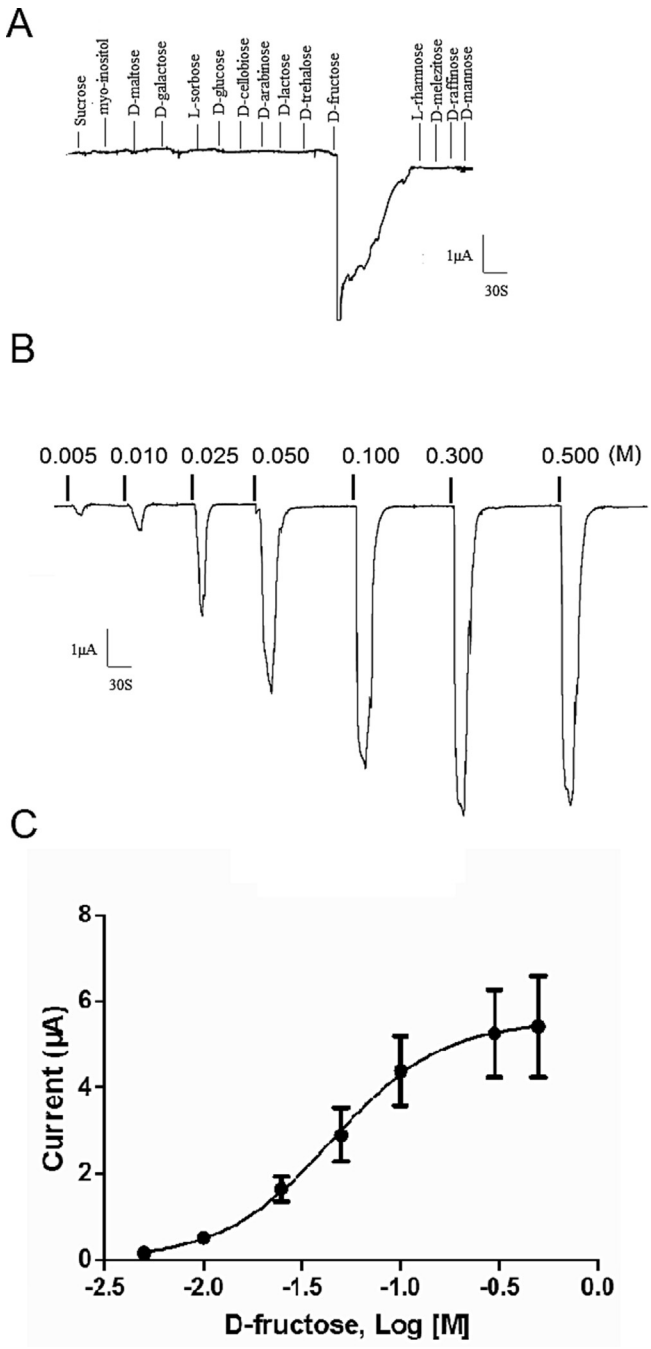

Fig. 4. Two-electrode voltage-clamp recordings of *Xenopus* oocytes expressing *HarmGR4* isolated in the present study. (A) Inward current responses of the oocytes expressing *HarmGR4* in response to 0.050 M solution of the 15 sugars. (B) The oocytes expressing *HarmGR4* stimulated with a range of D-fructose concentrations. (C) Dose-response curve of the oocytes expressing *HarmGR4* to D-fructose. EC_{50} = 0.045 M. Bars indicate SEM (n = 5).

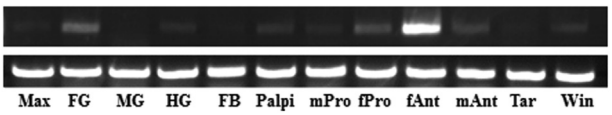

Fig. 5. RT-PCR analysis of *HarmGR4* expression in adult and larval tissues of *Helicoverpa armigera*. Max: maxilla, FG: foregut, MG: midgut, HG, hindgut, FB: fatbody, all from the fifth instar larva; Palpi: labial papli, mPro: male proboscis, fPro: female proboscis, fAnt: female antennae, mAnt: male antennae, Tar: tarsi, Win, wings, all from the female adult. The actin gene was used as a control to qualify and quantify cDNA samples.

participate directly in detection of sweet tastants (Freeman et al., 2014). Furthermore, we identified the full-length sequence of fructose receptor HarmGR4 in *H. armigera* by RACE PCR using the specific primers designed on the corresponding GR of *H. virescens*. Xu et al. also cloned a likely same gustatory receptor gene in *H. armigera*, called *HaGR9* (GenBank Accession number JX970522), but the coding sequence seems partial, only containing an ORF of 1398 bp, lacking the 5′ untranslated region (UTR), the 3′UTR and 27 bp nucleotides after start codon (Xu et al., 2012). *HarmGR4* consists of 108 bp 5′ UTR, 1425 bp ORF, and 126 bp 3′UTR. In addition, the GC content of HarmGR4-ORF is quite high (up to 54.9%), which may make isolation of its full-length coding sequence more difficult.

In the *Xenopus*-based functional studies, HarmGR4 cells showed a specific response to D-fructose and no response to 14 other sweet tastants, including D-galactose and D-maltose, indicating that HarmGR4 is the receptor protein for D-fructose. Using the same heterologous expression system, BmGR9 showed a response only to D-fructose but not to other chemicals, and was identified as fructose receptor in *B. mori* (Sato et al., 2011). However, in another *in vitro* study using Sf9 cells with calcium imaging, Xu et al. functionally characterized the responses of HaGR9 (HarmGR4 lacking the N-terminal sequence) to 7 chemicals and found that it showed dose-dependent responses not only to D-fructose, but also to D-galactose and D-maltose (Xu et al., 2012). One possible explanation is that the incomplete protein may fail to fold into its normal conformation, leading to reduced specificity and detection of also galactose and maltose. Alternatively, we can not exclude the possibility that HarmGR4 exhibits different ligand response properties in different expression systems.

Apart from the female antennae, HarmGR4 is highly expressed in the foregut, suggesting its involvement in the detection and regulation of nutrients during digestion. HarmGR4 is also expressed in the other tissues and organs of *H. armigera*, such as proleg tarsi, on which some contact chemosensilla are also sensitive to fructose (Zhang et al., 2010). Interestingly, three recent reports have provided evidence that GR43a, a HarmGR4 ortholog in *Drosophila*, not only detects fructose in the chemoreception structures, but is also used as a sensor of internal nutrients in other organs (Mishra et al., 2013; Miyamoto and Amrein, 2014; Miyamoto et al., 2012). In the adult female fly, the expression of GR43a in taste neurons is observed in the legs, labial palpi, labral and dorsal cibarial sense organs (Miyamoto and Amrein, 2014; Miyamoto et al., 2012). However, GR43a is also expressed in neurons of the proventricular ganglion, the brain and the uterus (Miyamoto and Amrein, 2014; Miyamoto et al., 2012). In *Drosophila* larvae, GR43a is the main sugar receptor, and is expressed in the taste organs as well as in the brain and the gastrointestinal system (Mishra et al., 2013). Remarkably, GR43a-expressing brain neurons are necessary and sufficient to sense hemolymph fructose and promote feeding in starved flies, but suppress feeding in satiated flies (Miyamoto et al., 2012). Although the function of fructose receptor in insect species has not been well characterized, the fact that GR43a is expressed in at least six organ systems and functions as a fructose receptor in the brain, indicates that this receptor may play diverse roles in insects.

In conclusion, we have provided behavioral and electrophysiological evidence that female antennae respond to fructose and identified the fructose receptor, HarmGR4, from *H. armigera*. Moreover, the expression of HarmGR4 also in larval foregut suggests that HarmGR4 may be also involved in the control of sugar consumption. In view of the wide distribution of HarmGR4 orthologs across various insect species, we think that the physiological function of fructose receptor is very important (Xu et al., 2012).

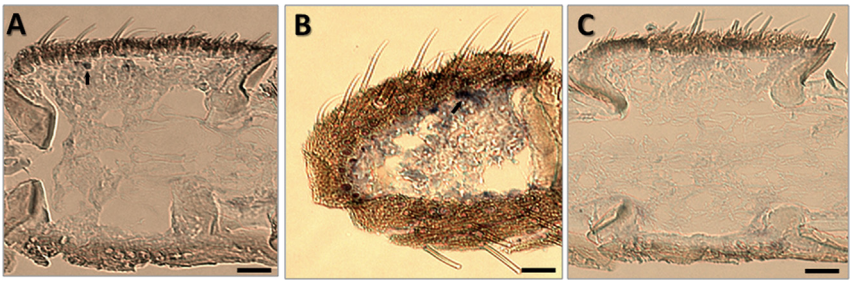

Fig. 6. Localization of HarmGR4 in the distal part of female antennae of *H. armigera*. DIG-labeled antisense RNA probes for HarmGR4 were hybridized to cryosections (A and B) of female antennae of *H. armigera*. No cells were labeled when sense riboprobes were applied on the control (C). Bold arrows point to the cell bodies stained by antisense riboprobes. Scale bar represents 20 μm.

Further study will be necessary to elucidate the role of HarmGR4 in the foregut and other related tissues and organs of larvae.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.03.002.

References

Abdel-latief, M., 2007. A family of chemoreceptors in *Tribolium castaneum* (Tenebrionidae: Coleoptera). PLoS One 2 (12), 31319.
Ambika, T., Sheshshayee, M.S., Viraktamathi, C.A., Udayakumar, M., 2005. Identifying the dietary source of polyphagous *Helicoverpa armigera* (Hübner) using carbon isotope signatures. Curr. Sci. 89 (12), 1982–1984.
Bengtsson, J.M., Trona, F., Montagne, N., Anfora, G., Ignell, R., Witzgall, P., Jacquin-Joly, E., 2012. Putative chemosensory receptors of the codling moth, *Cydia pomonella*, identified by antennal transcriptome analysis. PLoS One 7 (2), e31620.
Chyb, S., Dahanukar, A., Wickens, A., Carlson, J.R., 2003. Drosophila Gr5a encodes a taste receptor tuned to trehalose. Proc. Natl. Acad. Sci. U. S. A. 100 (Suppl. 2), 14526–14530.
Clyne, P.J., Warr, C.G., Carlson, J.R., 2000. Candidate taste receptors in *Drosophila*. Science 287 (5459), 1830–1834.
Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., Carlson, J.R., 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron 22, 327–338.
Dahanukar, A., Foster, K., van der Goes van Naters, W.M., Carlson, J.R., 2001. A Gr receptor is required for response to the sugar trehalose in taste neurons of *Drosophila*. Nat. Neurosci. 4, 1182–1186.
Dahanukar, A., Lei, Y.T., Kwon, J.Y., Carlson, J.R., 2007. Two Gr genes underlie sugar reception in *Drosophila*. Neuron 56 (3), 503–516.
Fitt, G.P., 1989. The ecology of *Heliothis* species in relation to agroecosystems. Annu. Revi. Entomol. 34 (1), 17–53.
Freeman, E.G., W, Z., D, A., 2014. Detection of sweet tastants by a conserved group of insect gustatory receptors. Proc. Nat.l Acad. Sci. U. S. A. 111 (4), 1598–1603.
Gottsberger, G., Schrauwen, J., Linskens, H., 1984. Amino acids and sugars in nectar, and their putative evolutionary significance. Plant Syst. Evol. 145, 55–57.
Grosse-Wilde, E., Kuebler, L.S., Bucks, S., Vogel, H., Wicher, D., Hansson, B.S., 2011. Antennal transcriptome of *Manduca sexta*. Proc. Nat.l Acad. Sci. U. S. A. 108 (18), 7449–7454.
Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A., Cravchik, A., Collins, F.H., Robertson, H.M., Zwiebel, L.J., 2002. G proten-coupled receptors in *Anopheles gambiae*. Science 298 (5591), 176–178.
Hodgson, E.S., Lettvin, J.Y., Roeder, K.D., 1955. Physiology of a primary receptor unit. Science 122, 417–418.
Howlett, N., Dauber, K.L., Shukla, A., Morton, B., Glendinning, J.I., Brent, E., Gleason, C., Islam, F., Izquierdo, D., Sanghavi, S., Afroz, A., Aslam, A., Barbaro, M., Blutstein, R., Borovka, M., Desire, B., Elikhis, A., Fan, Q., Hoffman, K., Huang, A., Keefe, D., Lopatin, S., Miller, S., Patel, P., Rizzini, D., Robinson, A., Rokins, K., Turlik, A., Mansfield, J.H., 2012. Identification of chemosensory receptor genes in *Manduca sexta* and knockdown by RNA interference. BMC Genomics 13, 211–220.
Isono, K., Morita, H., 2010. Molecular and cellular designs of insect taste receptor system. Front. Cell. Neurosci. 4, 20.
Jacquin-Joly, E., Legeai, F., Montagné, N., Monsempes, C., François, M.C., Poulain, J., Gavory, F., Walker, W.B., Hansson, B.S., Larsson, M.C., 2012. Candidate chemosensory genes in female antennae of the noctuid moth *Spodoptera littoralis*. Int. J. Biol. Sci. 8, 1036–1050.
Jiang, X.J., Guo, H., Di, C., Yu, S., Zhu, L., Huang, L.Q., Wang, C.Z., 2014. Sequence similarity and functional comparisons of pheromone receptor orthologs in two closely related *Helicoverpa* species. Insect Biochem. Mol. Biol. 48, 63–74.
Jiao, Y., Moon, S.J., Montell, C., 2007. A *Drosophila* gustatory receptor required for the responses to sucrose, glucose, and maltose identified by mRNA tagging. Proc. Natl. Acad. Sci. U. S. A. 104 (35), 14110–14115.
Jiao, Y., Moon, S.J., Wang, X., Ren, Q., Montell, C., 2008. Gr64f is required in combination with other gustatory receptors for sugar detection in *Drosophila*. Curr. Biol. 18 (22), 1797–1801.
Jorgensen, K., Kvello, P., Almaas, T.J., Mustaparta, H., 2006. Two closely located areas in the suboesophageal ganglion and the tritocerebrum receive projections of gustatory receptor neurons located on the antennae and the proboscis in the moth *Heliothis virescens*. J. Comp. Neurol. 496, 121–134.
Krieger, J., Raming, K., Dewer, Y., Bette, S., Conzelmann, S., Breer, H., 2002. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. Eur. J. Neurosci. 16, 619–628.
Kwon, J.Y., Dahanukar, A., Weiss, L.A., Carlson, J.R., 2007. The molecular basis of CO_2 reception in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 104 (9), 3574–3578.
Leal, W.S., 2013. Odorant reception in insects: roles of receptors, binding protein, and degrading enzymes. Annu. Rev. Entomol. 58, 373–391.
Lee, Y., Kang, M.J., Shim, J., Cheong, C.U., Moon, S.J., Montell, C., 2012. Gustatory receptors required for avoiding the insecticide L-canavanine. J. Neurosci. 32 (4), 1429–1435.
Lee, Y., Moon, S.J., Montell, C., 2009. Multiple gustatory receptors required for the caffeine response in *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 106 (11), 4495–4500.
Legeai, F., Malpel, S., Montagne, N., Monsempes, C., Cousserans, F., Merlin, C., Francois, M.C., Maibeche-Coisne, M., Gavory, F., Poulain, J., Jacquin-Joly, E., 2011. An Expressed Sequence Tag collection from the male antennae of the Noctuid moth *Spodoptera littoralis*: a resource for olfactory and pheromone detection research. BMC Genomics 12, 86.
Liu, Y., Gu, S., Zhang, Y., Guo, Y., Wang, G., 2012. Candidate olfaction genes identified within the *Helicoverpa armigera* antennal transcriptome. PLoS One 7 (10), e48260.
Mishra, D., Miyamoto, T., Rezenom, Y.H., Broussard, A., Yavuz, A., Slone, J., Russell, D.H., Amrein, H., 2013. The molecular basis of sugar sensing in *Drosophila* larvae. Curr. Biol. 23 (15), 1466–1471.
Miyamoto, T., Amrein, H., 2014. Diverse roles for the *Drosophila* fructose sensor Gr43a. Fly(Austin) 8 (1), 19–25.
Miyamoto, T., Chen, Y., Slone, J., Amrein, H., 2013. Identification of a *Drosophila* glucose receptor using Ca^{2+} imaging of single chemosensory neurons. PLoS One 8 (2), e56304.
Miyamoto, T., Slone, J., Song, X., Amrein, H., 2012. A fructose receptor functions as a nutrient sensor in the *Drosophila* brain. Cell 151 (5), 1113–1125.
Moon, S.J., KÖttgen, M., Jiao, Y., Xu, H., Montell, C., 2006. A taste receptor required for the caffeine response in vivo. Curr. Biol. 16 (18), 1812–1817.
Moon, S.J., Lee, Y., Jiao, Y., Montell, C., 2009. A *Drosophila* gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. Curr. Biol. 19 (19), 1623–1627.
Ozaki, K., Ryuda, M., Yamada, A., Utoguchi, A., Ishimoto, H., Calas, D., Marion-Poll, F., Tanimura, T., Yoshikawa, H., 2011. A gustatory receptor involved in host plant recognition for oviposition of a swallowtail butterfly. Nat. Commun. 2, 542.
Popescu, A., Couton, L., Almaas, T.J., Rospars, J.P., Wright, G.A., Marion-Poll, F., Anton, S., 2013. Function and central projections of gustatory receptor neurons on the antenna of the noctuid moth *Spodoptera littoralis*. J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol. 199 (5), 403–416.

Rajapakse, C.N.K., Walter, G.H., 2007. Polyphagy and primary host plants: oviposition preference versus larval performance in the lepidopteran pest *Helicoverpa armigera*. Arhr. Plan. Interact. 1, 17–26.

Robertson, H.M., Wanner, K.W., 2006. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. Genome Res. 16 (11), 1395–1403.

Robertson, H.M., Warr, C.G., Carlson, J.R., 2003. Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. 100 (Suppl. 2), 14537–14542.

Sarate, P.J., Tamhane, V.A., Kotkar, H.M., Ratnakaran, N., Susan, N., Gupta, V.S., Giri, A.P., 2012. Developmental and digestive flexibilities in the midgut of a polyphagous pest, the cotton bollworm, *Helicoverpa armigera*. J. Insect Sci. 12, 42.

Sato, K., Tanaka, K., Touhara, K., 2011. Sugar-regulated cation channel formed by an insect gustatory receptor. Proc. Natl. Acad. Sci. U. S. A. 108 (28), 11680–11685.

Sharm, H.C., 2001. Cotton Bollworm/legume Pod Borer, *Helicoverpa Armige*a (Hubner) (Noctuidae: Lepidoptera): Biology and Management. Crop Protection Compendium. Commonwealth Agricultral Bureau International Oxon, U. K, p. 77.

Slone, J., Daniels, J., Amrein, H., 2007. Sugar receptors in *Drosophila*. Curr. Biol. 17 (20), 1809–1816.

Smith, C.D., Zimin, A., Holt, C., Abouheif, E., Benton, R., Cash, E., Croset, V., Currie, C.R., Elhaik, E., Elsik, C.G., Fave, M.J., Fernandes, V., Gadau, J., Gibson, J.D., Graur, D., Grubbs, K.J., Hagen, D.E., Helmkampf, M., Holley, J.A., Hu, H., Viniegra, A.S., Johnson, B.R., Johnson, R.M., Khila, A., Kim, J.W., Laird, J., Mathis, K.A., Moeller, J.A., Munoz-Torres, M.C., Murphy, M.C., Nakamura, R., Nigam, S., Overson, R.P., Placek, J.E., Rajakumar, R., Reese, J.T., Robertson, H.M., Smith, C.R., Suarez, A.V., Suen, G., Suhr, E.L., Tao, S., Torres, C.W., van Wilgenburg, E., Viljakainen, L., Walden, K.K., Wild, A.L., Yandell, M., Yorke, J.A., Tsutsui, N.D., 2011. Draft genome of the globally widespread and invasive Argentine ant (*Linepithema humile*). Proc. Natl. Acad. Sci. U. S. A. 108 (14), 5673–5678.

Smith, J.J.B., Mitchell, B.K., Rolseth, B.M., Whitehead, A.T., 1990. SAPID tools: microcomputer programs for analysis of multiunit nerve recordings (Version 3.5: spike analysis programs for insect data). Chem. Senses 15, 253–270.

Tay, W.T., Soria, M.F., Walsh, T., Thomazoni, D., Silvie, P., Behere, G.T., Anderson, C., Downes, S., 2013. A brave new world for an old world pest: *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Brazil. PLoS One 8 (11), e80134.

Ueno, K., Ohta, M., Morita, H., Mikuni, Y., Nakajima, S., Yamamoto, K., Isono, K., 2001. Trehalose sensitivity in *Drosophila* correlates with mutations in an expression of the gustatory receptor gene Gr5a. Curr. Biol. 11 (18), 1451–1455.

Van Loon, J.J.A., 1990. Chemoreception of phenolic acids and flavonoids in larvae of two species of *Pieris*. J. Comp. Physiol. A 166, 889–899.

Vosshall, L.B., Amrein, H., Morozov, P., Rzhetsky, A., Axel, R., 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. cell 96, 725–736.

Wanner, K.W., Robertson, H.M., 2008. The gustatory receptor family in the silkworm moth *Bombyx mori* is characterized by a large expansion of a single lineage of putative bitter receptors. Insect Mol. Biol. 17 (6), 621–629.

Xu, W., Zhang, H.J., Anderson, A., 2012. A sugar gustatory receptor identified from the foregut of cotton bollworm *Helicoverpa armigera*. J. Chem. Ecol. 38 (12), 1513–1520.

Zalucki, M.P., Dagllsh, G., Firempong, S., Twine, P.H., 1986. The biology and ecology of *Helicoverpa armigera* (Hübner) and *H. punctigera* Wallengren (Lepidoptera: Noctuidae) in Australia: what do we know? Aust. J. Zool. 34, 779–814.

Zhang, H.J., Anderson, A.R., Trowell, S.C., Luo, A.R., Xiang, Z.H., Xia, Q.Y., 2011a. Topological and functional characterization of an insect gustatory receptor. PLoS One 6 (8), e24111.

Zhang, Y.F., Huang, L.Q., Ge, F., Wang, C.Z., 2011b. Tarsal taste neurons of *Helicoverpa assulta* (Guenee) respond to sugars and amino acids, suggesting a role in feeding and oviposition. J. Insect Physiol. 57, 1332–1340.

Zhang, Y.F., van Loon, J.J.A., Wang, C.Z., 2010. Tarsal taste neuron activity and proboscis extension reflex in response to sugars and amino acids in *Helicoverpa armigera* (Hübner). J. Exp. Biol. 213 (Pt 16), 2889–2895.