1 **Characterization of gustatory receptor 7 in the brown planthopper reveals functional** 2 **versatility**

3 Running title: *NI*Gr7 reveals functional versatility

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14 **Abstract**

15 Insect pests consume tastants as their necessary energy and nutrient sources. Gustatory
16 receptors play important roles in insect life and can form within an extremely complicated
17 regulatory network. However, there are still many gustatory genes that have a significant
18 impact on insect physiology, but their functional mechanism is still unknown. Here, we
19 purified and characterized a gustatory receptor (protein) coding gene, *NI*Gr7, from the brown
20 planthopper (BPH) *Nilaparvata lugens*, which is an important insect pest of rice. Our results
21 revealed that *NI*Gr7 has an active association with various ligands, such as lectins, lipids
22 (phospho- and sphingolipid) and copper. The mass-spectrometry result showed that *NI*Gr7 is
23 a sugar receptor, and *NI*Gr7 is validated by different types of insoluble polysaccharides and a
24 varied range of tastants. Furthermore, we observed that *NI*Gr7-bound ATP hydrolysed on the
25 ATPase activity assay, which indicated that *NI*Gr7 may be associated with important
26 biological functions in the BPH. The important *NI*Gr7 for chemoreception has now been
27 characterized in the BPH. We showed that *NI*Gr7 in the BPH is required for various protein-
28 ligands, as well as protein-sugars interactions, to play crucial roles in this pest. This study
29 will provide valuable information for further functional studies of chemoreception
30 mechanisms in this important agricultural pest.

31 **Keywords:** Sugar gustatory receptor, *Nilaparvata lugens*, Protein purification, Protein-
32 ligands binding, ATP hydrolysis.

33 Introduction

34 Arthropods' gustatory receptors (Grs) are active in leading insect feeding behaviours and in
 35 establishing the platform between insect gustation and the environment. The Grs are closely
 36 associated with insect olfactory receptors (Ors) (**Robertson et al., 2003**). Grs have been
 37 investigated as divergent domains of seven-TM (transmembrane) spanning proteins, which is
 38 a typical example of G protein-coupled receptors (GPCRs), but they are noticeably diverse
 39 and impart no sequence resemblance with Grs of mammals (**Clyne et al., 2000**). In addition,
 40 the topology of Gr' seven-TM helices are reversed in comparison to that of the GPCRs
 41 (**Zhang et al., 2011**). Although having well-established information on olfaction (**Benton et**
 42 **al., 2006; Robertson et al., 2006**) and increasing documentation of insect Grs, little known
 43 about the physiological mechanisms that include understanding of the gustation system.

44 The *Gr* genes were first established in *Drosophila* (**Clyne et al., 2000**), which is a
 45 polyphagous dipteran insect, and forty-three putative *Gr* genes have been revealed in this
 46 insect (**Amrein and Throne, 2005**). Grs have been documented into various clades and are
 47 named as sugar (**Slone et al., 2007**), bitter (**Wanner and Robertson, 2008; Lee et al., 2009**),
 48 carbon dioxide (CO₂) (**Xu and Anderson, 2015**), GR43a-like (**Kui et al., 2018**), sex
 49 attractant (**Shankar et al., 2015**), and unknown (**Kui et al., 2018**) receptors, based on their
 50 sequence homologies with receptors that have been discovered in *Drosophila* (**Robertson et**
 51 **al., 2003**) or on an important molecule to which they reacted (**Kui et al., 2018**). Seventy-six
 52 and 79 *Gr* genes have been respectively identified in *Anopheles gambiae* (**Hill et al., 2002**)
 53 and *Aedes aegypti* (**Kent et al., 2008**), which is a hematophagous dipteran insect. Sixty-five
 54 and 197 *Gr* genes have been noted in the lepidopteran insects *Bombyx mori* (**Wanner and**
 55 **Robertson, 2008**) and *Helicoverpa armigera* (**Xu et al., 2016**), respectively. The highest
 56 number of *Gr* genes, 220, has been observed in *Tribolium castaneum*, which is a Coleopteran
 57 insect (**Richards et al., 2008**). Currently, the majority of research consideration has been
 58 focused on the *Gr* genes of *Drosophila* (**Slone et al., 2007; Dunipace et al., 2001; Gardiner**
 59 **et al., 2008; Weiss et al., 2011; Dahanukar et al., 2001; Dahanukar et al., 2007**); however,
 60 with fast-growing genome examinations on an additional species of insects (**Robertson and**
 61 **Wanner, 2006; Wanner and Robertson, 2008; Richards et al., 2008; Smadja et al., 2009;**
 62 **Xue et al., 2014**), the investigation is approaching a different spectrum of species. However,
 63 there is a need to shed light on the *Gr* genes in an important agricultural insect, such as the
 64 brown planthopper. The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera:
 65 Delphacidae), is one of the most serious agricultural planthopper species and feeds on rice

66 plants (Ojha and Zhang, 2019). Recently, thirty-two putative *Gr* genes in this insect have
67 been reported from our research group (Kui et al., 2018). However, the gustatory perception
68 of these *Gr* genes, or the protein macromolecular platform, in the physiology of this insect
69 has remained largely unknown.

70 In the present investigation, the functional properties of BPH Gr7 (*N*Gr7, protein) have been
71 revealed after homologous expression in *E. coli*. Purified *N*Gr7 was examined for its
72 association with lectins, lipids, polysaccharides, metals, and ATPase activity assays. These
73 results are essential for understanding the molecular level of taste regulation within the BPH
74 feeding behaviour and improve our understanding of the insect sugar-receptor family, insect-
75 plant interaction and adaptation.

76 **Materials and Methods**

77 **Insect sample**

78 The BPH strain was maintained and reared on susceptible rice (Huang Hua Zhan) plants as
79 described by Kui et al (2018).

80 **Bioinformatics**

81 The *N*Gr7 (protein) sequence was examined for different parameters, including the
82 molecular weight, theoretical pI, amino acid composition, atomic composition, extinction
83 coefficient, estimated half-life, instability index, aliphatic index and grand average of
84 hydropathicity, by using an ExPASy ProtParam tool (<https://web.expasy.org/protparam/>).
85 The TOPCONS tool was used to predict a secretory signal sequence and the transmembrane
86 helix in the *N*Gr7 (<http://topcons.cbr.su.se/>). Furthermore, the *N*Gr7 sequence was searched
87 with other known annotated insect Grs for sequence identity and to build a phylogenetic tree
88 using protein BLAST tools from the NCBI weblink
89 (<https://www.ncbi.nlm.nih.gov/blast/treeview/treeView>).

90 **Cloning, expression, purification and biotin labelling of *N*Gr7**

91 The total RNA of the female adult BPH was isolated by using a TRIzol reagent (Invitrogen,
92 Carlsbad, CA), and one µg RNA was converted to cDNA by using the PrimeScript™ RT-
93 PCR Kit (Takara, Japan) following the manufacturer's instructions. Then, 10 ng cDNA was
94 used as a template for PCR to amplify *N*Gr7 using the forward primer 5'-CATATC-
95 ATGTTGTTACTGACAATTGTTTTGATT-3' containing an *Nde*I sequence and the reverse
96 primer 5'-GAATTC-TCACTGTGTTCTGCGCGTTT-3' containing an *Eco*RI sequence. A

PCR was carried out in 25 µl containing 200 µM dNTPs, 5 U *Taq* DNA polymerase (New England Biolabs, NEB, Inc, USA) and 13 µM of each of the primers. The PCR conditions were 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 5 min. Amplicon (~730 bp) was analysed on 1% (w/v) agarose gel, was gel-purified using HiPure Gel Pure DNA Micro kit (Magen, China) following the manufacturer's instructions and was quantified using the NanoVue Plus spectrophotometer. The PCR product was digested with *NdeI* and *EcoRI* and was ligated to pMAL-c5X (NEB, Inc, USA). *Escherichia coli* (*E. coli*, DH5α) was used for transformation of the pMAL-c5X-*NlGr7* plasmid. The transformed bacteria were selected by screening the colonies on ampicillin (50 µg/ml) containing media and plasmid purification. Then, the colonies were further analysed by restriction enzyme digestion and PCR. The *NlGr7* gene of the recombinant plasmid was sequenced by the Sanger method. The expression host *E. coli* BL21(DE3)pLysS was used as a transformation host for the pMAL-c5X-*NlGr7* vector. A single colony of transformed *E. coli* BL21(DE3)pLysS with pMAL-c5X-*NlGr7* was incubated overnight in a shaking incubator in 500 ml Luria Bertani (LB) medium containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) at 37°C with constant agitation (200 rpm). The transformed cells at 25°C (OD₆₀₀ = 0.6) were induced with 0.5 mM isopropyl b-D-thiogalactopyranoside (IPTG; Sigma, USA), which resulted in the expression of the maltose-binding protein (MBP)-tagged *NlGr7* protein. After 8 h of induction, the cells were harvested at 5,000g for 15 min. The pellet was resuspended in a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 3 mM β-ME, 10% v/v glycerol, and 0.15 mg/ml lysozyme. Cells were lysed by sonication (50 Hz, 20 cycles, with each cycle consisting of 20 s on- and 120 s off-time, 4°C), and the sonicated suspension was centrifuged at 20,000g for 30 min. The cleared supernatant was applied to amylose beads (NEB, Inc, USA), and protein was eluted with buffer 25 mM Tris-HCl pH 8.0, 200 mM NaCl, 3 mM β-ME and 10 mM maltose. Eluted *NlGr7* protein fractions were checked by SDS-PAGE (12%), and the pure fractions were pooled. The eluted *NlGr7* protein fractions were applied on a 10 kDa cut-off Centricon centrifugal device (Merck Pte. Ltd., Germany) for desalting against buffer (25 mM Tris-HCl, pH 8.0, 10 mM NaCl) and were concentrated to 56.75 mg/ml.

Cleavage of the MBP tag from *NlGr7* was completed according to manual instructions of the pMAL protein fusion and purification system (NEB, Inc, USA). The tag (MBP) was removed by incubating with Factor Xa at 4°C for 3 h. The cleaved *NlGr7* protein was concentrated by using a 10 kDa cut-off Centricon centrifugal device (Merck Pte. Ltd., Germany) and was purified by affinity chromatography on amylose resin (NEB, Inc, USA)

equilibrated with 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 2 mM DTT; then, it was purified by a benzamidine column (GE Healthcare, USA) equilibrated with 20 mM Tris-HCl, pH 8.0, 200 mM NaCl and 2 mM DTT. Unbound *N/Gr7* fractions were checked by SDS-PAGE and were concentrated to 10.00 mg/ml and stored at -80°C. Subsequently, the purified protein of *N/Gr7* was analysed using LC-MS/MS (Q-TOF). The labelling of *N/Gr7* with biotin was completed as described by Ojha et al (2014). The expression and purification of the MBP (as a control protein) was completed as described above. The protein was concentrated to 61.77 mg/ml and stored at -80°C.

Protein-lectin-binding assay

To study lectin binding with *N/Gr7*, 96-well Nunc microtiter plates (Fisher Scientific, Waltham, MA) were coated with *N/Gr7* in (100 ng; 50 µl/well) overnight (15-16 h, at 4°C). The plates were washed to remove unbound proteins, were blocked with 3% bovine albumin serum in Tris buffer and were incubated for 2 h at 100 rpm at room temperature. Additional biotinylated lectins (20 µg/ 50 µl; Vector Labs, Burlingame, CA) were added to each well and were incubated for 2 h at room temperature. Then, the plates were washed thrice with Tris buffer containing 0.05% Tween-20 to remove unbound or weakly bound biotinylated lectins, followed by incubation with streptavidin-HRP (1:2,000, 1 h, room temperature). The HRP activity was measured using a 50 µl TMB substrate solution (TransGen Biotech Co., LTD, China) containing 0.06% of 30% v/v hydrogen peroxide (H₂O₂) in each well. After 20 min, the reaction was stopped by adding 50 µl sulfuric acid (0.5 N) to each well. The absorbance was measured at 450 nm using an ELISA plate reader. The appropriate negative control was included, and the tests were run in quadruplicate.

Protein lipid overlay (PLO) assay

To test the binding ability of MBP-*N/Gr7* and MBP to various lipids, the well-established protein-lipid overlay (PLO) assay was performed using PIP and sphingo strips (Molecular Probes, USA) according to the manufacturer's instructions. These strips contained 100 pmol of various phospholipids and sphingolipids, which were spotted and immobilized on a nitrocellulose membrane. The purified MBP-*N/Gr7* was overlaid on PIP and sphingo strips. The purified MBP was used as an experimental control. Approximately 25 µg/ml protein was incubated with the strips in TBS-T (contained 3% BSA, Biotechnology Co. Ltd, Guangzhou, Xiang Bo) overnight at 4°C. The strips were then washed with TBS-T/BSA three times with gentle agitation for 10 min each wash, at room temperature. The MBP-*N/Gr7* and MBP interaction with spotted lipids were detected by subsequently blocking the strips in TBS-T buffer (10 mM Tris, pH 7.5, 70 mM NaCl, and 0.1% Tween) with 3% BSA and then

incubating the strips in 1:2000 dilution of monoclonal anti-MPB-HRP antibody (NEB, Inc, USA) in a blocking buffer for 1 h at room temperature. After thorough washing, a horse reddish peroxidase signal was detected by using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Thermo Scientific) to yield an insoluble brown product. The intensity of the signals was analysed by using ImageJ.

An electrophoretic mobility shift assay (EMSA) for a metal-binding assay

The metal-binding properties of *NIGr7* were confirmed by mixing purified *NIGr7* (approximately 0.75 µg/5 µl) with equal volumes of the following solutions: 0.5 mM (final concentration) EDTA or 0.015, 0.05, 0.15, 0.5, or 1.0 mM metals (CuSO₄, and CaCl₂). Each mixture was incubated for 30 min at 25°C, was added to 10 µl Laemmli sample buffer, and then was subjected to 12% SDS-PAGE under reducing/non-heating conditions. As a negative control, we used the maltose-binding protein for metal-binding assay.

ATPase activity assay with purified *NIGr7*

The ATPase activity assay was performed in 96-well microtitre plates by using an ATPase/GTPase activity assay kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. An aliquot of *NIGr7*-purified protein (6.25, 12.5, 25, 50, 100 µg/well) was mixed with a 5 µl assay buffer to make 10 µl of the ATPase activity assay sample. The phosphate standards and blank control for colorimetric detection were prepared according to the manufacturer's instructions of the ATPase/GTPase activity assay kit. An aliquot of 30 µl reaction mix (made with 20 µl assay buffer plus 10 µl 4 mM ATP solution) was added into each ATPase activity assay sample. After incubation at room temperature for 30 min, 200 µl reagent was added to each sample to terminate the enzyme reaction, and all samples were incubated for an additional 20 min. At this step, the transparent reaction mix suddenly changed to a fine green endpoint colour. The colour intensity was measured on a microtitre plate reader at 620 nm. All the assays were repeated five times. The ATPase activity of *NIGr7* was determined by using the mean value of the samples according to the linear regression of standards.

Polysaccharide (insoluble)-binding study

To identify the specific carbohydrate-binding conserved aromatic amino acid residues in the *NIGr7* sequence, *NIGr7* was aligned with known carbohydrate-binding homologs (Duan et al., 2016) to create multiple sequence alignments by using ClustalW (<http://www.genome.jp/tools-bin/clustalw>). Furthermore, *NIGr7* binding to various insoluble polysaccharides was determined as described by Duan et al (2016). The polysaccharides that

were tested were chitin, agarose, Sephadex G-100, raw cassava starch (tapioca starch), alpha-cellulose, and xylan from corn cobs.

Tastant-binding assay

To study the tastant-binding determination with *NIGr7*, the tastant-binding assay with *NIGr7* was performed in 96-well microtitre plates. Microtitre plates were coated with purified *NIGr7* protein (100 ng per well, 100 μ l) and incubated overnight (15-16 h, at 4°C). The unbound protein was washed off (flicking and flapping manner) thrice with Tris buffer (25 mM Tris, pH 8.0, 10 mM NaCl). Then, the unbound sites were blocked with 3% bovine albumin serum in Tris buffer and were incubated for 2 h at 100 rpm at room temperature. An additional 100 μ l tastants (50, 25, and 12.5 mM) were added into each well and were incubated for 2 h at room temperature. Then, free tastants were washed off thrice with Tris buffer containing 0.05% Tween-20. Subsequently, the wells were incubated with 100 μ l biotinylated *NIGr7* (100 ng), followed by incubation with streptavidin-HRP (1: 2000, 1 h, room temperature). Finally, the colour was developed. The bound enzyme activity was measured using 100 μ l TMB substrate solution (TransGen Biotech Co., LTD, China) containing 0.06% of 30% v/v hydrogen peroxide (H₂O₂) in each well. Due to the enzyme activity, a gradual increase in brilliant blue colour intensity with the increasing concentration of tastants was observed. After 20 min, the reaction was stopped by adding 100 μ l sulfuric acid (0.5 N) to each well. In this step, the brilliant blue colour suddenly changed to a fine yellow endpoint colour. The absorbance was measured at 450 nm using an ELISA plate reader.

Results

Characterization of *NIGr7*

The truncated cDNA of *NIGr7* consisted of 730 nucleotide bases coding for 243 amino acids with a predicted molecular mass of 28.70 kDa (**Fig. S1**). The cDNA clone was designated *NIGr7* (**Kui et al., 2018**). The estimated pI of the predicted protein *NIGr7* was found to be 9.19. There were eleven polar and nine non-polar amino acid residues. The instability index, as computed by the ExPASy ProtParam tool, was 36.27, which classified the protein as a stable protein (**Fig. S1**). Bioinformatics analysis using the TOPCONS tool predicted the absence of a secretory signal sequence and transmembrane helices in *NIGr7* (**Fig. S2**). *NIGr7* showed a 20.69-22.62% variation in sequence identity with the known Grs sequence of insect pests (**Table S1**). Phylogenetic analysis of *NIGr7* revealed the degree of relationship with respect to genes from other insects (beetle, flies, mosquitos, moths, and butterflies) (**Fig. 1**). However, this study clearly classified *NIGr7* and other insect taxa into two large clades. We

employed the polymerase chain reaction to amplify 730 bp *NIGr7* amplicons. We then cloned, expressed, and purified the amplified amplicons (data not shown). The apparent molecular weight of our protein of interest showed a mobility shift of ~55.428 kDa on SDS-PAGE due to a high (9.19) isoelectric point (pI) and the presence of hydrophobic amino acids in the *NIGr7* sequence. Furthermore, this purified ~55.428 kDa protein was confirmed to be *NIGr7* (**Fig. 2A**), which is a sugar transporter of the BPH, based on mass-spectrometry (LCMS/MS-Q-TOF) (**Fig. S3**).

Lectin affinity for *NIGr7*

To study the affinity of lectins, which are carbohydrate-binding proteins with *NIGr7*, a total of fourteen lectins, griffonia (*Bandeiraea*) *simplicifolia* lectin (GSL), *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Sophora japonica* agglutinin (SJA), wheat germ agglutinin (WGA), concanavalin A (ConA), soybean agglutinin (SBA), wheat germ agglutinin (WGA), *Dolichos biflorus* agglutinin (DBA), *Ulex europaeus* agglutinin 1 (UEA1), *Ricinus communis* agglutinin 1 (RCA1, Ricin), and peanut agglutinin (PNA), were demonstrated to have affinity for *NIGr7*. DBA revealed the highest affinity, while UEA1 showed the lowest affinity for *NIGr7* (**Fig. 2B**). The standard deviation (SD) of a set of absorbance values ranged from ± 0.001 to ± 0.08 (**Fig. 2B**). These lectins may combine with *NIGr7* and likely play numerous roles in biological recognition phenomena involving cells, carbohydrates, and proteins.

Lipid affinity for *NIGr7*

We screened the arrays of 30 lipids by using the protein-lipid overlay (PLO) assay. This lipid screen showed that recombinant *NIGr7* binds to several anionic phospholipids, including PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,5)P₂, PtdIns(4,5)P₂, PtdIns(3,4,5)P₃, phosphatidic acid (PtdOH), phosphatidyl-serine (PtdSer) (**Fig. 2C**) and a single strong signal of sphingolipid sulfatide (sulfogalactosylceramide; GalCerI3-sulfate) (**Fig. 2D**).

Metals affinity to *NIGr7*

The metal-binding capability of *NIGr7* was verified by a gel mobility shift assay. The purified *NIGr7* mixed with different concentrations of CuSO₄ and CaCl₂ was resolved on SDS-PAGE. Compared with the mobility of *NIGr7* in the presence of 1.0 mM EDTA, the mobility of *NIGr7* was slowed by the addition of 0.015 to 1.0 mM CuSO₄. The migration of *NIGr7* appeared to slow when the concentration of CuSO₄ was high (**Fig. 3A**), which suggested that *NIGr7* has the ability to bind Cu²⁺. Furthermore, the copper-binding site, as predicted by the RaptorX-binding server (<http://raptorx.uchicago.edu/BindingSite>), was interesting for the

observation of glutamine (Q)-64, asparagine (N)-117, lysine (K)-218, glutamine (Q)-222, threonine (T)-225, tyrosine (Y)-226, and isoleucine (I)-229 in the *N*/Gr7 domain (**Fig. 3B**). The mobility of *N*/Gr7 shifted with the addition of CaCl₂ in comparison with the mobility of *N*/Gr7 in the presence of 1.0 mM EDTA (**Fig. S4**). On SDS-PAGE, the calcium-*N*/Gr7 complex was stable in the presence of a low (0.015 and 0.05 mM) CaCl₂ concentration. At the same time, the calcium-*N*/Gr7 complex was reduced in the presence of increasing (0.15, 0.5, and 1.0 mM) concentrations of CaCl₂ (**Fig. S4**). This reduced intensity of the calcium-*N*/Gr7 complex on SDS-PAGE was due to the precipitation of the *N*/Gr7 protein in the presence of a high concentration of calcium chloride under incubation for 30 min at 25°C.

Hydrolysis of the *N*/Gr7-bound ATP

To understand how the predicted active site of *N*/Gr7 might relate to its biological function, we investigated its biochemical activities *in vitro*. A total of 4 phosphorylated sites (serine (S)-54, tyrosine (Y)-104, tyrosine (Y)-105, and threonine (T)-201) and their corresponding catalytic protein kinases were predicted from the *N*/Gr7 amino acid (1-243) sequence (<http://kinasePhos.mbc.nctu.edu.tw/>, **Fig. S5**). Furthermore, the developed phosphate standard curve showed that phosphate could be detected at a minimum of 500 pmol and maximum of 2000 pmol. The R² value was 0.992, as shown in the figure (**Fig. 3C, Table S2A**). Examination of the ATPase activity assay of bound phosphate in *N*/Gr7 showed that 217.68-1125.85 pmol of phosphate was liberated (**Fig. 3D, Table S2B**), and its respective stable dark green colour with free phosphate liberated by the enzyme in a colorimetric product is shown in the figure (**Fig. 3E**). The R² value was 0.994, as shown in the figure (**Fig. 3D**). These data are consistent with the finding that *N*/Gr7 displays its ATPase activity *in vitro*. We postulated that phosphate binding plays an important biochemical function.

***N*/Gr7 exhibited polysaccharide (insoluble) specificity**

The purified *N*/Gr7 showed a binding affinity for six insoluble polysaccharides. *N*/Gr7 showed the highest affinity for tapioca starch, while *N*/Gr7 showed the lowest affinity for Sephadex G-100 (**Fig. 4A**). Furthermore, seven carbohydrate-binding homologs sequences (**Duan et al., 2016**), belonging to accession numbers ADR64668-2, AFN57700-2, CBM_{C5614}-1, ADR64664, ADR64668-1, AFN57700-1, and CAJ19146, were aligned with *N*/Gr7 to observe conserved aromatic amino acids, which were accepted to play an essential role in identifying and binding to polysaccharides. Two (Tryptophan (W)-59, and W-128) aromatic amino acids were identified as being completely conserved in all the aligned-sequences (numbered according to amino acids in *N*/Gr7) (**Fig. 4B, Red box**). At the same time, phenylalanine (F)-91, tyrosine (Y)-104, F-108, and F-111 were observed to be partially

conserved aromatic amino acids in all the aligned sequences (**Fig. 4B**, Pink box). A phylogenetic tree revealed that *NIGr7* showed the least similarity with other known carbohydrate-binding homolog sequences (**Fig. 4C**).

***NIGr7* showed an interaction with various tastants**

Twelve tastants (D-(+) glucose, maltose, sucrose, D-(+) galactose, D-xylose, trehalose, D-(-) ribose, D-(-) melezitose, maltotriose, D-sorbitol, D-cellobiose, and myoinositol) were individually analysed, and all showed binding affinity for *NIGr7* (**Fig. 5**). The binding affinities of the tastant preparations at each of the 3 concentrations of 50, 25, and 12.5 mM are depicted in Figure 5. Among the tastants, 50 mM D-cellobiose showed the highest (absorbance 0.352) binding affinity for *NIGr7* (**Fig. 5K**), while 50 mM sorbitol showed the lowest (absorbance 0.065) binding affinity for *NIGr7* (**Fig. 5J**). The standard deviation (SD) of a set of absorbance values ranged from ± 0.0005 to ± 0.042 (**Fig. 5**).

Discussion

Here, we systematically characterized the functional properties of *NIGr7* using the macromolecular (*in vitro*) approach. Our study begins to overcome challenges in studying this highly divergent superfamily of insect Gr proteins and provides a systematic overview of ligand (metals, carbohydrates, lipids, lectins, and tastants) detection and ATP binding by this sugar receptor. The majority of gustatory receptor ligands in *Drosophila* have been studied by using electrophysiology recordings and/or behavioural investigations. However, there is, to some extent, a gap in the accurate interpretations of the electro-physiological data and behaviours that are recorded between wild-type and Gr mutant flies (**Miyamoto et al., 2013**). The lectin-binding proteins of the rice brown planthopper are still undefined. Primary information from the BPH gustatory receptor and the lectin(s)-binding study reveals that the structure that interacts with the lectin(s) consists of carbohydrate moieties (**Lis and Sharon, 1986**). A variety of carbohydrate specificity-lectin(s) were selected for study (**Fig. 2B**). In assays, the *NIGr7* protein interacted with a variety of sugar-specific lectins, which offers a complex oligosaccharide structure. Similar facts were found earlier for the human parotid salivary gland (**Rohringer and Holden, 1985**). The role that lectin-binding with *NIGr7* (protein) plays in the developmental stages of the BPH has yet to be determined.

Although phospholipids (PLs) are only a minor nutrient compared to starch and protein, they may have both nutritional and functional significance. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and their lyso forms are the major PLs in rice. Two PLs, PtdIns4P (phosphatidylinositol monophosphate) and PtdIns(4,5)P2

(phosphatidylinositol bisphosphate), have been reported as key intermediate sources of second messengers, and they directly affect the signal pathways in many animal cells and plants cells (**Chen et al., 1991**). In this study, *NIGr7* binds to various lipids with different affinities and specificities (**Fig. 2C, D**), which could be the result of non-specific electrostatic interactions. The noticed affinities of *NIGr7* for the lipids are specifically known as PH (pleckstrin homology) lipid-binding domains (**Stahelin, 2009**). However, various examples in prior studies show that low-affinity interactions have a distinct and influential impact on biological roles (**Becker et al., 1996; Irwin and Tan, 2014; Wilson, 2003**). Our rationale, which can be noticed in *NIGr7*-lipid interactions and in the lipids with their reported biological functions, reveals that lipid binding targets the chaperone proteins to specific cellular locations, where, in addition to participating in transmembrane targeting and protein folding, they also affect the physiological pathway of the membrane. Hence, the specific binding of lipids suggests that lipid binding has been a conserved function of *NIGr7* since its appearance (**Fig. 2C, D**).

Copper is an important micronutrient that plays an essential role in plant growth and development (**Roy, 1931**); little is known about copper in insects. Insects often consume heavy metal ions from their niche. Previous reports revealed that copper is related to both the developmental stage of the organism, when animals undergo the transition from larval to adult blood types (**Durstewitz and Terwilliger, 1997; Ye et al., 2015; Li et al., 2016**), and the moulting cycle, when large fluctuations in protein synthesis take place (**Ye et al., 2015; Li et al., 2016**). In the present study, *NIGr7* showed an interaction with copper. Therefore, taking this observation further, we speculate that copper may also play a role in the developmental stage (egg to adult) and moulting cycle of BPH. However, this has yet to be verified.

It has been noted that effectors from herbivores, which can bind to Ca^{2+} , are involved in suppressing the plant's defence (**Atamian et al., 2013**) and contracting forisomes (**Will et al., 2007**). However, the physiological mechanism of effector-mediated regulation of plant defences remains widely unexplained (**Consales et al., 2012**). The structure of the transduction pathway in gustation is unclear. The signalling pathways, including cGMP (**Amakawa et al., 1990**), inositol 1,4,5-triphosphate (IP_3) (**Koganezawa and Shimada, 2002**), and the sugar-receptor protein-gated channel (**Murakami and Kijima, 2000**), have been studied in few insects. Furthermore, GPCRs activate a temporal rise in intracellular Ca^{2+} because inositol 1,4,5-triphosphate activates delivery of Ca^{2+} from intracellular storage (**Torfs et al., 2002**). Therefore, we speculate that the GPCRs- IP_3 - Ca^{2+} -mediated network may

impact the regulation of ligand-mediated calcium flux in the BPH physiological mechanism, but this has yet to be elucidated.

Polysaccharides display a wide range of solubility in water. However, some are water insoluble, e.g., chitin, agarose, tapioca starch, alpha cellulose, and xylan. Cellulose and chitin polysaccharides play structural roles in plants, fungi, and insects. The interactions between proteins and cellulose (Georgelis et al., 2012; Boraston et al., 2004; Boraston, 2004), as well as between human chitin-binding proteins (YKL-39 and -40) (Schimpl et al., 2012), have been studied extensively. However, the physiological role of these proteins remains poorly understood. Our results revealed a new carbohydrate-binding protein, *NI*Gr7, which targets different insoluble polysaccharides that adopt varied conformations, including chitin, agarose, tapioca starch, alpha cellulose, and xylan. This study reveals that *NI*Gr7 may possess higher plasticity to accommodate varied ligand conformations. This varied binding specificity remains unexplored without a structural study of the *NI*Gr7-ligand complex, and it has yet to be a priority in future studies.

To study *NI*Gr7 as a sugar Gr, we tested three different concentrations of twelve sugars to reveal the receptor's ligands. The results revealed that all 12 sugars interacted with *NI*Gr7 (Fig. 5). A previous report demonstrated that galactose, xylose, glucose, and trehalose can regulate intracellular Ca^{2+} levels in insect cell lines (Chen et al., 2019; Xu et al., 2012). We speculate that expression of *NI*Gr7 within the BPH may regulate the expression, localization, and/or function of the endogenous taste receptors of the BPH, thus leading to an increase or decrease in the responses to tastants.

Conclusions

Currently, there is insufficient knowledge about the function of gustatory (sugar) receptors within the insect. In a previous study in *Drosophila*, Gr43a showed upregulation during the feeding experiences of hungry flies and downregulation during feeding experiences in satiated flies (Miyamoto et al., 2012). In the present study, we observed the functional versatility of *NI*Gr7. We assume that *NI*Gr7 binding with its ligands may likely stimulate the physiological pathways of the BPH for feeding behaviour and adaptation. These observations extended our knowledge of the Grs (proteins) mechanism in insect and pest control.

Author contributions

WZ and AO conceived and designed the experiments. AO performed the experiments and analyzed the data. AO and WZ wrote the manuscript. Both authors have read and approved the final manuscript.

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Competing interests

The authors declared that they have no competing or financial interest.

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Figure legends

Figure 1 | Phylogenetic tree showing the relation between orthologues of gustatory receptor reported from different organisms.

Figure 2 | *NlGr7* recombinant protein and *NlGr7*-ligands overlay assays. (A) Purified *NlGr7*, (B) *NlGr7*-lectin overlay assay shows that *NlGr7* binds to several lectins. Protein-lipid overlay assay shows that *NlGr7* binds to several anionic lipids. (C) *NlGr7* reveals interaction to phospholipids, and (D) interaction to sphingolipids.

Figure 3 | Overview of the *NlGr7*-copper complex and ATPase/GTPase activity assay for *NlGr7*. (A) The gel mobility shifts of the *NlGr7*-copper complex showed a varied range of copper. (B) Predicted binding amino acid residues Q64, N117, K218, Q222, T225, Y226, and I229 anchor copper within the *NlGr7*. (C) Standard curve prepared according to the ATPase/GTPase activity kit instructions. (D) The *NlGr7* (6.25, 12.5, 25, 50, or 100 µg) determined ATP. (E) This ATPase/GTPase activity assay is a colorimetric test that shows the amount of free inorganic phosphate (Pi). Controls that included no *NlGr7* were measured to observe for spontaneous ATP hydrolysis of ATP, and their optical density (OD) values were deducted from the samples' values.

Figure 4 | Characterization of *NlGr7* as a novel carbohydrate protein. (A) Binding of *NlGr7* to insoluble polysaccharides. Thirty micrograms of purified *NlGr7* was incubated with 200 µl 4% (wt/vol) insoluble polysaccharide including chitin, agarose, Sephadex G-100, raw cassava starch (tapioca starch), alpha-cellulose, or xylan from corn cobs. The same amount of protein used in the binding assay but without polysaccharide was included as a control (CK). (B) Multiple sequence alignment of *NlGr7* and selected carbohydrate-binding homologs. The

sequence similarity and identity are shown by dots and asterisks, respectively. Entirely conserved aromatic (tryptophan (W)-59, and W-128) amino acids are red, and partly conserved aromatic (phenylalanine (F)-91, tyrosine (Y)-104, F-108, and F-111) amino acids are pink. (C) Phylogenetic tree showing the relation between orthologues of the carbohydrate protein that was reported previously.

Figure 5| Tastants screening assay. The binding of *NIGr7* to different concentrations (50, 25, and 12.5 mM) of tastants. (A) D-(+) glucose, (B) maltose, (C) sucrose, (D) D(+) galactose, (E) D-xylose, (F) trehalose, (G) D-(-) ribose, (H) D-(-) melezitose, (I) maltotriose, (J) D-sorbitol, (K) D-cellobiose, and (L) myoinositol.

Supplementary materials

Figure S1. The computation of various physical and chemical parameters of *NIGr7*.

Figure S2. Consensus prediction of membrane protein topology using the TOPCONS server indicated no trans-membrane helices (blue in the graph) in the predicted amino acid sequence of *NIGr7*. TOPCONS predicted the topology of *NIGr7* from five different topology prediction algorithms: OCTOPUS, Philius, PolyPhobius, SCAMPI (multiple sequence mode), and SPOCTOPUS. The output of these five algorithms was used as input for the TOPCONS Hidden Markov Model (HMM) (shown in maroon), which provided a consensus prediction for the protein together with a reliability score based on the agreement of the included methods across the sequence. In addition, ZPRED was used to predict the Z-coordinate (i.e., the distance to the membrane centre) of each amino acid, and the G-scale was used to predict the free energy of the membrane insertion for a window of 19 amino acids that were centred around each position in the sequence.

Figure S3. LCMS/MS-Q-TOF-based identification of the *NIGr7*. (A) Parameters used in MASCOT search, (B and C) The green area is a significant threshold. This area of the score over the results of the identification of the mascot positive results, (D and E) decoy search summary, and (F) cross-examination of the LCMS/MS-QTOF-generated peptide sequence of the sugar transporter sequence blast in the NCBI protein database.

Figure S4. An electrophoretic mobility shift assay (EMSA) for the calcium-binding assay.

Figure S5. Computationally predicted catalytic kinase-specific phosphorylation sites within the *NIGr7* sequence using the KinasePhos tool.

605 **Table S1.** *NlGr7* protein sequence identity with known chemoreceptors of insects.

606 **Table S2.** ATPase activity assay with *NlGr7*. Hydrolysis of the *NlGr7*-bound ATP. (A)
 607 Standard curve of phosphate, (B) the concentration of released phosphate from the *NlGr7*-
 608 bound ATP. The released concentration of phosphate from the samples per well was
 609 calculated using the phosphate standard curve value 500 pmol.

Figure 1

Figure 2

A

B

C

D

Figure 3

Figure 4

A

C

B

Figure 5