Distribution of Gustatory Receptors and Their Co-Expression with FMRFamide-Related Peptides in Enteroendocrine Cells and Neurosecretory Cells of Larvae of the Silkworm *Bombyx mori*

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Contents

Abstract

Insects taste nonvolatile chemicals through gustatory receptors (Grs) and make choices for feeding, mating, and oviposition. To date, genome projects have identified 69 Gr genes in the silkworm, *Bombyx mori*. However, the expression sites of these Grs remain to be explored.

In the Chapter 1, I explored expression cells of BmGr6. I used reverse transcription- polymerase chain reaction (RT-PCR) to investigate expression of the *B. mori* Gr-6 (BmGr6) gene, a member of the putative sugar clade gene family in various tissues. *BmGr6* was expressed in the midgut, central nervous system, and oral sensory organs. Immunohistochemistry using an anti-BmGr6 antiserum demonstrated that BmGr6 is expressed in cells of the antenna, labrum, maxillary galea, maxillary palps, and labium of the oral sensory organs. Furthermore, immunohistochemistry showed that BmGr6 is expressed in putative midgut enteroendocrine cells and in cells of the central nervous system including putative neurosecretory cells of the brain and ganglia. These results demonstrated that BmGr6 is widely expressed in both gustatory and non-gustatory organs.

In the Chapter 2, I clarified whether BmGr6-expressing cells are midgut enteroendocrine cells and CNS neurosecretory cells. Double-immunohistochemistry indicated that BmGr6 is expressed in midgut enteroendocrine cells, also in CNS neurosecretory cells. In particular, a portion of BmGr6-expressing cells, in both midgut and CNS, secreting FMRFamide-related peptides (FaRPs). These results

suggest that BmGr6 functions not only as a taste receptor, but also as a chemical sensor such as for the regulation of gut movement, physiological conditions, and feeding behavior of larvae.

In the Chapter 3, I explored expression cells of BmGr9. In contrast to bmGr6, BmGr9 has been shown to respond specifically to fructose and function as a ligand-gated ion channel, but expression sites (cells) of this Gr are still unclear. I demonstrated using RT-PCR that *BmGr9* gene, was widely expressed in the CNS, as well as oral sensory organs. Additionally, using immunohistochemistry with an anti-BmGr9 antiserum, BmGr9 was shown to express in cells of the oral sensory organs including the maxillary galea, maxillary palps, labrum, labium, and also in CNS putative neurosecretory cells. Furthermore, by double-immunohistochemistry, most of BmGr9-expressing cells were co-localized with putative neuropeptide F1-expressing cells in the brain, suggesting that BmGr9 are involved in promotion of feeding behavior. In addition, a portion of BmGr9-expressing cells in the brain was indicated to be co-localized with cells expressing BmGr6, a molecule of the sugar receptor clade, suggesting that some other sugar may also regulate feeding behavior of *B. mori* larvae.

In the Chapter 4, the expression of putative bitter clade BmGrs genes in the midgut and CNS was also examined. By RT-PCR, I observed amplified products of 26 and 25 BmGrs genes in the midgut and CNS, respectively. In addition, *BmGr53* gene, was also found to be widely expressed in the organs and tissues of the *B. mori* larvae. Furthermore, by immunohistochemistry with an anti-BmGr53 antiserum, I

showed that BmGr53 is expressed in cells of the brain, suggesting that BmGr53 may play roles in modulating feeding behavior or physiological homeostasis.

Key words: *Bombyx mori*, Gustatory receptor, Oral sensory organs, Midgut, Central nervous system, Enteroendocrine cell, Neurosecretory cell, Co-expression.

General Introduction

Taste is a sensory modality found in most animals (Dunipace et al., 2001). The gustatory system endows animals with the ability to detect contact-chemical cues, distinguish the palatable from the unpalatable, and elicit taste acceptance or avoidance behaviors (Scott, 2005). Insects taste transduction is performed by gustatory receptor neurons (GRNs), several GRNs are held within bristles called taste sensilla, which located on the mouthparts, legs, wing margins, and some parts of the thorax and abdomen (Chapman, 2003). Gustatory receptors (Grs) which are members of a large family of proteins with multipass transmembrane domains and generally expressed in GRNs, and provide insects with the capability to detect chemical signals critical for feeding, mating and oviposition (Clyne et al., 2000; Robertson et al., 2003; Montell, 2009). Insect Grs genes were initially identified in *Drosophila melanogaster* (Clyne et al., 2000) and so far, 68, 76, 10, 62, 96 and 69 Grs genes in the *D. melanogaster* (Clyne et al., 2000), *Anopheles gambiae* (Hill et al., 2002), *Apis mellifera* (Honeybee Genome Sequencing Consortium, 2006), *Tribolium castaneum* (Abdel-latief, 2007), *Linepithema humile* (Smith et al., 2011) and *Bombyx mori* (Sato et al., 2011; Wanner and Robertson, 2008; Zhang et al., 2011) respectively have been identified through genome projects.

The expression profiles of some Grs in *D. melanogaster, A. gambiae, A. mellifera, T. castaneum,* and *A. aegypti* have been determined using reverse transcription-polymerase chain reaction (RT-PCR), the Gal4/UAS system, and in situ

hybridization. For example, in *D. melanogaster,* approximately 20 Grs genes were initially found to be expressed in gustatory organs using RT-PCR analysis (Clyne et al., 2000), but subsequent cellular expression studies using the GAL4/UAS system showed that Grs genes were also expressed in the neurons of wings and gustatory organs including the labial palps, pharynx, and legs. Gr21a and Gr 63a are also expressed in olfactory sensilla and Gr22e expression has been found in antennae (Dunipace et al., 2001; Hiroi et al., 2002; Jones et al., 2007; Marella et al., 2006; Scott et al., 2001; Suh et al., 2004; Thorne et al., 2004; Wang et al., 2004). In addition, some studies indicated that Grs are expressed in neurons in the brain, the subesophageal ganglion (SOG), abdominal ganglia (AG), and in peripheral sensory neurons (Miyamoto et al., 2012; Park and Kwon, 2011a; Thorne and Amrein, 2008). These studies showed that Grs-expressing cells are distributed widely in both gustatory and non-gustatory organs.

Molecular genetic studies of insect Grs in taste perception are mainly focused on the model organism *D. melanogaster*. According to previous studies, some Grs function in the detection of sweet and bitter chemicals. DmGr5a is required for trehalose sensing (Dahanukar et al., 2001; Ueno et al., 2001; Chyb et al., 2003) and DmGr64a is important for the detection of glucose, sucrose and other sugars (Dahanukar et al., 2007; Jiao et al., 2007; Slone et al., 2007). Additionally, DmGR66a and DmGR93a are necessary for sensing caffeine (Moon et al., 2006; Lee et al., 2009), and DmGR33a is required for detecting a wide range of bitter tasting chemicals (Moon et al., 2009; Miyamoto et al., 2013). However, some Grs appear to

have non-gustatory functions. For example, DmGr68a is expressed in the taste neurons of male forelegs and is possible to encode a receptor for a female pheromone (Bray and Amrein, 2003). DmGr32a, is expressed in taste neurons of the labellum and tarsal leg segments, it may function as a receptor for inhibitory male pheromones (Ebbs and Amrein 2007). Moreover, DmGr21a is coexpressed with DmGr63a, in olfactory neurons of the antennae and confers the CO_2 sensitivity (Jones et al., 2007; Kwon et al., 2007). Recently, DmGr43a, was shown not only detected fructose in the chemosensory organs, but is also used as a sensor of internal nutrients in other organs (Miyamoto et al., 2012). Signally, DmGr43a expressing in neurons of brain to sense hemolymph fructose and promote feeding in starved flies, but suppress feeding in satiated flies (Miyamoto et al., 2012; Mishra et al., 2013; Miyamoto and Amrein, 2014). Furthermore, DmGr28b is critical for light-induced responses (Xiang et al., 2010) and a DmGr28b.d paralog involve in rapid warmth avoidance (Ni et al., 2013). These studies showed that Grs are function in diverse sensory contexts, including sensing of sweet and bitter chemicals and nonvolatile pheromones, as well as CO_2, internal nutrients, light and temperature.

The silkworm, *B*. *mori*, which belongs to the Lepidopteran order, is an important model insect used in molecular genetics and structural and functional genomics studies. Recently, the *B. mori* genome was shown to contain genes encoding 69 Grs (Zhang et al., 2011). These BmGrs are classified as putative carbon dioxide receptors, sugar receptors, bitter receptors, and duplicated orthologs of the DmGr43a receptor (Sato et al., 2011; Wanner and Robertson, 2008; Zhang et al., 2011). To date,

functional analyses have only been performed on a few BmGrs. BmGr8 which was shown to be specific receptors for inositol (Zhang et al., 2011). Moreover, membrane topology studies on BmGr8 and BmGr53 showed that these two Grs have an inverted topology relative to that of typical G protein-coupled receptors (Zhang et al., 2011). In addition, BmGr9 has been shown to respond specifically to fructose as a ligand-gated ion channel (Sato et al., 2011). Recently, DmGr43a, a BmGr9 ortholog in *D. melanogaster*, was showed to detect fructose not only in the chemosensory organs as a tastant, but also in other organs as an internal nutrient (Miyamoto et al., 2012; Miyamoto and Amrein, 2014). DmGr43a expressing neurons in the brain sense hemolymph fructose and promote feeding in starved flies, but suppress feeding in satiated flies (Miyamoto et al., 2012). Additionally, possibility of the secretion of neuropeptide F (DmNPF) was discussed in the in the regulation of the feeding of the fly. Since Dm43a is an ortholog of BmGr9, it allowed us to speculate that BmGr9 may serve similar biological functions as DmGr43a in the *B. mori* brain, and feeding behavior of the silkworm might be also modulated by the neuropeptide F1 (BmNPF1), a *B. mori* orthologue of DmNPF. However, whether BmNPF1 is co-expressed with BmGr9 in the *B. mori*, is not known. Previous studies in *D. melanogaster* have showed that co-expression of multiple Grs is necessary to respond to various compounds (Moon et al., 2006; Kwon et al., 2007; Slone et al., 2007; Jiao et al., 2008; Lee et al., 2009). Although BmGr9 was appeared not to require the expression of other BmGrs to show the responsiveness to fructose in vitro, the possibility that BmGr9 co-expresses with others BmGrs in neurons and expand their ligand

spectrum was unable to be excluded. Up to present, however, whether BmGr9 is co-expressed with any other BmGrs in the *B. mori* larvae has not yet been reported.

In this work, I aimed to determine the distribution of BmGrs (especially focus on BmGr6 and BmGr9)-expressing cells in the *B. mori* larvae and explore the functions of these BmGrs. I used reverse transcription-polymerase chain reaction (RT-PCR) to investigate the expression sites of the *BmGrs* genes in the *B. mori* larvae. In addition, I explored BmGr6- (a molecule of the sugar receptor clade), BmGr9- (a fructose receptor), BmGr53- (a molecule of the bitter receptor clade) expressing cells in *B. mori* larval tissues using specific polyclonal antiserum raised against BmGr6, BmGr9 and BmGr53, respectively. Moreover, for BmGr6, I attempted to clarify whether BmGr6-expressing cells are midgut enteroendocrine cells and CNS neurosecretory cells by the double-immunohistochemistry using antisera raised against some peptide hormones. In the case of BmGr9, I also I attempted to clarify whether BmGr9-expressing cells are neurosecretory cells and payed attention especially to CNS including brain, a secretory center for the regulation of behavior and homeostasis. Furthermore, examined whether BmGr9-expressing cells express BmGr9 alone or not as a sensor for the nutrients in the hemolymph.

Chapter 1

Expression of a Sugar Clade Gustatory Receptor, BmGr6, in the Oral Sensory Organs, Midgut, and Central Nervous System of Larvae of the Silkworm *Bombyx mori*

1.1. Introduction

The gustatory system plays a critical role in insect survival and reproduction and, supports feeding, mating, and oviposition. Insects possess sensitive gustatory organs that can detect nonvolatile chemicals and aid in choosing nutritious food and avoiding harmful substances. The ability to respond to these substances is conferred largely by gustatory receptors (Grs) which are members of a large family of proteins with seven transmembrane domains. Insect Grs genes were initially identified in *Drosophila melanogaster* (Clyne et al., 2000) and additional Grs genes have been identified in subsequent studies from the genomes of other insects including *Anopheles gambiae*, *Apis mellifera*, *Tribolium castaneum*, *Aedes aegypti* and *Bombyx mori* (Abdel-latief, 2007; Hill et al., 2002; Honeybee Genome Sequencing Consortium, 2006; Kent et al., 2008; Sato et al., 2011; Wanner and Robertson, 2008; Zhang et al., 2011).

Previous studies have showed that gustatory receptors (Grs) are generally expressed in gustatory organs, such as the paired labella, labrum, mouthparts, wing margins, genitalia, and tarsal segments of the legs (Dunipace et al., 2001; Scott et al., 2001; Thorne et al., 2004; Wang et al., 2004; Dahanukar et al., 2007). However, some Grs are also expressed in non-gustatory organs. For example, the expression DmGr21a and DmGr63a have been found in the antennae (Jones et al., 2007; Kwon et al., 2007). DmGr43a is expressed not only in gustatory neurons, but also in defined sets of neurons of the proventricular ganglion, the brain and the uterus (Miyamoto et al., 2012). Moreover, some Grs were expressed in midgut enteroendocrine cells and

may have chemosensory roles in the intestine and regulate physiological functions (Park and Kwon, 2011b). These studies suggested that Grs are widely expressed in both gustatory and non-gustatory organs.

There are 69 Grs (BmGrs) were identified from *Bombyx mori* genome and five BmGrs (BmGr4–8) were classified as putative sugar receptors family. To date, however, functional analyses have only been performed on BmGr8 which was shown to be specific receptor for inositol (Zhang et al., 2011). In addition, membrane topology studies on BmGr8 showed that this Gr has an inverted topology relative to that of G protein-coupled receptors (Zhang et al., 2011). Despite these previous findings, research on the other putative sugar Grs of *B*. *mori* has been limited and little attention has been paid to their tissue distribution. Therefore, in this study, I aimed to determine the distribution of BmGr6, a member of the sugar receptor family, in various organs and tissues of the *B. mori* larval. RT-PCR analysis revealed that BmGr6 is expressed widely in various *B. mori* larval tissues and oral sensory organs. In addition, I generated a specific polyclonal antiserum against a 97-amino acid region of the BmGr6 protein and used it to visualize the Gr receptor protein in *B. mori* larval tissues. An immunohistochemistry study showed that BmGr6 was expressed in the larval oral sensory organs (antenna, labrum, maxillary galea, maxillary palps, and labium), midgut enteroendocrine cells, and the central nervous system (CNS). These findings on BmGr6 expression in tissues and cells may suggest that Gr6 functions in sugar sensing to regulate gut motility, feeding behavior, and metabolic homeostasis.

1.2. Materials and methods

1.2.1. Experimental animals

Eggs of silkworm *B. mori* (hybrid strain, Kinshu × Showa) were purchased from the silkworm egg producing company, Ueda Sanshu Ltd (Ueda, Japan). Larvae were reared on an artificial diet, Silkmate (Nihon-Nosanko, Yokohama, Japan) in plastic containers at 25 °C with 70% relative humidity and long-day lighting conditions (16 h Light, 8 h Dark).

1.2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Tissues including brain, central nervous ganglia, labrum, mandible, antenna, maxilla, labium, caudal leg, foregut, hindgut, testis, ovary of 20 larvae; thoracic leg, proleg, malpighian tubule of 15 larvae; anterior midgut, middle midgut, posterior midgut of five larvae; silk gland, fat body of two larvae were dissected from two-day-old fifth instar larvae. All collected tissues were homogenized in 0.5-2 ml cold ISOGEN II (Nippon Gene, Co., Ltd, Tokyo, Japan) using a Physcotron generator shaft, NS-10G (Microtec Nition, Co., Ltd, Chiba, Japan), motorized by a Drive Unit, NS-310E (Microtec Nition, Co., Ltd), and kept at -80 °C until use for RNA extraction. For cDNA synthesis, approximate 5 μg of total RNA was used in reverse transcript reaction containing 20 mM dNTPs, 5 pM oligo$(dT)_{20}$ primers, 800 units of Reverse Transcriptase Buffer (5×RT Buffer), 100 units of ReverTra Ace® (TOYOBO, Osaka, Japan) and 40 units of an RNase inhibitor for 90 min at 42 °C, followed by incubation at 99 °C for 5 min. For the double-stranded cDNA fragments amplification, 1 μl of

cDNA solution (<500 ng) was used as a template in a 25 µl final reaction volume containing 2.5 units of GoTaq polymerase (TaKaRa, Shiga, Japan) and 0.2 µM of BmGrs primers (Table 1.1). Primers were designed to produce the long amplicons including introns if genomic DNAs are used as templates. PCR was performed under the following reaction conditions: 95 °C for 3 min, then 35 cycles at 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min, followed by 72 °C for 5 min.

1.2.3. cDNA cloning of BmGrs genes

Total RNA from dissected *B. mori* larval maxillary galea was purified with ISOGEN II (Nippon Gene, Tokyo, Japan), and used to prepare cDNA with the ReverTra Ace® (Toyobo, Osaka, Japan) following the manufacturer's instructions. Full-length coding sequences of BmGr6, BmGr7 and BmGr9 were obtained through Two-Step PCR using gene specific primers (Table 1.2). The amplified DNA fragments of BmGr6 and BmGr9 were cloned into EcoRI and ApaI site of pcDNA3.1 vector. For BmGr7, the amplified DNA fragments was cloned into EcoRV site of pcDNA3.1 vector (Thermo Fisher Scientific) using GeneArt® Seamless Cloning and Assembly Enzyme Mix (Thermo Fisher Scientific). 3xFLAG®-tag (Sigma-Aldrich Japan, Tokyo, Japan) was previously introduced into the downstream of EcoRV site of pcDNA3.1 and fused to 3' end of the BmGr7 gene.

1.2.4. Cell culture and transfection

Human Embryonic Kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (D-MEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaillé, France), 4 mM GlutaMAX™ (Thermo Fisher Scientific), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under a humidified incubator containing 5% CO_2. 60-70% confluent cells were transfected with BmGr6/pcDNA3.1 in six-well plate (Iwaki Glass, Tokyo, Japan). 2 μg of the plasmid DNA were premixed with polyethylenimine (PEI Max, Polysciences, Inc., Warrington, PA) in 100 μl fresh Opti-MEM (Thermo Fisher Scientific) for 15-30 min at room temperature and cells was transfected by adding the mixture into medium. The medium was replaced with fresh D-MEM after 9 h. Transfected cells were cultured for 24 h before immunocytochemistry.

1.2.5. Production of recombinant proteins and antisera preparation

Nucleotide sequences coding 97 amino acid residues (^{353}QVNLAS-TFYNYS^{449}) at the end of C-terminal of BmGr6 were amplified by PCR using the original plasmid as a template and primer sets that contain BamHI or XhoI sites in the forward and reverse primers, respectively (forward, 5′-TTCGTTGATAGGATCCCAAGTAAAT-3′; reverse, 5′-CCGCTCGAGCTACGAGTAGTTGTAA-3′). PCR was performed with 35 cycles; 98 °C for10 s, 50 °C for15 s, and 72 °C for 1 min. The amplified fragments were digested with BamHI and XhoI and inserted into the GST fusion protein expression vector, pGEX4T-3 (GE Healthcare, Chalfont, UK). The *Escherichia coli*

BL21 competent cells (TaKaRa, Shiga, Japan) was transformed BmGr6-pGEX4T-3 construct by electroporation using Gene Pulser (BioRad Japan, Tokyo). Production of the fusion protein was induced with isopropyl-b-D-thiogalactoside (IPTG) at final concentration of 1 mM for overnight. The bacterial cells were collected by centrifugation and sonicated with VP-15S ultrasonic processor (TAITEC Co., Japan). The inclusion bodies containing the recombinant protein were washed by 1 % Triton-X 100 and were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then the gels were stained with Coomassie Brilliant Blue R250 (CBB-R250). The recombinant protein bands were determined by comparing with that of the cell homogenate from a negative control culture grown with an empty vector, and the bands were removed from the gels. The recombinant proteins were then electrically eluted from the gels using a model 422 Electroeluter (Bio-Rad laboratories, USA), and dialyzed with phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic, 1.8 mM potassium dihydrogen phosphate, pH 7.4) at 4 °C for 16 h. 100 mg of recombinant protein was injected into mice four times every 10 days.

In this study, nucleotide sequences coding the C-terminal end of BmGr5 and BmGr7 which were indicated in Supplementary Fig. 4C were amplified using specific primers (Table 1.3), and inserted into pGEX4T-3 vector to produce recombinant proteins. These peptide fragments have a high degree of identity with the C-terminal conserved region used to raise the anti-BmGr6 antiserum (Fig. 1.12). Subsequently,

cross-immunoreaction of anti-BmGr6 antiserum to recombinant proteins of BmGr5 and BmGr7 were performed by western blot.

1.2.6. Western blotting and immunohistochemistry of transfected cells

In the present study, specificity of antisera was confirmed first by western blotting analysis using total protein from recombinant *E. coli* cells, and further confirmed by immunocytochemistry using of BmGr6 gene expressing in HEK293T cells.

Western blotting analysis was carried out as described previously (Yoshizawa et al., 2011). In brief, the BmGr6 antigen peptide was produced by *E. coli* which was described above and the cells were directly mixed with SDS-PAGE sample buffer. The samples were boiled for 5 min and immediately loaded on the 12.5% SDS-PAGE gel. After SDS-PAGE, proteins contained in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 2% bovine serum albumin (BSA) in Tris-NaCl-Tween buffer (TNT) for 1 h at room temperature and incubated with anti-BmGr6 antiserum in TNT at a 1:10,000 dilution for 1 h. After three washes with TNT, the membrane was incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad Japan, Tokyo) in TNT at a 1:10,000 dilution for 1 h. After three washes with TNT, membrane was stained using ECL™ prime Western blotting detection reagent (GE Healthcare), and visualized by exposure to X-ray film (Fuji Co., Japan).

HEK 293T cells expressing the BmGr6 gene were seeded onto poly-D-lysine coated glass cover slips. Next, cells were transiently transfected as described above.

After 24 h at 37 °C, 5% CO_2, cells were washed with PBS, and fixed by 4% paraformaldehyde in PBS, then washed again with PBS and conducted immunocytochemistry. The incubation and washing were done like the immunohistochemistry of oral sensory organs and midgut cryosections (Detail in immunohistochemistry and microscopy).

As negative controls, the transfected cells were immunostained with secondary antibody alone and non-transfected HEK293T cells were immunostained by anti-BmGr6 antiserum.

1.2.7. Immunohistochemistry and microscopy

Heads of three-day-old fifth instar larvae were removed, washed with 70% ethanol, and fixed overnight (12-24 h) at 4 °C in 4% paraformaldehyde in PBS. After fixation, the antenna, labrum, maxilla and labium were separated, and each organ was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Co., Ltd., Japan) and frozen at -20 °C. The embedded specimens were mounted on an object holder and sectioned using a Tissue-Tek Cryo3® cryostat (Sakura Finetek Japan, Co., Ltd., Japan). 16 μm thick cryosections of each organ was placed on MAS-GP type-A coated slide glasses (Matunami glass, Japan) and air dried at RT for about 4 h. After wash with TNT for 5 min, sections were incubated in TNT with 0.01% Triton X-100 for 10 min and were blocked using TNT with 2% BSA for 1 h at RT and then incubate with primary antibody (anti-BmGr6 antiserum in TNT at a 1:1000 dilution) for 2 h. After three times 5 min washes with TNT, the sections were incubated in secondary

antibody, a goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, USA), at a 1:1000 dilution in TNT for 1 h. Next, twice rinses for 5 min each in TNT, the sections were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; l μg/ml, Sigma Aldrich, Schnelldorf, Germany) for 5 min at RT to stain the cell nuclei. After 5 min washes with TNT, the sections were mounted in 90% glycerol/PBS containing 1, 4-diazabicyclo [2.2.2] octane at RT and observed under a fluorescence microscope, LSM710/LSM710 NLO (CARL ZEISS) at RT. The same incubation and washing procedures were used for the HEK293T cells immunohistochemistry.

For whole mount immunocytochemistry, the midgut and nervous system from two-day-old third instar larvae were fixed at 4 °C overnight in 4% paraformaldehyde in PBS, containing 0.1% Triton X-100 (PBST). After fixation, tissues were washed 3 times for 10 min each in PBST, and then incubated for 1 h in TNT containing 2% BSA. The tissues were subsequently incubated with primary antiserum (anti-BmGr6 in TNT at a 1:1000 dilution) for 36 h. After washing 3 times for 1 h each, the tissues were incubated with a goat anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen) at a 1:1000 dilution in TNT at 4 °C overnight. After 3 times washing process was carried out as described above, tissues were also mounted in 90% glycerol/PBS containing 1,4-diazabicyclo [2.2.2] octane and observed under a fluorescence microscope, LSM710/LSM710 NLO (CARL EISS) at RT.

Estimation of the numbers of immunoreactive cells in whole midgut was conducted with the fluorescence microscope. Cells were counted in successive fields under low magnification. Negative control was prepared by application of the

preimmune mouse antiserum taken from the animal that was used to produce the primary antiserum. Secondary antibody incubation, and all subsequent processing, was performed in the dark. Likewise, all samples were stored in the dark at 4 °C until they were examined.

1.3. Results

1.3.1. RT-PCR analysis of *BmGr6* gene expression

To determine the sites of BmGr6 expression in the oral sensory organs, midgut, nervous system, and other *B*. *mori* tissues, RT-PCR experiments were performed using total RNA prepared from tissues taken from fifth instar larvae. Primers (see materials and methods) were designed to span an intron to differentiate between amplification of genomic DNA and cDNA. As shown in Fig. 1.1, RT-PCR produced intense BmGr6 bands from the antennae, maxillary, labium, thoracic leg, proleg, caudal leg, anterior midgut, middle midgut, posterior midgut, hindgut, malpighian tubule, and testis. Faint bands were detected in the brain, labrum, mandible, silk gland, and ovary (Fig. 1.1 A). Interestingly, no bands were detected in the foregut using a standard number of RT-PCR cycles; however, a weak band was detected when the products were amplified using 40 cycles suggesting that BmGr6 is expressed in the foregut at low levels. To analyze the detailed location of BmGr6 gene expression in the ventral nerve cord, I examined expression in each ganglion and found that all of the ganglia expressed BmGr6 (Fig. 1.1 B). These results indicate that BmGr6 is expressed broadly in *B*. *mori* larvae. On the other hand, *BmGr5* and *BmGr7*, which were most closely related to *BmGr6* in phylogenetic analysis (Fig. 1.4), were used as controls. The expression profile of *BmGr5* and *BmGr7* differed with *BmGr6*. *BmGr5* expression was limited to the antennae, maxillary, labium, proleg, testis, ovary and fat body. Whereas *BmGr7* was expressed in a number of tissues but scarcely in ventral nerve cord, and no amplimer was detected in maxillary, labium,

anterior and posterior midgut, silk gland, malpighian tubule and fat body (Fig. 1.1).

1.3.2. Antiserum specificity

To determine the distribution of BmGr6-expressing cells and validate the RT-PCR results, I produced a mouse antiserum to BmGr6 (anti-BmGr6 antiserum). Initially, I attempted to produce several different regions of BmGr6 as recombinant protein fragments in *Escherichia coli*, but the amounts of the recombinant protein produced were null or too small. A 97-amino acid residue (^{353}QVNLAS-TFYNYS^{449}) at located the C-terminal end of the BmGr6 sequence was used successfully as an immunogen and anti-BmGr6 mouse antiserum was generated. The specificity of the antiserum was confirmed by western blot analysis of total protein including a *glutathione-S-transferase* (GST)-BmGr6 fusion protein isolated from recombinant *E. coli* cells. A single band with a molecular mass of 38 kDa, consistent with the theoretical total mass of a fusion protein composed of the 97 BmGr6 amino acid residues and GST, was detected specifically suggesting that the specificity of the anti-BmGr6 antiserum was adequate (Fig. 1.2 A).

The specificity of the antiserum was further verified by immunohistochemistry using HEK293T cells expressing BmGr6. BmGr6-expressing HEK293T cells were stained with the anti-BmGr6 antiserum (Fig. 1.2 B-a) and no stained cells were observed when the primary antibody was omitted (Fig. 1.2 B-b). In addition, no immunoreactivity was detected in untransfected HEK293T cells, even when immunostained with an anti-BmGr6 antiserum (Fig. 1.2 B-c). However, an amino acid

sequence alignment of BmGr6 with other BmGrs showed that the amino acids used to create the Gr6 antibody were conserved in other BmGrs [BmGr4 to BmGr10 (Wanner and Robertson, 2008)] which belong to sugar and fructose receptor clades (Figs. 1.3 and 1.4); thus, the anti-BmGr6 antibody may cross-react with other BmGrs. I performed immunohistochemistry to exclude this possibility.

Homology and phylogenetic analyses revealed that BmGr6 is most closely related to BmGr5 (C-terminus, 68%), followed by BmGr7 (C-terminus, 51%) (Figs. 1.3 and 1.4). Unfortunately, I was unable to acquire HEK293T cells expressing BmGr5 to examine whether anti-BmGr6 antiserum cross-reacts with BmGr5 because the BmGr5 gene lacks the first exon (Wanner and Robertson, 2008). However, I was able to acquire BmGr7-expressing HEK293T cells. I stained HEK293T cells expressing flag tag appended-BmGr7 with anti-BmGr6 antiserum to determine the specificity of the anti-BmGr6 antiserum and no stained cells were observed, although anti-flag antiserum did detect BmGr7 expression (Fig. 1.5 A). To further deny the possibility of cross-reactivity of the anti-BmGr6 antiserum to other BmGrs, *E. coli* produced recombinant BmGr5 and BmGr7 protein fragments, which have a high degree of identity with the C-terminal conserved region used to raise the anti-BmGr6 antiserum were prepared and western blot was conducted (Fig. 1.6). A prominent band was detected from the recombinant proteins of BmGr6, however, not any band was detected from BmGr5 and BmGr7 recombinant proteins (Fig. 1.6 B). In addition, anti-BmGr9 antiserum, which was raised against a partial fragment of BmGr9 (a D-fructose receptor), ^{209}SLRARL-DYDYGL^{306}, stained BmGr9-expressing HEK293T

cells, but anti-BmGr6 antiserum did not (Fig. 1.5 B). Taken together, these results showed that the antiserum was specific to BmGr6 and could be used for immunohistochemistry to detect BmGr6 expression in *B*. *mori* tissues.

1.3.3. Distribution of BmGr6-expressing cells in oral sensory organs

I performed immunohistochemistry analysis with the anti-BmGr6 antiserum to determine the distribution of BmGr6-expressing cells in the oral sensory organs of *B*. *mori* larvae. Negative control experiments were conducted using pre-immune mouse serum taken from the animal that was subsequently immunized with the Gr6 peptide to produce the primary antiserum, and no immunoreactive cells were observed (Fig. 1.15).

Previous studies have shown that the olfactory chemosensilla in lepidopteran larvae are located in the antennae and maxillary palp (Schoonhoven and Van Loon, 2002) and that the gustatory chemosensilla are located in the maxillary galea, maxillary palps, and the epipharynx (Albert, 1980; Asaoka and Shibuya, 1995; Schoonhoven and Van Loon, 2002). Therefore, to investigate which cells express BmGr6 in the sensilla of oral sensory organs, cryosections of antenna, labrum, maxillary galea, maxillary palpus, and labium from fifth instar larvae were incubated with anti-BmGr6 antiserum. As depicted in Fig. 1.7A, clusters of immunostained cells were observed in the second and third antenna segments. The cytoplasm around the nuclei of cells was stained intensely as rings or sometimes rings of granules. Labrum cryosections also exhibited immunostained cells beneath the epipharyngeal sensilla

(Fig. 1.7C, upper) and clusters of immunostained cells were also observed in the base of the labrum (Fig. 1.7C, bottom). In maxillary galea, a few closely placed cells were immunostained beneath the lateral styloconic sensillum, but no immunostained cells were found beneath the medial sensillum (Fig. 1.7D). This was confirmed using sections derived from three different maxillary galea. A few randomly scattered cells were also stained in the maxillary palps (Fig. 1.7E), but many stained cells were found in labium, which plays roles in both gustation and olfaction (Fig. 1.7F). These results confirmed the RT-PCR results.

1.3.4. Distribution of BmGr6-expressing cells in the midgut

The *B*. *mori* larval midgut can be divided into three different regions, the anterior, middle, and posterior midgut, based on the folding and color of the tissue (Giordana et al., 1998). Our RT-PCR experiments showed that BmGr6 was expressed in all regions of the midgut (Fig.1.1 A). Moreover, the onset and duration of *BmGr6* expression during different development stages were compared. cDNAs prepared from midgut of different larval stages (1st to 5th instars) were analyzed and PCR products were obtained with slight different intensities (Fig. 1.8). This result indicated that *BmGr6* was expressed in midgut throughout all developmental stages from the first instar to fifth instar. Previous studies have shown that mammalian taste receptors are not only expressed in taste cells of the tongue, but also in gut enteroendocrine cells (Dyer et al., 2005; Mace et al., 2007; Margolskee et al., 2007). Likewise, some insect Grs are also expressed in midgut enteroendocrine cells (Park and Kwon,

2011b). I therefore asked whether BmGr6 was also expressed in midgut enteroendocrine cells. To visualize the BmGr6-expressing cells in the midgut, I performed immunohistochemistry experiments using whole-mount midguts from third instar larvae. Numerous immunostained cells were found that were located mainly in the middle and posterior midgut (Fig. 1.9, MMa-c and PMa-c) and only a few immunostained cells were found in the anterior midgut (Fig. 1.9, AMa-b). Stained cells were observed clearly and could be counted in the midgut allowing us to estimate the number of stained cells in the guts of 35, two-day-old third instar larvae in successive fields under low magnification using a fluorescence microscope. As shown in Fig. 1.10A, the numbers of immunostained cells differed between individuals. The number of immunostained cells ranged from 0 to 23, 11 to 69, and 29 to 172 in the anterior, middle, and posterior midgut, respectively. In addition, the number of immunostained cells increased from the anterior to posterior midgut. On average, the midgut contained 122 cells per larva with six cells in the anterior region, 33 cells in the middle region, and 83 cells in the posterior region (Fig. 1.10B).

As shown in Fig. 1.11A, some immunostained cells observed in the whole-mount midgut exhibited a triangular shape with a broad base directed toward the basement membrane of the midgut and the peak directed toward the gut lumen. A triangular shape is a fundamental characteristic of midgut enteroendocrine cells. To better observe the morphology of these immunostained cells, I performed immunohistochemistry on cryosections from the anterior, middle, and posterior midgut. All immunostained cells in these cryosections exhibited one of three typical

shapes: pyramidal-flask shaped cells characterized by a narrow apical membrane facing the lumen and a broader basolateral membrane (Fig. 1.11 B-a, -b, and -e), bottle-shaped cells with a slender oval shape and a long apical extension and usually without a wide base (Fig. 1.11 B-c left and -d), and gourd-shaped cells with a wide cell body positioned on the basal lamina and an apical extension to the gut lumen (Fig. 1.11 B-c right).

1.3.5. BmGr6 expression in the nervous system of *B. mori* larvae

In *D. melanogaster,* some Grs are expressed in neurons in the brain, SOG, AG, and in peripheral sensory neurons (Miyamoto et al., 2012; Park and Kwon, 2011a; Thorne and Amrein, 2008). RT-PCR analysis demonstrated that BmGr6 was expressed in the brain, SOG, and thoracic-abdominal ganglia (Fig. 1.1 B). Moreover, cDNAs prepared from CNS of different larval stages (1st to 5th instars) were analyzed. Using these as templates, PCR products were obtained with slightly different intensities (Fig. 1.8 B). This result indicated that *BmGr6* was expressed in the CNS throughout development from the first instar to fifth instar. Thus, BmGr6 may also be expressed in the *B*. *mori* nervous system. To validate our RT-PCR results and visualize the cells which express BmGr6 in the nervous system, I performed immunohistochemistry using whole-mount nervous organs. Immunostained cells or axons were present throughout the larval nervous system (Figs. 1.12 and 1.13).

Based on anti-BmGr6 staining, I counted more than 11 pairs of immunostained cells in the larval brain (BR) and no cells were stained using the secondary antiserum

alone. These immunostained cells were observed in clusters located in the medial and lateral regions. Two pairs of cells with large somata (indicated by open, thick arrows in Fig. 1.12 A) and two pairs of median cells with ellipsoidal somata (indicated by solid arrows in Fig. 1.12 A) were observed in the intercerebralis. Other stained cells were found in the lateral region of the brain (indicated by arrowheads in Fig. 1.12 A).

The frontal ganglion (FG) contained two pairs of immunostained cells (Fig. 1.12 B). In the middle region of the SOG, a cluster of dorsomedial cells was stained (arrowheads in Fig. 1.12 C) and a dorsomedial cell in the anterior region of the SOG was also stained by the antiserum (superimposed in Fig. 1.12 C). The prothoracic ganglion (TG1) contained one pair of dorsolateral cells with relatively weak immunostaining (Fig. 1.12 D). The mesothoracic ganglion (TG2) and the metathoracic ganglion (TG3) contained two pairs of cells with relatively strong immunostaining (Fig. 1.12 E and F), that were typically located in the dorsolateral region.

All of the AG contained either one or two large immunostained cells (Fig. 1.12 G–L). The first abdominal ganglion (AG1) and the fifth abdominal ganglion (AG5) contained two large immunostained dorsomedial cells in the middle region of the ganglion. The second abdominal ganglion (AG2) and the fourth abdominal ganglion (AG4) contained a large dorsomedial immunostained cell in the middle region of the ganglion. In addition, the third abdominal ganglion (AG3) and the sixth abdominal ganglion (AG6) contained a large dorsolateral stained cell in the anterior region and a

large dorsomedial stained cell in the posterior region of the ganglion, respectively. AG6 also exhibited several weakly stained cells in the anterior region. In AG7 (the anterior neuromere) of the terminal abdominal ganglia (TAG), at least two pairs of large dorsomedial immunostained cells were observed and, in the anterior region of AG8 (the posterior neuromere of TAG), four large dorsomedial immunostained cells were observed. AG8 contained two pairs of dorsolateral immunostained cells (arrowheads in Figure 1.12 M–N) and a cluster of immunostained cells in the posterior region of the ganglion (Fig. 1.14).

The corpora cardiaca (CC), corpora allata (CA), and nervus corporis cardiaca (NCC) 1 and 2 were covered with immunostained axons that originated from the brain, but no stained cells were observed inside the organs (Fig. 1.13, A1–A3). Although no stained cells were found in the foregut or hindgut, immunostaining was observed in nerves innervating the foregut and esophagus (Fig. 1.13 C) and immunoreactive neuropiles were observed on the surface of the hindgut (Fig. 1.13, B1–B2). In addition, immunostained axons within the proctodeal nerves protruding from the last abdominal ganglion (AG8) were also observed (Fig. 1.14)

1.4. Discussion

1.4.1. *BmGr6*-expression is not restricted to the gustatory organs

In this study, RT-PCR analysis demonstrated that, BmGr6, a member of the sugar clade in the phylogenic tree of gustatory receptors (Fig. 1.4), was expressed ubiquitously in larval sensing organs and mouthparts including the antennae, labrum, maxillary galea, maxillary paplus, and labium in *B. mori* (Fig. 1.1 A). This observation partly differs, however, from a previous report which showed that BmGr6 expression was limited to the maxilla and proleg and was not detected in the antennae, labrum, mandible, labium, thoracic leg, or gut (Sato et al., 2011). The differences between these observations may be due to differences in the primers used and the total RNA concentrations of the samples.

1.4.2. BmGr6 expression in the oral sensory organs of *B. mori* larvae

In the maxillary gustatory system of silkworm larvae, three gustatory receptor neurons (GRNs) in the lateral styloconic sensillum are sensitive to sucrose, *myo*-inositol, and glucose (Ishikawa 1963, 1966; Ishikawa and Hirao 1963). In the medial sensillum, only one GRN has been found and this GRN appears to be sensitive to bitter compounds (Descoins and Marion-Poll 1999; Tanaka et al. 1994). In this study, immunohistochemistry analyses showed that a few closely placed cells were immunostained in the lateral styloconic sensillum, but not in the medial sensillum. These results suggested that BmGr6 is involved in sugar detection. Moreover, in *D. melanogaster,* several sugar clade Grs were expressed in a single

cell (Dahanukar et al., 2007; Montell, 2009; Thorne et al., 2004). Several replications of our single cell RT-PCR analyses showed that both the BmGr6 and BmGr9 genes were expressed in the same cell dissected from the lateral styloconic sensillum by laser capture microdissection (LCM, paper in preparation) and BmGr9 was recently identified as a D-fructose receptor (Sato et al., 2011). These results also suggest that BmGr6 is involved in sugar detection. Aside from the maxillary galea, immunohistochemistry analysis showed that BmGr6 was expressed other oral sensory organs including the antennae, labrum, maxillary papilla, and labium. These results in combination with the RT-PCR results allow us to speculate that BmGr6 may be expressed in many oral sensory organs.

1.4.3. BmGr6 expression in the midgut of *B. mori* larvae

In mammals, taste receptors are not only expressed in taste cells of the tongue, but also in gut enteroendocrine cells (Dyer et al., 2005; Mace et al., 2007; Margolskee et al., 2007). Taste receptors expressed in mammalian luminal epithelium are thought to function as food sensors to avoid harmful substances and to regulate nutrient uptake in the gut (Breer et al., 2012; Sclafani, 2007). The digestive systems of insects and vertebrates share many similarities including cell types and development (Hartenstein et al., 2010; Park and Kwon, 2011b; Stainier, 2005). Park and Kwon (2011) reported that 12 Grs including DmGr43a (the fructose receptor) and DmGr64a (a member of the sugar receptor family) are also expressed in *D. melanogaster* adult gut enteroendocrine cells (Park and Kwon, 2011b). In addition, they showed that

DmGr43a and DmGr64a colocalized with neuropeptide F (NPF)-expressing enteroendocrine cells and another 10 Grs (members of the non-sugar receptor family) also colocalized with locustatachykinin (LTK) or diuretic hormone 31 (DH31) in enteroendocrine cells (Park and Kwon, 2011b). In this study, RT-PCR analysis showed that *BmGr6* was expressed in the midgut (Fig. 1.1 A) and, more importantly, immunochemistry revealed the typical cell shapes of BmGr6-expressing enteroendocrine cells in the midgut of *B. mori* larvae (Figs. 1.9–1.11). Furthermore, the number of BmGr6 immunostained cells was shown to increase from the anterior to the posterior portions of the midgut. (Figs. 1.9 and 1.10). The distribution of BmGr6-expressing cells is similar to that of allatostatin C-secreting enteroendocrine cells in the *D. melanogaster* midgut (Veenstra et al., 2008). Allatostatin C is expressed abundantly in the proximal part of the posterior midgut, but sparsely in the anterior and middle midgut. As described above, BmGr6 is a member of the sugar receptor family (Fig. 1.4), therefore, BmGr6 may be involved in perceiving information about sugars in the gut lumen during digestive processes and may control midgut motility through hormonal secretion. Studies on colocalization of BmGr6 with regulatory peptides including allatostatin C, sulfakinin, neuropeptide 1 (NPF1), and NPF2 are in progress. I also attempted to perform a ligand assay for BmGr6 using Ca^{2+} imaging of HEK293T cells with GCaMP3-pcDNA3.1 and by examining the electrophysiology of *Xenopus* oocytes using a two-electrode voltage-clamp; however, only BmGr9 and BmGr10 ligands were indicated and BmGr6 did not give positive results.

1.4.4. BmGr6 expression in the nervous system of *B. mori* larvae

Insects possess a complex nervous system for incorporating a variety of internal physiological information as well as external sensory information (Gullan and Cranston, 2005). The gustatory neurons of insects are chemosensory neurons that express gustatory receptors. Previous studies in *D. melanogaster* reported that DmGr28a and five alternatively spliced forms of DmGr28b were not only expressed in the chemosensory neurons of the sensory organs, but also in neurons of the brain (Thorne and Amrein, 2008). Park and Kwon (2011) reported that some Grs are expressed in abdominal neurons which project to the abdominal ganglia of the CNS (Park and Kwon, 2011a). A previous study also reported that DmGr43a is expressed in the brain, particularly in SOG neurons, the proventriculus, and in uterus-associated neurons (Miyamoto et al., 2012). In this study, our RT-PCR and immunohistochemistry experiments both showed that BmGr6 is expressed in the *B*. *mori* larval nervous system. In insects, the CNS consists of the brain, the SOG, and thoracic-abdominal ganglia. In this study, BmGr6 was expressed in cells in the larval *B*. *mori* brain (Fig. 1.12 A). These cells might be neurosecretory cells because they have the typical shape, size, and location of neurosecretory cells (Ichikawa, 1991). Previous studies showed that more than 20 neuropeptides including bommo-myosuppressin (BMS), bommo-FMRFamide (BRFa), and prothoracicostatic peptide (PTSP) are expressed in the CNS of *B*. *mori* larvae (Roller et al., 2008; Yamanaka et al., 2005; Yamanaka et al., 2006; Yamanaka et al., 2009). For example, BMS, which suppresses ecdysteroidogenesis in the prothoracic gland (PG) during

insect development, is produced in two pairs of medial neurosecretory cells in the larval *B*. *mori* brain and released into the hemolymph from the CC (Yamanaka et al., 2005). In this study, BmGr6 was expressed in cells which, based on their location and shape, appeared similar to these two pairs of medial neurosecretory cells (Fig. 1.12 A). Moreover, BRFa, which suppresses ecdysteroidogenesis in the PG, was expressed in two pairs of neurons with large cell bodies in each thoracic ganglion and these large thoracic neurons were identified as ventrolateral neurosecretory cells 1 and 2 (NS-$VTL_{1,2}$) (Yamanaka et al., 2006). BmGr6 was expressed in two pairs of large ventrolateral neurons in TG2 and TG3 (Fig. 1.12 E and F). In addition, PTSP, a multifunctional neuropeptide, was detected in cells in the brain, frontal ganglion, abdominal ganglia, and terminal abdominal ganglion in *B*. *mori* larvae (Yamanaka et al., 2009). BmGr6-expressing cells were also observed in the frontal ganglion, abdominal ganglia, and the terminal abdominal ganglion (Fig. 1.12 B and D–N). The size and location of the four large, anterior dorsomedial cells and the posterior-medial cluster of cells appeared remarkably similar to those of PTSP-expressing cells. Apart from BmGr6, the expression of two other Grs, DmGr28b.b and DmGr28b.c, was also reported in parts of intercerebralis-associated cells of the *D. melanogaster* brain. These cells are median neurosecretory cells which release *Drosophila* insulin-like peptides and they play a role similar to mammalian insulin in the regulation of sugar levels in the body (Thorne and Amrein, 2008). The expression of some Grs has been reported in the *D. melanogaster* SOG (Miyamoto et al., 2012; Thorne et al., 2004; Thorne and Amrein, 2008; Wang et al, 2004) and BmGr6 expression was also

observed in several SOG neurons (Fig. 1.12 C). These results suggest that the insect CNS has several types of gustatory-expressing neurosecretory cells. In addition, the thoracic ganglia in insects controls locomotion by innervating the legs and wings and the abdominal ganglia control respiration, circulation, heartbeat, diuresis, hindgut movement, abdominal posture, and functions of the genitalia and the ovipositor (Nässel, 1996). Therefore, although which of these functions is mediated by BmGr6 is not yet known, some signals, such as sugar sensing, appear likely to be processed by these ganglia.

BmGr6 is expressed not only in the CNS but also in the sympathetic nervous system, including the frontal ganglion (FG) (Fig. 1.12 B), which is a central pattern generator, may play a role in autonomous functions in circadian regulation, and may regulate foregut movement through allatostatins and allatotropin (Ayali et al., 2002; Duve et al., 1999; Sehadová et al., 2004). Previous studies have shown that the allatostatin and allatotropin axons exit the *Helicoverpa armigera* FG and extend through the recurrent nerve which is on the surface of esophagus. In addition, other allatostatin and allatotropin axons originating from neurones in the brain pass through the FG and extend to the foregut and midgut through the recurrent nerve (Duve et al., 1999; Davey et al., 2005). In this study, recurrent nerves stained with anti-BmGr6 antisera were observed to traverse the surface of the foregut (Fig. 1.13 C). These findings suggest the possibility that BmGr6-expressing cells in the brain and FG include allatostatin- and allatotropin-secreting cells, and that Gr6 plays a role in the regulation of feeding behavior. In addition, these results together suggest that Gr6

plays a role in the regulation of feeding behavior because the FG is a central pattern generator which regulates foregut peristalsis (Ayali et al., 2002). In this study, axons immunostained with anti-BmGr6 antiserum were also observed to traverse the surface of the hindgut (Fig. 1.13, B1and B2) and immunostained proctodeal nerves protruding from the last abdominal ganglion (AG8) were also observed (arrows in Fig. 1.14). Because previous studies reported that the midgut and hindgut are supplied with proctodeal neurons from the last abdominal ganglion (Duve et al., 1999; Davey et al., 2005), BmGr6 may also be involved in regulating hindgut movement by sensing information from the last abdominal ganglion.

Table 1.1 Primers used for RT-PCR

Gene	S/AS	Primer sequences (5'-3')
β-actin	S	AACTGGGATGACATGGAGAAGATCTGGC
	AS	GAGATCCACATCTGCTGGAAGGTGGA
BmGr5	S	ACCGTTCTGGCGAACATTAC
	AS	CAATAGTGCCAGCTACCGAC
BmGr6	S	CCTTCAGCTGTTTATTGTGTAGAGGT
	AS	AAATGAATCCGAAGCTGGTG
BmGr7	S	CAAATGGACGAGCAGCTAGA
	AS	AAGATGTTTCGGCAAATAATAATCTC

S, sense primer; AS, antisense primer.

Table 1.2 Primers used for cloning into pcDNA3.1 in this study

Gene	PCR (1st/2nd)	S/AS	Primer sequences (5'-3')
BmGr6	1st	S	GGATTTGTTCGTGATTTGTATGAGTT
	1st	AS	CTGAAGCGGGTGCTTGATTG
	2nd	S	CCGGAATTCATGCTTCTGAGAAACTACAA
	2nd	AS	CCGCGGGCCCCTACGAGTAGTTGTAAAATG
BmGr7	1st	S	CAGTACTGTACAGCGAAAGCCA
	1st	AS	GAATCAGCACTAATTCGTATGTCAC
	2nd	S	CCACCCGGATCCGATATGGTTTTGGAAGCGCATAC
	2nd	AS	GTCTTTGTAGTCGATTATAAGATGTTTCGGCAAAT
BmGr9	1st	S	CCTCTGAGGTTCGAGCCAC
	1st	AS	CTAAATAAGTGGTGACCGCTCC
	2nd	S	CGCGGATCCATGCCTCCTTCGCCAGATCT
	2nd	AS	CCGGAATTCTTAACTATCATATCGCTGGA

S, sense primer; AS, antisense primer. Underline indicate restriction endonuclease recognition sites.

Table 1.3 Primers used for recombinant proteins production

Gene	S/AS	Primer sequences (5'-3')
BmGr5	S	GACGAATTCGAAGGAGATGTGCCCAAAC
	AS	GTCCTCGAGAGCTGAATTGAAGCAACAC
BmGr6	S	TTCGTTGATAGGATCCCAAGTAAAT
	AS	CCGCTCGAGCTACGAGTAGTTGTAA
BmGr7	S	GAGGGATCCATTTATGATGCGAACGATG
	AS	GCGCTCGAGGTTAATTGAATCAGCACT

S, sense primer; AS, antisense primer. Underline indicate restriction endonuclease recognition sites.

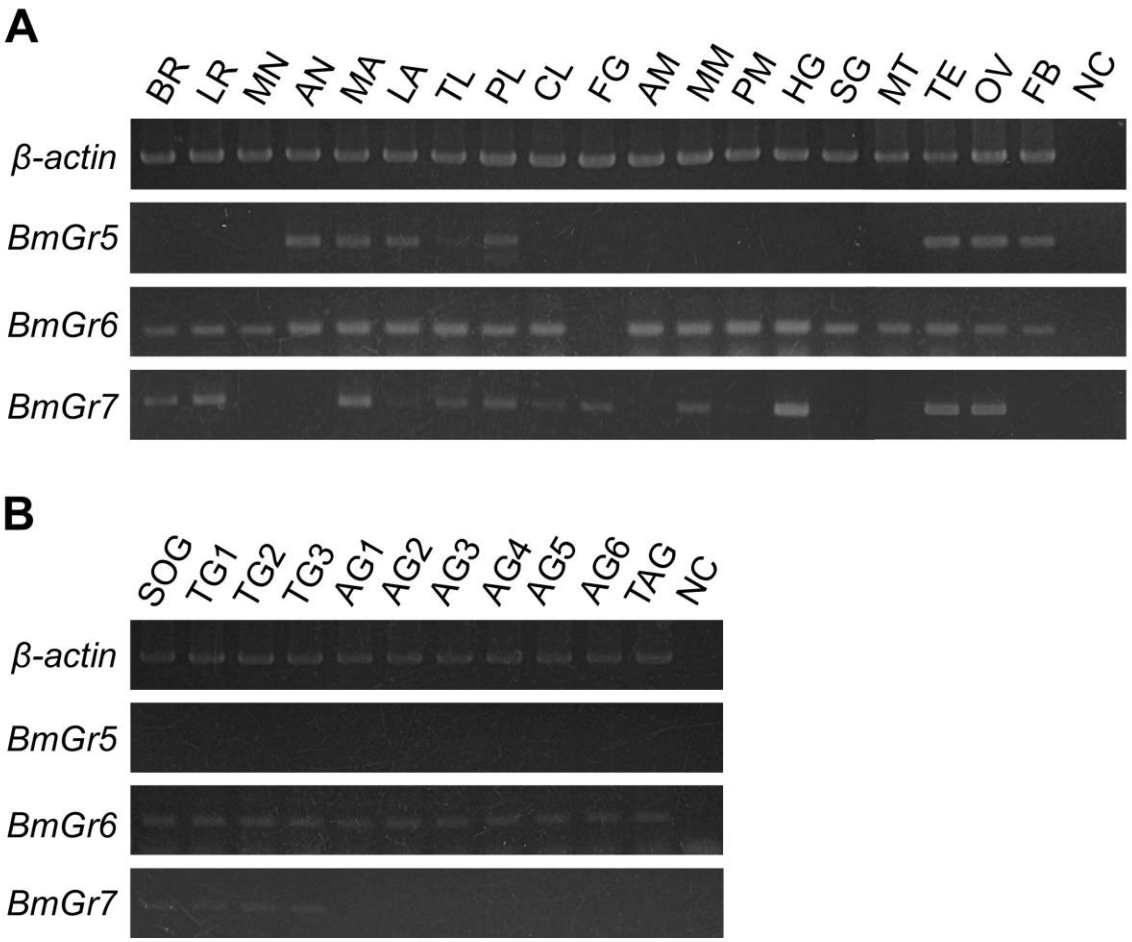


Fig. 1.1. RT-PCR analysis of *BmGr6* gene expression.

β-actin was used as an internal control; NC, negative control with no template in the reaction. (A) Tissues were dissected from two-day-old fifth instar larvae. BR, brain; LR, labrum; MN, mandible; AN, antenna; MA, maxilla; LA, labium; TL, thoracic leg; PL, proleg; CL, caudal leg; FG, foregut; AM, anterior midgut; MM, middle midgut; PM, posterior midgut; HG, hindgut; SG, silk gland; MT, malpighian tubule; TE, testis; OV, ovary; FB, fat body. (B) RT-PCR analysis of BmGr6 gene expression in each ganglion from two-day-old fifth instar larvae. SOG, suboesophageal ganglion; TG1, prothoracic ganglion; TG2, mesothoracic ganglion; TG3, metathoracic ganglion; AG, unfused abdominal ganglia; TAG, terminal abdominal ganglion.

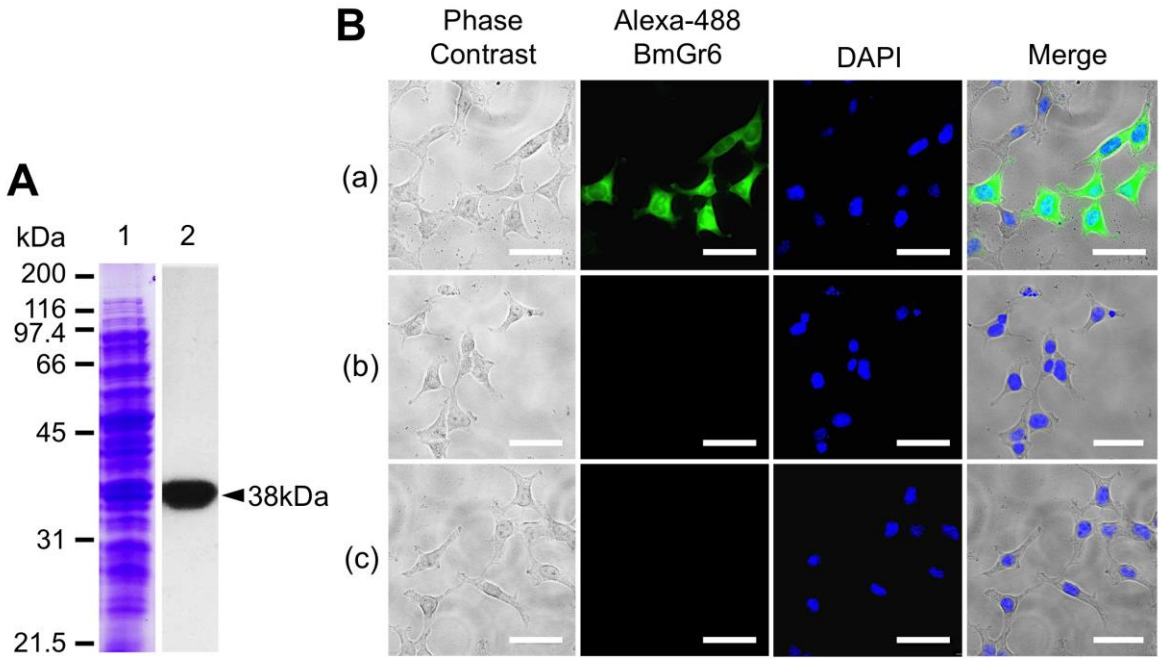


Fig. 1.2. Confirmation of anti-BmGr6 antiserum specificity and immune staining of HEK293T cells expressing BmGr6.

(A) Total proteins of BmGr6-expressing *E. coli* cells were separated by 12% (w/v) SDS-PAGE followed by Coomassie brilliant blue (CBB) staining or immunoblotting. The theoretical molecular mass of BmGr6 protein is 37.7 kDa. Lane 1, CBB staining of total protein isolated from *E. coli* BL21 (DE3) pLysS transformed with pGEX-4T-3 plasmid harboring a BmGr6 cDNA; Lane2, immunoblotting using 10 μg/ml anti-BmGr6 mouse antiserum. (B) Immunocytochemistry analysis of BmGr6-expressing HEK293T cells. HEK293T cells transfected with pcDNA3.1 plasmid harboring a BmGr6 cDNA were immunostained with anti-BmGr6 and secondary antibody (a) or with secondary antibody alone (b). (c) HEK293T cells without transfection were immunostained with anti-BmGr6 and secondary antibody. Scale bars = 20 μm.

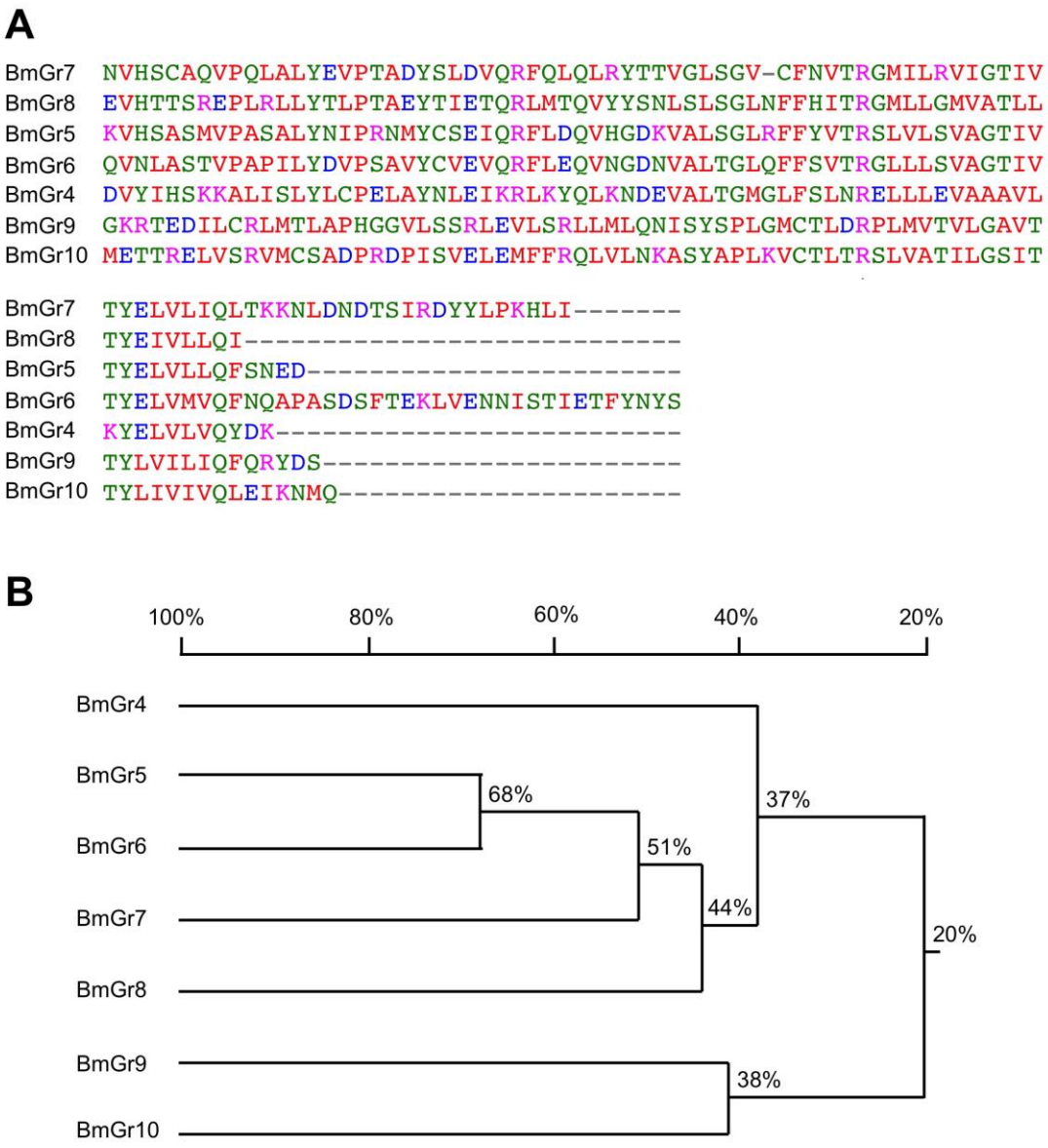


Fig. 1.3. Amino acid sequence alignment (A) and homology three (B) of the BmGr6 protein region used for immunization and the corresponding regions of other BmGr proteins. BmGr amino acid sequences were obtained from Wanner et al. (2008) and used to generate a multiple sequence alignment using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). To understand the identities among BmGrs, homology three was constructed using software DNAMAN, based on the multiple sequence alignment. Percentages of identity among BmGrs are indicated on the nodes.

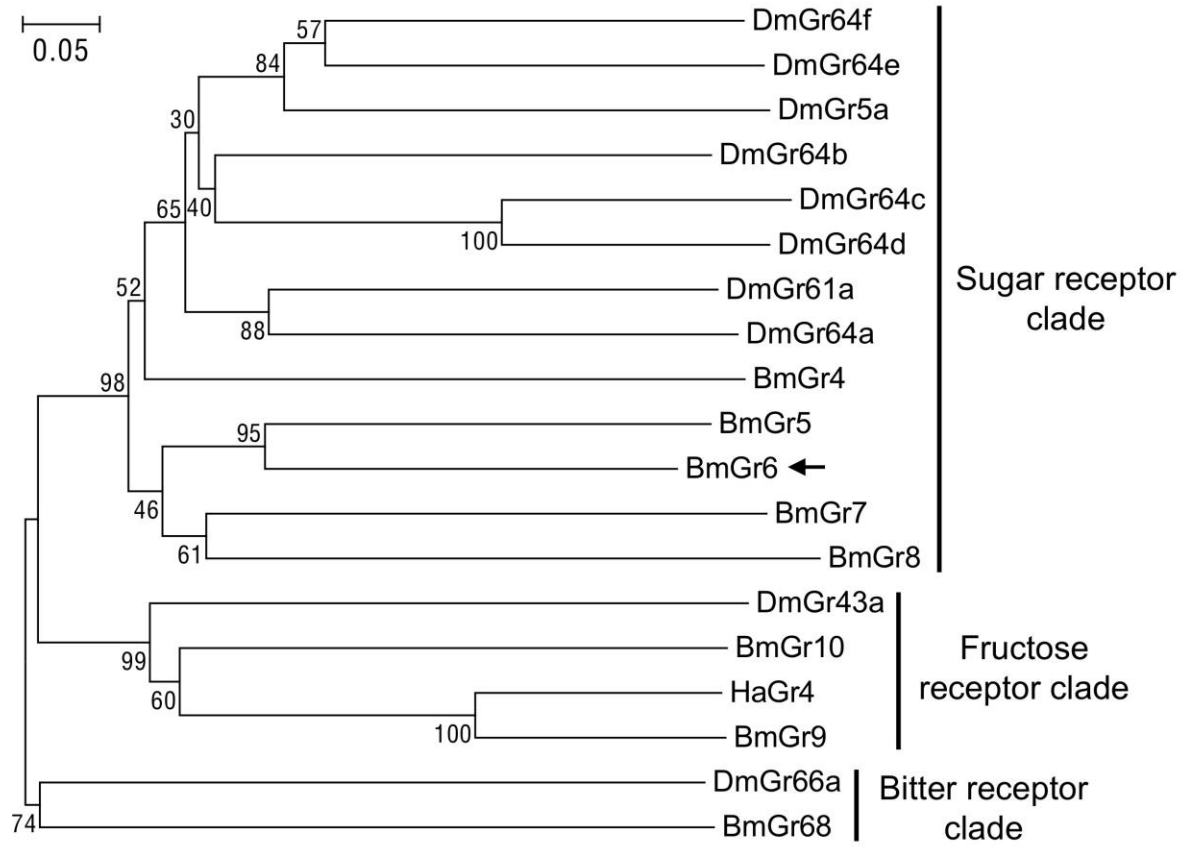


Fig. 1.4. Phylogenetic tree of selected gustatory receptors including BmGr6.

Total amino acid sequences of eleven *Bombyx mori* Grs, ten *Drosophila melanogaster* Grs, and one *Helicoverpa armigera* Gr were aligned using the ClustalW program and phylogenetic analysis was conducted using the neighbor-joining method (Mega 6.06 software). Numbers at the nodes indicate bootstrap values (1000 replicates). Molecules belonging to the bitter receptor clade were used as outgroup members to ascertain the putative sugar receptor and fructose receptor clades. BmGr6 is indicated by an arrow. The amino acid sequences of BmGr4 through BmGr10 (BmGr5 lacks the first exon) were obtained from Wanner et al. (2008) and the *D. melanogaster* Grs, HaGr4 and BmGr68, were obtained from NCBI. Accession numbers: DmGr5a, BAB68265; DmGr64f, AAF47826; DmGr64e, AAS64969;

DmGr64b, AAN11577; DmGr64a, AAN11576; DmGr61a, CBA14269; DmGr64c, CBA14213; DmGr64d, AAS64968; DmGr43a, NP_001286158; HaGr4 (HarmGR4, Jiang et al., 2015), JX982536; DmGr66a, AAF50447; and BmGr68, NP_001233217.

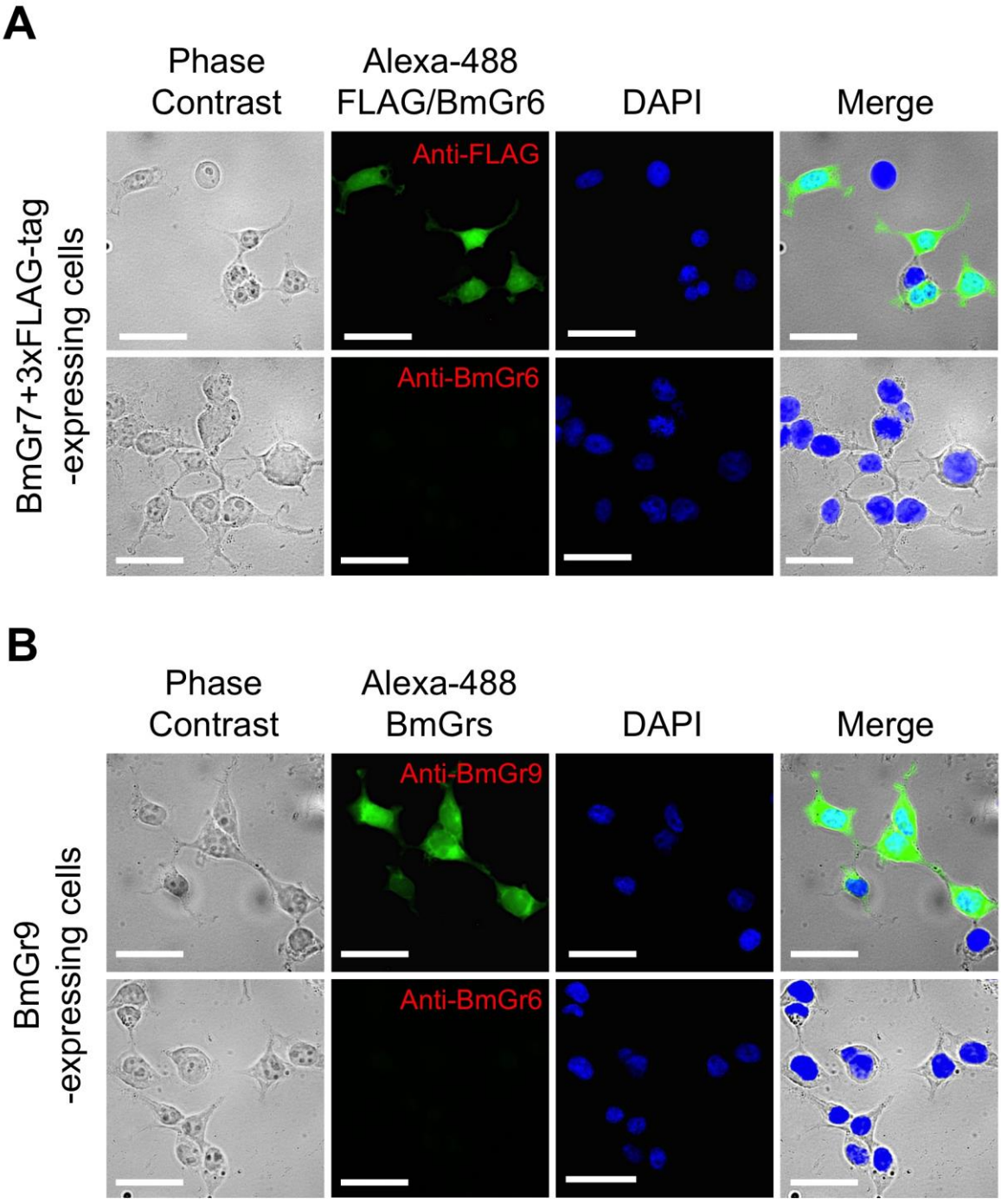
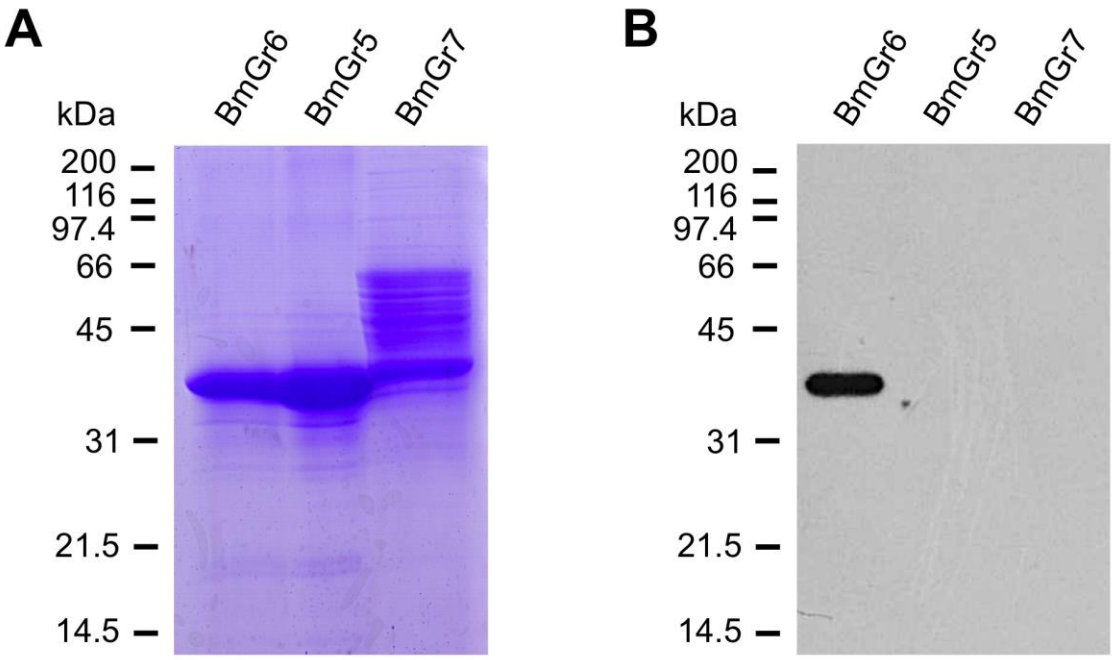


Fig. 1.5. Confirmation of anti-BmGr6 antiserum specificity by immune staining of HEK293T cells expressing BmGr7 (A) or BmGr9 (B).

(A) HEK293T cells expressing BmGr7 with a 3xFLAG-tag at the C-terminus were immunostained with anti-FLAG mouse antibody and anti-mouse IgG conjugated to

Alexa Fluor 488 (upper) or with anti-BmGr6 mouse antiserum and anti-mouse IgG conjugated to Alexa Fluor 488 (bottom). (B) HEK293T cells expressing BmGr9 were immunostained with anti-BmGr9 mouse antiserum and secondary antibody (upper) or with anti-BmGr6 and secondary antibody (bottom). Scale bars = 20 μm.



C

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BmGr7  IYDANDVCNENMDIIATASVTYLTYYVFSFLFLVTRALLLSIMAANVHSCAQVPQLALYE
BmGr5  ----KEMCPNY-PSGPLGGYEQIMYLLFSLSFLLGRSLVVSLVAAKVHSASMVPASALYN
BmGr6  ----------------------------------------------QVNLASTVPAPILYD

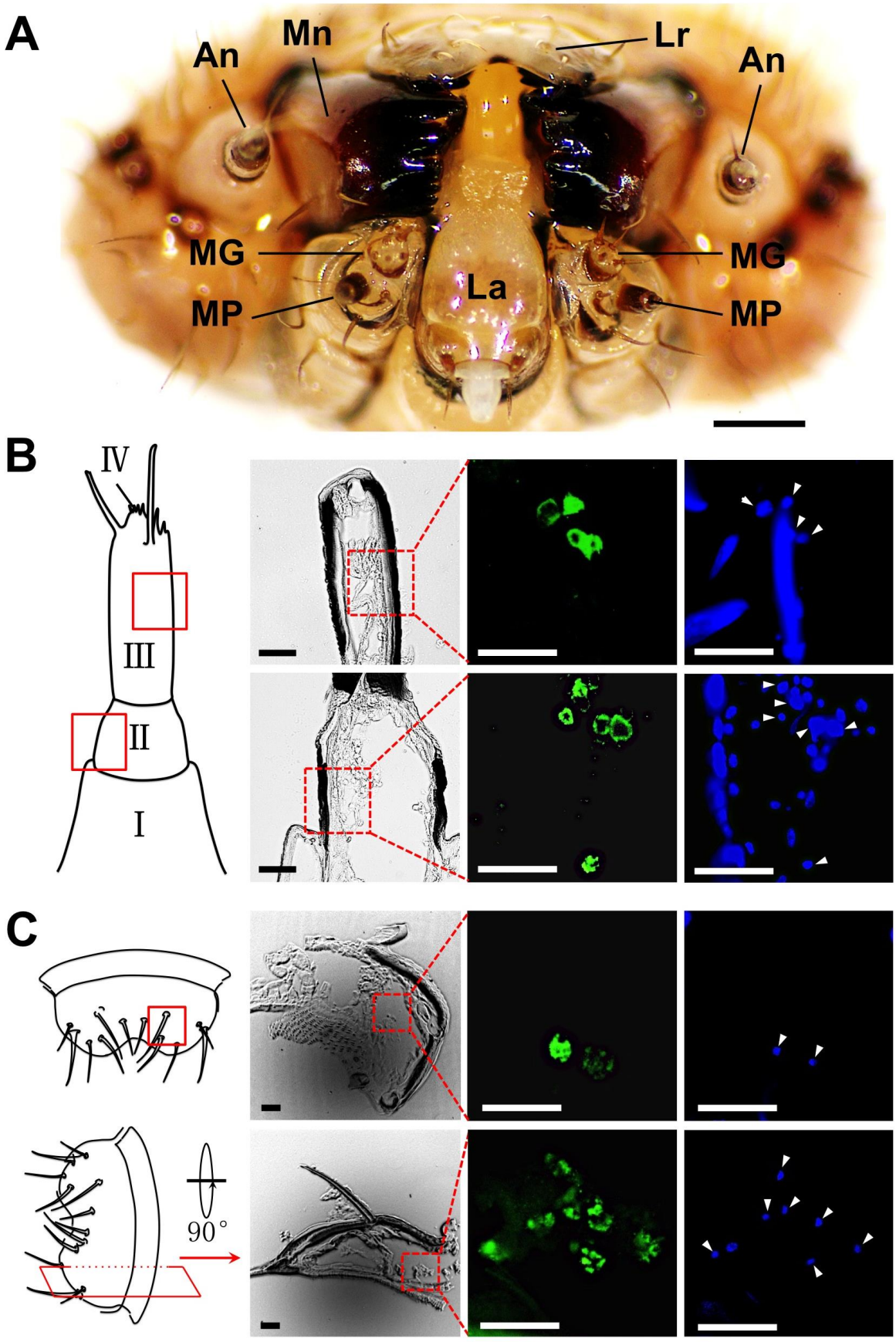
BmGr7  VPTADYSLDVQRFQLQLRYTTVGLSGVC-FNVTRGMILRVIGTIVTYELVLIQLT-----
BmGr5  IPRNMYCSEIQRFLDQVHGDKVALSGLRFFYVTRSLVLSVAGTIVTYELVLLQFS-----
BmGr6  VPSAVYCVEVQRFLEQVNGDNVALTGLQFFSVTRGLLLSVAGTIVTYELVMVQFNQAPAS

BmGr7  ----------------------
BmGr5  ----------------------
BmGr6  DSFTEKLVENNISTIETFYNYS
```

Fig. 1.6. Confirmation of anti-BmGr6 antiserum specificity by western blot.

The GST fusion protein of BmGr6, BmGr5 and BmGr7 were separated by 12% SDS-PAGE followed by Coomassie brilliant blue (CBB) staining (A) or immunoblotting (B). The theoretical molecular mass of BmGr6, BmGr5 and BmGr7 protein is 38 kDa, 39 kDa and 40 kDa, respectively. (A) CBB staining of GST fusion protein of BmGr6, BmGr5 and BmGr7. (B) Immunoblot analysis of BmGr6, BmGr5 and BmGr7 recombinant proteins using mouse anti-BmGr6 antiserum. (C) Amino

acid sequence alignment of the BmGr6 protein C-terminal region used for immunization and the corresponding regions (high similarity) of BmGr5 and BmGr7 proteins used for recombinant proteins production. Amino acid sequences were obtained from Wanner et al. (2008) and used to generate a multiple sequence alignment using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).


A
An
Mn
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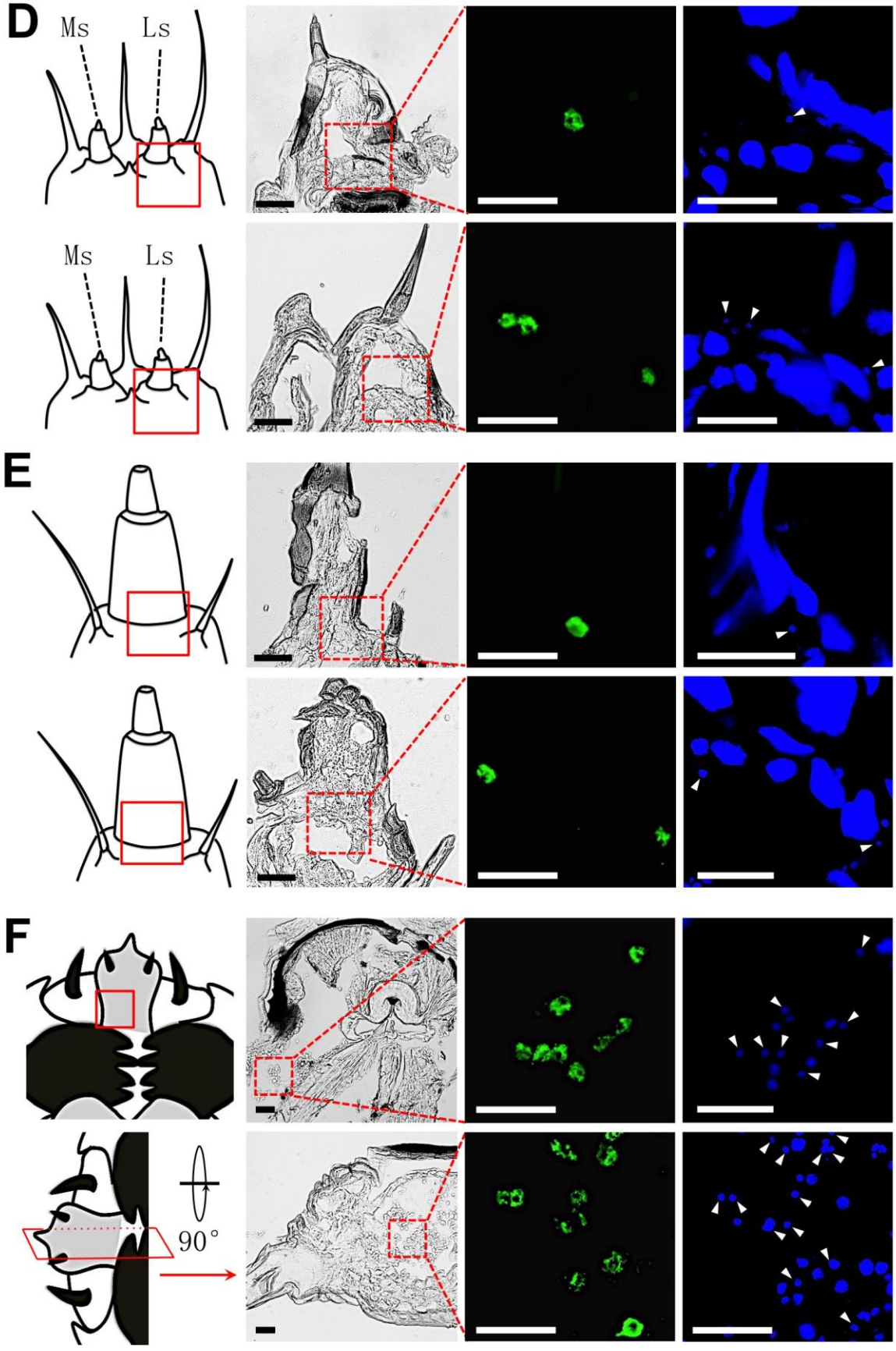
D
Ms
Ls
Ms
Ls
E
F
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Fig. 1.7. BmGr6 expression in larval oral sensory organs.

Cryosections of larval oral sensory organs were stained with anti-BmGr6 mouse antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488. (A) Stereoscopic microscopy image of a fifth instar larval head showing the antennae (An), labrum (Lr), mandible (Mn), maxillary galea (MG), maxillary palpus (MP) and labium (La). Scale bar = 300 μm. (B–F) Fluorescence microscopy images of BmGr6-expressing cells in cryosections of antennae (B), labrum (C), maxillary galea (D), maxillary palps (E), and labium (F). Panels on the left show schematic drawings of oral sensory organs, second panels show transmitted light microscopy images, third panels show fluorescence images of higher magnification of the areas boxed in the second panels, and panels on the right are images of DAPI staining of the same cryosections. Scale bars = 30 μm.

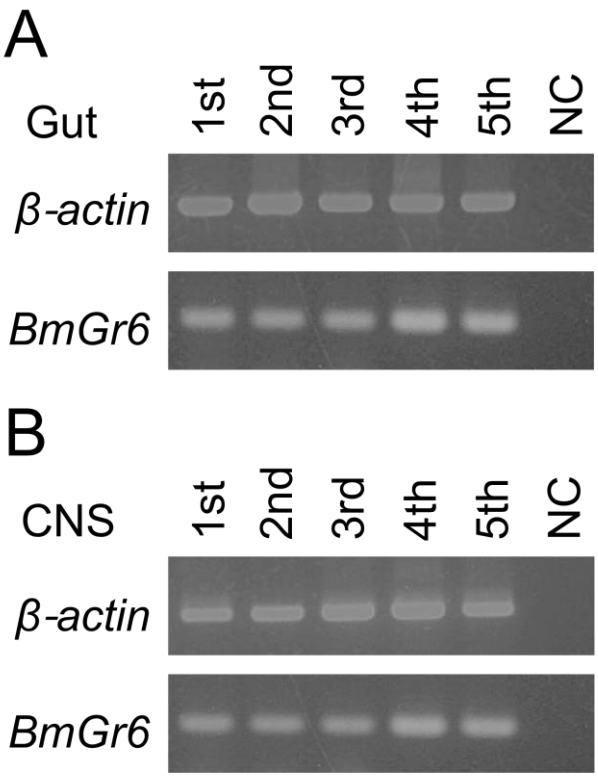


Fig. 1.8. Comparison of the *BmGr6* gene expression in the midgut and CNS of different instar stages.

Tissues were dissected from two-day-old of 1st to 5th instar larvae, respectively. *β-actin* was used as the internal control. NC, negative control, indicates no template in the reaction. Amplification products were analyzed on agarose gels and visualized by UV illumination after ethidium bromide staining.

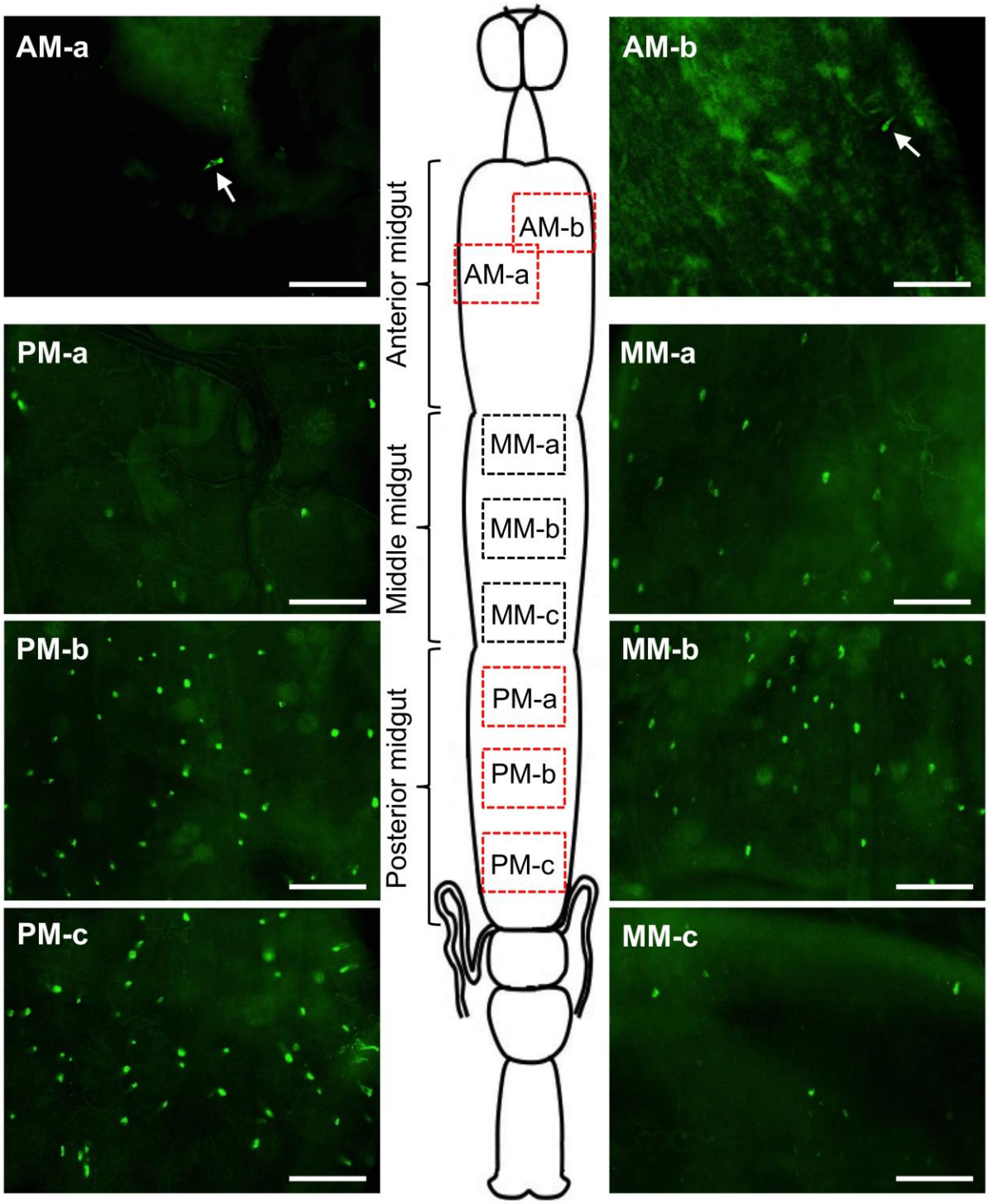


Fig. 1.9. Whole-mount immunohistochemistry analysis of BmGr6-expressing cells in a *B. mori* third instar larval midgut.

Whole-mount guts were stained with anti-BmGr6 mouse antiserum in combination with anti-mouse IgG conjugated with Alexa Fluor 488. Immunoreactive cells were

observed in the anterior (AM) (arrows), middle (MM), and posterior portions (PM) of the midgut. An illustration of the digestive tract of a *B*. *mori* larva depicting the locations of all the images in this figure is shown in the middle panel. Scale bars = 100 μm.

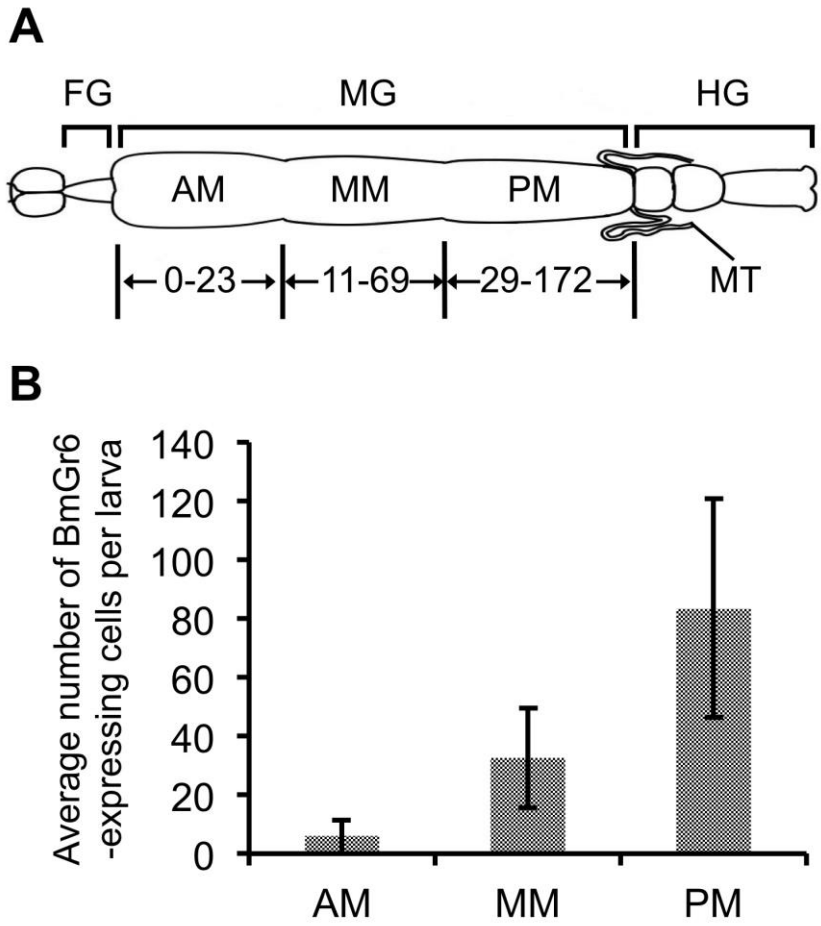


Fig. 1.10. BmGr6-expressing cells in the *B. mori* third instar larval midgut.

Whole-mount guts were stained with anti-BmGr6 mouse antiserum in combination with anti-mouse IgG conjugated with Alexa Fluor 488. (A) The lowest and highest numbers of immunostained cells were observed in the anterior and posterior midgut, respectively. Numbers were calculated based on whole-mount staining of 35 two-day-old third instar larvae using anti-BmGr6 antiserum. (B) The average number of immunostained cells in the anterior, middle, and posterior midgut based on the experiment in (A). Data are represented as mean ± standard deviation. FG, foregut; MG, midgut; AM, anterior MG; MM, middle MG; PM, posterior MG; HG, hindgut; MT, malpighian tubules.

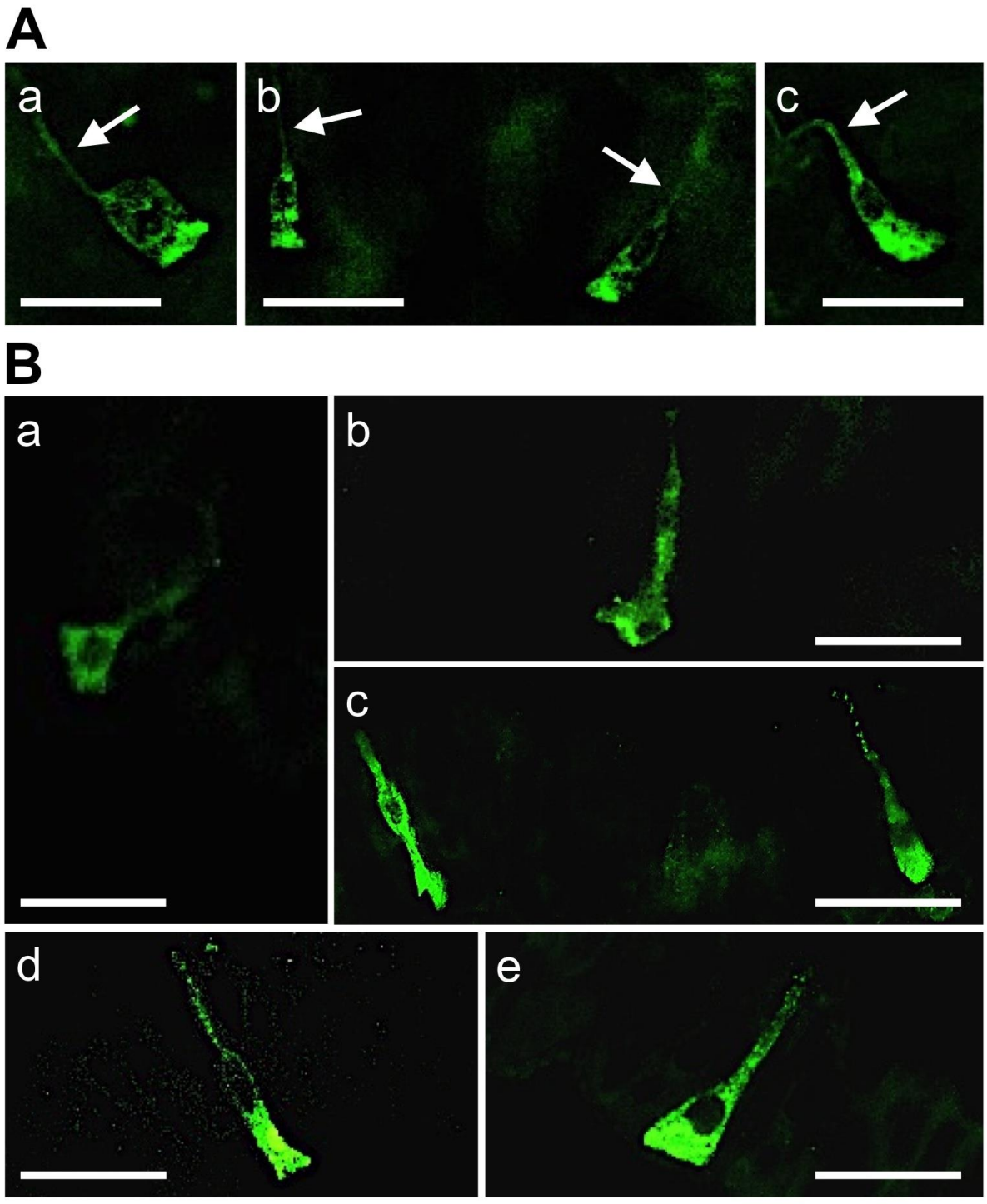


Fig. 1.11. Detailed view of anti-BmGr6 immunostained cells in the *B. mori* larval midgut.

Whole-mount tissues and sections were stained with anti-BmGr6 mouse antiserum in combination with anti-mouse IgG conjugated with Alexa Fluor 488. (A) Detailed view

of anti-BmGr6 immunostained cells observed in the anterior (a), middle (b), and posterior midgut (c). (B) Anti-BmGr6 immunostained cells observed in cryosections from anterior (a), middle (b and c), and posterior midgut (d and e). (a, b, and e) Pyramidal-flask shaped cells with a wide base abutting the basal lamina and a long apical extension of the gut lumen. (c left and d) Bottle-shaped cells characterized by a narrow apical membrane in contact with the lumen and a much broader basolateral membrane. (c right) Gourd-shaped cells with a long apical extension and lacking a wide base. Scale bars = 30 μm.

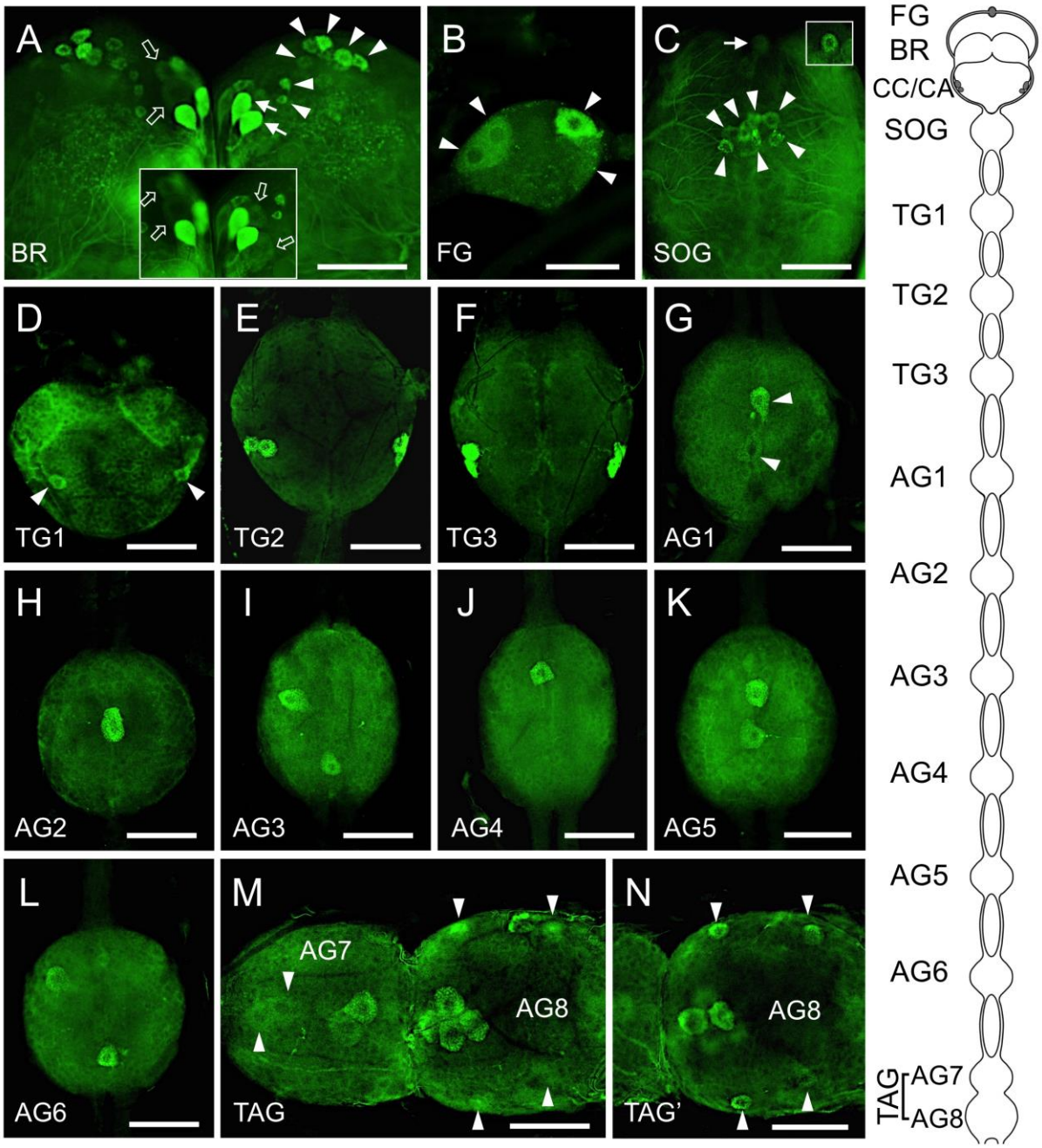


Fig. 1.12. BmGr6 expression in the central nervous system of *B. mori* larvae.

Whole-mount tissues were stained with anti-BmGr6 mouse antiserum in combination with anti-mouse IgG conjugated with Alexa Fluor 488. (A) Numerous cells were immunostained by anti-BmGr6 in the brain. Cells with large somata are indicated by open thick arrows (a brighter photo image of the same immunostained cells is superimposed) and median cells with ellipsoidal somata are indicated by solid arrows.

Other stained cells are indicated by arrowheads. (B) Frontal ganglion exhibiting two pairs of immunostained cells (arrowheads). (C) Immunostained dorsomedial cell in the anterior region (arrow, a brighter photo image of the same immunostained cell is superimposed) and several immunostained dorsomedial cells in the middle region of the SOG (arrowheads). (D) A pair of immunostained ventrolateral cells in TG1. (E and F) Two pairs of immunostained ventrolateral cells in TG2 and TG3. (G–L) Immunostained cells in the AG. (M–N) Two pairs of large immunostained dorsomedial cells in the anterior and posterior regions of AG7 and four immunostained large dorsomedial cells in the anterior region and two pairs of dorsolateral cells in the lateral of AG8 of the TAG. BR, brain; FG, frontal ganglion; CC, corpus cardiacum; CA, corpus allatum; SOG, suboesophageal ganglion; TG, thoracic ganglia; AG, abdominal ganglia; TAG, terminal abdominal ganglion. Scale bars = 100 μm (A and C-N) or 50 μm (B)

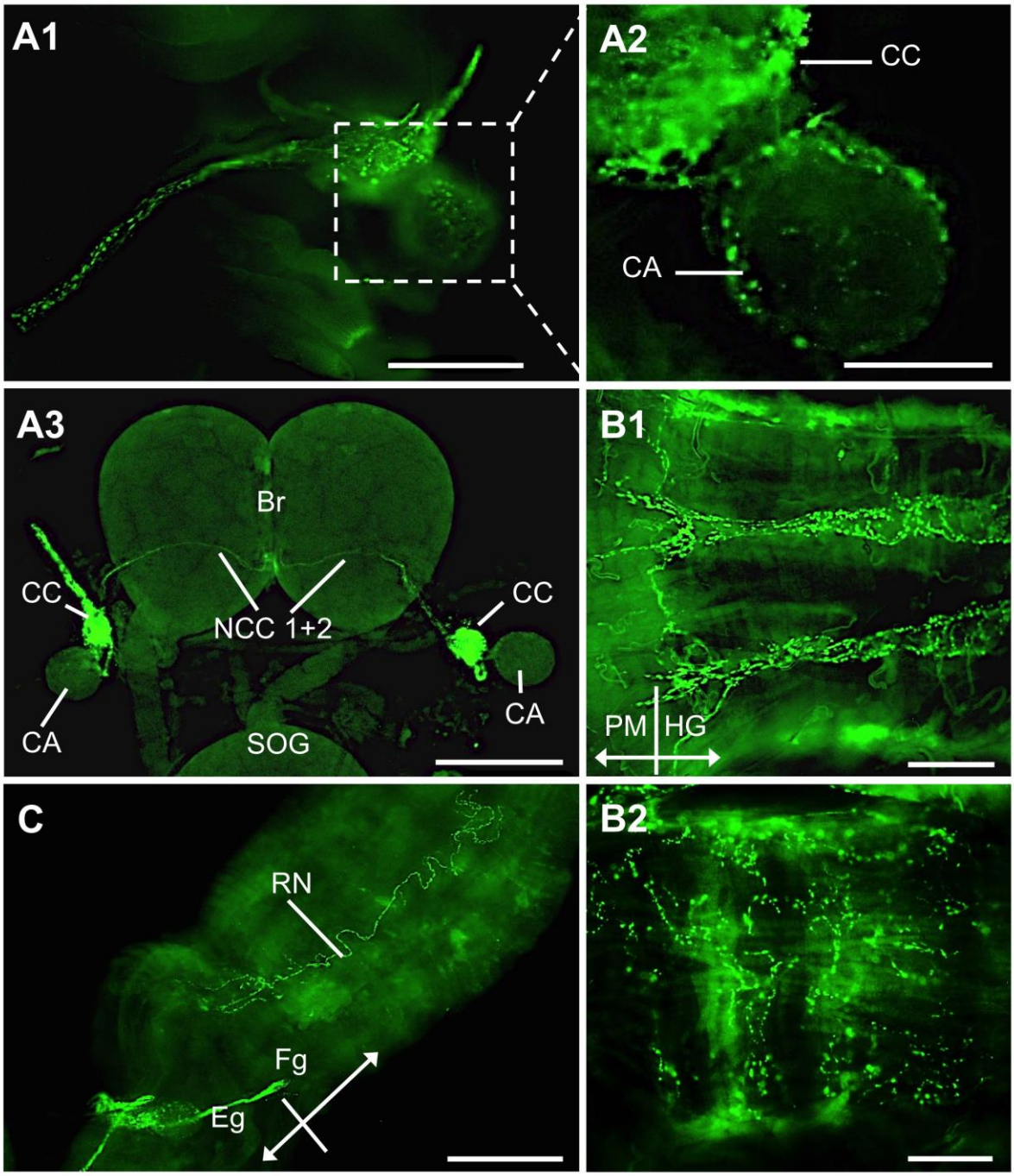


Fig. 1.13. BmGr6 immunoreactivity in the larval nervous system.

Whole-mount tissues were stained with anti-BmGr6 mouse antiserum in combination with anti-mouse IgG conjugated with Alexa Fluor 488. (A1–A2) Immunoreactive axons covering the corpus cardiacum (CC) and corpus allatum (CA) of a larva. A2 shows a higher magnification of the area enclosed by the dashed square in A1. Scale

bars = 100 μm (A1) or 50 μm (A2). (A3) Nervus corporis cardiac (NCC) 1 and 2 and CC were stained. Scale bar = 100 μm. (B1–B2) Immunostained neuropiles on the hindgut of a larva. Scale bars = 20 μm. (C) Dorsal view of the foregut and a part of the esophagus. Immunoreactivity is evident in the recurrent nerve innervating the foregut. Br, brain; SOG, suboesophageal ganglion; PM, posterior midgut; HG, hindgut; Eg, esophagus; Fg, foregut; RN, recurrent nerve. Scale bar = 100 μm.

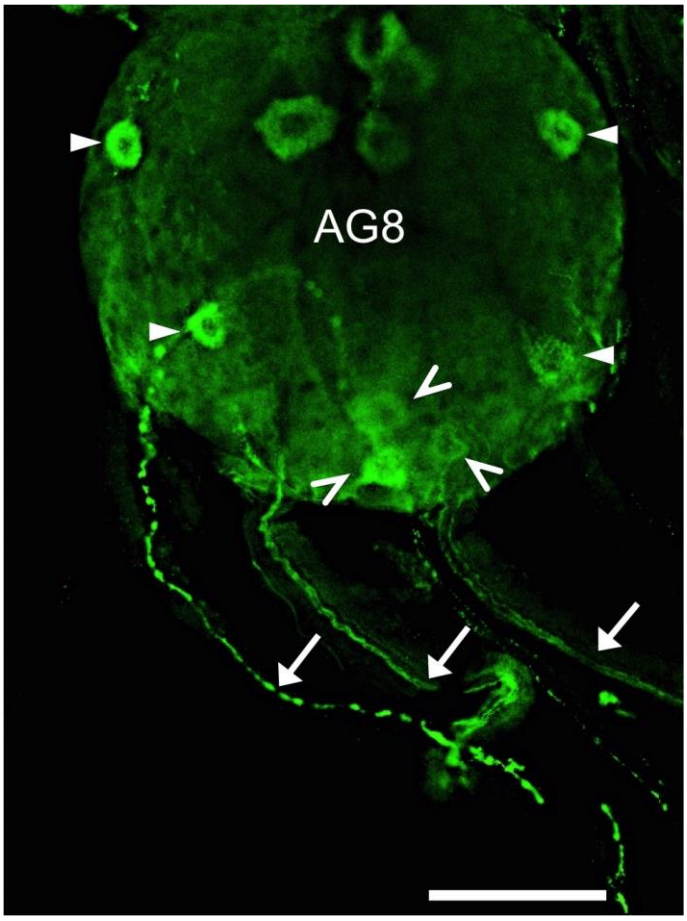
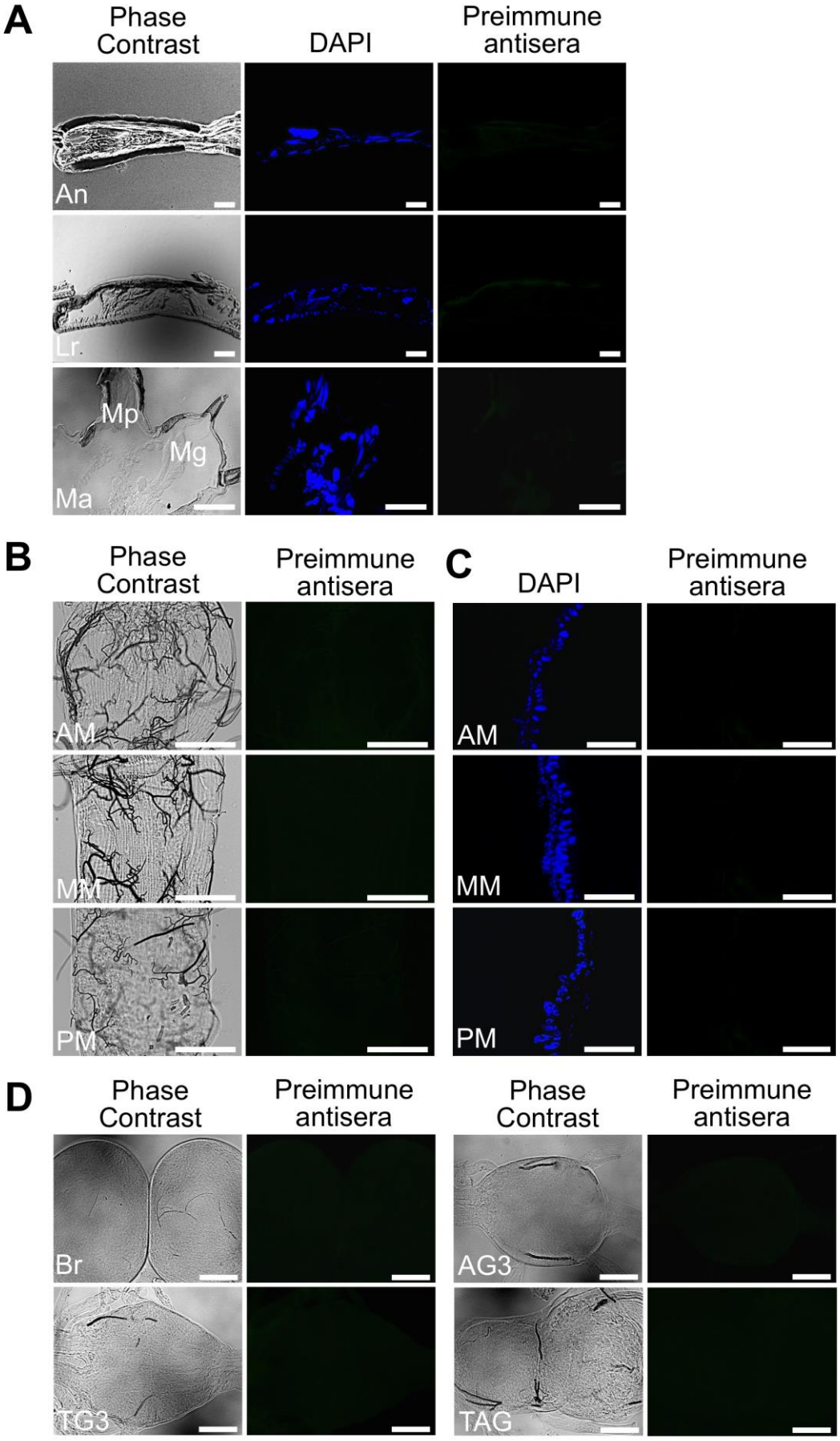


Fig. 1.14. Nerve system adjacent to abdominal ganglion 8 stained with anti-BmGr6.

Whole-mount tissues were stained with anti-BmGr6 mouse antiserum and anti-mouse IgG conjugated to Alexa Fluor 488. Two pairs of large dorsomedial immunostained cells located in the anterior region, two pairs of dorsolateral immunostained cells (closed arrowheads), and a cluster of immunostained cells (opened arrowhead) were stained in the posterior region of AG8 (the posterior neuromere of TAG). Proctodeal nerves protruding from the last abdominal ganglion were also stained (arrows). Scale bars = 100 μm.


A
Phase Contrast
DAPI
Preimmune antisera
An
Lr
Mp
Mg
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B
Phase Contrast
Preimmune antisera
AM
MM
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C
DAPI
Preimmune antisera
AM
MM
PM
D
Phase Contrast
Preimmune antisera
Phase Contrast
Preimmune antisera
Br
AG3
TG3
TAG

Fig. 1.15. Negative controls for immunohistochemistry.

Negative control experiments were conducted using pre-immune mouse antiserum from the animal that was subsequently immunized with BmGr6 to produce the primary antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488. No immunoreactive cells were observed. (A) Oral sensory organs (An, antennae; Lr, labrum; Ma, maxillary; Mg, maxillary galea; Mp, maxillary palpus). (B) Whole-mount midgut (AM, anterior midgut; MM, middle midgut; PM, posterior midgut). (C) Cryosection of midgut (AM, anterior midgut; MM, middle midgut; PM, posterior midgut). (D) Nervous system (Br, brain; TG, thoracic ganglia; AG, abdominal ganglia; TAG, terminal abdominal ganglia). Scale bars = 100 μm (A, B, and D) or 50 μm (C).

Chapter 2

Function of BmGr6-Expressing Cells Suggested by Co-Localization with FMRFamide-Related Peptides (FaRPs)-Expressing Cells

2.1. Introduction

Recent studies in mammals have shown that taste receptors were expressed not only in the oral epithelium but also in intestinal enteroendocrine cells (Dyer et al., 2005; Mace et al., 2007; Margolskee et al., 2008; Wu wt al., 2005). The gut enteroendocrine cells are chemosensory cells which produce many kinds of regulatory peptides to detect luminal nutrients or chemicals (Sternini et al., Raybould, 2010; Steinert and Beglinger, 2011). Then functional responses were induced by peptides in a paracrine manner (by acting on nearby cells in the gut), or in an endocrine manner (by acting on distant targets such as the brain) (Sternini et al., Raybould, 2010; Steinert and Beglinger, 2011; Park and Kwon, 2011b). According to previous studies, the roles of T1R (receptors that perceive sweet substances and L-amino acids) in enteroendocrine cells are mainly sensing glucose sensing in the gut (Raybould, 2010; Steinert and Beglinger, 2011; Kokrashvili et al., 2009). On the other hand, T2Rs (a family of bitter taste receptors) were found to function in enteroendocrine STC-1 cells (the intestinal secretin tumor cell line) (Chen et al., 2006). However, the specific function of T2R in the intestine is unknown.

The insect midgut is an important organ because it not only occupies a large space in their hemocoel, but also is a major part of the digestive tract. The insect midgut plays critical roles in the physiological regulation including metabolism, immune response, homeostasis of electrolytes, osmotic pressure, circulation (Takeda 2010). A previous study in *Drosophila melanogaster* showed that 14 *D. melanogaster* gustatory receptors (DmGrs) are expressed in the midgut of *D. melanogaster* (Park

and Kwon, 2011). Moreover, the co-localization of the Gr-Gal4 drivers with peptides including neuropeptide F (NPF), diuretic hormone 31 (DH31), and locustatachykinin (LTK) was also observed (Park and Kwon, 2011). This study demonstrated that some Grs are expressed in midgut enteroendocrine cells, it is possible that Grs may be involved in the detection and regulation of nutrients during digestion.

Insects possess a complex nervous system for incorporating a variety of internal physiological information as well as external sensory information (Gullan and Cranston, 2005). The gustatory neurons of insects are chemosensory neurons that express gustatory receptors. The insect central nervous system (CNS) comprises of the brain, ventral ganglia and associated neurons. Insect nervous systems have many neurosecretory cells (NSCs) that can release their products into the haemolymph (Rowell, 1976; Raabe, 1983). Previous studies in *D. melanogaster* reported that DmGr28a and five alternatively spliced forms of DmGr28b were not only expressed in the chemosensory neurons of the sensory organs, but also in neurons of the brain (Thorne and Amrein, 2008). Park and Kwon (2011) reported that some Grs are expressed in abdominal neurons which project to the abdominal ganglia of the CNS (Park and Kwon, 2011a). A previous study also reported that DmGr43a is expressed in the brain, particularly in SOG neurons, the proventriculus, and in uterus-associated neurons (Miyamoto et al., 2012).

Give the information that BmGr6 is expressed in cells of midgut and CNS (Chapter 1), I therefore asked whether these midgut cells which express BmGr6 are enteroendocrine cells; and also, whether CNS BmGr6-expressing cells are

nerosecretory cells. If so, whether any neuropeptides or peptide hormones that do exist in these cell. Another question is what function do BmGr6-expressing cells have in the midgut and CNS? To address these questions, I employed double-immunohistochemistry.

FMRFamide (Phe-Met-Arg-Phe-amide) is a neuropeptide from a broad family of FMRFamide-related peptides (FaRPs) all sharing an -RFamide sequence at their C-terminus, and the *Drosophila* anti-FMRFamide (anti-*Dm*FMRFamide) antiserum cross-reacts with FaRPs in various animals such as silkworm and hydrozoan (Chiori er al., 2009; Jager et al., 2011; López-Vera et al., 2008; Yamanaka et al., 2006). Thus, to confirm whether BmGr6-expressing cells are midgut enteroendocrine cells and CNS neurosecretory cells, and to speculate what hormones are the BmGr6-expressing cells excreting, double-immunohistochemistry using both whole mount midgut and midgut cryosections were performed. I found that a portion of BmGr6-expressing cells was co-localized with the cells stained by anti-DmFMRFamide antiserum. Besides, there were many anti-DmFMRFamide alone staining cells. These suggest that BmGr6 was expressed in a portion of midgut enteroendocrine cells which express FaRPs. Furthermore, I also find that some of BmGr6-expressing cells co-localized with FMRFamdie-expressing cells in the brain, SOG, as well as thoracic-abdominal ganglia. These findings indicated that BmGr6 was expressed in neurosecretory cells and a portion of these cells expressing FaRPs.

2.2. Materials and methods

2.2.1. Experimental animals

Eggs of silkworm *B. mori* (hybrid strain, Kinshu × Showa) were purchased from the silkworm egg producing company, Ueda Sanshu Ltd (Ueda, Japan). Larvae were reared as described in section 1.1,1.

2.2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Anterior, middle and posterior midgut were dissected from two of 3-day-old fifth instar larvae. All collected tissues were homogenized in 0.5–2 ml cold ISOGEN II (Nippon Gene, Tokyo, Japan) and kept at -80 °C until use for RNA extraction. For cDNA synthesis, double-stranded cDNA fragments amplification, and PCR was performed as described in section 1.1.2.

2.2.3. Immunohistochemistry and microscopy

For cyrosection, the posterior midgut of 3-day-old 3rg-instar larvae were removed, washed with 70% ethanol, and fixed overnight (12–24h) at 4 °C in 4% paraformaldehyde in PBS. After fixation, samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan) and frozen at -20 °C. The embedded specimens were mounted on an object holder and sectioned using a Tissue-Tek Cryo3® cryostat (Sakura Finetek, Tokyo, Japan). 12 μm thick cryosections of each organ was placed on MAS-GP type-A coated slide glasses (Matsunami Glass, Osaka, Japan) and air-dried at RT for about 4 h. Then the immunohistochemistry was

performed as described in section 1.1.7. After immunohistochemistry, samples were mounted in 90% glycerol/PBS containing 1, 4-diazabicyclo [2.2.2] octane at RT and observed under a fluorescence microscope, LSM710/LSM710 NLO (CARL ZEISS) at RT.

For whole mount immunocytochemistry, the midgut and nervous tissues from 2-day-old 3rd-instar larvae were fixed at 4 °C overnight in 4% paraformaldehyde in PBS, containing 0.1% Triton X-100 (PBST). After fixation, tissues were washed 3 times for 10 min each in PBST, and then incubated for 1 h in TNT containing 2% BSA. The tissues were subsequently incubated with primary antiserum (anti-DmFMRFamide and anti-DmNPF antisera) as described in section 1.1.7. After immunohistochemistry, tissues were also mounted in 90% glycerol/PBS containing 1,4-diazabicyclo [2.2.2] octane and observed under a fluorescence microscope, LSM710/LSM710 NLO (CARL EISS) at RT.

For double-immunohistochemistry, a mouse polyclonal antiserum (anti-BmGr6) and rabbit anti-DmFMRFamide (ABcam) or anti-DmNPF antiserum (ABcam) were used. Tissues were incubated in a primary antisera mixture of anti-BmGr6 antiserum diluted 1:1000 and a 1:1000 dilution of either anti-DmFMRFamide or anti-DmNPF antiserum. After the incubation and washing procedures as described above, the specimens were incubated with a secondary antisera mixture of a goat anti-rabbit IgG conjugated to Alexa Fluor 488 diluted 1:1000 and a goat anti-mouse IgG coupled to Alexa Fluor 555 (Invitrogen) diluted 1:1000.

Negative controls for double-immunohistochemistry were performed using anti-BmGr6 alone as a primary antiserum, and incubated with secondary antisera mixture as noted above (Fig. 2.5).

2.3. Results

2.3.1. BmGr6 is expressed in midgut enteroendocrine cells

To further confirm that BmGr6-expressing cells are actually midgut enteroendocrine cells, and to speculate what hormones are the BmGr6-expressing cells excreting, double-immunohistochemistry using both whole mount midgut and midgut cryosections were performed. Previous studies have reported that FMRFamide (Phe-Met-Arg-Phe-amide) is a neuropeptide from a broad family of FMRFamide-related peptides (FaRPs) all sharing an -RFamide sequence at their C-terminus, and the *Drosophila* anti-FMRFamide (anti-DmFMRFamide) antiserum cross-reacts with FaRPs in various animals such as silkworm and hydrozoan (Chiori er al., 2009; Jager et al., 2011; López-Vera et al., 2008; Yamanaka et al., 2006). I therefore used this antiserum to identify FaRPs expressing in *B. mori* midgut enteroendocrine cells, and observed that the anti-DmFMRFamide antiserum labeled enteroendocrine cells along the length of the midgut (Fig. 2.1). Double-immunohistochemistry showed that a portion of BmGr6-expressing cells was co-localized with the cells stained by anti-DmFMRFamide antiserum (Fig. 2.2 A and B). Besides, there were many anti-DmFMRFamide alone staining cells. These suggest that BmGr6 was expressed in a portion of midgut enteroendocrine cells which express FaRPs. Roller et al. (2008) showed that four peptide hormones including CCHamide, neuropeptide F1 (NPF1), neuropeptide F2 (NPF2) and sulfakinin were expressed in enteroendocrine cells of the *B. mori* larval midgut. Among these four peptide hormones, NPF1 and sulfakinin have -RFamide at the

C-terminus (Roller et al., 2008), suggesting the possibility that BmGr6 may co-express with these two peptides in the midgut enteroendocrine cells. Since NPF1 appear to be closely related to *D. melanogaster* and *A. aegypti* NPF (Roller et al., 2008), and the *Drosophila* anti-NPF (anti-DmNPF) antiserum could be used to identify NPF orthologue in *A. aegypti* (Stanek et al., 2002), I therefore performed double-immunohistochemistry to determine whether BmGr6 and BmNPF1 are co-expressed in the same enteroendocrine cells, using a mouse anti-BmGr6 and a rabbit anti-DmNPF antisera. However, no co-localization of BmGr6-expressing cells and putative BmNPF1-expression cells was observed (Fig. 2.4), even though numerous putative BmNPF1-expressing cells were observed to be distributed in the middle and posterior midgut (Fig. 2.3).

2.3.2. BmGr6 is expressed in CNS neurosecretory cells

To determine whether BmGr6-expressing cells are neurosecretory cells, I performed double-immunohistochemistry of whole mount central nervous tissues, using anti-BmGr6 antiserum and anti-DmFMRFamide antiserum. As I have already indicated in (Fig. 1.7 A) (Chapter 1), two clusters of cells were observed to be stained with anti-BmGr6 antiserum in the lateral region of the brain. A portion of BmGr6-expressing cells was confirmed to be co-localized with the cells stained by anti-DmFMRFamide antiserum in the lateral region of the brain (Fig. 2.6 A). The other cells with large somata were observed to be stained with anti-BmGr6 in the medial region of the brain (Fig. 1.7 A). Some of these cells were also stained with

anti-DmFMRFamide antiserum (Fig. 2.6 B). In the suboesophageal ganglion (SOG), a cluster of cells located in the middle region of this ganglion were stained with anti-BmGr6 antiserum (Fig. 1.7 C), and most of these cells were co-localized with anti-DmFMRFamide-stained cells (Fig.2.7 A_1–A_3). In the case of thoracic ganglia (TG), one pair of BmGr6-expression dorsallateral cells located in the posterior region of TG1, two pairs of ganglion BmGr6-expression dorsallateral cells located in the posterior region of TG2 and TG3 were observed (Figs.1.7 D–F and 2.7 B_1–B_3), these cells were found to co-localize with anti-DmFMRF-stained cells (Fig. 2.7 B_1–B_3). In addition, BmGr6-expressing cells in abdominal ganglia (AG) 1–8 were also observed to be co-localized with the cells stained by anti-DmFMRFamide antiserum (Figs. 1.7 G–N and 2.7 C_1–D_3). These findings suggest that BmGr6 was expressed in CNS neurosecretory cells, and these cells express FaRPs.

2.3.3. Co-expression of BmGr6- and putative BmNPF1-expressing cells in CNS

A previous study has shown that neuropeptide F1 (NPF1) which was expressed in both midgut enteroendocrine cells and CNS neurosecretory cells of the *B. mori* larval (Roller et al., 2008). Although putative NPF1-expressing cells were found not co-expression with BmGr6-expressing cells in the midgut, the possibility that NPF1-expressing cells co-localize with BmGr6-expressing cells in the CNS cannot be excluded. I therefore next performed double-immunohistochemistry to determine whether BmGr6 and NPF1 are co-expressed in the same neurosecretory cells or not, using a mouse anti-BmGr6 and a rabbit anti-DmNPF antisera. As mentioned above,

two clusters of BmGr6-expressing cells were observed to be located in the lateral region of the brain (Fig.1.7 A). Most of these cells were observed to be co-localized with the cells stained by anti-DmNPF antiserum (Fig.2.8 A). Moreover, many BmGr6-expressing cells with large somata in the medial region of the brain, were also stained with anti-DmNPF antiserum (Figs. 1.7 A and 2.8 B). Apart from the brain, a cluster of BmGr6-expressing cells in the SOG were found co-localization with anti-DmNPF-stained cells (Figs.1.7 C and 2.9 A_1–A_3). Besides, four large BmGr6-expressing cells in the AG8 were also observed to be stained with anti-DmNPF antisrerum (Figs. 1.7 M–N and 2.9 D_1–D_3). However, although BmGr6-expressing cells were found in all thoracic-abdominal ganglia (Figs. 1.7 D–M), no co-localization of BmGr6-expressing cells and anti-DmNPF-stained cells was observed (Fig. 2.9 B_1–C_3).

2.4. Discussion

In mammals, taste receptors are not only expressed in taste cells of the tongue, but also in gut enteroendocrine cells (Dyer et al., 2005; Mace et al., 2007; Margolskee et al., 2007). Taste receptors expressed in mammalian luminal epithelium are thought to function as food sensors to avoid harmful substances and to regulate nutrient uptake in the gut (Breer et al., 2012; Sclafani, 2007). The digestive systems of insects and vertebrates share many similarities including cell types and development (Hartenstein et al., 2010; Park and Kwon, 2011b; Stainier, 2005). Park and Kwon (2011) reported that 12 DmGrs including DmGr43a (the fructose receptor) and DmGr64a (a member of the sugar receptor family) are also expressed in *D. melanogaster* adult gut enteroendocrine cells (Park and Kwon, 2011b). In addition, they showed that DmGr43a and DmGr64a co-localized with neuropeptide F (NPF)-expressing enteroendocrine cells and another 10 Grs (members of the non-sugar receptor family) also co-localized with locustatachykinin (LTK) or diuretic hormone 31 (DH31) in enteroendocrine cells (Park and Kwon, 2011b). In this study, double-immunohistochemistry showed that a portion of BmGr6-expressing cells co-localized with enteroendocrine cells which were indicated to produce some kinds of FaRPs (Fig. 2.2), suggesting that BmGr6 was expressed in midgut enteroendocrine cells. Moreover, since only a portion of BmGr6-expressing cells co-localized with FaRPs-expressing cells, it might be suggested that BmGr6 is expressed in at least two types of enteroendocrine cells. Additionally, a previous study has showed that several peptides including peptides with -RFamide at the

C-teminal, sulfakinin and neuropeptide F1 (NPF1) are expressed in midgut enteroendocrine cells of *B. mori* larval (Roller et al., 2008). Surprisingly, no co-localization was observed between BmGr6- and NPF1-expression cells (Figs. 2.4 and 2.5). Thus, it is possible that BmGr6 is expressing in sulfakinin-prodicing enteroendocrine cells. However, I cannot exclude the possibility that BmGr6 is also expressing in the other types of enteroendocrine cells which are producing unknown hormone, since there are many hormones were also expressed in the midgut of *B. moro* larvae (Fig.2.10).

In the present study, BmGr6 was confirmed expression in the CNS neurosecretory cells of *B. mori* larvae using double-immunohistochemistry (Figs. 2.6 and 2.7). In addition, double-immunohistochemistry showed that partial BmGr6-expressing cells also express FMRFamide-related peptides (FaRPs) (Figs. 2.6 and 2.7). Previous studies have showed that more than 20 neuropeptides were expressed in the CNS of *B*. *mori* larvae (Roller et al., 2008; Yamanaka et al., 2005; Yamanaka et al., 2006; Yamanaka et al., 2009). Among these neuropeptides, for example, bommo-FMRFamide (BRFa) belong to FaRPs (Roller et al., 2008; Yamanaka et al., 2006). BRFa, which suppresses ecdysteroidogenesis in the PG, was expressed in two pairs of neurons with large cell bodies in each thoracic ganglion and these large thoracic neurons were identified as ventrolateral neurosecretory cells 1 and 2 (NS-$VTL_{1,2}$) (Yamanaka et al., 2006). BmGr6 was also expressed in ventrolateral neurosecretory cells (NS-$VTL_{1,2}$) in TG2 and TG3 (Figs. 1.7 E–F and 2.7

B_1–B_3), suggestting that BmGr6 may serve similar biological functions BRFa in the thoracic ganglia of *B. mori* larvae.

On the other hand, co-localization of BmGr6-expressing cells and anti-DmNPF-stained cells were also found in the brain (Fig. 2.6). Since neuropeptide F1 (BmNPF1) is an ortholog of DmNPF in *B. mori* (Roller et al., 2008), we believe that anti-DmNPF-stained cells in the *B. mori* brain probably produced BmNPF1, and therefore the anti-DmNPF-stained cells are putative BmNPF1-expressing cells. Such an expression would be consistent with findings in a previous study reported that BmNPF1-expression cells in the brain of *B. mori* larvae (Roller et al., 2008). Although the specific physiological role of BmNPF1 in *B. mori* is still unknown, the expression of this neuropeptide in the CNS supports its suggested functions in feeding behavior. Moreover, the ortholog of BmNPF1 in *D. melanogaster*, DmNPF is known to increase food consumption. Additionally, in *Helicoverpa zea*, an orexigenic action to promote feeding behaviors were suggested by high circulating levels of NPF1-like material in actively feeding larvae. Thus, it could speculate that BmGr6 may function involve in promote feeding behavior of *B. mori* larvae.

Table 2.1 Primers used for RT-PCR in this study

Gene	S/AS	Primer sequences (5'-3')
Neuropeptide F 1	S	AGCAAGAACCTAGCCGTGGT
	AS	TCCGGCTTCTCAACATCTTT
Neuropeptide F 2	S	GTCGGCCATTTTACTGTTCG
	AS	TATTGGTGGTAAGCGCAATG
CCHamide	S	GTATCAACGCAGAGTACGCG
	AS	GAAGCAGGAGTGCCCAAAT
Sulfakinin	S	GATTGTTATAATCCAGTATGACATTCGAG
	AS	TCAGTCATCAGAACGACCAAACCG
Tachykinin	S	CAGCGATACCAACGAGACAGAAG
	AS	CAGCGATACCAACGAGACAGAAG
Allatostatin A	S	GCCTACAGCTATGTCTCCGAGTACAAG
	AS	TTCACTGGTGTCGTCTTCCGATC
Allatostatin C	S	CATAAGGAACAATCTGGGCTTGGC
	AS	TAGCACTGCCGGAAACGGACTT
Short Neuropeptide F	S	GCTCAGGCGTTATCGAACTACGATG
	AS	GCTCAGGCGTTATCGAACTACGATG
Allatropin	S	GAATCTGACAATGCAACTGGAAGTG
	AS	GAATCTGACAATGCAACTGGAAGTG
Diuretic hormone 31	S	AAGTTCACCTGCGTGTTGGC
	AS	TTAGCAGCCGCCAATCCCAT
FMRFamide	S	GAATCACCCTCGATCCATAGCGAT
	AS	GAATCACCCTCGATCCATAGCGAT
IMFamide	S	TCAGATGTCGAGAGCTGTAATGA
	AS	TATTCAAGTCGCCGTTGTTG

S, sense primer; AS, antisense primer.

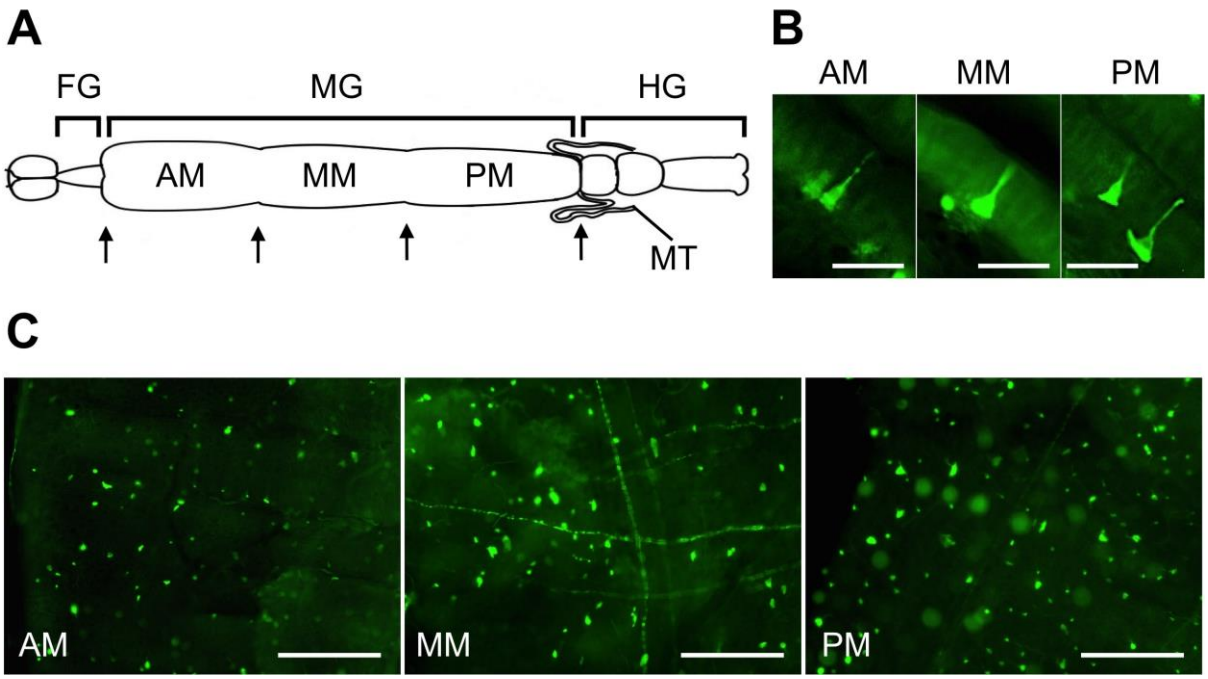


Fig. 2.1. Whole-mount immunohistochemistry analysis of FaRPs-expressing cells in a *B. mori* third instar larval midgut.

(A) An illustration of the digestive tract of a *B. mori* larva. (B) Detailed view of anti-DmFMRFamide immunostained cells observed in the anterior (AM), middle (MM), and posterior midgut (PM). (C) Whole-mount guts were stained with anti-*Dm*FMRFamide rabbit antiserum in combination with anti-rabbit IgG conjugated with Alexa Fluor 488. Immunoreactive cells were observed in the anterior (AM), middle (MM), and posterior portions (PM) of the midgut. Scale bars = 30 μm in (A) or 100 μm in (C).

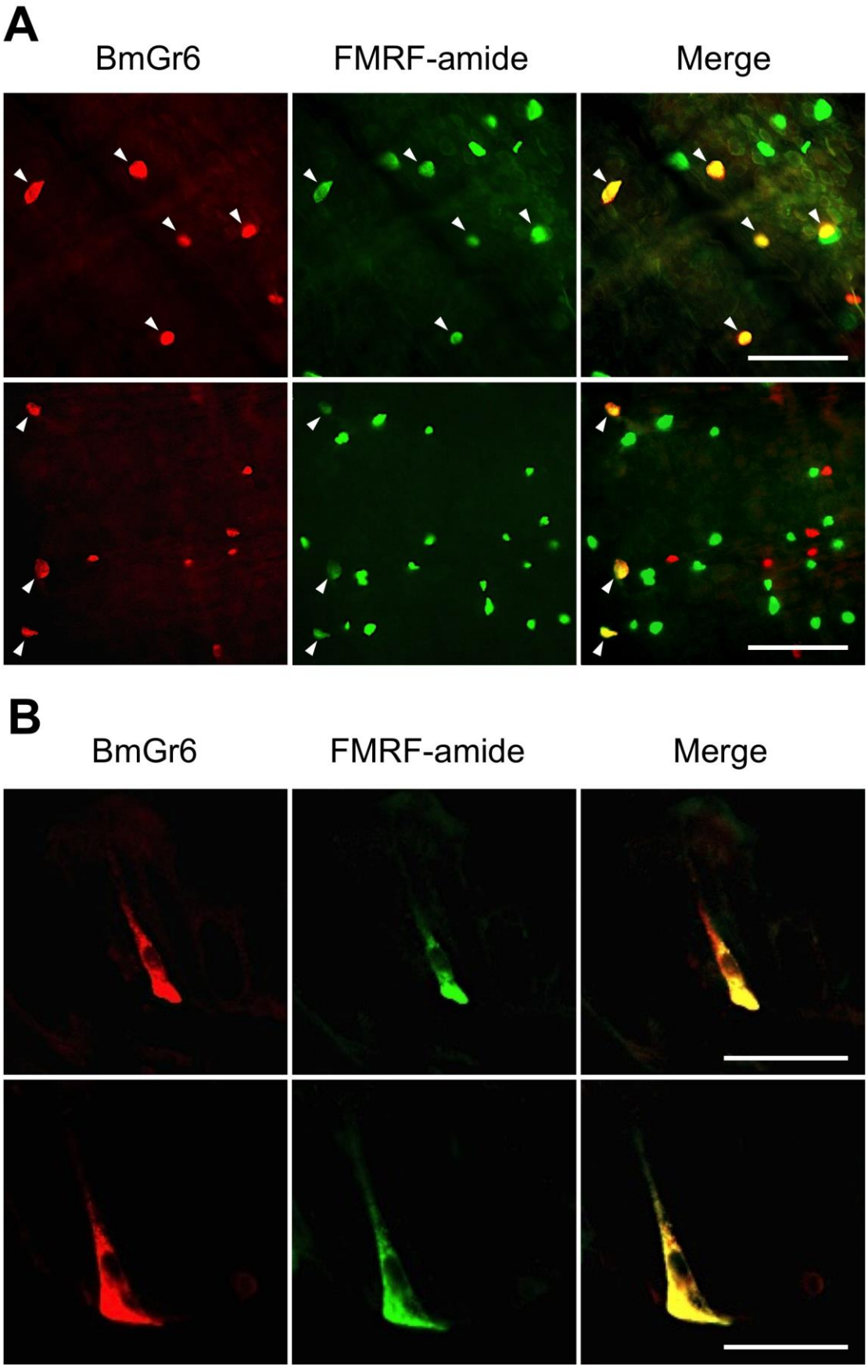

A
BmGr6
FMRF-amide
Merge
B
BmGr6
FMRF-amide
Merge

Fig. 2.2. Double-immunohistochemistry of the whole mount midgut and midgut cryosections of third instar *B. mori* larvae.

Whole mount midgut (A) and midgut cryosections (B) of the larvae were double stained with anti-BmGr6 and anti-*Dm*FMRFamide (A and B) antisera. As secondary antisera, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. The left panels are results stained with mouse anti-BmGr6 antiserum. The second panels are results stained with rabbit anti-*Dm*FMRFamide antiserum. The third panels are merged images of the left panels and the second panels. For negative controls, anti-BmGr6 was used alone as a primary antiserum and stained with secondary antisera mixture noted above. (a) Results use excitation and emission wavelengths selective for Alexa Fluor 555 fluorescence. (b) The same focal plane as in (a), but now using excitation and emission wavelengths selective for Alexa Fluor 488 fluorescence. Scale bars = 100 μm in (A) and 30 μm in (B).

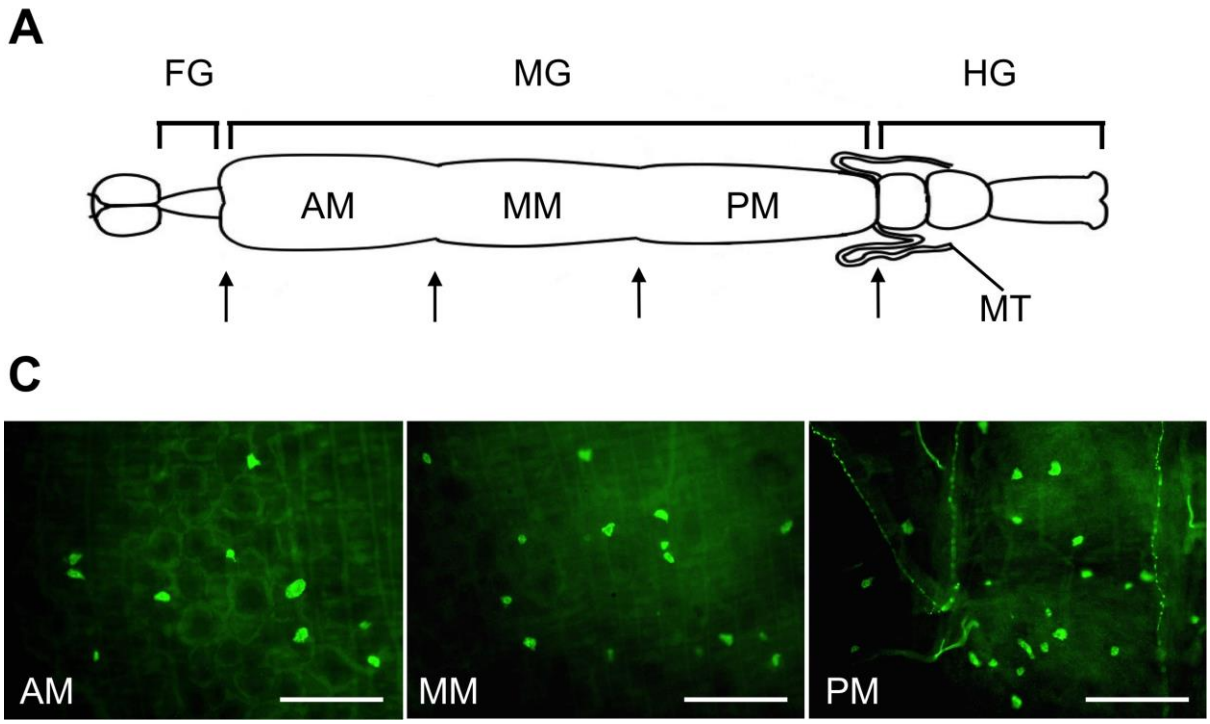


Fig. 2.3. Whole-mount immunohistochemistry analysis of NPF-expressing cells in a *B. mori* third instar larval midgut.

(A) An illustration of the digestive tract of a *B*. *mori* larva. (B) Whole-mount guts were stained with anti-*Dm*NPF rabbit antiserum in combination with anti-rabbit IgG conjugated with Alexa Fluor 488. Immunoreactive cells were observed in the anterior (AM), middle (MM), and posterior portions (PM) of the midgut. Scale bars = 30 μm in (A) or 100 μm in (C).

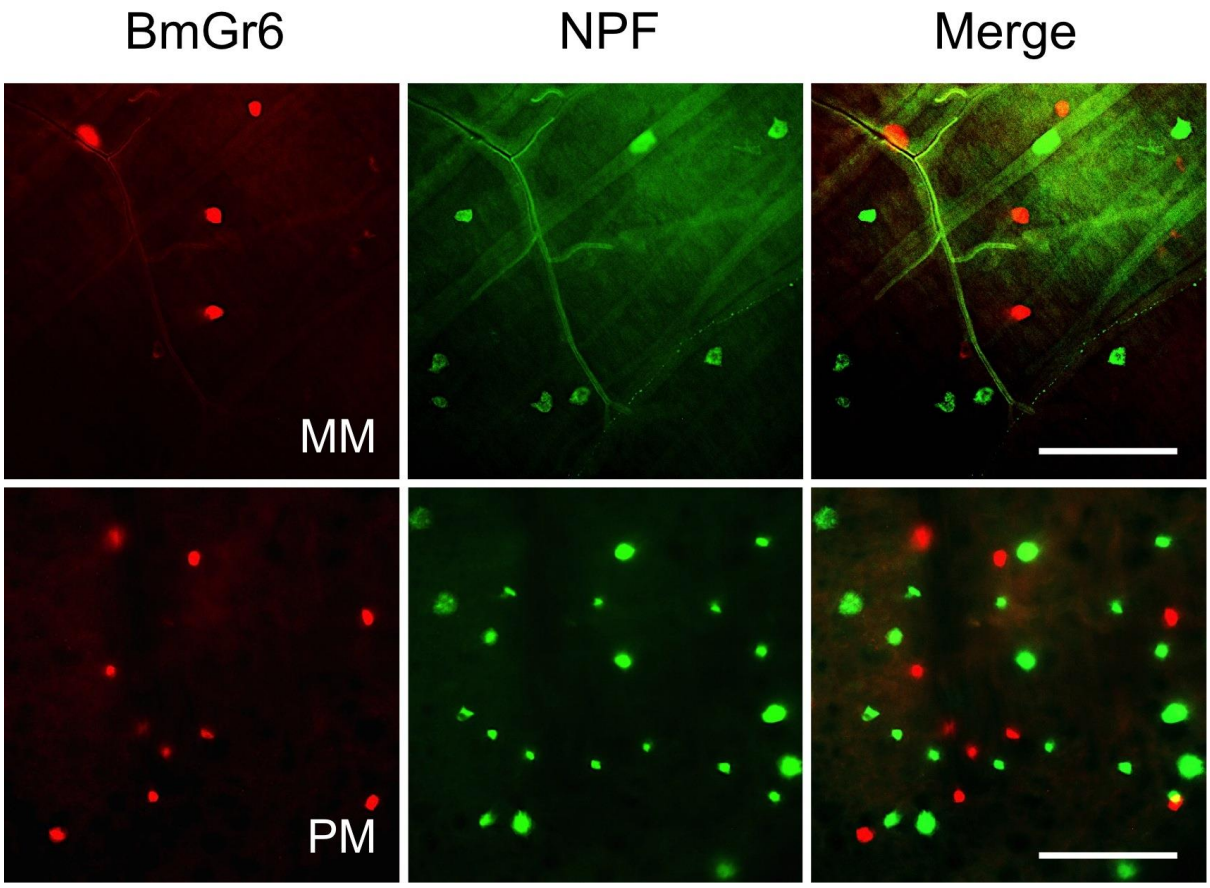


Fig. 2.4. Double-immunohistochemistry of the whole mount midgut of third instar *B. mori* larvae.

Whole mount midgut of the larvae were double stained with anti-BmGr6 and anti-*Dm*NPF antiserum. As secondary antisera, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. The left panels are results stained with mouse anti-BmGr6 antiserum. The second panels are results stained with rabbit anti-*Dm*NPF antiserum. The third panels are merged images of the left panels and the second panels.

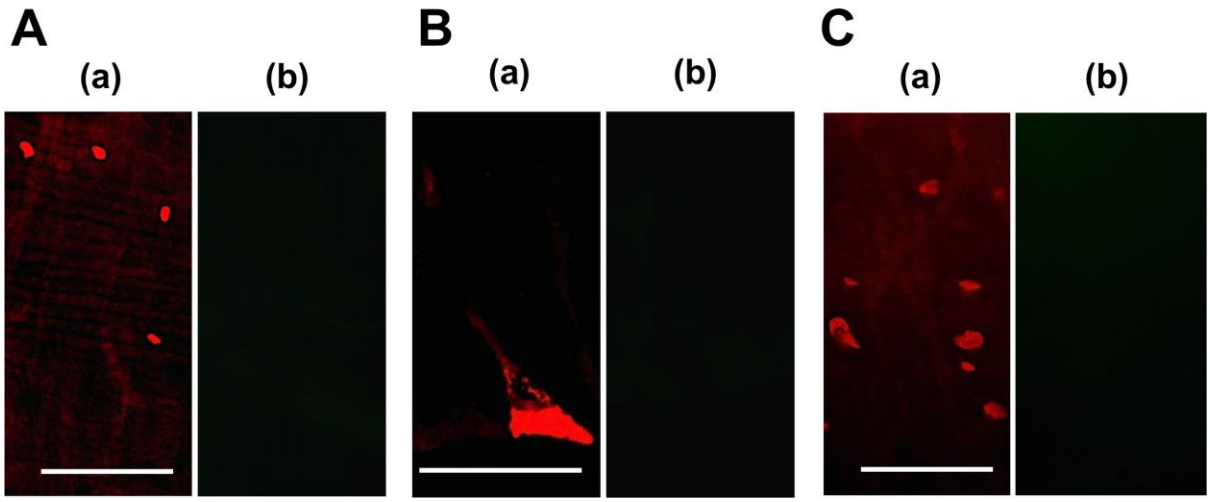


Fig.2.5. Negative controls for anti-DmFaRP (A and B) and anti-DmNPF antisera, anti-BmGr6 was used alone as a primary antiserum and stained with secondary antisera mixture noted above. (a) Results use excitation and emission wavelengths selective for Alexa Fluor 555 fluorescence. (b) The same focal plane as in (a), but now using excitation and emission wavelengths selective for Alexa Fluor 488 fluorescence. Scale bars = 100 μm in (A) and 30 μm in (B).

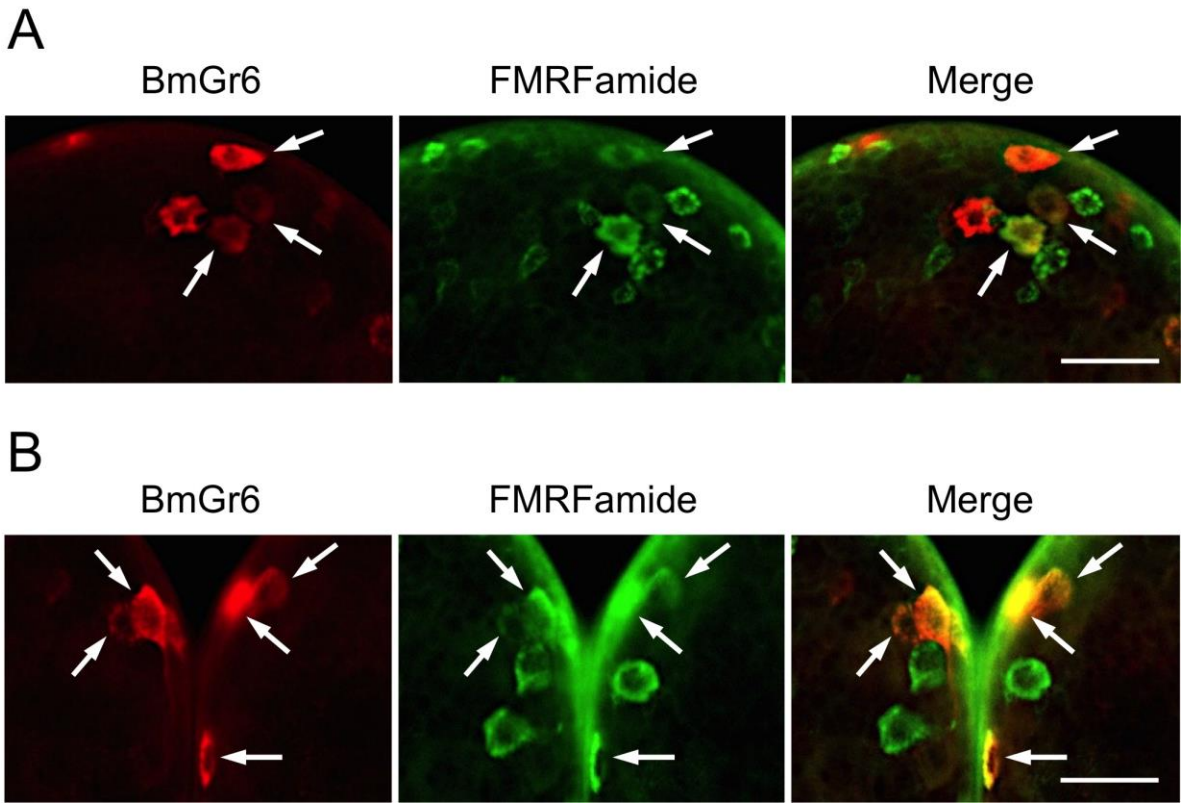


Fig. 2.6. Double immunohistochemical staining of whole mount brain of third instar *B. mori* larvae.

Whole mount brain was double stained with anti-BmGr6 and anti-*Dm*FMRFamide antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. BmGr6-expressing cells are co-localized with anti-*Dm*FMRFamide-stained cells in lateral (A) and medial (B) regions of the brain. Arrows indicate cells which were stained with both anti-BmGr6 and anti-*Dm*FMRFamide antisera. Scale bars = 50 μm.

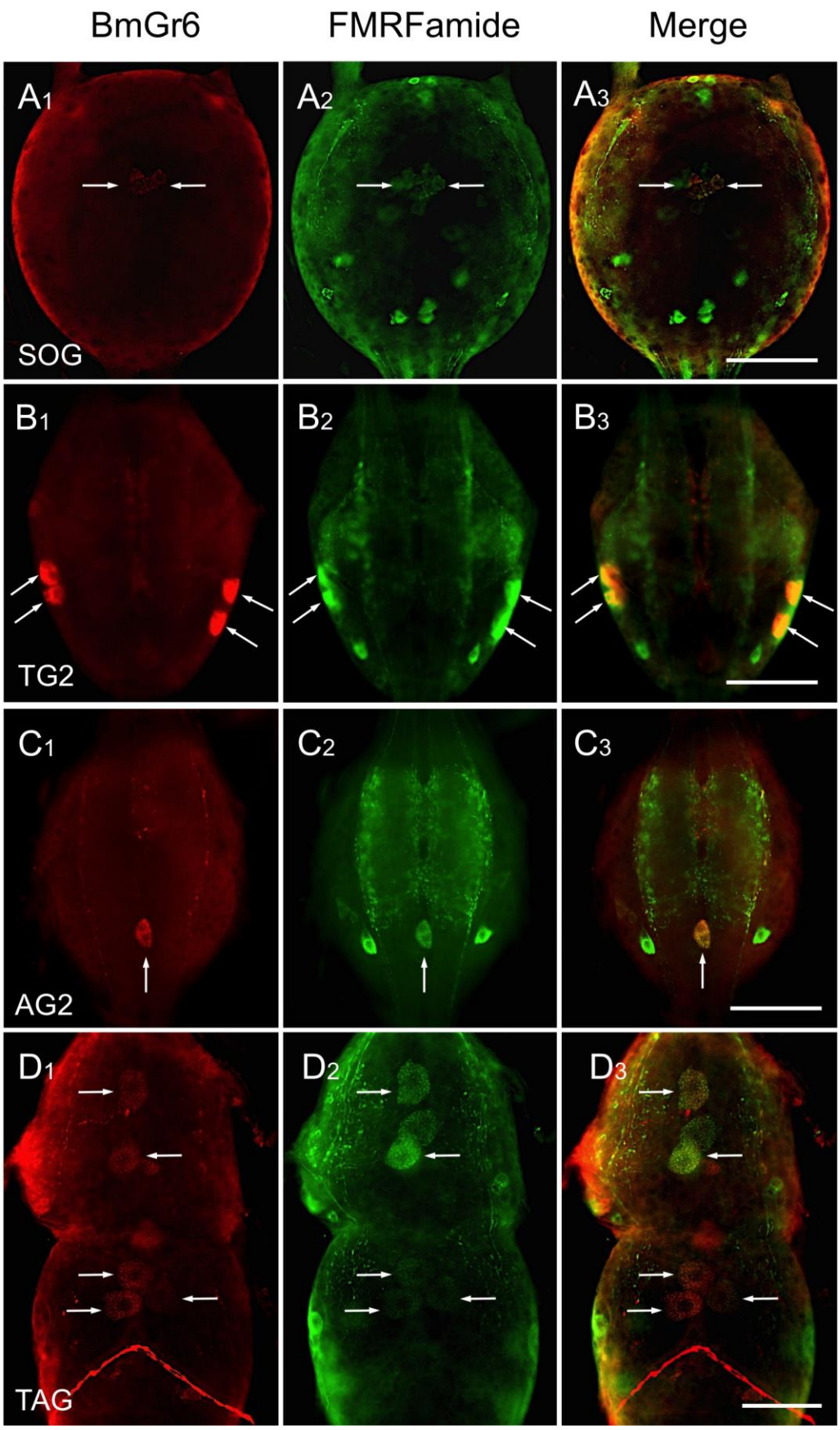

BmGr6
FMRFamide
Merge
A1
A2
A3
SOG
B1
B2
B3
TG2
C1
C2
C3
AG2
D1
D2
D3
TAG

Fig. 2.7. Double immunohistochemical staining of whole mount ganglia of third instar *B. mori* larvae.

Whole mount central nervous tissues of the larvae were double stained with anti-BmGr6 and anti-*Dm*FMRFamide antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. The left panels are results stained with mouse anti-BmGr6 antiserum. The middle panels are results stained with rabbit anti-*Dm*FMRFamide antiserum. The right panels are merged images of the left panels and the middle panels. Arrows indicate BmGr6-expressing cells, arrowheads indicate FMRFamide-expressing cells, open arrows indicate cells which were stained with both anti-BmGr6 and anti-*Dm*FMRFamide antisera. Scale bars = 100 μm.

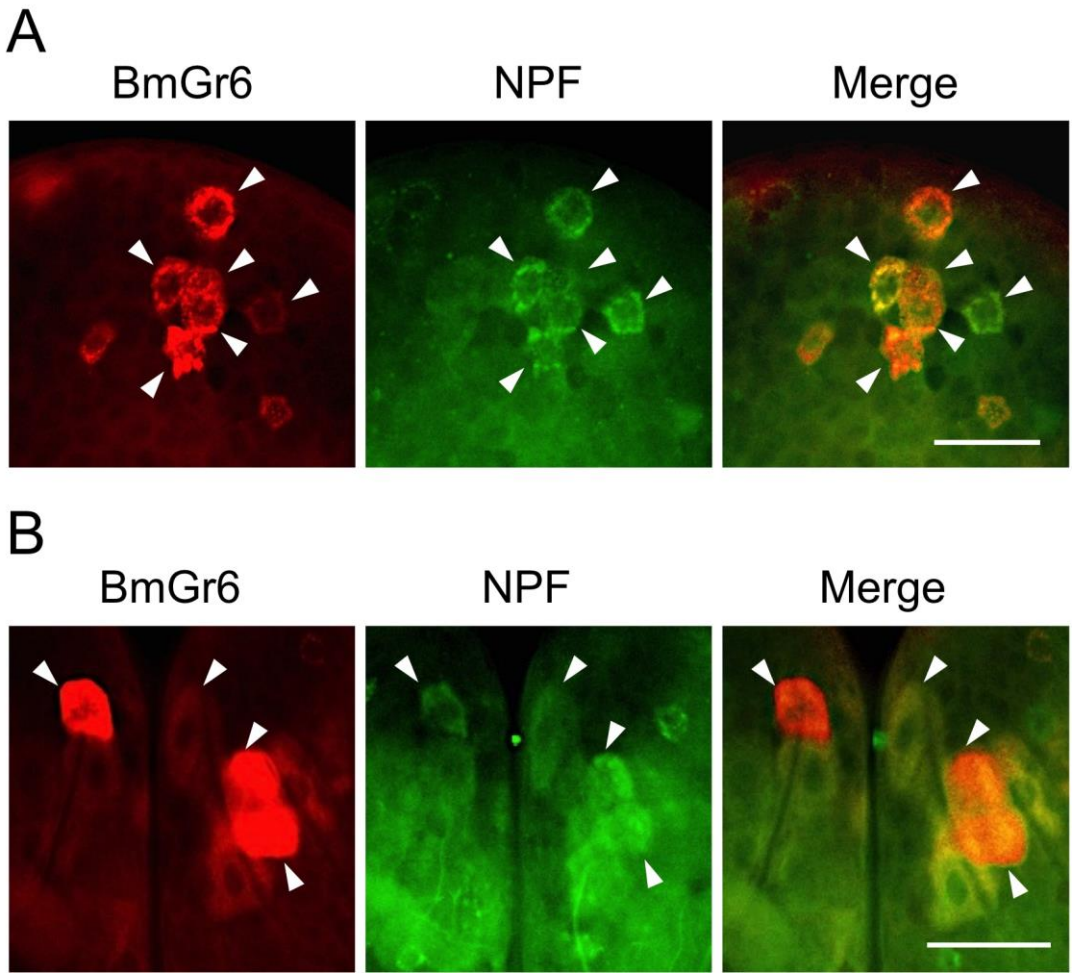


Fig. 2.8. Co-localization of NPF1-expressing cells and BmGr6-expressing cells in the brain of instar *B. mori* larvae.

Whole mount brain was double stained with anti-BmGr6 and anti-DmNPF antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. (A and B) BmGr6-expressing cells co-localization with anti-*Dm*NPF stained cells in the lateral (A) and medial (B) regions.Arrowaheads indicate cells which were stained with both anti-BmGr6 and anti-DmNPFamide antisera Scale bars = 50 μm.

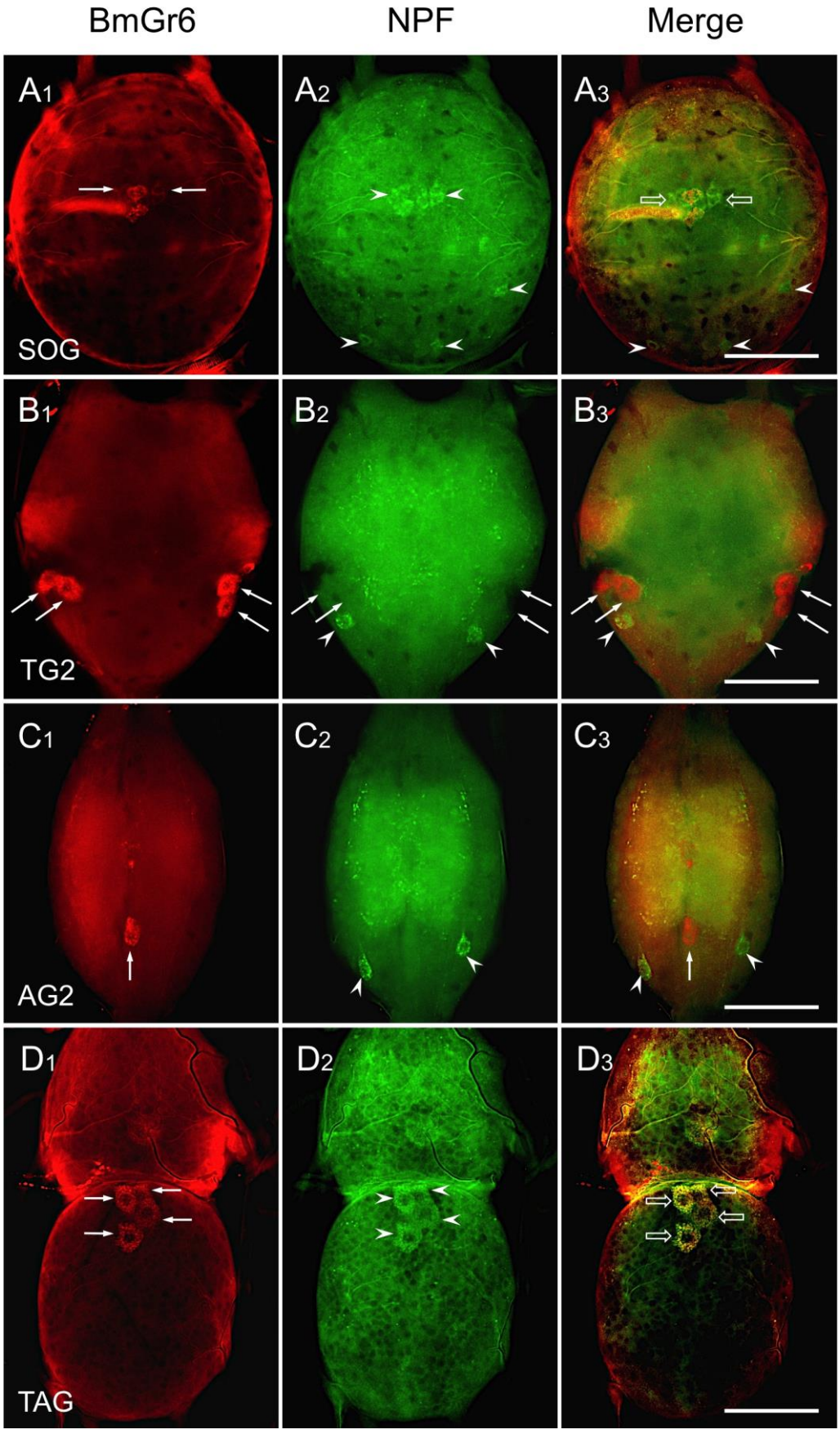

BmGr6
NPF
Merge
A1
A2
A3
SOG
B1
B2
B3
TG2
C1
C2
C3
AG2
D1
D2
D3
TAG

Fig. 2.9. Double immunohistochemical staining of whole mount ganglia of third instar *B. mori* larvae.

Whole mount central nervous tissues of the larvae were double stained with anti-BmGr6 and anti-DmNPF antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. The left panels are results stained with mouse anti-BmGr6 antiserum. The middle panels are results stained with rabbit anti-DmNPF antiserum. The right panels are merged images of the left panels and the middle panels. Arrows indicate BmGr6-expressing cells, arrowheads indicate anti-DmNPF-stained cells, open arrows indicate cells which were stained with both anti-BmGr6 and anti-DmNPF antisera. Scale bars = 100 μm.

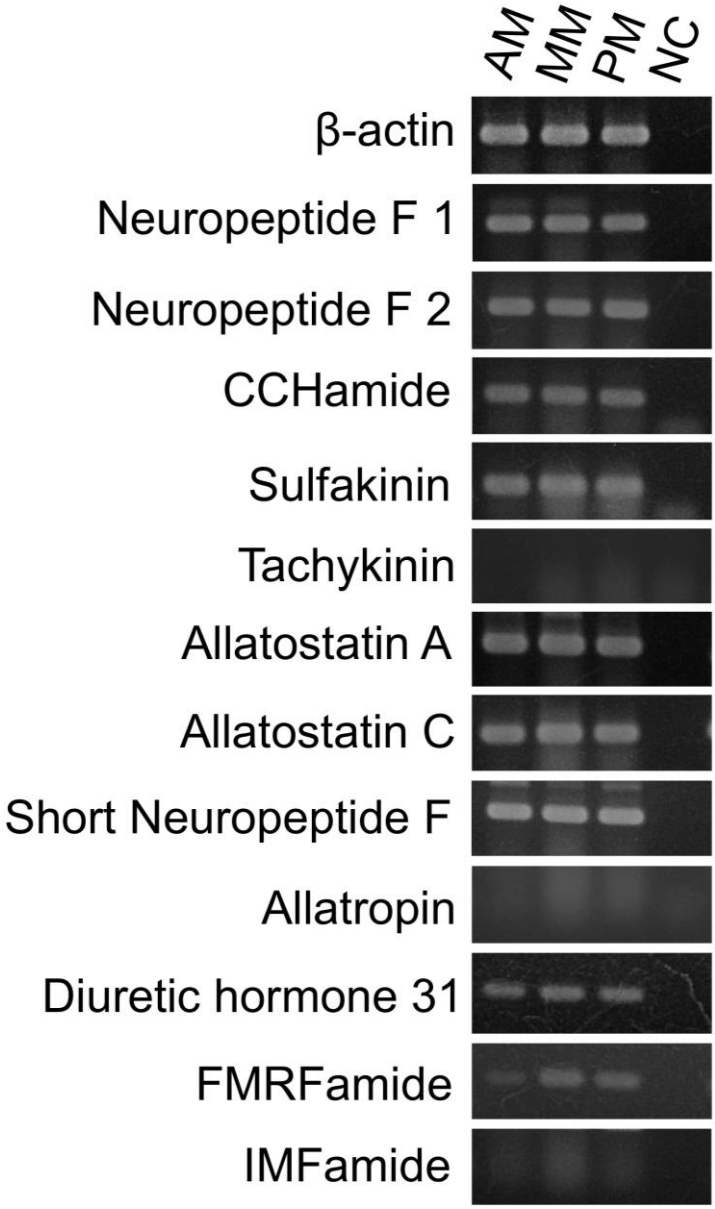


Fig. 2.10. Expression of neuropeptides and peptide hormones genes in *B. mori* midgut by RT-PCR analysis.

Tissues were dissected from two-day-old fifth instar larvae. *β-actin* was used as the internal control; NC, negative control, indicates no template in the reaction. Amplification products were analysed on agarose gels and visualized by UV illumination after ethidium bromide staining.

Chapter 3

Expression Cells of a Fructose Receptor, BmGr9 and Its Involvement in Promotion of Feeding Suggested by Co-expression with Neuropeptide F1

3.1. Introduction

Based on homology with *D. melanogaster* Grs (DmGrs), insect Grs have been putatively classified into sugar and bitter families; however, not all Grs are restricted to taste sensing functions. DmGr21a and DmGr63a, are implicated in the CO_2 response in the antenna (Jones et al., 2007; Kwon et al., 2007), DmGr68a is essential for *D. melanogaster* courtship behavior (Bray and Amrein, 2003), and DmGr28b is critical for light-induced responses in *D. melanogaster* larvae (Xiang et al., 2010). In addition, some *D. melanogaster* Grs are expressed in midgut enteroendocrine cells (Park and Kwon, 2011b) suggesting that Grs may have chemosensory roles in the intestine and may regulate physiological functions. More recently, DmGr43a, an ortholog of the *B*. *mori* BmGr9 protein, was shown to function as a nutrient sensor for hemolymph fructose in *D. melanogaster* brains (Miyamoto et al., 2012). In addition, a recent study showed that a DmGr28b.d paralog is involved in rapid warmth avoidance in *D. melanogaster* (Ni et al., 2013). Based on these findings, it is possible to Imagine that some Grs may play roles in functions other than gustation including olfaction, light sensing, sensing for homeostasis, and sensing relevant to motility.

BmGr9, a silkworm Gr, has been shown to respond specifically to fructose and as a ligand-gated ion channel (Sato et al., 2011), but the distribution of this Gr-expressing cells in tissues of the *B. mori* is so far not known. Recently, DmGr43a, a BmGr9 ortholog in *D. melanogaster,* was shown to detect fructose not only in the chemosensory organs, but also in the brain as a sensor for hemolymph fructose (Miyamoto et al., 2012; Miyamoto and Amrein, 2014). DmGr43a was indicated to

function to promote feeding in starved flies, but suppress feeding in flies satiated with diet (Miyamoto et al., 2012). Additionally, possibility of the secretion of neuropeptide F (DmNPF) was discussed in the promotion of the feeding of the starved fly (Miyamoto et al., 2012), since DmNPF and the mammalian ortholog, neuropeptide Y (NPY) are known to increase food consumption (Nassel and Winther, 2010; Valassi et al., 2008; Wu et al., 2003, 2005). Because Dm43a is an ortholog of BmGr9, it allowed us to speculate that BmGr9 may serve similar biological functions as DmGr43a in the *B. mori* brain, and feeding behavior of *B. mori* might be regulated by *B. mori* ortholog of DmNPF, neuropeptide F1 (BmNPF1) (Roller et al., 2008). However, whether BmNPF1 is co-expressed with BmGr9 expressing cells in the *B. mori*, is unknown. Previous studies in *D. melanogaster* showed that co-expression of multiple Grs is necessary to respond to various compounds (Moon et al., 2006; Kwon et al., 2007; Slone et al., 2007; Jiao et al., 2008; Lee et al., 2009). Although BmGr9 was appeared not to require the expression of other BmGrs to show the responsiveness to fructose in vitro, the possibility that BmGr9 co-expression with others BmGrs in neurons cannot be excluded (Sato et al., 2011). Up to the present, however, whether BmGr9 is co-expressed with any other BmGrs in the B. mori larvae has not yet been reported. Recently, I described the distribution of the cells expressing BmGr6 which is a molecule of the sugar receptor clade and was confirmed to be expressed in midgut enteroendocrine cells and CNS neurosecretory cells as well as cells in the chemosensory organs (Chapter 2). BmGr6 may function not only as a taste receptor, but also as a chemical sensor such as those for the regulation of gut movement,

physiological conditions, and feeding behavior of larvae (Chapter 2). However, it is also not currently known whether BmGr6 is co-expressed with any other BmGrs in the *B. mori* larvae and, if it is present, whether BmGr6 is co-expressed with BmGr9.

In the present study, I used reverse transcription (RT)-PCR to investigate the expression sites of the *BmGr9* gene in the *B. mori* larvae. In addition, I explored BmGr9 expressing cells in *B. mori* larval tissues paying attention especially to CNS including brain, a secretory center for the regulation of behavior using a specific polyclonal antiserum raised against BmGr9. I attempted to clarify whether BmGr9 expressing cells are neurosecretory cells or not by the double-immunohistochemistry. Furthermore, I examined whether BmGr9-expressing cells express BmGr9 alone or not as a sensor for the nutrients in the hemolymph.

3.2. Materials and methods

3.2.1. Experimental animals

Eggs of silkworm *B. mori* (hybrid strain, Kinshu × Showa) were purchased from the silkworm egg producing company, Ueda Sanshu Ltd (Ueda, Japan). Larvae were reared on as described in section 1.1.1.

3.2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Tissues including brain, central nervous ganglia, labrum, mandible, antenna, maxilla, labium, caudal leg, foregut, hindgut, testis, ovary of 20 larvae; thoracic leg, proleg, malpighian tubule of 15 larvae; midgut of five larvae; silk gland, fat body of two larvae were dissected from two-day-old fifth instar larvae. All collected tissues were homogenized in 0.5–2 ml cold ISOGEN II (Nippon Gene, Co., Ltd, Tokyo, Japan) using a Physcotron generator shaft, NS-10G (Microtec Nition, Co., Ltd, Chiba, Japan), motorized by a Drive Unit, NS-310E (Microtec Nition, Co., Ltd), and kept at -80 °C until use for RNA extraction. For cDNA synthesis, double-stranded cDNA fragments amplification and PCR were performed as described in section 1.1.2.

3.2.3. cDNA cloning of *BmGrs* genes

Total RNA from dissected *B. mori* larval maxillary galea was purified with ISOGEN II (Nippon Gene, Tokyo, Japan), and used to prepare cDNA with the ReverTra Ace® (Toyobo, Osaka, Japan) following the manufacturer's instructions. Full-length coding sequences of BmGr6, BmGr7 and BmGr9 were obtained through

Two-Step PCR using gene specific primers (Table 3.2). The amplified DNA fragments of BmGr9 and BmGr6 were cloned into EcoRI and ApaI site of pcDNA3.1 vector. For BmGr10, the amplified DNA fragments was cloned into EcoRV site of pcDNA3.1 vector (Thermo Fisher Scientific) using GeneArt® Seamless Cloning and Assembly Enzyme Mix (Thermo Fisher Scientific). 3xFLAG®-tag (Sigma-Aldrich Japan, Tokyo, Japan) was previously introduced into the downstream of EcoRV site of pcDNA3.1 and fused to 3' end of the BmGr10 gene.

3.2.4. Cell culture and transfection

Cell culture and transfection were performed as described in section 1.1.4.

3.2.5. Production of recombinant proteins and antisera preparation

Nucleotide sequences coding 106 amino acid residues (^{208}SLRARL–ADGYGL^{315}) of BmGr9 were amplified by PCR using the original plasmid as a template and primer sets that contain BamHI or XhoI sites in the forward and reverse primers, respectively (forward, 5′-TGCGTTTACAGGATCCTCACTGCG-3′; reverse, 5′-TGATGACCAGCTCGAGGCCGTAACCG-3′) (also seen in Table 3.2). PCR was performed with 35 cycles; 98 °C for10 s, 50 °C for15 s, and 72 °C for 1 min. The amplified fragments were digested with BamHI and XhoI and inserted into the GST fusion protein expression vector, pGEX4T-3 (GE Healthcare, Chalfont, UK). The *Escherichia coli* BL21 competent cells (TaKaRa, Shiga, Japan) was transformed BmGr6-pGEX4T-3 construct by electroporation using Gene Pulser (BioRad Japan,

Tokyo). Production of the fusion protein was induced with isopropyl-b-D-thiogalactoside (IPTG) at final concentration of 1 mM for overnight. The bacterial cells were collected by centrifugation and sonicated with VP-15S ultrasonic processor (TAITEC Co., Japan). The inclusion bodies containing the recombinant protein were washed by 1% Triton-X 100 and were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then the gels were stained with Coomassie Brilliant Blue R250 (CBB-R250). The recombinant protein bands were determined by comparing with that of the cell homogenate from a negative control culture grown with an empty vector, and the bands were removed from the gels. The recombinant proteins were then electrically eluted from the gels using a model 422 Electroeluter (Bio-Rad laboratories, USA), and dialyzed with phosphate-buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic, 1.8 mM potassium dihydrogen phosphate, pH 7.4) at 4 °C for 16 h. 100 mg of recombinant protein was injected into mice four times every 10 days.

3.2.6. Western blotting and immunohistochemistry of transfected cells

In the present study, specificity of antisera was confirmed first by western blotting analysis using total protein from recombinant *E. coli* cells, and further confirmed by immunocytochemistry using of *BmGr9* gene expressing in HEK293T cells.

Western blotting analysis was carried out as described in section 1.1.6

HEK 293T cells expressing the *BmGr9* gene were seeded onto poly-D-lysine coated glass cover slips. Next, cells were transiently transfected as described above. After 24 h at 37 °C, 5% CO_2, cells were washed with PBS, and fixed by 4% paraformaldehyde in PBS, then washed again with PBS and conducted immunocytochemistry. The incubation and washing were done like the immunohistochemistry of oral sensory organs and midgut cryosections (Detail in section 1.1.7).

As negative controls, the transfected cells were immunostained with secondary antiserum alone and non-transfected HEK293T cells were immunostained by anti-BmGr9 antiserum.

3.2.7. Immunohistochemistry and microscopy

Heads of two-day-old fifth instar larvae were removed, washed with 70% ethanol, and fixed overnight (12–24 h) at 4 °C in 4% paraformaldehyde in PBS. After fixation, the antenna, labrum, maxilla and labium were separated, and each organ was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Co., Ltd., Japan) and frozen at -20 °C. The embedded specimens were mounted on an object holder and sectioned using a Tissue-Tek Cryo$_3$® cryostat (Sakura Finetek Japan, Co., Ltd., Japan). 16 µm thick cryosections of each organ was placed on MAS-GP type-A coated slide glasses (Matunami glass, Japan) and air dried at RT for about 4 h. Then, sections were used to performed immunohistochemistry as described in section 1.1.

6 but now incubate with primary antiserum (anti-BmGr9 antiserum in TNT at a 1:1000 dilution).

For whole mount immunocytochemistry, the nervous system from two-day-old third instar larvae were fixed at 4 °C overnight in 4% paraformaldehyde in PBS, containing 0.1% Triton X-100 (PBST). After fixation, tissues were used to performed immunohistochemistry as described in section 1.1.6.

Negative control was prepared by application of the pre-immune mouse antiserum taken from the animal that was used to produce the primary antiserum. Secondary antiserum incubation, and all subsequent processing, was performed in the dark. Likewise, all samples were stored in the dark at 4 °C until they were examined.

For double-immunohistochemistry, a mouse polyclonal antiserum (anti-BmGr9) and rabbit anti-*Dm*FMRFamide (ABcam) or anti-*Dm*NPF antiserum (ABcam) were used. Tissues were incubated in a primary antisera mixture of anti-BmGr9 antiserum diluted 1:1000 and a 1:1000 dilution of either anti-*Dm*FMRFamide or anti-*Dm*NPF antiserum. After the incubation and washing procedures as described above, the specimens were incubated with a secondary antisera mixture of a goat anti-rabbit IgG conjugated to Alexa Fluor 488 diluted 1:1000 and a goat anti-mouse IgG coupled to Alexa Fluor 555 (Invitrogen) diluted 1:1000. Negative controls (Fig. 3.14 A and B) for double-immunohistochemistry were performed using anti-BmGr9 alone as a primary antiserum, and incubated with secondary antisera mixture as noted above.

For antisera labeling, the anti-BmGr9 and -BmGr6 antisera were first affinity purify according to the Protein A Kit (Aproscience, Tokushima, Japan). After purification, these antiseta were labeling with Alexa Fluor® 555 or 488 respectively using Alexa Fluor® Antibody Labeling Kit (Thermo Fisher Scientific, Kanagawa, Japan). Double whole-mount immunohistochemistry was performed on the same tissues using antisera that label specifically with Alexa Fluor® dye in the dark. Negative control was prepared by application of the preimmune mouse antiserum taken from the animal that was used to produce the primary antiserum. Secondary antibody incubation, and all subsequent processing, was performed in the dark. Likewise, all samples were stored in the dark at 4 °C until they were examined. Negative control (Fig. 3.14 C) for labeling anti-BmGr6 antisetum, labeling anti-BmGr9 antiserum was used alone. As mentioned above, all samples were stored in the dark at 4 °C until they were examined.

3.3. Results

3.3.1. RT-PCR for the analysis of BmGr9 gene expression in tissues

To determine the expression sites of *BmGr9* gene in the tissues including oral sensory organs and central nervous system (CNS), RT-PCR experiments were performed using specific primers which were designed considering the intron region of the genome to make it possible to differentiate the amplicon of target cDNA from that of contaminating genomic DNA. cDNA was prepared from various organs and tissues in fifth instar day 2 silkworm larvae. As shown in Fig. 3.1A, RT-PCR products of BmGr9 from the labrum, mandible, maxillary, labium, thoracic leg, proleg, midgut, hindgut, testis and ovary formed intense bands in the agarose gel electrophoresis. Interestingly, a significant level of *BmGr9* expression was also observed in the CNS, a non-chemosensory tissue that has been shown to express Grs (Miyamoto et al., 2012; Park and Kwon, 2011a; Thorne and Amrein, 2008). In addition, faint bands were also detected in the foregut and fat body, whereas no band was detected from antenna and malpighian tubule (Fig. 3.1 A). These results indicate that *BmGr9* gene is widely expressed in some chemosensory organs as well as in non-chemosensory organs.

3.3.2. Antiserum specificity

To validate the RT-PCR results and identify BmGr9 expressing cells, a mouse antiserum to BmGr9 was raised and used in immunohistochemistry studies. I attempted to produce several different regions of BmGr9 molecule as recombinant

protein fragments in *Escherichia coli*, while the amounts of the most of recombinant proteins produced were null or too small. However, a 106-amino acid residue (^{208}SLRARL-ADGYGL^{315}) of the BmGr9 sequence was successfully produced and used as an immunogen for anti-BmGr9 mouse antiserum. The specificity of the raised antiserum was confirmed first by western blot analysis of total proteins including a *glutathione-S-transferase* (GST)-BmGr9 fusion protein isolated from recombinant *E. coli* cells. Consistent with the theoretical molecular mass of the fusion protein, a single band with a molecular mass of 39 kDa, was detected specifically (Fig. 3.2 A).

The specificity of the antiserum was further verified by immunohistochemistry using HEK293T cells expressing BmGr9. BmGr9-expressing HEK293T cells were stained with the anti-BmGr9 antiserum (Fig. 3.2 B-a) and no stained cells were observed when the primary antibody was omitted (Fig. 3.2 B-b). In addition, no immunoreactivity was detected in untransfected HEK293T cells, even when they were immunostained with the anti-BmGr9 antiserum (Fig. 3.2 B-c).

On the other hand, homology and phylogenetic analyses revealed that BmGr9 is most closely related to BmGr10 which is considered to be an another ortholog of DmGr43a (Figs. 3.3 and 3.4). Thus, a possibility that the anti-BmGr9 antiserum cross-react with BmGr10 existed. To exclude this possibility, I next stained HEK293T cells expressing flag tag appended-BmGr10 with anti-BmGr9 antiserum and no stained cells were observed (Fig. 3.2 C-b), although anti-flag antiserum did detect BmGr10 expression (Fig. 3.2 C-a). Furthermore, to exclude the possibility that anti-BmGr9 antiserum may cross-react with other BmGrs protein, we also performed

immunohistochemistry using HEK293T cells expressing BmGr6 with anti-BmGr9 antiserum. I found that anti-BmGr6 antiserum (Chapter 1), stained BmGr6-expressing HEK293T cells (Fig. 3.2 D-a), but anti-BmGr9 antiserum did not (Fig. 3.2 D-b). Taken together, these results showed that anti-BmGr9 antiserum is specific to BmGr9 and can be used for immunohistochemistry to detect BmGr9 expression in *B*. *mori* tissues.

3.3.3. Distribution of BmGr9-expressing cells in oral sensory organs

Given the observation that *BmGr9* gene is expressed in the oral sensory organs (Fig. 3.1), I sought to indicate the cells which express BmGr9 in these organs, and immunohistochemistry experiments were performed. Cryosections from the maxillary galea, maxillary palpus, labrum and labium from fifth instar day 3 larvae were incubated with anti-BmGr9 antiserum (Fig. 3.5). Negative control studies were conducted using pre-immune serum taken from the animal that was subsequently immunized with the BmGr9 peptide to produce the primary antiserum, and no immunoreactive cell was observed (Fig. 3.13 A).

As depicted in Fig. 3.5 A, a cell stained with anti-BmGr9 was apparent in the maxillary galea. In the case of maxillary palpus, a cell stained with anti-BmGr9 was also observed in the bottom region of this organ (Fig. 3.5 B). A section which was shown in Fig. 3C represents one pair of anti-BmGr9 staining cell in the labrum. Furthermore, a cell stained with anti-BmGr9 was also observed in sections of the labium (Fig. 3.5 D). Taken together, consistent with the results of RT-PCR analysis

which indicated that *BmGr9* gene is expressed in oral sensory organs, BmGr9 protein is also localized in cells of these organs.

3.3.4. Expression of BmGr9 gene and protein in the nervous system of B. mori larvae

RT-PCR analysis using specific primers for the BmGr9 revealed that this Gr gene was expressed in the CNS (Fig. 3.1 A). Next, location of BmGr9 gene expression in the brain and each ganglion was examined. By the RT-PR, I found that brain and all ganglia expressed the *BmGr9* gene (Fig. 3.1 B). Moreover, the information about onset or duration of *BmGr9* expression during different developmental stages (1st to 5th instars) were compared by RT-PCR (Fig. 3.1 C). Results indicated that *BmGr9* was constantly expressed in CNS throughout all developmental stages from the first instar to fifth instar.

To further validate our RT-PCR results and visualize the cells which express BmGr9 in the nervous system, I performed immunohistochemistry using whole-mount central nervous tissues. Small numbers of cells or axons stained with anti-BmGr9 were present symmetrically throughout the larval central nervous system (Figs. 3.6 and 3.7). Based on anti-BmGr9 staining, I counted more than 8 pairs of anti-BmGr9 staining cells in the larval brain (Fig. 3.6 A) and no cell was stained with the secondary antiserum alone (Fig. 3.13 B). The cells were observed in clusters and located in the medial and lateral regions. A cluster consisting of at least three cells with large somata were located in the medial region of the brain and the cells were

weakly stained (indicated by open, thick arrows in Fig. 3.6 A). The other cluster consisting of five cells was located in the lateral region of the brain and cells were more intensely stained (indicated by arrowheads in Fig. 3.6 A).

Cells clearly stained with anti-BmGr9 were seen in whole-mount ventral nerve tissues of *B. mori* larvae. In the subesophageal ganglion (SOG), two pairs of dorsolateral cells were stained intensely. They were located in the middle region and posterior region, respectively (arrows in Fig. 3.6 B). In each thoracic ganglion (TG) 1–3, a pair of dorsolateral cells showed BmGr9 expression (Fig. 3.6 C_1–C_3). Furthermore, one pair of posterior dorsomedial cells was detected in each larval abdominal ganglion, AG1–6 (Fig. 3.6 D_1–D_6). In AG7 (the anterior neuromere) of the terminal abdominal ganglia (TAG), one pair of posterior dorsomedial cells were stained (closed arrows in Fig. 3.6 D_7,) and in the anterior region of AG8 (the posterior neuromere of TAG), three to four large dorsomedial cells were observed to be stained (open arrows in Fig. 3.6 D_7 and D_8). Besides, in the posterior region of AG8, a cluster of cells was stained with anti-BmGr9 (arrowheads in Fig. 3.6 D_8).

In the frontal ganglion (FG), one pair of cells was stained intensely with anti-BmGr9 (arrows in Fig. 3.7 A) and some other cells were also stained faintly. The corpora cardiaca (CC) and corpora allata (CA) were covered with anti-BmGr9 staining axons that originated from the brain, but no staining cell was observed inside the organs (Fig. 3.7 B and C).

3.3.5. Co-expression of BmGr9 and FaRPs in neurosecretory cells of the CNS

To determine whether BmGr9-expressing cells are neurosecretory cells, I performed double-immunohistochemistry of whole mount central nervous tissues, using anti-BmGr9 antiserum and anti-*Dm*FMRFamide antiserum which has been used to identify the BmGr6-expression neurosecretory cells (Chapter 2). As I already indicated (Fig. 3.6 A), two clusters of cells were observed to be stained with anti-BmGr9 antiserum in the lateral region of the brain. At least three BmGr9-expressing cells were confirmed to be co-localized with the cells stained by anti-*Dm*FMRFamide antiserum in the lateral region of the brain (Fig. 3.8 A). The other cells with large somata were observed to be stained with anti-BmGr9 in the medial region of the brain (Fig. 3.6 A and 3.8 B). All anti-BmGr9 positive cells were also stained with anti-DmFMRFamide antiserum (Fig. 3.8 B). Moreover, one pair of BmGr9-expression cells located in the posterior region of each abdominal ganglion (AG) 1–7 (Fig. 3.6 D_1–D_7) were also observed to be co-localized with the cells stained by anti-*Dm*FMRFamide antiserum (Fig. 3.9 C_1–C_3). Three to four large dorsomedial cells were stained with anti-BmGr9 antiserum in the AG8 (open arrows in Fig. 3.6 D_7–D_8). These large cells were also observed to be co-localized with the cells stained by anti-*Dm*FMRFamide antiserum (yellow arrows and arrowheads in Fig. 3.9 D_1–D_3). In contrast, although two pairs and one pair of dorsolateral cells showed BmGr9 expression in SOG and thoracic ganglia respectively (Fig. 3.6 B and C_1–C_3), no co-localization was observed between BmGr9-expression cells and *Dm*FMRFamide-expression cells in these ganglia (Fig. 3.9 A_1–A_3, B_1–B_3). These

findings suggest that BmGr9 was expressed in a portion of neurosecretory cells which express FaRPs. These findings raise the possibility that BmGr9 functions not only in detection of fructose in the gustatory organs, but also as a sensor of internal nutrients in the brain and other internal tissues;

3.3.6. Co-expression of BmGr9 and NPF1 in the brain neurosecretory cells

A previous study showed that DmGr43a expresses in neurons of brain to sense hemolymph fructose and promote feeding in starved flies and possibility of the secretion of neuropeptide F (DmNPF) in the state of promotion of the feeding was discussed (Miyamoto et al., 2012). Since Dm43a is an ortholog of BmGr9, this allowed us to speculate that BmGr9 may serve similar biological functions as DmGr43a in the *B. mori* brain, and feeding behavior of *B. mori* larvae might be modulated by the DmNPF ortholog. Because neuropeptide F1 (BmNPF1) appears to be an orthologue of *D. melanogaster* NPF (Roller et al., 2008), and the *Drosophila* anti-NPF (anti-DmNPF) antiserum could be used to identify NPF1 in *B. mori* (Chapter 2). I therefore next performed double-immunohistochemistry to determine whether BmGr9 and putative BmNPF1 are co-expressed in the same neurosecretory cells, using a mouse anti-BmGr9 and a rabbit anti-DmNPF antisera. As I mentioned above, two clusters of BmGr9-expressing cells were observed to be located in the lateral region of the brain (Fig. 3.6 A). Most of these cells were observed to be co-localized with the cells stained by anti-NPF antiserum (Fig. 3.10 A) as the case of anti-DmFMRFamide antiserum staining (Fig. 3.8 A). Moreover, all BmGr9-expressing

cells with large somata in the medial region of the brain, were stained with anti-DmNPF antiserum (Figs. 3.6 A and 3.10 B) as the case of anti-DmFMRFamide antiserum staining (Fig. 3.8 B). Apart from the brain, four large BmGr9-expressing cells in the AG8 were also observed to be stained with anti-DmNPF antisrerum (Figs. 3.6 D_7–D_8 and 3.10 C) as the case of anti-DmFMRFamide antiserum staining (Fig. 3.9 D). However, although BmGr9-expressing cells were found in all thoracic-abdominal ganglia (Figs. 3.6 C_1–C_3, D_1–D_8), no co-localization of BmGr9-expressing cells and anti-DmNPF-stained cells was observed in thoracic ganglia and the other abdominal ganglia (AG) 1–7 (Fig. 3.11).

3.3.7. Co-expression of BmGr9 and BmGr6 in the brain neurosecretory cells

I recently described that a portion of BmGr6-expression cells are co-localized with FMRFamide-expression cells in brain of the *B. mori* larvae (Chapter 2). Moreover, as I mentioned above, some of BmGr9-expression cells are also co-localized with anti-DmFMRFamide-stained cells in the brain. According to these findings, a possibility that BmGr9 and BmGr6 could be co-expressed in the same brain neurosecretory cells exists. To scrutinize this view, double immunohistochemistry experiments were performed. As results are depicted in Fig. 3.6A, two clusters of BmGr9-expressing cells were observed to be located in the lateral region of the brain (Fig. 3.6 A). Most of these cells were observed to be co-localized with BmGr6-expressing cells (Fig. 3.12 A). Moreover, all BmGr9-expressing cells with large somata in the medial region of the brain, were also co-localized with

BmGr6-expressing cells (Figs. 3.6 A and 3.12 B). These results demonstrated that most of BmGr9-expressing neurosecretory cells in the brain also express BmGr6.

Apart from the brain, it is intriguing to note that almost all BmGr9-expressing cells also express BmGr6 in the frontal ganglion (FG) (Fig. 3.12 C) which constitutes a major source of innervation to foregut muscles and plays a key role in the control of foregut movements (Ayali, 2004).

3.4. Discussion

3.4.1. BmGr9-expression is not restricted to the gustatory organs

Sato et al. (2011) reported that BmGr9 gene was expressed in the maxilla, labium, thoracic leg and gut, but they did not examine whether this Gr gene was expressed in CNS, silk gland, malpighian tubule, testis, ovary and fat body (Sato et al., 2011). In this study, I indicated by RT-PCR that *BmGr9* gene was expressed not only in the sensory organs including labrum and proleg, but in CNS and other tissues including silk gland, testis, ovary and fat body (Fig. 3.1). The differences between these observations with regard to the *BmGr9* gene expression in labrum and proleg may be due to differences in the primers used and the total RNA concentrations of the samples.

The gustatory organs of insect play an important role in feeding behaviour during larval life. In lepidopteran larvae, the gustatory sensilla are located in gustatory organs including maxillary galea, maxillary palpus, and labrum (Albert, 1980; Asaoka and Shibuya, 1995; Schoonhoven and Van Loon, 2002). BmGr9 expression was examined at the level of both gene and its protein. Our findings indicate that BmGr9 is localized in cells in the maxillary galea, maxillary palps, and labrum, as well as in the labium (Fig. 3.5), which are similar to previous study reported that most of *T. castaneum* Grs were expressed in the labium, maxillae (Abdel-latief, 2007). Our results demonstrated that BmGr9 is broadly expressed throughout oral sensory organs. This suggests that BmGr9 may play an important role in taste perception and feeding behaviors in *B. mori* larvae.

3.4.2. BmGr9-expression in the nervous system of *B. mori* larvae

Insect nervous system plays a significant role in incorporation of a variety of internal physiological information as well as external sensory information (Gullan and Cranston, 2005). Insect gustatory receptors are generally expressed in gustatory neurons. Previous studies showed in *D. melanogaster*, that Grs are expressed in neurons in the brain, the subesophageal ganglion (SOG), and abdominal ganglia (AG) (Thorne and Amrein, 2008; Park and Kwon, 2011a; Mishra et al., 2013; Miyamoto et al., 2012). Our RT-PCR results also indicated that *BmGr9* gene is expressed in the CNS of *B. mori* larvae (Fig. 3.1). More importantly, the results of immunohistochemistry showed that BmGr9 protein was expressed in cells of CNS of the *B. mori* larvae (Figs. 3.6–7). It was reported that DmGr43a is expressed in brain neurons, and are necessary to sense fructose in the hemolymph to regulate feeding behavior (Miyamoto et al., 2012). These findings have raised the possibility that BmGr9 serves similar biological functions as DmGr43a in the *B. mori* brain.

Similar to our recent report with BmGr6 (Chapter 1), BmGr9 is also expressed in cells located in ganglia of CNS (Fig. 3.6). Moreover, the thoracic ganglia (TG) in insects controls locomotion by innervating the legs and wings and the abdominal ganglia (AG) regulate functions such as respiration, heartbeat, hindgut movement, abdominal posture, and functions of the genitalia and ovipositor (Nässel, 1996). Therefore, it seems likely that some information, such as fructose sensing, is conveyed to be processed by these ganglia.

3.4.3. BmGr9 involvement in promotion of feeding suggested by co-expression with BmNPF1

Sato et al. (2011) showed that BmGr9 respond specifically to fructose (Sato et al., 2011). Later, a BmGr9 ortholog in *D. melanogaster,* DmGr43a was indicated expressing in neurons of brain to sense hemolymph fructose and promote feeding in starved flies, but suppress feeding in satiated flies (Miyamoto et al., 2012). As mentioned above, BmGr9 is an ortholog of Dm43a, and BmGr9 is also expressed in brain neurosecretory cells (Figs. 3.6 A and 3.8). These findings allowed us to speculate that BmGr9 may serve similar biological functions as DmGr43a in the *B. mori* brain. I find that most of BmGr9-expressing cells were co-localized with anti-DmFMRFamide-stained cells in the brain (Fig. 3.8). Previous works on *Drosophila* and other insect species suggested that FMRFamide-immunoreactive cells in the insect could produce several neuropeptides such as NPF, sNPF and sulfakinins (Boer et al. 1980; Veenstra et al., 2008), suggesting that BmGr9 was expressed in a portion of brain neurosecretory cells which express NPF, sNPF or sulfakinins. Moreover, co-localization of BmGr9-expressing cells and anti-DmNPF-stained cells were also found in the brain (Fig. 8 A and B). Since all were co-localized with BmGr9 positive cells (Figs. 3.6, 3.8 and 3.10), anti-FMRFamide serum stained cells and anti-DmNPF-stained cells were considered to be the same. Because neuropeptide F1 (BmNPF1) is an ortholog of DmNPF in *B. mori* (Roller et al., 2008), I believe that anti-DmNPF-stained cells in the *B. mori* brain probably produced

BmNPF1, and therefore the anti-DmNPF-stained cells are putative BmNPF1-expressing cells.

Although the specific physiological role of BmNPF1 in *B. mori* is still unknown, the co-expression of this neuropeptide with BmGr9 in the CNS suggests its regulating role in feeding behavior. By the way, its ortholog in *D. melanogaster*, DmNPF is known to increase food consumption. Previous studies showed that *DmNPF* gene is highly expressed in the fly larvae attracted to food (Wu et al., 2003), and over-expression of DmNPF prolongs feeding episodes (Wu et al., 2005). Over-expression of the DmNPF receptor (DmNPFR) caused well-fed larvae to consume aversive, bitter tasting food (Garczynski et al., 2002; Wu et al., 2005). Accordingly, DmNPF in the central nervous system of *D. melanogaster* promote feeding and related behaviors. Moreover, high level concentration of NPF1-like peptides was observed in the circulating hemolymph of actively feeding *Helicoverpa zea* larvae, suggesting that NPF1 of lepidopteran insects have orexigenic activity. From these, it is reasonable to speculate that BmGr9 may function as a fructose sensor in the neurosecretory cells of the brain to promote feeding behavior of *B. mori* larvae.

3.4.4. BmGr9 co-expression with BmGr6 in the brain

BmGr9 does not require any expression of other BmGrs in the same cell to show the responsiveness to fructose in vitro (Sato et al., 2011). However, the possibility that BmGr9 co-express others BmGrs in the same neurons is unable to be excluded,

since DmGr43a was reported to co-express DmGr64a, a molecule of the sugar receptor clade in the same neurons of the brain (Fujii et al., 2015). In this study, I showed that most of BmGr9-expressing cells in the *B. mori* larvae brain also expressed BmGr6 (Fig. 3.12 A and B), a molecule of the sugar receptor clade. Previous studies in *D. melanogaster* have showed that eight DmGrs (DmGr64a-f, DmGr61a and DmGr5a Gr) are co-expressed in gustatory receptor neurons (GRNs), and DmGr64a is required for detecting multiple sugars including sucrose, maltose and glucose (Jiao et al., 2007). It is possible that except D-fructose, BmGr9 may exhibit different ligand response properties in the *B. mori* larvae. On the other hand, as shown in Fig. 3.12A, some small cells were stained with anti-BmGr6 antiserum alone, suggesting that BmGr6 may response ligands which are different from the compounds that were detected by the combination of BmGr9 and BmGr6; and BmGr6 may play roles distinct combinations of BmGr9 and BmGr6. In addition, previous studies showed that seven DmGrs which most related to DmGr5a were expressed in DmGr5a-expressing cells, and these cells recognize many sugars and may thus express additional Grs (Chyb et al., 2003; Jiao et al., 2007; Wang et al., 2004). Therefore, the possibility that BmGr6-expressing cells may express other sugar clade BmGrs cannot be excluded.

An unexpected finding in this study is that I was unable to detect any positive cell for BmGr9 in gut via immunohistochemistry using the anti-BmGr9 antiserum. *BmGr9* gene expression in the gut was reported by Sato et al. (2011). In this study, I also observed *BmGr9* gene expression (Fig. 3.1) by PCR with materials prepared from the

midgut, hindgut, and foregut (Fig. 3.1 A). However, no BmGr9 expressing cells were found by immunohistochemistry using both whole mount gut (1st to 3rd instar larvae) and gut cryosections (4th and 5th instar larvae), even though I used the same method as that used for the observation of BmGr6 expressing enteroendocrine cells of the midgut (Chapter 1). One possible interpretation of these results is that low levels of expression of this Gr protein exist in the cells of *B. mori* larvae gut. So, in contrast to BmGr6, it is unlikely that BmGr9 plays important roles in the gut.

Table 3.1 Primers used for RT-PCR

Gene	S/AS	Primer sequences (5'-3')
BmGr9	S	CAGAAGACATACTGTGCCGACT
	AS	CATATCGCTGGAATTGAATGAG
β-actin	S	CGTACCACCGGTATCGTGCT
	AS	GAGGATCTTCATGAGGTAGTCGGTC

Table 3.2 Primers used for cloning into pcDNA3.1 in this study

Gene	PCR 1st/2nd	S/AS	Primer sequences (5'-3')
BmGr9	1st	S	CCTCTGAGGTTCGAGCCAC
	1st	AS	CTAAATAAGTGGTGACCGCTCC
	2nd	S	CGCGGATCCATGCCTCCTTCGCCAGATCT
	2nd	AS	CCGGAATTCTTAACTATCATATCGCTGGA
BmGr10	1st	S	CGTCTACAGTGCATGGTCCCG
	1st	AS	CCAGCTGAACTATCACTATTAGGTATG
	2nd	S	CCACCCGGATCCGATATGACAATGTCAATTAAACC
	2nd	AS	GTCTTTGTAGTCGATTTGCATATTTTTAATTTCCA
BmGr6	1st	S	GGATTTGTTCGTGATTTGTATGAGTT
	1st	AS	CTGAAGCGGGTGCTTGATTG
	2nd	S	CCGGAATTCATGCTTCTGAGAAACTACAA
	2nd	AS	CCGCGGGCCCCTACGAGTAGTTGTAAAATG

S, sense primer; AS, antisense primer. Underline indicate restriction endonuclease recognition sites.

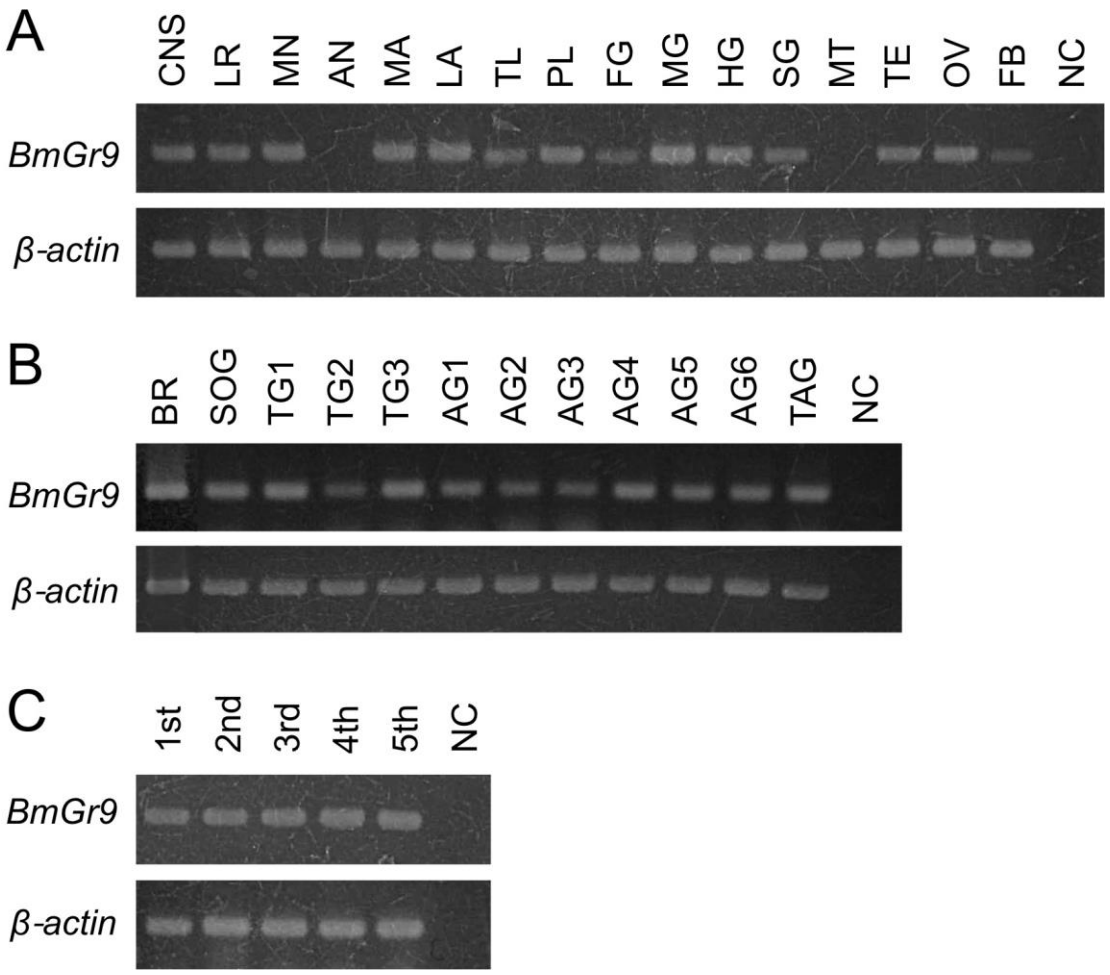


Fig. 3.1. RT-PCR analysis of expression of *BmGr9* gene in *B. mori* larval.

(A) Tissues were dissected from two-day-old fifth instar larvae. ***β-actin*** was used as the internal control. Amplification products were analysed on agarose gels and visualized by UV illumination after ethidium bromide staining. CNS, central nervous system; LR, labrum; MN, mandible; AN, antenna; MA, maxilla; LA, labium; TL, thoracic leg; PL, proleg; FG, foregut; MG, midgut; HG, hindgut; SG, silk gland; MT, malpighian tubule; TE, testis; OV, ovary; FB, fat body; NC, negative control, indicating no contamination by the DNA with template in the reaction reagents and water. (B) RT-PCR analysis of *BmGr9* gene expression in the brain and ganglia from two-day-old fifth instar larvae. BR, brain; SOG, suboesophageal ganglion; TG1,

prothoracic ganglion; TG2, mesothoracic ganglion; TG3, metathoracic ganglion; AGs, unfused abdominal ganglia; TAG, terminal abdominal ganglion. (C) *BmGr9* gene expressing in the CNS of different instar stages. Tissues were dissected from two-day-old 1st to 5th instar larvae, respectively.

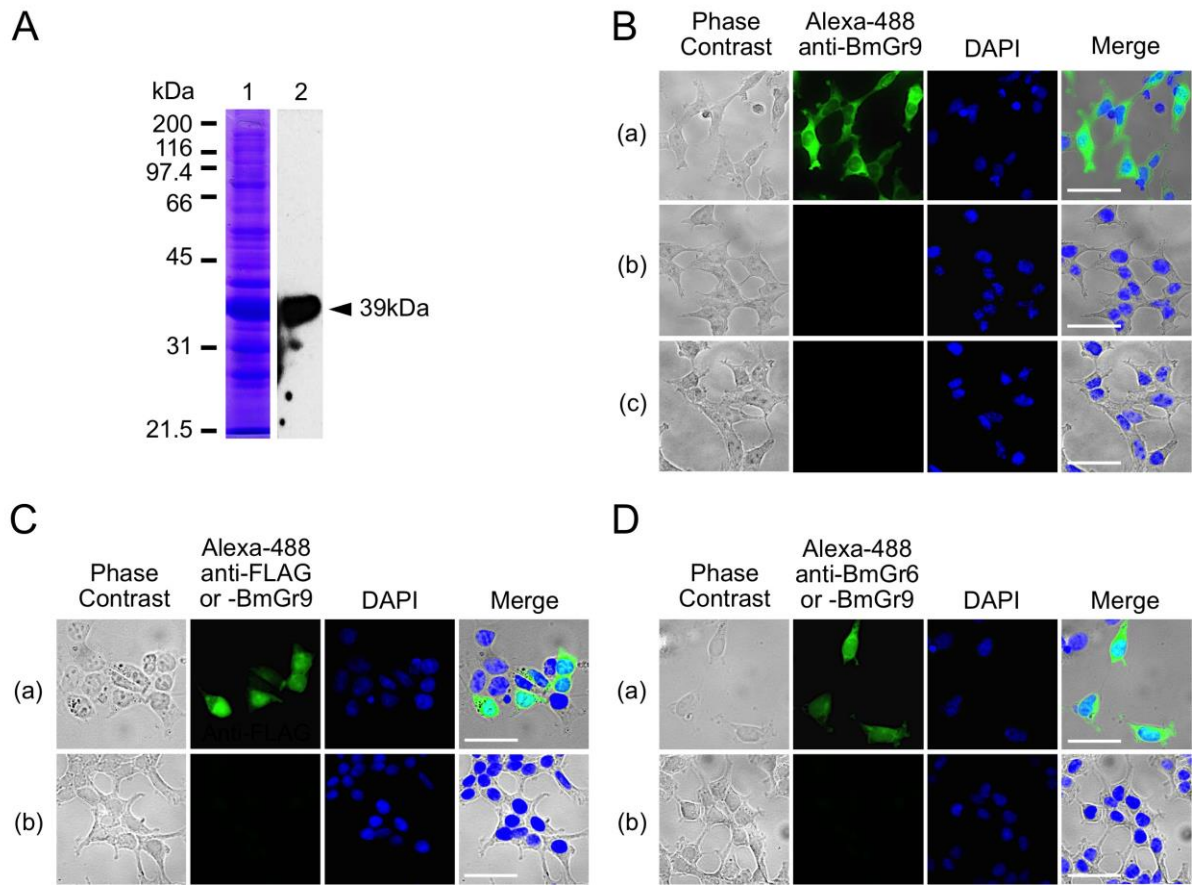


Fig. 3.2. Specificity confirmation of anti-BmGr9 antiserum by immunoblotting and immunostaining.

(A) Total proteins of BmGr9-expressing *E. coli* cells were separated by 12% SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining or immunoblotting. The theoretical molecular mass of BmGr9 protein is 39 kDa. Lane 1, CBB staining of total proteins of *E. coli* BL21 (DE3) pLysS transformed with BmGr9 cDNA-harboring pGEX-4T-3; Lane2, immunoblotting using anti-BmGr9 mouse antiserum. (B) Immunostaining of BmGr9-expressing HEK293T cells. HEK293T cells transfected with BmGr9 cDNA-haboring pcDNA3.1 were immunostained with anti-BmGr9 and secondary antibody (anti-mouse IgG conjugated with Alexa Fluor 488) (a), or with

secondary antibody alone (b), or HEK293T cells without transfection were immunostained with anti-BmGr9 and secondary antibody (c).

(C) HEK293T cells expressing BmGr10 with 3xFLAG-tag in the C-terminal were immunostained with mouse anti-FLAG antibody and anti-mouse IgG conjugated to Alexa Fluor 488 (a), or mouse anti-BmGr9 antiserum and anti-mouse IgG conjugated with Alexa Fluor 488 (bottom). (D) HEK293T cells expressing BmGr6 were immunostained with mouse anti-BmGr6 antiserum and secondary antibody (a), or anti-BmGr9 and secondary antibody (b). Scale bars = 20 μm.

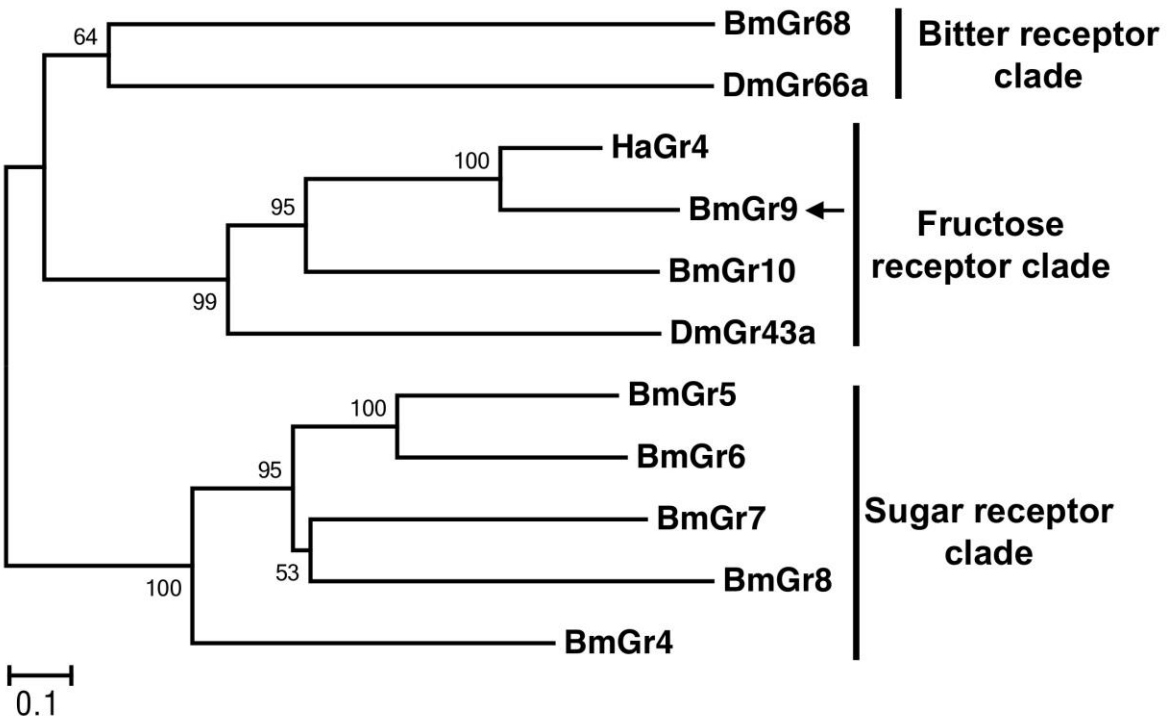


Fig. 3.3. Phylogenetic tree of selected gustatory receptors including BmGr9.

Total amino acid sequences of eight *Bombyx mori* Grs, two *Drosophila melanogaster* Grs, and one *Helicoverpa armigera* Gr were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and phylogenetic analysis was conducted using the neighbor-joining method (Mega 6.06 software). Numbers at the nodes indicate bootstrap values (1000 replicates). Molecules belonging to the bitter receptor clade were used as outgroup members to ascertain the putative sugar receptor and fructose receptor clades. BmGr9 is indicated by an arrow. The amino acid sequences of BmGr4 to 10 were obtained from Wanner and Robertson (2008) and the *D. melanogaster* Grs, HaGr4 and BmGr68, were obtained from NCBI (http://www.ncbi.nlm.nih.gov)

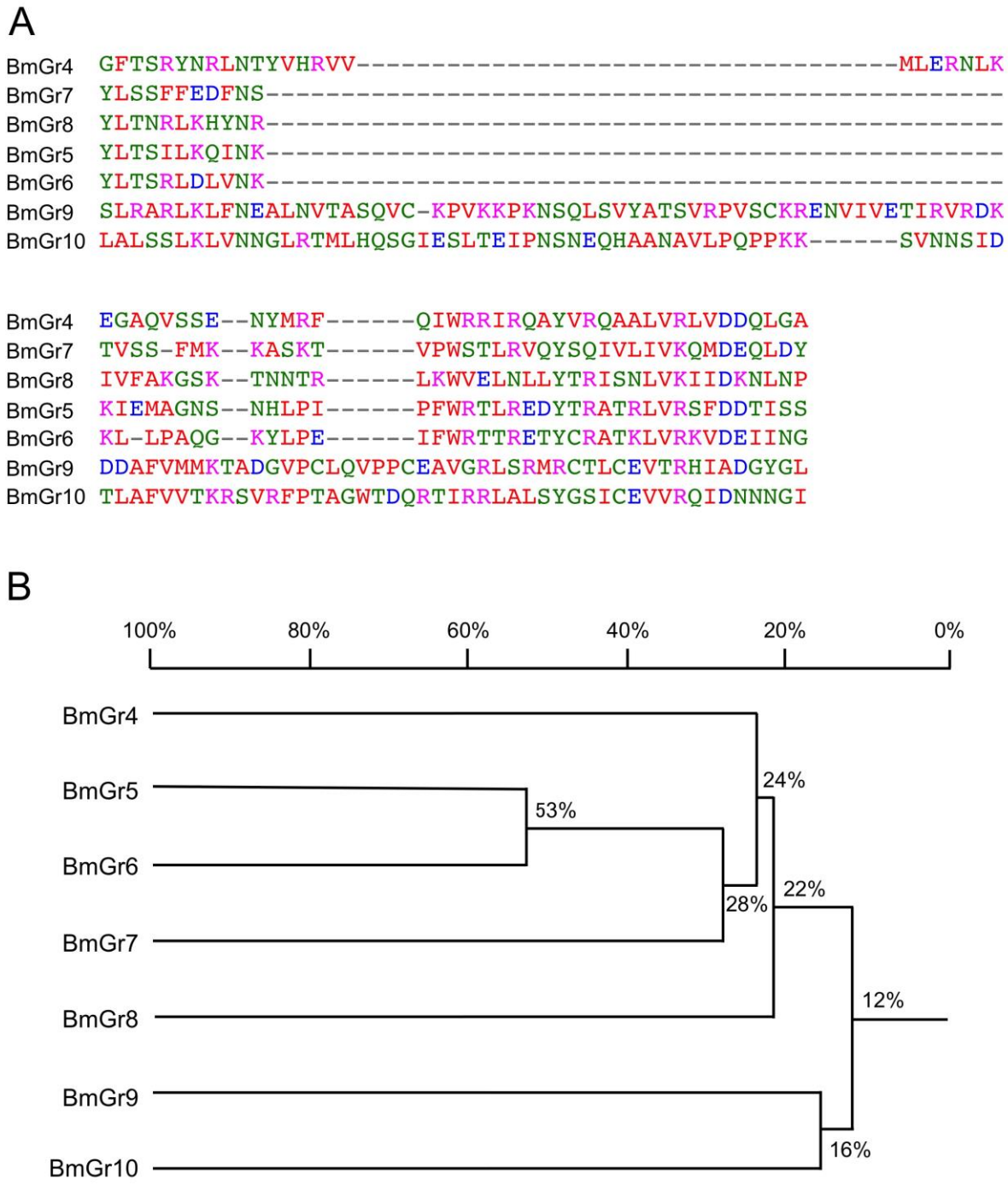


Fig. 3.4. Amino acid sequence alignment (A) and homology three (B) of the BmGr9 protein region used for immunization and the corresponding regions of other BmGrs.

BmGrs amino acid sequences were obtained from Wanner and Robertson (2008) and used to generate a multiple sequence alignment using the ClustalW2 program

(http://www.ebi.ac.uk/Tools/msa/clustalw2/). To understand the identities among BmGrs, homology three was constructed using software DNAMAN, based on the multiple sequence alignment. Percentages of identity among BmGrs are indicated on the nodes.

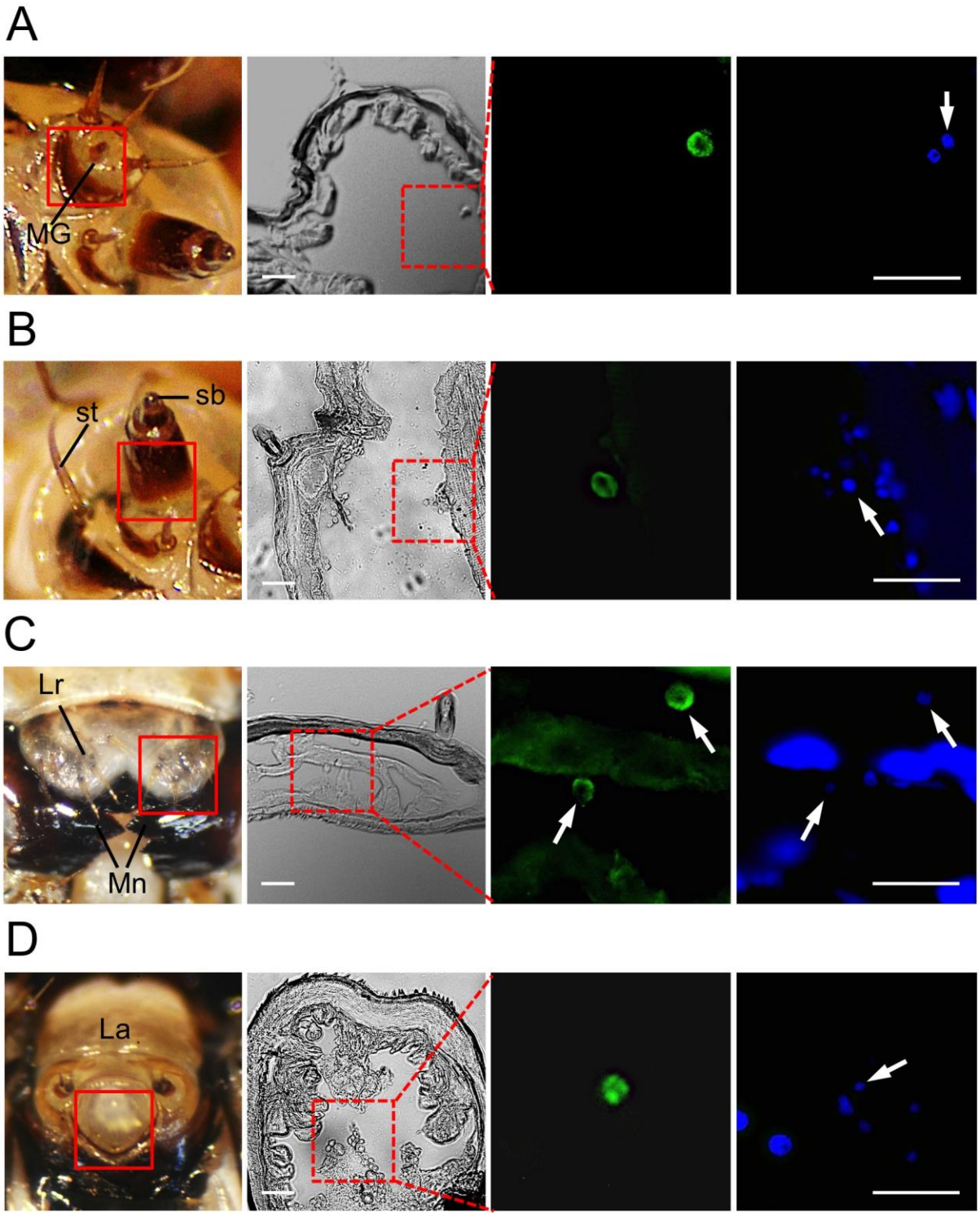


Fig. 3.5. BmGr9-expression in larval oral sensory organs.

Cryosections of larval oral sensory organs were stained with mouse anti-BmGr9 antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488®. BmGr9 expressing cells in cryosections of maxillary galea (A), maxillary palpus (B),

labrum (C) and labium (D). The left panels show stereoscopic microscopy image of a fifth instar larvae. The second panels show transmitted light microscopy images of cryosections from regions boxed in the left panels. The third panels showing fluorescence images of higher magnification of the area boxed in the second panels, and the right panels show DAPI staining images of the same cryosections as the third ones. st, sensilla trichodea; sb, sensilla basiconica. Scale bars = 30 μm.

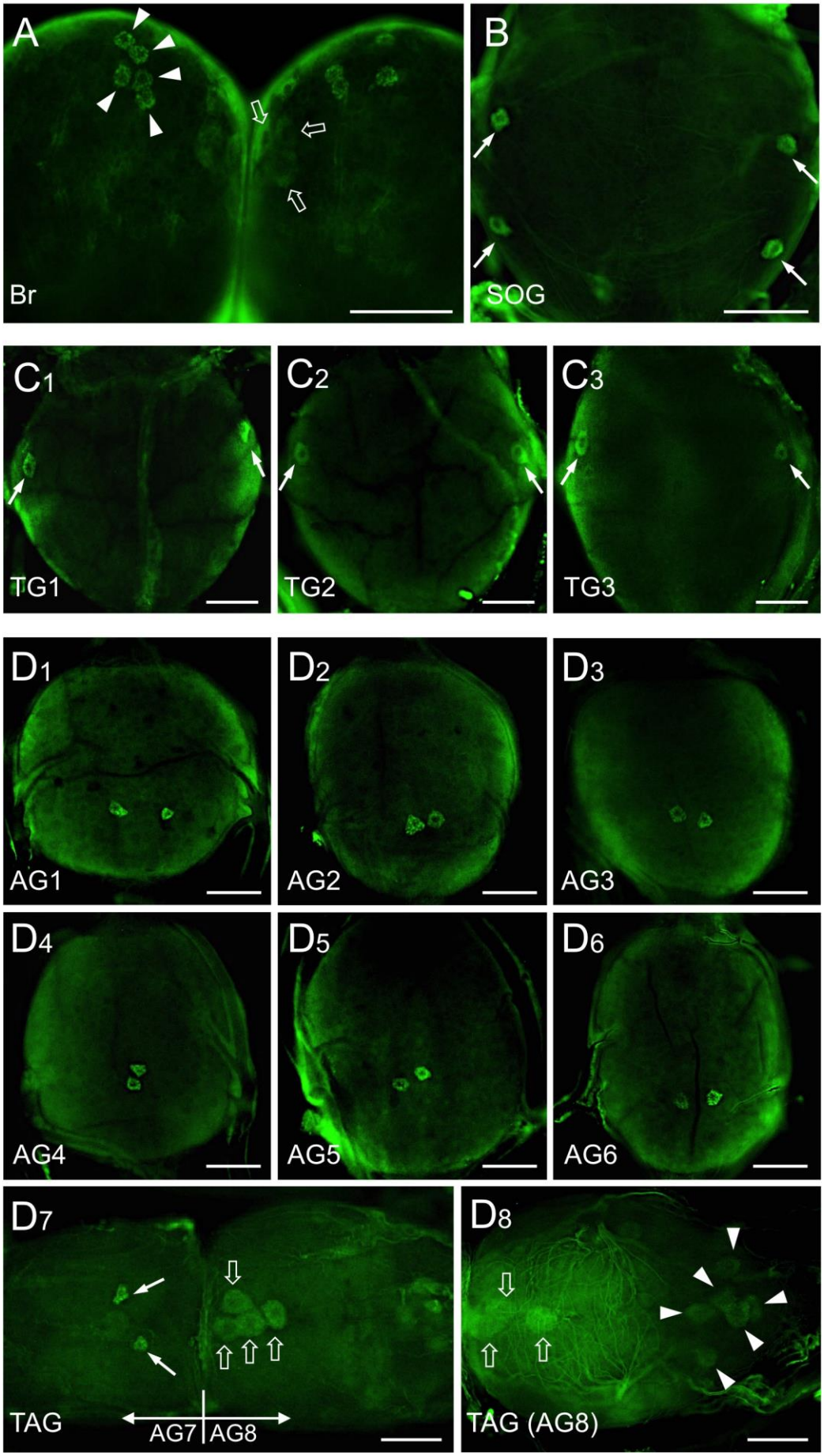

A
Br
B
SOG
C1
TG1
C2
TG2
C3
TG3
D1
AG1
D2
AG2
D3
AG3
D4
AG4
D5
AG5
D6
AG6
D7
TAG
AG7
AG8
D8
TAG (AG8)

Fig. 3.6. BmGr9-expression in the central nerves system of *B. mori* larvae.

Whole-mount tissues were stained with mouse anti-BmGr9 antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488®. (A) Numerous cells immunostained by anti-BmGr9 antiserum in the brain. Open thick arrows indicate cells with large somata and arrowheads indicate other stained cells. (B) One pair of immunostained dorsolateral cells in middle and posterior region of SOG, respectively (arrows). (C_1–C_3) One pair of immunostained dorsolateral cells in TG1, TG2 and TG3, respectively. (D_1–D_6) Immunostained cells in the AGs.

(D7 and D8) AG7 shows one pair of immunostained dorsomedial cells in posterior region; AG8 shows four immunostained large dorsomedial cells in the anterior region (Open thick arrows), one pair of immunostained dorsolateral cells in the lateral (arrows) and a cluster of immunostained cells (arrowheads) in the posterior region. Br, brain; SOG, suboesophageal ganglion; TG, thoracic ganglia; AG, abdominal ganglia; TAG, terminal abdominal ganglion. Scale bars = 100 μm in (A) or 50 μm in (B–D_8).

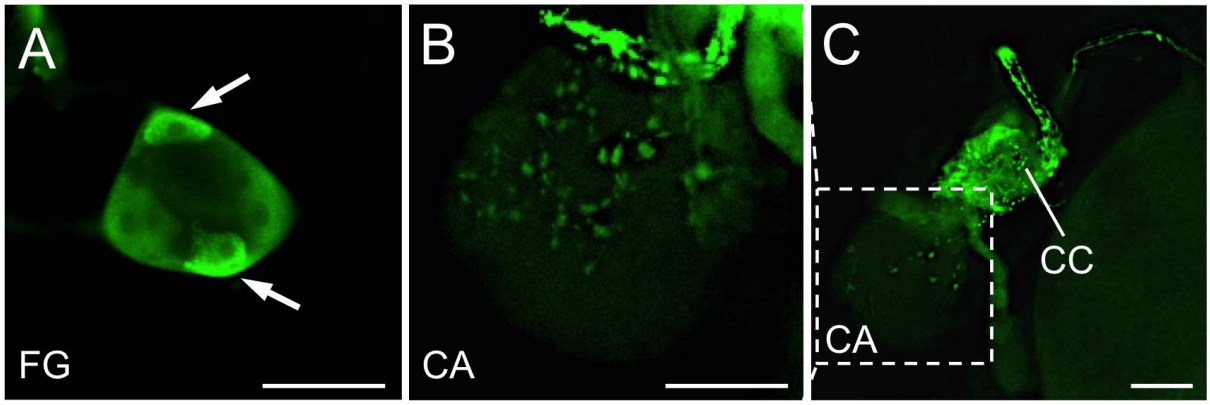


Fig. 3.7. BmGr9 immunoreactivity in the frontal ganglion, corpus cardiacum and corpus allatum.

Whole-mount tissues were stained with mouse anti-BmGr9 antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488®. **(A)** Frontal ganglion (FG) exhibiting a pair of immunostained cells (arrows). (B and C) Immunoreactive axons covering the corpus cardiacum (CC) and corpus allatum (CA) of a larval. (B) shows a higher magnification of the area enclosed by dashed square in (C). Scale bars = 100 μm (A) or 50 μm in (B and C).

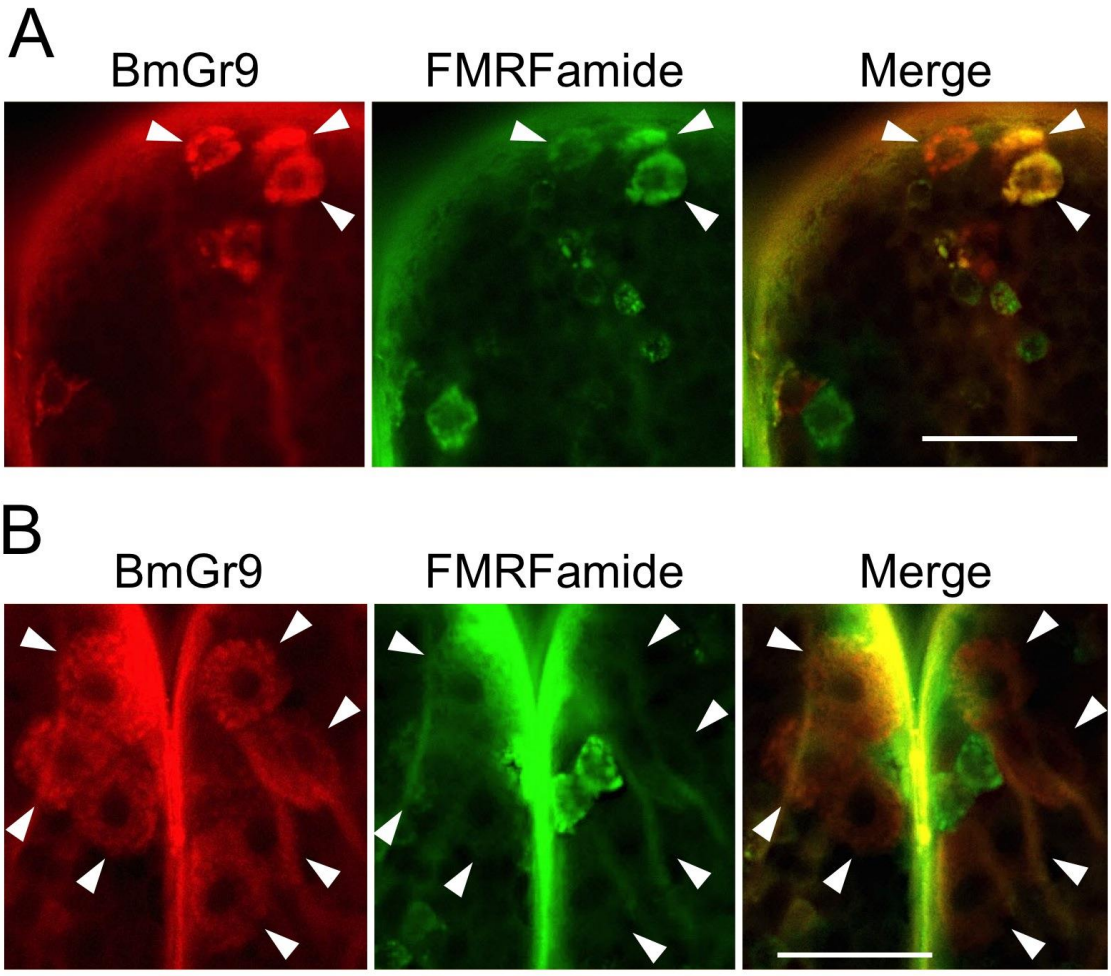


Fig. 3.8. Double immunohistochemical staining of whole mount brain of third instar *B. mori* larvae.

Whole mount brain was double stained with anti-BmGr9 and anti-*Dm*FMRFamide antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. BmGr9-expressing cells are co-localized with anti-*Dm*FMRFamide-stained cells in lateral (A) and medial (B) regions of the brain. Arrowheads indicate cells which were stained with both anti-BmGr9 and anti-*Dm*FMRFamide antisera. Scale bars = 50 μm.

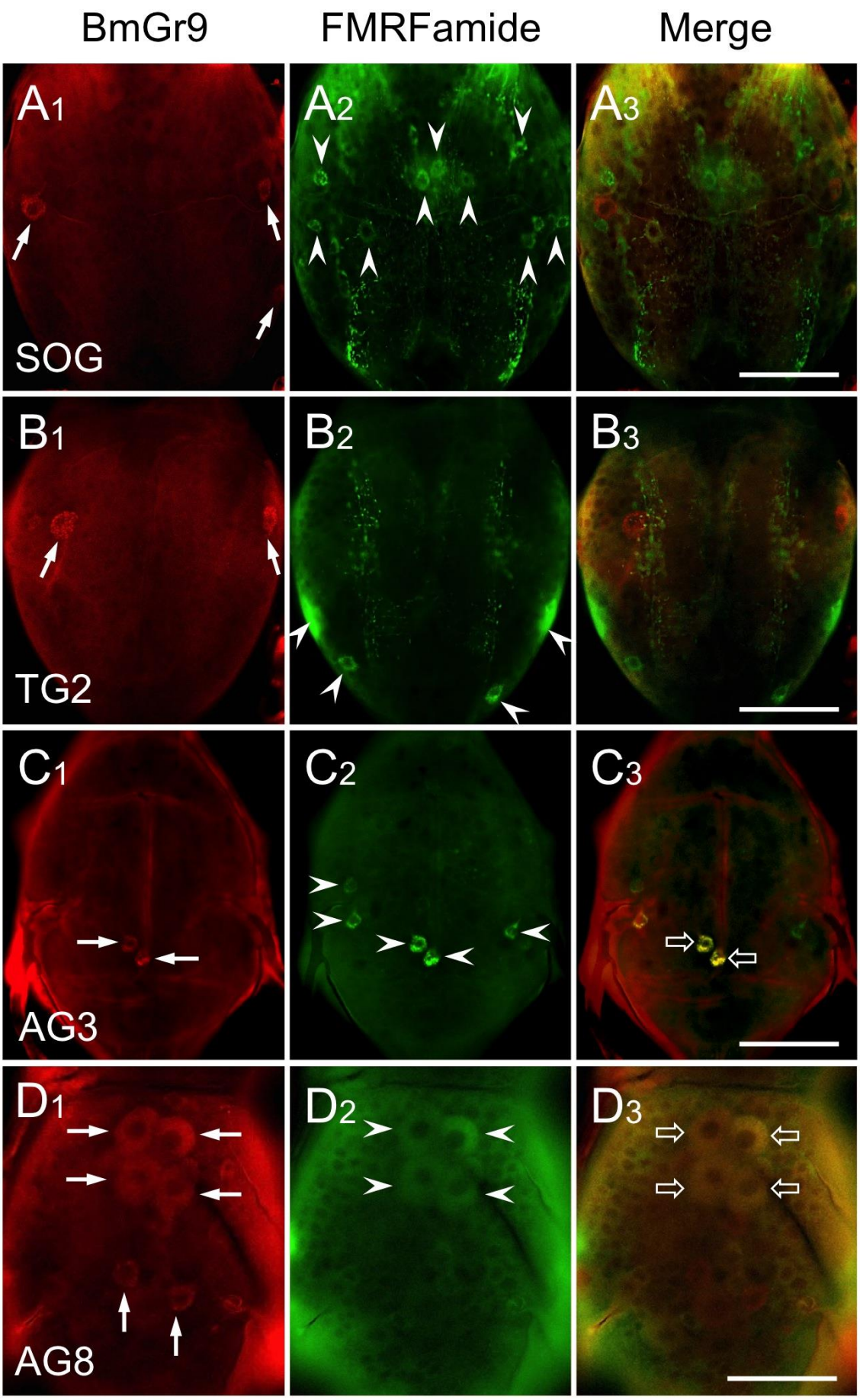

BmGr9
FMRFamide
Merge
A1
A2
A3
SOG
B1
B2
B3
TG2
C1
C2
C3
AG3
D1
D2
D3
AG8

Fig. 3.9. Double immunohistochemical staining of whole mount ganglia of third instar *B. mori* larvae.

Whole mount central nervous tissues of the larvae were double stained with anti-BmGr9 and anti-*Dm*FMRFamide antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. The left panels are results stained with mouse anti-BmGr9 antiserum. The middle panels are results stained with rabbit anti-*Dm*FMRFamide antiserum. The right panels are merged images of the left panels and the middle panels. Arrows indicate BmGr9-expressing cells, arrowheads indicate FMRFamide-expressing cells, open arrows indicate cells which were stained with both anti-BmGr9 and anti-*Dm*FMRFamide antisera. Scale bars = 100 μm.

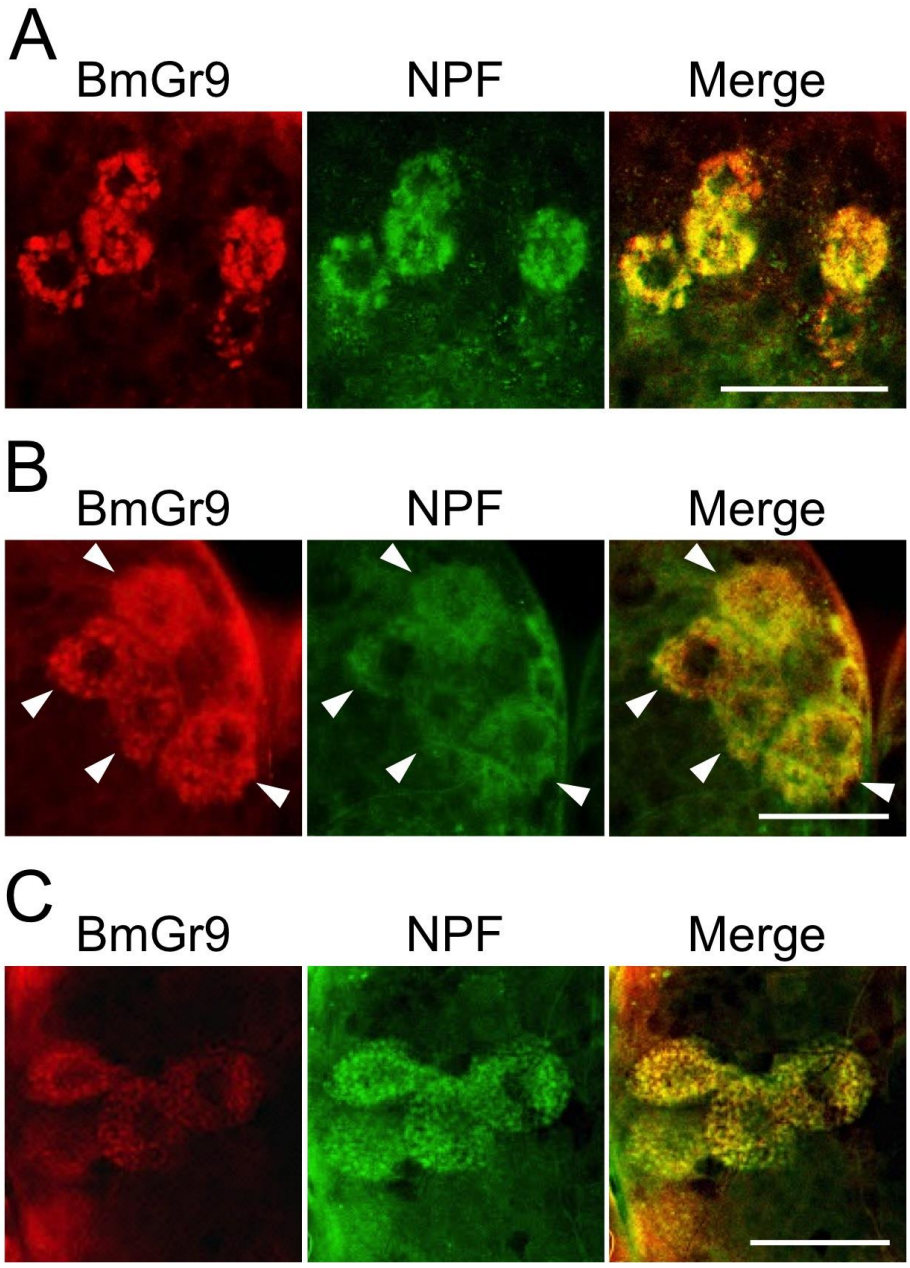


Fig. 3.10. Co-localization of NPF1-expressing cells and BmGr9-expressing cells in the brain and AG8 of instar *B. mori* larvae.

Whole mount brain was double stained with anti-BmGr9 and anti-*Dm*NPF antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. (A and B) BmGr9-expressing cells co-localization with anti-*Dm*NPF stained cells in

the lateral (A) and medial (B, arrows) regions. (C) Co-localization of BmGr9-expressing cells and anti-*Dm*NPF-stained cells in the abdominal ganglion (AG) 8. Scale bars = 50 μm.

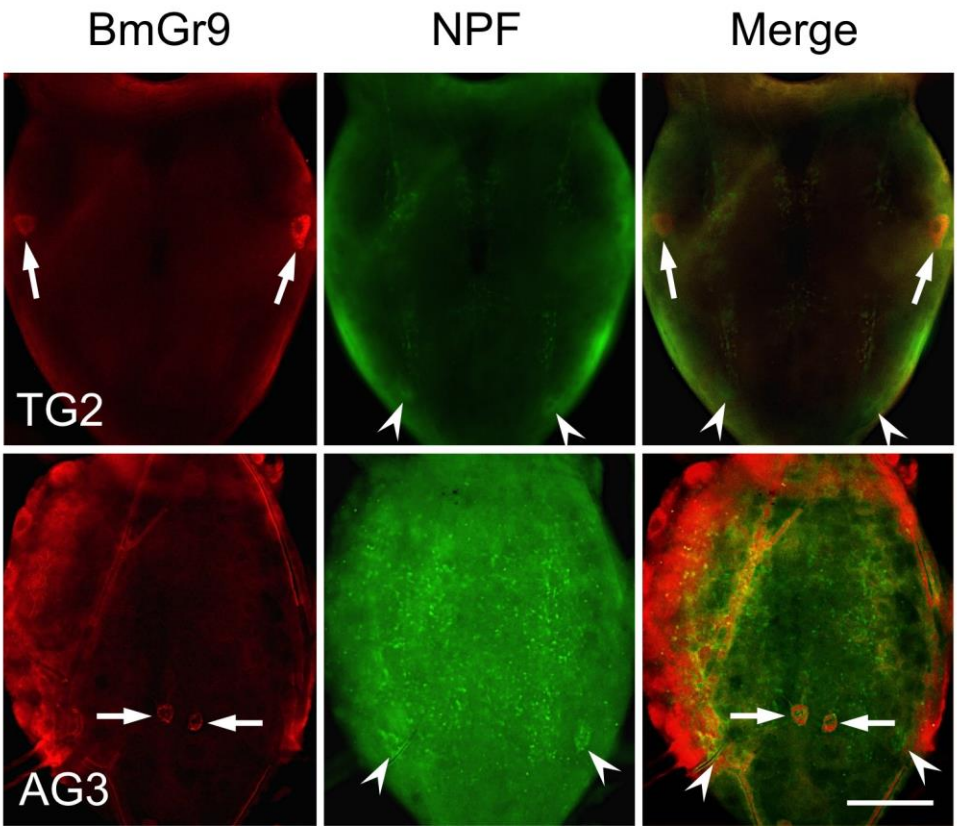


Fig. 3.11. Double immunohistochemical staining of whole mount thoracic-abdominal ganglia of third instar *B. mori* larvae.

Whole mount thoracic-abdominal ganglia of the larvae were double stained with anti-BmGr9 and anti-*Dm*NPF antisera. As secondary antibodies, a mixture of goat anti-mouse IgG coupled to Alexa Fluor 555 (red) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (green) were used. Arrows indicate BmGr9-expressing cells, arrowheads indicate *Dm*NPF-stained cells. Scale bars = 100 μm.

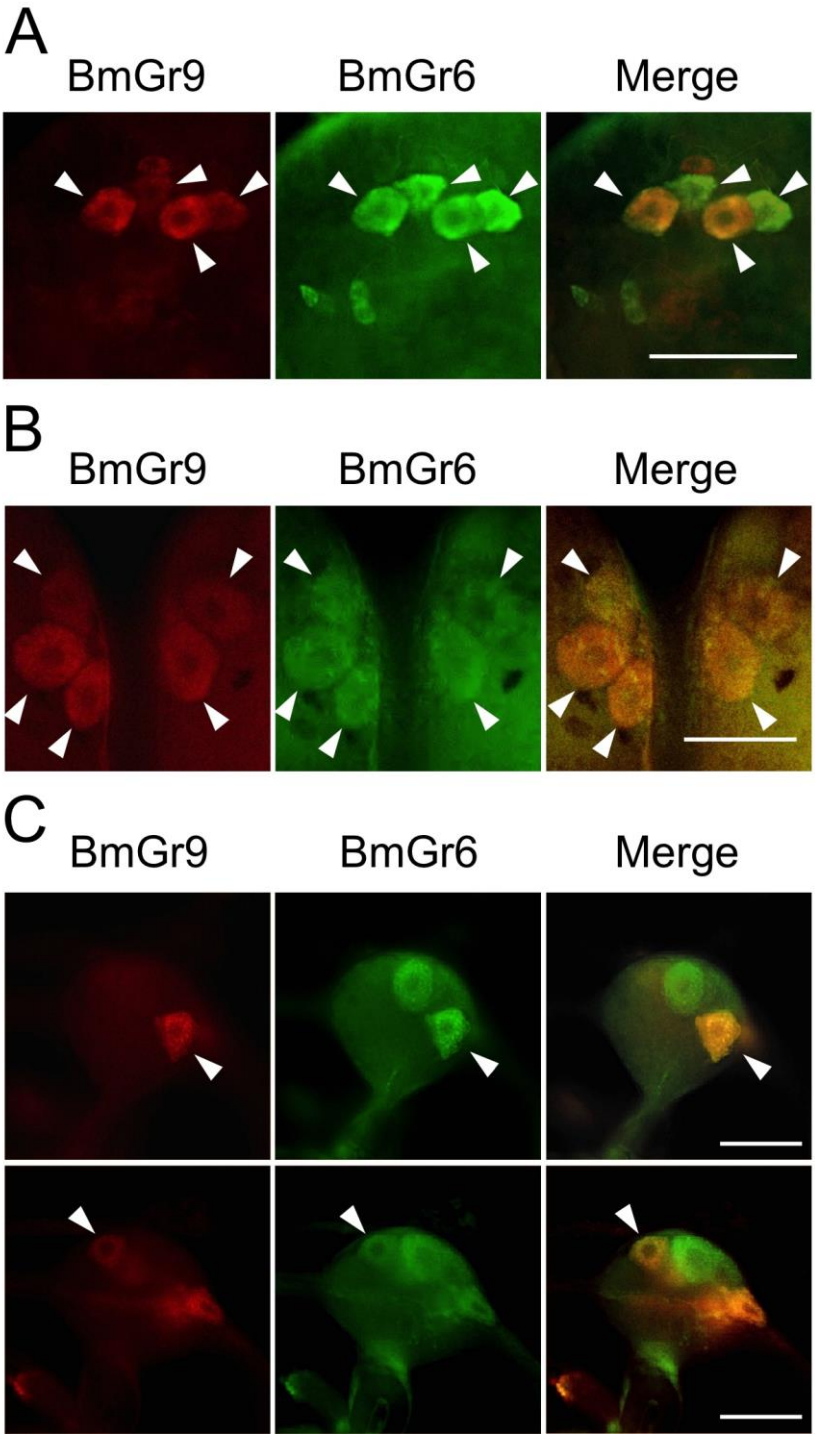


Fig. 3.12. Co-localization of BmGr9-expressing cells and BmGr6-expressing cells in the brain of *B. mori* larvae.

Whole mount brain of third (A) and fourth (B) instar larvae were stained with fluorescence labeled anti-BmGr9 (red fluorescence) and anti-BmGr6 (green

fluorescence) antisera. BmGr9-expressing cells co-localization with BmGr6-expressing cells in the lateral (A) and medial (B) regions of brain. (C) Co-localization of BmGr9-expressing cells and BmGr6-expressing cells in the frontal ganglion (FG). Arrowheads indicate the cells which expressed both BmGr9 and BmGr6. Scale bars = 50 μm.

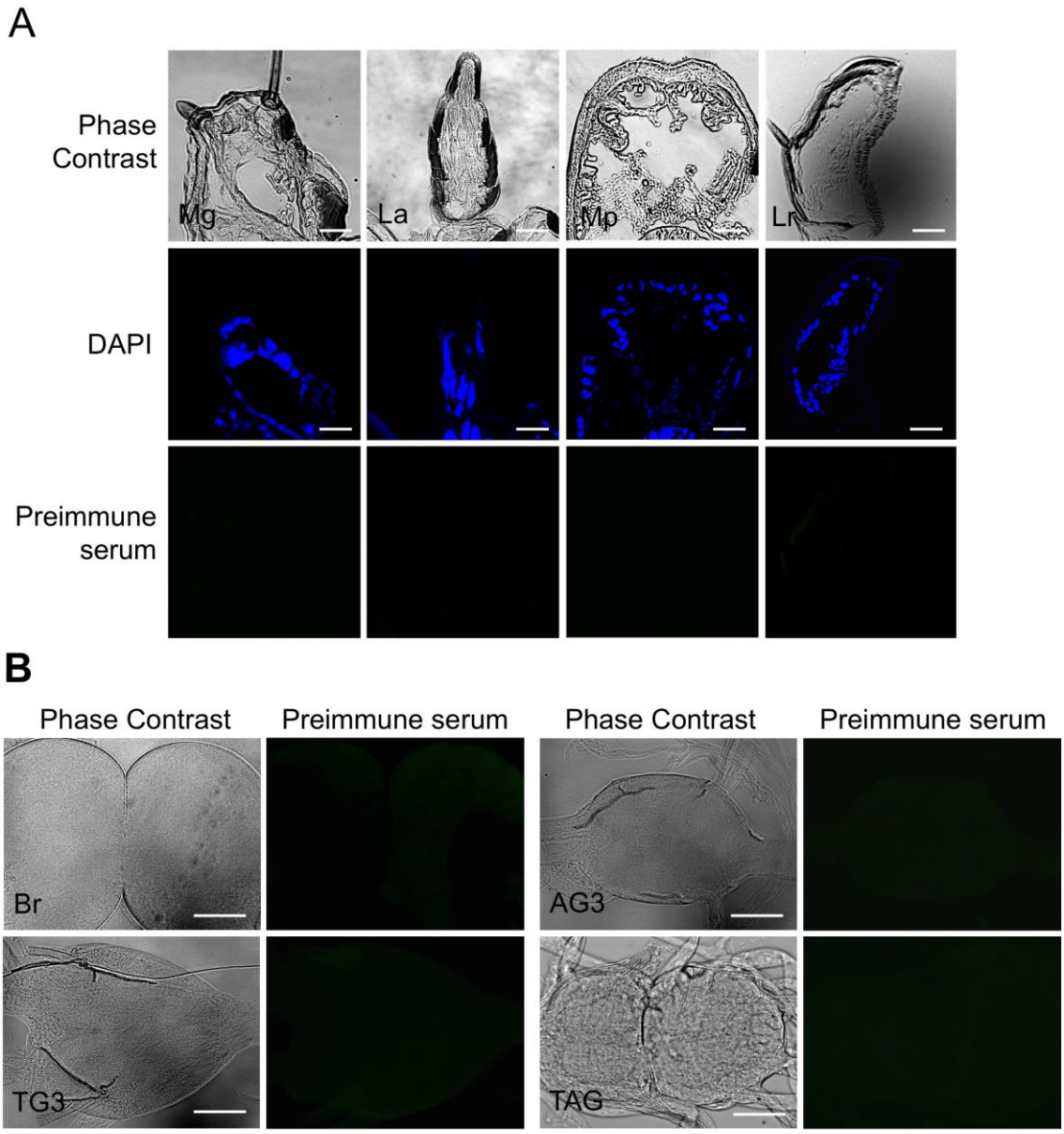


Fig. 3.13. Negative controls for immunohistochemistry.

Negative control experiments were conducted by application of the pre-immune mouse serum taken from the animal that was immunized with BmGr9 peptide later to produce the primary antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488, and no immunoreactive cells were observed. (A) Oral sensory organs (Mg, maxillary galea; Mp, maxillary palpus; La, labium; Lr, labrum). (B)

Nervous system. Br, brain; TG, thoracic ganglia; AG, abdominal ganglia; TAG, terminal abdominal ganglia). Scale bars = 30 μm in (A) or 100 μm in (B).

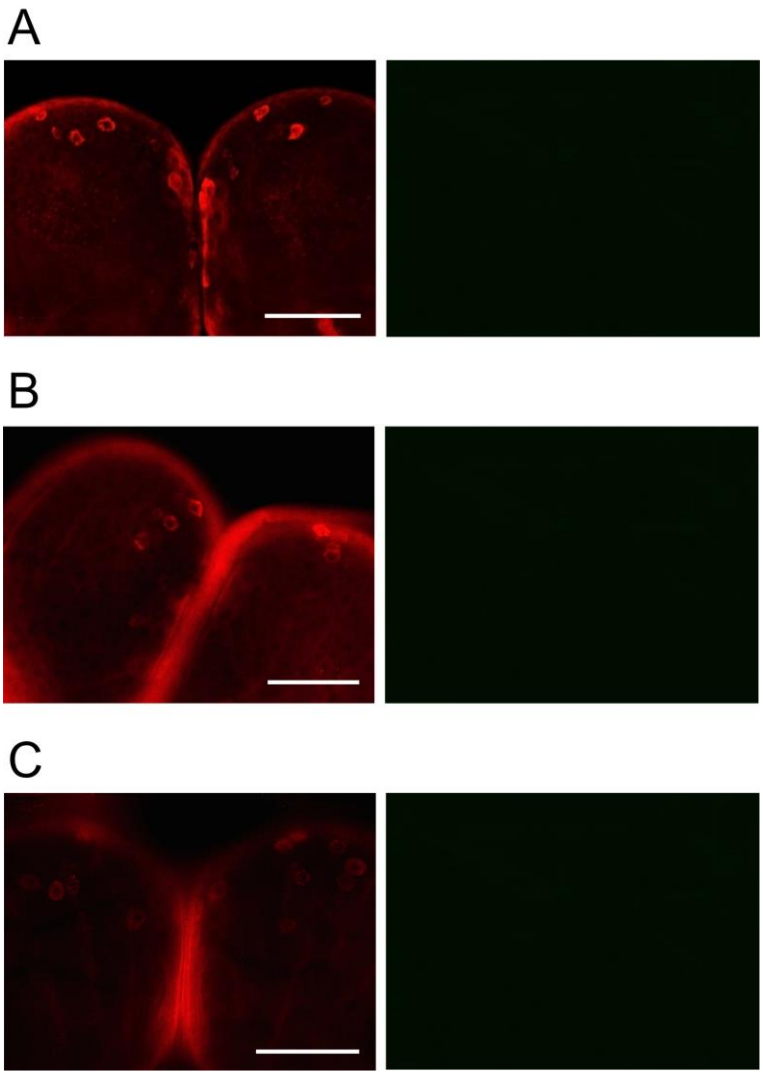


Fig. 3.13. Negative controls for immunohistochemistry.

Negative control experiments were conducted by application of the pre-immune mouse serum taken from the animal that was immunized with BmGr9 peptide later to produce the primary antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488, and no immunoreactive cells were observed. (A) Oral sensory organs (Mg, maxillary galea; Mp, maxillary palpus; La, labium; Lr, labrum). (B) Nervous system. Br, brain; TG, thoracic ganglia; AG, abdominal ganglia; TAG, terminal abdominal ganglia). Scale bars = 30 μm in (A) or 100 μm in (B).

Chapter 4

Expression of Bitter Clade Gustatory Receptors in the Midgut and Central Nervous System of the Silkworm *Bombyx mori* Larval

4.1. Introduction

The ability to sense food that is nutritious or toxic substances is essential for animal's survival (Scott, 2005). By sensation of bitter chemicals, an animal can be alerted that a specific food is harmful and should not be ingested (Apostolopoulou et al., 2014). Insect gustatory receptors (Grs) include receptors specific for sugar and bitter chemicals, and cuticular hydrocarbons, as well as some odorants such as carbon dioxide. In *Drosophila melanogaster*, the sensation of sugar and bitter chemicals is mediated, by distinct DmGrs expressed in sugar and bitter gustatory receptor neurons, respectively (Chen, 2015). According to previous studies in *D. melanogaster*, some Grs function in the detection of bitter chemicals. For example, DmGr66a is widely expressed in most of bitter-sensing neurones and specifically involved in the sensing of caffeine (Thorne et al., 2005; Marella et al., 2006). DmGr33a is required for detecting a wide range of bitter tasting chemicals (Moon et al., 2009; Miyamoto et al., 2013).

In mammals, about 45 Grs were classified into two T1R and T2R families. Previous studies have shown that Multiple T2Rs function as bitter receptors, leading to the proposal that all T2Rs may be homomeric bitter receptors (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). These Grs are not only expressed in taste cells of the tongue, but also in gut enteroendocrine cells (Dyer et al., 2005; Mace et al., 2007; Margolskee et al., 2007). For example, T2Rs (a family of bitter taste receptors) were found to function in enteroendocrine STC-1 cells (the intestinal secretin tumor cell line) (Chen et al., 2006). In *D. melanogaster,* some bitter

Grs such as DmGr93a and DmGr33a were also showed to express not only in gustatory organs but also in midgut enteroendocrine cells. However, whether these Grs are expressed in the central nervous system is still unclear.

In the *Bombyx mori*, based on the membrane phylogenetic analysis of all *B. mori* Grs (BmGrs) and previously identified insect GRs, 59 BmGrs were classified to the putative bitter receptor family (Wanner and Robertson, 2008; Zhang et al., 2011). Up to present, however, the distribution of all BmGrs of bitter receptor family in organs and tissues of the *B. mori* is remain to be explored. By topological studies, BmGr53, a bitter clade receptor, was shown that has an inverted topology relative to that of G protein-coupled receptors (Zhang et al., 2011). Despite the previous finding, research on this Grs has been limited and little attention has been paid to its tissue distribution. Therefore, in this study, I systematically examined the expression of 58 BmGrs genes in the gut (paying attention especially to the midgut) and central nervous system (CNS) was examined by reverse transcription-polymerase chain reaction (RT-PCR). In addition, *BmGr53* gene, was examined in various organs and tissues of the *B. mori* larvae. Furthermore, I explored BmGr53-expressing cells in *B. mori* larval tissues paying attention especially to the brain, a secretory center for the regulation of behavior and homeostasis using a specific polyclonal antiserum raised against BmGr53.

4.2. Materials and methods

4.2.1. Experimental animals

Eggs of silkworm *B. mori* (hybrid strain, Kinshu × Showa) were purchased from the silkworm egg producing company, Ueda Sanshu Ltd (Ueda, Japan). Larvae were reared as described in section 1.1.1.

4.2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Tissues were dissected from 3-day-old fifth instar larvae. Then all collected tissues were homogenized in 0.5–2 ml cold ISOGEN II (Nippon Gene, Tokyo, Japan) and kept at -80 °C until use for RNA extraction. For cDNA synthesis, double-stranded cDNA fragments amplification, and PCR was performed as described in section 1.1.2.

4.2.3. Immunohistochemistry and microscopy

The whole mount midgut and brain from 2-day-old 3rd-instar larvae were fixed at 4 °C overnight in 4% paraformaldehyde in PBS, containing 0.1% Triton X-100 (PBST). After fixation, tissues were washed 3 times for 10 min each in PBST, and then incubated for 1 h in TNT containing 2% BSA. The tissues were subsequently incubated with primary antiserum (anti-DmFMRFamide and anti-DmNPF antisera) as described in section 1.1.7. After immunohistochemistry, tissues were also mounted in 90% glycerol/PBS containing 1,4-diazabicyclo [2.2.2] octane and observed under a fluorescence microscope, LSM710/LSM710 NLO (CARL EISS) at RT.

Negative control was prepared by application of the preimmune mouse antiserum taken from the animal that was used to produce the primary antiserum. Secondary antibody incubation, and all subsequent processing, was performed in the dark. Likewise, all samples were stored in the dark at 4 °C until they were examined.

4.3. Results

4.3.1. Putative bitter BmGrs genes expression in the gut of *B. mori* larvae

This work started from examination with regard to the expression of *BmGrs* genes in the foregut, midgut and hindgut of *B. mori* larval by RT-PCR with primers specific for each 58 BmGrs which belong to the putative bitter receptor clade (Fig.4.1). Results indicated as seen in Fig.4.2, that 15 bitter receptors were found to be expressed in foregut, midgut and hindgut. In detail, I identified amplification products of 19, 27, and 18 receptors genes in the foregut, midgut and hindgut, respectively. Of the 19 BmGrs expressed in foregut, *BmGr61* was foregut specific (Fig. 4.2 B and C); among the 27 BmGrs expressed in midgut, nine *BmGrs* (*BmGr30, 34, 38, 41, 43, 45, 46, 48, 57)* were midgut specific. Among the 18 BmGrs in hidgut, *BmGr61* was foregut specific. In addition, I found that *BmGr11* and *BmGr19* were expressed in both foregut and hindgut, but not in the midgut. *BmGr49* was expressed in both midgut and hindgut, but not in foregut. However, *BmGr16* was expressed in both foregut and midgut, but not in hindgut. In all 58 BmGrs, there are 28 BmGrs genes were not found expressed in foregut, midgut and hindgut.

Next, expression of 27 *BmGrs* genes which were found to express in the midgut, was examined in the anterior, middle and posterior midgut respectively. As shown in Fig. 4.3, 19 *BmGrs* genes (*BmGr12, 19, 21, 22, 23, 24, 35, 41, 45, 46, 48, 53, 56, 57, 60, 62, 63, 66, 67*) were observed to be expressed in all regions of the midgut. Moreover, *BmGr30* and BmGr38 gene were expressed only in the posterior midgut; while *BmGr16,* BmGr*34* and *BmGr43* gene was expressed only in the middle midgut.

In addition, *BmGr27* gene was mainly expressed in the anterior and posterior midgut, but not in the middle midgut; while *BmGr57* gene was expressed in the middle and posterior midgut, but not in the anterior midgut. From these, it was indicated that even in the midgut, expression member of BmGrs is different in each position from anterior to posterior, suggesting that function of BmGrs expressing cells in each site is different.

4.3.2. Putative bitter BmGrs genes expression in the CNS of *B. mori* larvae

Expression of these 58 BmGrs genes in central nervous system (CNS) of the *B, mori* larvae was examined by RT-PCR. As shown in Fig. 4.4, RT-PCR products of 19 *BmGrs* (*BmGr19, 21, 23, 27, 38, 40, 42, 44, 47, 49, 53, 56, 58, 59, 60, 62, 63, 66,* and *67*) showed intense bands in agarose gel electrophoresis. Faint bands of 6 BmGrs (*BmGr11, 12, 22, 24, 30* and *57*) were also observed. However, the other BmGrs genes were not found to be expressed in the CNS. In addition, by comparing this result with the BmGrs expressing in the midgut, I found that only 18 BmGrs (*BmGr11, 12, 19, 21, 22, 23, 24, 27, 30, 38, 53, 56, 57, 60, 62, 63, 66 and 67*) genes were expressed in both midgut and CNS (Fig. 4.5).

4.3.3. RT-PCR analysis of *BmGr53* gene expression

To determine the distribution of putative bitter clade BmGrs-expressing cells, we attempted to prepare mouse or rabbit polyclonal antisera specific to each of these BmGrs. However, only antiserum against BmGr53 (anti-BmGr53 antiserum) was

successfully prepared. Next, to investigate the detail sites of BmGr53 expression in the oral sensory organs, midgut, nervous system, and other *B*. *mori* tissues, RT-PCR experiments were performed using total RNA prepared from tissues taken from fifth instar larvae. Primers were designed to span introns to differentiate between amplicons from genomic DNA and cDNA. As shown in Fig. 4.6, RT-PCR produced intense *BmGr53* bands from the antennae, maxillary, labium, thoracic leg, proleg, caudal leg, anterior midgut, middle midgut, posterior midgut, hindgut, malpighian tubule, and testis. Faint bands were detected in the brain, labrum, mandible, foregut, silk gland, and ovary (Fig. 4.6 B). These results indicate that *BmGr53* is expressed broadly in *B*. *mori* larvae. To analyze the detailed location of *BmGr53* gene expression in the ventral nerve cord, each ganglion was further examined by RT-PCR and found that *BmGr53* gene was expressed scarcely in ventral nerve cord (Fig. 4.6 C).

4.3.4. BmGr53 expression in the brain of *B. mori* larvae

RT-PCR analysis demonstrated that *BmGr53* gene was expressed in the brain (Fig. 4.3). Thus, it was expected that these are several BmGr53 expressing cells in the *B*. *mori* brain. To confirm the hypothesis, whole-mount nervous tissues were immunostained using anti-BmGr53 antiserum and BmGr53 expressing cells were visualized.

Based on anti-BmGr53 staining, I counted more than 8 pairs of immunostained cells in the larval brain (BR). In contrast, no cells were stained when the secondary

antiserum alone was used (Fig. 4.8). Those immunostained cells were observed in clusters located in the medial and lateral regions. As shown in Fig. 4.7, four pairs of cells with large somata were observed in the medial region of the brain (Fig. 4.7B). Other four pairs of anti-BmGr53 stained cells were also found in the lateral regions of the brain (Fig. 4.7C).

4.4. Discussion

In mammals, taste receptors are not only expressed in taste cells of the tongue, but also in gut enteroendocrine cells (Dyer et al., 2005; Mace et al., 2007; Margolskee et al., 2007). Taste receptors expressed in mammalian luminal epithelium are thought to function as food sensors to avoid harmful substances and to regulate nutrient uptake in the gut (Breer et al., 2012; Sclafani, 2007). For example, T2Rs (a family of bitter taste receptors) were found to function in enteroendocrine STC-1 cells (the intestinal secretin tumor cell line) (Chen et al., 2006). In insects, the midgut is an important organ for insects because it occupies a large space in their hemocoel and plays critical roles relevant to physiological regulation. Park and Kwon (2011b) found that DmGrs were expressed in enteroendocrine cells of the *D. melanogaster* gut (Park and Kwon, 2011b). Moreover, they determined that some of these DmGrs are co-localized with enteroendocrine cells including neuropeptide F (NPF), locustatachykinin (LTK) or diuretic hormone 31 (DH31) (Park and Kwon, 2011b). For example, DmGr93a and DmGr33a, two bitter receptors, and were also showed to express in the fly gut and co-localized with NPF in the gut enteroendocrine cells (Park and Kwon, 2011b). In this study, RT-PCR showed that 27 putative bitter *BmGrs* genes were expressed in the midgut. It is possible that also these putative bitter BmGrs are expressed in enteroendocrine cells of the *B. moti* midgut, and may co-express with some neuropeptides in the midgut enteroendocrinde cells. This study may provide a basis for predicting the functions of these putative bitter BmGrs, which likely include bitter compounds sensing in the midgut and play roles in feeding behavior.

On the other hand, 25 putative bitter *BmGrs* genes were also found expressed in CNS. Of these 25 BmGrs, especially regarding *BmGr53*, which was expressed in not only oral sensory organs, but also brain as well as midgut (Fig. 4.6), these results are in good agreement with previous study that showed that *BmGr53* gene is widely expressed in various organs and tissues of the *B. mori* (Sato et al., 2011). More importantly, immunohistochemistry was also used to show the BmGr53 protein which was expressed in cells of brain of the *B. mori* larvae (Figs. 4.7). These cells have the typical shape, size, and location of neurosecretory cells (Ichikawa, 1991). These findings are similar to the observations of BmGr6 and BmGr9 which are expressed in cells of the brain (Chapter 1–3). As mentioned in chapter 1 and 3, most of BmGr6- and BmGr9-expressing cells were co-localized with putative neuropeptide F1 (BmNPF1)-expressing cells. Although whether the BmGr53-expressiion cells co-express with BmNPF1-expressing cells remains unknown, the expression of this Gr in the brain suggests its functions in feeding behavior.

An unexpected finding in this study is that I was unable to detect any positive cell for BmGr53 in the gut via immunohistochemistry using the anti-BmGr53 antiserum. *BmGr53* gene expression in the gut was reported by Sato et al. (2011). In this study, we also observed *BmGr53* gene expression by PCR with materials prepared from the foregut, midgut (including anterior, middle and posterior midgut), and hindgut (Figs. 4.2–3 and 4.6). However, no BmGr53-expressing cells were found by immunohistochemistry using both whole mount gut (1st to 3rd instar larvae) and gut cryosections (4th and 5th instar larvae), even though I used the same method as that

used for the observation of BmGr6 expressing enteroendocrine cells of the midgut (Chapter 1). One possible interpretation of these results is that low levels of expression of this BmGr protein exist in the cells of *B. mori* larvae gut.

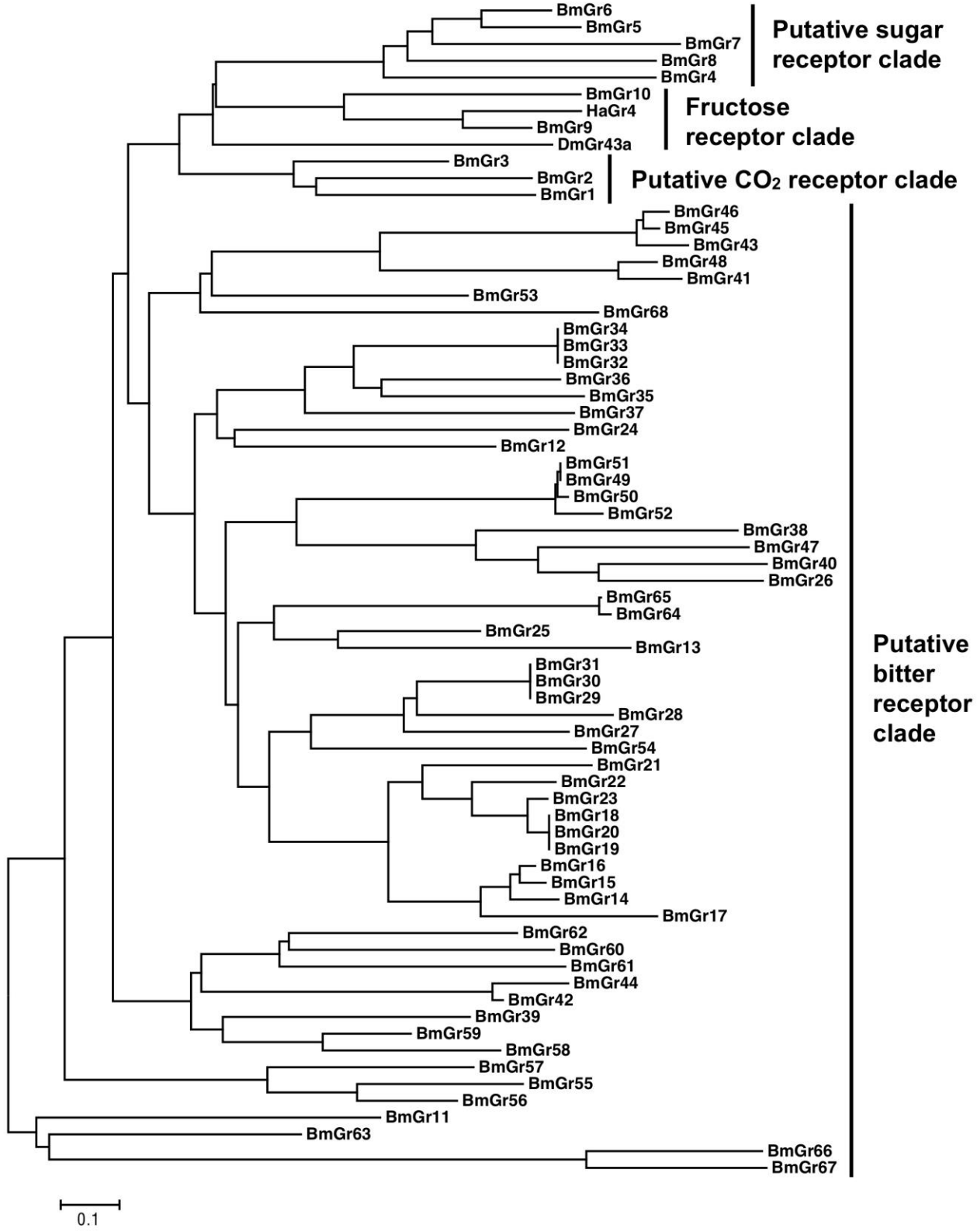
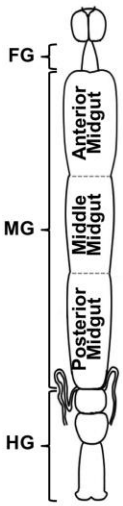


Fig. 4.1. Phylogenetic tree of *B. mori* gustatory receptors.

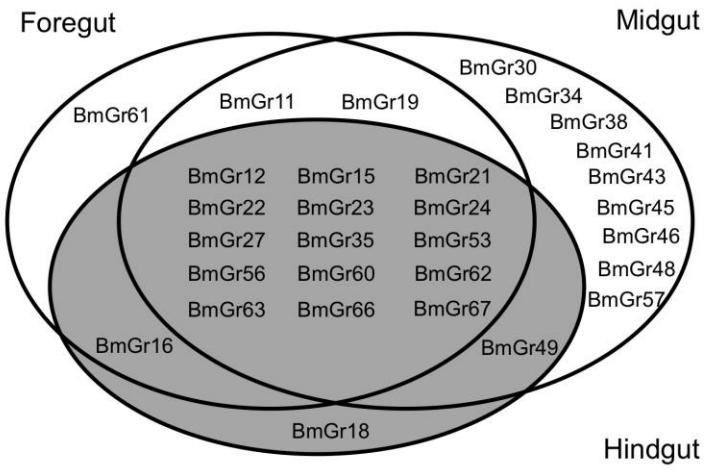
Total amino acid sequences of 69 *Bombyx mori* Grs, ONR *Drosophila melanogaster*

Grs were aligned using the ClustalW program and phylogenetic analysis was conducted using the neighbor-joining method (Mega 6.06 software). Numbers at the nodes indicate bootstrap values (1000 replicates).

A



C



No expression

BmGr13	BmGr14	BmGr17
BmGr20	BmGr25	BmGr26
BmGr28	BmGr29	BmGr31
BmGr32	BmGr33	BmGr36
BmGr37	BmGr39	BmGr40
BmGr42	BmGr44	BmGr47
BmGr50	BmGr51	BmGr52
BmGr54	BmGr55	BmGr58
BmGr59	BmGr64	BmGr65
BmGr68		

B

BmGr	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
FG	+	+	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-
MG	+	+	-	-	+	-	-	-	+	-	+	+	+	+	-	-	+	-	-	+	-	-	-	+	+	-	-	+	-
HG	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-

BmGr	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
FG	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+	+	+	-	-	+	+	-
MG	-	+	-	+	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+	-
HG	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-	-	+	-	+	+	-	-	+	+	-

Fig. 4.2. BmGrs genes expression in the midgut of *B. mori* larval

(A) An illustration of the gut of a 5th instar *B. mori* larvl. (B) RT-PCR analysis of BmGrs expression in the foregut, midgut and hindgut of the 5th instar larvae. (C) A comprehensive list of BmGrs expressed in the foregut, midgut and hindgut.

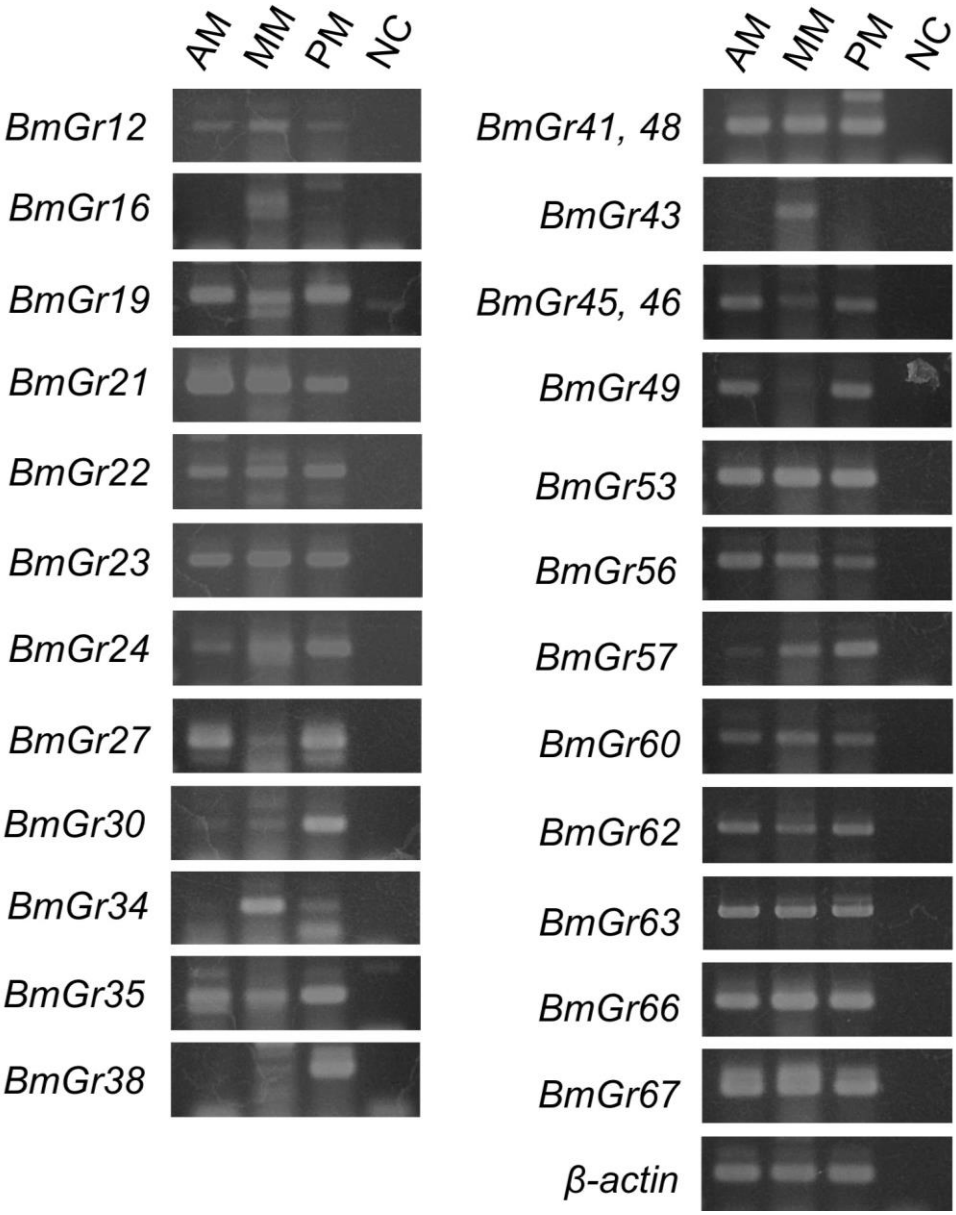


Fig. 4.3. RT-PCR analysis of 26 bitter clade BmGrs genes expression in the midgut of *B. mori* larval.

Anterior, middle and posterior midgut were dissected from two-day-old fifth instar larvae. *β-actin* was used as the internal control. Amplification products were analyzed on agarose gels and visualized by UV illumination after ethidium bromide staining.

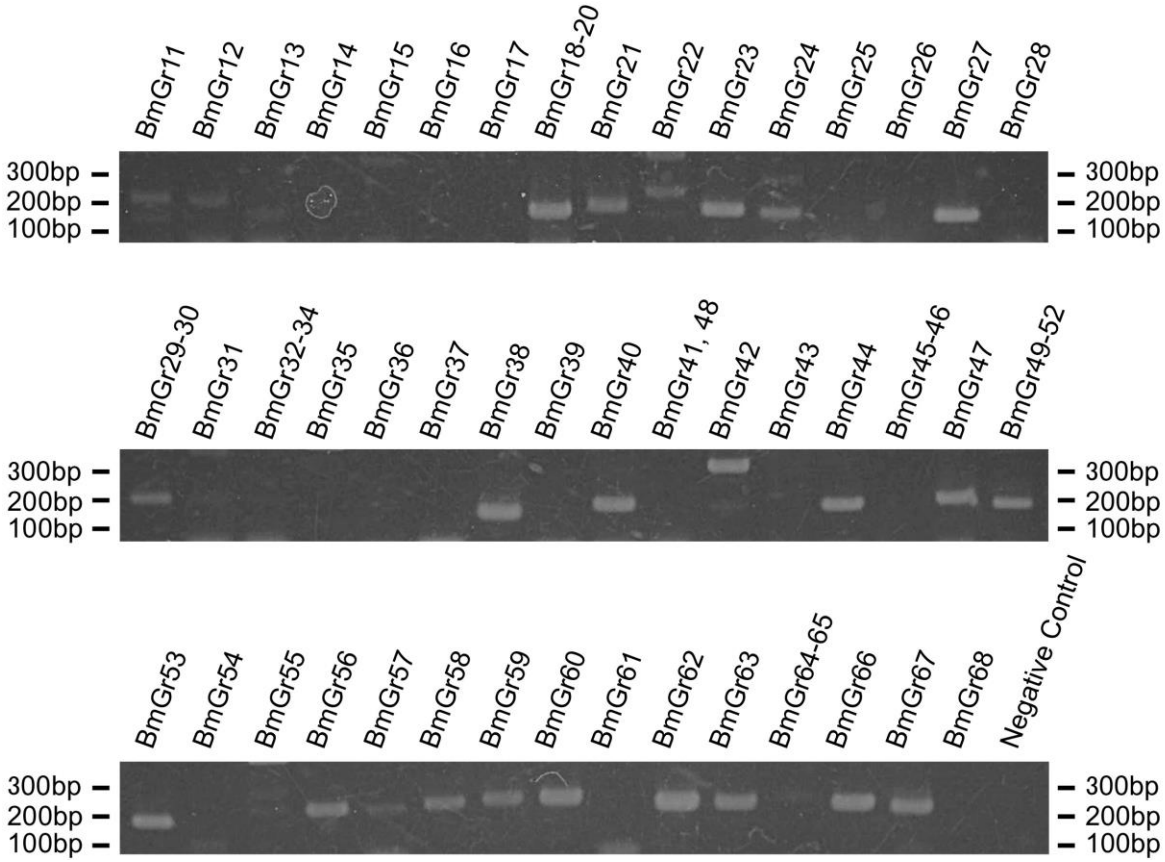


Fig. 4.4. RT-PCR analysis of bitter clade BmGrs genes expression in the CNS of *B. mori* larval.

Tissues were dissected from two-day-old fifth instar larvae. *β-actin* was used as the internal control. Amplification products were analyzed on agarose gels and visualized by UV illumination after ethidium bromide staining.

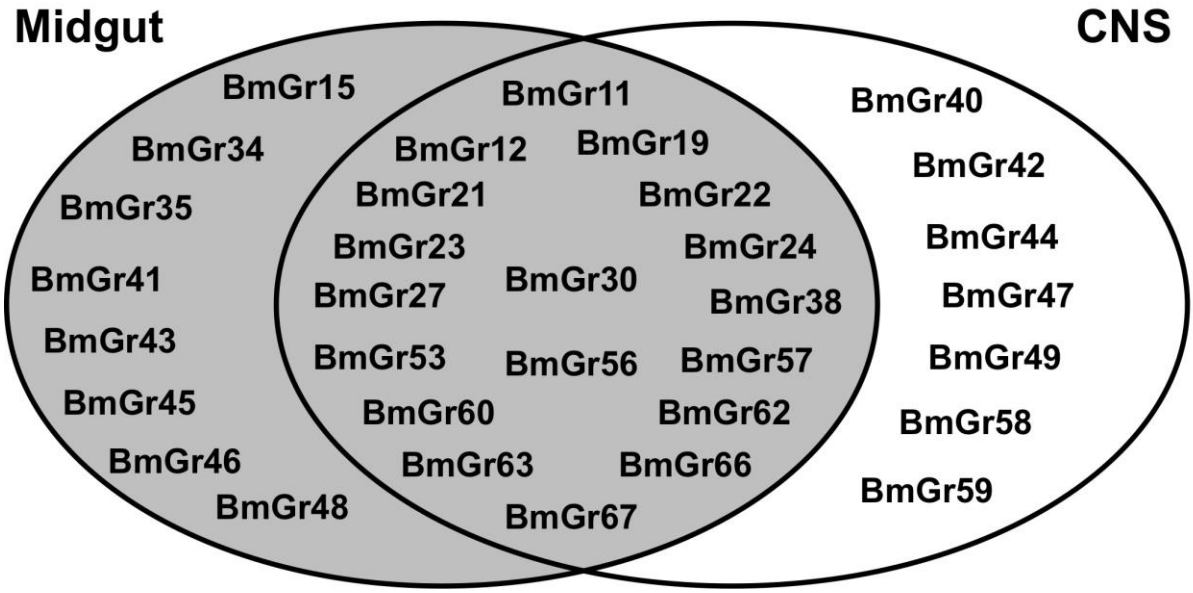


Fig. 4.5. A comprehensive of bitter clade BmGrs expressed in the midgut and centtal nervous system (CNS).

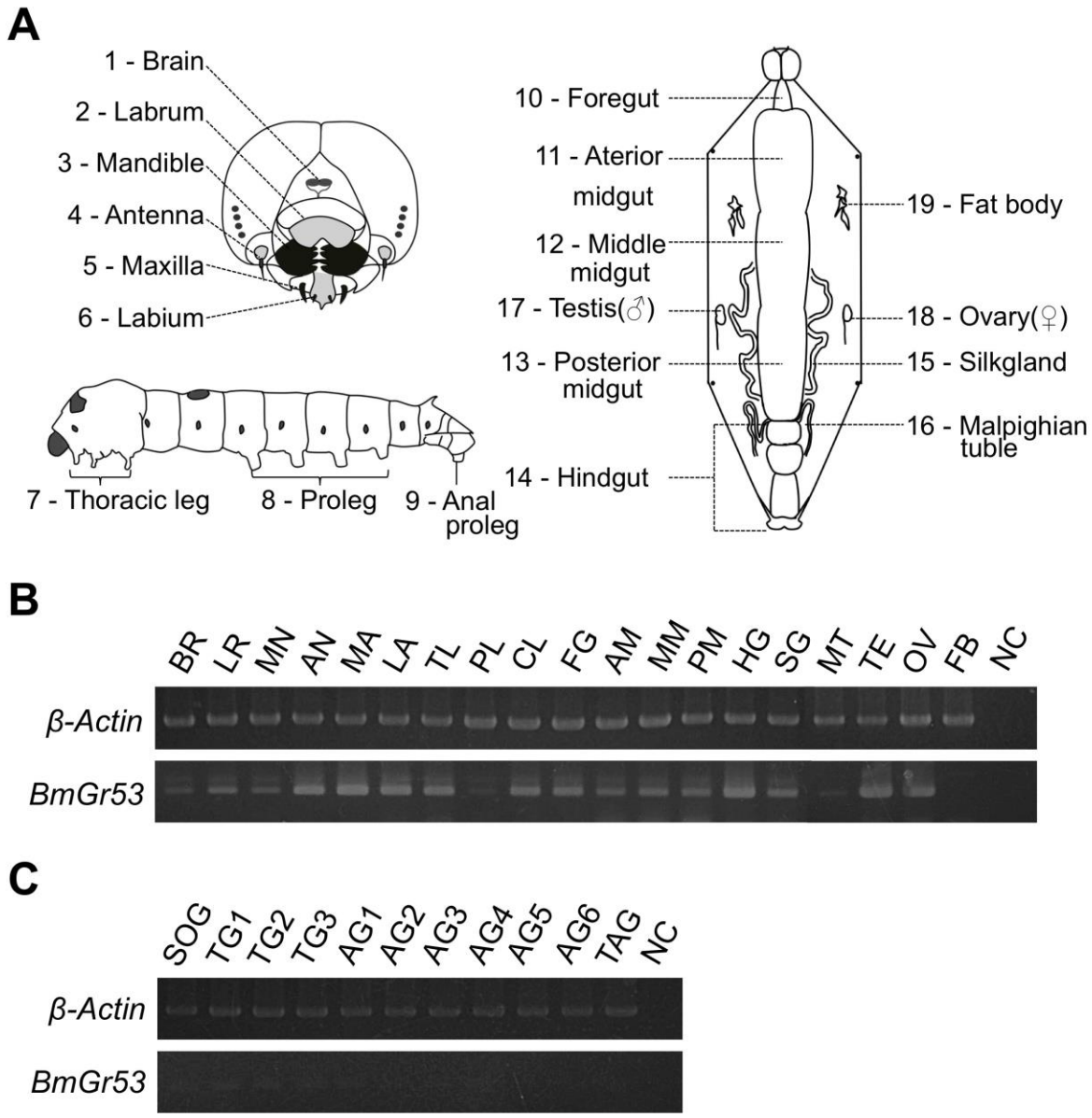


Fig. 4.6. RT-PCR analysis of *BmGr53* gene expression.

β-actin was used as an internal control; NC, negative control with no template in the reaction. (A) Illustrations of various tissues and organs of the fifth instar larva in which chemosensory and non-chemosensory organs are depicted. (B) Tissues were dissected from two-day-old fifth instar larvae. BR, brain; LR, labrum; MN, mandible; AN, antenna; MA, maxilla; LA, labium; TL, thoracic leg; PL, proleg; CL, caudal leg; FG, foregut; AM, anterior midgut; MM, middle

midgut; PM, posterior midgut; HG, hindgut; SG, silk gland; MT, malpighian tubule; TE, testis; OV, ovary; FB, fat body. (C) RT-PCR analysis of BmGr6 gene expression in each ganglion from two-day-old fifth instar larvae. SOG, suboesophageal ganglion; TG1, prothoracic ganglion; TG2, mesothoracic ganglion; TG3, metathoracic ganglion; AG, unfused abdominal ganglia; TAG, terminal abdominal ganglion.

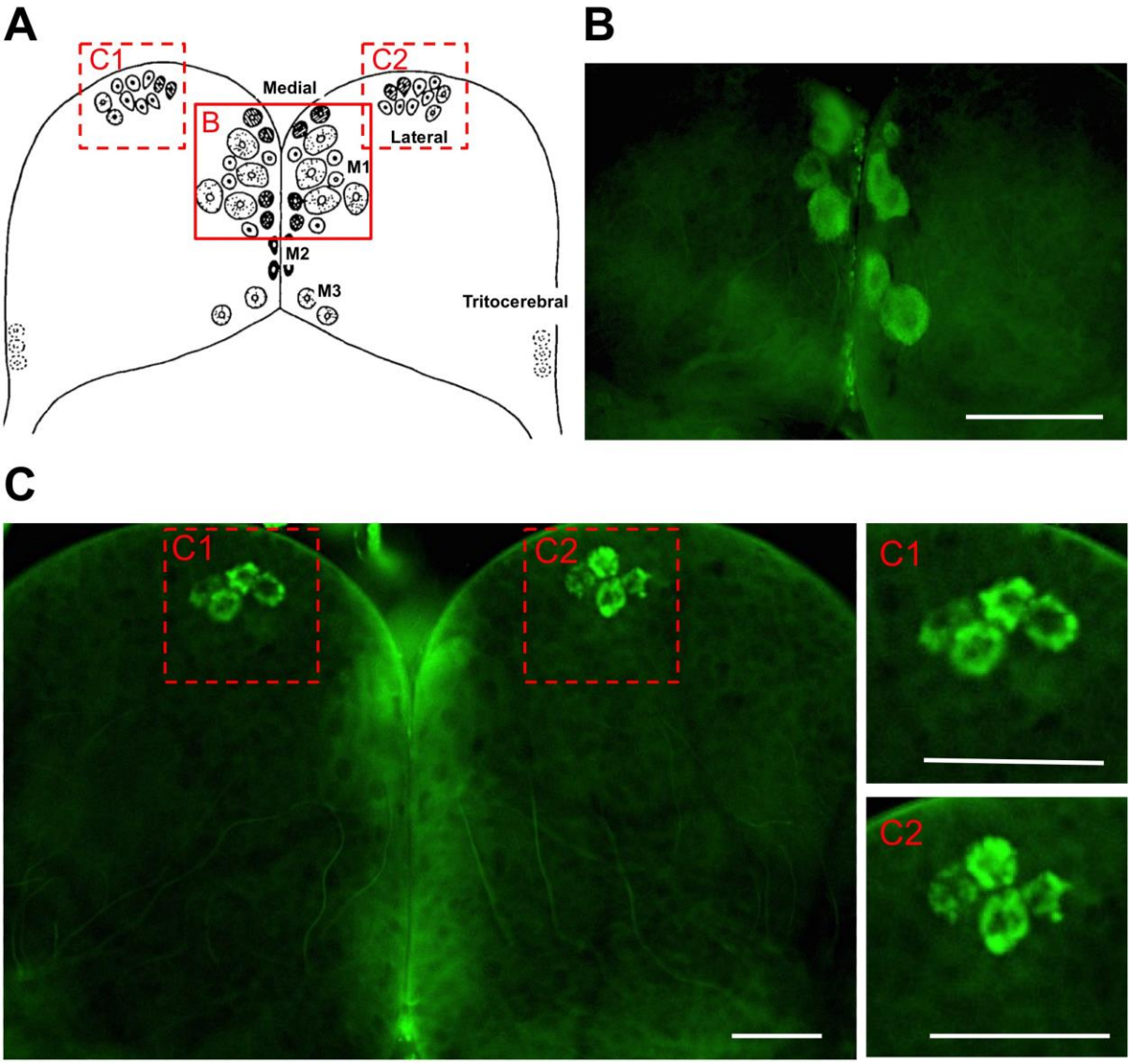


Fig. 4.7. BmGr6 expression in the brain of *B. mori* larvae.

Whole-mount brain were stained with anti-BmGr53 mouse antiserum in combination with anti-mouse IgG conjugated with Alexa Fluor 488. (A). Highly diagrammatic representation of the distribution of somata of putative neurosecretory cells (NSCs) distinguishable by their opalescent appearance in living brains or from their chromophilic cytoplasmic inclusions. Frontal view. Ml, M2 and M3 represent the first, second and third groups of medial cells, respectively. Different shadings of Ml cells and lateral cells represent different staining properties. Three tritocerebral cells

(interrupted circles) were visualized by retrogradefillsof Lucifer Yellow *via* the NCC3 (Ichikawa, 1991). (B) Numerous cells were immunostained by anti-BmGr6 in the brain. Cells with large somata are indicated by arrowaheads. Other stained cells are indicated by arrows. (C) Cells with large somata in the intercerebralis. Scale bars = 100 μm.

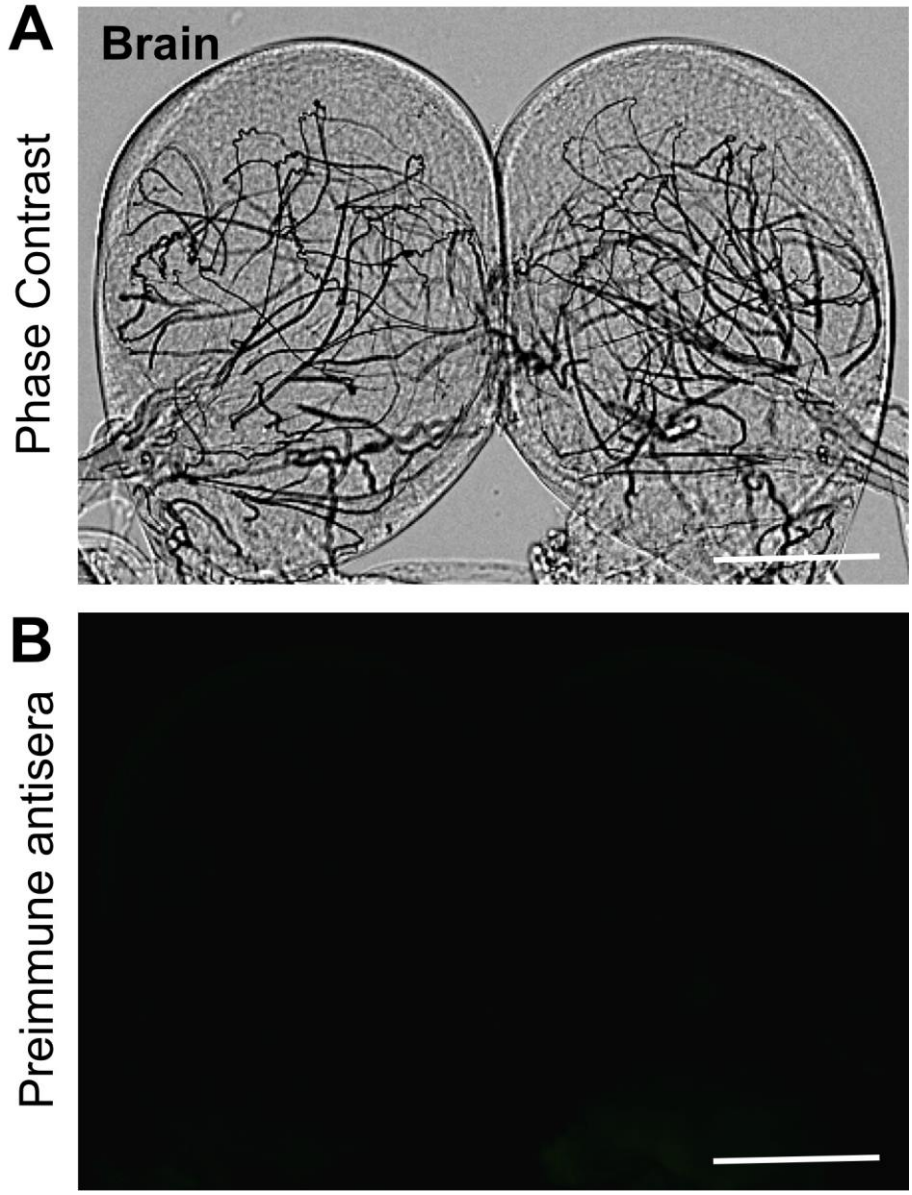


Fig. 4.8. Negative controls for immunohistochemistry.

Negative control experiments were conducted by application of the pre-immune mouse serum taken from the animal that was immunized with BmGr53 peptide later to produce the primary antiserum in combination with anti-mouse IgG conjugated to Alexa Fluor 488, and no immunoreactive cells were observed. Scale bars = 100 μm.

Conclusions

As described in this work, the distribution of BmGr6-expressing cells was visualized in oral sensory organs including antenna, labrum, labium, maxillary galea and maxillary palps (Fig. 1.3). More importantly, immunohistochemistry showed that a few closely placed cells were immunostained in the lateral styloconic sensillum (LS), but not in the medial sensillum (MS) (Fig. 1.3 D and E). Previous studies have shown that in the maxillary gustatory system of *B. mori* larvae, three gustatory receptor neurons (GRNs) in the LS are sensitive to sucrose, *myo*-inositol, and glucose (Ishikawa 1963, 1966; Ishikawa and Hirao 1963). Thus, it could be speculating that BmGr6 is involved in sugar detection. Moreover, BmGr6-expressing cells also occurred on the midgut, and the number of BmGr6 immunostained cells was shown to increase from the anterior to the posterior portions of the midgut (Figs. 1.4 and 1.5). The distribution is similar to that of allatostatin C-secreting enteroendocrine cells in the *D. melanogaster* midgut (Veenstra et al., 2008). Due to the BmGr6 is a member of the sugar receptor family, therefore, this Gr may be involved in perceiving information about sugars in the gut lumen during digestive processes and may control midgut motility through hormonal secretion. In addition, BmGr6-expressing cells were present throughout the CNS and had immunoreactive axons from the brain through over the corpora allata (CC)/corpora cardiaca (CA) complex, foregut and hingut (Figs. 1.7 and 1.8). The nervous system provides insects of abilities to incorporate a variety of internal physiological information as well as external sensory information (Gullan

and Cranston, 2005). It is possible that BmGr6 functions not only as a taste receptor, but also as a chemical sensor for the regulation of gut movement, physiological conditions, and feeding behavior.

The observation of some BmGr6-expressing cells were co-localized with anti-DmFMRFamide-stained cells in the midgut (Fig. 2.2) demonstrated that BmGr6 is expressed in midgut enteroendocrine cells, indicating that BmGr6-expressing cells produce some kinds of FMRFamide-related peptides (FaRPs). Moreover, since only a portion of BmGr6-expressing cells co-localized with anti-DmFMRFamide-stained cells, it might be suggested that BmGr6 is expressed in at least two types of midgut enteroendocrine cells. In addition, no co-localization of BmGr6-expressing cells and anit-DmNPF-stained cells was observed (Fig. 2.4), suggesting that BmGr6 is also expressed in the other types of enteroendocrine cells which are producing unknown hormone.

A portion of BmGr6-expressing cells were also observed to co-localize with anti-DmFMRFamide-stained cells in the brain as well as in ganglia, indicating that BmGr6 is expressed in CNS neurosecretory cell, and these cells also secrete some kinds of FaRPs. Moreover, BmGr6 was found to express in ventrolateral neurosecretory cells (NS-VTL$_{1,2}$) in TG2 and TG3 (Figs. 1.7 E–F and 2.7 B$_1$–B$_3$). A previous study has showed that bommo-FMRFamide (BRFa) which suppresses ecdysteroidogenesis in the PG, was expressed in cells of NS-VTL1,2. It is possible that BmGr6 serve similar biological functions BRFa in the thoracic ganglia of *B. mori* larvae. Furthermore, the co-localization of BmGr6-expressing cells and

anti-DmNPF-stained cells were also found in the brain (Fig. 2.6). Neuropeptide F1 (BmNPF1) is an ortholog of DmNPF in *B. mori* (Roller et al., 2008), and DmNPF is known to increase food consumption. Thus, it could speculate that BmGr6 may function involve in promote feeding behavior of *B. mori* larvae.

BmGr9, in contrast to bmGr6, has been shown to respond specifically to fructose and function as a ligand-gated ion channel (Sato et al., 2011). Immunohistochemistry demonstrated that BmGr9 is expressed in cells of the oral sensory organs including the maxillary galea, maxillary palps, labrum, labium, and also in CNS putative neurosecretory cells. In addition, double-immunohistochemistry indicated that BmGr9-expressing cells in the brain are neurosecretory cells; besides, most of BmGr9-expressing cells were co-localized with putative neuropeptide F1-expressing cells in the brain. As mentioned above, Neuropeptide F1 (BmNPF1) is an ortholog of DmNPF in the *B. mori* (Roller et al., 2008), and DmNPF is known to increase food consumption, suggesting that BmGr9 are involved in promotion of feeding behavior. Furthermore, a portion of BmGr9-expressing cells in the brain was co-localized with cells expressing BmGr6, it is possible that except D-fructose, BmGr9 may exhibit different ligand response properties in the *B. mori* larvae. On the other hand, some cells were stained with anti-BmGr6 antiserum alone (Fig. 3.12A), suggesting that BmGr6 may response ligands which are different from the compounds that were detected by the combination of BmGr9 and BmGr6; and BmGr6 may play roles distinct combinations of BmGr9 and BmGr6. In addition, BmGr6-expressing cells might also express other sugar clade BmGrs.

RT-PCR showed that 27 putative bitter *BmGrs* genes were expressed in midgut of the *B. mori* larvae. A previous study in *D. melanogaster* showed that DmGrs including bitter DmGrs were expressed in enteroendocrine cells of the gut (Park and Kwon, 2011b). Moreover, they determined that some of these DmGrs are co-localized with enteroendocrine cells including neuropeptide F (NPF), locustatachykinin (LTK) or diuretic hormone 31 (DH31) (Park and Kwon, 2011b). For example, DmGr93a and DmGr33a, two bitter receptors, and were also showed to express in the fly gut and co-localized with NPF in the gut enteroendocrine cells (Park and Kwon, 2011b). It is possible that also these putative bitter BmGrs are expressed in enteroendocrine cells of the *B. moti* midgut, and may co-express with some neuropeptides in the enteroendocrinde cells. On the other hands, 25 putative bitter *BmGrs* genes were also found expressed in CNS, especially regarding *BmGr53*, which was found to express not only in oral sensory organs, but also in the brain (Fig. 4.6). More importantly, immunohistochemistry showed the BmGr53 protein which was expressed in cells of brain of the *B. mori* larvae, suggests this Grs may function in feeding behavior.

Future studies should identify the ligands of BmGr6 and examine whether BmGr6 plays a role of co-receptor. Moreover, combining the in situ hybridization (ISH) and immunohistochemistry (IHC) might be employed to investigate the neuropeptides which is co-expressed with BmGr6 in midgut enteroendocrine cells and CNS neurosecretory cells should be resolved. Also, determination of whether other BmGrs were also expressed in BmGr6-expressing cells or BmGr9-expressing cells should be

resolved. In the case of BmGr53, ligands of this putative bitter Gr remain to be explored. In addition, except brain, the distribution of BmGr53-expressing cells in other tissues such as the oral sensory organs and legs may also be evaluated.

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