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Numerous peptidoglycan recognition protein genes expressed in the bacteriome of the green rice leafhopper *Nephotettix cincticeps* (Hemiptera, Cicadellidae)

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Abstract

Peptidoglycan recognition proteins (PGRPs) contribute to the immune response of vertebrates and higher invertebrates. Animals have a varying number of *PGRP* genes: 4 in human and mouse, 13 in *Drosophila*, and 0 in an aphid. Transcriptome analyses revealed that numerous *PGRP* genes (> 300 in an RNA-Seq analysis) were expressed in the green rice leafhopper *Nephotettix cincticeps* (Uhler) (Hemiptera, Cicadellidae). *PGRP* genes were mostly expressed in the bacteriomes, in which two bacterial endosymbionts, *Sulcia* and *Nasuia*, are harbored. *E. coli* inoculation upregulated antimicrobial peptide gene expression in the leafhopper, whereas *PGRP* gene expression was not affected by the bacterial challenge. High and constant expression of *PGRP* genes in the bacteriomes and the lack of an immune response against invading bacteria suggest that these PGRPs play a hitherto unknown role in the bacteriomes, implicating a relationship with symbiotic association in *N. cincticeps*.

Keywords PGRP · Bacteriome · Symbiont · Leafhopper · Innate immunity

Introduction

Peptidoglycan recognition proteins (PGRPs) function as pattern recognition receptors (PRRs), and were first discovered as hemolymph proteins of the silkworm *Bombyx*

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mori (Linnaeus) (Yoshida et al. 1996). PGRPs recognize pathogens and contribute to the process of their elimination in vertebrates and higher invertebrates (Boneca 2009; Dziarski 2004; Michel et al. 2001; Steiner 2004; Takehana et al. 2002). Peptidoglycan (PGN), a major structural component of the cell wall of almost all bacterial species, activates signal transduction pathways for antimicrobial defense after being recognized by PGRPs (Dziarski and Gupta 2006; Guan and Mariuzza 2007; Royet and Dziarski 2007). Some PGRPs exhibit amidase activity that enables them to work as PGN scavengers, resulting in suppressing a heightened immune response to PGN (Bischoff et al. 2006; Kim et al. 2003; Mellroth et al. 2003; Persson et al. 2007; Zaidman-Remy et al. 2006, 2011). PGRPs thus work for both activation and suppression of immune response in vertebrates and invertebrates alike (Kurata, 2014; Royet et al. 2011).

Humans and mice possess four *PGRP* genes (Royet and Dziarski 2007). In insects, PGRPs activate the proPO cascade (Yoshida et al. 1996), Toll and Imd pathway (Royet et al. 2005), and cellular autophagy (Yano et al. 2008). In *Drosophila melanogaster* Meigen, PGRP-SD and -SA function as PRRs for Gram-positive bacteria upstream of the Toll pathway (Bischoff et al. 2004; Michel et al. 2001) and PGRP-LC and -LE as PRRs for Gram-negative and some of Gram-positive bacteria in the Imd pathway (Choe et al.

2002; Gottar et al. 2002; Leulier et al. 2003; Rämet et al. 2002; Takehana et al. 2002). PGRP-LE is also an intracellular recognition molecule inducing macroautophagy in *Drosophila* (Yano and Kurata 2011). The number of *PGRP* genes varies among insect species: *D. melanogaster* has 13 *PGRP* genes (Ferrandon et al. 2007); the mosquito *Anopheles gambiae* Giles has 7 (Christophides et al. 2002); the honeybee *Apis mellifera* Linnaeus has 4 (Evans et al. 2006); the beetle *Tribolium castaneum* (Herbst) has 8 (Zou et al. 2007); and the silkworm *B. mori* has 12 (Tanaka et al. 2008) but the aphid *Acrythosiphon pisum* Harris lacks *PGRP* genes (Gerardo et al. 2010).

The green rice leafhopper *Nephotettix cincticeps* (Uhler) (Hemiptera, Cicadellidae) (Online Resource 1A), a common rice pest found in Asian countries with moderate climates, harbors two bacterial endosymbionts and a symbiotic *Rickettsia* (Mitsuhashi and Kono 1975). A pair of symbiotic organs called the bacteriome, which is located on both sides of the anterior part of the abdomen (Online Resource 1B), stores *Sulcia* and *Nasuia*, members of the phylum Bacteroidetes and the class betaproteobacteria, respectively (Online Resource 1C and D) (Noda et al. 2012). *Sulcia* is present in an outer region of the bacteriome, whereas *Nasuia* is restricted to an inner region of the bacteriome. The bacteriome symbionts are vertically transmitted via the ovaries from generation to generation (Nasu 1965). The whole-genome analyses of *Sulcia* and *Nasuia* species revealed that these endosymbiotic bacteria have very small sized genomes and provide essential nutrients to their host insects (Bennett and Moran 2013; McCutcheon 2010; McCutcheon and Moran 2007, 2012). While on the other hand, expressed host genes in the bacteriome are poorly understood.

In this study, we report the discovery and expression of *PGRP* genes of the green rice leafhopper *N. cincticeps* (hereafter referred to as "*NcPGRP*"). An unexpected large number of *PGRP* genes was discovered from expressed sequence tag (EST) and RNA-Seq analyses of this species, and their expression was mostly restricted to the bacteriome. The results of our study indicate that there is an unusual function of these *PGRP* genes with regards to the symbiotic association between the leafhopper and bacteriome symbionts.

Materials and methods

Insects and antibiotic treatment

The green rice leafhopper *N. cincticeps*, collected at Yawara, Ibaraki, were reared on rice seedlings at 26 °C under a 16:8 h light: dark cycle in a plastic box (30×28×24 cm). The leafhoppers were individually reared in glass test tubes (130 mm length and 16 mm diameter) containing rice seedlings and their developmental stages were examined. Antibiotics

(tetracycline, rifampicin, and ampicillin) were individually administered to each leafhopper to kill symbionts. Antibiotics solution (0.05%) was given to rice seedlings in the glass test tubes from 0-day-old 1st instar nymphal stage (Noda et al. 2001).

Expressed sequence tag analysis

EST analysis was performed according to the method prescribed by Noda et al. (2008). mRNA was extracted from each sample; approximately 20 insects were used for whole-body libraries, ca. 50 individuals for dissected tissue or body part libraries, and ca. 200 for egg libraries (https://ncest.dna.affrc.go.jp/yokobai_lib_name.html). cDNA was synthesized using an oligo-dT primer along with a SMART II oligonucleotide in a SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., Palo Alto, CA, USA). Amplification was performed by using SMART technology, and the nested universal primer (NUP) and the 3′-polymerase chain reaction (PCR) primer available in the kit. The PCR products were cloned into pGEM®-T cloning vectors (Promega Corp., Madison, WI, USA). The NUP primer was used for the sequencing reaction by using colony PCR products amplified with SP6 and T7 primers as the PCR template. Sequencing was performed using Big Dye Terminator v3.0 or v3.1 (Applied Biosystems, USA) and a DNA analyzer (model 3700, PE Applied Biosystems). Sequencing data were first manually processed and examined, and the following EST sequences were eliminated after blast analyses: cloning vector, *Escherichia coli* genome, mitochondrial genome, and ribosomal rRNA gene sequences. The EST sequences of *N. cincticeps* are available on https://ncest.dna.affrc.go.jp.

PCR, sequencing, and RT-PCR

Full-length cDNA sequences were determined using the SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc.) or 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen). PCR products were cloned into pGEM®-T cloning vectors (Promega Corp.) and the sequences were determined according to the abovementioned method using SP6, T7, and sequence-specific primers.

The expression of 18 *NcPGRP* genes was determined by reverse transcription PCR (RT-PCR) using specific primers (Online Resource 2). The head, thorax, abdomen, midgut, and ovary were dissected from five 3-day-old adult females and the testis was from five 3-day-old adult males. Bacteriomes were dissected from 8 adults each of both the sexes. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized with SuperScript II-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The conditions for RT-PCR were as follows: 1 cycle of 95 °C for 1 min; 30–35 cycles of 95 °C for 30 s, 54–60 °C for 30 s,

and 72 °C for 90 s; and final extension of 72 °C for 5 min. *Ribosomal protein L10* (*NcRpL10*) gene was also amplified for validating the cDNA samples.

The expression levels of *NcPGRP1* and *NcPGRP12* genes were determined based on each developmental stage of the leafhopper by real-time reverse transcription PCR (RRT-PCR). The stages tested were 5- to 7-day-old eggs (50 eggs), 1st instar nymph (30 nymphs), 2nd instar nymph (20 nymphs), 3rd–5th instar nymph (10 nymphs), and 0-, 3-, 7-day-old males and females (5 adults each from both the sexes). cDNA templates were prepared as described above and RRT-PCR was performed using LightCycler 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN, USA) at the thermal conditions of 1 cycle of 95 °C for 5 min; 50 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. Absence of undesirable byproducts was confirmed by automated melting curve analysis. The details of the primers used for RRT-PCR are provided in Online Resource 2.

Computing data and phylogenetic analyses

Clustering analysis of the EST sequences was performed using CLOBB and CLOBB2 algorithms (Parkinson et al. 2002). The parameters used for clustering were 95% identity and 50-bp coverage. Blast analysis was performed using NCBI blast 2.2.26 (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/). Signal peptides were predicted using SignalP 4.1 (https://www.cbs.dtu.dk/services/SignalP-4.1/), SignalP 5.0 (https://www.cbs.dtu.dk/services/SignalP/), and Phobius (https://phobius.sbc.su.se), and transmembrane regions were examined using TMHMM ver. 2.0 (https://www.cbs.dtu.dk/services/TMHMM/) and Phobius. Multiple alignments were performed using Clustal X ver. 1.83 and the alignment was manually corrected. Phylogenetic analysis was performed by the Neighbor-Joining method using MEGA ver. 4.1. Bootstrap analysis was performed with 1000 replicates.

Illumina sequencing and de novo assembly

Total RNAs were isolated from the whole bodies of 10 adult males, adult females, and mature (4th–5th instar) nymphs each using an RNeasy Mini kit (Qiagen). mRNA was purified by passing the total RNA through a chromatographic column of beads with oligo (dT)-cellulose, and was subsequently fragmented into short sequences of ca. 200 bp. The fragmented mRNA was ligated with the adaptor mix and was transcribed to cDNA by reverse transcriptase before sequencing (Hokkaido System Science Co., Ltd., Japan). Sequencing was performed on an Illumina HiSeq-2000 sequencing system, which generated 40.3 million pairs of 100 base length paired-end reads (8.07 Gb). The raw sequence data were submitted to the DNA Data Bank

of Japan (DDBJ) Sequence Read Archive (DRA) database and is accessible through the BioProject [DRA008970]. Prior to assembly, the sequenced raw data were filtered with ea-utils (version 1.1.2) to obtain clean reads by removing adaptors, and this process resulted in a total of 39,951,943 pairs of high-quality reads (99.05% of total raw reads). De novo assembly of the quality-processed reads was performed using the Trinity assembly program (version r2013-02-25).

Calculation of bacterial density

The number of bacteria in the leafhoppers was counted by calculating the copy number of the rRNA gene of *Nasuia* and *Sulcia* by quantitative PCR (qPCR). DNA was individually isolated from antibiotic-treated 0-day-old 5th instar nymphs by using the DNeasy Blood and Tissue Kit (Qiagen). qPCR was performed as mentioned earlier using LightCycler 480 (Roche Diagnostics). The details of the primers used for the qPCR are provided in Online Resource 2. Since *Nasuia* and *Sulcia* possess one copy of the rRNA gene in the genome (Bennett and Moran 2013; McCutcheon and Moran 2007), the copy number of the gene calculated in qPCR was regarded as the number of bacteria in one leafhopper individual.

Western blotting

Mouse anti-NcPGRP12 antibody was prepared using a recombinant protein expressed in *E. coli*. The sequence corresponding to the open reading frame of the *NcPGRP12* gene was cloned into Novagen's pET42a plasmid (Merck-Millipore), and expressed in the *E. coli* BL21. Polyclonal antibody was obtained by injecting the recombinant protein into a mouse after His-tag purification. Protein samples for Western blotting were prepared from the head, thorax, 1st–4th segments of the abdomen, the rest of the segments of the abdomen, and the bacteriome obtained from 3-day-old females. The bacteriomes are found in the 2nd–4th segments of the abdomen (Online Resource 1B). Samples were homogenized with the phosphate buffer with 1% triton X-100 and sonicated using the ultrasonic homogenizer ML-2000 (Misonix Inc, Farmingdale, NY, USA). Water-soluble fraction was electrophoresed in a 12.5% polyacrylamide gel (e-PAGEL E-T 12.5L, ATTO Corporation, Tokyo, Japan). Proteins were transferred from the polyacrylamide gel to the PVDF sheet (Hybond-P, GE Healthcare, Buckinghamshire, UK) using the transfer blotting apparatus Trans-Blot SD (Bio-Rad, Hercules, CA, USA) and made to react with the antiserum to NcPGRP12 (1000-fold dilution). After treatment with anti-mouse IgG-HRP (MBL Co., Ltd, Nagoya, Japan) (2000-fold dilution), the proteins were detected with the HistoMark TrueBlue Peroxidase System (KPL, Gaithersburg, MD, USA).

Microarray analysis

The leafhopper 15 K oligo-microarray slide (60-mer oligonucleotides on 15,118 spots for *Nephotettix* genes, Agilent Technologies, Palo Alto, CA, USA; #2527323) was constructed using EST sequences. One or 2 oligo-probe sequences were designed from the 9180 gene clusters from the EST sequences. cDNA and labeled cRNA were synthesized using the Quick Amp Labeling Kit according to the manufacturer's instruction (Agilent Technologies). Total RNA 400 ng each for Cyanine 3-CTP (Cy3) and Cyanine 5-CTP (Cy5) was converted to cDNAs with the T7 promoter oligo-dT primer and Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT). Second-strand cDNA was transcribed to cRNA using T7 RNA polymerase together with Cy3 or Cy5. The labeled cRNA was purified using the RNeasy Mini Kit (Qiagen). The labeled cRNA (825 ng) was hybridized to a microarray slide for 17 h at 65 °C. The arrays were then washed using the Wash Buffer Kit (Agilent Technologies) and scanned at 5 μm resolutions using Agilent Microarray Scanner. The scanned signals were calculated with Feature Extraction Software v. 9.5 (Agilent Technologies). Signal data were analyzed using GeneSpring software v. 9.0 (Agilent Technologies). Statistical analysis was performed using *t* test in the GeneSpring.

Two-color dye-swap microarray analyses were performed for the bacteria (*E. coli*)-injected female adults and distilled water-injected female adults to elucidate the effect of bacterial challenge on *PGRP* gene expression. *E. coli*-suspended water was infused into a glass capillary tube and set on a manipulator model MYN-1 (Narishige, Tokyo, Japan). Leafhoppers were anesthetized by putting them on ice before injection. The capillary was inserted at the intersegmental region between thorax and abdomen on the ventral side under a microscope, and the bacteria-suspended water (0.03 μl) was injected into the body cavity using Transjector 5246 (Eppendorf, Hamburg, Germany). Three or 12 h after injection, total RNA was extracted from the abdomen of 5 leafhoppers using RNeasy Mini Kit (Qiagen).

Another set of the same analyses was also performed between *Rickettsia*-infected and *Rickettsia*-eliminated leafhopper adults (Watanabe et al. 2014) for comparing the *PGRP* gene expression level. The microarray analysis data were submitted to NCBI (GEO accession numbers, GSE135974 and GSE135973, respectively).

Results

PGRP genes in the ESTs of *N. cincticeps*

EST analyses were performed for each tissue of the leafhopper (https://ncest.dna.affrc.go.jp). Genes dominantly- and specifically-expressed in the bacteriome were selected from *Nephotettix* ESTs for obtaining insect genes related to symbiosis. Genes were first surveyed in 22,097 EST sequences that were generated from the bacteriome, ovary, testis, salivary glands, and a cell line (Nc-24, Kimura and Omura 1988). The total EST sequences from the different tissues were grouped together using the clustering script CLOBB (Parkinson et al. 2002) under the parameters of 95% identity and 50-bp coverage, resulting in the identification of 6773 clusters including 3895 singlets. The gene clusters that exclusively contained clones from the bacteriome library were then manually surveyed. Together with many undefined genes and housekeeping genes, *PGRP* genes were noticeably expressed in the bacteriome. Eighteen gene clusters, which include 5 or more clones whose sequences highly showed homology to the *PGRP* genes, were selected. *PGRP* gene clusters that contained 4 or less clones were discarded for further analyses. Complete sequences of the 18 *PGRP* genes were determined by 5′ rapid amplification of cDNA ends (5′ RACE) and 3′ RACE. The genes were labeled as *NcPGRP1* to *NcPGRP18* in the order of descending prevalence of the number of EST clones in each cluster and then in the order of the descending prevalence of the size of coding sequence (accession numbers LC484294–LC484311). The size of an amino acid sequence of the NcPGRPs ranged from 148 to 300 with an average size of 195, which is equivalent to or smaller than those of *Drosophila* PGRPs-S (~ 203) and PGRPs-L (~ 520). The evolutionary relationship between NcPGRPs and PGRPs of other insects was elucidated by the phylogenetic analysis of the amino acid sequences; the phylogenetic tree indicated that NcPGRPs formed a monophyletic group together with one PGRP from *T. castaneum* (PGRP-LF) (Fig. 1).

Bacteriome-specific expression of *PGRP* genes

The level of *NcPGRP* gene expression in the head, thorax, abdomen (without the bacteriome and gut), midgut, ovary, testis, female bacteriome, and male bacteriome was determined by RT-PCR. The thorax and abdomen included the fat body. All the 18 genes were almost exclusively expressed in the bacteriome (Fig. 2). PGRPs are generally found in immune-competent organs and hemolymph in insects, and *PGRP* genes are mainly expressed in the fat body, and lesser in the cuticular epidermis, midgut, and hemocytes (Dziarski and Gupta 2006). However, in the samples of our study, the 18 *NcPGRP* genes were not expressed in the thorax and abdomen that include the fat body and epidermis. Expression was not also observed in the midgut (Fig. 2). The expression profile throughout the leafhopper developmental stages was examined by RRT-PCR in 2 *NcPGRP* genes, *NcPGRP1* that was highly expressed based on the number of EST (described below) and *NcPGRP12* that had
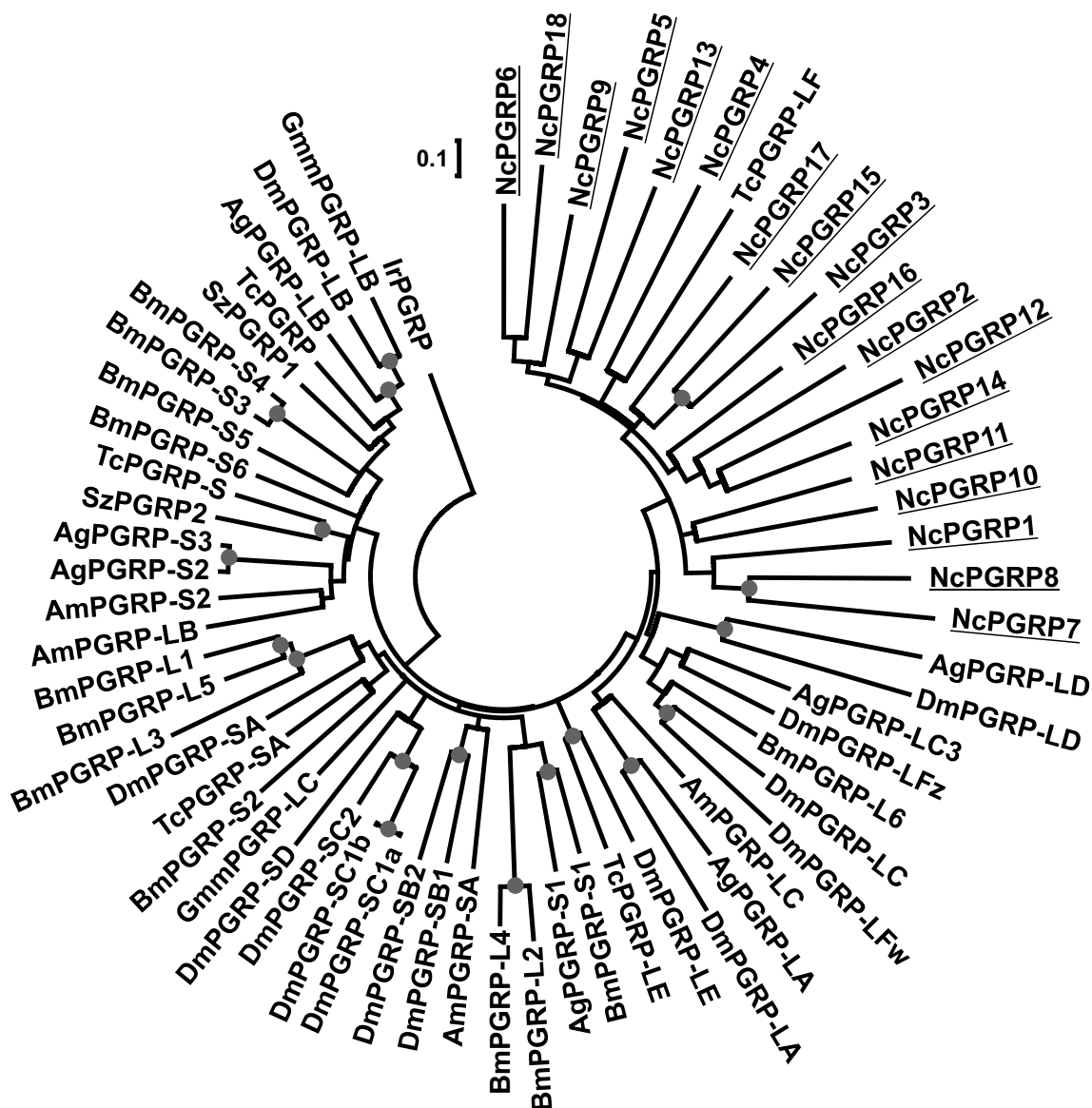
Fig. 1 Phylogenetic tree of PGRP in insects. NJ-tree of PGRP domain (164 sites) was constructed using MEGA ver. 4. Amino acid alignment was performed using ClustalW. Closed circles show the nodes whose bootstrap values were above 50%. Initial letters show the insect species: Ag *Anopheles gambiae*, Am *Apis mellifera*, Bm *Bombyx mori*, Dm *Drosophila melanogaster*, Gmm *Glossina morsitans morsitans*, Nc *Nephotettix cincticeps* (underlined), Sz *Sitophilus zeamais*, Tc *Tribolium castaneum*, Ir *Ixodes ricinus* (outgroup)

the longest translation size (300 amino acids) among the 18 genes. These *NcPGRP* genes were ubiquitously expressed in the leafhopper, showing moderate fluctuation in expression level through the developmental stages (Online Resource 3).

Bacteriome-specific localization of PGRP

Prediction analyses of signal peptides and the transmembrane region of NcPGRPs suggested that NcPGRPs were located in the bacteriome rather than being secreted from the organ. The deduced amino acid sequences were analyzed using SignalP 4.0 (https://www.cbs.dtu.dk/servi ces/SignalP/), Phobius (https://phobius.sbc.su.se/), and TMHMM Server v. 2.0 (https://www.cbs.dtu.dk/services/ TMHMM-2.0/). All NcPGRPs did not possess any signal peptide sequences and transmembrane region, except NcPGRP10. NcPGRP10 was predicted to contain a signal peptide sequence from the 1–17 residues and a transmembrane region in 20–45 residues by Phobius, with their provability value 0.6–0.7. However, SinalP and TMHMM did not support the presence of signal peptide and a transmembrane region in NcPGRP10.

To detect the location of PGRP proteins, mouse antisera against PGRP1 and PGRP12 were prepared for Western

Fig. 2 Expression of 18 *PGRP* genes in various tissues of *Nephotettix cincticeps*. All the tissue samples were prepared from 3-day-old adult females except the testis and male bacteriome samples, which were prepared from 3-day-old adult males. The sample from the abdomen did not include the bacteriome. The *ribosomal protein L10* (*NcRpL10*) gene was used as a control for RT-PCR
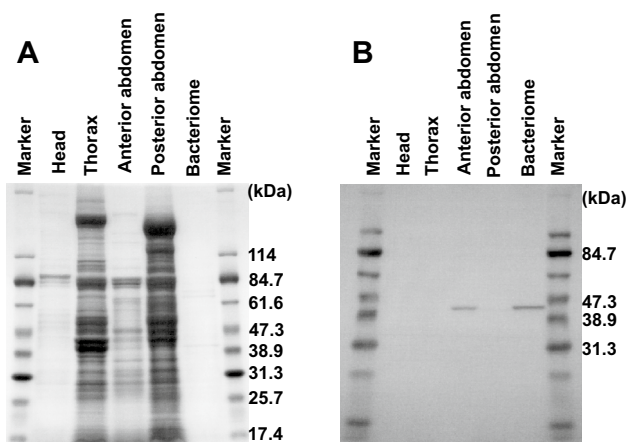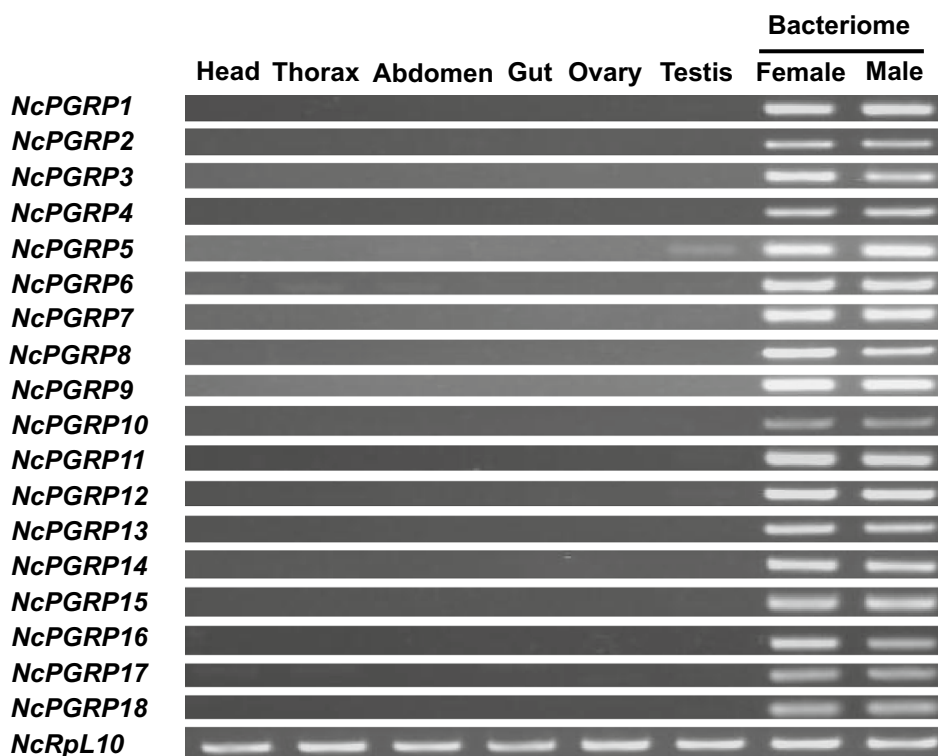

A

B

Fig. 3 Localization of NcPGRP12 in an adult female. Body parts of ten 3-day-old adult females were used for analysis. **a** Stained with Coomassie Brilliant Blue. **b** Western blotting using anti-NcPGRP12 antiserum. Anterior abdomen corresponds to the 1st to 4th segments of the abdomen and posterior abdomen corresponds to the 5th segments onwards to the tail. Anterior abdomen contains the bacteriome

blot analysis. Since the antiserum against PGRP1 did not show high antibody titre, the location of PGRP was determined using the antiserum against NcPGRP12. NcPGRP12 was detected in the bacteriome and the anterior part of the abdomen that includes the bacteriome; no signal was observed in the head, thorax, and posterior part of the abdomen (Fig. 3).

Immune response in *N. cincticeps*

Bacterial inoculation into insects by microinjection usually upregulates *PGRP* gene expression together with an increase in antibacterial peptide gene expression (Irving et al. 2001; Lee et al. 2007). Therefore, *E. coli* was injected into female adults and the expression profiles of immune system-related genes were analyzed using the *Nephotettix* microarray (8×15 K, Agilent). The microarray included 297 PGRP gene probes from 150 PGRP genes; 2 probes were designed for most of the *PGRP* gene sequences. Total RNAs were isolated from the abdomen of 5 female adults.

Antibacterial protein genes were clearly upregulated by the bacterial challenge after 12 h (Online Resource 4); *defencin* gene (probe number NCNY2028) showed a 2.2-fold change at 3 h ($p > 0.05$, t test based on 3 biological replicates) and 8.3 at 12 h ($p < 0.01$, t test based on 4 biological replicates), and *diptericin* gene (probe numbers NCMG2043 and NCMG4985) 2.1 on average at 3 h (1.97–2.26, $p > 0.05$) and 5.0 on average at 12 h (3.46–6.62, $p < 0.01$) after bacterial injection. Although immune response pathways have not been elucidated in *N. cincticeps*, *E. coli* was recognized by the leafhopper. However, the *E. coli* challenge did not upregulate any *NcPGRP* genes after 3 h and 12 h with one exception among 297 probes on the microarray at 12 h (NC_Bac4333-1, $p < 0.05$, t test based on 4 biological replicates) (Online Resource 4).

Nephotettix cincticep used in the experiments harbors symbiotic *Rickettsia* in the whole body (Noda et al. 2012; Watanabe et al. 2014), which is considered to possess PGN in its outer membrane. A *Rickettsia*-uninfected colony was created by administrating rifampicin (Noda et al. 2001; Watanabe et al. 2014). Microarray analysis was performed for *Rickettsia*-infected and -uninfected young adults and the infection status of all examined individuals was confirmed by diagnostic PCR. Antimicrobial peptide genes (NCNY2028, NCMG2043, and NCMG4985) were slightly upregulated but the extent of upregulation was not significant (Online Resource 5). The expression level of many *PGRP* genes (246 out of 297 probes) were not affected by *Rickettsia* infection ($p > 0.05$, t test based on 4 biological replicates), although 45 probes were upregulated and 6 probes were downregulated ($p < 0.05$) (data not shown).

The effect of antibiotic treatments on symbiont populations and *PGRP* gene expression

Microarray analysis did not detect the activation of *NcPGRP* genes in the bacterial challenge. Together with the fact that most of NcPGRPs are considered to be located in the bacteriome, they may not participate in an immune response against invading microorganisms. Therefore, the bacteriome symbionts were exposed to antibiotics to disrupt the normal balance between the host and the symbionts in the bacteriome, and PGRP expression level was examined. Tetracycline, rifampicin, or ampicillin was administered from the 1st instar nymph stage and 16S rRNA gene copy numbers of *Nasuia* and *Sulcia* were calculated in 0-day-old 5th instar nymphs by qPCR. *Sulcia* population significantly decreased after tetracycline treatment (Online Resource 6A), and *Nasuia* population in the bacteriome was significantly decreased after tetracycline and rifampicin treatments (Online Resource 6B). The expression levels of 18 *NcPGRP* genes were examined by RRT-PCR in the antibiotic-treated 5th instar leafhoppers in comparison with untreated 5th instar leafhoppers. The expression levels of *NcPGRP* genes were reduced after antibiotic treatments, with the extent of reduction being in the following order: tetracycline > rifampicin > ampicillin (Online Resource 6C–E). Significant reduction was observed in 12, 11, and 7 *PGRP* genes after tetracycline, rifampicin, or ampicillin treatment, respectively. In tetracycline-treated samples, some *PGRP* genes showed less than 10% expression level compared to those of the untreated ones.

An abundance of *PGRP* genes in *N. cincticeps*

The genes specifically expressed in the bacteriome were again examined after the analysis of the last EST library. A total of 38,309 ESTs were finally generated from 14 plasmid libraries made from 10 different organs or tissues (the mycetome, midgut, ovary, testis, salivary glands, compound eye, egg, nymphal whole body, cell line, and bacteria-challenged female adult) (https://ncest.dna.affrc.go.jp). The number of EST sequences obtained from the libraries of the bacteriome NcMYA and NcMYB was 3095. The 3095 ESTs from the bacteriome were clustered into 1293 groups containing 832 singletons (under the parameters of 95% identity and 50 bp coverage). The top 20 largest clusters are shown in Online Resource 7. The top 3 cluster genes were undefined and the 4th one was the *PGRP* gene (*NcPGRP1*). *PGRP* genes also ranked 18th and 20th on the list (*NcPGRP2* and *NcPGRP3*, respectively). *PGRP* genes were only found in the libraries of bacteriome and bacteriome-containing tissues, except two *PGRP* genes, NCTE-1686 and NCTE-0899, which were found from the testis library. Other highly expressed genes in the bacteriome were those for bis5-nucleosyl-tetraphosphatase, arginine kinase, ferritin, lipoyltransferase, bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase, mitochondrial SarDH, and ribosomal protein L19, and 7 uncharacterized genes.

The abundance of *PGRP* genes in *N. cincticeps* was also confirmed by the RNA-Seq data of the whole leafhopper body. De novo assembly of the RNA-Seq sequences by Trinity (https://trinityrnaseq.sourceforge.net/) yielded 102,723 contigs with an average length of 1,115 bp and a median length of 456 bp. Tblastn analysis was performed against the contig nucleotide sequences using the putative amino acid sequences of the *Drosophila* 12 PGRP as queries (DmPGRP-LA [Q95T64], DmPGRP-LB [Q8INK6], DmPGRP-LC [Q9GNK5], DmPGRP-LD [Q9GN97], DmPGRP-LE [Q9VXN9], DmPGRP-LF [Q8SXQ7], DmPGRP-SA [Q9VYX7], DmPGRP-SB1 [NP_648917], DmPGRP-SB2 [NP_648916], DmPGRP-SC1 [NP_610407], DmPGRP-SC2 [NP_610410], and DmPGRP-SD [NP_648145]). The blast analysis found 572 contig sequences with PGRP similarity (E value < 10). Preliminary homology searches (blastx) of the 572 sequences against the non-redundant NCBI DNA database were performed to eliminate sequences that were not related to the *PGRP* gene, indicating that 554 sequences showed the best homology to PGRP. Among the 554 sequences, isoform sequences with the same Trinity gene ID but different isoform ID were combined. The longest or moderately sized contigs that included PGRP domain were selected, resulting in 317 independent sequences. Blastx homology searches were performed again against the most recent database of non-redundant GenBank CDS translations (e value < 1e−03). Finally, 307 contigs were selected as *PGRP* or *PGRP*-related genes of *N. cincticeps* (Online Resource 8). The corresponding genes in the EST libraries were blast-searched and 155 ESTs were found (Online Resource 8).

Eighteen NcPGRP did not possess signal peptides as previously described. The 307 sequences were then tested for signal peptides using SignalP 5.0. Two genes, comp41637_c0_seq1 and comp85737_c0_seq1 possessed signal peptides. These two genes were not found in ESTs (Online Resource 8).

Some *NcPGRP* sequences among the 307 contigs showed irregular features compared to the *PGRP* genes. For example, a plausible consensus amino acid sequence "YNFXXXXXXXXYEGRGW" or similar amino acid sequences were found in the putative amino acid sequences of most of the contigs (Online Resource 9). However, such sequences were not found in some contig sequences. Moreover, some contigs were too short as a *PGRP* gene or included stop codons in the PGRP domain. Such irregular features were found in at least 35 contigs, which suggests that these genes cannot be translated into functional PGRP proteins.

Discussion

The present study on *N. cincticeps* revealed 2 unusual features of the *PGRP* genes. One, *NcPGRP* genes were mostly expressed in the bacteriome. Most *NcPGRP* genes were found in the libraries of bacteriome and bacteriome-containing tissues and a few in those of other tissues in EST analyses. Their expression in other tissues was highly insignificant as far as at least 18 genes identified are concerned (Fig. 2). High level of PGRP gene expression in *Sulcia* and *Nasuia* bacteriocytes and low level of expression in the insect body could be actually found in an RNA-Seq study reporting the expression levels of 11 *PGRP* genes from the aster leafhopper *Macrosteles quadrilineatus* Forbes (Mao et al. 2018, Table S2 in supplementary information). The other unusual feature is that an extremely large variety of *PGRP* genes were expressed in *N. cincticeps* (Online Resource 8, 9). This large number of PGRP genes in leafhoppers has not been reported so far. These inexplicable facts of *PGRP* genes posed some questions that have been discussed below.

It is well established that the most important role of PGRPs in insects is to recognize the invading bacteria. However, it is questionable whether NcPGRPs work as PRR molecules against the bacteria. Many *NcPGRP* genes were expressed in the bacteriome. NcPGRP molecules, which did not have a signal peptide, seem to work in the cytoplasm of bacteriome cells. Actually, Western blot analysis showed the presence of NcPGRP12 in the bacteriome (Fig. 3). NcPGRPs, therefore, do not easily reach the invading bacteria in the hemolymph. Two genes in the contigs of RNA-Seq analysis, comp41637_c0_seq1 and comp85737_c0_seq1, which possessed a signal peptide, require further examination. PGRP genes are usually known to be upregulated in response to invading bacteria, for example in *Drosophila*

(Irving et al. 2001), *Bombyx* (Lee et al. 2007), the maize weevil *Sitophilus zeamais* Motschulsky (Anselme et al. 2006), and the brown planthopper *Nilaparvata lugens* (Stål) (Bao et al. 2013). No significant upregulation was observed in 150 *NcPGRP* genes used for microarray analysis upon the bacterial challenge (Online Resource 4). No noticeable upregulation in many *PGRP* genes including the 18 highly expressed genes was also observed in *Rickettsia*-infected leafhopper (Online Resource 5). The *Rickettsia* symbiont infects the cytoplasm and nuclei of most leafhopper tissues including the bacteriome (Mitsuhashi and Kono 1975; Noda et al. 2012; Watanabe et al. 2014), apparently having PGN as well as other rickettsiae.

The *PGRP* genes expressed in the symbiotic organs are so far reported in 2 groups of insects: weevils (Anselme et al. 2006, 2008; Heddi et al. 2005; Maire et al. 2019; Vigneron et al. 2012) and the tsetsefly (Wang and Aksoy 2012; Wang et al. 2009). The maize weevil *S. zeamais* expressed a *PGRP* gene in the bacteriome, which is located at the junction of foregut and midgut (Nardon and Grenier 1989). The weevil *PGRP* gene, an ortholog of *Drosophila PGRP-LB*, shows a high steady-state level expression in the bacteriome and its expression is upregulated by Gram-negative bacteria (Anselme et al. 2006). Symbiont infection in the bacteriome also causes upregulation of the *PGRP* gene. Moreover, the symbionts outside the bacteriome are recognized as microbial intruders by the host, inducing antibacterial peptide production (Anselme et al. 2008). A notable point is that overexpression of the *PGRP* gene in the bacteriome would prevent the activation of the immune pathway by its putative enzyme activity as a scavenger, similar to the *Drosophila PGRP-LB* gene (Anselme et al. 2006). A similar case has been reported in the tsetse fly *Glossina morsitans morsitans* Westwood, which possesses the obligate endosymbiont *Wigglesworthia* in the midgut bacteriome. The antibacterial peptides of this fly killed the symbionts. The tsetse *PGRP-LB* gene is considered to degrade the PGN released by the bacterial endosymbionts (Bing et al. 2017; Wang and Aksoy 2012; Wang et al. 2009) through *N*-acetylmuramyl-L-alanine amidase activity of the PGRP molecule (Zaidman-Remy et al. 2006), resulting in preventing the activation of the host's immune responses, which damages the symbionts in the bacteriome. The involvement of PGRP in microbial association in animals has also been observed in some invertebrates such as the bobtail squid (Troll et al. 2009) wherein a PGRP neutralizes a PGN-derived toxin of the luminescence-producing bacterial symbiont, *Vibrio fischeri* (Troll et al. 2010). All these examples indicate that PGRPs exhibit enzymatic activity and degrade the PGN of symbionts that may trigger the host's immune response.

PGRPs that function as PGN scavengers are known to have 5 important amino acid residues for their enzymatic activity, namely, His-17, Tyr-46, His-122, Lys-128,

and Cys-130 compared with the T7 lysozyme amino acid structure (Cheng et al. 1994; Mellroth et al. 2003; Persson et al. 2007). *Drosophila* PGRPs that exhibit amidase activity, e.g., DmPGRP-LB, -SB1, -SB2, -SC1a, -SC1b, and SC2, all possess 4 amino acids, namely, His-17, Tyr-46, His-122, and Cys-130. The abovementioned PGRPs of the weevils, tsetsefly, and squid possess most of these amino acids (Anselme et al. 2006; Wang et al. 2009; Troll et al. 2010). In contrast, NcPGRPs do not have these amino acid residues; Tyr-46 was found in some NcPGRPs (*NcPGRP1*, *3*, *7*, *8*, *9*, *10*, *11*, *12*, *14*, *15*, *16*, and *17*) and His-122 in *NcPGRP16*, which suggests that NcPGRPs do not work as PGN scavengers for protecting symbionts from the host's immune attack. NcPGRPs appear to have a function different from that of PGRPs of the weevils, tsetsefly, and squid. An important feature of the bacteriome symbionts of *Nephotettix* when compared with the symbionts of the weevils, tsetsefly, and squid is the absence of PGN; *Nasuia* and *Sulcia* do not possess PGN. The peptidoglycan biosynthesis pathway is not found in the five species of *Sulcia* (Bennett and Moran 2013; McCutcheon and Moran 2007, 2010; McCutcheon et al. 2009; Woyke et al. 2010) and the *Nasuia* species of *Macrosteles quadrilineatus* (Bennett and Moran 2013), thus lacking most of the related enzymes. Because of no possession of PGN, *Nasuia* and *Sulcia* are amorphous in shape (Mitsuhashi and Kono 1975; Nasu 1965; Noda et al. 2012) (Online Resource 1C and D). The presence of *Rickettsia*, a probable facultative symbiont in *N. cincticeps*, also appeared to be unrelated to the high level of expression of *PGRP* genes (Online Resource 5). These results indicate that *N. cincticeps* does not need PGN clearance mediated by PGRPs in the bacteriome.

In *Nephotettix*, bacterial challenge caused the induction of antimicrobial peptide genes, indicating that this species has innate immune pathway(s) as other insect species, although the details of the pathway(s) remain unclear. The expression of many *PGRP* genes in this leafhopper remained unaffected in the face of a bacterial invasion (Online Resource 4), although this observation does not necessarily deny the presence of *PGRP* gene(s) that are involved in the immune response pathway. Among hemipteran insects, the brown planthopper *N. lugens* is reported to possess 2 *PGRP* genes (Bao et al. 2013) while the pea aphid *A. pisum* does not have any *PGRP* genes (Gerardo et al. 2010). *A. pisum* also lacks some genes of the Imd pathway. In *N. cincticeps*, 3 genes involved in the Imd pathway, *imd*, *Dredd*, and *Relish*, were blast-surveyed into *Nephotettix* RNA-Seq sequences using *Drosophila* gene sequences as queries. Although the gene *imd* was not found (least *e* value = 0.023), *Dredd* (*e* value 2e−13) and *Relish* (2e−53) genes were found in the RNA-Seq data (data not shown). *N. cincticeps* may possess the Imd or Imd-related pathway, and if so, the PRRs working for the pathway should be explored.

The remaining questions are why *N. cincticeps* possesses more than 300 *PGRP* genes and what is the function of the NcPGRPs. The phylogenetic tree of insect PGRPs (Fig. 1) suggests that multiple gene duplication has occurred in an evolutionary process in the leafhopper. New genes are often created by gene duplication, and some duplicated genes are maintained in the genome for a long time (Nei and Rooney 2005). Montaño et al. (2011) indicates that the mode of *PGRP* gene evolution was characterized by birth and death processes. The high level of expression of *NcPGRP* genes in the bacteriome strongly suggests that PGRPs have an important function in the bacteriome. However, some *NcPGRP* genes show irregular features. Nei and Rooney (2005) indicated that some duplicate genes become nonfunctional through deleterious mutations and are deleted from the genome. Even if we suppose that a large number of *PGRP* genes is the result of birth, why has the death of *PGRP* genes, especially in truncated or nonfunctional genes, not occurred in the past evolutionary process?

Antibiotic treatments that killed some of the symbionts suppressed *PGRP* gene expression (Online Resource 6), indicating an as-yet-unknown relationship among symbionts and PGRPs in the bacteriome. Many *PGRP* genes are retained in the leafhopper and mostly expressed in the bacteriome. These unusual features of *NcPGRP* genes are apparently related to the function of this specialized organ for endosymbiosis of the leafhopper. However, the physiological function of the bacteriome is less well understood. Studies of other genes highly expressed in the bacteriome undoubtedly contribute to elucidating the function of the PGRPs in *N. cincticeps*, because some highly- and bacteriome specifically expressed genes are expected to have novel function important for bacteriome maintenance.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest directly relevant to the content of this article.

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