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ORIGINAL ARTICLE

Comparative analysis of peptidoglycan recognition proteins in endoparasitoid wasp *Microplitis mediator*

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Abstract Peptidoglycan recognition proteins (PGRPs) are a family of innate immune receptors that specifically recognize peptidoglycans (PGNs) on the surface of a number of pathogens. Here, we have identified and characterized six PGRPs from endoparasitoid wasp, *Microplitis mediator* (MmePGRPs). To understand the roles of PGRPs in parasitoid wasps, we analyzed their evolutionary relationship and orthology, expression profiles during different developmental stages, and transcriptional expression following infection with Gram-positive and -negative bacteria and a fungus. *MmePGRP-S1* was significantly induced in response to pathogenic infection. This prompted us to evaluate the effects of RNA interference mediated gene specific knockdown of *MmePGRP-S1*. The knockdown of *MmePGRP-S1* (iMmePGRP-S1) dramatically affected wasps' survival following challenge by *Micrococcus luteus*, indicating the involvement of this particular PGRP in immune responses against Gram-positive bacteria. This action is likely to be mediated by the Toll pathway, but the mechanism remains to be determined. MmePGRP-S1 does not play a significant role in anti-fungal immunity as indicated by the survival rate of iMmePGRP-S1 wasps. This study provides a comprehensive characterization of PGRPs in the economically important hymenopteran species *M. mediator*.

Key words endoparasitoid wasp; insect immunity; microbial infection; *Microplitis mediator*; peptidoglycan recognition protein

Introduction

Innate immunity is the first line of host defense in both mammals and insects (Akira *et al.*, 2006; Lemaitre & Hoffmann, 2007). The invasion of pathogens and parasites elicits both cellular and humoral responses, which together provide efficient protection against harmful non-self entities (Hultmark, 2003). Cellular responses generally involve phagocytosis, encapsulation and nodulation that are mediated by various types of hemocytes, including plasmatocytes and granulocytes (Strand, 2008). The humoral response, on the other hand, is involved in the production of antimicrobial peptides (AMPs) and melanization. Two principle signaling pathways, Toll and immune-deficiency (IMD), mediate the induction of the transcription factor nuclear factor kappa B (NF-κB), ultimately leading to the synthesis of AMPs in the fat body, the major immune organ of insects (Janeway

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& Medzhitov, 2002; Lemaitre & Hoffmann, 2007). Apart from immune signaling pathways, melanization is a universal defense mechanism in insects. Activation of a cascade of serine proteases and prophenoloxidase (PPO) results in the formation of melanin and other toxic substances, subsequently sequestering and killing invading microorganisms or parasites (Jiang, 2008).

Immune responses are initiated by the recognition of microbial-associated molecular patterns (MAMPs) by a group of host proteins termed pattern recognition receptors (PRRs) (Yu *et al.*, 2002; Hetru & Hoffmann, 2009). MAMPs, such as lipopolysaccharide and peptidoglycans (PGNs) of bacteria and β-1,3-glucan of fungi, are present on the surface of microbes but not in the hosts. In insects, PRRs, including peptidoglycan recognition protein (PGRP), β-glucan recognition protein (βGRP) and C-type lectin (CTL) (Janeway & Medzhitov, 2002) serve as essential components of a surveillance mechanism for the detection of MAMPs of pathogens. The interaction between PRRs and MAMPs is the first event triggering downstream humoral and cellular responses.

PGRPs, a protein family of PRRs, contain a conserved PGRP domain similar to bacteriophage T7 lysozyme. PGRPs are capable of detecting PGN, an essential cell wall-specific component found in nearly all bacterial species (Rosenthal & Dziarski, 1994). Based on the third amino acid residue of the PGN short peptide, they are divided into two major types: L-lysine-type (Lys) and meso-diaminopimelic acid-type (DAP). Most Gram-negative bacteria and few Gram-positive bacteria have DAP-type PGNs on their surface, whereas most Gram-positive bacteria contain Lys-type PGNs. In *Drosophila melanogaster*, Lys-type PGNs are recognized by PGRP-SA while DAP-type are recognized by PGRP-LC, leading to the subsequent activation of the Toll and IMD pathways, respectively (Hetru & Hoffmann, 2009).

PGRPs are evolutionarily conserved over vast evolutionary distances starting from insects to mammals (Werner *et al.*, 2000; Dziarski & Gupta, 2006). First purified from the hemolymph and cuticle of the silkworm *Bombyx mori*, PGRPs were shown to elicit PPO activation and initiate the silkworm immune response against bacteria (Yoshida *et al.*, 1996). Homologues of PGRP were then identified in a variety of species ranging from echinoderms to vertebrates, including humans (Dziarski & Gupta, 2006). PGRPs have also been characterized in several other insects, including *Trichoplusia ni* (Kang *et al.*, 1998), *D. melanogaster* (Werner *et al.*, 2000; Dziarski & Gupta, 2006), *Anopheles gambiae* (Christophides *et al.*, 2002), *Manduca sexta* (Yu *et al.*, 2002), *Nasonia vitripennis* and *Ostrinia nubilalis* (Khajuria *et al.*, 2011). However, there are some insects like the pea aphid *Acyrthosiphon pisum*, where no PGRPs are as yet reported (Gerardo *et al.*, 2010). Additionally, no PGRPs have been identified in nematodes and plants (Wang & Wang, 2013). Based on transcript size and domain architecture, insect PGRPs are divided into two broad groups: short PGRPs, which are small extracellular proteins; and long PGRPs, which are either intercellular or transmembrane proteins (Royet *et al.*, 2011).

While extensive studies in *Drosophila* and moth PGRPs have been performed, little is known regarding this group of proteins in any hymenopteran insect. Parasitic wasps, belonging to the order hymenoptera, can be used as biological agents to control their host insects (Lu *et al.*, 2014; Schellhorn *et al.*, 2015). *Microplitis mediator* is an important natural enemy of many lepidopteran insect pests, such as the cotton bollworm *Helicoverpa armigera* and the oriental armyworm *Pseudaletia separata*, making it a potential biocontrol agent (Dong *et al.*, 2000). In this study, we have identified and characterized six *PGRP* genes in *M. mediator*. The phylogenetic reconstruction and structural modeling of MmePGRPs were performed. Additionally, we examined expression of these six *PGRP* genes during development and in response to microbial challenge. Further RNA interference and survival assays provided additional evidence that suggest the role of MmePRGP-S1 in the immune response against Gram-positive bacteria. Overall, we present a study on *M. mediator* PGRPs and provide clues to the functional roles of PGRPs in hymenopteran immunity.

Materials and methods

Experimental insects

The endoparasitoid wasp, *M. mediator* (Hymenoptera: Braconidae), and its host insect oriental armyworm, *P. separata* (Lepidopera: Noctuidae), were kindly provided by the Hebei Academy of Agricultural and Forestry Sciences. Wasps were reared at 26 $\pm$ 1°C under a 14 : 10 h light : dark photo period with 60% $\pm$ 10% relative humidity. Adult wasps were fed with 10% honey solution. The *P. separata* colony was maintained under the same conditions, and the second instar larvae were used for parasitism. Larvae and adults were fed with wheat seedlings and a 10% honey solution, respectively.

Septic injury

The Gram-positive bacterium *Micrococcus luteus*, Gram-negative bacterium *Enterobactor cloacae* and fungus *Beauveria bassiana* were purchased from China

General Microbiological Culture Collection Center (CGMCC). *M. luteus* and *E. cloacae* were cultured overnight in lysogeny broth medium (1% tryptone, 0.5% yeast extract, 1% NaCl w/v), harvested by centrifugation at 2500 × *g* for 5 min, washed three times with sterile 1× phosphate-buffered saline (PBS), and suspended at a density of 5×10^7 colony forming units (CFUs) in PBS. *B. bassiana* was cultured in potato dextrose agar (PDA) medium (5% potato extract, 0.5% dextrose, 1.7% agar) at 25°C for 7 days. Conidia were diluted to a concentration of 1×10^8 per mL with sterile 1 × PBS for septic injury (Zou *et al.*, 2010). Freshly prepared microbes were used for all experiments. Septic injuries were carried out by pricking the rear end of the wasp abdomen with an acupuncture needle pre-dipped in a suspension of bacteria or fungal conidia. Control wasps were pricked with sterile PBS only.

Identification and characterization of M. mediator PGRPs

The PGRP sequences from other insect species, including *D. melanogaster*, *Tribolium castaneum* and *Apis mellifera*, were used as templates to search the *M. mediator* assembled unigene sequence. The reference *M. mediator* unigene sequences were assembled from 10 different immune challenged complementary DNA (cDNA) libraries sequenced using Illumina HiSeq™ 2000 platform. For immune challenge, insects inoculated with the *M. luteus*, *E. cloacae* or *B. bassiana* were collected 6 h, 24 h and 48 h post-infection (Table S1). The potential MmePGRP protein sequences were further analyzed for the presence of putative functional domains using InterProScan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) for signal peptides using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and for transmembrane domains using TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Sequence alignments and phylogenetic analysis

Predicted MmePGRP protein sequences were aligned with PGRPs from *D. melanogaster*, *Aedes aegypti*, *T. castaneum*, *B. mori*, *Nasonia vitripennis*, *Microplitis demolitor* and *A. mellifera* using CLUSTALX 2.0 (Thompson *et al.*, 1997). PGRP contain one domain (cd06583) by NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The phylogenetic tree of amino acid sequences of the PGRP domain was built using the neighbor-joining method. The protein weight matrix, BLOSUM 30, with a respective gap opening and extension penalty of 10 and 0.1, respectively, was used to perform the multiple sequence alignment. A phylogenetic tree was constructed by the neighbor-joining analysis with bootstrap method, using 1 000 repetitions and visualized by Treeview software (TreeView version 1.6.6, University of Glasgow, Glasgow, UK). Multiple sequence alignment was displayed and edited using Genedoc software (http://www.psc.edu/index.php/user-resources/software/genedoc).

Tertiary structure prediction

Putative tertiary structures of MmePGRP domains were established by SWISS-MODEL workspace (http://swissmodel.expasy.org/), a web-based tool for 3D structure prediction (Biasini *et al.*, 2014). Homology models were built based on optimally satisfying spatial restraints obtained from evolutionarily related structures. Experimental structures of *Drosophila* PGRP-SA (1sxr.1A, 1s2j.1A), PGRP-LB (1oht.1.A) and PGRP-LE (2cb3.1.A) were selected as templates for modeling the structures of all MmePGRPs. The quality of the final model was evaluated using QMEAN Z-score (Benkert *et al.*, 2009, 2011). All selected structures with Z-score greater than –4 were considered as reliable. The representative model chosen for the production of molecular graphics was displayed using PyMOL (http://www.pymol.org/).

RNA isolation and cDNA preparation

Ten female adult wasps per treatment were collected at 6 h, 24 h and 48 h post-injection. Total RNA was extracted using an RNeasy mini kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. The RNA concentration was determined by measuring A_{260nm} using a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). For each reaction, 1 μg total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove genomic DNA. These RNAs were then used to synthesize cDNA using an Maloney murine leukemia virus (M-MLV) reverse transcriptase kit according to the protocol provided (Promega, Madison, WI, USA).

Synthesis and microinjection of double-stranded RNA (dsRNA)

MmePGRP-S1 cDNA templates were generated by reverse transcription polymerase chain reaction

(RT-PCR) using both sense and antisense primers fused with T7-phage promoter sequences (Table S2). Gene-specific cDNA fragments (500 bp in length) were generated and used for subsequent dsRNA synthesis reactions. dsRNA synthesis was accomplished by simultaneous transcription of both strands of template DNA using T7 RNA polymerase from the T7 RiboMAX Express RNAi kit (Promega, USA). Green fluorescence protein (GFP) was used to generate control interdomain GFP (iGFP) dsRNA. A Nanoliter 2000 injector (World Precision Instruments, Sarasota, FL, USA) was used to introduce the corresponding dsRNA (0.2 μL, 5 μg/μL) into the abdomen of CO_2-anesthetized newly emerged 1-day-old wasps. MmePGRP-S1 knockdown efficiency was confirmed by RT-PCR.

RT-PCR and quantitative real-time PCR (qPCR) analysis

RT-PCR reactions were performed using specific primers (Table S2) using the TaKaRa ExTaq® with 1 μL cDNA, and 1 μL of each primers (10 μmol/L). The nucleotide sequences of MmePGRPs have been deposited in the GenBank™/EBI with accession numbers from KT390715 to KT390720.

qPCR reactions for all samples were performed on an MX3000P system (Stratagene, La Jolla, CA, USA). Reactions were prepared according to the protocol provided with the SuperReal PreMix Plus SYBR green (Tiangen Biotech, Beijing, China). All primers used for qPCR were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and are listed in Table S2. Thermal cycling conditions used are as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 59°C for 20 s and extension at 68°C for 20 s. Amplification was performed at a final volume of 20 μL containing 1 μL cDNA, 0.6 μL of each primer (10 μmol/L), and 10 μL 2 × SuperReal PreMix Plus (SYBR green). All reactions were performed in quadruplicate. *Actin* expression was used as a housekeeping reference (Ramet *et al.*, 2002). Data were collected by MxPro qPCR software (Stratagene, La Jolla, CA, USA), and exported to EXCEL for further analysis. Figures were generated using Graphpad6 software (Graphpad Software, San Diego, CA, USA). The time course expression levels of MmePGRPs injected with various pathogens were performed using one-way analysis of variance (ANOVA), followed by *post-hoc* Tukey Honestly Significant Difference (HSD) test for multiple comparisons. Differences were considered significant when the *P*-value was less than 0.05. The two-way ANOVA was used to determine the combined effects of infection and time. For Figure 7, Student's *t*-test was conducted to analyze the significance of the differences between the iGFP and the iPGRP-S1 groups. The difference was considered statistically significant at $P < 0.05$ and quite significant at $P < 0.01$. Values are reported as mean ± standard deviation (mean ± SD). All qPCR statistical data were analyzed with the SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA).

Gene expression profiling

We generated 15 RNAseq libraries from *M. mediator* – six developmental stage samples and nine immune challenge samples. *M. mediator* passes through four distinct life stages: egg, larva, pupa and adult, of which the first two stages are spent in *P. separata*. Parasitized second instar *P. separata* were dissected at 24 h for the collection of eggs ($n = 400$), at 2 days first instar larvae ($n = 200$), 5 days for second instar larvae ($n = 20$) and 7 days for third instar larvae ($n = 20$). In addition, pupal ($n = 15$) stage and adult ($n = 10$) female wasps were also collected. After septic injury, we selected 10 female wasps from each immune treatment. cDNA libraries, representing mRNA samples from the parasitoid wasp from either various developmental stages or different immune challenges, were constructed and sequenced by Illumina Technology (Z. Lin & Z. Zou, unpublished data). The RSEM software package (Li & Dewey, 2011; Grabherr *et al.*, 2011) was used to map library reads to specific transcripts (in this case, the six PGRP transcripts). Changes in PGRP expression were calculated by the number of reads that mapped to the genes and are represented in the form of fragments per kb per million mapped reads (FPKM). Hierarchical clustering of FPKM value was performed using the Heatmap 2 suite in R (https://www.r-project.org/) with the Pearson correlation-based metric and average linkage clustering method.

Survival assay

Two-day-old adult female iGFP and iMmePGRP-S1 wasps were infected with various pathogens and analyzed for survival rate at various time points. After dsRNA injection, we moved all the wasps into the 14 ºC freezer for 3 days. Control (iGFP) and MmePGRPS1 dsRNA treated (iPGRP-S1) wasps were divided into two subgroups, then challenged with PBS (control), or the pathogens (*E. cloacae*, *M. luteus* or *B. bassiana*) separately. After septic injury, the wasps were placed into new plastic containers. Fifty organisms per treatment were maintained

in individual containers and were continuously fed with 10% honey solution. The viability of wasps was recorded at 10 h, 20 h, 30 h, 40 h, 50 h and 60 h post-infection. The survival analysis was repeated three times using three independent cohorts of wasps. Survival curves of wasps with different treatments were compared using Kaplan-Meier estimator. Meanwhile, curves of different groups were compared by log-rank test.

Results

Overview of the M. mediator PGRP genes

To identify *PGRP* genes in *M. mediator*, known PGRP sequences from *D. melanogaster* (Werner *et al.*, 2000), *T. castaneum* (Zou *et al.*, 2007), *M. demolitor* (Burke *et al.*, 2014) and *A. mellifera* (Evans *et al.*, 2006) were individually blasted against *M. mediator* assembled unigene sequences (Z. Lin & Z. Zou, unpublished data) using Basic Local Alignment Search Tool (BLASTx). For each novel PGRP, additional iterative BLAST searches were performed until no more sequences were found. As a result, six PGRPs were obtained from the transcriptome of *M. mediator*. Full-length sequences of all MmePGRPs were confirmed by RT-PCR and sequencing (Table S3). The total PGRP gene count was comparable to those of *T. castaneum* (7), *N. vitripennis* (8), *A. aegypti* (8), and *A. gambiae* (8), but is less than that of *D. melanogaster* (13).

All MmePGRPs contain a PGRP domain (approximately 160 residues in length) at the carboxyl termini and are divided into short and long forms, characterized by the presence or absence of a transmembrane (TM) domain. Three short PGRPs, MmPGRP-S1-3, contain a secretory signal peptide; whereas the long PGRPs, MmePGRP-L1, 2 and 3, contain a TM domain. Additionally, the Rip homotypic interaction motif (RHIM) was identified in PGRP-L3 (Fig. 1A).This newly identified RHIM domain is similar to those found in *D. melanogaster* PGRP-LA, -LC and -LE and has been reported to mediate a key interaction in downstream signaling pathways (Kajava *et al.*, 2014). Most PGRPs are 190–216 residues in length (average size 205 aa). However, due to the amino terminal extension, PGRP-L3 is composed of 473 residues. Our molecular modeling indicates that the additional sequence in PGRP-L3 does not affect its folding (Fig. S1). The additional sequence is rich in hydrophilic residues and contains an external loop. Since both the sequence and domain structures are similar to those of DmePGRP-LC and -LE, it can be assumed that MmePGRP-L3 is involved in the IMD pathway (Takehana *et al.*, 2002).

Multiple sequence alignment of DmePGRPs, AmePGRPs and the deduced MmePGRP proteins indicates a moderate conservation of PGRPs in these insects (Fig. 1B). Sequence identity of MmePGRPs with DmePGRP and AmePGRP sequences are shown in Table S4. Comparative analysis showed that the C-terminal regions of all PGRPs are highly conserved, whereas the N terminal regions are relatively diverse (Fig. 1B). MmePGRP-S1 and -S2 contain five conserved amino acids (His, Trp, His, Thr and Cys) required for Zn^{2+} binding and amidase activity (Fig. 1B). Other MmePGRPs have lost these catalytic residues as a result of mutations. The conserved amino acid, Arg, which is required for the recognition of DAP-type peptidoglycans (Lim *et al.*, 2006), is present in all MmePGRPs except -S3, suggesting their involvement in the immune response against Gram-negative bacteria (Fig. 1B). Three α-helices and seven β-sheets connected by less conserved loop structures were predicted to be present in MmePGRPs (Reiser *et al.*, 2004).

Structural features and phylogenetic analysis of M. mediator PGRPs

Crystal structures of several *Drosophila* PGRPs have been solved previously, including that of DmePGRP-LB, -SA, -SD and -LC (Reiser *et al.*, 2004; Chang *et al.*, 2005; Guan *et al.*, 2005; Lim *et al.*, 2006; Leone *et al.*, 2008). Using these PGRPs as templates (1oht.1.A, 2cb3.1.A, 1s2j.1.A, 2cb3.1.A, 1sxr.1A and 2cb3.1.A, respectively), theoretical tertiary structures of MmePGRPs (S1, S2, S3, L1, L2 and L3) were established using the SWISS-MODEL workspace prediction algorithm. All the six MmePGRPs share more than 34% identity with their respective templates. The quality of the protein models were assessed using the QMEAN Z-score (Benkert *et al.*, 2009, 2011) (Fig. S1). The QMEAN Z-score of the six MmePGRPs (S1, S2, S3, L1, L2 and L3) are -3.49, -2.45, -2.87, -2.78, -3.82 and -2.77, respectively. The final model is presented initially colored by model quality assigned using QMEAN (Fig. S1). Overall, the six MmePGRPs adopted a typical PGRP domain structure with a structural fold similar to that of bacteriophage T7 lysozyme (red) (Chang *et al.*, 2005) (Fig. 2A). The conserved region consists of three α-helices on the outer side and five β-sheets at the center. The Zn^{2+} binding sites and amidase activity sites are well-conserved in MmePGRP-S1 and -S2 (Fig. 2B). The specific cleft on the back of DmePGRP-LB associated with oligomerization necessary for downstream pathway activation was not identified in MmePGRP-S1 (Kim *et al.*, 2003). However, when the PGRP domain of MmePGRP-S3 was compared with those in

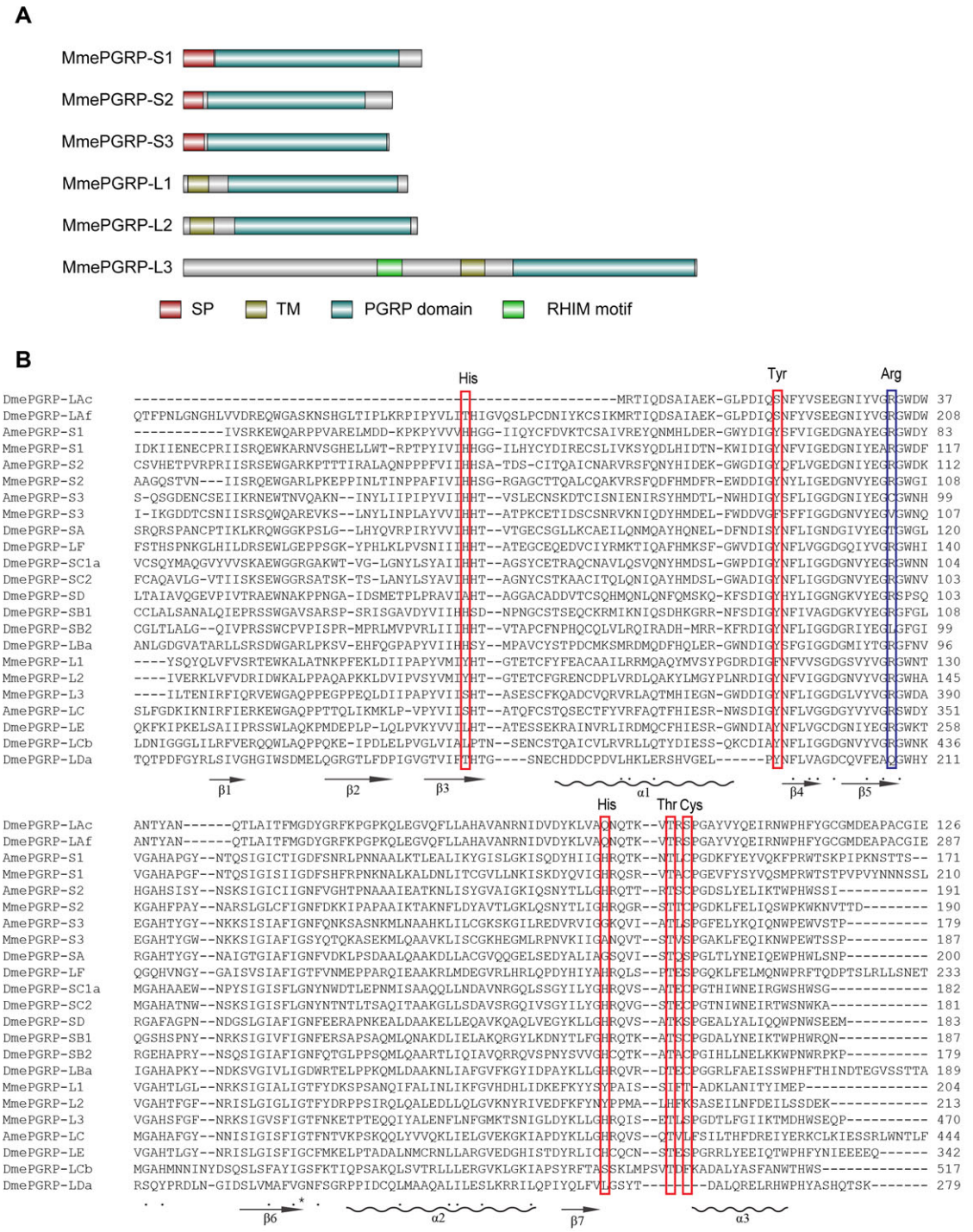

Fig. 1 Overview of *Microplitis mediator* peptidoglycan recognition protein (PGRP) genes (MmePGRPs). (A) Schematic representation of the MmePGRPs. Putative PGRP domains signal peptides (SP), Rip homotypic interaction motif (RHIM) and transmembrane domains (TM) are indicated. MmePGRP-L1, -L2 and -L3 harbor a TM domain, whereas MmePGRP-S1, -S2 and -S3 contain a secretory signal peptide. (B) Multiple sequence alignment of the PGRP domain amino acid sequences from *M. mediator*, *Apis mellifera* and *Drosophila melanogaster*. Asterisk, identical; dot, conservative substituted. Red indicates the residues required for Zn^{2+}-binding/amidase activity. Blue indicates amino acids required for recognition/binding of diaminopimelic acid (DAP)-type peptidoglycans (PGNs). Regions of protein secondary structural elements are marked under the sequences.

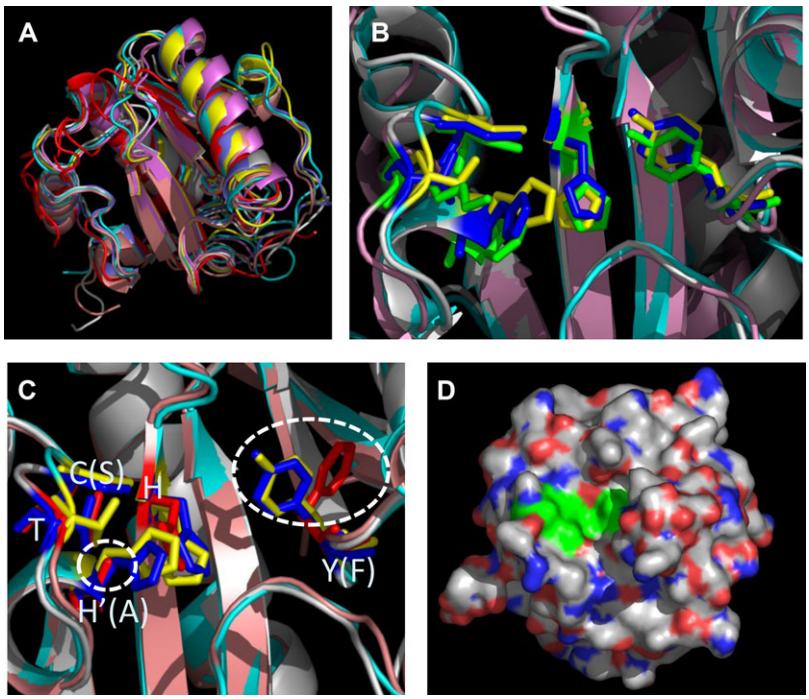

Fig. 2 Predicated tertiary structures of *Microplitis mediator* peptidoglycan recognition protein (PGRP) genes (MmePGRPs). (A) Structural alignment of all six MmePGRPs and T7lys (lysozyme structure from the bacteriophage T7). (B) The Zn^{2+}-binding and amidase catalytic sites of MmePGRP-S1, -S2 and T7 lysozyme are indicated in blue, yellow and green, respectively. (C) Zn^{2+}-binding and amidase catalytic sites of MmePGRP-S1 (blue), -S2 (yellow) and -S3 (red) are shown. Mutated sites of PGRP-S3 are indicated by circles and residues are shown in parentheses. (D) Surface representation of MmePGRP-S1. The surface is color-coded according to the amino acid residues in the protein sequence. A cleft-like structure can be clearly visualized in this structural model. Critical amino acid residues responsible for Zn^{2+}-binding and amidase activity (marked in green) are located at the bottom of the cleft.

MmePGRP-S1 and -S2, only two amino acids (His and Thr) involved in Zn^{2+} binding and amidase activity were present (Fig. 2C–D).

To assess the evolutionary relatedness of PGRPs, a phylogenetic tree was constructed using sequences of PGRP domain from different insect species (Saitou & Nei, 1987). A close orthology was observed between MmePGRP-S1, -S2 and MdePGRP-S1, -S2, respectively (Fig. 3). Of special interest is MmePGRP-S1 which shares 86%, 60% and 61% amino acid sequences identity with MdePGRP-S1, AmePGRP-S1 and Nvi-PGRP-S1 peptides. Based on our phylogenetic analysis, MmePGRP-S3 also shares a 1 : 1 : 1 orthology with NviPGRP and AmePGRP-3 (Fig. 3). All the short PGRPs in these two clades harbor the key residues required for Zn^{2+} binding and amidase activity. On the other hand, all the three long MmePGRPs, are closely related to each other and are clustered together into one clade along with AmePGRP-LC. This similarity of the long MmePGRPs suggests a possible gene duplication event in *M. mediator*. Our phylogenetic tree gives an overall idea about the evolutionary position of the six newly characterized MmePGRPs in comparison to PGRPs from other related insects.

MmePGRP transcriptional expression during different developmental stages and under various pathogenic challenges

Compared to other insects, the parasitoid wasp *M. mediator* has a particular life cycle, spending the egg and the larval stages inside the host and subsisting off host nutrition. MmePGRP FPKM values from our RNAseq libraries for various developmental stages of wasp showed some interesting trends. The mRNA abundance of all the short MmePGRPs (-S1, -S2 and -S3) and long MmePGRP-L2 and L3 were relatively low in eggs and the early larval stages. MmePGRP-S2, -S3, -L2 and -L3 were induced only during the third instar larvae and finally peaking in the adults, where the expression level is significantly higher in comparison to other developmental stages (Fig. 4A). For MmePGRP-S1, no larval induction was observed, but like the above-mentioned MmePGRPs, it was

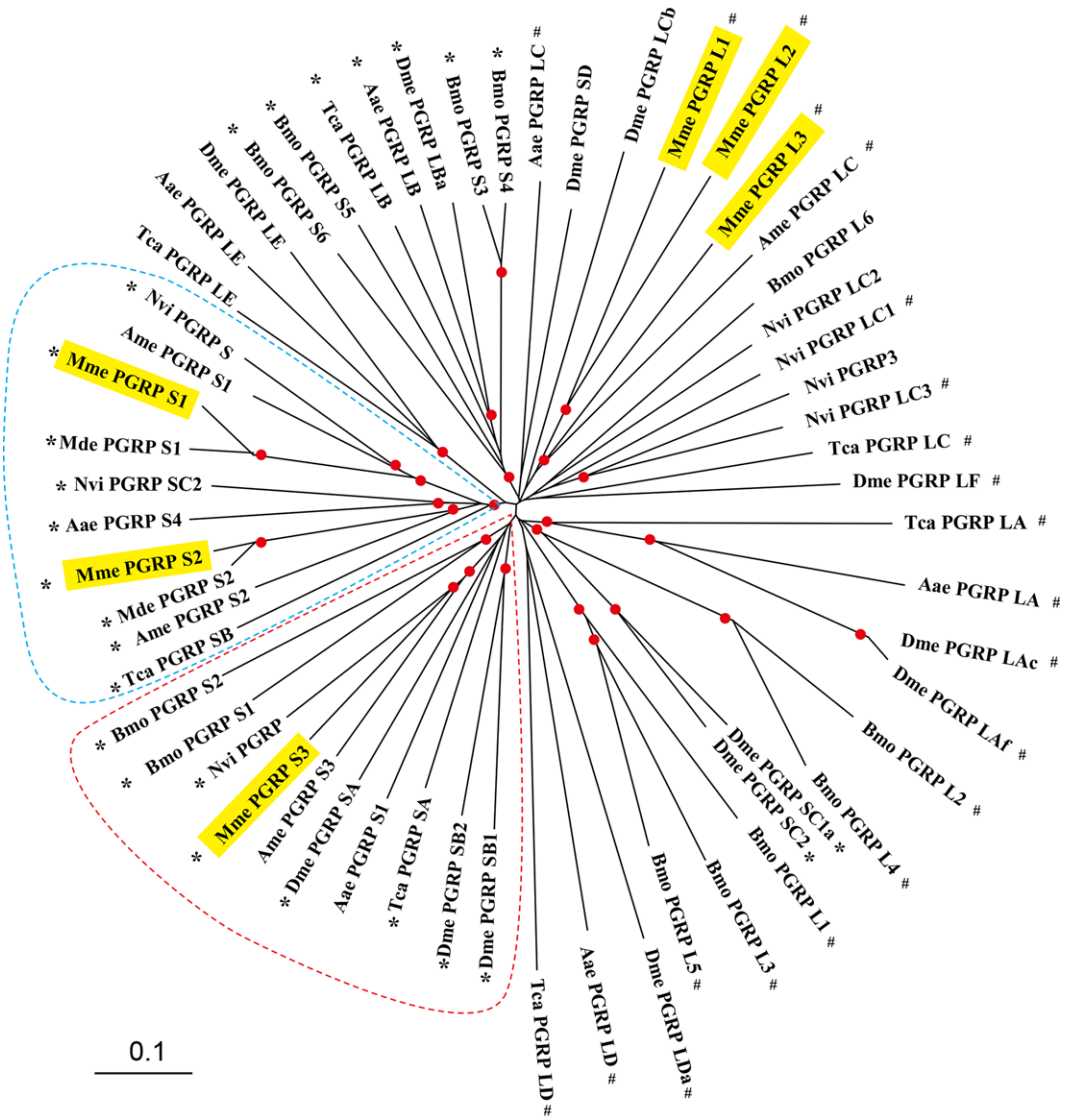

Fig. 3 Phylogenetic analysis of insect peptidoglycan recognition proteins (PGRPs). Amino acid sequences of six *Microplitis mediator* (Mme), two *M. demolitor*, 13 *Drosophila melanogaster* (Dme), seven *Tribolium castaneum* (Tca), 12 *Bombyx mori* (Bmo), seven *Aedes aegypti* (Aae), four *Apis mellifera* (Ame), and eight *Nasonia vitripennis* (Nvi) PGRPs were examined. Red spots at the nodes denote bootstrap values greater than 600 from 1000 trials. The group enclosed by the red dotted line includes candidates lacking the conserved amino acid Arg required for recognition of diaminopimelic acid (DAP)-type peptidoglycans (PGNs) except for DmePGRP-SB1. *, PGRP containing signal peptide; #, PGRP containing transmembrane domain.

remarkably up-regulated in the adult stage (Fig. 4A). The induction of MmePGRP-S1, -S2, -S3, -L2 and -L3 in adults indicates their role in wasp immunity at a stage when it is most vulnerable to pathogenic attack. In contrast, MmePGRP-L1 has a stage-specific response completely different from the rest of MmePGRPs. It is highly expressed in egg and early larval instar but not other developmental stages, including adults (Fig. 4A). This interesting observation suggests that *M. mediator* has evolved specific PGRPs for pathogen recognition during early and late developmental stages.

FPKM values of MmePGRPs from our pathogen-challenged RNAseq libraries revealed that various MmePGRPs responded differentially to bacterial and fungal infection. As a result of *E. cloacae* (Gram-negative bacterium) inoculation, an induction in the expression of all the short MmePGRPs were noted in at least one time point post-injection that we processed. The long

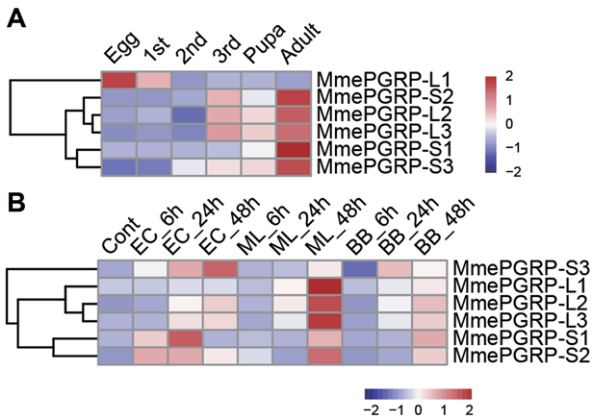

Fig. 4 RNA-Seq based transcriptomic analysis of *Microplitis mediator* peptidoglycan recognition proteins (MmePGRPs). (A) Expression profiles of MmePGRPs at various developmental stages from egg to pupae (1st–first larval stage, 2nd–second larval stage, 3rd–third larval stage). (B) Expression of MmePGRPs at different time points post-infection with either the Gram-positive bacterium *Micrococcus luteus*, Gram-negative bacteria *Enterobactor cloacae*, or fungus *Beauveria bassiana.* Time points are indicated along the *x*-axis. The level of expression is color-coded in a red/blue scheme provided to the right of each graph. MmePGRPs are clustered based on their expression trend.

MmePGRPs responded differentially to the infection, with -L2 and -L3 showing a mild induction and -L1 levels remaining unchanged (Fig. 4B). A clear and dramatic induction of all the MmePGRPs were observed 48 h post-*M. luteus* (Gram-positive bacterium) infection (Fig. 4B). However, in comparison to the level of induction of other MmePGRPs, the expression level of MmePGRP-S3 was moderate. As a result of *B. bassiana* (fungus) infection all the MmePGRPs except -L1 was activated in at least one time point assayed (Fig. 4B).

Next, we collected independent sample sets for qPCR validation of our RNAseq data on MmePRGR expression levels after pathogenic challenge. Our qPCR analysis of MmePGRP expression post pathogen challenge confirmed our results from RNAseq in general (Fig. 5, Fig. S3). MmePGRP-S1 and -S2 were significantly induced by all three pathogenic forms at one or more time points assayed (Fig. 5, top left and center). MmePGRP-S3 was mostly not effected by *E. cloacae* infection with some mild effect observed 24 and 48 h post -inoculation (Fig. 5, top right), indicating that MmePGRp-S3 might not be efficient enough in eradicating the infection. Of the long MmePGRPs, L2 and L3 are variably induced by all three pathogens at one time point post-injection (Fig. 5, bottom center and right). However, MmPGRP-L1 was only sensitive to *M. luteus* challenge with respect to mRNA levels (Fig. 5, bottom left). Taken together these results suggest important immune roles for MmePGRP-S1, -S2, -L2, -L3 against all kinds of pathogens, and a role of MmPGRP-L1 against Gram-positive bacteria.

MmePGRP-S1 plays a critical role to against Gram-positive bacteria

Our transcriptomic data and qPCR results indicated that the MmePGRP-S1 transcript is significantly up-regulated following infection with either *E. cloacae* or *M. luteus* or *B. bassiana*. To specifically evaluate the role of MmePGRP-S1 in immune response against microbial infection, dsRNA-mediated knockdown was performed. The efficiency of RNA interference (RNAi) was evaluated by measuring transcript levels of MmePGRP-S1 3d post-dsRNA injection. The RNA abundance of MmePGRP-S1 was significantly reduced in iPGRP-S1 insects, indicating a successful knockdown (Fig. S2).

Next, the survival of iPGRP-S1 wasps under *E. cloacae* or *M. luteus*, or *B. bassiana* challenge was assayed. No clear difference in the survival rates between control and iMmePGRP-S1 was observed post *E. cloacae* infection (Fig. 6A). By 60 h post-infection both the control and treatment showed a 100% mortality, indicating that the Gram-negative bacteria is able to overcome the host defense irrespective of the presence or absence of PGRP-S1. This result suggests that in wasps, PGRP-S1 is probably inactive against Gram-negative bacteria. However, a clear difference in the survivability of the iMmePGRP-S1 wasps were observed (40%) in comparison to iGFP controls (90%) 50 h post-*M. luteus* infection, indicating an immune role of MmePGRP-S1 against Gram-positive bacteria (Fig. 6B). No difference in the survival rates of iPGRP-S1 and control wasp groups was observed after *B. bassiana* infection, indicating that MmePGRP-S1 may not play a role in anti-fungal defense (Fig. 6C).

Therefore, MmePGRP-S1 most likely helps the wasp in detecting the bacterial pathogen, which presumably triggers the immune signaling pathway. The production of antimicrobial peptides (AMPs), which is controlled by the Toll pathway, could result in the effect we see in the survival rate (Fig. 6). To further support our hypothesis about the immune potency of PGRP-S1 against pathogens, we tested the effects of MmePGRP-S1 knockdown on the expression of AMP-defensin1, which is involved in defense against the infection of bacteria. Both iPGRP-S1 wasps and iGFP controls were challenged with *M. luteus*.

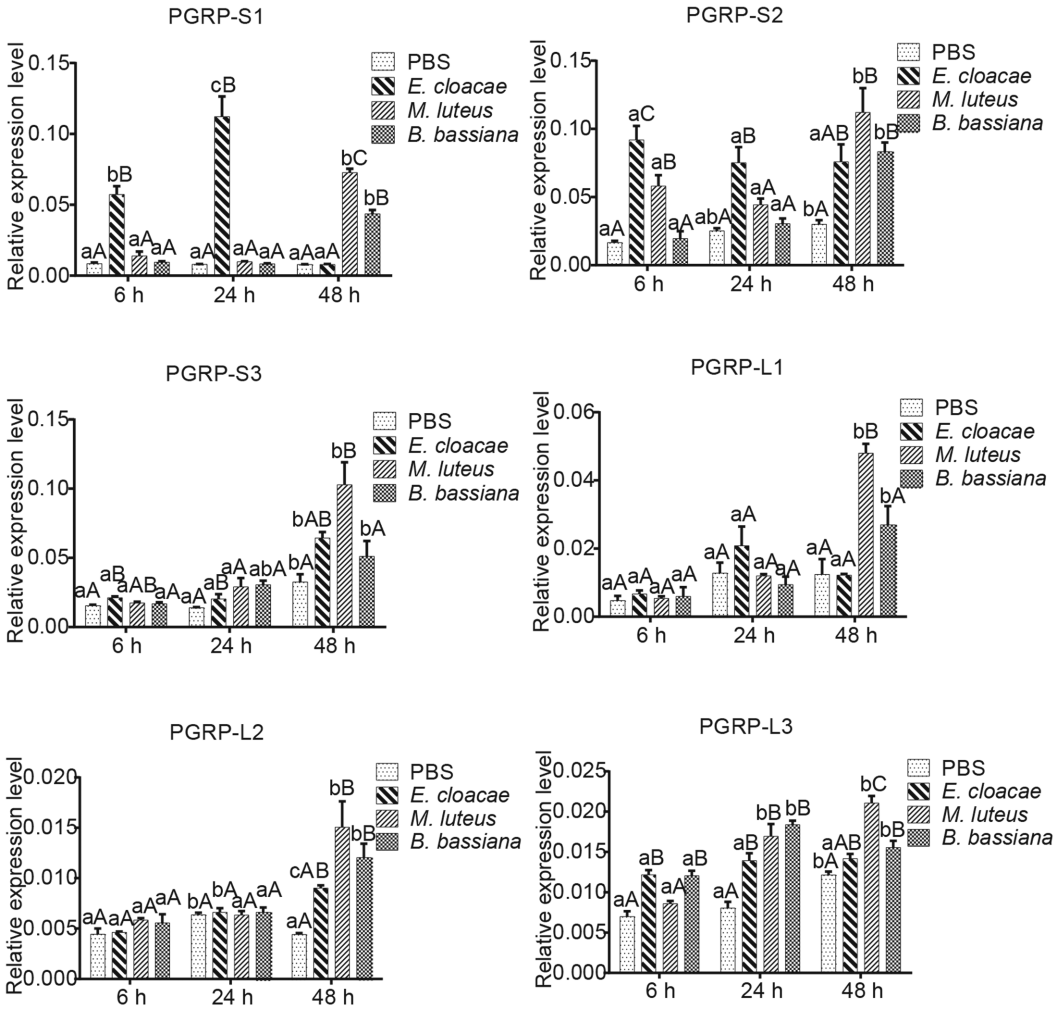

Fig. 5 Expression levels of *Microplitis mediator* peptidoglycan recognition proteins (MmePGRPs) messenger RNA (mRNA) following either Gram-negative (*Enterobactor cloacae*) or Gram-positive (*Micrococcus luteus*) bacterial challenge or fungal (*Beauveria bassiana*) infection as measured by quantitative polymerase chain reaction analysis. Time points along the *x* -axis represent the hours post-infection. *Actin* was used as a housekeeping gene. Error bars represent the means ± standard deviations (SD) from three replicates. The different lowercase letters (a–c) represented the significant difference at the different time points after infection with the same pathogen ($P < 0.05$), and the capital letters (A–D) indicate the significant difference at the same time points after different pathogenic infections ($P < 0.05$).

A clear reduction in the level of defensin1 was observed 48 h post-injection in iPGRP-S1 wasps as compared to the iGFP controls (Fig. 7).

Discussion

Hymenoptera comprises many beneficial insects, including the honey bee and parasitoid wasps. These insects are integral parts of the ecosystem functioning as effective pollinators and biocontrol agents for agricultural pests. However, ecological imbalances and the spread of pathogens endanger this economically and agriculturally important insect group. Drastic climate changes have significantly decreased parasitism, thereby increasing the frequency of herbivore outbreaks (Stireman *et al.*, 2005). A recently reported decline in honey bee colonies could be caused by either one or a combination of the following factors: environmental stress, viral attacks, pesticides and insect parasites (Nazzi & Pennacchio, 2014). The innate immune system of the insect is a potent defense

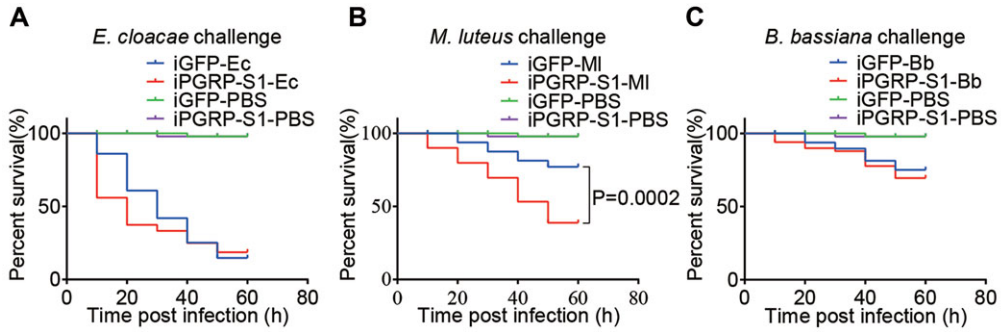

Fig. 6 Survival rates of *Microplitis mediator* peptidoglycan recognition protein (MmePGRP)-S1 depleted adult female *Microplitis mediator* insects variously challenged with *Enterobactor cloacae* (A), *Micrococcus luteus* (B) or *Beauveria bassiana* (C). Interdomain green fluorescent protein (iGFP) injected wasps infected with these pathogens were used as controls. Significant differences were observed in survivability of PGRP-S1 depleted wasps when infected with Gram-positive bacteria *M. luteus*. Some differences were observed between control and treatment 24 h post *E. cloacae* infection. iPGRP-S1, PGRP-S1 RNAi depleted wasps; iGFP, GFP RNAi treated control wasps. Data were analyzed using the Kaplan-Meier method.

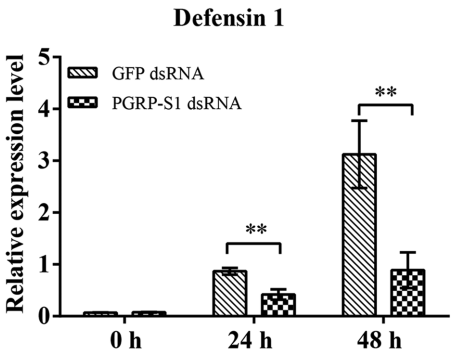

Fig. 7 The effect of *Microplitis mediator* peptidoglycan recognition protein (MmePGRP)-S1 on the expression of defensin1. The messenger RNA (mRNA) level of defensin1 was measured by quantitative polymerase chain reaction in MmePGRP-S1 depleted wasps infected by Gram-positive (*Micrococcus luteus*) bacterial challenge. Error bars represent the means ± standard deviations (SD) from three replicates. $*P < 0.05$, $**P < 0.01$.

mechanism against all of these threats. Thus, detailed studies on hymenopteran immunity are essential for developing novel approaches for the protection of this economically important insect group.

Activation of the innate immune response is mediated by recognition of pathogens by the PRRs. Insect PGRPs are the most versatile PRRs that have the ability to bind PGNs present on the surface of bacteria. *D. melanogaster* has 13 PGRP genes, encoding 20 transcript variants (Werner *et al.*, 2000). In this study, we have identified a total of six PGRP genes from *M. mediator* transcriptome. All short PGRPs (MmePGRP-S1-3) are secretory proteins containing a signal peptide. On the other hand, all long MmePGRPs harbor a TM domain but no signal peptide motif, indicating that they are membrane-bound proteins. The number of PGRP in *M. mediator* is higher than reported PGRPs from the social insect honey bee. It is hypothesized that the honey bee, being a social insect, depends more on colony level defense, thus reducing the burden on individual immune needs (Evans *et al.*, 2006). Vertebrates, on the other hand, also have a lower number of PGRP genes in their genome (4) due to the existence of a large array of other forms of membrane-bound or soluble PRRs.

The role of PGRPs in anti-microbial defense has been thoroughly studied across various insect species. *D. melanogaster* PGRP-SA, -SC1 and -SD are involved in the recognition of Gram-positive bacteria, leading to the activation of Toll pathway and ultimately the production of AMPs. Moreover this mechanism is conserved in other organisms. Among the long PGRPs, PGRP-LC, and -LE function synergistically in the recognition of Gram-negative bacteria, leading to activation of the IMD pathway (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002), whereas *D. melanogaster* PGRP-LB and -LF act as potentially negative regulators in the PGRP-LC-mediated IMD pathway (Zaidman-Remy *et al.*, 2006; Persson *et al.*, 2007; Maillet *et al.*, 2008). In addition to their roles in immune signaling pathways, some PGRPs are members of the PPO system and trigger the PPO-mediated host defense system (Yoshida *et al.*, 1996). In *D. melanogaster*, overexpression of DmePGRP-LE gives rise to spontaneous melanization (Takehana *et al.*, 2002). In this study, we have demonstrated that apart from sharing a close evolutionary relationship with other hymenopteran PGRPs, MmePGRPs have tertiary structures similar to those of

PGRP molecules with known functions. This study allows us to make assumptions about the possible immune function of *M. mediator* PGRPs, which deserves further investigation.

Some members of the PGRP superfamily, such as mammalian PGLYRP-2 and *D. melanogaster* PGRP-SC1, -SC2, -LB and -SB1, have amidase activity and thus the ability to degrade PGN. PGRP amidases are zinc-dependent enzymes, and the Zn^{2+} binding site along with the amidase catalytic sites are essential for amidase activity (Dziarski, 2004). PGRP-SC1 may be one of the factors that trigger phagocytosis of *Staphylococcus aureus* (Garver *et al.*, 2006). Additionally, two *H. armigera* PGRPs with amidase activity have been shown to agglutinate Gram-positive and -negative bacteria and inhibit the growth of these two bacteria in the presence of Zn^{2+} ion (Yang *et al.*, 2013). In the present study, well-conserved Zn^{2+}-binding and amidase catalytic sites were identified in MmePGRP-S1 and -S2. Furthermore, MmePGRP-S1 and -S2 shared a similar tertiary structure with that of bacteriophage T7 lysozyme, with high conservation of the motifs required for amidase activity. All of these characteristics suggest that MmePGRP-S1 and -S2 are members of the amidase-type PGRPs and are involved in the degradation of PGN during the immune response against microbial infection. There is a possibility that other MmePGRPs lacking amidase activity have lost critical catalytic residues over the course of evolution. On the other hand, all MmePGRPs, except for -S3, have the conserved Arg required for specific recognition of DAP-type peptidoglycans (Lim *et al.*, 2006), suggesting that these PGRPs are capable of DAP-type PGN recognition.

In this study, we investigated the mRNA expression profiles of six MmePGRPs throughout various developmental stages, including egg, larvae and pupae. None of the MmePGRP mRNAs, except that of -L1, were detected during the early larval stage, suggesting that the growth of *M. mediator* inside the host protects it from bacterial infection. We also characterized the expression of MmePGRPs as a result of immune challenge with two types of bacteria, *E. cloacae* and *M. luteus*, and a fungus, *B. bassiana*. *E. cloacae* or *M. luteus* infection resulted in increased mRNA abundance of both MmePGRP-S1 and -S2 transcripts, suggesting their importance in the immune response of *M. mediator* to Gram-positive and -negative bacteria. However, our detailed survival analysis of iMmePGRP-S1 wasps revealed that it plays an immune role only against Gram-positive bacteria. Furthermore, MmePGRP-S1 RNAi had significantly induced the expression of AMP-defensin1, which might play a role in conferring immunity to the wasp against *M. luteus* challenge. Thus, we have reported for the first time, a direct role of MmePGRP-S1 in combating Gram-positive bacteria. MmePGRP-S1 is expected to recognize PGN in the bacterial cell wall, and owing to the presence of residues required for amidase activity, may be involved in PGN degradation as well. Based on the existing details about the immune signaling pathways in other insects, we speculate that this immune action in *M. mediator*, particularly the induction of Def1, may be mediated by the Toll pathway. This hypothesis requires further validation in the future.

Acknowledgments

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Disclosure

The authors declare no conflict of interest.

References

Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, 124, 783–801.

Benkert, P., Biasini, M. and Schwede, T. (2011) Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics*, 27, 343–350.

Benkert, P., Kunzli, M. and Schwede, T. (2009) QMEAN server for protein model quality estimation. *Nucleic Acids Research*, 37, W510–514.

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T.G., Bertoni, M., Bordoli, L. and Schwede, T. (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research*, 42, W252–258.

Burke, G.R., Walden, K.K., Whitfield, J.B., Robertson, H.M. and Strand, M.R. (2014) Widespread genome reorganization of an obligate virus mutualist. *PLoS Genetics*, 10: e1004660.

Chang, C.I., Ihara, K., Chelliah, Y., Mengin-Lecreulx, D., Wakatsuki, S. and Deisenhofer, J. (2005) Structure of the ectodomain of *Drosophila* peptidoglycan-recognition protein LCa suggests a molecular mechanism for pattern recognition. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 10279–10284.

Choe, K.M., Werner, T., Stoven, S., Hultmark, D. and Anderson, K.V. (2002) Requirement for a peptidoglycan recognition

protein (PGRP) in relish activation and antibacterial immune responses in *Drosophila*. *Science*, 296, 359–362.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N.T., Hoffmann, J.A., Kanzok, S.M., Letunic, I., Levashina, E.A., Loukeris, T.G., Lycett, G., Meister, S., Michel, K., Moita, L.F., Muller, H.M., Osta, M.A., Paskewitz, S.M., Reichhart, J.M., Rzhetsky, A., Troxler, L., Vernick, K.D., Vlachou, D., Volz, J., von Mering, C., Xu, J.N., Zheng, L.B., Bork, P. and Kafatos, F.C. (2002) Immunity-related genes and gene families in *Anopheles gambiae*. *Science*, 298, 159–165.

Dong, W.X., Zhang, Z.N., Fang, Y.L., Zhang, F. and Kan, W. (2000) Response of parasitoid *Microplitis mediator* to plant volatiles in an olfactometer. *Insect Science*, 7, 344–350.

Dziarski, R. (2004) Peptidoglycan recognition proteins (PGRPs). *Molecular Immunology*, 40, 877–886.

Dziarski, R. and Gupta, D. (2006) The peptidoglycan recognition proteins (PGRPs). *Genome Biology*, 7, 232.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z. and Hultmark, D. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology*, 15, 645–656.

Garver, L.S., Wu, J. and Wu, L.P. (2006) The peptidoglycan recognition protein PGRP-SC1a is essential for Toll signaling and phagocytosis of *Staphylococcus aureus* in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 660–665.

Gerardo, N.M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S.M., de Vos, M., Duncan, E.J., Evans, J.D., Gabaldon, T., Ghanim, M., Heddi, A., Kaloshian, I., Latorre, A., Moya, A., Nakabachi, A., Parker, B.J., Perez-Brocal, V., Pignatelli, M., Rahbe, Y., Ramsey, J.S., Spragg, C.J., Tamames, J., Tamarit, D., Tamborindeguy, C., Vincent-Monegat, C. and Vilcinskas, A. (2010) Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. *Genome Biology*, 11, R21.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D. and Royet, J. (2002) The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature*, 416, 640–644.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q.D., Chen, Z.H., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N. and Regev, A. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, 29, 644–652.

Guan, R., Roychowdury, A., Ember, B., Kumar, S., Boons, G.J. and Mariuzza, R.A. (2005) Crystal structure of a peptidoglycan recognition protein (PGRP) in complex with a muramyl tripeptide from Gram-positive bacteria. *Journal of Endotoxin Research*, 11, 41–46.

Hetru, C. and Hoffmann, J.A. (2009) NF-kappa B in the immune response of *Drosophila*. *Cold Spring Harbor Perspectives in Biology*, 1: a000232.

Hultmark, D. (2003) *Drosophila* immunity: paths and patterns. *Current Opinion in Immunology*, 15, 12–19.

Janeway, C.A. and Medzhitov, R. (2002) Innate immune recognition. *Annual Review of Immunology*, 20, 197–216.

Jiang, H.B. (2008) The biochemical basis of antimicrobial responses in *Manduca sexta*. *Insect Science*, 15, 53–66.

Kajava, A.V., Klopffleisch, K., Chen, S. and Hofmann, K. (2014) Evolutionary link between metazoan RHIM motif and prion-forming domain of fungal heterokaryon incompatibility factor HET-s/HET-s. *Scientific Report*, 4, 7436.

Kang, D., Liu, G., Lundstrom, A., Gelius, E. and Steiner, H. (1998) A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 10078–10082.

Khajuria, C., Buschman, L.L., Chen, M.S., Zurek, L. and Zhu, K.Y. (2011) Characterization of six antibacterial response genes from the European corn borer (*Ostrinia nubilalis*) larval gut and their expression in response to bacterial challenge. *Journal of Insect Physiology*, 57, 345–355.

Kim, M.S., Byun, M. and Oh, B.H. (2003) Crystal structure of peptidoglycan recognition protein LB from *Drosophila melanogaster*. *Nature Immunology*, 4, 787–793.

Lemaitre, B. and Hoffmann, J. (2007) The host defense of *Drosophila melanogaster*. *Annual Review of Immunology*, 25, 697–743.

Leone, P., Bischoff, V., Kellenberger, C., Hetru, C., Royet, J. and Roussel, A. (2008) Crystal structure of *Drosophila* PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan. *Molecular Immunology*, 45, 2521–2530.

Li, B. and Dewey, C.N. (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, 12, 323.

Lim, J.H., Kim, M.S., Kim, H.E., Yano, T., Oshima, Y., Aggarwal, K., Goldman, W.E., Silverman, N., Kurata, S. and Oh, B.H. (2006) Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. *Journal of Biological Chemistry*, 281, 8286–8295.

Lu, Z.X., Zhu, P.Y., Gurr, G.M., Zheng, X.S., Read, D.M., Heong, K.L., Yang, Y.J. and Xu, H.X. (2014) Mechanisms for flowering plants to benefit arthropod natural enemies of insect pests: prospects for enhanced use in agriculture. *Insect Science*, 21, 1–12.

Maillet, F., Bischoff, V., Vignal, C., Hoffmann, J. and Royet, J. (2008) The *Drosophila* peptidoglycan recognition protein

PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. *Cell Host & Microbe*, 3, 293–303.

Nazzi, F. and Pennacchio, F. (2014) Disentangling multiple interactions in the hive ecosystem. *Trends in Parasitology*, 30, 556–61.

Persson, C., Oldenvi, S. and Steiner, H. (2007) Peptidoglycan recognition protein LF: a negative regulator of *Drosophila* immunity. *Insect Biochemistry and Molecular Biology*, 37, 1309–1316.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B. and Ezekowitz, R.A.B. (2002) Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. *Nature*, 416, 644–648.

Reiser, J.B., Teyton, L. and Wilson, I.A. (2004) Crystal structure of the *Drosophila* peptidoglycan recognition protein (PGRP)-SA at 1.56 A resolution. *Journal of Molecular Biology*, 340, 909–917.

Rosenthal, R.S. and Dziarski, R. (1994) Isolation of peptidoglycan and soluble peptidoglycan fragments. *Methods in Enzymology*, 235, 253–285.

Royet, J., Gupta, D. and Dziarski, R. (2011) Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nature Review Immunology*, 11, 837–851.

Saitou, N. and Nei, M. (1987) The Neighbor-Joining Method–a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406–425.

Schellhorn, N.A., Parry, H.R., Macfadyen, S., Wang, Y. and Zalucki, M.P. (2015) Connecting scales: Achieving in-field pest control from areawide and landscape ecology studies. *Insect Science*, 22, 35–51.

Stireman, J.O. III, Dyer, L.A., Janzen, D.H., Singer, M.S., Lill, J.T., Marquis, R.J., Ricklefs, R.E., Gentry, G.L., Hallwachs, W., Coley, P.D., Barone, J.A., Greeney, H. F., Connahs, H., Barbosa, P., Morais, H.C. and Diniz, I.R. (2005) Climatic unpredictability and parasitism of caterpillars: implications of global warming. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 17384–17387.

Strand, M.R. (2008) The insect cellular immune response. *Insect Science*, 15, 1–14.

Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki, T. and Kurata, S. (2002) Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 13705–13710.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882.

Wang, X.W. and Wang, J.X. (2013) Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. *Fish Shellfish Immunology*, 34, 981–989.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H. and Hultmark, D. (2000) A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 13772–13777.

Yang, D.Q., Su, Z.L., Qiao, C., Zhang, Z., Wang, J.L., Li, F. and Liu, X.S. (2013) Identification and characterization of two peptidoglycan recognition proteins with zinc-dependent antibacterial activity from the cotton bollworm, *Helicoverpa armigera*. *Developmental and Comparative Immunology*, 39, 343–351.

Yoshida, H., Kinoshita, K. and Ashida, M. (1996) Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. *Journal of Biological Chemistry*, 271, 13854–13860.

Yu, X.Q., Zhu, Y.F., Ma, C., Fabrick, J.A. and Kanost, M.R. (2002) Pattern recognition proteins in *Manduca sexta* plasma. *Insect Biochemistry and Molecular Biology*, 32, 1287–1293.

Zaidman-Remy, A., Herve, M., Poidevin, M., Pili-Floury, S., Kim, M.S., Blanot, D., Oh, B.H., Ueda, R., Mengin-Lecreulx, D. and Lemaitre, B. (2006) The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. *Immunity*, 24, 463–473.

Zou, Z., Evans, J.D., Lu, Z., Zhao, P., Williams, M., Sumathipala, N., Hetru, C., Hultmark, D. and Jiang, H. (2007) Comparative genomic analysis of the *Tribolium* immune system. *Genome Biology*, 8, R177.

Zou, Z., Shin, S.W., Alvarez, K.S., Kokoza, V. and Raikhel, A.S. (2010) Distinct melanization pathways in the mosquito *Aedes aegypti*. *Immunity*, 32, 41–53.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Predicated tertiary structures of MmePGRPs. (A–C) Predicated tertiary structures of short PGRPs: MmePGRP-S1 (A), MmePGRP-S2 (B) and MmePGRP-S3 (C). (D–F) Putative tertiary structures of long PGRPs, MmePGRP-L1 (D), MmePGRP-L2 (E) and MmePGRP-L3 (F). The final model is presented initially colored by model quality assigned using QMEAN. N represents the N-terminal extension.

Fig. S2 Confirmation of the MmePGRP-S1 RNAi silencing efficiency. The mRNA abundance of MmePGRP-

S1 abolished after MmePGRP-S1 dsRNA treatments in wasp. Control wasps were injected with GFP dsRNA. actin was used as an internal control of RT-PCR.

Fig. S3 Expression levels of MmePGRPs mRNA following either Gram-negative (*E. cloacae*) or Gram-positive (*M. luteus*) bacterial challenge or fungal (*B. bassiana*) infection as measured by qPCR analysis. A two-way ANOVA was used to determine the combined effects of infection and time. The different lowercase letters (a–d) represent the significant difference at the group of different infection. Data are expressed as mean ± SD.

Table S1 FPKM values of MmePGRPs from 15 transcriptomes.

Table S2 Primer sequences used in gene cloning, qPCR, and dsRNA synthesis.

Table S3 Deduced amino acid sequences of MmePGRPs.

Table S4 Percentages of identity of MmePGRPs amino acid sequences with their orthologs from *D. melanogaster* and *A. mellifera*.