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RESEARCH

Peptidoglycan Recognition Protein S2 From Silkworm Integument: Characterization, Microbe-Induced Expression, and Involvement in the Immune-Deficiency Pathway

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ABSTRACT. Peptidoglycan recognition protein (PGRP) binds specifically to peptidoglycan and plays an important role as a pattern recognition receptor in the innate immunity of insects. The cDNA of a short-type PGRP, an open reading frame of 588 bp encoding a polypeptide of 196 amino acids, was cloned from *Bombyx mori*. A phylogenetic tree was constructed, and the results showed that *BmPGRP-S2* was most similar to *Drosophila melanogaster PGRP* (*DmPGRP-SA*). The induced expression profile of *BmPGRP-S2* in healthy *Escherichia coli*- and *Bacillus subtilis*-challenged *B. mori* was measured using semiquantitative reverse transcriptase polymerase chain reaction analysis. The expression of *BmPGRP-S2* was upregulated at 24 h by *E. coli* and *Ba. subtilis* challenge. In addition, in the integument of *B. mori*, RNAi knockdown of *BmPGRP-S2* caused an obvious reduction in the transcription expression of the transcription factor *Relish* and in antibacterial effector genes *Attacin*, *Gloverin*, and *Moricin*. The results indicated that *BmPGRP-S2* participates in the signal transduction pathway of *B. mori*.

Key Words: *Bombyx mori*, innate immunity, peptidoglycan recognition protein, RNA interference

Insects are the most abundant species on the earth and can combat a variety of pathogens mainly through a sophisticated innate immune system. The insect defense system consists of three major parts: structural barriers and cellular and humoral immune responses (Lemaitre and Hoffmann 2007). Structural barriers, the first protective lines of defense, comprise the cuticle, midgut epithelium, and trachea. Cellular immune responses involve phagocytosis, nodulation, and encapsulation rather than antibodies and are mediated by several types of hemocytes (Lavine and Strand 2002, Sideri et al. 2007). Humoral immune responses include the melanization of the hemolymph and secretion of antimicrobial peptides (AMPs) (Hoffmann 1995). A characteristic of insect immunity is the rapid and transient activation of immune genes to produce effectors in response to microbial infection. When microorganisms penetrate the hemocoel, they are first recognized by factors for cellular and humoral immune reactions (Andrew and Powell 2007). Next, modulating and signaling factors are stimulated, and signals are transduced in specific tissues (Akria et al. 2001). Genes that encode effectors are activated through signaling cascades, which produce a battery of these molecules in specific tissues, and secreted into the hemolymph (Wang et al. 2010).

Peptidoglycan recognition proteins (PGRPs) have been shown to play a central role in insect immunity by recognizing invading microorganisms (Takeda and Akira 2005, Anselme et al. 2006). PGRPs were isolated as a recognition protein for peptidoglycan (PGN), which could activate the prophenoloxidase cascade (Yoshida et al. 1996), and the cDNA of the gene encoding this protein was cloned (Ochiai and Ashida 1999). The first PGRP identified by that study (Ochiai and Ashida 1999) was designated *Bombyx mori* (Bm) PGRP-S1. The homology search showed that PGRP is a protein homologous to bacteriophage T7 lysozyme, in which Cys-130 is essential for N-acetylmuramoyl-L-ala amidase (NAMLAA) activity. NAMLAA is an enzyme that cleaves the lactyl–amide bond between muramic acid and the peptide chain in PGN (Gelius et al. 2003, Kim et al. 2003, Mellroth et al. 2003, Wang et al. 2003). Mutant forms of PGRP that lack a potential zinc ligand are enzymatically inactive but retain their PGN affinity (Kaneko et al. 2004, Steiner 2004, Royet et al. 2005).

Genome-wide analysis showed that *B. mori* has 12 distinct PGRP genes with conserved PGRP domains (Tanaka et al. 2008). Six of them belong to the short (S) subfamily, which is mainly secreted proteins, and six belong to the long (L) subfamily, mainly transmembrane or intracellular proteins. The biological functions of the short and long PGRP subfamilies might not be the same (Guan et al. 2004, Dziarski and Gupta 2006). Chen et al. (2014) suggested that *B. mori PGRP-S5* functions as a pattern recognition receptor for the initiation of the prophenoloxidase pathway and as an effecter to inhibit bacterial growth; however, the integumentary immune system involving *B. mori PGRP-S5* is still not known.

AMPs, the major and best-known immune effectors induced by infection, are synthesized by the fat body, hemocytes, and other tissues and are regulated by the Toll and immune deficiency (IMD) pathways (Bulet et al. 1999, Lemaitre and Hoffmann 2007). At least 150 insect AMPs have been purified or identified. Most are small and cationic and show activities against bacteria or fungi (Hoffmann 1995, Bulet and Stocklin 2005). Based on the sequences, structures, and activities, insect AMPs can be classified into four families: the α-helical peptides (e.g., cecropin, moricin, and sarcotoxin), cysteine-rich peptides (e.g., insect defensin, drosomycin, and heliomicin), proline-rich peptides (e.g., apidaecin, drosocin, and lebocin), and glycine-rich peptides (e.g., attacin and gloverin) (Laszlo-Otvos 2000, Bulet and Stocklin 2005). In *B. mori*, four gloverin genes (*Bmglv1–4*) have been identified, and *Bmglv2–4* are derived from duplicating *Bmglv1* (Cheng et al. 2006, Kaneko et al. 2007, Kawaoka et al. 2008, Mrinal and Nagaraju 2008). In 1995, moricin, a 42-amino-acid peptide, was initially isolated from the hemolymph of *B. mori* (Hara and Yamakawa 1995a). This peptide is highly basic, and its amino acid sequence has no significant similarity to the sequences of other AMPs (Hara and Yamakawa 1995b). Moricin-like AMPs have been exclusively found in Lepidoptera (Oizumi et al. 2005). It has also been reported that moricin has antibacterial properties against Gram-negative and Gram-positive bacteria (Hara and Yamakawa 1995a; Sato and Feix 2006).

Studies on the systematic immunity of *Drosophila melanogaster* found that Gram-positive bacteria can activate the Toll signaling pathway by secreting members of the PGRP family, such as PGRP-SA and

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SD (Gottar et al. 2002, Kaisho and Akira 2001, Ranjiv et al. 2002), whereas the IMD pathway is activated by membrane-bound or intracellular receptors, such as PGRP-LC and LE (Govind 2008). In *Drosophila*, three mammalian Rel/NF-κB homologs that control antibacterial and antifungal peptide genes have been identified. Two Rel/NF-κB proteins, dorsal (Steward 1987) and dorsal-related immunity factor (Dif) (Ip et al. 1993), are activated by the Toll pathway in response to infection with fungi and Gram-positive bacteria. These factors are localized in the cytoplasm and interact with the mammalian IκB homolog, Cactus, in unstimulated cells. In response to an infection, Dif and Dorsal translocate into the nucleus by dissociating from Cactus and activate antifungal peptide genes, such as *drosomycin* (Lemaitre et al. 1997). Another Rel/NF-κB protein, Relish, is activated by the IMD pathway in response to infection by Gram-negative bacteria (Dushay et al. 1996, Hedengren et al. 1999). Relish comprises the N-terminal Rel homology domain (RHD) and C-terminal ankyrin repeat domain. An N-terminal fragment, including RHD of Relish, is released by endoproteolytic cleavage in response to bacterial infection and translocates from the cytoplasm to the nucleus, activating antibacterial peptide genes, such as *diptericin* (Stoven et al. 2000). Recently, several Rel/NF-κB homologs from other insects have been cloned and characterized. Insect Rel/NF-κB protein can be categorized into two types on the basis of structural features: Dif-Dorsal or Relish.

Tzou et al. (2000) showed that all seven *Drosophila* AMPs could be induced in surface epithelia in a tissue-specific manner and that *imd* played a critical role in the activation of this local response to infection. In particular, *drosomycin* expression, which was regulated by the Toll pathway during the systemic response, was regulated by *imd* in the respiratory tract, thus demonstrating the existence of distinct regulatory mechanisms for local and systemic induction of AMP genes in *Drosophila*.

In this study, the complete open reading frame (ORF) of *BmPGRP-S2* was successfully cloned. A phylogenetic tree was constructed, and the results showed that *BmPGRP-S2* was most similar to *DmPGRP-SA*. The induced expression profile of *BmPGRP-S2* in healthy, *Escherichia coli*-, *Bacillus subtilis*-, and *Saccharomyces cerevisiae*-challenged *B. mori* was measured by semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis. The expression of *BmPGRP-S2* was upregulated at 24 h by *E. coli*, *Ba. subtilis*, or *S. cerevisiae* challenge in the head, integument, and midgut of *B. mori*. In addition, RNAi knockdown of *BmPGRP-S2* caused an obvious reduction in the Relish and *Attacin*, *Gloverin*, and *Moricin*. The results indicate that *BmPGRP-S2* has an effect on the transcription of antibacterial peptide genes and participates in the signal transduction pathway in *B. mori*.

Materials and Methods

Bombyx mori. The *B. mori* variety, Jingsong × Haoyue, was provided by the department of pathology and physiology at the Sericultural Research Institute, Chinese Academy of Agricultural Sciences at Zhenjiang city of Jiangsu province, China. *E. coli* and *Ba. subtilis* were available at our laboratory.

Cloning the ORF of BmPGRP-S2. RNA was extracted from the integument of *B. mori* larvae (third day of fifth instar) 24 h after injection with 10 µl *Ba. subtilis* (1.0×10^9 cells). First-strand cDNA was synthesized using oligo dT primers (TaKaRa Biotechnology Co., Ltd., Dalian, China). Reverse transcription was performed according to the manufacturer's instructions using M-MLV reverse transcriptase (TaKaRa Biotechnology Co., Ltd., Dalian, China) with total RNA as a template.

The sequences of the two primers, 5′-ATGTTGGTTGCACCGTCTC-3′ (forward) and 5′-AACCCAGTACTTTTCGAGCA-3′ (reverse) were selected to amplify the ORF of *BmPGRP-S2*. Semiquantitative RT-PCR was carried out in a 25-µl reaction volume containing 2.5 µl 10 × PCR buffer, 4.0 µl dNTP, 1.0 µl each primer, 0.25 µl *Taq* polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China), and 2.0 µl cDNA mix. PCR was initiated with a denaturation step at 94°C for 3.0 min, followed by 30 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 1.0 min), and a final step at 72°C for 10 min. The PCR produced was separated using 1.0% agarose gel, purified from the gel and ligated into a pMD18-T (TaKaRa Biotechnology Co., Ltd., Dalian, China) vector, and transformed into *E. coli* DH10B using the standard heat-shock method. The sequence of the inserted cDNA was confirmed by DNA sequencing.

Sequence and Structure Analysis of the ORF of BmPGRP-S2. Sequence alignment and phylogenetic analyses were performed using ClustalX1.8 and MEGA4.0. Domain analysis of the retrieved protein sequences was carried out using Pfam (http://www/sanger.ac.uk/Software/Pfam/) and SMART (http://smart.embl-heidelberg.de/). Signal peptides and transmembrane domains were analyzed using the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Induced Expression Analysis by Semiquantitative RT-PCR. Semiquantitative RT-PCR of the mRNA extracted from *B. mori* larvae was conducted by extracting total RNA from the integument of third-day fifth-instar larvae 24 h after injection with 5.0 µl ddH_2O (control), 5.0 µl *E. coli* (2.8×10^8 cells/ml), 5.0 µl *Ba. subtilis* (10×10^8 cells/ml), and 5.0 µl *S. cerevisiae* (6.5×10^8 cells/ml) and was performed according to the manufacturer's instructions.

The semiquantitative RT-PCR method was the same as that used in the above-mentioned cloning method.

RNA Interference. For RNAi, dsRNAs were generated using the T7 RiboMAX Express RNAi System (Promega Corp., Madison, WI) according to the manufacturer's instructions. For the production of the templates, the ORF of *BmPGRP-S2* was amplified using two primers with a T7 promoter sequence at the 5′ ends (S2-T7-F1, GGATCC TAATACGACTCACTATAGGATGTTGGTTGCACCGTCTC; S2-T7-R1: GGATCCTAATACGACTCACTATAGGAACCCAGTACT TTTCGAGCA). PCR was performed under the following conditions: 94°C for 3.0 min, followed by 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.0 min; another 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1.0 min; and a final step at 72°C for 10 min.

Newly exuviated (day 0) fifth-stage larvae were used for the injection experiment. Larvae were injected with 5.0 µg ds-RNA specific for *BmPGRP-S2* and 6.0 h later with 5.0 µl *E. coli* (7×10^8 cells/ml), 5.0 µl *Ba. subtilis* (5.2×10^8 cells/ml), and 5.0 µl *S. cerevisiae* (6×10^8 cells/ml). Controls involving 5.0 µl phosphate-buffered saline (PBS) without dsRNA in the primary injections and the corresponding microorganism in the secondary injections were also used. After microorganism injection, the treated insects were held at 28°C for 6.0 h and then dissected as before to isolate the integument. RT-PCR using extracted RNA as a template, and a suitable pair of primers was performed to determine the mRNA transcription patterns of each gene.

Primers used were as follows: 5′-GGCTCGCTCTAGACAACG TA-3′ (forward primer for *BmAttacin*), 5′-ACATTGGGCTCCCACG AAG3′ (reverse primer for *BmAttacin*), 5′-TACTCGATCAGCGGGC AATC-3′ (forward primer for *BmGloverin*), 5′-GACCATACCACC GGCAGAAA-3′ (reverse primer for *BmGloverin*), 5′-GCAAAAACA GTAAACCGCGCA-3′ (forward primer for *BmMoricin*), 5′-CACAGT TGTCGTTGCAATCAAT-3′ (reverse primer for *BmMoricin*), 5′-TAG GATGTTGGTCATCGCCG-3′ (forward primer for *BmCactus*), 5′-CA GACTCTCGTTGTCGCTGT-3′ (reverse primer for *BmCactus*), 5′-CA GATGCGAACGATACCGGA-3′ (forward primer for *BmRel*), 5′-TCG CTTGTGGAGAGCTTGTT-3′ (reverse primer for *BmRel*), 5′-TACAA AGTCGGGTTCGGTGG-3′ (forward primer for *BmRelish*), and 5′-TG AAACTCCCGTTAGCCGTC-3′ (reverse primer for *BmRelish*).

Semiquantitative RT-PCR was conducted under the following conditions: initial denaturation at 94°C for 3.0 min, amplification for 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1.0 min, followed by a final extension at 72°C for 10 min. The resulting amplified cDNA was electrophoresed by agarose gel electrophoresis.

Results

Nucleotide and the Deduced Amino Acid Sequence of BmPGRP-S2. ORF of *BmPGRP-S2* was deposited in GenBank under accession number KF906541. The *BmPGRP-S2* nucleotide and deduced amino acid sequences are shown in Fig. 1.

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Fig. 1. Nucleotide and the deduced amino acid sequences of the encoding region of *BmPGRP-S2*. The signal peptide is underlined, the PGRP domain is gray, the Ami_2 domain is boxed, the star is the stop codon, the initiation codon is double underlined, and there is a different base between the results of cloning and the draft sequence of the *Bombyx* genome, which is indicated by a circle.

ORF of *BmPGRP-S2* was 588 bp encoding a polypeptide of 196 amino acids with an estimated molecular mass of 21.47 kDa and a predicted isoelectric point of 5.35. There were a signal peptide (1–21 amino acids), a PGRP domain (25–167 amino acids; $E = 2.23e\text{-}52$), and an Ami 2 domain (36–173 amino acids; $E = 2.34e\text{-}18$). The hydrophobic and hydrophilic regions of the protein amino acid sequence were staggered. *BmPGRP-S2* had no transmembrane domains, which suggested that this protein might be a secreted protein. The conserved PGRP and Ami 2 domains were involved in the metabolic process of PGN catabolism and with NAMLAA activity. This suggested that *BmPGRP-S2* might destruct the cellular function through hydrolyzing the PGN bridge chain, as with the T7 lysozyme.

An analysis performed by comparing the sequence of the cloned fragment with the corresponding draft sequence of the *Bombyx* genome showed that there was one difference in the first base encoding the 46th amino acid of the gene, which was changed from adenine to guanine; the amino acid encoded was changed from serine to proline accordingly, as indicated in Fig. 1 (circle).

Homologous and Phylogenetic Character of BmPGRP-S2. Basic Local Alignment Search Tool (BLAST) analysis revealed that the deduced amino acid sequence of BmPGRP-S2 was highly similar to BTL-LP2, bacteriophage T7 lysozyme-like protein 2 of *B. mori*. The PGRP family was related to the PGN-degrading bacteriophage T7 lysozyme. In the T7 lysozyme, His-17, His-122, and Cys-130 are Zn^{2+}-binding ligands, and Tyr-46 and Lys-128 are needed for enzymatic activity. Site-directed mutagenesis of T7 lysozyme has shown that activity is retained when Lys-128 is replaced by Thr. The latter amino acid residue, as well as the other four residues needed for T7 lysozyme activity, was found in BmPGRP-S2, BmPGRP-S1, and BTL-LP2. The results are shown in Fig. 2.

A phylogenetic tree (Fig. 3) was constructed using the neighbor-joining method with a 1,000 bootstrap test based on the multiple alignments of BmPGRP-S2 and PGRPs from *D. melanogaster*. Compared with *D. melanogaster*, BmPGRP-S2 was most similar to DmPGRP-SA. Because *D. melanogaster DmPGRP-SA* was inducible after infection, we questioned whether *BmPGRP-S2* is also inducible after infection. The microbe-induced expression of *BmPGRP-S2* was then conducted as follows.

***BmPGRP-S2* is Upregulated in Response to Infection.** The expression profile of *BmPGRP-S2* in the integument of *B. mori* after microorganism injection is shown in Fig. 4. Both *Ba. subtilis* and *E. coli* could induce the upregulation of *BmPGRP-S2* compared with the insects injected with ddH_2O in the control group. Twenty-four hours after injection, *BmPGRP-S2* expression was significantly upregulated in the *Ba. subtilis*-injected group compared with that of the *E. coli*-injected group. This suggested that *BmPGRP-S2* was participating in *B. mori* immunity to a bacteria challenge.

Silencing BmPGRP-S2 Expression in B. mori Results in a Significant Reduction of E. coli- and Ba. subtilis-Induced Expression of AMPs and Transcription Factor Relish in Integument. To demonstrate that RNAi of *BmPGRP-S2* could knock down the microorganism-induced expression of potential effectors, insects were pretreated with ds-RNA specific for *BmPGRP-S2*. Following RNAi, injection of *E. coli* could detect the expression of genes *Relish*, *Attacin*, *Gloverin*, and *Moricin*; however, the previous injection of ds-RNA specific for *BmPGRP-S2* led to a substantial reduction in Relish and in *Attacin*, *Gloverin*, and *Moricin*. Similarly, *Ba. subtilis* injection detected the expression of *Relish* and of *Attacin*, *Gloverin*, and *Moricin*, which, in turn, could be knocked down by previous injection with the specific ds-RNA for *BmPGRP-S2*. The transcription expression of the transcription

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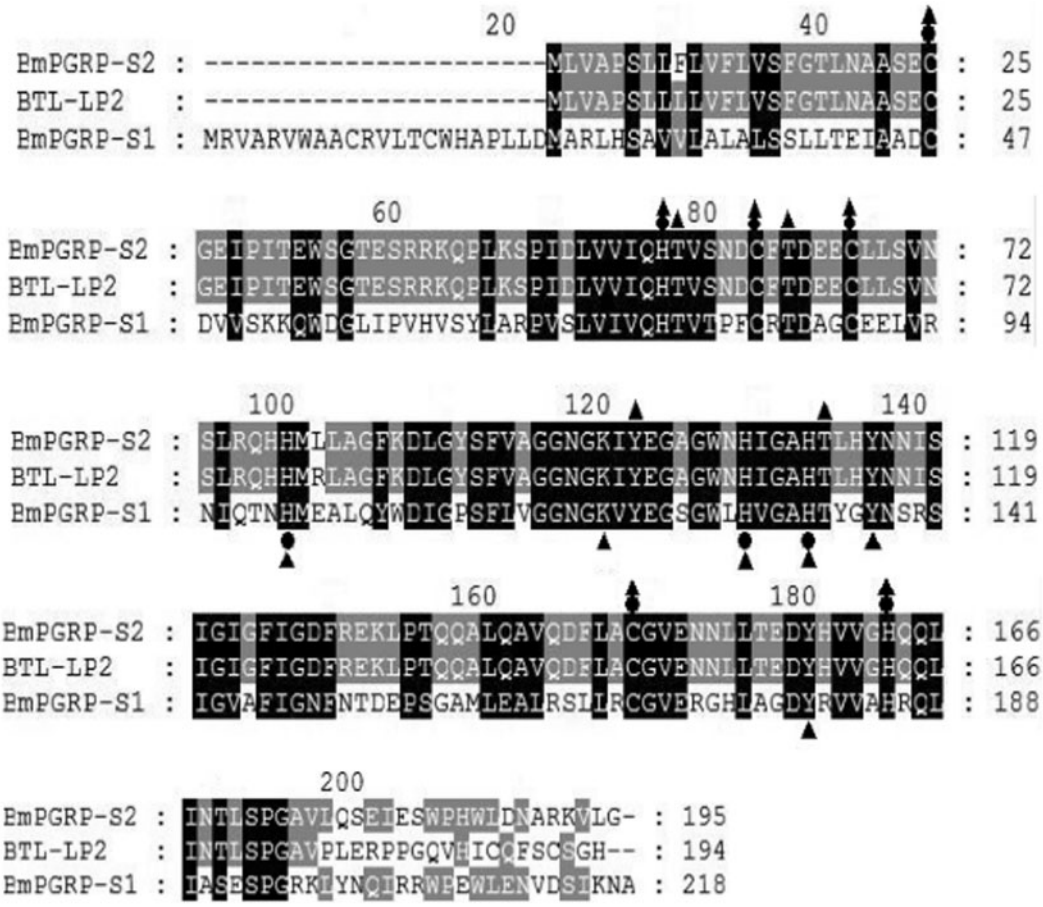

Fig. 2. Multisequence alignment of BmPGRP-S1 (NP_001036836) and BmPGRP-S2 with BTL-LP2, *B. mori* mRNA for bacteriophage T7 lysozyme-like protein 2 (BAB33295). Amino acid residues that are conserved sequences are shaded dark, and similar amino acids are shaded gray. Zn^{2+} binding sites and amidase catalytic sites are marked with a circle and triangle, respectively.

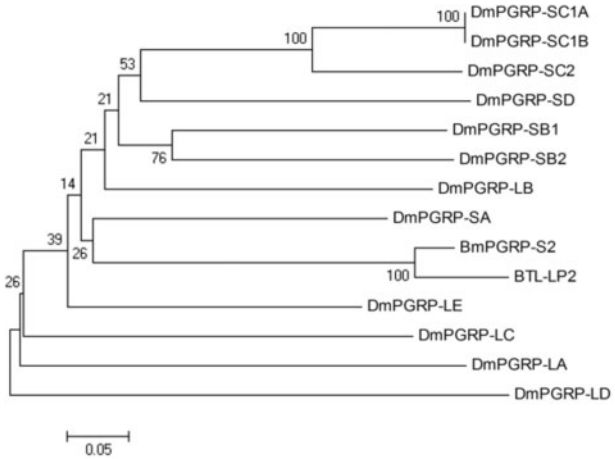

Fig. 3. Phylogenetic tree of BmPGRP-S2 with PGRPs from *Drosophila*. The tree is constructed by the neighbor-joining (NJ) algorithm using Mega 4.0 based on the multiple-sequence alignment by ClustalW. The numbers above the branch represent bootstrap percentages. The topology was tested using bootstrap analyses (500 replicates). The protein sequences used for phylogenetic analysis were as follows: DmPGRP-SA (AAF48056.1), DmPGRP-SB1 (CAD89138.1), DmPGRP-SB2 (CAD89150.1), DmPGRP-SC1A (CAD89163.1), DmPGRP-SC1B (CAD89174.1), DmPGRP-SC2 (CAD89187.1), DmPGRP-SD (CAD89 193.1), DmPGRP-LA (AGB94318.1), DmPGRP-LB (AFH06372.1), DmPGRP-LC (AFH04364.1), DmPGRP-LD (AAO41277.1), DmPGRP-LE (AAG32064.1), and BTL-LP2 (BAB33295.1).

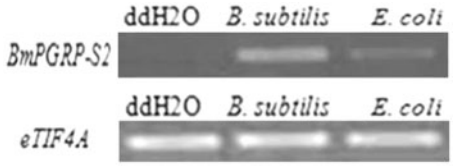

Fig. 4. Upregulation of BmPGRP-S2 mRNA as determined by RT-PCR 24 h after injection of *Ba. subtilis* and *E. coli* into *B. mori* larvae on day 3 of the fifth instar compared with the ddH2O-injected control. *eTIF4A* was used as an internal control gene.

factors *Rel* and *Cactus* (data not shown) was not detected, which indicated that *BmPGRP-S2* has an effect on the expression of Relish and on *Attacin*, *Gloverin*, and *Moricin*. Thus, microorganisms induce the over-transcription of immune response genes in *B. mori*, and this can be specifically knocked down by RNAi of *BmPGRP-S2*.

Discussion

In this study, *BmPGRP-S2* was found to play an important role in the innate immunity of *B. mori*. Analysis on the induced expression of *BmPGRP-S2* showed that *BmPGRP-S2* transcription was upregulated significantly in *B. mori* integument after bacterial infection. By using RNA interference, *BmPGRP-S2* was shown to regulate the expression of AMPs and participate in the activation of the IMD signal transduction pathway in *B. mori* integument.

Drosophila PGRP-SA circulates in the hemolymph, detects the Lys-type PGs of most Gram-positive bacteria, and activates a serine protease cascade to induce AMP gene transcription through the Toll

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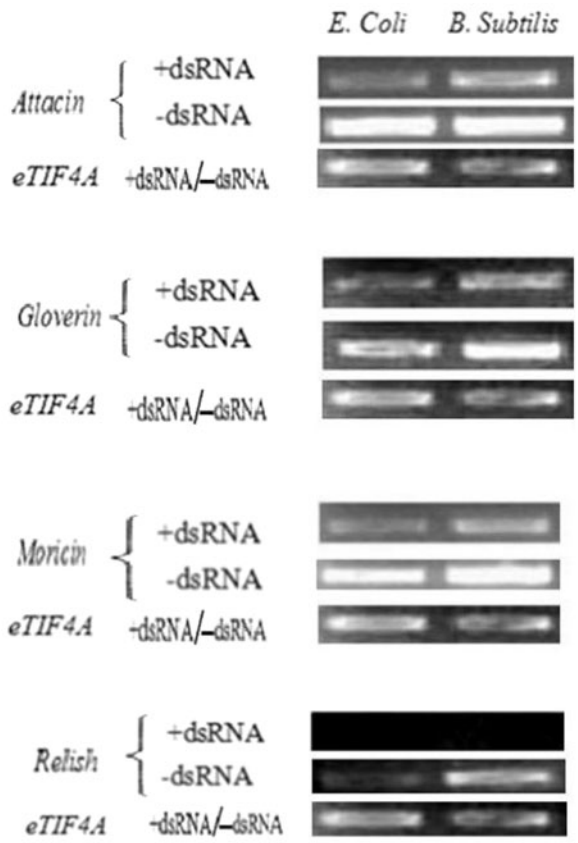

Fig. 5. Downregulation of Relish (B) and of the AMP genes *Attacin*, *Gloverin*, and *Moricin* (C) by RNAi knockdown of BmPGRP-S2 (A). Newly exuviated fifth-instar larvae were used for the experiment. The mRNA levels of the genes in the integument of silkworms were determined by RT-PCR. +dsRNA, *BmPGRP-S2* ds-RNA injection; -dsRNA, no *BmPGRP-S2* ds-RNA injection; *eTIF4A* was used as an internal control gene.

pathway in the fat body (Michel et al. 2001, Bischoff et al. 2004, Charroux et al. 2009). In our study, *BmPGRP-S2* expression in the *B. mori* integument was significantly upregulated after injection with *Ba. subtilis*, compared with that after injection with *E. coli*. As an important pathogen-associated molecular pattern, PGN is an essential cell wall component of nearly all bacteria, especially Gram-positive bacteria (Doyle and Dzianski 2001). Gram-positive and Gram-negative PGNs show subtle but important differences in chemical structure and localization. The Gram-positive PGN (Lys-type) is multilayered and exposed on the surface of the bacteria, whereas the Gram-negative PGN (DAP-type) is single layered and found within the periplasmic space under the outer membrane of the bacteria. Both of these differences are considered to be important in the specificity of pathogen recognition. They indicate that the cell wall component of microorganisms has an effect on *BmPGRP-S2* expression.

In *Drosophila*, the Rel/NF-κB protein, Relish, is activated by the IMD pathway in response to infection with Gram-negative bacteria (Dushay et al. 1996, Hedengren et al. 1999). Several AMP genes such as *Attacin* (M. Sugiyama et al. 1995) and *Moricin* (Furukawa et al. 1999) have been identified from *B. mori*. These genes contain conserved κB motifs in the 5′-upstream region, and nuclear factors that bind to κB motif were found by electrophoresis mobility shift assay (Taniai et al. 1995), suggesting that the Rel/NF-κB homologs also control AMP genes in *B. mori*. In addition, Furukawa et al. (2009) showed that BmCactus was involved in the Toll signal transduction pathway in *B. mori*. In our study, the injection of *E. coli* led to overexpression of Relish and of *Attacin*, *Gloverin*, and *Moricin* in the integument of *B. mori*; however, previous injection of ds-RNA specific for *BmPGRP-S2* led to a greater reduction in Relish and in *Attacin*, *Gloverin*, and *Moricin*. In addition, injection of *E. coli* showed no detectable effect on the transcription expression of the transcription factors *Rel* and *Cactus*. Taken together, this indicated that *BmPGRP-S2* participated in the activation of the IMD signal transduction pathway and had an effect on the transcription of an antibacterial peptide gene in *B. mori*.

Davis et al. (2012) showed that in *Drosophila*, the IMD pathway was required for the expression of larval epidermal AMP. Similarly, in our experiment, *BmPGRP-S2* regulated the expression of AMPs and participated in the activation of the IMD signal transduction pathway in *B. mori* integument.

Tanaka et al. (2008) showed that the expression of *BmPGRP-S2* in the fat body of *B. mori* was strongly stimulated by *E. coli* (Gram-negative bacteria) but not at all by *Staphylococcus aureus* (Gram-positive bacteria). In our experiment, *BmPGRP-S2* expression was enriched in *B. mori* integument after *Ba. subtilis* infection. These results indicated that *BmPGRP-S2* expression is differentially regulated in different tissues after infection by different microorganisms.

Chen et al. (2014) suggested that *B. mori* PGRP-S5 serves as a receptor for PGN, leading to the proPO activation pathway, and might act as an antimicrobial protein as well. Whether it is the receptor for the AMP gene induction pathway remains unknown and needs further investigation. In our experiment, injection of *E. coli* could detect the expression of Relish and AMP genes *Attacin*, *Gloverin*, and *Moricin*, compared with insects injected with PBS, from which RT-PCR detected none of these mRNAs (data not shown); however, previous injection of ds-RNA specific for *BmPGRP-S2* led to a great reduction of Relish and of *Attacin*, *Gloverin*, and *Moricin*. This indicated that *BmPGRP-S2* might serve as a receptor for PGN leading to the IMD activation pathway in the integument. Although both *BmPGRP-S2* and *BmPGRP-S5* belong to the short PGRP, their biological functions might not be the same.

In this report, *BmPGRP-S2* was cloned from *B. mori* integument. The induced expression showed that *BmPGRP-S2* transcription is upregulated after bacterial infection. Analysis on RNA interference revealed that *BmPGRP-S2* regulates the expression of AMPs and participates in the activation of the IMD signal transduction pathway in *B. mori* integument. The results indicated that *BmPGRP-S2* plays an important role in the innate immunity of *B. mori*.

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References Cited

Akria, S., K. Takeba, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2: 675–680.

Andrew, F. R., and A. Powell. 2007. Invertebrate immune systems specific, quasi-specific, or nonspecific? J. Immunol. 179: 7209–7214.

Anselme, C., A. Vallier, S. Balmand, M. O. Fauvarque, and A. Heddi. 2006. Host PGRP gene expression and bacterial release in endosymbiosis of the weevil *Sitophilus zeamais*. Appl. Environ. Microbiol. 72: 6766–6772.

Bischoff, V., C. Vignal, I. G. Boneca, T. Michel, J. A. Hoffmann, and J. Royet. 2004. Function of the *Drosophila* pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat. Immunol. 5: 1175–1180.

Bulet, P., and R. Stocklin. 2005. Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept. Lett. 12: 3–11.

Bulet, P., C. Hetru, J. L. Dimarcq, and D. Hoffmann. 1999. Antimicrobial peptides in insects: structure and function. Dev. Comp. Immunol. 23: 329–344.

Charroux, B., T. Rival, K. Narbonne-Reveau, and J. Royet. 2009. Bacterial detection by *Drosophila* peptidoglycan recognition proteins. *Microbes Infect*. 11: 631–636.

Chen, K., C. Liu, Y. He, H. Jiang, and Z. Lu. 2014. A short-type peptidoglycan recognition protein from the silkworm: expression, characterization and involvement in the prophenoloxidase activation pathway. Dev. Comp. Immunol. 45:1–9.

Cheng, T., P. Zhao, C. Liu, P. Xu, Z. Gao, Q. Xia, and Z. Xiang. 2006. Structures, regulatory regions, and inductive expression patterns of antimicrobial peptide genes in the silkworm *Bombyx mori*. Genomics 87: 356–365.

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Davis, M. M., and Y. Engstrom. 2012. Immune response in the barrier epithelia: lessons from the fruit fly *Drosophila melanogaster*. J. Innate Immun. 4: 273–283.

Doyle, R. J., and R. Dzianski. 2001. Molecular medical microbiology, pp. 137–153. Academic Press, Waltham, Massachusetts, USA.

Dushay, M. S., B. Asling, and D. Hultmark. 1996. Origins of immunity: relish, a compound Rel-like gene in the antibacterial defense of *Drosophila*. Proc. Natl. Acad. *Sci*. USA. 93: 10343–10347.

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

Furukawa, S., H. Tanaka, H. Nakazawa, J. Ishibashi, T. Shono, and M. Yamakawa. 1999. Inducible gene expression of moricin, a unique antibacterial peptide from the silkworm (*Bombyx mori*). Biochem. J. 340: 265–271.

Furukawa, S., H. Tanaka, J. Ishibashi, S. Imanishi, and M. Yamakawa. 2009. Functional characterization of a cactus homolog from the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem. 73: 2665–2670.

Gelius, E., C. Persson, and J. Karlsson. 2003. A mammalian peptidoglycan recognition protein with N-acetylmuramoyl-L-alanine amidase activity. Biochem. Biophys. Res. Commun. 306: 988–994.

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

Govind, S. 2008. Innate immunity in *Drosophila*: pathogens and pathways. Insect Sci. 15: 29–43.

Guan, R., E. L. Malchiodi, and Q. Wang. 2004. Crystal structure of the C-terminal peptidoglycan-binding domain of human peptidoglycan recognition protein Ialpha. J. Biol. Chem. 279: 31873–31882.

Hara, S., and M. Yamakawa. 1995a. A novel antibacterial peptide family isolated from the silkworm, *Bombyx mori*. Biochem. J. 310: 651–656.

Hara, S., and M. Yamakawa. 1995b. Moricin, a novel type of antibacterial peptide isolated from the silkworm, *Bombyx mori*. J. Biol. Chem. 270: 29923–29927.

Hedengren, M., B. Asling, M. S. Dushay, I. Ando, S. Ekengren, M. Wihlborg, and D. Hultmark. 1999. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Mol. Cell 4: 827–837.

Hoffmann, J. A. 1995. Innate immunity of insects. Immunology 7: 4–10.

Ip, Y. T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzales-Crespo, K. Tatei, and M. Levine. 1993. Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. Cell 75: 753–763.

Kaisho, T., and S. Akira. 2001. Bug detectors. Nature 414: 701–703.

Kaneko, T., W. E. Goldman, and P. Mellroth. 2004. Monomeric and polymeric Gram-negative peptidoglycan but not purified LPS stimulate the *Drosophila* IMD pathway. Immunity 20: 637–649.

Kaneko, Y., S. Furukawa, H. Tanaka, and M. Yamakawa. 2007. Expression of antimicrobial peptide genes encoding Enbocin and Gloverin isoforms in the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem. 71: 2233–2241.

Kawaoka, S., S. Katsuma, T. Daimon, R. Isono, N. Omuro, K. Mita, and T. Shimada. 2008. Functional analysis of four Gloverin-like genes in the silkworm, *Bombyx mori*. Arch. Insect Biochem. Physiol. 67: 87–96.

Kim, M. S., M. J. Byun, and B. H. Oh. 2003. Crystal structure of peptidoglycan recognition protein LB from *Drosophila melanogaster*. Nat. Immunol. 4: 787–793.

Laszlo-Otvos, J. R. 2000. Antibacterial peptides isolated from insects. J. Pept. Sci. 6: 497–511.

Lavine, M. D., and M. R. Strand. 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32: 1295–1309.

Lemaitre, B., and J. Hoffmann. 2007. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25: 697–743.

Lemaitre, B., J. M. Reichhart, and J. A. Hoffmann. 1997. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. USA. 94: 14614–14619.

Mellroth, P., J. Karlsson, and H. Steiner. 2003. A scavenger function for a *Drosophila* peptidoglycan recognition protein. J. Biol. Chem. 278: 7059–7064.

Michel, T., J.-M. Reichhart, J. A. Hoffmann, and J. Royet. 2001. *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414: 756–759.

Mrinal, N., and J., Nagaraju 2008. Intron loss is associated with gain of function in the evolution of the gloverin family of antibacterial genes in *Bombyx mori*. J. Biol. Chem. 283: 23376–23387.

Ochiai, M., and M. Ashida. 1999. A pattern recognition protein for peptidoglycan. J. Biol. Chem. 274: 11854–11858.

Oizumi, Y., H. Hemmi, M. Minami, A. Asaoka, and M. Yamakawa. 2005. Isolation, gene expression and solution structure of a novel moricin analogue, antibacterial peptide from a lepidopteran insect, *Spodoptera litura*. Biochim. Biophys. Acta 1752: 83–92.

Ranjiv, S. K., F. Leulier, and B. Lemaitre. 2002. Pathogen surveillance-the flies have it. Science 296: 273–275.

Royet, J., J. M. Reichhart, and J. A. Hoffmann. 2005. Sensing and signaling during infection in *Drosophila*. Curr. Opin. Immunol. 17: 11–17.

Sato, H., and J. B., Feix 2006. Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic α-helical antimicrobial peptides. Biochim. Biophys. Acta 1758: 1245–1256.

Sideri, M., S. Tsakas, E. Markoutsa, M. Lampropoulou, and V. J. Marmaras. 2007. Innate immunity in insects: surface-associated dopa decarboxylase-dependent pathways regulate phagocytosis, nodulation and melanization in medfly haemocytes. Immunology 123: 528–537.

Steiner, H. 2004. Peptidoglycan recognition proteins: on and off switches for innate immunity. Immunol. Rev. 198: 83–96.

Steward, R. 1987. Dorsal, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-rel. Science 238: 692–694.

Stoven, S., I. Ando, L. Kadalayil, Y. Engstrom, and D. Hultmark. 2000. Activation of the *Drosophila* NF-κB factor Relish by rapid endoproteolytic cleavage. EMBO Rep. 1: 347–352.

Sugiyama, M., H. Kuniyoshi, E. Kotani, K. Taniai, K. Kadono-Okuda, Y. Kato, M. Yamamoto, M. Shimabukuro, S. Chowdhury, J. Xu, et al. 1995. Characterization of a *Bombyx mori* cDNA encoding a novel member of the Attacin family of insect antibacterial proteins. Insect Biochem. Mol. Biol. 25: 385–392.

Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. Int. Immunol. 7: 1–14.

Tanaka, H., J. Ishibashi, K. Fujita, Y. Nakajima, A. Sagisaka, K. Tomimoto, N. Suzuki, M. Yoshiyama, Y. Kaneko, and T. Iwasaki, et al. 2008. A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. Insect Biochem. Mol. Biol. 38: 1087–1110.

Taniai, K., K. Kadono-Okuda, Y. Kato, M. Yamamoto, S. Shimabukuro, S. Chowdhury, J. Xu, E. Kotani, S. Tomino, and M. Yamakawa. 1995. Structure of two cecropin B-encoding genes and bacteria-inducible DNA binding proteins which bind to the 5′-upstream regulatory region in the silkworm, *Bombyx mori*. Gene 163: 215–219.

Tzou, P., S. Ohresser, D. Ferrandon, M. Capovilla, J. M. Reichhart, B. Lemaitre, J. A. Hoffmann, and J. L. Imler. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. Immunity 13: 737–748.

Wang, Z. M., X. Li, R. R. Cocklin, M. Wang, K. Fukase, S. Inamura, S. Kusumoto, D. Gupta, and R. Dziarski. 2003. Human peptidoglycan recognition protein-L is an N-acetylmuramoyl-L-alanine amidase. J. Biol. Chem. 278: 49044–49052.

Wang, J. H., S. Valanne, and M. Ramet. 2010. *Drosophila* as a model for antiviral immunity. World J. Biol. Chem. 1: 151–159.

Yoshida, H., K., Kinoshita, and M. Ashida. 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. J. Biol. Chem. 271: 13854–13860.

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