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 B. 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, Grovlin, 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 (Lemaître 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 hemocoelea, 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 (NAMLLA) activity. NAMLLA 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-SA functions as a pattern recognition receptor for the initiation of the prophenoloxidase pathway and as an effector to inhibit bacterial growth; however, the 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, Lemaître 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 grovlin) (Laszlo-Otovos 2000, Bulet and Stocklin 2005). In B. mori, four grovlin genes (Bmglv1–4) have been identified, and Bmglv1–4 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...
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, B. 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 B. 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 B. subtilis (1.0 × 10⁸ 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'-ATGGTTGGTGGCAAGCTTC-3' (forward) and 5'-AAACCAGTACTTTTCTGAGCTTA-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 product 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 ddH₂O (control), 5.0 μl E. coli (2.8 × 10⁸ cells/ml), 5.0 μl B. subtilis (10 × 10⁸ cells/ml), and 5.0 μl S. cerevisiae (6.5 × 10⁸ 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, GATCC TAATACGACTCACTATA; S2-T7-R1, GATATCTAATACGACTCACTATA) using M-MLV reverse transcriptase (TaKaRa Biotechnology Co., Ltd., Dalian, China) vector, and transformed into DH10B using the standard heat-shock method. For the production of the expression plasmid, the treated insects were held at 28°C for 0.5 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′-GGCTCGTCTTCAGAACAAGTA-3′ (forward primer for BmAttacin), 5′-ACATTGGGCTCCACGAAAG-3′ (reverse primer for BmAttacin), 5′-TACCTGATACGCGGGGAAATC-3′ (forward primer for BmGloverin), 5′-GACCATCCACCGGAGCAGAA-3′ (reverse primer for BmGloverin), 5′-TATACCGCGCGGGATCTTAC-3′ (forward primer for BmMoricin), 5′-CAAGTGTGTACAGCTTACCTGCAATT-3′ (reverse primer for BmMoricin), 5′-TAGGATGTGTGTCATCGCGCGG-3′ (forward primer for BmCactus), 5′-CAAGTACTCGTCTTCAGCAGT-3′ (reverse primer for BmCactus), 5′-CAAGTGTGTGTCATCGCGCGG-3′ (forward primer for BmRelish), 5′-TCTCGCTGTGAGGCTTTCCTTGC-3′ (reverse primer for BmRelish), 5′-TATACCGGATTGGAGCGTTACCA-3′ (forward primer for BmRelish), and 5′-TCTCGAGTCTCGTCACTTTTCTGAGCA-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.
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; $E = 2.23 \times 10^{-52}$), and an Ami_2 domain (36–173 amino acids; $E = 2.34 \times 10^{-18}$). 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 neighboring-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$_2$O 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...
Relish 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 pathway.
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 (Tanai 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, Gloverinin 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, Gloverinin, 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, Gloverinin, 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, Gloverinin, 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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