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Methods paper

Cloning and expression analysis of a peptidoglycan recognition protein in silkworm related to virus infection

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ABSTRACT

In this study, the full-length cDNA of a peptidoglycan recognition protein named *BmPGRP-S3* was identified from the silkworm, *Bombyx mori* by rapid amplification of cDNA ends. It is 807 bp and comprises the following: a 5′-untranslated region (UTR) with a length of 112 bp, a 3′-UTR with a length of 92 bp including a polyadenylation signal sequence (AATAAA) and a poly(A) tail. The longest open reading frame (ORF) of *BmPGRP-S3* is 603 bp and encodes a polypeptide of 200 amino acids with a predicted molecular weight of 22.3 kDa including a PGRP domain. Sequence similarity and phylogenic analysis results indicated that BmPGRP-S3 belongs to the group of insect PGRPs and is closer to BmPGRP-S4 with the highest identity of 68%. Fluorescent quantitative real-time PCR results revealed that the mRNA transcripts of *BmPGRP-S3* were presented in all of the tissues, but were highest in the midgut. In the silkworm larvae infected with *B. mori* cytoplasmic polyhedrosis virus (BmCPV), the relative expression level of *BmPGRP-S3* was upregulated. The DNA segment of a mature BmPGRP-S3 peptide was inserted into the expression plasmid pET-28a(+) to construct a recombinant expression plasmid. Western blot results revealed that mature BmPGRP-S3 could be detected in the hemolymph and midgut which were the most important immune tissues in silkworm. All the results suggested that BmPGRP-S3 may play an important role in the immune response of silkworm to BmCPV infection and provided helpful information for further studying the function of BmPGRP-S3 in silkworm.

1. Introduction

Peptidoglycan recognition proteins (PGRPs) are pattern-recognition molecules that can identify peptidoglycan (PGN) in bacteria with PGN as a unique cell wall component. PGRPs function in innate immunity activities and regulation (Steiner, 2004). PGRPs can regulate phenol oxidase system, activate Toll and Imd pathways, and participate in melanotic encapsulation response and phagocytosis (Gottar et al., 2002; Maillet et al., 2008). In insects, PGRPs are grouped into short-type PGRPs (PGRP-S) and long-type PGRPs (PGRP-L) based on predicted protein structures (Leone et al., 2008). PGRP-Ss contain signal peptides and can be secreted as small extracellular proteins (approximately 20 kDa to 25 kDa). PGRP-Ls have long transcripts with several different splicing types and are either intracellular or integral membrane proteins (Wei et al., 2007). *Drosophila* contains only 13 PGRP genes but with 19 PGRP protein products (Christophides et al., 2002). For example, the PGRP-LC gene in *Drosophila* contains three alternative splicing sites of the primary transcript. The encoded transmembrane proteins have an identical intracellular part but different extracellular PGRP domains, namely, *x*, *y*, and *a*. Thus, PGRP-LC exhibits three isoforms (Schmidt et al., 2001).

PGRPs have been used to identify the PGN of Gram-positive bacteria in the innate immune system of various invertebrates, including insects, and vertebrates, such as mammals. PGRPs contain a conserved PGRP domain with a sequence similar to N-acetylmuramyl-alanine amidase. During evolution, catalytic activity in PGRPs has been lost because key residues have mutated (Girardin and Philpott, 2006). A PGN recognition activity has been established for some PGRPs such as PGRP-SA and PGRP-SD in *Drosophila*. These PGRPs are involved in the activation of the Toll pathway (Leone et al., 2008). However, other molecules can also be identified by PGRPs. For example, PGRP-S in cattle can respond to Gram-positive bacteria, Gram-negative bacteria and some fungi (Tydell et al., 2002, 2006). PGRP-LC in *Drosophila* exhibits distinct functions in responding to lipopolysaccharide (LPS) and PGN (Werner et al., 2006). Tsai et al. (2008) found that *Sigma virus* (SIGMA; family *Rhabdoviridae*) infection upregulates the expressions of PGRP-SB1 and

Abbreviations: PGRPs, peptidoglycan recognition proteins; RACE, rapid amplification of cDNA ends; ORF, open reading frame; BmCPV, *Bombyx mori* cytoplasmic polyhedrosis virus; EST, expressed sequence tag; qRT-PCR, quantitative real-time polymerase chain reaction; DEPC, diethylpyrocarbonate; UTR, untranslated region; PGN, peptidoglycan; LPS, lipopolysaccharide; SIGMA, *Sigma virus*; DCV, *Drosophila* C virus; PGRP-L, long-type peptidoglycan recognition protein; PGRP-S, short-type peptidoglycan recognition protein; DAB, diaminobenzidine; PGLYRP1, peptidoglycan recognition protein 1; PGLYRP2, peptidoglycan recognition protein 2; DAP, diaminopimelic acid; EGFP, enhanced green fluorescence protein.

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PGRP-SD in *Drosophila*; the expression level of PGRP-SB1 is also particularly high. PGRP-SA transcription is induced by *Drosophila* C virus (DCV; family *Dicistroviridae*) infection (Tsai et al., 2008).

Xu et al. (2010) investigated the pattern recognition receptors of PGRPs in silkworm and identified 10 BmPGRPs (BmPGRP-L1–5 and BmPGRP-S1–5). Silkworm larval samples were collected 3, 6, 12, and 24 h after these larvae were subjected to *Escherichia coli*, *Bacillus bombysepticus*, and *Beauveria bassiana* challenge; these samples were then analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), showing that two long-type PGRPs (BmPGRP-L1 and BmPGRP-L3) and three short-type PGRPs (BmPGRP-S1–3) can be upregulated by Gram-positive bacteria, Gram-negative bacteria, and fungi. In this study, an upregulated PGRP, which has been named as BmPGRP-S3 by Xu et al. (2010), was derived from a microarray system to compare differently expressed genes in the midguts of *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV)-infected silkworm larvae and normal silkworm larvae (Wu, 2010; Wu et al., 2011). To study the function of BmPGRP-S3, we cloned the full-length cDNA from the EST sequence data of sw22599 by rapid amplification of cDNA ends. qRT-PCR was performed to analyze the expression characteristics of *BmPGRP-S3* in different tissues and in the midgut at different periods after BmCPV infection was induced. The recombinant protein of BmPGRP-S3 was also expressed in vitro to raise the antibody against BmPGRP-S3.

2. Materials and methods

2.1. Silkworm strain

The silkworm strain *P50*, provided by the National Center for Silkworm Genetic Resources Preservation of the Chinese Academy of Agricultural Sciences, was used in the present study. The larvae were cultured at standard temperature of 25 °C under a photoperiod of 12 h light and 12 h dark up to fourth molting for virus inoculation.

2.2. Virus inoculation

BmCPV virus stock was suspended in distilled water to a concentration of 10^7 polyhedra/ml. 10 μl virus suspension was fed to newly molted fifth instar larvae of silkworm. The infection dose was calculated to be 1×10^5 polyhedra per larva. The control larvae were fed with 10 μl distilled water.

2.3. Tissue collection

The different tissues of the fifth instar larvae at 3 days and the midgut of both BmCPV-infected and control silkworm larvae were collected by dissecting the larvae on ice. The isolated tissues were quickly washed with diethylpyrocarbonate-treated water to remove attached leaf pieces and then were immediately frozen in liquid nitrogen. The hemolymph from five larvae was pooled as one sample, to which Phenylthiourea antioxidant was added. Samples were immediately stored at −80 °C after addition of 1 ml Trizol reagent (TaKaRa) for RNA extraction.

2.4. Cloning and sequencing of BmPGRP-S3

Total RNA from silkworm midgut was extracted with Trizol reagent (TaKaRa) and the polyA$^+$ RNA was purified using polyA tract mRNA isolation system II (Promega) according to the instructions of the manufacturer. Full-length cDNA sequences of *BmPGRP-S3* were obtained using SMART™ RACE cDNA Amplification Kit (Clontech). The primers for the specific 5′RACE and 3′RACE were designed according to the EST sequence of sw22599.

SMART™ RACE cDNA Amplification Kit (Clontech) was used to prepare the 3′-RACE-ready cDNA according to the instructions of the manufacturer. The gene-specific primer P1 was used in the RACE reactions for the cloning of 3′-end cDNA. P2 (Table 1) was used to amplify the 5′-end using the first-strand cDNA synthesized as template to obtain the 5′-end of cDNA. PCR amplification was conducted using the following parameters: denaturation at 95 °C for 1 min; followed by 28 cycles of 95 °C for 15 s, 68 °C for 3 min; and then a final extension at 72 °C for 10 min.

The DNA products were gel-purified and cloned into pMD19-T simple vector (TaKaRa, Japan). After being transformed into competent cells of *E. coli* Top10, the positive recombinants were identified through ampicillin selection and PCR screening with M13− and M13+ primers (Table 1). The positive clone was sequenced on an ABI3730 Automated Sequencer (Applied Biosystems).

The sequences of *BmPGRP-S3* were assembled with the obtained fragments from 5′RACE and 3′RACE and analyzed using the BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.gov/blast). To exclude the possibility that composed sequence might be a misassembled artifact, P3 and P4 were designed according to the composed sequence (Table 1) and used in PCR to obtain the full length of *BmPGRP-S3*. The PCR amplification was performed as follows: denaturation at 94 °C for 4 min, followed by 33 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, and one final cycle of 72 °C for 7 min. And then DNA products were gel-purified, sub-cloned and sequenced as described above.

2.5. Sequence analysis, multiple sequence alignment, and phylogenetic analysis

BmPGRP-S3 sequence obtained was analyzed using the BLAST algorithm at NCBI for comparative analysis. The deduced amino acid sequence of *BmPGRP-S3* was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and SMART program (http://smart.embl-heidelberg.de/). The signal peptide was predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The potential sites of Asn-linked glycosylation were predicted with NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). Multiple protein sequences were aligned using the MegAlign program by CLUSTAL W method in DNASTAR software package. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 5 software package (Tamura et al., 2011). Bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

2.6. Quantitative real-time PCR (qRT-PCR)

The presence and expression level of *BmPGRP-S3* in different tissues of silkworm were detected using qRT-PCR method. Total RNAs were

Table 1
Names and sequences of primers used in *BmPGRP-S3* study.

Primer name	Sequence (5′–3′)
Cloning primers	
P1 (reverse)	CTAACTAATTTTATTGGACTAGGC
P2 (forward)	TTCGTAAGCCGAGAGGAATGGGG
P3 (forward)	TCTCTGTGCTGTGAATAACT
P4 (reverse)	ACAAACAACAATAGCCTTT
Oligo (dG)-adaptor	GGCCACGCGTCGACTAGTACG$_{10}$
Expression primers	
P5	CGC**GGATCC**TTGCCTAGTCCAATAAAATTAG
P6	CCG**CTCGAGTTA**ATCATCCCAATTCAGTG
RT primers	
RTF	GTCCAGTGCCCATAGTAGTGAT
RTR	CGATGCTTCGGGTGTTG
Oligo (dT)-adaptor	GGCCACGCGTCGACTAGTACT$_{17}$
Actin primers	
AF (forward)	CCGTATGCGAAAGGAAATCA
AR (reverse)	TTGGAAGGTAGAGAGGGAGG
Vector primers	
M13–21 (forward)	TGTAAAACGACGGCCAGT
M13− (reverse)	CAGGAAACAGCTATGACC