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Enhanced antiviral immunity against *Bombyx mori* cytoplasmic polyhedrosis virus via overexpression of peptidoglycan recognition protein S2 in transgenic silkworms

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ABSTRACT

In insect innate immunity, peptidoglycan recognition proteins act as pattern recognition receptors, helping hosts combat invasive microorganisms. *Bombyx mori* cytoplasmic polyhedrosis virus (BmCPV) is the main silkworm pathogen that invades the midgut columnar cell layer. We previously reported that *B. mori* peptidoglycan recognition protein S2 (BmPGRP-S2) was upregulated in silkworm larvae after BmCPV infection. Here, we constructed a transgenic vector overexpressing BmPGRP-S2 under the control of a midgut-specific promoter. Transgenic silkworm lines (PGRPS2-1 and PGRPS2-2) were generated via embryonic microinjection. BmPGRP-S2 was successfully overexpressed in transgenic silkworms and BmE cells. After oral inoculation with BmCPV, the mortality of PGRPS2-1 and PGRPS2-2 decreased by approximately 36% and 32%, respectively, compared with that of the non-transgenic line, and BmCPV mRNA contents were significantly lower. In the PGRPS2-1 line, *imd*, *relish*, and the antimicrobial peptide (AMP) genes *attacin2*, *gloverin2*, and *moricin* showed increased expression after viral infection; however, the Toll pathway was not activated. These results indicate that BmPGRP-S2 overexpression can activate the Imd pathway and induce AMP upregulation, enhancing silkworm antiviral resistance.

1. Introduction

Two types of molecules are involved in pathogen identification by the innate immune system: pathogen-associated molecular patterns (PAMPs) and host pattern recognition receptors (PRRs) (Medzhitov and Jr, 2000). PAMPs are conserved molecules on microbial surfaces, such as lipopolysaccharide, peptidoglycan (PGN), and various metabolic products. PRRs are specific host molecules that recognize different PAMPs, such as Toll-like receptors, collectins, and peptidoglycan recognition protein (PGRPs). PGRPs specifically recognize bacterial PGN, and are conserved from insects to mammals (Hoffmann et al., 1999). According to structural differences, PGRPs are divided into long-type PGRPs (PGRP-Ls) and short-type PGRPs (PGRP-Ses), which play different roles in innate immunity. For example, in *Drosophila*, PGRP-LE activates the prophenoloxidase (proPO) cascade (Takehana et al., 2002); PGRP-LC can interact with PGRP-LE to activate the Imd signaling pathway (Choe et al., 2002; Kaneko et al., 2006); and the Toll pathway is usually activated by PGRP-Ses like PGRP-SA and PGRP-SC1a (Garver et al., 2006; Michel et al., 2001). Activation of the Imd and Toll pathways can induce the production of downstream antimicrobial peptide genes (AMPs) to resist pathogenic microorganism. (Dziarski and Gupta, 2004; Kurata, 2014). Twelve PGRP families have been identified in silkworm (Tanaka et al., 2008). The first identified PGRP was BmPGRP-S1, which is involved in proPO activation (Yoshida et al., 1996). BmPGRP-S5 plays multiple roles, i.e., as a receptor for activation of the proPO pathway, as a negative regulator for the Imd pathway, and as a bactericide (Chen et al., 2014, 2016). BmPGRP-L1 is a player in the Imd pathway of *B. mori* (Zhan et al., 2017). BmPGRP-L6 overexpression enhances AMP promoter activity through the Imd pathway, although it also binds to Lysine-type PGNs that activate the Toll pathway (Tanaka and Sagisaka, 2016).

A few studies have suggested that PGRPs may be involved in antiviral immune defense. Expressed sequence tags (ESTs) were generated from the gut tissues of *Peregrinus maidis* after viral infection, and 202 ESTs were predicted to be related to the innate immune response, including 8 PGRP genes (Whitfield et al., 2011). After honey bees were

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infected with Chinese Sacbrood Virus, PGRP-S2 and PGRP-LC expression was downregulated (Zhang et al., 2013). In *Drosophila*, Sigma virus infection resulted in increased expression of PGRP-SB1, PGRP-SD, and some antibacterial peptide genes (Tsai et al., 2008). *Bemisia tabaci* PGRP (BtPGRP) has a potential Tomato yellow leaf curl virus (TYLCV) binding site, as an interaction between BtPGRP and TYLCV was detected *in vitro* by immunocapture PCR, and their colocalization was observed in the midgut (Wang et al., 2016). Maize fine streak rhabdovirus inhibited the expression of PGRP-SB1, -SD, and -LC, and PGRP-LC in *Graminella nigrifrons,* leading to significant mortality (Chen and Michel, 2012; Chen et al., 2015).

Bombyx mori is a model insect from the order Lepidoptera with important economic value which faces serious threats from diseases (Xia et al., 2004, 2009). *B. mori* cytoplasmic polyhedrosis virus (BmCPV; family Reoviridae) is one of its main pathogens, and invades the midgut columnar (Liu et al., 2012). BmCPV is a double-stranded RNA virus with a genome consisting of 10 discrete segments: S1, S2, S3, S4, S6, and S7 encode structural proteins; S5, S8, S9, and S10 encode nonstructural proteins (Cao et al., 2012; Hill et al., 1999). Little research has been performed examining the interactions between silkworms and BmCPV, and silkworm defensive genes against BmCPV have not been identified. The expression of BmPGRP-S3 was upregulated after infecting silkworm larva with BmCPV (Kun et al., 2014). In our previous study, BmPGRP-S2 was induced by BmCPV, and was mainly expressed in the midgut and weakly expressed in the silk gland or fat body (Jiang et al., 2016).

Overexpressing resistance genes can improve the antiviral capacity of silkworms against *B. mori* nuclear polyhedrosis virus (BmNPV) (Jiang et al., 2012a, 2012b). In this study, we generated two the transgenic silkworm lines, PGRPS2-1 and PGRPS2-2, with increased expression of BmPGRP-S2 under the control of the midgut-specific promoter P3 + 5UI (Jiang et al., 2015). After BmCPV infection, components of the Imd pathway were upregulated and mortality was reduced in the transgenic strains compared to the control strain. This is the first report of the antiviral action of PGRPs during CPV infection.

2. Materials and methods

2.1. Silkworm strains, cells, and viruses

The Dazao (DZ) silkworm strain and BmE cell line were maintained at the Gene Resource Library of Domesticated Silkworms and the State Key Laboratory of Silkworm Genome Biology (Southwest University, China), respectively. Larvae were orally inoculated with wild BmCPV (Guangdong strain, China).

2.2. Multiple sequence alignment

The amino acid sequence of BmPGRP-S2 was analyzed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool, and the sequences of 10 PGRPs with and without amidase activity were downloaded as the most similar sequences. The amino acid sequence of Enterobacteria phage T7 lysozyme was also downloaded. Multiple protein sequences were aligned using the multiple sequence alignment program in the DNAMAN software package.

2.3. Vector construction

BmPGRP-S2 was cloned from the midgut cDNA of fifth instar 3-day DZ larvae. After detection by sequencing, *BmPGRP-S2* was amplified using a forward primer encoding an N-terminal FLAG tag. A midgut specific promoter (P3 + 5UI) and SV40 termination signal (Jiang et al., 2015) were inserted along with *BmPGRP-S2* into the transgenic vector *piggyBac*[3 × p3 dsRed afm] with the reporter gene 3 × p3-dsRed-SV40 to generate the transgenic vector pb-PGRPS2. We subsequently added FLAG and His tags to the N and C termini of BmPGRP-S2, respectively. The vector 1180-P3P + 5UI-FLAG-BmPGRP-S2-His-SV40 (named PGRPS2-FH) was constructed using the basic vector 1180 (Jiang et al., 2017a).

2.4. Transgenic silkworm generation and insertion site analysis

As previously described (Jiang et al., 2012a, 2012b), the pb-PGRPS2 vector plasmid was microinjected into non-diapausing DZ embryos. Two transgenic silkworm lines, PGRPS2-1 and PGRPS2-2, were obtained. The transposon-specific primers pBacL and pBacR were used to analyze the genomic DNA of the transgenic lines as a template for inverse PCR analysis (Jiang et al., 2012a, 2013).

2.5. Detection of BmPGRP-S2 expression in transgenic silkworms

RNA and protein were extracted from the midguts of PGRPS2-1, PGRPS2-2, and non-transgenic DZ (Nm) day 3 fifth instar larvae. Total RNA of each line was extracted using a Total RNA Extraction Kit (Promega), and digested with 20 U of RNase-free DNase I (Promega). RNA was reverse-transcribed to cDNA, which was used as a template in quantitative polymerase chain reaction (qPCR) analysis (Jiang et al., 2012b). qPCR analysis was performed with PGRPS2 primers, and TIF-4A primers were used as a control (Guo et al., 2016). Detection of each gene was repeated in triplicate. The Student's t-test was used for data analysis. The protein was used for western blotting with antibodies against the FLAG tag and tubulin (as a control). The PGRPS2-FH and 1180 (control) plasmids were transfected into BmE cells. RNA was extracted 24 h after transfection and used for reverse transcription (RT)-PCR analysis using PGRP-S2 and TIF-4A primers. Proteins and cell culture medium were extracted 48 h post transfection for western blot analysis using anti-FLAG, -His, and -tubulin antibodies.

2.6. Detection of antiviral capacity in transgenic silkworms

The mortality of PGRPS2-1, PGRPS2-2, and Nm larvae was investigated after oral inoculation with 3×10^5 occlusion bodies (OBs) of wild BmCPV per larva at the newly exuviated fourth instar. Each line was infected in three replicates of 60 larvae. Cumulative mortality rates were recorded daily until the wandering stage (Jiang et al., 2012b). RNA was obtained from the midgut of PGRPS2-1, PGRPS2-2, and Nm larvae 72 h post infection. qPCR analysis was performed using primers targeting S2, S4, S5, and S10 to detect viral mRNA contacts (Jiang et al., 2017b). TIF-4A was used as a control. Detection of each gene was repeated in triplicate. The Student's t-test was used for data analysis.

2.7. Detection of Toll and Imd pathway activation

Using the cDNA of the PGRPS2-1 and Nm lines 72 h post infection as the template, *spatzle*, *rel*, *imd*, *relish*, *attacin2*, *gloverin2*, and *moricin* expression were detected by qPCR. The expression of *imd* and *relish* genes was detected by qPCR analysis using the cDNA of uninfected PGRPS2-1 and Nm larvae. *TIF-4A* was used as an internal control to standardize the variance among the different templates. Detection of each gene was repeated in triplicate. The Student's t-test was used for data analysis.

3. Results

3.1. BmPGRP-S2 does not display amidase activity

The 20 most similar sequences to BmPGRP-S2 were found in the NCBI database. The amino acid sequences of typical PGRPs with and without amidase activity and Phage T7 lysozyme were used for multiple sequence alignment. A cysteine residue is key for amidase activity in T7 lysozyme, BmPGRP-S5, and DmPGRP-SC1b, which have amidase