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Expression of antimicrobial peptide genes in *Bombyx mori* gut modulated by oral bacterial infection and development

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ABSTRACT

Although *Bombyx mori* systematic immunity is extensively studied, little is known about the silkworm's intestine-specific responses to bacterial infection. Antimicrobial peptides (AMPs) gene expression analysis of *B. mori* intestinal tissue to oral infection with the Gram-positive (*Staphylococcus aureus*) and -negative (*Escherichia coli*) bacteria revealed that there is specificity in the interaction between host immune responses and parasite types. Neither Att1 nor Leb could be stimulated by *S. aureus* and *E. coli*. However, CecA1, Glo1, Glo2, Glo3, Glo4 and Lys, could only be trigged by *S. aureus*. On the contrary, *E. coli* stimulation caused the decrease in the expression of CecA1, Glo3 and Glo4 in some time points. Interestingly, there is regional specificity in the silkworm local gut immunity. During the immune response, the increase in Def, Hem and LLP3 was only detected in the foregut and midgut. For CecB1, CecD, LLP2 and Mor, after orally administered with *E. coli*, the up-regulation was only limited in the midgut and hindgut. CecE was the only AMP that positively responses to the both bacteria in all the testing situations. With development, the expression levels of the AMPs were also changed dramatically. That is, at spinning and prepupa stages, a large increase in the expression of CecA1, CecB1, CecD, CecE, Glo1, Glo2, Glo3, Glo4, Leb, Def, Hem, Mor and Lys was detected in the gut. Unexpectedly, in addition to the IMD pathway genes, the Toll and JAK/STAT pathway genes in the silkworm gut can also be activated by microbial oral infection. But in the developmental course, corresponding to the increase in expression of AMPs at spinning and prepupa stages, only the Toll pathway genes in the gut exhibit the similar increasing trend. Our results imply that the immune responses in the silkworm gut are synergistically regulated by the Toll, JAK/STAT and IMD pathways. However, as the time for approaching pupation, the Toll pathway may play a role in the AMPs expression.

1. Introduction

Epithelia constitute the first line of defense in the innate immunity of both vertebrates (Ganz and Lehrer, 1998) and invertebrates (Tzou et al., 2000), and antimicrobial peptides (AMPs) are essential components of this epithelial immunity (Boulanger et al., 2002). The gut epithelium is an essential interface in insects that is in intimate contact with a countless number of microbes (Hooper and Gordon, 2001). The direct contact between gut epithelia and ingested pathogens activates the immune deficiency (IMD)/NF-κB pathway, which results in de novo synthesis of innate immune effectors including AMPs (Ryu et al., 2008). Despite the central role of the NF-κB/AMP pathway in host survival during the systemic immune response, which follows microbial infection in the hemocoel, its exact physiological function in intestinal innate immunity has not yet been convincingly demonstrated (Lemaitre, 2004; Ryu et al., 2006).

The insect gut is divided into three regions: foregut, midgut and hindgut (Fig. S1). The foregut and the hindgut are of ectodermal origin and are therefore covered by a cuticle that is continuous to the external one (Vallet-Gely et al., 2008). The midgut is of endodermal origin and is not lined by a cuticle; it comprises an epithelium layer that is bordered by a peritrophic membrane on its luminal surface (Vallet-Gely et al., 2008). Although the basic anatomical sub-divisions of insect gut were established almost 50 years ago (Christophers, 1960), information regarding their roles in innate immunity is rather scarce. Several studies about local immune response in the insect gut were mostly focused on the section of midgut (Boulanger et al., 2002; Crampton and Luckhart, 2001; Herrera-Ortíz et al., 2004; Freitak et al., 2007).

From their initial isolations and characterizations, AMPs were observed to exhibit a broad-spectrum of activities against

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microorganisms, capable of responding very generally to different classes of pathogens, including Gram-positive and -negative bacteria, fungi, mycoplasma, and viruses (Diamond et al., 2009). But when induced in surface epithelia, AMPs are also expressed in a tissue-specific manner (Imler and Bulet, 2005; Tzou et al., 2002).

In nature, insects gut might be infected by many pathogens with food at any time. Therefore, it is necessary to get a clear picture of insect gut immune response to the invaded bacteria. As the silkworm genome sequencing has been completed, it is clear about genes involved in innate immunity (Tanaka et al., 2008). In this paper, to determine the AMP genes specifically induced in the gut, we investigated transcriptome variation in dissected three sections of *Bombyx mori* larvae gut (minus the Malpighian tubules) after oral infection with both Gram-positive (*Staphylococcus aureus*) and -negative (*Escherichia coli*) bacteria. The expression levels of 19 AMPs were measured using real-time RT-qPCR. Several critical genes involved in the silkworm Toll, IMD and JAK/STAT signaling pathways were also tested. In addition, the real-time RT-qPCR analysis of the AMP and signaling pathway genes was also performed during the different larva developmental stages.

2. Materials and methods

2.1. Insect, immune challenge and sample preparation

B. mori (Nistari) larvae were reared on mulberry leaves at 25 °C with a 12 h light and 12 h dark cycle. All the silkworm guts dissected from the larvae from the 4th instar in the molting stage (4LM), the 5th instar immediately after molt (5L0h), the 5th instar 12 h after molt (5L12h), the 5th instar 72 h after molt (5L72h), the spinning stage and prepupa were used for a developmental time course assay (at least five larvae or prepupa for each developmental stage). For gut section specific assay and immune challenge assay, the silkworm guts were only dissected from the 5th instar larvae 72 h after molt.

Microbes used in these experiments were *E. coli* (DH5α, Gram-negative bacteria) and *S. aureus subsp. aureus* (Strain Number: CICC 10201, China Center of Industrial Culture Collection, Gram-positive bacteria). *E. coli* and *S. aureus* were precultured in LB and Broth Medium, respectively. Pellets were obtained by centrifugation at 8000 rcf at the time when the cultures were in the log phase of growth. Living bacteria were resuspended in NaCl solution (0.85%, w/v, autoclaved). To induce the immune responses in the guts, the silkworm larvae were reared on mulberry leaves pre-coated with *S. aureus* (10^8/larva) or *E. coli* (10^9/larva), respectively, and incubated at 25 °C for challenge. The guts, including foregut, midgut and hindgut, from the larvae fed on mulberry leaves pre-coated with the same volume of NaCl solution, were used as the control. The above immune challenged larvae were still alive before sampling at the scheduled times and were suitable for analysis of AMPs expression. At each time point, at least five silkworm larvae were sampled. The gut parts including foregut, midgut, hindgut were harvested at 1, 2, 4, 8, 12 and 24 h after bacteria feeding. And over the 24-h sampling period, expression level of any AMP gene did not significantly vary ($p < 0.05$) in the control silkworm (Table S1). The guts for both developmental time course assay and immune challenge assay were washed in 0.85% NaCl solution three times to remove any attached hemocytes, pulverized in liquid nitrogen and treated with Trizol agent (Invitrogen, Carlsbad, CA, USA) so as to stabilize the RNA, then stored at −80 °C for further use.

2.2. RNA extraction, cDNA synthesis

Total RNA was extracted by using UNIQ-10 (Sangon, Shanghai, China) according to the manufacturer's instructions. The RNA samples were further treated with DNase I (RNase free) (TaKaRa, Dalian, China) to remove any contaminating DNA following the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a 20 μl reaction mixture with a First-Strand cDNA Synthesis Kit according to the manufacture's instruction (TaKaRa, Dalian, China).

2.3. Quantitative real-time RT-PCR (real-time RT-qPCR)

Quantitative RT-PCR was performed on a 7500 real-time system (Applied Biosystems, Carlsbad, CA, USA). The 20 μl mixture including 1 μl of cDNA, 0.4 μl of each primer (10 μM), 0.4 μl of ROX reference Dye II, 10 μl of SYBR Premix ExTaq (TaKaRa, Dalian, China) and 7.8 μl H_2O was placed in 96 well plates. The PCR set with an initial denaturation of 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 34 s. A dissociation curve analysis was performed for all primer pairs, and all experimental samples yielded a single sharp peak at the amplicon's melting temperature. Primers for the AMP genes and the eIF4A gene were designed using the online Primer3 internet based interface (http://frodo.wi.mit.edu) and presented in Tables S2 and S3. The dynamic range of a given primer system and its normalizer were examined by running triplicate reactions of 10-fold dilution series (10 different cDNA concentrations). As targets and normalizer had similar efficiency ranges (from 1.990 to 2.015), the comparative quantitation method ($\Delta\Delta$Ct) was used to contrast the different treatments and tissues, and transformed to absolute values with $2^{-\Delta\Delta Ct}$ for obtaining relative fold expressions (Livak and Schmittgen, 2001). All the real-time RT-qPCR experiments were repeated for at least three times with similar results observed. Relative fold expressions for each gene were set to 1 for the control treatment (calibrator). Post-test for significance of expression differences compared with the control or other calibrators were calculated in Excel (Microsoft) by ANOVA test. When the p value <0.05, it was considered significant.

2.4. Qualification of the reference gene

The eukaryotic initiation factor 4A (eIF4A) gene was used for normalization of cDNA templates. The standard deviation (SD) of Ct was determined from triplicate reactions using 100 ng of cDNA from the naive silkworms with the six developmental stages and immune challenge ones with various time points (Table S4). Since all the three SD's were less than 1.0, eIF4A was considered to be appropriate for use as reference gene in the gut of silkworm (Taylor et al., 2008).

2.5. Bioinformatic information of B. mori AMP genes and signal pathway genes

All the *B. mori* AMP-related protein sequences and several IMD pathway genes were cited from previous results (Tanaka et al., 2008). The corresponding RNA sequences were predicted by BLAST searches using *B. mori* genomic database (http://silkworm.genomics.org.cn, http://kaikoblast.dna.affrc.go.jp/, http://blast.ncbi.nlm.nih.gov/Blast.cgi). And several key genes involving in Toll and JAK/STAT signal transduction pathways were cited from Huang et al. (2009).

3. Results

To locate the expression of AMPs in the different parts of the silkworm gut and to determine if the relative expressions of AMPs are altered in the silkworm gut during the developmental time course and immune challenges, 19 pairs of real-time RT-qPCR primers were designed based on sequences available in previous results (Tanaka et al., 2008) and the BLAST result. The expression levels of