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Gene expression profiling of resistant and susceptible *Bombyx mori* strains reveals nucleopolyhedrovirus-associated variations in host gene transcript levels

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ABSTRACT

We investigated variations in the gene expression of *Bombyx mori* following infection with a nucleopolyhedrovirus (BmNPV). Two *B. mori* strains, *KN* and *306*, which are highly resistant and susceptible to BmNPV infection, respectively, were used in this study. The infection profiles of BmNPV in the *B. mori KN* and *306* larvae revealed that the virus invaded the midguts of both these strains. However, its proliferation was notably inhibited in the midgut of the resistant strain. By using the suppression subtractive hybridization method, two cDNA libraries were constructed in order to compare the BmNPV responsive gene expressions between the two silkworm lines. In total, 62 differentially expressed genes were obtained. Real-time qPCR analysis confirmed that eight genes were significantly up-regulated in the midgut of the *KN* strain following BmNPV infection. Our results imply that these up-regulated genes may be involved in the *B. mori* immune response against BmNPV infection.

Introduction

Bombyx mori nucleopolyhedrovirus (BmNPV) is a principal pathogen of the domestic silkworm, and its host range is restricted to *B. mori* larvae [1]. BmNPV causes severe losses in sericulture, but there are currently no therapeutic agents that can effectively control BmNPV infection. Among the seven-hundred silkworm strains in the National Center for Silkworm Genetic Resources Preservation of the Chinese Academy of Agricultural Sciences, most *Bombyx* strains are susceptible to BmNPV infection. Only a few strains show high resistance to BmNPV infection.

In the nucleopolyhedrovirus (NPV) replication cycle, there are two different virion phenotypes, which are the occlusion-derived virus (ODV) and the budded virus (BV). The ODV initiates infection within the insect midgut columnar epithelial cells, whereas the BV is responsible for systemic infection throughout the host [2,3]. The two viral forms are essential for NPV natural propagation. The replication cycle of *Autographa californica* multinucleocapsid NPV (AcMNPV), which is the most extensively characterized example, begins immediately after the ODV nucleocapsid delivers the viral genome into the host cell nucleus. In the first 6 h postinfection (h pi), viral immediate early genes begin to express in host cells for DNA replication, and the virogenic stroma, which is the site of viral RNA transcription, DNA replication and nucleocapsid assembly, begins to form in the center of the host cell nucleus. At about 12 h pi, the virogenic stroma expands to fill most of the nucleus. Between 12 and 20 h pi, the BV virion phenotype is produced [4]. BmNPV is the second most widely studied baculovirus after AcMNPV. The systemic process of infection by BmNPV in *B. mori* larvae revealed that the virus replication occurred in the midgut epithelial cells within 24 h pi [1]. In general, the growth kinetics of BmNPV in host cells is slower than those of AcMNPV [4].

NPVs require interaction with their hosts to accomplish virus replication in the host insect cells. NPV infection typically causes a global shutoff of host gene expression, and protein synthesis in insect cells begins at around 12–18 h pi [5]. Conversely, particular host genes are induced or remain stably expressed until the late stage of the infection. Inducible gene expression by NPV occurs both *in vivo* and *in vitro*. For example, the *heat shock protein cognate 70* (*hsc70*) gene was up-regulated in *Spodoptera frugiperda* cells that were infected with AcNPV [6], and the *suppressor of profilin 2* (*sop2*) gene was up-regulated in the silkworm midgut that was infected with BmNPV [7]. However, the mechanism of viral modulation of host mRNA levels during their infection remains largely unknown.

In order to gain a better understanding of the mechanisms that underlie resistance to BmNPV in some *Bombyx* strains, we first investigated the infection profiles of BmNPV in the larval midguts of susceptible and resistant *B. mori* strains by performing real-time quantitative PCR (qPCR) at different time points. Our results revealed that BmNPV invaded the midgut tissue of both the susceptible and resistant strains. However, viral proliferation in the midgut of the resistant strain was inhibited by unknown mechanisms.

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Based on the current understanding of the interactions between BmNPV and its host, *B. mori*, we focused on clarifying which host genes were up-regulated during the early phase of BmNPV infection by suppression subtractive hybridization (SSH), which is an effective technique by which differentially expressed cDNA fragments can be selectively amplified [8]. We chose 12 h pi as the time point to determine the resistance-related genes in the *B. mori* midgut, as they might be associated with BmNPV replication and both ODV and BV virion formation. Two silkworm strains of *B. mori*, *KN* and *306*, were used for comparing differentially expressed genes. The *KN* line is highly resistant to BmNPV infection, and the *306* line is susceptible to BmNPV infection. Two subtracted cDNA libraries were constructed using a BmNPV-infected and phosphate-buffered saline (PBS)-treated *KN* strain and a BmNPV-infected and PBS-treated *306* strain. A total of 101 cDNA clones that were expressed in response to BmNPV infection were obtained from the libraries. Real-time qPCR analysis confirmed that eight genes were significantly up-regulated following BmNPV infection in the larval midgut of the resistant *KN* strain.

The aim of this study is to identify *B. mori* genes with potential functions against BmNPV infection in the larval midgut and to use these genes to understand the antiviral mechanisms that are involved in the immune responses of insects.

Results

Determination of BmNPV proliferation by performing real-time qPCR

We monitored the dynamic proliferation of BmNPV in the midguts of the *B. mori* resistant *KN* and susceptible *306* strains by performing real-time qPCR, in order to gain a better understanding of the invasion properties of BmNPV. Melting curve analysis confirmed that specific amplification was achieved using one pair of primers against BmNPV *GP41* gene, for which no non-specific amplification or primer–dimer artifacts were observed (data not shown). In an early stage of BmNPV infection (6 h pi), the viral proliferation was detected in the susceptible larval midgut with relative copy numbers of 14.42 but was almost undetectable in the resistant strain (Fig. 1). As the infection progressed, the viral proliferation levels increased in the midguts of both *B. mori* strains. The relative copy numbers that corresponded to the BmNPV *GP41* gene rapidly increased from approximately 14.42 to nearly 7.3×10^5 copies within 6–72 h pi in the susceptible *306* larvae (Fig. 1). In contrast, the viral proliferation rate was extremely low in the larval midgut of the resistant *KN* strain, with the relative copy numbers remaining below 10 until 24 h pi, gradually increasing beyond 10 at 48 h pi and achieving 15.73 at 72 h pi, with no significant variations between 48 and 72 h pi (Fig. 1). These results revealed that BmNPV invaded the midgut tissue of both *KN* and *306* strains, but the viral proliferation in the midgut of the resistant strain was significantly inhibited by unknown mechanisms.

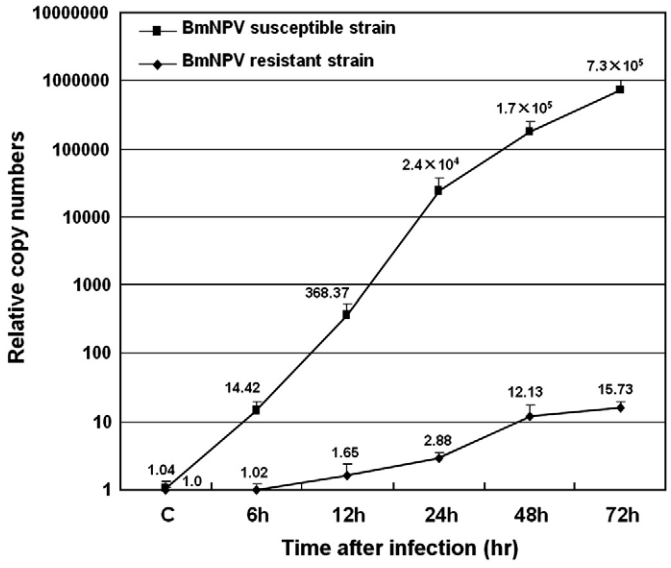

Fig. 1. BmNPV proliferation in the midguts of *B. mori* resistant *KN* and susceptible *306* strains. Total DNA was extracted from the fifth instar larval midguts of *KN* and *306* strains at the indicated times after BmNPV infection and subjected to real-time qPCR analysis using BmNPV *GP41* primers. PBS-treated samples were used as controls (C). The relative copy numbers were calculated by using *Bombyx GAPDH* gene as an internal control. Samples from each time point were tested in triplicate, and the mean value was used for analysis relative to BmNPV genomic copies.

Transmission electron microscopic (TEM) observation

Further evidence of the inhibition of BmNPV proliferation in resistant *B. mori* larval midguts was obtained by examining the BmNPV-infected *KN* and *306* larval midguts during the late phase of infection using TEM. As the relative copy numbers of BmNPV differed most dramatically (10^4 fold) at 72 h pi in the two *B. mori* strains (Fig. 1), we focused our observations at this time point. As a result, the polyhedral and ODV structures were clearly observed in the enlarged nucleus of the tracheal epithelial cells that surrounded the midgut cells in the susceptible larvae. The virogenic stroma, which is the putative site of nucleocapsid assembly, was also observed in the nucleus of the tracheal epithelial cells (Figs. 2a and b). However, similar structures were not found in the nucleus of both midgut and tracheal epithelial cells of the resistant larvae (Figs. 2c and d). Thus, the inhibition of BmNPV proliferation appeared to be restricted to the resistant strain.

Isolation and sequence determination of subtractive cDNA clones

Following two SSH experiments, a total of 101 cDNA clones that were specifically expressed in the tester strains were isolated. There were 34 genes (62 clones) in the R library and 30 genes (39 clones) in the S library, which has a total of 62 genes (101 clones) in the two libraries. The genes that were up-regulated following BmNPV infection in the midgut of the susceptible (S) and resistant (R) strains when compared to the PBS treatment are listed in Table 1. According to the annotation of the *Spodoptera frugiperda* sequences [9], the subtractive genes were classified into five groups, which were genes that encoded proteins that were ubiquitously expressed by many cell types (AI–AIX), genes that were responsible for cell–cell communication (BIII), genes that encoded transcription factors and gene-regulatory proteins (C), genes that encoded molecules that were expressed in insects (DI–DIV), and other genes (EI–EIII) (Table 1).

The distribution of the up-regulated genes was significantly different in the two libraries. There were only two overlapping genes between the libraries, which encoded *B. mori serine protease (SP-2)* and a *serine protease precursor* (Table 1). Thirty-two genes were isolated in the R library but not in the S library, such as *B. mori cytochrome C oxidase subunit II* and *B. mori transgelin* genes. Twenty-eight genes were detected in the S library but not in the R library, such as *B. mori 14-3-3 zeta* and *B. mori surfeit 4-like protein* genes. These observations implied that the differential expression of these genes may be correlated with the resistance and susceptibility of these strains to BmNPV infection (Table 1).

The differences in the gene distributions were also reflected by the most abundant transcripts in each library. At 6.45% each, *arylphorin*, *gloverin-4* and *actin A3* were the most abundant transcripts in the R library. At 7.69% each, *B. mori triacylglycerol lipase* and *leukotriene A4 hydrolase* were the most abundant transcripts in the S library. In terms of functional gene distribution, the most significant differences were seen in the serine protease and related inhibitors group, which comprised 14.5% of all the clones in the R library and was significantly lower in the S library (7.68%). In addition, none of serine proteinase inhibitor transcripts were found in the S library, which suggested that