Genomics 94 (2009) 138–145

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

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

Yan-Yuan Bao [a], Xu-Dong Tang [a], Zu-Yao Lv [a], Xiu-Ye Wang [b], Cai-Hong Tian [a], Yi Peng Xu [a], Chuan-Xi Zhang [a,*]

[a] Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Insect Sciences, Zhejiang University, Hangzhou, 310029, China
[b] Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, 212018, China

ARTICLE INFO

Article history:
Received 24 December 2008
Accepted 12 April 2009
Available online 21 April 2009

Keywords:
Suppression subtractive hybridization
BmNPV
Bombyx mori
Antiviral response

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.

* Corresponding author. Fax: +86 571 86971697.
E-mail address: chxzhang@zju.edu.cn (C.-X. Zhang).

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doi:10.1016/j.ygeno.2009.04.003

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.

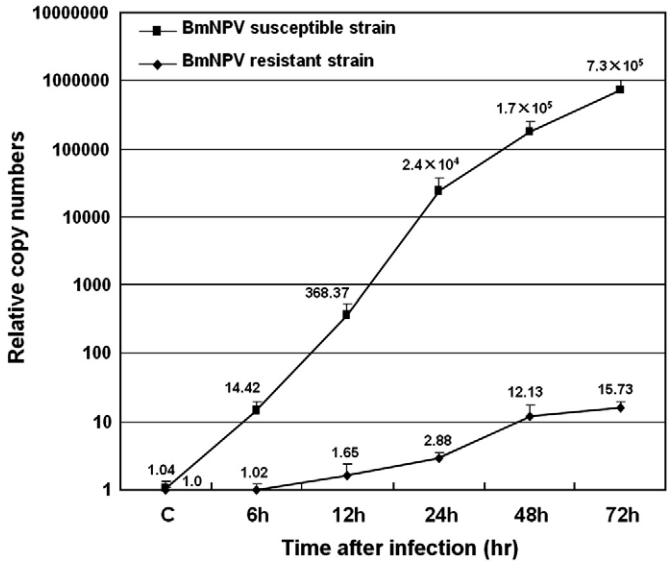

Fig. 1. BmNPV proliferation in the midguts of *B. mori* resistant *KN* and susceptible *306* strains. Total DNA was extracted from the fifth instar larval midguts of *KN* and *306* strains at the indicated times after BmNPV infection and subjected to real-time qPCR analysis using BmNPV *GP41* primers. 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

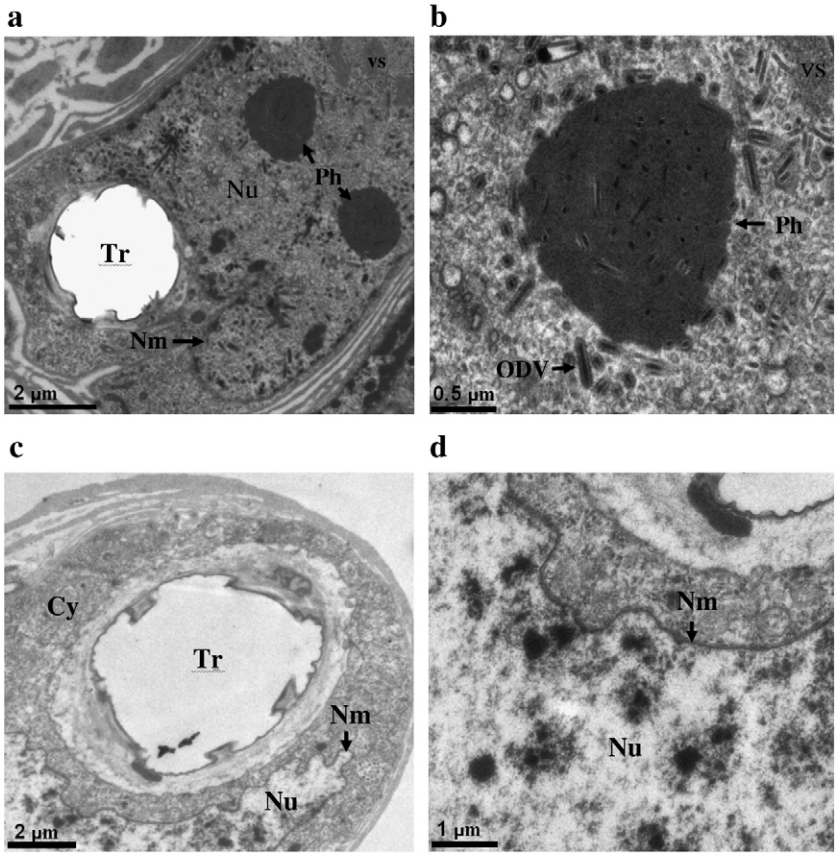

Fig. 2. Electron microscopic observations of BmNPV-infected susceptible and resistant larval midgut tissues. (a and b) BmNPV-infected susceptible midgut tissue at 72 h pi and enlarged image; (c and d) BmNPV-infected resistant midgut tissue at 72 h pi and enlarged image. Ph, Polyhedra; ODV, occlusion-derived virus; Cy, cytoplasm; Nu, nucleus; Nm, nuclear membrane; Tr, Trachea; VS, virogenic stroma. Scale bars: 2 μm (a, c); 1 μm (d); 0.5 μm (b).

the serine protease and related inhibitors might play more important roles in the *B. mori* resistant strain than in the susceptible strain. The antibacterial peptide transcripts were highly abundant in the R library, comprising 14.5% of the clones, but only comprised 2.56% of the transcripts in the S library. Some gene transcripts showed a relative high abundance, such as the transcripts for the ribosomal protein gene group that comprised a total of 4.83% and 10.24% of all the clones in the R and S libraries, respectively. The transcripts of *arylphorin* and *SP1*, which are two hymolymph storage proteins, comprised a total of 8.06% of all the clones in the R library but were not present in the S library.

Determination of differentially expressed cDNAs by performing real-time qPCR

In order to determine the up-regulated expression of those genes that are activated by BmNPV infection and to understand the gene expression variations that are coupled with viral proliferation, we performed time course analysis in the larval midgut by real-time qPCR (Fig. 3). We focused on the genes that were present in the R library but not in the S library, as they were most likely BmNPV responsive genes in the resistant strain and might contribute to the resistance against BmNPV infection. Based on this consideration, we selected eight genes, which had the highest abundance in the R library, to determine their transcript levels following different treatment of the two *B. mori* strains. The three genes that encoded *B. mori arylphorin*, *actin A3* and *gloverin-4* had the highest abundance at 6.45% each, the four genes that encoded *B. mori promoting protein*, *cathepsin B*, *serpin-5* and *lebocin* showed high abundance at 4.84% each and the *B. mori gloverin-3* gene had an abundance of 3.23% in the R library. These genes are implicated in multiple biological processes, but little is known with regard to their mechanisms of action in antiviral responses.

The expression patterns of these genes differed between the strains. The transcripts of *gloverin-3*, *lebocin* and *arylphorin* genes were detected at extremely low levels in the midguts of the PBS-treated susceptible *306* and resistant *KN* strains (Figs. 3b, c, e). The expression levels of the *B. mori arylphorin* transcripts, within 6–12 h pi, did not change in susceptible larvae but significantly increased in resistant larvae at 12 h pi, then quickly increased in both strains from 24 h pi, and attained the highest levels at 48 h and 72 h pi in resistant and susceptible strains, respectively (Fig. 3e). The *B. mori gloverin-3* and *lebocin* genes shared a similar expression pattern. Their expressions were strongly induced in both *B. mori* strains at 6 h pi by BmNPV infection and quickly decreased until 24 h pi. Notably, their expression levels increased again at 48 h pi only in resistant larvae (Figs. 3b, c). A similar expression pattern of the *B. mori Gloverin-4* gene was restricted to the resistant larval midgut. In susceptible larvae, its transcripts were consistently detected at extremely low levels throughout the time course (Fig. 3a). Similarly, in resistant larvae, *B. mori serpin-5* transcript levels also quickly increased until 6 h pi, decreased until 24 h pi and then increased again at 48 h pi. However, the *serpin-5* transcript levels were consistently higher in both PBS-treated and BmNPV-infected resistant larvae than in susceptible larvae (Fig. 3d). *B. mori actin A3* gene expression showed a similar pattern to that of the *gloverin-4* gene in the susceptible strain, and its transcripts consistently remained at very low levels (Fig. 3h). Therefore, the expression levels of *B. mori actin A3* and *gloverin-4* were clearly not activated by BmNPV infection in the *Bombyx 306* strain. However, in resistant strain throughout the entire time course, the expression level of *actin A3* gene was consistently higher in the resistant larvae when compared to the susceptible larvae, and its expression achieved a maximum at 12 h pi (Fig. 3h).

Table 1
Distribution of subtracted cDNA clones in the major functional categories.

Gene name	Accession no.	Functional class	Clone number (percentage) Of subtracted cDNA clones in each library	
			Library R	Library S
Bombyx mori NADPH oxidoreductase	ABK30932	AI electron transport	2 (3.23)	0 (–)
B. mori cytochrome C oxidase subunit II	AAF33752	AI electron transport	2 (3.23)	0 (–)
B. mori Rab7	ABF51264	AI GTP-binding protein	1 (1.61)	0 (–)
B. mori GTP-binding nuclear protein Ran	ABD36346	AI GTP-binding protein	0 (–)	1 (2.56)
B. mori chlorophyllide A binding protein precursor	CAJ34654	AI small molecule transportation	2 (3.23)	0 (–)
B. mori fatty acid binding protein	ABD36301	AI small molecule transportation	1 (1.61)	0 (–)
B. mori ADP/ATP translocase	AAO32817	AI carrier protein	0 (–)	1 (2.56)
B. mori mRNA cap-binding protein eIF4E	ABF51281	AII RNA processing protein	1 (1.61)	0 (–)
B. mori reverse transcriptase	BAD86652	AII RNA processing protein	0 (–)	1 (2.56)
Aedes aegypti zinc finger protein	EAT45966	AII transcription factor	0 (–)	2 (5.13)
A. aegypti sap18	EAT33351	AIII repression of transcription	2 (3.23)	0 (–)
B. mori actin A3	AAC47446	AIV actin	4 (6.45)	0 (–)
B. mori alpha-tubulin	BAB86849	AIV tubulin	2 (3.23)	0 (–)
B. mori ribosomal protein L18	AAV34829	AV ribosomal protein	1 (1.61)	0 (–)
B. mori ribosomal protein L37	AAV34850	AV ribosomal protein	1 (1.61)	0 (–)
B. mori ribosomal protein L23A	AAV34835	AV ribosomal protein	0 (–)	1 (2.56)
B. mori ribosomal protein L27A	AAV34839	AV ribosomal protein	1 (1.61)	0 (–)
B. mori ribosomal protein S2	ABX57502	AV ribosomal protein	0 (–)	1 (2.56)
B. mori ribosomal protein S7	AAV34863	AV ribosomal protein	0 (–)	1 (2.56)
B. mori ribosomal protein P0	AAV34809	AV ribosomal protein	0 (–)	1 (2.56)
B. mori chaperonin subunit 6a zeta	ABD36094	AV Chaperonin	0 (–)	1 (2.56)
B. mori aminopeptidase N	BAA33715	AVI hydrolysis	2 (3.23)	0 (–)
B. mori leukotriene A4 hydrolase	ABF51345	AVI hydrolysis	0 (–)	3 (7.69)
B. mori lipase-1	BAC00960	AVI lipolysis	0 (–)	1 (2.56)
B. mori triacylglycerol lipase	ABD36173	AVI lipolysis	0 (–)	3 (7.69)
B. mori glutathione S-transferase omega 1	ABD36128	AVI biosynthesis	1 (1.61)	0 (–)
B. mori uricase	ABA39861	AVI biosynthesis	0 (–)	1 (2.56)
B. mori H+ transporting ATPase V0 subunit D	ABF51370	AVI ATP synthesis enzyme	1 (1.61)	0 (–)
B. mori glucosidase	AAP13852	AVI carbohydrate metabolism	2 (3.23)	0 (–)
B. mori mitochondrial aldehyde dehydrogenase	ABF51464	AVI carbohydrate metabolism	0 (–)	1 (2.56)
Culicoides variipennis heat shock protein 60	AAB94640	AVII stress response	1 (1.61)	0 (–)
B. mori cathepsin B	BAB40804	AVIII protein degradation	3 (4.84)	0 (–)
Culex quinquefasciatus dipeptidyl peptidase 4	EDS45210	AVIII proteolysis	0 (–)	1 (2.56)
B. mori 30 kDa protein precursor	CAA30432	AIX transport protein	1 (1.61)	0 (–)
B. mori 30 kDa lipoprotein PBMHPC-19 precursor	CAA30435	AIX transport protein	1 (1.61)	0 (–)
B. mori 14-3-3 zeta	BAG38533	AIX molecular adaptor	0 (–)	1 (2.56)
B. mori transgelin	ABF51271	AIX actin binding protein	1 (1.61)	0 (–)
B. mori secreted protein acidic and rich in cysteine	ABF51358	BIII extracellular matrix protein	1 (1.61)	0 (–)
Helicoverpa armigera intestinal mucin-2	ACB54933	BIII extracellular matrix protein	0 (–)	1 (2.56)
H. armigera intestinal mucin-4	ACB54955	BIII extracellular matrix protein	0 (–)	1 (2.56)
B. mori gloverin-4	BAF63528	DI AMP	4 (6.45)	0 (–)
B. mori gloverin-3	BAF63527	DI AMP	2 (3.23)	0 (–)
B. mori lebocin	BAA22883	DI AMP	3 (4.84)	0 (–)
B. mori attacin	AAB34519	DI AMP	0 (–)	1 (2.56)
B. mori arylphorin	ABF51447	DII storage molecule	4 (6.45)	0 (–)
B. mori SP-1	CAA31417	DII storage molecule	1 (1.61)	0 (–)
B. mori promoting protein	BAA89306	DIII binding protein	3 (4.84)	0 (–)
B. mori surfeit 4-like protein	ABD36168	DIII unknown function	0 (–)	2 (5.13)
B. mori serine protease (SP-2)	AAX39409	DIV protease	2 (3.23)	1 (2.56)
B. mori highly basic serine protease precursor	BAA20136	DIV protease	1 (1.61)	1 (2.56)
Heliothis virescens serine protease SP-1	ABR88240	DIV protease	0 (–)	1 (2.56)
B. mori serpin-5	AAS68506	DIV protease inhibitor	3 (4.84)	0 (–)
B. mori serpin-33	ACI24664	DIV protease inhibitor	1 (1.61)	0 (–)
B. mori chymotrypsin inhibitor CI-8A	AAK52495	DIV protease inhibitor	1 (1.61)	0 (–)
B. mori antitrypsin precursor	BAA00639	DIV protease inhibitor	1 (1.61)	0 (–)
A. aegypti similar to acid phosphatase-1	EAT44756	EI	0 (–)	1 (2.56)
B. mori putative cholesterol transporter BmStart1	BAE44462	EII	0 (–)	1 (2.56)
Drosophila melanogaster CG8351-PA	AAF54292	EII	0 (–)	2 (5.13)
D. melanogaster GM15606p	AAM50709	EII	0 (–)	1 (2.56)
D. melanogaster RE04191p	AAL48570	EII	0 (–)	1 (2.56)
Tribolium castaneum similar to CG8326-PA	XP_972936	EII	0 (–)	1 (2.56)
D. erecta GG16090	EDV52476	EII	0 (–)	1 (2.56)
No similarity		EIII	1 (1.61)	0 (–)
No similarity		EIII	1 (1.61)	0 (–)
No similarity		EIII	0 (–)	1 (2.56)
No similarity		EIII	0 (–)	1 (2.56)
Clone numbers			62	39

List of *Bombyx mori* genes responsive to BmNPV infection in library R and S. Gene name: annotation of the subtracted cDNA sequences was based on GenBank database using the BLASTX algorithm. Functional class: classes and subclasses to which the sequences belong to, according to classification for *Spodoptera frugiperda* described in Barat-Houari et al. [9]. Number and percentage of clones: distribution of the cDNA clones corresponding to the given gene with available sequences in the two subtracted libraries (library R and S), respectively.

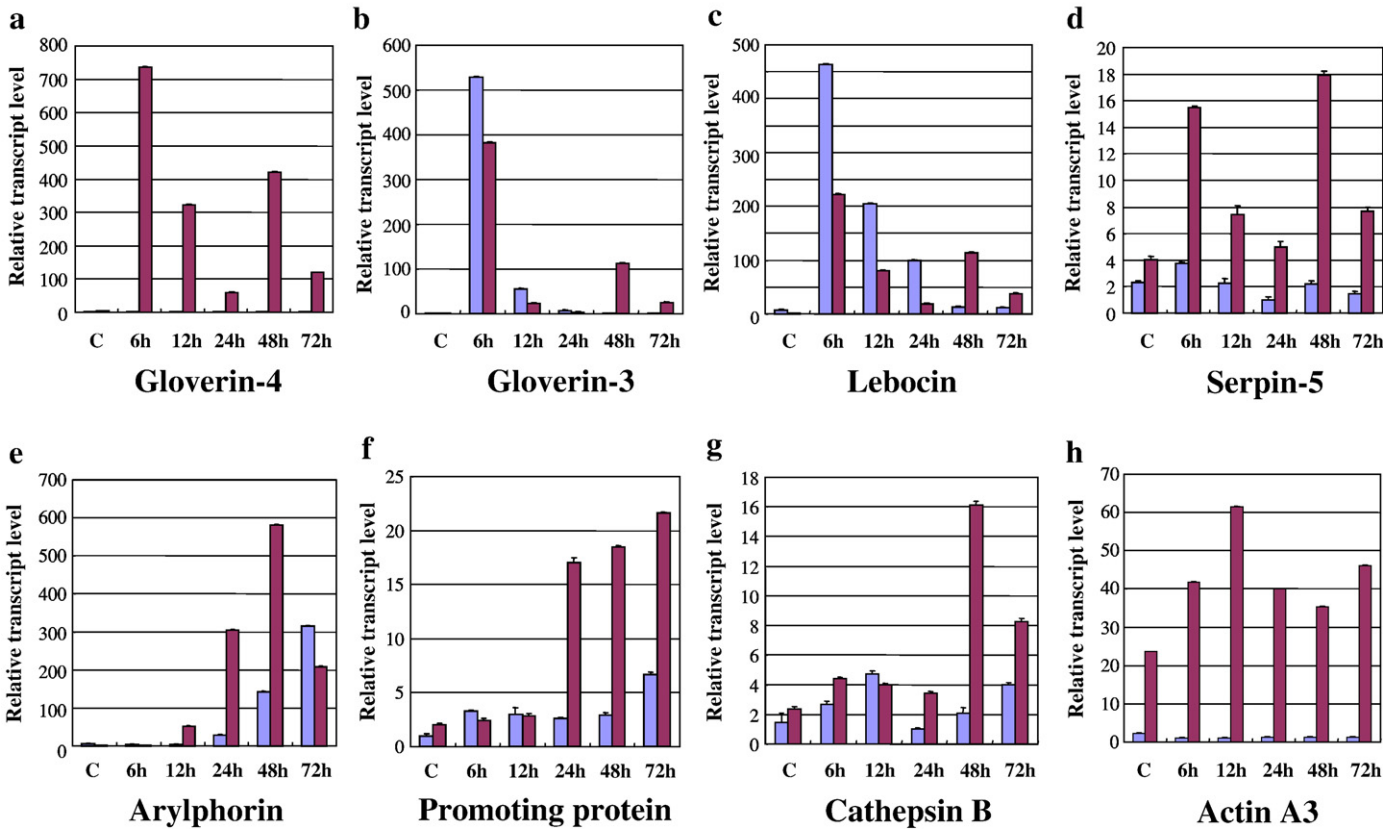

Fig. 3. Expression time course of BmNPV responsive genes in *Bombyx* midguts. Fifth instar larvae of *KN* and *306* strains were infected with BmNPV. Total RNA was extracted from the midgut at the indicated times after infection and subjected to DNase I treatment and reverse transcription. PBS-treated samples were used as controls (C). Two microliters of each 10-fold diluted first-strand cDNA (20 ng) reaction was analyzed in each real-time qPCR reaction. The reaction was performed with the specific primers for amplifying *Bombyx* (a) *gloverin-4*, (b) *gloverin-3*, (c) *lebocin*, (d) *serpin-5*, (e) *arylphorin*, (f) *promoting protein*, (g) *cathepsin B* and (h) *actin A3*. The relative expression levels of each gene at different time points were normalized using the C_t values that were obtained for the *GAPDH* amplifications run on the same plate. In each assay, the expression level is shown relative to the lowest expression level, which is arbitrarily set at one. All samples were tested in triplicate. The mean value ± SD was used for analysis of relative transcript levels for each time point using the $\Delta\Delta C_t$ method. The *Bombyx 306* and *KN* strains are shown on the left (blue) and right (purple), respectively.

The transcripts of *B. mori promoting protein* and *cathepsin B* genes showed low expression levels in the PBS-treated susceptible and resistant larvae (Figs. 3f, g). Following BmNPV infection, increased transcript levels were observed in the two *B. mori* strains (Figs. 3f, g). In the resistant strain, the transcript levels of the *promoting protein* gene significantly increased from 24 h pi and achieved the maximum at 72 h pi (Fig. 3f), while the maximal level of *cathepsin B* transcripts peaked at 48 h pi (Fig. 3g). In contrast, the variations in *promoting protein* and *cathepsin B* transcript levels with BmNPV infection in susceptible larval midgut were not significant (Figs. 3f, g).

Discussion

Although the mechanisms that underlie the regulation of immune responses in the silkworm have been studied extensively, its antiviral immune mechanism remains unclear. So far, only a few molecules that have been isolated from the *B. mori* larval midgut, such as *lipase* [10], *serine protease* [11] and *NADPH oxidoreductase* [12], have been reported to exhibit antiviral activity against BmNPV infection. Many other antiviral factors may be involved in the complex immune responses of insects, but these remain undefined. In this study, we monitored the BmNPV proliferation in the midguts of highly susceptible and resistant *B. mori* strains. Our qPCR results revealed that BmNPV invaded the midgut tissues of both *B. mori* strains, but its proliferation was greatly slowed down in the resistant strain (Fig. 1). This indicated that the antiviral mechanism that occurred in the *B. mori* resistant strain was not due to resistance against BmNPV entry but rather to the inhibition of BmNPV proliferation by unknown mechanisms. TEM analysis indicated that polyhedra and ODVs were present in the nucleus of tracheal epithelial cells surrounding the midgut cells at 72 h pi in the susceptible larvae but not in the resistant strain, after scanning many ultrathin sections (Fig. 2). Keddie and Rahman et al. reported that the spread of BmNPV during the systemic infection process in *B. mori* larvae occurred through the tracheae and that BmNPV multiplication was detected in larval tissues that were tightly associated with tracheae [1,3]. They also reported that polyhedra were present in the tracheal epithelial cells at 72 h pi or later. In this study, a few copies of the BmNPV genome were obtained in the resistant midgut at 72 h pi by real-time PCR (Fig. 1), but we did not observe polyhedra, ODVs or any similar structures in either the nucleus of tracheal epithelial cells or the midgut cells at 72 h pi. The fact that there were so few BmNPV virions may account for our inability to locate the polyhedral or ODV structures in the examined tissues. However, this observation confirmed that BmNPV proliferation was inhibited in the resistant larvae.

BmNPV infection in *B. mori* larvae through the oral route progressed in a temporal manner, as seen with AcMNPV, but the BmNPV infection process was slower [4]. *In vivo*, BmNPV infects *Bombyx* larval midgut columnar epithelial cells within 12 h pi and mainly undergoes replication within 24 h pi [1]. *In vitro*, Katsuma et al. determined that the inhibitors of the extracellular signal-regulated kinases, ERK and JNK, markedly reduced ODV and BV production through the inhibition of the expression of delayed-early, late and very late viral genes around 12 h pi in BmNPV-infected BmN cells [5]. In order to identify the potential host genes that are involved in the inhibition of the viral proliferation that occurs in the larval midgut, we chose 12 h pi as a

time point, since it was closely linked to viral genomic replication and virion production. We constructed two 12 h pi cDNA libraries, which are complementary. The R and S libraries display BmNPV responsive genes upon the viral infection of the resistant and susceptible strains, respectively. We focused on the identification of the genes that were present in the R library but not the S library, as it increases the possibility that these genes are involved in the response and the resistance to BmNPV.

Gloverin and lebocin seem to be lepidopteran-specific antibacterial peptides [13], and have expression levels that are strongly induced in *Bombyx* larval fat body by *Escherichia coli* immune challenge [14–16]. In this study, BmNPV infection caused a strong induction of *B. mori* gloverin and lebocin gene expression in larval midguts at 6 h pi, and this induction occurred in both *B. mori* strains for gloverin-3 and lebocin genes but only in resistant larval midguts for *gloverin-4* gene. Among these genes, the regulatory motifs, such as the transcription factor κB binding site and GATA sequences, were highly conserved in the upstream promoter regions, which have been shown to play an essential role in full gene expression [10,14]. This conservation suggests that the BmNPV-induced expression of the *B. mori* antibacterial peptide genes may be activated through an NF-κB signaling cascade [17]. However, the regulatory mechanisms upstream of the NF-κB signaling cascade are complex, which can be reflected by the significantly different expression levels of the *gloverin-4* gene in the two *B. mori* strains (Fig. 3a). One possible explanation for this result is that certain regulatory factors were activated or blocked by BmNPV invasion, which caused a differential induction of antibacterial peptide genes in the *B. mori* susceptible and resistant strains. In resistant larval midguts, these genes promptly responded to BmNPV infection at an early stage (6 h pi) of viral infection, which indicated that they might act with each other and that this cooperation may help in developing an effective defense mechanism against BmNPV infection.

A similar expression tendency in resistant larval midguts was seen for the *B. mori serpin-5* gene (Fig. 3d). Serpins are serine protease inhibitors that function in regulating the prophenol oxidase activation cascade [18]. *B. mori serpin-5* has a high sequence homology with an immune-responsive serpin, *Manduca sexta serpin-5*, that was constitutively expressed at a low level in larval hemocytes and the fat body and increased dramatically upon bacterial or fungal challenge [18]. In this study, we determined that *B. mori serpin-5* expression was significantly activated by BmNPV infection in resistant larval midguts at an early stage (6 h pi) and also at a late stage (48 h pi), but that it was not activated in the susceptible strain. This suggests that *serpin-5* was a quick immune-responsive gene and was probably involved in the host's antiviral mechanisms. In addition, several serine proteases and inhibitors were isolated from the R and S libraries (Table 1). Although insect genomes are rich in serine proteases and inhibitors, is not well understood, in any arthropod species, how they are regulated or how microbial components trigger the activation [18]. Understanding the physiological functions of these proteases and inhibitors will require further characterization in order to determine their roles in immunity or other processes.

The expression patterns of the other host genes were different. For example, the *B. mori arylphorin*, *promoting protein* and *cathepsin B* genes were constitutively expressed at low levels in the midguts of both *B. mori* strains, and were significantly up-regulated from 24 or 48 h pi in resistant larvae (Figs. 3e–g). A similar expression pattern for *arylphorin* was shown in the interaction between BmDNV-Z and its host *B. mori* larvae [19], which indicated that the up-regulated expression of the *arylphorin* gene was possibly a defense strategy against *Bombyx* viral infection. Promoting protein, which is a silkworm hymolymph protein, enhanced susceptibility to BmNPV infection and resulted in high budded virus production in silkworm cells cultured *in vitro* [20,21]. However, its biological function in the silkworm larvae is totally unknown. As indicated by its binding to bacterial and fungal cell wall components, such as PGN, LPS and chitin (β-1,4-glucans) [22], the promoting protein may act in defense against various pathogens. In this experiment, significantly increased *promoting protein* gene expression was observed within 24–72 h pi only in the resistant midguts (Fig. 3f), but it is currently difficult to assume whether this protein facilitates BmNPV proliferation during the late stage of the infection or contributes to other functions in the silkworm larvae. Similarly, the transcript levels of *cathepsin B*, a cysteine proteinase [23], were significantly increased in resistant larval midgut at 48 h pi, which indicated a potential defensive function.

The expression of *B. mori actin A3* was significantly different between the two *B. mori* strains upon viral infection, with its transcripts showing an extremely low level in susceptible strains but a high level in both PBS-treated and BmNPV-infected resistant larval midguts (Fig. 3h). These observations indicate that *actin A3* might play an important role in the host immune system of the resistant strain.

In summary, the antiviral mechanism that occurs in the resistant *B. mori* strain is not due to resistance against the BmNPV invasion but rather to the inhibition of BmNPV proliferation in the larval midgut. The defense processes against BmNPV infection that occur in the resistant larvae might be regulated via interactions involving multiple genes. The pathways involved in these interactions are currently unclear. Our data provide a global view of host responses to viral infection and insights for further investigations on the complex interactions between BmNPV and its host, *B. mori*.

Materials and methods

Insects and virus

The silkworm, *B. mori*, resistant (*KN*) and susceptible (*306*) strains were provided by the Sericultural Research Institute of the Chinese Academy of Agricultural Sciences (Zhenjiang, China). The larvae of both the strains were reared at 27 °C on fresh mulberry leaves. The newly exuviated fifth instar larvae were used for these experiments.

The BmNPV virus (T3 strain) was propagated in BmN-5 cells, which were maintained at 27 °C in TC-100 insect medium (Gibco, NY, USA) that was supplemented with 10% (v/v) fetal bovine serum (Gibco). The titration of virus and other routine manipulations were performed according to standard protocols [24]. One milliliter of BmNPV viral suspension was added with 100 μl of kanamycin (50 mg/ml) and 100 μl of gentamicin (7 mg/ml). Each newly exuviated fifth instar larva of the susceptible and resistant strains was orally administered 10 μl of the BmNPV viral suspension (7.5×10^8 polyhedra/ml PBS) by using an Eppendorf pipette, while the susceptible and resistant control larvae were administered 10 μl of PBS, individually. Oral administration of BmNPV volume ensured 100% infection in the susceptible *306* strain.

Collection of tissue

BmNPV-infected fifth instar larvae were dissected, and the midguts were removed at different intervals post infection (pi) (6, 12, 24, 48 and 72 h pi). They were quickly washed in diethylpyrocarbonate (DEPC)-treated PBS solution (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4 (pH 7.4)) and immediately frozen in liquid nitrogen.

Investigation of BmNPV proliferation by performing real-time quantitative PCR

Total DNA was extracted from the midguts of the BmNPV-infected *B. mori KN* and *306* larvae at 6, 12, 24, 48 and 72 h pi as well as from the PBS-treated larvae by using the Universal Genomic DNA Extraction Kit Ver 3.0 (TaKaRa, Dalian, China). DNA samples that were obtained from three larvae per treatment group were used as the template for PCR amplification.

One pair of *GP41* primers (forward primer: 5′CGTAGTAGTAGTA-ATCGCCGC3′ and reverse primer: 5′AGTCGAGTCGCGTCGCTTT3′) were

designed based on the BmNPV *GP41* gene sequence (GenBank accession no. BAA03365). As an internal control, the *B. mori GAPDH* gene (GenBank accession no. ABA43638) was also analyzed using the following primers: 5′GCTGCCTCCTTGACCTTTTGC3′ (forward primer) and 5′CATTCCGCGTCCCTGTTGCTAAT3′ (reverse primer). The specificity of the primers was confirmed by using NCBI BLAST algorithms (http://www.ncbi.nlm.nih.gov/).

The reactions were conducted on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA, USA), using the SYBR *Premix Ex Taq* Kit (TaKaRa), according to the manufacturer's protocol. Total DNA and specific BmNPV primers were used to amplify the *BmNPV GP41* gene sequence. Each amplification reaction was performed using a 20-μl reaction mixture, under the following conditions: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and at 60 °C for 34 s. The fluorescent signals yielded by the PCR products were detected by subjecting the products to a heat-dissociation protocol (temperature range, 60–95 °C) during the last step of each cycle. Following amplification, melting curves were constructed, and data analysis was performed by using the ABI 7500 system SDS software. Two microliters of genomic DNA from BmNPV-infected samples and PBS-treated negative controls (20 ng each), and 2 μl of no-template control (NTC) were used as templates. The reaction was carried out in triplicate, and the average threshold cycle (C_t) value was used to quantify the relative BmNPV copy number. The results were standardized using the expression level of the *B. mori glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene.

Transmission electron microscopy (TEM)

The newly exuviated fifth instar larvae of the susceptible and resistant strains were orally administered with PBS and infected with BmNPV as described earlier, in the Insects and virus section. Midguts were dissected out at 72 h pi, fixed in 4% (v/v) glutaraldehyde overnight at 4 °C and then post-fixed with 1% (v/v) osmium tetroxide for 1 h at room temperature. The fixed midguts were dehydrated through an ethanol series (50–100%, v/v) and soaked in acetone for 20 min. Infiltration was performed in 50%, 75% and 100% (v/v) spurr resin (Sigma, St. Louis, MO, USA) and maintained at 70 °C for 16 h. Ultrathin sections that were contrasted with uranyl acetate and lead citrate were observed under a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. Three larvae per treatment group were subjected to TEM analysis.

Construction of the subtracted cDNA library through SSH

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the midguts of the BmNPV-infected and PBS-treated *KN* and *306* larvae at 12 h pi. Poly $(A)^+$ RNA was purified by using an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), and 2 μg of Poly $(A)^+$ RNA was used as the starting material for reverse transcription to construct the subtracted cDNA libraries. SSH was performed using a PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, USA). In keeping with the manufacturer's protocol, we designated the cDNA samples that contained specifically expressed transcripts, which were namely those that responded to BmNPV infection, as “testers” and the reference cDNA samples as “drivers.” The following two SSH libraries were constructed by performing hybridizations between strains that had been subjected to different treatments. The R library, which corresponds to the resistant line, was constructed using the PBS-treated (driver) and BmNPV-infected (tester) *KN* strains, and the S library, which corresponds to the susceptible line, was constructed using the PBS-treated (driver) and BmNPV-infected (tester) *306* strains. The subtracted cDNA libraries were generated by inserting the differentially expressed cDNA fragments into pGEM-T Easy vectors (Promega, Madison, WI, USA) and transforming these vectors in JM109 competent cells. Aliquots (100 μl) of the transformation mixture were then spread on Luria–Bertani (LB) agar plates that contained 100 μg/ml ampicillin, 80 μg/ml X-gal and 50 μM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and were incubated at 37 °C overnight.

All subtractive clones were subjected to sequencing. The nucleotide and amino acid sequence homologies were determined by searching the GenBank database using the BLASTX algorithm (http://www.ncbi.nlm.nih.gov/).

Confirmation of differentially expressed genes by performing real-time qPCR

Trizol reagent (Invitrogen) was used to extract total RNA from the midguts of the BmNPV-infected *KN* and *306* larvae at 6, 12, 24, 48 and 72 h pi as well as from PBS-treated larvae. RNA was treated with 10 U of DNase I (TaKaRa), following the manufacturer's instructions. The concentration of DNase-treated RNA was adjusted with DEPC H_2O to 1 μg/μl, and 1 μg of DNase I-treated RNA was reverse transcribed in a 10 μl reaction system using the AMV RNA PCR Kit (TaKaRa). Real-time qPCR was performed using 2 μl of diluted first-strand cDNA (1/10) in each 20 μl reaction mixture. Specific primer sets were designed for genes that encoded *Bombyx* arylphorin, gloverin-3, gloverin-4, lebocin, serpin-5, promoting protein, cathepsin B and actin A3 (Table 2). The conditions used for the real-time qPCR were as described in Investigation of BmNPV proliferation by performing real-time quantitative PCR. The results were standardized to the expression level of the constitutive *Bombyx GAPDH* gene. An NTC sample was run to detect contamination and to determine the degree of dimer formation. A relative quantitative method ($\Delta\Delta C_t$) was used to evaluate quantitative variation.

Table 2
Primers used in real-time qPCR for confirmation of differentially expressed genes.

Gene	Accession no.	Forward primer	Reverse primer
Bm arylphorin	ABF51447	5′GCCACGGTTTCGTTGTTC3′	5′GCTGCGGCTTCAGGATTA3′
Bm gloverin-4	BAF63528	5′ACTATTATATTTCTTCGCCACGGT3′	5′ACCAAAAAGCCCATCATCATT3′
Bm gloverin-3	BAF63527	5′ATTGCTGTTTTTCATCGCTAC3′	5′GAGTCCATCGTCGTTCTGTCC3′
Bm lebocin	BAA22883	5′CCGTTTAACCCCAAGCCAATA3′	5′TCCCTCGGAATCAGAAAGTGC3′
Bm serpin-5	AAS68506	5′CGTGATCAGTGCAGGATGTATTC3′	5′CCACTAAATATCACAGGGGTGGT3′
Bm promoting protein	BAA89306	5′GCTGGCAAAACTCAAACCTTAGACT3′	5′TGTAGCAGCACATCTGGCTCTCATT3′
Bm cathepsin B	BAB40804	5′GTTGCTGCCCTATTTGTGGA3′	5′TGTTGCCGGGTACGTGATGT3′
Bm actin A3[a]	AAC47446	5′AATGGCTCCGGTATGTGC3′	5′TTGCTCTGTGCCTCGTCT3′
Bm GAPDH[b]	ABA43638	5′CATTCCGCGTCCCTGTTGCTAAT3′	5′GCTGCCTCCTTGACCTTTTGC3′

[a] Actin A3: cytoplasmic actin (A3).
[b] GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Acknowledgments

This work was supported by the National Program of High-tech Research and Development (863 High-Tech Program, No. 2006AA10A119) from the Ministry of Science and Technology of China, National Natural Science Foundation of China (Grant No. 30570074); Natural Science Foundation of Zhejiang Province, China (Grant No.Y307049) and the Specialized Research Fund for the Doctoral Program of Higher Education (Grant No. 20070335102).

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