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RESEARCH REPORT

Virus-induced opposite effect on *Bombyx mori* gene transcriptions

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Abstract

Bombyx mori bidensovirus (BmBDV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV) are serious pathogens of *Bombyx mori*. In this study, we reported the changes of transcription level of several immune genes, including *bmi*, *argo*, *dicer*, *cap1*, *cap3* and *car*, in *Bombyx mori* midgut after exposure to BmBDV or BmNPV. Silkworm strains 798 (anti-BmBDV) and 306 (susceptible to BmBDV) were subjected to BmBDV infection, and NB (anti-BmNPV) and HUABA (35) (susceptible to BmNPV) were subjected to BmNPV infection. The results showed that the transcription levels differ largely among different silkworm strains, and that the extent to which the gene transcriptions were affected by the viruses was different. However, both BmNPV and BmBDV viruses can reverse the transcription patterns of these genes when the silkworms were administered with the viruses compared with those control groups. The transcript levels of *bmi* and *dicer* were decreased in 798 and 306 strains that were inoculated with BmBDV compared with their respective controls, but were increased in NB and HUABA (35) inoculated with BmNPV. The transcript levels of *argo* and *cap3* were risen in 798, 306 and NB strains when inoculated with their respective viruses, but were decreased in HUABA (35) strain. The transcript levels of *cap1* were risen in all silkworm strains, while the levels of *car* were decreased in 798, 306 and HUABA (35) strains, and increased in NB strain when inoculated with their respective viruses. These findings may contribute to more in-depth understanding on functions of these genes in virus infection and proliferation.

Key Words: BmBDV; BmNPV; *Bombyx mori*; qPCR; immune genes; expression level

Introduction

Microorganisms, especially viruses, are harmful to the growth and propagation of *Bombyx mori*. Virus results in approximately 70 % of damage to the sericulture industry due to viral diseases. *Bombyx mori* viruses can be divided into four categories: nuclear polyhedrosis (BmNPV), midgut polyhedrosis, virus flacherie and densovirus (BmBDV). BmBDV is a non-enveloped spherical virus that is composed of two single stranded DNA segments, and it replicates mainly in the columnar cells of the midgut epithelium (Hu *et al.*, 2013). BmNPV is a circular, double stranded DNA virus, and similar to BmBDV, BmNPV also enters through the columnar cells of the midgut epithelium and replicates in this type of cells (Gomi *et al.*, 1999). However, BmBDV infection shows up as a chronic disease while BmNPV acts acutely, indicating that these two viruses differ pathogenically in their viral infection mechanisms.

Bombyx mori and other invertebrates use innate immunity as defense strategy against various pathogens. Many molecules and biological processes are involved in the resistance to insect viruses, such as antimicrobial peptides, phenol oxidase-dependent melanization and encapsulation, apoptosis, phagocytosis and RNAi (Lipardi *et al*., 2003; Galiana-Arnoux *et al*., 2006; Liu *et al*., 2006; Yao *et al*., 2006; Wang *et al*., 2006). *NADPH-oxidoreductase*, *lipase-1* and *serine protease-2* genes have been reported to be anti-viral genes (Uchida *et al*., 1984, Ponnuvel *et al*., 2003, Nakazawa *et al*., 2004). Peptides genes that have been reported to have antimicrobial properties are *gloverin-1*, *lebocin* and *attacin* (Bao *et al*., 2009, 2010). Several heat shock protein genes, including *small heat shock protein*, *HSP70 cognate* and *Hsc70/Hsp90-organizing protein* were reported to be related to viral resistance (Bao *et al*., 2010).

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Molecules that are involved in the prophenoloxidase cascade system, such as trypsin-like serine protease, serine protease-1, serpin-5 and retinoid-inducible serine carboxypeptidase, also relate to viral resistance (Bao *et al*., 2008, 2020; Qin *et al.,* 2012; Zhou *et al*., 2013). Cytochrome c oxidase subunit II, ribosomal s3a and death associated protein (DAP), which are involved in the apoptotic pathway, relate to the resistance to virus (Chi *et al*., 2009; Bao *et al*., 2010; Xu *et al*., 2010). Molecules that involve in pattern recognition, such as the sialic acid binding Ig-like lectins, relate to viral resistance. Molecules involved in oxidative stress, such as thiol peroxiredoxin, glutathione S-trasferase omega and thioredoxin relate to the resistance to virus (Zhao, 2007; Bao *et al*., 2009; Zhou *et al*., 2013). Molecules involved in energy metabolism, such as arginine kinase, V-ATPase c subunit, V-APTase B subunit and V-ATPase H subunit relate to the resistance to virus (Zhao, 2007; Yang *et al*., 2009; Bao *et al*., 2010). Actin, myosin, or tubulin family proteins, such as suppressor of profiling 2, transgelin, actin-depolymerizing factor 1 and myosin heavy chain, relate to the resistance to virus (Xu *et al.*, 2005; Zhao, 2007; Bao *et al.*, 2009). In the ubiquitin pathway, ubiqutin-conjugating enzyme E2 and 26S protease regulatory subunit 6B relate to the resistance to virus (Bao *et al.*, 2010). Other molecules related to the resistance to virus include Rab7, beta-N-acetylglucosaminidase 2 (GlcNACase 2), amino acid transporter, potassium coupled amino acid transporter, insect intestinal mucin 3 and juvenile hormone epoxide hydrolase (Bao *et al.*, 2009; Qin *et al.*, 2012).

It is reported that in *Bombyx mori*, BmCaspase1 (cap1), BmCaspase3 (cap3), BmICE (bmi), BmDcr-like (dicer), BmAgo2 (argo), carboxylesterase (car) may be related to the resistance to virus (Tanaka *et al.*, 2008). cap1, cap3 and bmi all belong to capases, a cysteine aspirate protease family. These proteins relates to apoptosis, necrosis and inflammation (Alnemri *et al.*, 1996). The family is important for maintaining a stable number of T cells, for tissue differentiation and regeneration, and also plays a significant role in neural development in mammals (Shalini *et al*., 2015). Some members of the family also have an effect on tumor resistance, and have a potential role in maintaining the stability of the genome, metabolism, autophagy and aging (Bernstein *et al*., 2001). dicer, belonging to RNase III family, can recognize and incise foreign dsRNA (Jaskiewicz *et al*., 2008). It is also reported that the family has other functions like facilitating activation of RNA-induced silencing complex (RISC), repairing damaged DNA and playing an important role in the reproduction and development of animals (Ketting *et al*., 2001; Wei *et al*., 2012). argo, belonging to argonaute protein family, is the necessary catalytic part of RISC which plays important role in RNAi (Kai, 2013). Besides, some reports suggest that argonaute protein can inhibit the expression levels of some genes, and stimulate the expression of others (Chu *et al*., 2010). car belongs to caboxylesterase family which is a sublevel of the hydrolase family. Current published studies mainly focus on its functions of hydrolyzing exogenous compounds and activating drugs. It is reported that carboxylesterase has the function of anti-virus (Gao *et al*., 2007). However, it may also play an important role in virus multiplication (Blais *et al*., 2010).

This study aims at examining the transcription levels of these immune related gens, namely *cap1, cap3, bmi, dicer, argo and car*, in different strains of the silkworm after inoculation with BmBDV or BmNPV virus, in order to explore the effects of these viruses on the transcription levels of the virus-related genes.

Materials and Methods

The Bombyx mori *breeding and virus inoculation*

Bombyx mori strains 798 (anti-BmBDV), 306 (susceptible to BmBDV), NB anti-BmNPV) and HUABA (35) (susceptible to BmBDV) were all preserved in the Institute of Life sciences, Jiangsu University. The silkworm strains 798, 306, NB and HUABA (35) were raised routinely with fresh mulberry leaves till the fifth instar before inoculation of viruses. The NB and Huaba silkworm strains were each administered through the mouths with 5 μL of the BmNPV virus at a concentration of 2×10^8 PIB/mL, and 306 and 798 strains were each administered with 5 μL of BmBDV virus at a concentration of 20 mg/mL. The silkworm midgut was isolated from the body at the time points of 24, 48, 72 and 96 h post inoculation of the viruses, washed quickly using PBS buffer and stored in an Eppendorf tube with Sample Protector for RNA/DNA (TaKaRa, Dalian, China). The tube was immersed in liquid nitrogen and then stored at -70 ^{o}C for further use. For comparison, a control group was set up for each strain of the silkworm without the virus treatment.

RNA extraction and reverse transcription into cDNA

TRIzol reagent (Sangon Biotech, Shanghai, China) was used to extract total RNA, and then transcribed into cDNA using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Specifically, the midgut was grinded into powder in liquid nitrogen and then transferred into an RNase-free Eppendorf tube. After the addition of 1 mL TRIzol reagent, the solution was mixed and rested for 5 min before centrifugation at 12,000*g* for 5 min at 4 ^{o}C. The supernatant was transferred into a new RNase-free Eppendorf tube, and 200 μL chloroform was added, mixed and incubated for 2 min. After centrifugation at 12,000*g* for 15 min at 4 ^{o}C, the supernatant was again transferred to a new RNase-free Eppendorf tube. The solution was added with 500 μL isopropanol, mixed, and incubated for 10 min. The supernatant was discarded through centrifugation at 12,000*g* for 10 min at 4 ^{o}C. The precipitate was dried and added with 100 μL DEPC treated pure water. The solution was mixed gentlely and incubated for 10 min at 55 ^{o}C to obtain the RNA solution. The cNDA synthesis was carried out according to the manufacturer's guide. Briefly, an aliquot of 1 μL of oligo dT primer at 50 μM, 1 μL of dNTP mixture (10 mM each), 5 μL of RNA template, and 3 μL of RNase-free H_2O were mixed in a microtube, and then incubated at 65 ^{o}C for 5 min. The tube was then immediately quenched on ice. To

Table 1 The primer sequences used for qPCR

Gene name	Sense primer	Anti-sense primer
bmi	5' CTGCCGACCAACCATACAAG 3'	5' GAACATACCAACCAGCCGTC 3'
argo	5' CCATCGCCAGATCAGAGTAATA 3'	5' GTCACGGAACACGAACACCT 3'
dicer	5' GAAGTTCTGAAGCCAGTTTCGTTAT 3'	5' TGAATGTCTTGAGTTGTGGGAGC 3'
cap1	5' TCTCGCACGGGCACCAAT 3'	5' AACACAGCAACCAGCAGACAAT 3'
cap3	5' GAAATACGCTACGACATACGA 3'	5' TCTACGACTTCAAAGCCAAAC 3'
car	5' TGGAGGAAGTAGTATCAGC 3'	5' AGTGTATCACGGGCAATC 5'
tif-3	5' AGATGACGGGGAGCTTGATGGT 3'	5' GAGGGCGGAATGTACTTGTTGC 3'

the tube, 4 μL of 5×PrimeScript II buffer, 0.5 μL of RNase inhibitor, 1 μL of PrimeScript II RTase, and 4.5 μL of RNase-free H_2O was added and then mixed gentlely. The reverse transcription reaction was carried out at 45 ^oC for 30 min, and then incubated at 95 ^oC for 5 min before cooled down on ice.

qPCR

CDS (BGIBMGA006131-TA) of *bmi*, CDS (BGIBMGA006946-TA) of *cap1*, CDS (BGIBMGA004420-TA) of *cap3*, CDS (BGIBMGA010406-TA) of *argo*, CDS (BGIBMGA011542-TA) of *dicer* and CDS (GIBMGA010988-TA) of *car* were obtained from the Silkworm Genome Database. The primer sequences used for qPCR were listed in Table 1. *tif-3* was used as internal reference. The qPCR was performed with an Applied Biosystems 7300 according to AceQ® qPCR (Vazyme, Naning, China) specifications. The qPCR data was analyzed using $2^{-\Delta\Delta CT}$.

Results

The transcriptional levels of the immune genes in silkworm strains 798 and 306 inoculated with BmBDV

The transcription levels of *bmi* in the control groups increased steadily and significantly from 0 h to 72 h (Fig. 1a). The highest level was reached at 72 h, and then the level began to decrease, which was probably because the virus replication rate had decreased after this time point. It is interesting that when the silkworms are inoculated with BmBDV, the expression level of *bmi* was significantly inhibited, maintaining at similar or lower level than on the first day (Fig. 1a).

On the contrary to *bmi*, the transcription levels of *argo* in the control groups decreased sharply during the first 24 h and then maintained essentially unchanged at very low levels (Fig. 1b). However, for silkworms that were inoculated with the virus, the level began to increase notably after 24 h and remain at a relatively high level (Fig. 1b). We notice a slight decrease of the expression in the virus-treated silkworms after 72 h, which may be contributed to the decreased virus replication rate similar to the case in *bmi* (Fig. 1b).

The transcription levels of *cap1* was mostly unchanged in control 798 and decreased in control 306, while it increased notably in virus-treated 306 and increased briefly and then decreased in the virus treated 798 (Fig. 1c). The data indicate that virus invasion can stimulate the expression of *cap1*, temporarily for 798 and relatively longer for 306. Also, *cap1* expression differs significantly between virus-resistant and -susceptible strains, both in untreated and virus-treated groups.

The transcription levels of *cap3* kept low in control 798 and even lower in 306. However, its level increased notably in the virus-susceptible 306 treated with BmBDV, and much more significantly in the resistant strain 798 treated with the virus (Fig. 1d). This is quite different from the case for *cap1* in the 798 strain, whose expression raised briefly and then remained below the starting level. These observations indicate that *cap1* and *cap3*, though both belong to the caspase family, may function very differently during viral invasion.

The transcription levels of *dicer* increased significantly in control 798 and even more significantly for 306 on the second day (24 h) of the fifth instar and remained at high levels thereafter (Fig. 1e). Like *bmi*, the up-regulation of *dicer* during the fifth instar indicates that these two genes may be required during metamorphosis. However, after virus treatment, *dicer* expression in 306 increased slightly on the second day (24 h) and then decreased to below the starting level after this time point. For virus-treated 798 strain, *dicer* expression remained always below the starting level (Fig. 1e). BmBDV appears to inhibit the transcription levels of both genes in either virus-resistant or susceptible strains.

The transcription levels of *car* increased significantly in control 306 and decreased in 798. After virus treatment, the expression in both 306 and 798 remained basically below the starting level (Fig. 1f).

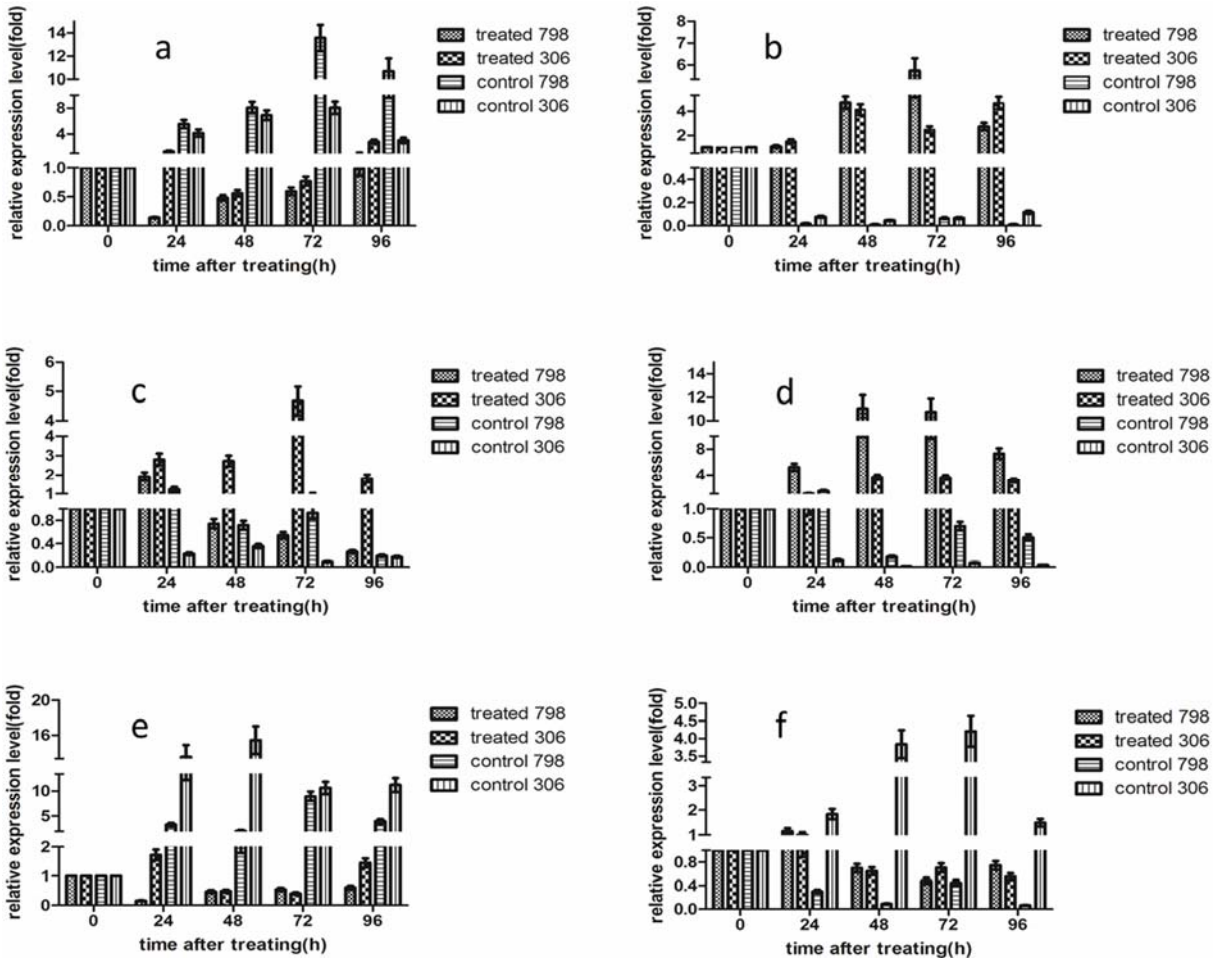

Fig. 1 The expression levels of different genes in midguts of 798 and 306 infected with BmBDV at different time points. a: *bmi*; b: *argo*; c: *cap1*; d: *cap3*; e: *dicer*; f: *car*.

The transcription levels of immune genes in silkworm strains NB and HUABA (35) inoculated with BmNPV

The transcription levels of *bmi* decreased steadily in control silkworm strains NB (viral-resistant) and HUABA (35) (viral susceptible), while increased notably for NB and significantly for HUABA (35) after BmNPV treatment (Fig. 2a). Compared with BmBDV treatment, which inhibited *bmi* expression, BmNPV can stimulate the expression. Although the two viruses have different effect on the gene expression, both have reversed the original expression patterns.

The transcription levels of *argo* was enhanced dramatically in control HUABA (35) strain, and decreased markedly in control NB (Fig. 2b). After virus treatment, the overall expression increased for both strains (Fig. 2b).

The transcription levels of *cap1* enhanced in control HUABA (35) but decreased in control NB, while it increased dramatically in virus-treated HUABA (35) and NB (Fig. 2c). Similar to BmBDV treatment, BmNPV can also stimulate the gene expression, but much more effectively.

The transcription levels of *cap3* decreased slightly in control NB and increased dramatically in control HUABA (35) (Fig. 2d). The overall expression after viral treatment generally increased and remained relatively stable, slightly above the starting level for both strains.

The transcription levels of *dicer* decreased and remained below the starting level in untreated NB and HUABA (35), while it increased notably in both strains after BmNPV treatment (Fig. 2e). Compared with BmBDV treatment, which inhibited *dicer* expression, BmNPV can stimulate the expression. Although the two viruses have different effect on the gene expression, both have reversed the original expression patterns.

The transcription levels of *car* enhanced markedly in control HUABA (35) but decreased in control NB (Fig. 2f). After BmNPV treatment, the expression decreased and remained below the starting level for HUABA (35). For virus-treated NB, the expression increased slightly after 24 h and kept basically unchanged after that (Fig. 2f). The results suggest that *car* function differently in NB and HUABA (35), with NB down-regulated and HUABA (35) up-regulated, whereas BmNPV virus can inhibit both the down-regulation of the former and up-regulation of the latter.

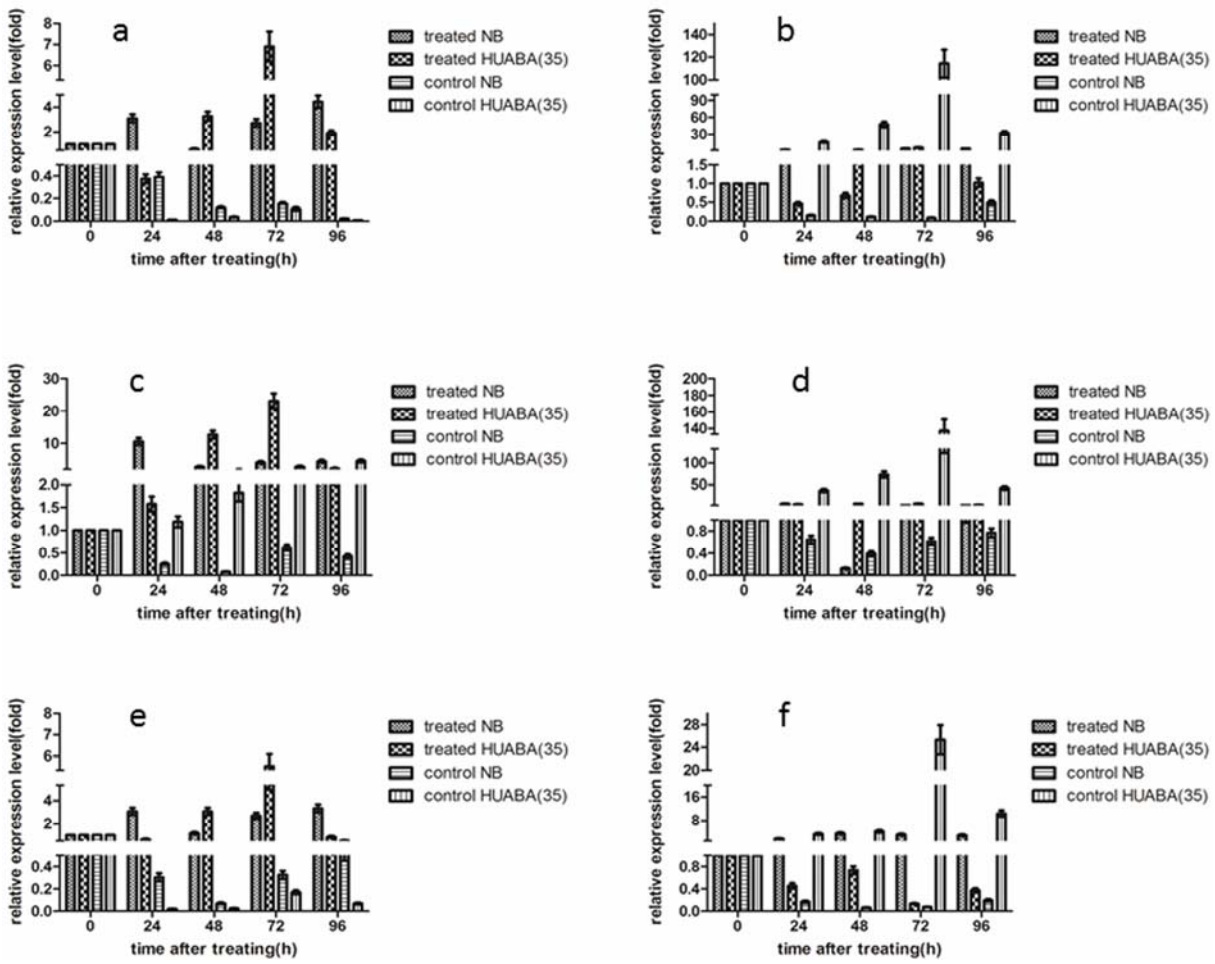

Fig. 2 The expression levels of different genes in midguts of 798 and 306 infected with BmNPV at different time points. a: *bmi*; b: *argo*; c: *cap1*; d: *cap3*; e: *dicer*; f: *car*.

Discussion

The transcriptional changes of six immune genes related to virus infections have been systematically investigated by qPCR. Two different silkworm viruses BmBDV and BmNPV were used to introduce viral infections. Both BmBDV and BmNPV can initiate viral infections to the silkworm midgut, thus the midgut was collected and analyzed, and a time course analysis was used to examine the transcriptional changes.

The results clearly showed that the transcriptional levels of these genes were quite different in different silkworm strains, and transcriptional changes upon virus treatment were also quite different. When infected with viruses, the transcription levels of *bmi* and *dicer* decreased in 798 and 306, but enhanced in NB and HUABA (35); the transcriptional levels of *argo* and *cap3* enhanced in 798, 306 and NB, but decreased in HUABA (35); the transcriptional levels of *cap1* enhanced in 798, 306, NB and HUABA (35); the transcription levels of *car* decreased in 798, 306 and HUABA (35) and increased in NB. However, a conclusion could be drawn based on the results, that viral treatment can adversely affect the gene expression patterns. That is, no matter the gene was up-regulated or down-regulated in the silkworm, viral treatment can inhibit this up-regulation or down-regulation.

It is interesting to note the transcription patterns of *bmi* and *dicer* are virus-specific. Compared with BmBDV treatment, which inhibited *bmi* expression, BmNPV can stimulate the expression. The transcriptional levels of *cap1* and *cap3* were enhanced briefly and then decreased in BmNPV treated NB strain, which may be caused by the apoptosis-inhibitory genes encoded by Baculovirus (Beidler *et al*., 1995, Roy *et al*., 1995). The transcriptional levels of *car* enhanced in BmNPV treated NB, the resistant strain, and decreased in BmNPV treated HUABA (35), the susceptible stain, suggesting that *car* has anti-BmNPV function.

dicer belongs to RNase III which can recognize and cut dsRNA. The transcription level of *dicer* increased in control silkworm strains but decreased when BmBDV virus-treated strains (Fig. 1e), indicating that the gene is related to virus infection. Similar to *dicer*, the transcription of *bmi*, an

interleukin-1-beta converting enzyme that belongs to capase1, also decreased in the virus treated silkworms (Fig. 1a). By contrast, the transcription levels of *argo* and *cap3* enhanced in BmBDV treated silkworms (Figs 1b, d). The anti-virus function of argonaute protein is mainly executed by RNAi, and *cap3* may involve in the resistance to diseases via translation to the corresponding protein.

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