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RESEARCH REPORT

Reference gene selection for quantitative real-time polymerase chain reaction analysis in *Bombyx mori* nucleopolyhedrovirus-infected silkworms

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Abstract

Bombyx mori nucleopolyhedrovirus (BmNPV) is the most serious viral disease in silkworms. To investigate the mechanisms of the immune responses of *B. mori* to a BmNPV infection, a suitable reference gene (RG) is necessary for normalizing data when studying the expression of genes in BmNPV-infected silkworms or cells. Thus, quantitative real-time PCR polymerase chain reaction was used to compare the stability of expression of nine potential RGs, including the *actin A3*, *translation initiation factor 3* (*TIF-3*), *TIF-A4*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *18S RNA*, *28S RNA*, *TATA-binding protein* (*TBP*), *ribosomal protein L3* (*Rpl3*), and *α-tubulin* genes, in silkworms infected with BmNPV. The results were analyzed by BestKeeper, geNorm, and NormFinder software. Overall, *α-tubulin* exhibited the most stable gene expression in BmNPV-infected silkworms, and this was verified by western blotting of the α-tubulin protein. Moreover, we detected the expression of some genes involved in the immune signaling pathways of silkworms after BmNPV infection using *α-tubulin* as an internal RG.

Key Words: *Bombyx mori*; reference genes; BmNPV; qPCR; immune genes

Introduction

The domesticated silkworm (*Bombyx mori*) is a lepidopteran model insect and an important economic insect for silk production. Sericulture is a principal source of income for farmers in many developing countries (Jiang and Xia, 2014). China has the most prominent sericulture history of any country. However, the disease caused by *B. mori* nucleopolyhedrovirus (BmNPV) is the most serious viral infectious disease of silkworms, and it is difficult to control, which has resulted in 60 % decreases in silk production in major sericultural areas worldwide.

Silkworms have an efficient and potent innate immune system to discriminate and eliminate invading pathogens and parasites. There are four signaling pathways that mediate immune responses against different pathogens, including the kinase/signal transducer and activator of transcription Janus (JAK-STAT) signaling pathway, the Toll signaling pathway, and the immunodeficiency (IMD) signaling pathway. Moreover, most silkworm strains are highly susceptible to BmNPV, while only a few are resistant (Chen *et al.*, 2003). Therefore, it is important to screen resistance genes and elucidate the immune mechanisms of silkworms against BmNPV. However, the mechanisms underlying the immune response to BmNPV are still unknown.

Many studies have examined the immune responses of silkworms after BmNPV infection. However, they have generated conflicting results. For example, some studies indicated that the Toll pathway is activated after BmNPV infection (Yang *et al.,* 2013), whereas others did not (Liu *et al.,* 2015). While the JAK-STAT pathway of silkworms is activated after silkworms challenged with BmNPV (Liu et al., 2015), overexpression of BmSTAT in BmN cells does not enhance anti-BmNPV activity (Zhang, 2011). We believe that the use of different reference genes (RGs) by different laboratories may account for these discrepancies.

Gene expression analysis has become a hot topic in many research fields. Quantitative methods of analyzing gene expression at the transcriptional

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Table 1 Candidate RGs and their primers for qPCR analysis

Primer name	Sequence(5'-3')	Length (bp)
Actin A3	F:CGGAATCGTCACTAACTGGG R:GCGGGCGTGTTGAATGT	173
TIF-A4	F: GAATGGACCCTGGGACACTT R: CTGACTGGGCTTGAGCGATA	186
GAPDH	F: TGTTGAGGGCTTGATGAC R: ACCTTACCCACAGCTTTG	150
α-tubulin	F: CTCCCTCCTCCATACCCT R: ATCAACTACCAGCCACCC	186
28sRNA	F:CCCAGTGCTCTGAATGTCAAC R:AGATAGGGACAGTGGGAATCTC	150
18sRNA	F: CGATCCGCCGACGTTACTACA R: GTCCGGGCCTGGTGAGATTT	201
TBP	F:GGTTGTGCCTGGGACTGT R:CACTCACCCGAAGTTTTCC	210
RpL3	F:GAAGATGATCCGCTACTGT R:TATCCTTTGCCCTTGGTG	232
TIF3	F:AGATGACGGGGAGCTTGATGGT R:GAGGGCGGAATGTACTTGTTGC	200

level, such as quantitate polymerase chain reaction (qPCR), RNA blotting, RNase protection analysis, and gene chip technology, as well as quantitative methods that analyze expression at the protein level, such as western blotting, all require the calculation of a RG to target gene expression ratio to obtain reliable results.

In this study, we screened nine candidate RGs (*actin A3*, *translation initiation factor 3* (*TIF-3*), *TIF-A4*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *18S RNA* (*18s*), *28S RNA* (*28s*), *TATA-binding protein (TBP), ribosomal protein L3 (Rpl3)*, and *α-tubulin*) using qPCR, and we analyzed the data with BestKeeper, geNorm, and NormFinder software. The results suggested that *α-tubulin* is the most appropriate RG for gene expression analyses when challenging silkworms with BmNPV. Moreover, we detected the expression of some genes involved in the immune signaling pathways of silkworms after BmNPV infection using *α-tubulin* as an internal RG.

Materials and Methods

Silkworm strains and virus

The BmNPV-resistant silkworm strain NB (median lethal dose [LD_{50}] = 2.5×10^8 polyhedral inclusion bodies [PIBs]/larva), the BmNPV-susceptible silkworm strain 306 (LD_{50} = 3.4×10^5 PIBs/larva), and the BC8 strain, which is also BmNPV-resistant and nearly isogenic to the NB strain, were used in this study. The near-isogenic (BC8) line was prepared in accordance with the method (Chen *et al*., 2003). These strains were preserved in our laboratory, and newly exuviated fifth-instar larvae were used for these experiments. BmNPV was propagated in silkworm strain 306, and the occlusion bodies (OBs) of BmNPV were isolated and purified from the infected *B. mori* larvae. The numbers of obtained OBs and cells were examined using a hemocytometer under light microscope. (Peng *et al*., 2013).

Virus inoculation and midgut collection

All larvae were infected orally with 5 μL of the BmNPV virus at a concentration of 2×10^8 PIBs/mL. Then, the midgut was collected for RNA extraction at 0, 12, 24, 48, and 96 h post-infection, washed quickly using phosphate-buffered saline, and stored in an Eppendorf tube containing Sample Protector for RNA/DNA (TaKaRa, Dalian, China). The tube was immersed in liquid nitrogen and then stored at −70 ℃ for further use. For comparison, a control group was set up for each silkworm strain without the virus treatment.

RNA extraction and cDNA synthesis

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed from 3 μg of total RNA using the Moloney murine leukemia virus reverse transcriptase (Vazyme, Nanjing, China) according to the manufacturer's instructions.

Table 2 *Bombyx mori* genes and their primers for qPCR analysis

Primer name	Sequence (5'-3')	Length
BmToll9-1	F:ATGAGTCAGTGGTGCCAGTTC R:ATCAGATAGTGGAGGGTCGTT	133
BmMyD88	F:TTATACCTAGCGATGGACCTGAT R:CTTATTGCTACACTGGTGGATGG	139
BmSTAT	F:GCCGAGATGCTGGACGACA R:TCCGCCAACCAACAGACGA	200
BmJun	F:CGCTTCCAAATGTAGACGACG R:ACCTGCTCCTTTAGCCTGTGC	140
BmIMD	F:ACGAAGAAGTTATCATTGAGGAA R:TTATGGTTGTTAGGGTCAGGTTT	169

Vector construction and qPCR

cDNAs of the nine RGs were amplified by PCR and individually cloned into the pMD18-T vector (Takara, Dalina, China). The positive recombinant plasmids were used as standard quantitative templates to construct standard curves for absolute transcript quantifications. The templates were diluted to different concentrations for qPCR. qPCR was performed using a 7300 Fast system (Applied Biosystems, Foster City, CA, USA) with the SYBR Green Master Mix Kit (Vazyme, Nanjing, China), according to the manufacturer's instructions. The primers used for qPCR are listed in Tables 1 and 2. The reaction volume was 20 μL with 2 μL diluted cDNA, 10 μL 2×SYBR Master Mix, and 200 nM of each primer. All samples were amplified in triplicates and three biological replicates were performed. The Ct values and the corresponding numerical value were imported into Microsoft Excel and used for further analysis.

Calculation of the stability of expression of the RGs

The stability analysis was dependent on the qPCR quantification using the standard curves, and the data were analyzed with the BestKeeper, GeNorm, and Normfinder tools. The relative expression of immune genes was calculated with the $\Delta\Delta C_T$ value. First, the ΔC_T for each sample of the virus-infected and control groups was calculated. Second, the maximal differences between the values were calculated as the $\Delta\Delta C_T$. The concrete data analysis strategies were described in results.

Western blotting

The midgut of silkworms were collected and mixed with 5×loading buffer and boiled for 5 min. The protein samples were separated on SDS-PAGE, transferred on to a nitrocellulose membrane and incubated with mouse α-tubulin (1:2,000; Proteintech, China), Then, the membrane was further incubated with HRP labeled goat anti-mouse IgG (1:20,000; Proteintech, China). The protein bands were identified by exposure to the Clarity Western ECL Substrate (Bio-Rad, USA).

Results

qPCR analysis of candidate RGs after BmNPV infection

We used *actin A3*, *TIF-3*, *TIF-A4*, *GAPDH*, *18s*, *28s*, *TBP*, *Rpl3*, and *α-tubulin* as candidate RGs. qPCR primers were designed based on the gene sequences in the National Center for Biotechnology Information, and PCR results showed that all the qPCR primers exhibited a high degree of specificity (Fig. 1).

To assess the expression stabilities of the nine candidate RGs in BmNPV-infected samples, we individually cloned them into the pMD18-T vector. Positive recombinant plasmids were used as templates to construct standard curves for absolute transcript quantifications. Because of the various behaviors of the candidate RGs, we evaluated their expression stability in BmNPV-infected silkworms. To identify the optimal RG, we used the BestKeeper, geNorm, and NormFinder data analysis software tools, which are used most frequently for analyzing the stability of RGs (Vandesompele *et al.,* 2002; Pfaffl *et al.,* 2004; Mallona *et al.,* 2010).

Table 3 Analysis of the candidate reference genes in view of the stability values estimated by NormFinder

Gene name	Rank	Stability value
Actin-A3	1	0.009
TIF-3	2	0.055
α-tubulin	3	0.081
28sRNA	4	0.101
TIF-A4	5	0.104
RpL3	6	0.105
18sRNA	7	0.117
GAPDH	8	0.131
TBP	9	0.349

Table 4 BestKeeper analysis results of the candidate reference genes

Gene name	Rank	r	P-value	SD(±CP)
α-tubulin	1	0.95	0.001	1.271
Actin-A3	2	0.93	0.001	0.802
28sRNA	3	0.92	0.001	1.179
RpL3	4	0.92	0.001	1.708
TIF-A4	5	0.91	0.002	1.160
GAPDH	6	0.89	0.003	1.288
TIF3	7	0.88	0.004	0.724
18sRNA	8	0.82	0.012	1.617
TBP	9	0.20	0.628	3.036

NormFinder analysis

NormFinder is one of the Visual Basic application tools for Microsoft Excel. It is an Add-In for Microsoft Excel; namely, the NormFinder function is added directly to the Microsoft Excel software package. For this algorithm, more stable genes have lower stability values (Vandesompele *et al.,* 2002). Additionally, NormFinder can estimate intra- and inter-group variations as well. The NormFinder results are shown in Table 3. *Actin-A3*, *TIF-3*, and *α-tubulin* were estimated to be the most stable RGs, with stability values of 0.009, 0.055, and 0.081 respectively, while the least stable gene was *TBP*, with a stability value of 0.349.

geNorm analysis

geNorm software is also a Visual Basic application tool for Microsoft Excel. It identifies the most stable reference genes from a given sample and determines the gene expression normalization factors according to the geometric mean values of candidate genes. The parameter employed by geNorm to measure the stability of candidate genes is the average expression stability (*M*) value. The *M* value is calculated according to the average pairwise variation among all detected genes. A lower *M* value indicates higher stability of gene expression (Bustin *et al.,* 2009). The geNorm results are shown in Figure 2. For the BmNPV-infected samples, *TIF-4A* and *α-tubulin* were the most stable RGs, while *TBP* was the least stable RG.

BestKeeper analysis

BestKeeper is an Excel-based spreadsheet software application. Different from the above two tools, BestKeeper can analyze raw Ct values, without any conversion (Tang *et al*., 2015). When the original Ct values were imported, the descriptive statistics of each candidate gene were computed. The BestKeeper results are shown in Table 4. The

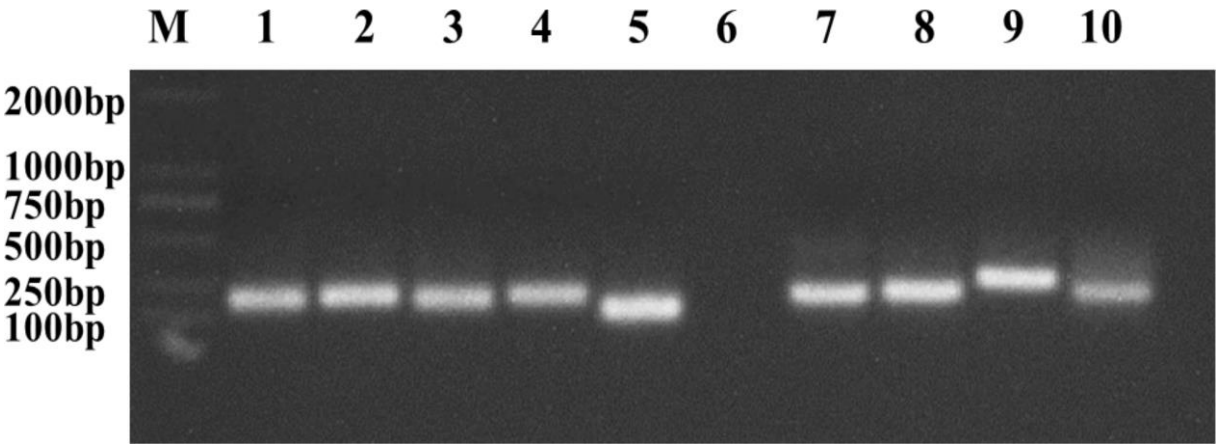

Fig. 1 Identification of primer specificity for qPCR amplification by PCR. 1.0 % agarose gel electrophoresis displayed the PCR products of each primer pair. M: Marker; Line1: Actin-A3; Line2: TIF-A4; Line3: GAPDH; Line4: α-tubulin; Line5: 28sRNA; Line7: 18sRNA; Line8: TBP; Line9: RpL3; Line10: TIF3.

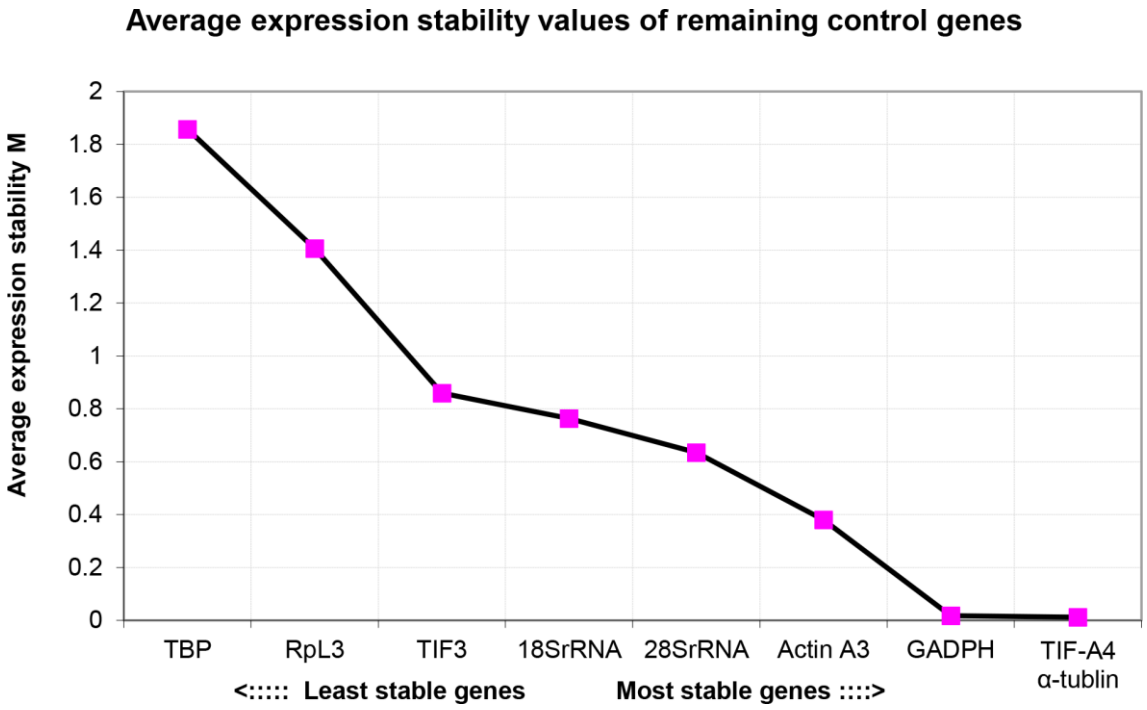

Fig. 2 Average expression stability (M) values of the candidate genes. The average expression stability (M) values are acquired through the stepwise exclusion of the least stable reference gene. Starting from the least stable gene at the left, the genes are ranked according to the ascending expression stability, ending with the two most stable genes at the right.

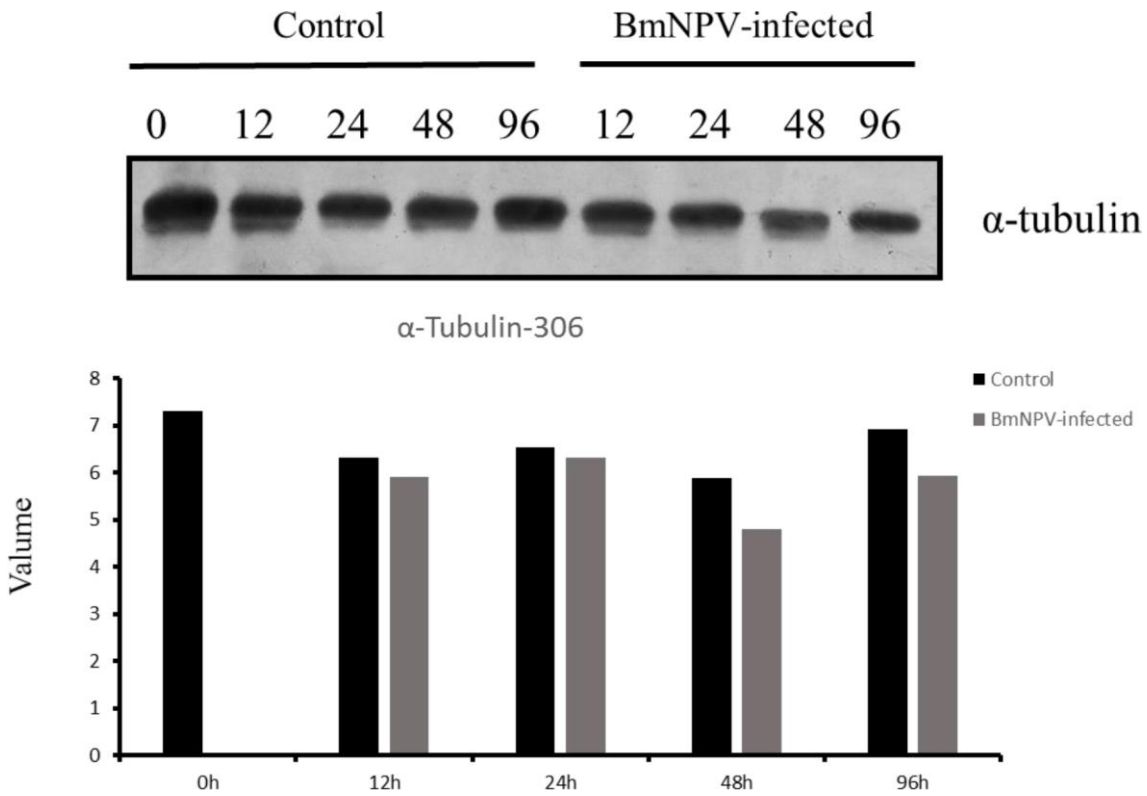

Fig. 3 Protein expression of α-tubulin in midgut of silkworm after BmNPV infected and no-infected. The Black columns represent control group and the gray columns represent treatment group. Control represents no viral infection.

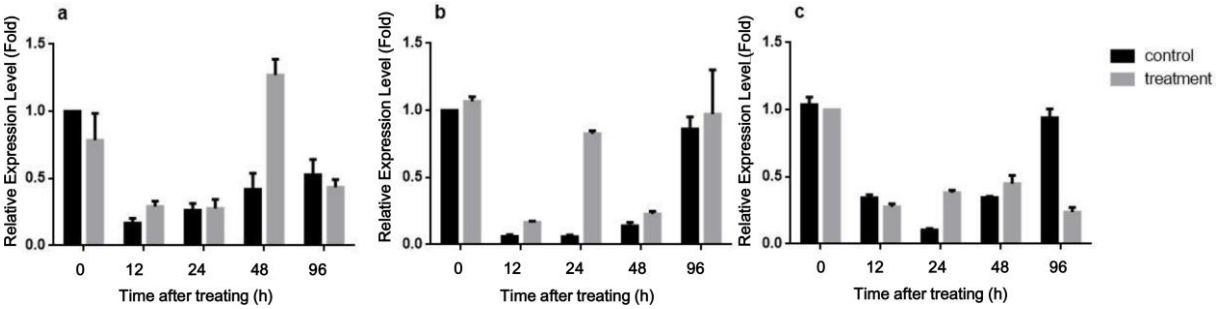

Fig. 4 The expression levels of BmToll9-1 in midguts 306, BC8 and NB infected with BmNPV at different time points. a: 306; b: BC8; c: NB.

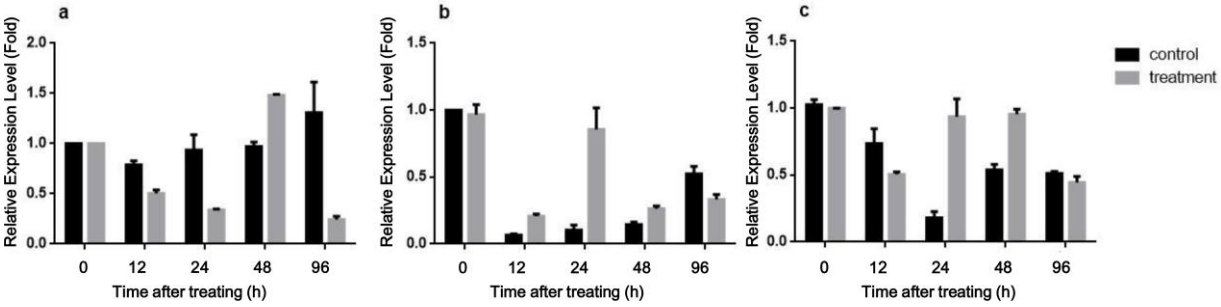

Fig. 5 The expression levels of BmMyD88 in midguts 306, BC8 and NB infected with BmNPV at different time points. a: 306; b: BC8; c: NB.

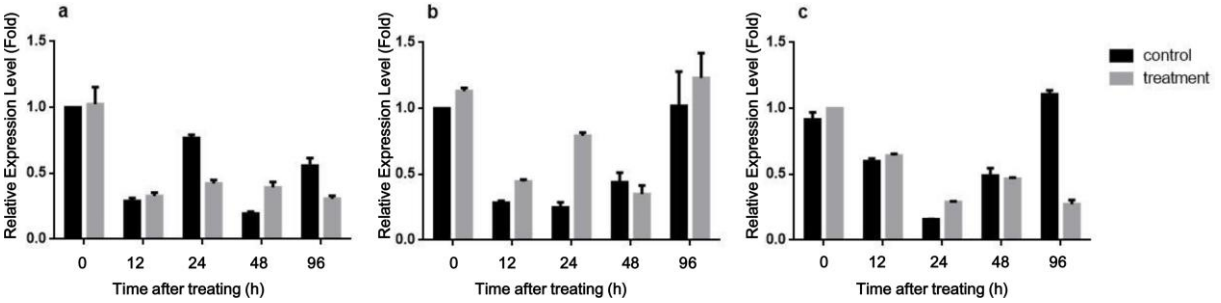

Fig. 6 The expression levels of BmSTAT in midguts 306, BC8 and NB infected with BmNPV at different time points. a: 306; b: BC8; c: NB

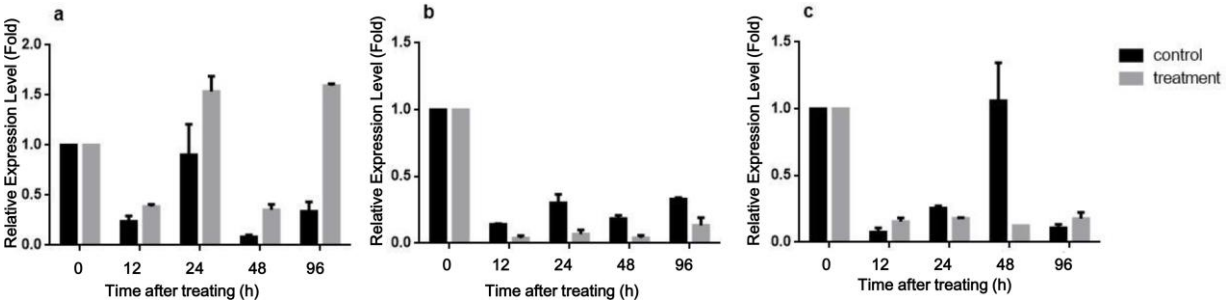

Fig. 7 The expression levels of BmJun in midguts 306, BC8 and NB infected with BmNPV at different time points. a: 306; b: BC8; c: NB

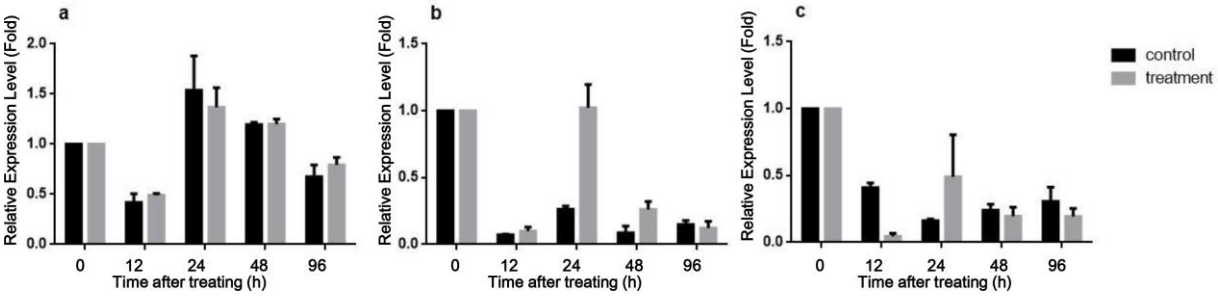

Fig. 8 The expression levels of BmIMD in midguts 306, BC8 and NB infected with BmNPV at different time points. a: 306; b: BC8; c: NB

results indicated that *α-tubulin* was the optimal RG, while TBP was the least stable RG. In brief, the BestKeeper result were almost identical to those obtained using NormFinder and geNorm.

Protein expression level of α-tubulin

To verify the stability of α-tubulin expression in the midgut of silkworms after BmNPV infection, we examined its expression at the protein level. Western blotting results showed that the expression of α-tubulin did not differ significantly between the control and treatment groups (Fig. 3). Thus, we selected *α-tubulin* as the optimal RG for further validation.

Expression of immune genes in silkworm strains 306, NB, and BC8 infected with BmNPV

The transcription level of *BmToll9-1* in the BmNPV-infected group increased steadily and significantly from 0 h to 96 h. In the 306 strain (which is susceptible to BmNPV), the highest transcription level was reached at 48 h, and then it began to decrease, which was probably because the virus replication rate decreased after this time point (Fig. 4a). When the BmNPV-resistant NB and BC8 strains were infected with BmNPV, the expression levels of *BmToll9-1* increased notably in the virus-treated groups (Figs 4b, c). The highest expression levels were reached at 24 h. The *BmMyD88* transcription level of the BmNPV-infected group decreased compared with that of the control group in strain 306 (Fig. 5a), but it increased sharply during the first 24 h and remained at a relatively high level in the BmNPV-infected NB and BC8 strains (Figs 5b, c). The data indicate that virus invasion can stimulate the expression of *BmToll9-1* and *BmMyD88* early in the NB and BC8 strains, and relatively late in strain 306. We observed a slight decrease of *BmMyD88* expression in the virus-treated silkworms after 96 h, which may have contributed to the decreased virus replication rate, similar to the case of *BmToll9-1*.

In contrast to *BmToll9-1*, the transcription level of *BmSTAT* mostly slightly decreased in the BmNPV-infected 306 strain at 24 h (Fig. 6a), while it increased briefly at 48 h. However, its level increased slightly in the BmNPV-resistant NB and BC8 strains infected with BmNPV at 24 h (Figs 6b, c). This differed substantially from the *BmToll9-1* and *BmMyD88* genes in the 306, NB, and BC8 strains, whose expression increased significantly. *BmToll9-1* and *BmMyD88* belong to the Toll signaling pathway, while *BmSTAT* belongs to the JAK-STAT signaling pathway, which indicates that the Toll signaling pathway can be activated rapidly after virus invasion, while virus invasion only has a small effect on the JAK-STAT signaling pathway.

The transcription level of *BmJun* decreased and remained below the starting level in the untreated 306 strain (Fig. 7a), while it increased notably after BmNPV treatment. For the NB and BC8 strains, *BmJun* expression also always remained below the starting level; it is interesting that it decreased notably after virus treatment (Figs 7b, c). These results suggest that BmNPV can upregulate gene expression in a BmNPV-susceptible strain, while downregulating expression in BmNPV-resistant strains.

The transcription level of *BmIMD* was mostly unchanged in the 306 control group (Fig. 8a), and it decreased in the NB and BC8 control groups (Fig. 8b). It remained steady in the virus-treated 306 strain and increased briefly and then decreased in the virus-treated NB and BC8 strains (Fig. 8c). The data indicate that virus invasion can stimulate the expression of *BmIMD* temporarily in virus-resistant strains.

Discussion

Bombyx mori is a lepidopteran model insect, and it is an important economic insect for silk production. Antiviral studies in silkworms are essential to improve sericulture production and control insect pests (Jiang and Xia, 2014; Zhang *et al*., 2014a). Therefore, to understand silkworms at the molecular level and to determine the specific mechanisms of BmNPV resistance, the selection of stable RGs for gene expression normalization in silkworms is imperative. In previous studies, different traditional RGs (such as *actin-3* (Gao *et al.,* 2014b; Wang *et al*., 2014, 2015; Kolliopoulou *et al*., 2015), *TIF-3* (Zhou, *et al*., 2013), *GAPDH* (Bao *et al*., 2009), *TIF-4A* (also known as *sw22934*) (Jiang *et al*., 2012a, b, 2013b), and *18s*) were used as internal RGs in BmNPV-infected silkworms. Although (Guo *et al*., 2014) selected an internal RG for an expression analysis, using relative expression ananlysis method, when challenging silkworms with BmNPV, BmCPV, and BmBDV, it is necessary to select **a** more optimal RG when silkworms are infected only with BmNPV. Thus, in the present study, the BestKeeper, geNorm, and NormFinder tools were employed to calculate

the stabilities of nine candidate RGs in *B. mori* after BmNPV infection. The results suggested that *α-tubulin* is a suitable RG for gene expression analyses after BmNPV infection in silkworms.

Using this RG, the transcriptional changes of five immune genes related to virus infections were investigated systematically by qPCR. BmNPV can initiate viral infections in the silkworm midgut; thus, the midgut was collected and analyzed, and a time-course analysis was used to examine the transcriptional changes.

When *α-tubulin* was used as the RG after BmNPV infection, the transcription levels of *BmToll9-1* and *BmJun* were upregulated in the silkworm strain 306, whereas there were no significant increases, and even slight decreases, of *BmMyD88*, *BmSTAT*, and *BmIMD* expression in the BmNPV-infected 306 group, which is consistent with previous conclusions, except for *BmSTAT*. The use of *α-tubulin* as a RG in the previous study may explain why the expression of *BmSTAT* was upregulated when silkworms were infected with BmNPV and BmBDV; however, overexpression of *BmSTAT* could not obviously enhance the anti-BmNPV activity of the silkworms. It is known that baculovirus can alter cellular filamentous actin and affect the *actin* expression level during viral infection and replication (Volkman, 2007); thus, *actin* is not a suitable RG for qPCR analysis in BmNPV infection experiments. Moreover, the Toll and JAK-STAT pathways should be further analyzed to determine whether they are activated in silkworms after BmNPV infection, because we did not observe any significant changes in the expression of *BmMyD88* of the Toll pathway and *BmSTAT* of the JAK-STAT after BmNPV infection, although we demonstrated that *BmToll9-1* and *BmJun* were upregulated.

In addition, we identified a highly BmNPV-resistant strain, NB, and constructed a BmNPV-resistant near-isogenic line, BC**8**. Our results showed that the expression of *BmToll9-1*, *BmMyD88*, *BmJun*, *BmSTAT*, and *BmIMD* were upregulated significantly in both BmNPV-resistant strains, indicating that both the Toll and JAK-STAT innate immune pathways may be activated in the BmNPV-resistant NB strains.

It is interesting to note that the JNK pathway, also named the stress-activated protein kinase pathway (SAPK), is essential for providing a cellular response to extracellular changes such as ultraviolet and reactive oxygen species-induced DNA damage, mechanical stress, and osmolality changes (Neganova et al., 2016). Many researchers have reported that the JNK/SAPK signaling pathway can be activated by stress responses, but few indicated that virus invasion can stimulate this pathway. In this study, we found that BmNPV can stimulate the upregulation of the *BmJun* gene in a viral-susceptible strain, while *BmJun* expression was downregulated in virus resistant strains. All these results are deserving of further study.

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