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ORIGINAL ARTICLE

# *Bm59* is an early gene, but is unessential for the propagation and assembly of *Bombyx mori* nucleopolyhedrovirus

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**Abstract** *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a major pathogen that specifically infects the domestic silkworm and causes serious economic loss to sericulture around the world. The function of BmNPV *Bm59* gene in the viral life cycle is inconclusive. To investigate the role of *Bm59* during viral infection, the transcription initiation site and temporal expression of *Bm59* were analyzed, and *Bm59*-knockout virus was generated through homologous recombination in *Escherichia coli*. The results showed that *Bm59* is an early transcription gene with an atypia early transcriptional start motif. Budded virion (BV) production and DNA replication in the BmN cells transfected with the *Bm59*-knockout virus bacmid were similar to those in the cells transfected with the wild-type virus. Electron microscopy revealed that the occlusion-derived virus can be produced in cells infected with the *Bm59*-knockout virus. These results indicated that *Bm59* is an early gene and is not essential for viral replication or assembly of BmNPV. These findings suggested that non-essential gene (*Bm59*) remained in the viral genome, which may interact with other viral/host genes in a certain situation.

**Keywords** *Bombyx mori* nucleopolyhedrovirus (BmNPV) · *Bm59* gene · Early gene

**Abbreviations**

| | |
|---|---|
| BmNPV | *Bombyx mori* nucleopolyhedrovirus |
| BV | Budded virion |
| ODVs | Occlusion-derived virions |
| OBs | Occlusion bodies |
| PM | Peritrophic membrane |
| bp | Base pairs |
| ORFs | Open reading frames |
| *Ac73* | AcMNPV ORF73 |
| cDNA | Complementary DNA |
| RACE | Rapid-amplification of cDNA ends |
| LB | Luria–Bertani |
| *Polh* | *Polyhedron* |
| *egfp* | Green fluorescent protein |
| PBS | Phosphate-buffered saline |
| IgG | Immunoglobulin G |
| qRT-PCR | Quantitative reverse transcription PCR |
| pI | Isoelectric point |
| *E. coli* | *Escherichia coli* |
| *CAT* | Chloramphenicol acetyl transferase |
| h p.t | Hours post-transfection |
| h p.i | Hours post-infection |
| $TCID_{50}$ | 50 % tissue culture infective dose |
| RNA polII | RNA polymerase II |

## Introduction

*Bombyx mori* nucleopolyhedrovirus (BmNPV) is a major pathogen that specifically infects the domestic silkworm and causes serious economic loss to sericulture around the world (Shen et al. 2009). The function of baculoviral genes

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has been studied comprehensively in baculoviruses such as BmNPV; however, the function of more than one-third of viral genes is still unknown (Ono et al. 2012). During its life cycle, this baculovirus produces two phenotypes of virions; budded virions (BVs) and occlusion-derived virions (ODVs). The former are produced in the early phase of infection and are responsible for horizontal spread to various types of cells in the host. In the late phase of the virus life cycle, numerous nucleocapsids are enveloped in the nucleus, to form ODVs. These are occluded into polyhedra. The two types of virions have similar nucleocapsid structure and identical genetic material but the origin and composition of their envelopes are different (Blissard 1996; Braunagel and Summers 2007). Occlusion bodies (OBs) in contaminated foods are normally ingested by the silkworm. The alkaline environment of the insect midgut triggers the dissolution of OBs and the release of ODVs into the midgut lumen. Once an ODV is released into the midgut, it breaches the peritrophic membrane (PM) and fuses to the midgut columnar cells, which causes the primary infection in the silkworm (Haas-Stapleton et al. 2004; Slack and Arif 2007). Newly formed nucleocapsids are transported from the nucleus to the cell membrane, where they bud and acquire a new envelope. The BVs spread by the tracheal system and hemolymph, into the other tissues of the insect, cause the secondary infection (Engelhard, et al. 1994).

The genome of BmNPV (T3 strain) is 128,413 base pairs (bp) in length and has 136 open reading frames (ORFs) (Gomi et al. 1999). To date, many of the functions of baculovirus genes have been resolved by the gene knockout technique and there are only a few viral genes, such as BmNPV ORF59 (*Bm59*), in which the features and functions are still obscure (Ono et al. 2012). The *Bm59* gene is a homolog of AcMNPV ORF73 (*Ac73*) but the functions of both *Ac73* and *Bm59* remain unclear. MALDI-TOF analysis results showed that *Ac73* is associated with AcMNPV BV but not with ODV (Wang et al. 2010). Illumina sequencing analysis has been used to show that the transcription start time of the *Bm59* gene is 12-h post-infection in cells infected by BmNPV (Xue et al. 2012).

In this study, we characterized the *Bm59* gene by mapping the transcription start sites and analyzed the possible role of *Bm59* in the virus life cycle. The BmNPV bacmid was used to construct a *Bm59*-knockout virus ($Bm^{Bm59KO-PG}$) through homologous recombination in *Escherichia coli*. We also reconstructed the Bm59-repair virus ($Bm^{Bm59RE-PG}$) and wild-type virus ($Bm^{Bm59WT-PG}$). Our results indicated that *Bm59* is an early gene and is not essential for the propagation or assembly of BmNPV.

## Materials and methods

### Cells and viruses

The BmN cells were maintained at 27 °C in TC-100 insect medium supplemented with 10 % (V/V) fetal calf serum (Gibco, USA). The *Escherichia coli* strain BW25113, which contains plasmid pKD46, was kindly provided by Dr. Mary Berlyn (Yale University). The *E. coli* strain BmDH10Bac, which contained the BmNPV genome and a helper plasmid pMON7124, was constructed previously in our laboratory.

### Validation of the transcription initiation site of *Bm59* using the RACE technique

In accordance with the manufacturer's protocol, total RNA was isolated from the BmN cells infected with BmNPV at 24-h post-infection (h p.i) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Digestion with RNase-free DNase I (Promega, USA), for 15 min at 37 °C, was used to remove contaminating genomic DNA. The quality of the total RNA was determined using the 260/280 absorbance ratio and electrophoresis. The full length of *Bm59* was cloned with rapid-amplification of cDNA ends (RACE), in accordance with the manufacturer's instructions (TaKaRa). The gene-specific primers, 5′ race-Bm59 and 3′ race-Bm59, were designed using the BmNPV T3 genome sequence (all the primers are listed in Table 1). The 5′ and 3′ cDNA (complementary DNA) ends were created using the RACE kit, in accordance with the manufacturer's handbook (TaKaRa, Dalian, China). The PCR products were inserted into the pMD-19 T vector for sequence analysis (Sangon, Shanghai).

### Sequence analysis

The translation and prediction of characteristics of the *Bm59* gene were performed with ExPASy (http://au.expasy.org). Secretory signal sequences were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane domain searches were performed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

### Bm59-knockout bacmid generation

The λ-Red recombination system was used for generation of the *Bm59*-knockout BmNPV bacmid in *E. coli*, as previously described (Datsenko and Wanner 2000; Vanarsdall et al. 2004; Wu et al. 2006; Xiang et al. 2011). The homologous recombinant fragment, which contained a chloramphenicol resistance gene cassette with *Bm59* flanking

**Table 1** Primers used for viral constructs and analyses

| Primer name | Sequence (5′–3′) |
|---|---|
| Bm59KO-1 | ATGAACACGTCCGTGGACGCGGTGACAAAGCTCATTCGTTTGCAGAACTAACATATGAATATCCTCCTTAG |
| Bm59KO-2 | TTATTGTACATAATGTTTTATTGTAATTAAATTTATACAGCGATTTAAGTGTAGGCTGGAGCTGCTTC |
| Bm59Re-1 | GAATTC ATGTACGTTAGCAAAGAGACC |
| Bm59Re-2 | GCGGCCGCTTAAGCGTAATCTGGAACATCGTATGGGTATTGTACATAATGTTTTAT |
| 5′ race-Bm59 | TCAAACTGTTTGCCCGGCGCGTTTAAGG |
| 3′ race-Bm59 | GACCAATATCTAAACAGCGAC |
| QBm59-1 | GACCAATATCTAAACAGCGAC |
| QBm59-2 | TTCGTCAAACACCATGGCGTT |
| Bm59-promoter-kpnI | GGTACCCAACAATACAGACAAAATTTTAG |
| Bm59-promoter-smaI | CCCGGGGTTTCTTTTTTGAAAACTAAATTG |
| M13F | GTTTTCCCAGTCACGAC |
| M13R | CAGGAAACAGCTATGAC |
| QBV-F | TTTGGCAAGGGAACTTTGTC |
| QBV-R | ACAAACCTGGC AGGAGAGAG |
| QDNA-F | CGTAGTGGTAGTAATCGCCGC |
| QDNA-R | AGTCGAGTCGCGTCGCTTT |

regions, was amplified by PCR using the Bm59KO-1/Bm59KO-2 primer pair and pKD3 as the template. The primers contained 48 bp sequences that were homologous to the 5′ and 3′-flanking coding regions of *Bm59* (Table 1). A termination codon (TAA) was artificially introduced into the primer to terminate the translation of the original open reading frame. The linear PCR fragment was electroporated into *E.coli* BW25113-pKD46 competent cells, which contained the BmNPV bacmid. Transformed cells were incubated at 37 °C for 4 h in 800 µl of Luria–Bertani (LB) medium. They were then placed on an LB agar medium that contained kanamycin (50 µg/ml) and chloramphenicol (7 µg/ml) for screening. Confirmation of transformation was performed by PCR analysis.

### Construction of knockout ($Bm^{Bm59KO-PG}$), repair ($Bm^{Bm59RE-PG}$), and wild-type ($Bm^{Bm59WT-PG}$) bacmids that contained *polyhedrin* and *egfp*

To investigate of the effect of the *Bm59* gene knockout on occlusion morphogenesis and virus infection, the *polyhedron* (*polh*) and green fluorescent protein (*egfp*) genes were inserted into the *polyhedrin* locus using a site-specific transposon (Wu et al. 2006; Xiang et al. 2013).

The *Bm59*-knockout bacmid and helper plasmid were electroporated into *E. coli* DH10Bac cells. The electrocompetent DH10Bac cells were then transformed with the vector that contained the *polyhedrin* and *egfp* expression cassette to generate the *Bm59*-knockout bacmid, $Bm^{Bm59KO}$. A 656 bp repair fragment, which contained the *Bm59* native promoter and the total ORF, with an HA-tag prior to the stop codon for the further protein expression validation, was amplified by PCR using the BmNPV bacmid as the template and the Bm59Re-1/Bm59Re-2 primer pair. The PCR product was cloned into pFastBac-PH-GFP (constructed by our laboratory) to generate the repair vector, pFastBac-PH-GFP-*Bm59*RE. The *Bm59*-knockout bacmid; $Bm^{Bm59KO}$, *Bm59*-repair bacmid; *Bm59*-RE and helper plasmid were transformed into the electrocompetent BmDH10Bac cells to obtain $Bm^{Bm59KO-PG}$ and $Bm^{Bm59RE-PG}$. The BmDH10Bac cells, which contained a BmNPV bacmid, were transformed with pFastBac-PH-GFP to produce the *Bm59*-knockout control virus, $Bm^{Bm59WT-PG}$. The presence of the transposon from each bacmid was verified with the M13F/M13R primer pair. The bacmid DNA from the correct recombinant products, $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$ and $Bm^{Bm59WT-PG}$, was extracted and purified with a QIAGEN large-construct kit and quantified by optical density.

### Transcriptional analysis and temporal expression analysis of the *Bm59* gene

Total RNA was isolated from BmN cells infected with BmNPV at various times points after transfection (0, 3, 6, 9, 12, 24, 48, and 72 h). The RNA samples were treated with RNase-free DNase I (Promega, USA) for 15 min at 37 °C. 1000 ng portion of total RNA was used as the template for the first-strand cDNA synthesis. The cDNA was synthesized with an oligo-dT primer, and the PCR was performed with the QBm59-1/QBm59-2 primer pair. The PCR products were analyzed on a 3.0 % agarose gel.

The BmN cells were also treated with the above procedure when the cells were transfected with the repair virus of $Bm^{Bm59RE-PG}$. Cells were washed three times with cold

phosphate-buffered saline (PBS). Cell lysates were analyzed by SDS-PAGE (15 % gel) and subsequently subjected to a Western blotting assay using anti-HA antibody (Huaan, China). Goat anti-rabbit Immunoglobulin G (IgG), conjugated with horseradish peroxidase (Sigma, USA), was used as the secondary antibody.

### Cloning of *Bm59* gene promoter region and construction of dual-luciferase report system for detection promoter

*Bm59* gene promoter region (upstream of initiation codon 216 nt) was cloned from the BmNPV genome, and then it was inserted into the pGL3-Basic vector for the detection of promoter activity. The promoter of ovarian tumor gene (potu) was inserted into the pGL3-Basic vector for the positive control (unpublished data). The negative control was pGL3. These three recombinant vectors (2 μg) with PRL-TK (0.2 μg) were co-transfected into the BmN cell with lipofectin, respectively. The promoter activity was detected with Dual-Luciferase®Reporter Assay System (Promega, USA). The experiment was repeated three times.

### Time course analysis of BV production in BmN cells

The BmN cells ($1.0 \times 10^6$ cells/35-mm-diameter plate) were transfected in triplicate with 2.0 μg of each bacmid using liposome reagent (Invitrogen, Carlsbad, CA, USA). The supernatants that contained BV were collected at selected time points (6, 12, 24, 48, 72, and 96 h). The DNA from the supernatants was extracted using the OMEGA Viral DNA Kit. The QBV-F/QBV-R primer pair was designed for quantitative reverse transcription PCR (qRT-PCR), and the reaction was carried out using the SYBR Premix ExTaq kit (TaKaRa, Dalian, China) on the ABI 7300 Fluorescent Quantification PCR System.

### qRT-PCR analysis of viral DNA replication

A qRT-PCR assay was used to detect viral DNA replication, as previously described (Vanarsdall et al. 2006). The BmN cells were treated as described above. At designated times after transfection (6, 12, 24, 48, 72, and 96 h), cells were collected for examination. The QDNA-F/QDNA-R primer pair for the *gp41* gene was used for the analysis. The extracted DNA was digested with the *Dpn* I enzyme and used for qRT-PCR.

### Electron microscopy analysis

The BmN cells ($2.0 \times 10^6$ cells/35-mm-diameter plate) were transfected with 5.0 μg of the $Bm^{Bm59WT\text{-}PG}$, $Bm^{Bm59KO\text{-}PG}$, and $Bm^{Bm59RE\text{-}PG}$ bacmids and harvested 72 h after transfection. The supernatant was removed, cells were washed with 1 × cacodylate buffer (0.1 M NaCl; 0.05 M cacodylate pH 7.5,) and then fixed in 2.5 % glutaraldehyde, with 0.1 M sodium cacodylate for 30 min. The cells were then washed twice with 1 × cacodylate buffer and fixed with 1:1 osmium tetroxide: cacodylate buffer for 30 min, followed by a wash with 1 × cacodylate buffer, then stained with 2 % uranyl acetate for 30 min. After dehydration through a series of 30–100 % ethanol washes, cells were embedded in Spur resin. Ultra thin sections were obtained and stained with 1 % uranyl acetate and lead citrate. Images were obtained using a JEM-1230 transmission electron microscope.

## Results

### The transcriptional start site of *Bm59*

The *Bm59* gene is located from nucleotide 56,377 to nucleotide 56,676 in the BmNPV genome. To design mutant viruses that have the *Bm59* gene deleted from the genome, it was necessary to identify the transcriptional start site of the gene. A RACE analysis was performed to identify the transcription start and termination sites of *Bm59*. At 24-h post-infection (h p.i), a 5′ RACE product of approximately 360 bp were obtained. Open reading frame (ORF) of *Bm59* contains 300 nucleotides and encodes a 99 amino acid residue, with a predicted molecular mass of 11.5 kDa and a predicted isoelectric point (pI) of 4.96. The transcriptional start motif (CAAC) is 216 nucleotides upstream of the start codon (Fig. 1), which indicated that *Bm59* may be an early transcription gene with an atypia transcriptional start motif.

The 3′ RACE results gave a 241 bp product that was specific to cells infected with the virus at 24 h p.i. This product had a typical polyadenylation signal (AATAAA) located three nucleotides downstream of the stop codon, TAA (Fig. 1). No signal peptide sequence, transmembrane region, or nuclear localization signals were found in the amino acid sequence.

### Transcriptional analysis and temporal expression analysis of *Bm59*

The qRT-PCR analysis indicated that *Bm59* transcription starts from 3 h p.i. and lasts until 96 h p.i. (Fig. 2a). This result demonstrated that *Bm59* is an early gene, which is consistent with the presence of the early gene motif in the putative promoter region. Western blotting also showed that, from 3 to 96 h p.i., Bm59 transcript could be detected (Fig. 2b). Protein expression results were consistent with the results of the transcriptional analysis. To verify early gene of *Bm59*, we cloned the promoter region of *Bm59* and

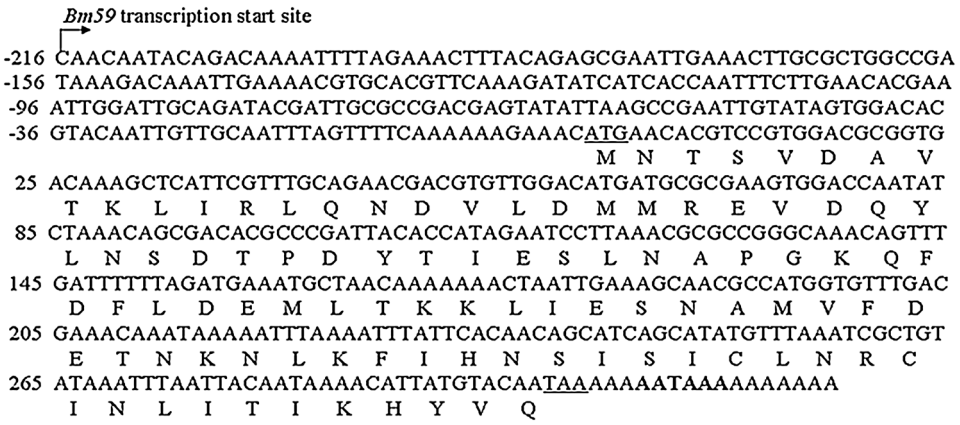



**Fig. 1** A RACE analysis of the *Bm59* gene transcription start site. Localization of the *Bm59* transcription start site, as determined by 5′ RACE. The *arrowhead* shows the initiation site of *Bm59* transcription, the baculovirus early promoter motif (CAAC), and *Bm59* translation start codon (ATG). The stop codon (TAA) is indicated with a *single line*

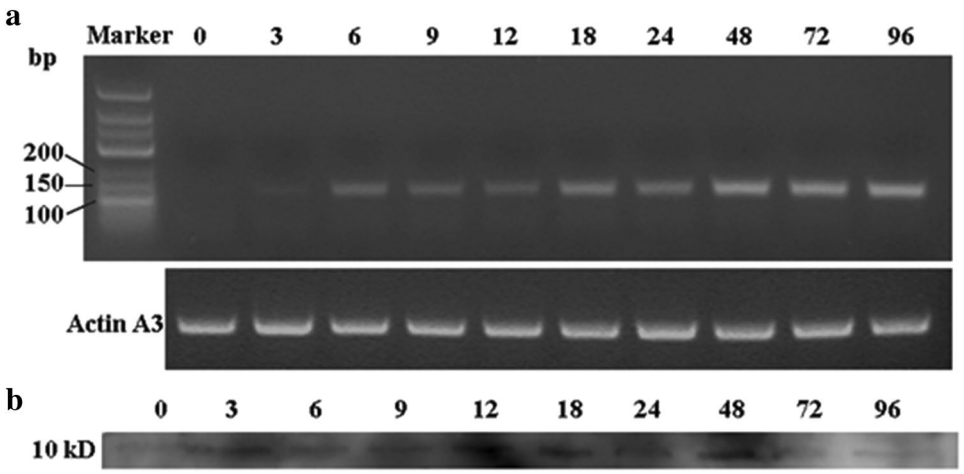



**Fig. 2** Analysis of Bm59 transcription and protein expression in BmN cells infected with BmNPV. **a** Total RNA was isolated from cells at 0, 3, 6, 9, 12, 18, 24, 48, 72, and 96 h p.i. The size of the transcript is indicated in bp on the *right of the panel*. The β-actin qRT-PCR product was used as the control. The products were separated on a 3.0 % agarose gel. **b** Western blotting analysis of Bm59 was performed on BmN cells infected with BmNPV. The cells were collected for the mock infection control at 3, 6, 9, 12, 18, 24, 48, 72, and 96 h p.i., and 20 μg of cell lysate was subjected to Western blotting analysis using anti-HA antibody

inserted into the pGL3-Basic vector for the detection of promoter activity. From the luciferase activity comparison results, we found strong luciferase activity was detected from the recombinant vector transfected cells (Fig. 3). It was indicated that this promoter can be transcribed by the RNA polymerase II (RNA polII) from the BmN cells.

### Construction of *Bm59* deletion and repaired bacmids

The function of *Bm59* in the viral life cycle was determined by evaluating the phenotypes of three recombinant bacmids; $Bm^{Bm59WT\text{-}PG}$, $Bm^{Bm59KO\text{-}PG}$, and $Bm^{Bm59RE\text{-}PG}$ (Fig. 4a, b). The Bm59-knockout bacmid was generated via the λ-Red homologous recombination system in *E. coli*, as described previously (Datsenko and Wanner 2000). All of the recombinant bacmids were verified by PCR analysis. As expected, the Bm59-Re-F/Bm59-Re-R PCR primer pair

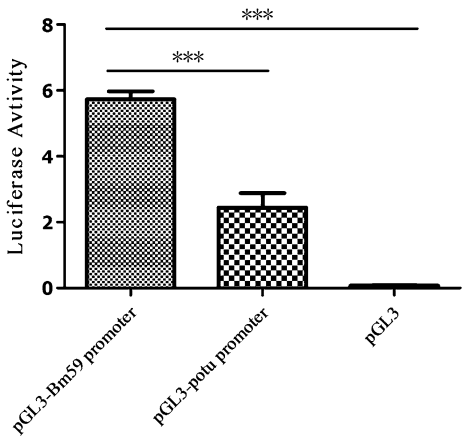
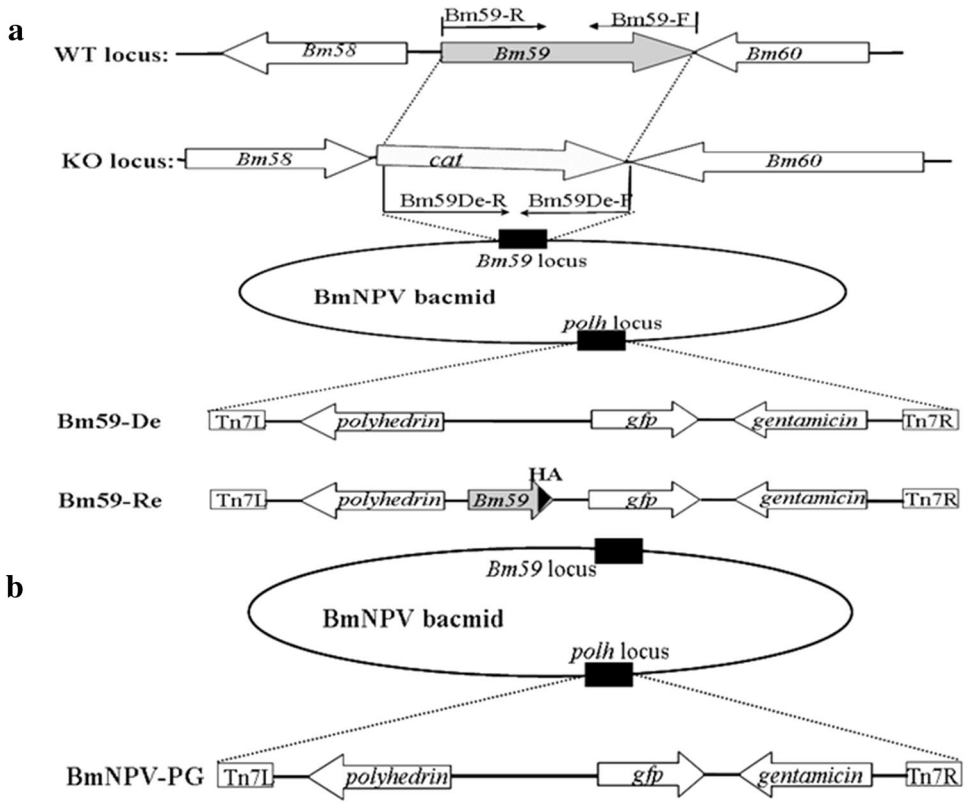



**Fig. 3** Luciferase expression analyses for the promoter of *Bm59* gene. Tested recombinant vector: pGL3-Bm59 promoter, positive recombinant vector: pGL3-potu promoter and negative vector: pGL3



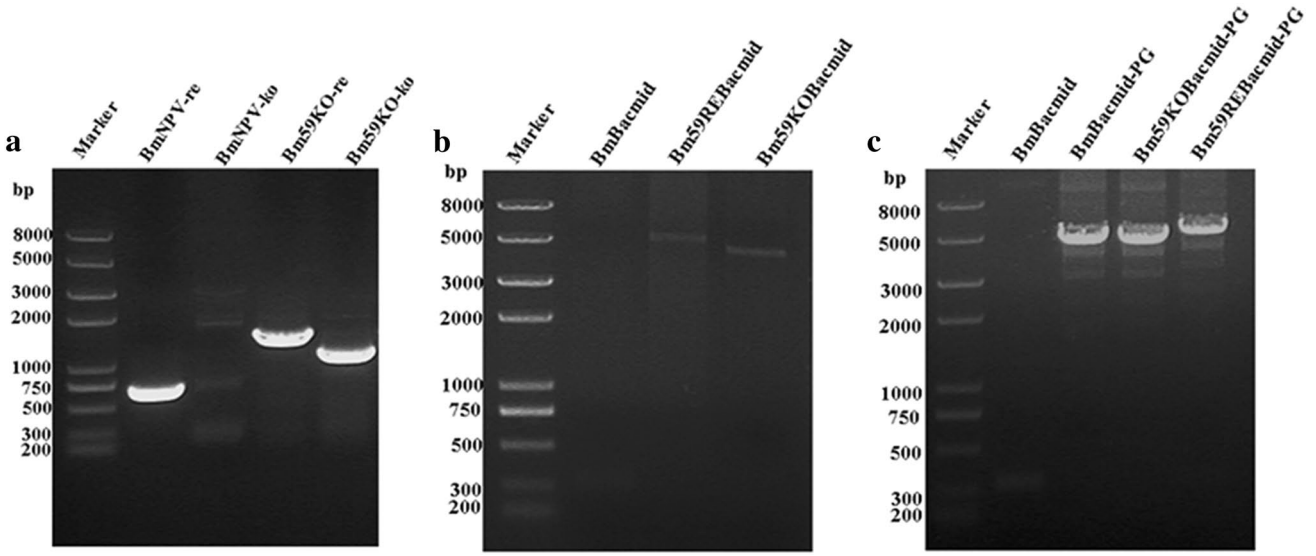




**Fig. 4** Construction the bacmids of $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$, and $Bm^{Bm59WT-PG}$. **a** Schematic diagram for construction of the *Bm59* disrupted and *Bm59* rescued bacmids by recombination in *E. coli*. A chloramphenicol resistance gene cassette was inserted into the *Bm59* ORF to give the disrupted mutant. The *polh*, *egfp*, and *Bm59* repaired genes, along with the gentamicin resistance gene (*Gm*), were inserted into the polyhedrin locus of the BmNPV bacmid to generate $Bm^{Bm59KO-PG}$ and $Bm^{Bm59RE-PG}$ bacmids. **b** The BmNPV bacmids, which contained *polh*, *Gm*, and *egfp* in the polyhedron locus, was used as a positive control virus

**Fig. 5** PCR validation of the $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$, and $Bm^{Bm59WT-PG}$ bacmids. **a** PCR analysis of the *Bm59* deletion bacmid. **b** Verification of the recombinant virus with the pUC/M13 primers. **c** Verification of the *egfp* and *Bm59* transposition by PCR with pUC/M13 primers

produced a single fragment of 1600 bp from the $Bm^{Bm59KO}$ bacmid and a 650 bp fragment from the BmNPV bacmid (Fig. 5a). The Bm59-De-F/Bm59-De-R primer pair did not generate a PCR product from the BmNPV bacmid but gave a 1100 bp fragment from the $Bm^{Bm59KO}$ bacmid (Fig. 5a). These results revealed that *Bm59* had been successfully replaced with the chloramphenicol acetyl transferase (*CAT*) gene by homologous recombination. To enable virus propagation and occlusion body formation to be observed, *ie1* promoter derived *egfp* and *polyhedrin* promoter derived

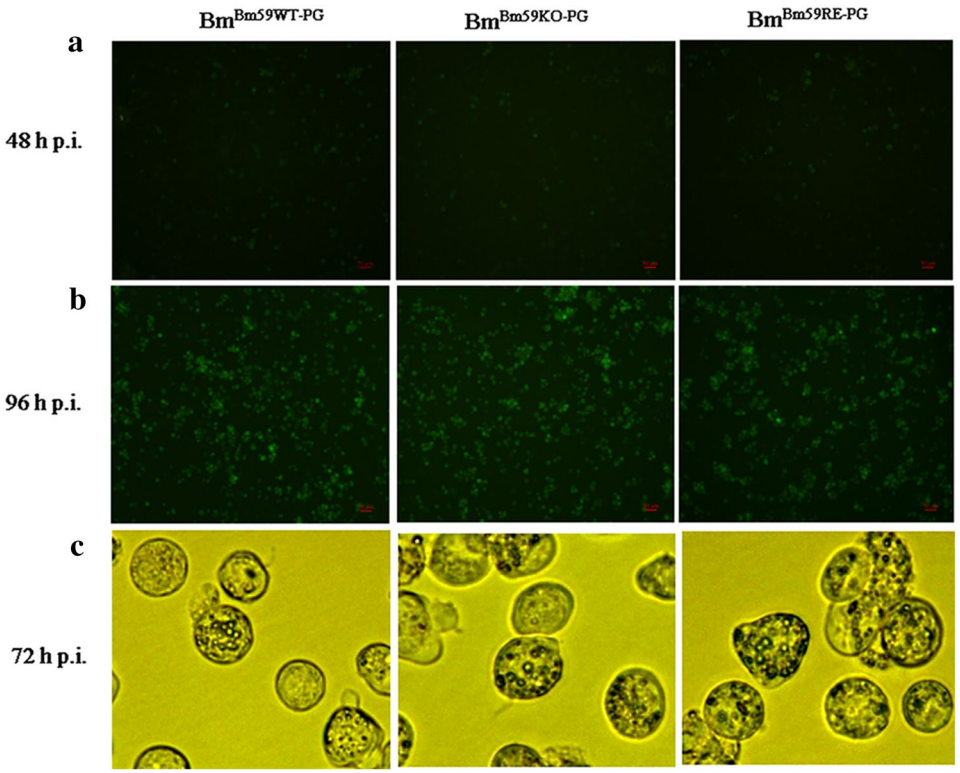



**Fig. 6** Analysis of viral replication in BmN cells. **a** and **b** Fluorescence microscopy shows the progression of the infection in BmN cells transfected with $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$, and $Bm^{Bm59WT-PG}$ bacmids, at 48- and 96-h p.t. **c** Light microscopy shows polyhedra production in BmN cells transfected with $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$, and $Bm^{Bm59WT-PG}$ bacmids, at 72-h p.t

*polyhedrin* were transposed into the polh locus of the BmNPV bacmid using Tn7-mediated transposition in *E. coli*. The presence of the $Bm^{Bm59WT-PG}$, $Bm^{Bm59KO-PG}$, and $Bm^{Bm59RE-PG}$ bacmids was confirmed by PCR, with M13F and M13R primers, and produced single fragments of 300, 5500, and 6000 bp, respectively (Fig. 5b, c). The results indicated that the recombinant bacmids had been successfully constructed.

## Virus growth curves and replication analysis

The BmN cells were transfected with three types of bacmid DNA constructs and were monitored with fluorescence microscopy. A similar number of transfected cells displayed fluorescence at 48 and 96-h post-transfection (h p.t), for each of the three bacmids (Fig. 6a,b). At 72 h p.t, normal polyhedra appeared in all three types of transfected cells (Fig. 6c). To confirm whether the BV in the supernatants carried the infection, the supernatants were removed from the $Bm^{Bm59KO-PG}$ transfected cells and added to freshly plated BmN cells. Florescence was detected 24-h p.t in the BmN cells incubated with the supernatant obtained from cells transfected with $Bm^{Bm59KO-PG}$. Widespread fluorescence was also observed 24-h p.t in cells incubated with the supernatants from cells transfected with $Bm^{Bm59WT-PG}$ or $Bm^{Bm59KO-PG}$ (data not shown).

To understand the effect of *Bm59* knockout on infectious BV production and DNA replication, qRT-PCR and 50 % tissue culture infective dose ($TCID_{50}$) were used for analysis. The BmN cells were transfected with three types of bacmid DNA and, at selected time points, qRT-PCR was performed to detect viral genomes. The assay was performed on the supernatants and cells to detect BV production (Fig. 7a) and DNA replication (Fig. 7b), respectively. The results showed that *Bm59* gene knockout did not affect the production of budded virions and viral DNA replication in cells. A $TCID_{50}$ endpoint dilution was used for the virus titer analysis at 6- and 72-h p.i (Fig. 7c). There was no significant disparity in the growth curves of these viruses at 6, 12, 24, 48, 72, and 96-h p.t. The titers of different viruses at 6- and 72-h p.i were equivalent and the results were similar to those obtained by qRT-PCR. This indicated that *Bm59* is not required for the production of infectious BV and the replication process in cells is not affected by *Bm59* knockout.

## Electron microscopy analysis

To evaluate the effect of *Bm59*-knockout on polyhedra formation and nucleocapsid morphogenesis, electron microscopy was used to visualize thin sections of BmN cells transfected with *Bm59*-knockout bacmid at 96-h p.t. The results showed polyhedra, rod-shaped nucleocapsids, and virogenic stroma in the nucleus of the cells. Numerous enveloped virions, which contained nucleocapsids, were found in the ring zone, before being assembled into

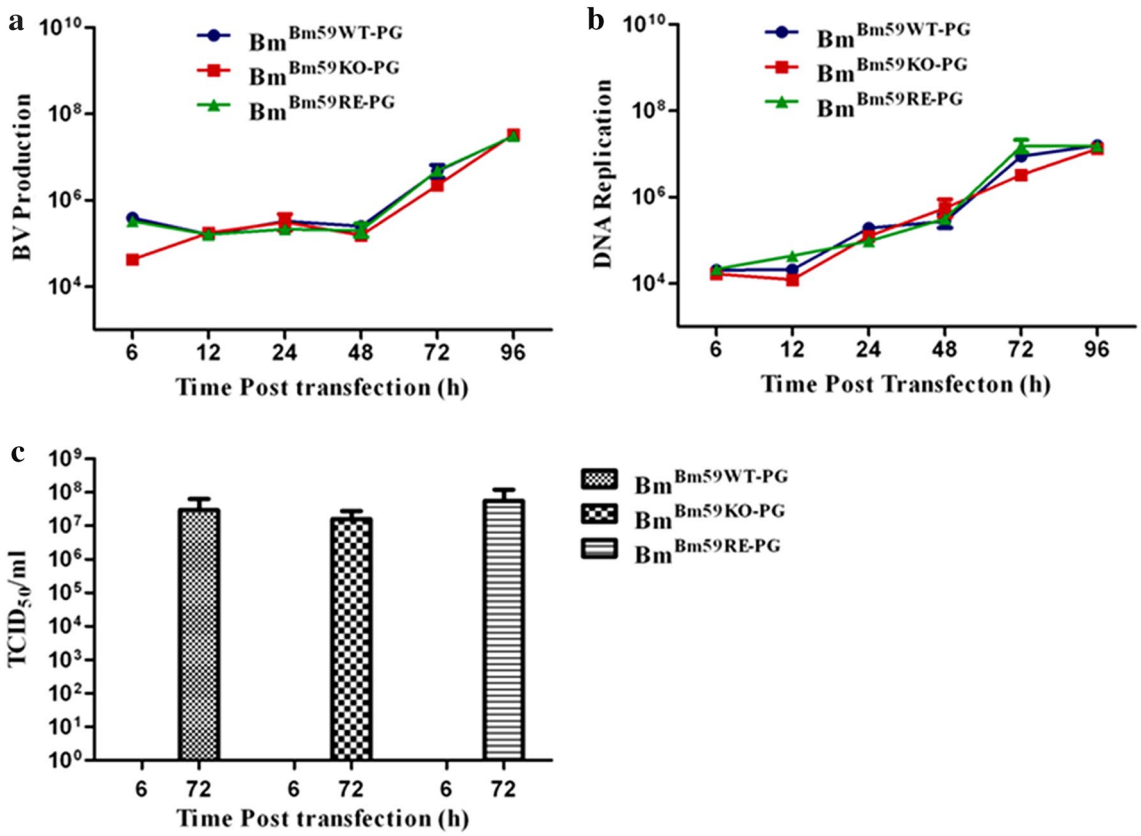



**Fig. 7** BV production, viral DNA replication, $TCID_{50}$, and virus gene expression level analysis in BmN cells transfected with $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$, and $Bm^{Bm59WT-PG}$ bacmids. **a** BV growth curves assayed by quantifying the number of viral genomes using real-time PCR analysis of supernatants of BmN cells transfected with $Bm^{Bm59KO-PG}$, $Bm^{Bm59RE-PG}$, and $Bm^{Bm59WT-PG}$ bacmids, at designated time points. **b** Real-time PCR analysis of viral DNA replication. BmN cells were transfected with 10 μg of each bacmid DNA. At the designated time points, total cellular DNA was extracted and analyzed by qRT-PCR. The results of three independent replication assays, with *error bars* to indicate standard deviation, are shown. **c** Infectious virus titers at 6- and 72-h p.t, determined by a $TCID_{50}$ end-point dilution assay. *Error bars* represent the standard errors

polyhedra. We also found numerous nucleocapsids assembled in the envelope (Fig. 8). These results indicated that *Bm59*-knockout did not affect the morphogenesis of polyhedra and ODV assembly into polyhedra.

## Discussion

Of the 57 complete genomes of baculoviruses in Genbank (Thiem 2009), *Bm59* gene homologs only appear in several types of baculovirus in the group of alpha baculoviruses. This indicates that the function of this gene in many other baculovirus species is not indispensable or is compensated by other viral genes or host cellular factors (Yang et al. 2009). In this study, BmNPV ORF59 was analyzed. First, in order to understand potential roles in the viral life cycle, we analyzed the full length of the *Bm59* gene and examined its temporal expression. We also investigated its effect on BV production, DNA replication, $TCID_{50}$ in BmN cells, viral assembly. The 5′RACE results showed that the N-terminal of *Bm59* has two transcriptional start sites. The transcriptional start motif (CAAC) of ORF is 216 nucleotides upstream of the start codon (ATG). This implied that *Bm59* is an early transcriptional gene with an atypia early transcriptional start motif.

Transcriptional analysis of the *Bm59* gene demonstrated that it is an early gene, with transcripts initially observed at 3-h p.i. This result is consistent with the predicted transcription start site. The gene may be associated with the transcriptional regulation of late genes (Tang et al. 2013). However, Illumina sequencing analysis has validated that the transcription start time of the *Bm59* gene is 12-h post-infection in cells infected by BmNPV (Xue et al. 2012). This difference may be attributed to the different methods taken. To elaborate *Bm59* gene is an early gene, we cloned the promoter region from the BmNPV genome. If it was an early gene, it was transcribed by RNA polymerase II (RNA polII) from the BmN cells. Therefore,

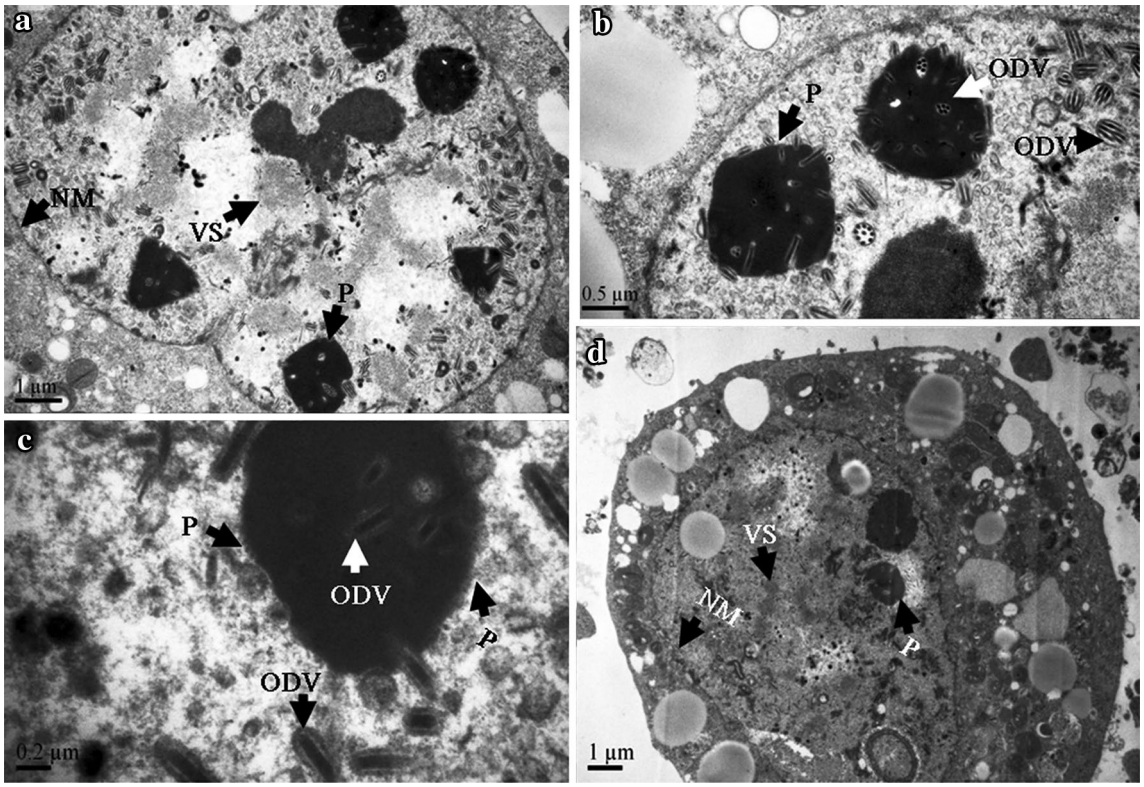



**Fig. 8** Transmission electron microscopy (TEM) analysis of BmN Cells transfected with $Bm^{Bm59WT\text{-}PG}$ and $Bm^{Bm59KO\text{-}PG}$ bacmid at 72-h p.t. **a** Polyhedra, virogenic stroma, and nucleocapsids within the nucleus of the BmN cell transfected with $Bm^{Bm59KO\text{-}PG}$. **b** Mature ODV with numerous nucleocapsids within the nucleus of the BmN cell transfected with $Bm^{Bm59KO\text{-}PG}$. **c** Mature ODV with signal nucleocapsid within the nucleus of the BmN cell transfected with $Bm^{Bm59KO\text{-}PG}$. **d** Polyhedra, virogenic stroma, nucleocapsids, and mature ODV within the nucleus of the BmN cell transfected with $Bm^{Bm59WT\text{-}PG}$. *P* polyhedrin, VS virogenic stroma, NM nuclear membrane, ODV occlusion-derived virion

Dual-Luciferase®Reporter Assay System was used. We found this promoter had strong activity, when it was compared with positive control and negative control. We concluded that *Bm59* is an early gene in the BmNPV genome.

Deletion of the *Bm59* gene did not affect viral spread in BmN cells, which showed that *Bm59* is not essential for viral replication. The *Bm59*-knockout bacmid transfection assay demonstrated that viral BV production and DNA replication were not affected by *Bm59* gene knockout, and the BV titers were not significantly different between $Bm^{Bm59WT\text{-}PG}$, $Bm^{Bm59KO\text{-}PG}$, and $Bm^{Bm59RE\text{-}PG}$. We concluded that *Bm59* is dispensable for viral BV production and DNA replication, at the cellular level. Non-essential genes remained in the viral genome during passage. These genes may interact with other viral/host genes for the viral propagation in certain situations (Ono et al. 2012). Electron microscopy analysis indicated that the virogenic stroma, rod-shaped nucleocapsids, ODVs, and ODVs that contained polyhedrins were formed in cells infected with the $Bm^{Bm59KO\text{-}PG}$ virus and $Bm^{Bm59WT\text{-}PG}$ virus. This indicated that *Bm59* is dispensable for occlusion body morphogenesis in cells infected with the BmNPV virus. Generally, these findings suggested that non-essential gene (*Bm59*) remained in the viral genome, which may interact with other viral/host genes in a certain situation.

#### Compliance with ethical standards

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**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** This article does not contain any studies with human participants.

## References

Blissard GW (1996) Baculovirus–insect cell interactions. Cytotechnology 20(1–3):73–93

Braunagel SC, Summers MD (2007) Molecular biology of the baculovirus occlusion-derived virus envelope. Curr Drug Targets 8(10):1084–1095

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97(12):6640–6645

Engelhard EK, Kam-Morgan LN, Washburn JO, Volkman LE (1994) The insect tracheal system: a conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. Proc Natl Acad Sci USA 91(8):3224–3227

Gomi S, Majima K, Maeda S (1999) Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. J Gen Virol 80(Pt 5):1323–1337

Haas-Stapleton EJ, Washburn JO, Volkman LE (2004) P74 mediates specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to primary cellular targets in the midgut epithelia of *Heliothis virescens* Larvae. J Virol 78(13):6786–6791

Ono C, Kamagata T, Taka H, Sahara K, Asano S, Bando H (2012) Phenotypic grouping of 141 BmNPVs lacking viral gene sequences. Virus Res 165(2):197–206

Shen H, Chen K, Yao Q, Zhou Y (2009) Characterization of the Bm61 of the *Bombyx mori* nucleopolyhedrovirus. Curr Microbiol 59(1):65–70

Slack J, Arif BM (2007) The baculoviruses occlusion-derived virus: virion structure and function. Adv Virus Res 69:99–165

Tang Q, Li G, Yao Q, Chen L, Feng F, Yuan Y, Chen K (2013) Bm65 is essential for the propagation of *Bombyx mori* nucleopolyhedrovirus. Curr Microbiol 66(1):22–29

Thiem SM (2009) Baculovirus genes affecting host function. Vitro Cell Dev Biol Anim 45(3–4):111–126

Vanarsdall AL, Okano K, Rohrmann GF (2004) Characterization of a baculovirus with a deletion of vlf-1. Virology 326(1):191–201

Vanarsdall AL, Okano K, Rohrmann GF (2006) Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing. J Virol 80(4):1724–1733

Wang R, Deng F, Hou D, Zhao Y, Guo L, Wang H, Hu Z (2010) Proteomics of the *Autographa californica* nucleopolyhedrovirus budded virions. J Virol 84(14):7233–7242

Wu W, Lin T, Pan L, Yu M, Li Z, Pang Y, Yang K (2006) *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38 K gene. J Virol 80(23):11475–11485

Xiang X, Chen L, Hu X, Yu S, Yang R, Wu X (2011) *Autographa californica* multiple nucleopolyhedrovirus *odv-e66* is an essential gene required for oral infectivity. Virus Res 158(1–2):72–78

Xiang X, Shen Y, Yang R, Chen L, Hu X, Wu X (2013) *Bombyx mori* nucleopolyhedrovirus BmP95 plays an essential role in budded virus production and nucleocapsid assembly. J Gen Virol 94(Pt 7):1669–1679

Xue J, Qiao N, Zhang W, Cheng RL, Zhang XQ, Bao YY, Xu YP, Gu LZ, Han JD, Zhang CX (2012) Dynamic interactions between *Bombyx mori* nucleopolyhedrovirus and its host cells revealed by transcriptome analysis. J Virol 86(13):7345–7359

Yang ZN, Xu HJ, Thiem SM, Xu YP, Ge JQ, Tang XD, Tian CH, Zhang CX (2009) *Bombyx mori* nucleopolyhedrovirus ORF9 is a gene involved in the budded virus production and infectivity. J Gen Virol 90(Pt 1):162–169