

## Functional characterization of *Bombyx mori* nucleopolyhedrovirus mutant lacking late expression factor 9

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**Summary.** – *Baculoviridae* is a family of invertebrate viruses with large double-stranded DNA genomes. Proteins encoded by some late expression factor (*lef*) genes are involved in the regulation of viral gene expression. *Lef-9* is one of four transcription-specific *Lefs*, which are components of the virus-encoded RNA polymerase, and can initiate and transcribe late and very late genes. As a multifunctional protein encoded by the *Bombyx mori* nucleopolyhedrovirus (BmNPV), *Lef-9* may be involved in the regulation of viral propagation. However, the underlying mechanism remains unclear. To determine the role of *lef-9* in baculovirus infection, *lef-9*-knockout virus (*lef-9*-KO-Bacmid virus) was constructed using the Red recombination system, and the Bac-to-Bac system was used to prepare *lef-9*-repaired virus (*lef-9*-Re-Bacmid virus). The *lef-9*-KO virus did not produce infectious viruses or show infection activity, while the *lef-9*-repaired virus recovered both. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of the transcription levels in wild-type-Bacmid, *lef-9*-KO-Bacmid, and *lef-9*-Re-Bacmid viruses showed that the *lef-9*-KO bacmid had little effect on viral genome replication. However, the transcription levels of the early and late viral genes, *lef-3*, *ie-1*, *vp39*, and *p10*, were significantly lower in BmN cells transfected with *lef-9*-KO-Bacmids than in the controls. Electron microscopy showed no visible enveloped virions in cells transfected with *lef-9*-KO-Bacmids, while many mature virions in cells transfected with *lef-9*-Re-Bacmid and wt-Bacmid were present. Thus, *lef-9* was not essential for viral genome replication, but significantly affected viral gene transcription and expression in all periods of cell life cycle.

**Keywords:** *Bombyx mori* nucleopolyhedrovirus; *lef-9* gene; virus replication; virus transcription; virus assembly

### Introduction

*Baculoviridae* is a family of viruses with large double-stranded DNA genomes. To date, more than 600 baculoviruses have been described (Herniou and Jehle, 2007; Yin *et al.*, 2015), and the genomes of 47 types of baculoviruses have been sequenced. The sizes of these genomes are between 80–180 kb, with 90% of genes involved in protein coding (Ayres *et al.*,

1994). The *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a model baculovirus. Its genome structure is very similar to the *Autographa californica* multiple NPV (AcMNPV) genome. AcMNPV is the first completely sequenced and well-studied baculovirus (Ayres *et al.*, 1994; Bulach *et al.*, 1999; Herniou *et al.*, 2003; Jehle *et al.*, 2006). The genome of this virus contains 133,894 bp, with an average A-T content of 59%, and 154 open reading frames (ORFs). In 1999, the BmNPV (T3 strain) genome was identified to contain 128,413 bp and 135 ORFs encoding 60 or more amino-acid residues (Gomi *et al.*, 1999). Comparison of the ORFs in the genomes of the two viruses revealed that 115 ORFs were highly conserved, with more than 90% homology (Yu *et al.*, 2013).

Proteins called late expression factors (LEFs) are transcribed and translated by 19 viral late genes identified by

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**Abbreviations:** BmNPV = *Bombyx mori* nucleopolyhedrosis virus; BmN = *Bombyx mori* cells; BV = budding virus; Lef = late expression factor

transient expression assays (Hang *et al.*, 1995; Rapp *et al.*, 1998). Among the 19 genes, *p143*, *ie-2*, *dnapol*, *lef-2*, *ie-1*, *p35*, *lef-1*, *lef-3*, and *lef-7* were found to influence viral DNA replication (Iyengar *et al.*, 1999; Vanarsdall *et al.*, 2006), while *lef-4*, *lef-5*, *lef-6*, *lef-8*, *lef-9*, *lef-10*, *lef-12*, *p47*, and *pp31* were found to act as late transcription genes (Lu and Miller, 1995). The four subunits of *lef-4*, *lef-8*, *lef-9*, and *p47* (Guarino *et al.*, 1998) were found to encode viral RNA polymerase (Vanarsdall *et al.*, 2005), while *lef-5*, *lef-6*, *lef-10*, *lef-12*, and *pp31* were found to be directly involved in the regulation of late gene transcription (Lu and Miller, 1995). Late genes are expressed during viral DNA replication and are soon afterwards regulated by RNA polymerase.

The *lef-9* gene is a highly conserved gene in insect baculoviruses (Rose *et al.*, 2013). The site of *lef-9* in the BmNPV genome is at the position 44,402–45,874 with length of 1473 bp. The gene encodes 491 amino acids, and the predicted molecular mass of the Lef-9 protein is 54 K. The predicted amino acid sequences of *lef-9* contain motifs that are similar to those that participate in the catalytic centers of known RNA polymerases (Crouch *et al.*, 2007). Although one study has shown that *lef-9* is a subunit of RNA polymerase (Guarino *et al.*, 1998), which regulates the transcription of late and very late genes. Several reports have shown that *lef-9* takes part in many other functions. Wu *et al.* found

that it could interact with IE-2 in silkworm cells to regulate viral gene expression and viral replication (Wu *et al.*, 2013). These results implied that *lef-9* might have multiple functions involved in the regulation of viral replication, but the details of the process and mechanism of viral gene replication and transcription are still not clear.

Many experimental studies have shown that gene knock-out technology is a powerful tool for determining gene function. It has also been used to study baculovirus genes (Su *et al.*, 2011; Lin and Blissard, 2002; Shi *et al.*, 2015). In this study, *lef-9*-KO bacmids were constructed using gene knockout technology with a Red recombination system, and were used to study the effect of this defect virus on infectious virus production, and viral replication and transcription, in comparison with wild-type virus (BmNPV) and *lef-9*-repaired virus.

## Materials and Methods

**Viruses and cells.** *Bombyx mori* cells (BmN, preserved in our laboratory) were cultured at 27°C in Sf-900 medium (Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum. Virus (*Bombyx mori* nucleopolyhedrosis virus, BmNPV) was preserved in our laboratory.

**Construction of *lef-9*-KO and *lef-9*-repaired bacmids.** To construct the *lef-9*-KO bacmid, we PCR-amplified linearized *lef-9* gene fragments. The 1100 bp long target fragment, named *lef-9*-C, was amplified by using the plasmid pKD3 (encoding a chloramphenicol acetyltransferase [*cat*] gene) (Youbio, China) as a template, and *lef-9*-CF and *lef-9*-CR as primers (Table 1). The *lef-9*-C fragment was transformed into DH10Bac competent cells (Takara, Japan) containing the plasmid pKD46 (Youbio, China) for the expression of homologous recombination enzymes required for the Red recombination system (Copeland *et al.*, 2001; Costantino and Court, 2003), and induced by *L*-Arabinose (Promega, USA). Bacteria were cultured for 24 hr at 37°C on agar plates with chloramphenicol and kanamycin. The positive colonies were selected and confirmed by PCR using the following primer pairs: *lef-9*F and *cat*R, *lef-9*R and *cat*F, and *lef-9*F and *lef-9*R (Table 1).

To construct repaired bacmids (*lef-9*-Re-Bacmid), the *lef-9* gene was cloned into the vector pFastBacHTB (Tiandz Inc., China) and then inserted downstream of the polyhedrin promoter by using the Bac-to-Bac system (preserved in our laboratory). A fragment containing the *lef-9* gene with its native promoter was amplified by PCR using the primers pFblef-9F and pFblef-9R (Table 1). The repaired fragment was cloned into pFastBacHTB to obtain recombinant plasmid pFastBacHTB-*lef-9*, which was transformed into DH10Bac competent cells containing the *lef-9*-KO bacmid prepared previously. Plates containing kanamycin, 5-bromo-

Table 1. Primer sequences for PCR amplification

Primers	Primer sequences
<i>lef-9</i> -CF*	Forward: 5'-TGTTTTCTTTTTGGATAAACT CCTACTGAGTT TGACCTCATAT TAGACGTGTAGGCTGGAGCTGC TTC-3'
<i>lef-9</i> -CR*	Reverse: 5'-TGATAAAATTTTTAAAATCACTTTAAA TTCTTCA TTGGTAAAAAATGCCATGGGAATTAGCCA TGGTCC-3'
<i>lef-9</i> F	Forward: 5'-CACGTTTTTCGTCGATTG-3'
<i>lef-9</i> R	Reverse: 5'-CGGCAAATCCGTGACTAT-3'
<i>cat</i> F	Forward: 5'-CACGTTTTAAATCAAACCTGGTG-3'
<i>cat</i> R	Reverse: 5'-CAATATGGACAACCTTCTTCG-3'
pFblef-9F	Forward: 5'-CGGGATCCTCAGCCTGTTGTCTG TGAATACC-3'
pFblef-9R	Reverse: 5'-CGGAATTCGGTATTGAGATTTTCC GCAGTT-3'
<i>gp41</i> F	Forward: 5'-CGTAGTGGTAGTAATCGCCGC-3'
<i>gp41</i> R	Reverse: 5'-AGTCGAGTCGCGTTCGCTTT-3'
<i>β-actin</i> F	Forward: 5'-GCGCGGCTACTCGTTCCTACTACC-3'
<i>β-actin</i> R	Reverse: 5'-TGCCGCAAGCTTCCATACCC-3'
M13F	Forward: 5'-GTTTTCCAGTCACGAC-3'
M13R	Reverse: 5'-CAGGAAACAGCTATGAC-3'

\*These two primers were composed of 50-bp *lef-9* homology arms (underlined) and a 20-bp *cat* homologous zone (underlined). Boxed sequences represent the restriction enzymes *Bam*HI and *Eco*RI.

4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), gentamicin, tetracycline, and isopropyl  $\beta$ -D-1-thiogalactoside (IPTG) were used to screen positive colonies. The primer combinations M13F and M13R, M13F and pFblef-9F, and M13R and pFblef-9R and pFblef-9F/R (Table 1) were used to confirm positive colonies. The construction map is shown in Fig. 1.

**Real-time quantitative PCR assay.** To analyze the effects of the *lef-9* gene on viral DNA replication, we transfected *lef-9*-KO-Bacmid, wt-Bacmid (wild type), and *lef-9*-Re-Bacmid DNA (1  $\mu$ g each) into BmN cells (1  $\times$  10<sup>6</sup>/35-mm dish), and collected the transfected cells after 6, 12, 18, 24, and 48 hr. We then analyzed the transcription level of the baculovirus *gp41* gene in the infected cells by performing quantitative real-time PCR (qRT-PCR) at various time points, and the viral *gp41* gene-specific primers gp41F and gp41R were used (Table 1).  $\beta$ -Actin was used as an internal control. To analyze early and late viral gene transcription at different transcription times after cell transfection, we used *ie-1* and *lef-3* as early gene controls (Kejju *et al.*, 1999). The *vp39* and *p10* genes were used as late and very late gene controls, respectively. The BmN cells transfected with *lef-9*-KO-Bacmid, wt-Bacmid, and *lef-9*-Re-Bacmid DNA (1  $\mu$ g each) were collected at 12, 24, 48, and 72 hr. Total RNA was extracted using Trizol for qRT-PCR and treated with DNase I to remove residual BmNPV genomic DNA. Oligo dT<sub>(18)</sub> was used as the primer for first-strand cDNA synthesis (Thermo Fisher Scientific, USA) by reverse transcription.

All primers were synthesized by the Shanghai Sangon Biotech Company (China) and PCR reagents were from TaKaRa Bio (Japan).

**Virus titration assay.** BmN cells were transfected with *lef-9*-KO-Bacmid, *lef-9*-Re-Bacmid, and wt-Bacmid (1  $\mu$ g each), and then, the cell culture supernatants were carefully collected after 12, 18, 24, 48, 72, and 96 hr. At the logarithmic growth phase, BmN cells were transferred to 96-well plates (10<sup>3</sup> cells/well), and the virus supernatant was added at different time points, at 10 $\times$  dilutions ranging from 10<sup>-1</sup> to 10<sup>-9</sup>. Then, 10  $\mu$ l of viral suspension were added into each well, and 8-well repeats were used for each dilution gradient. Wells without any virus were used as negative controls. After incubation for 4 days, the cell incidence of each dilution was recorded, and the 50% tissue culture infective dose (TCID<sub>50</sub>) was calculated using the Reed and Muench method.

**Western blot assay.** To analyze the effect of *lef-9* deficiency on the expression of viral genes, we studied the early protein Lef-3, the late protein VP39, and the very late protein P10 by using western blot analysis. The *lef-9*-KO virus, *lef-9*-repaired virus, and wild-type virus were used to transfect BmN cells, and cell lysates were collected after 72 hr for sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis and western blot analysis. Briefly, the cells were washed twice with PBS and lysed in lysis buffer (Beyotime Institute of Biotechnology, China) at 4 $^{\circ}$ C and centrifuged at 12,000 rpm. The lysate (10  $\mu$ g of protein per lane) was separated on 12% SDS-polyacrylamide gel, blotted onto nitrocellulose membrane and incubated with specific primary antibodies Lef-3, VP39, and P10 (1:1000) (Abmart Company, China). The expression of Lef-9 in cells transfected with the bacmids was analyzed by 6 $\times$ His antibody (Zexiyuan Biotechnology Company, China). The antibodies were detected by a horseradish peroxidase-

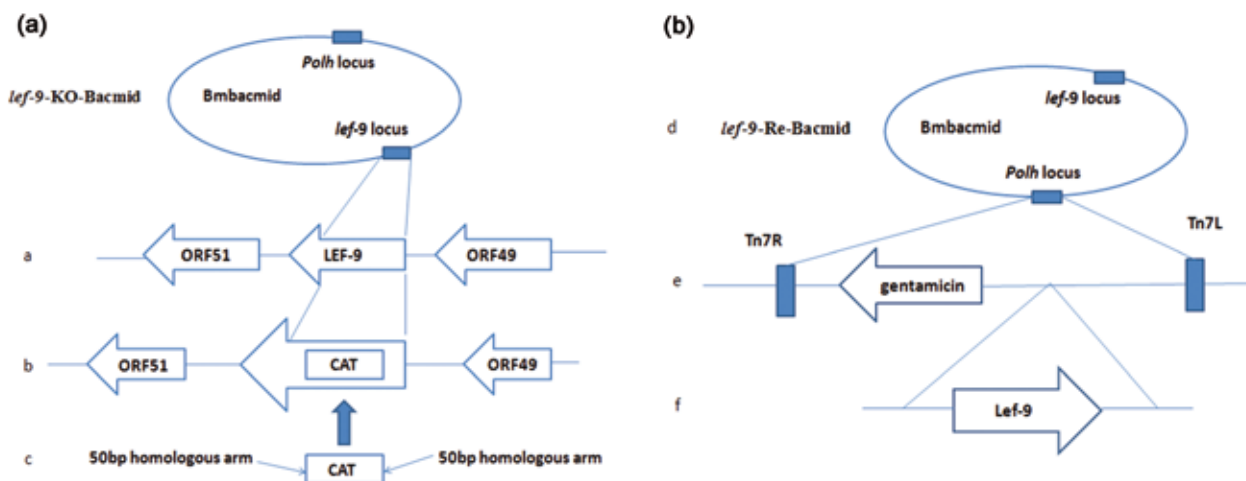


Fig. 1

#### Construction of *lef-9* knockout (a) and *lef-9*-repaired (b) BmNPV bacmids

(a) Location of *lef-9* in the *lef-9*-KO-Bacmid genome; (b) recombination between *cat* and *lef-9*; (c) PCR amplification of the target fragment using the pKD3 plasmid as a template and the primers *lef-9*-CF/*lef-9*-CR; (d) location of *polh* in the *lef-9*-Re-Bacmid genome; (e) insertion of *lef-9* together with its promoter into the transposon; (f) PCR product of *lef-9* and its promoter using the wild-type bacmid as a template.

conjugated goat anti-mouse IgG antibody (DingGuo Biotechnology Company, China) using Western Bright™ ECL system (Advansta, USA).

**Electron microscopy.** BmN cells ( $1 \times 10^5$ /35-mm dish) were transfected by 1  $\mu$ g each of *lef-9*-KO-Bacmid, wt-Bacmid, and *lef-9*-Re-Bacmid and collected after 24 hr. Cells were incubated overnight in 2.5% glutaraldehyde at 4°C. Cell samples were prepared, and 70–90 nm sections were made using a microtome (Reichert, USA). The sections were treated with lead citrate solution and 50% ethanol, and a saturated solution of uranyl acetate for 15 min for staining, and analyzed with a Hitachi H-7650 (Japan) transmission electron microscope.

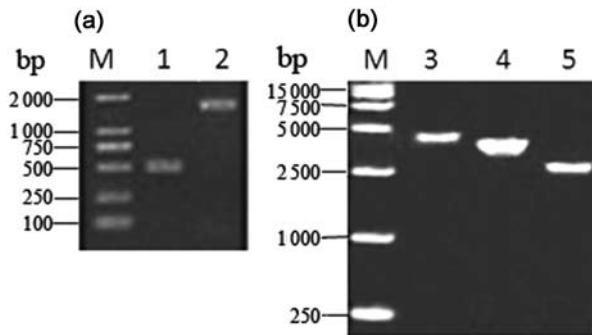


Fig. 2

#### Validation of *lef-9* knockout and repaired bacmids by PCR

Lane M, DNA marker; lane 1, *lef-9F* and *catR* PCR product; lane 2, *lef-9F* and *lef-9R* PCR product; lane 3, M13F and M13R PCR product; lane 4, M13F and pFblef-9R PCR product; lane 5, pFblef-9F and M13R PCR product.

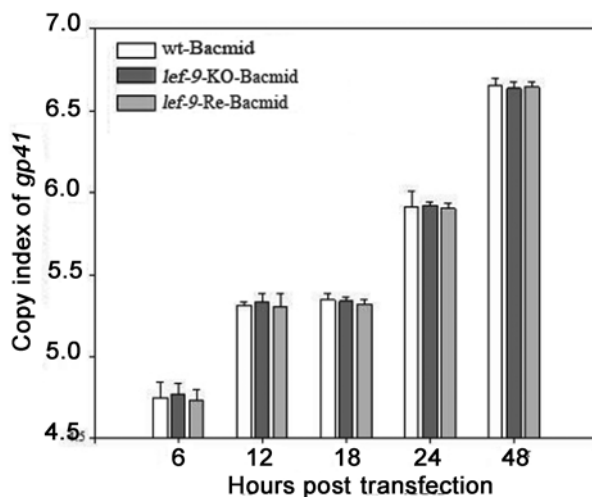


Fig. 3

#### Quantitative real-time PCR analysis of viral DNA replication

Cells transfected with bacmids were harvested after 6–48 hr and assayed for *gp41* by real-time PCR. Values are expressed as means  $\pm$  SEM.

## Results

### Identification of *lef-9*-KO and *lef-9*-repaired bacmids

Positive colonies proliferating on plates containing chloramphenicol and kanamycin were selected and confirmed by PCR, with extracted bacmid DNA as a template. The results showed that the PCR yielded 500 bp long product by using the *lef-9F/catR* primers, and 1500 bp long product when using the *lef-9F/lef-9R* primers (Fig. 2a). Size of the products was consistent with the theoretical sizes, which indicated that the *lef-9*-KO-Bacmid had been constructed successfully. The *lef-9*-repaired bacmid-positive colonies, were selected from plates containing kanamycin, gentamicin, tetracycline, IPTG, and X-gal, and confirmed by PCR. The primer combinations used were M13F/M13R, M13F/pFblef-9R, and pFblef-9F/M13R (Table 1). PCR yielded a 4430 bp long product with M13F/M13R primers, 3830 bp long product with M13F/pFblef-9R primers, and 2690 bp long product with pFblef-9F/M13R primers (Fig. 2b). The PCR products were consistent with the theoretical sizes, indicating that *lef-9*-Re-Bacmid had been successfully constructed.

### Viral replication analysis

The total RNA extracted from the transfected cells collected at different time points was subjected to qRT-PCR. The Ct values related to *gp41* determined using qRT-PCR were converted to absolute copy numbers after calculations with a standard curve, with time after transfection on the abscissa and the copy number of the BmNPV-*gp41* index on the or-

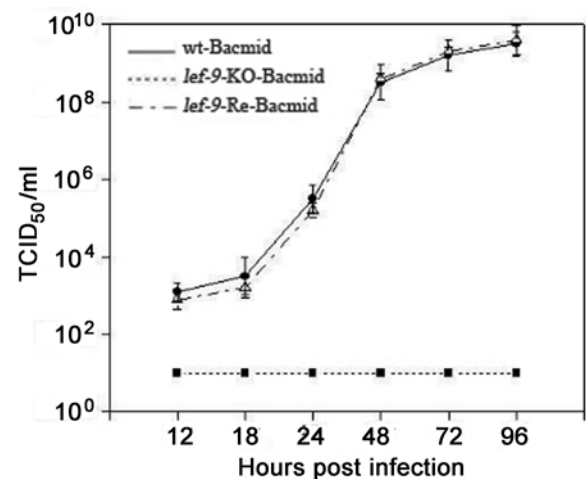


Fig. 4

#### Virus titers in BmN cells transfected with bacmids

Cells transfected with the bacmids were harvested after 12–96 hr and assayed for virus titers. Values are expressed as means  $\pm$  SEM.

dinate (Fig. 3). As shown in Fig. 3, there was no significant difference in viral DNA copy numbers ( $P > 0.05$ ) between the silkworm BmN cells transfected with *lef-9*-KO-Bacmid, wt-Bacmid, and *lef-9*-Re-Bacmid, suggesting that the BmNPV *lef-9* gene was not essential for genome replication.

The *lef-9*-KO-Bacmid had a TCID<sub>50</sub> of 0 at all time points, indicating that the *lef-9*-KO virus did not produce contagious budding virus (BV; Fig. 4). The *lef-9*-KO-Bacmid lost the ability to produce infectious BV. In contrast, the wt-Bacmid and *lef-9*-Re-Bacmid retained this function, and generated increasing numbers of BVs with time after transfection. This

result further confirms that *lef-9* is not indispensable for viral genome replication.

*Viral transcription analysis*

To study the effects of *lef-9*-KO bacmids on BmNPV gene transcription, we measured the transcription levels of the early genes *lef-3* and *ie-1*, the late gene *vp39*, and the very late gene *p10*. The qRT-PCR results showed that at 12 hr, the *lef-3* transcription level in the *lef-9*-KO-Bacmid-transfected BmN cells showed little difference from the levels in the wt-Bacmid- and

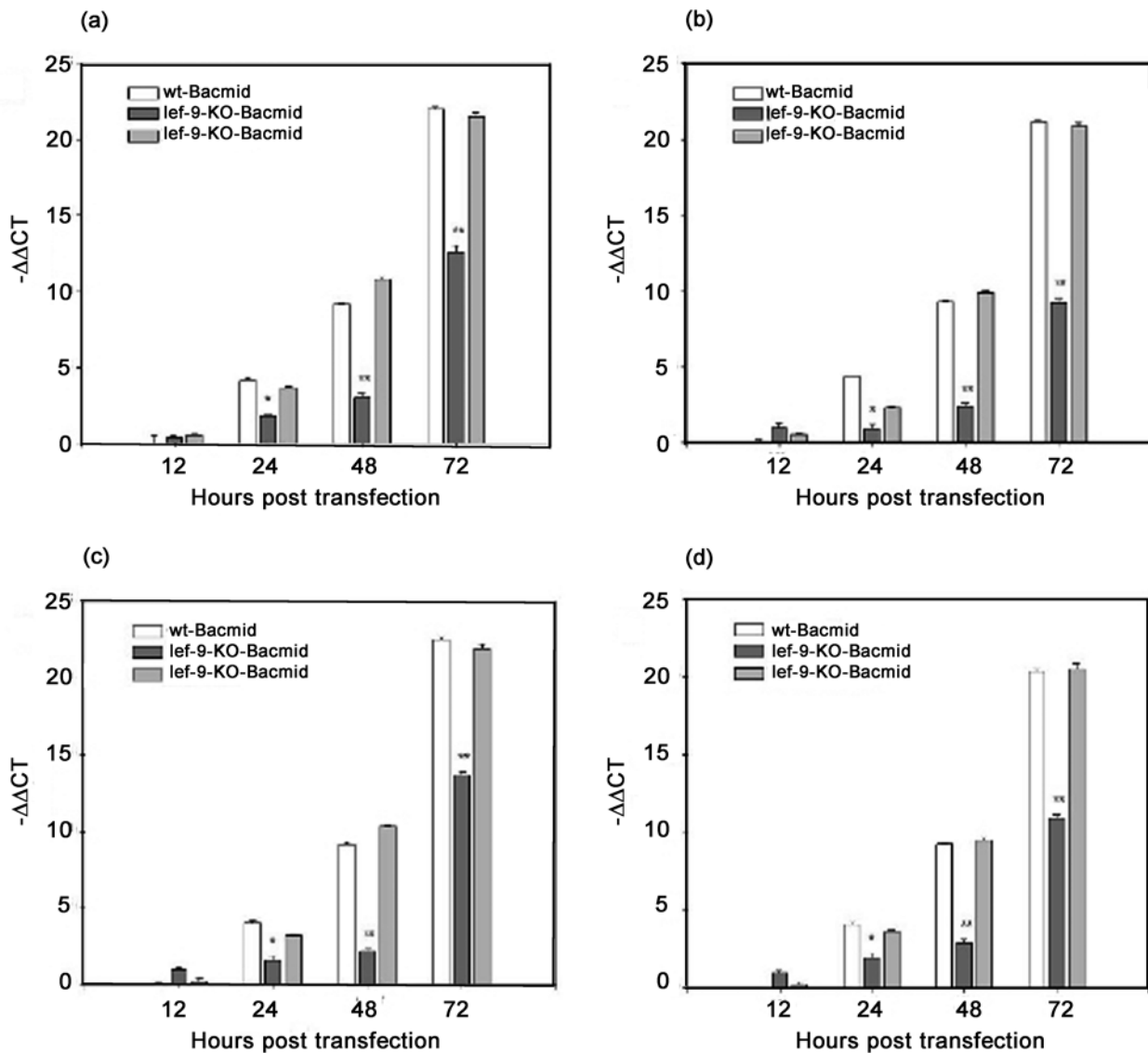


Fig. 5

Transcription levels of *lef-3* (a), *ie-1* (b), *vp39* (c) and *p10* (d) in cells transfected with bacmids

Transcription levels were analyzed by real-time PCR. \* $P < 0.05$ , \*\* $P < 0.01$  vs. wt-Bacmid or *lef-9*-Re-Bacmid at 12, 24, 48, and 72 hr. Values are expressed as means  $\pm$  SEM. Similar results were obtained in three independent experiments.

*lef-9*-Re-Bacmid-transfected cells (Fig. 5a). At 24 hr however, *lef-3* expression was significantly lower in the *lef-9*-KO cells than in the wt-Bacmid- and *lef-9*-Re-Bacmid-transfected cells ( $P < 0.05$ ). Furthermore, these significant differences retained at 48 and 72 hr ( $P < 0.01$ ). Analysis of the early gene *ie-1* (Fig. 5b), the late gene *vp39* (Fig. 5c), and the very late gene *p10* (Fig. 5d) showed similar trends to those observed with *lef-3*. Therefore, we speculated that the *lef-9* gene was positively correlated with virus genome transcription, including the transcription of early, late, and very late genes. The deficiency of the *lef-9* gene had a great influence on the viral genome transcription level.

#### Western blot analysis

The western blot (Fig. 6a) results showed that the lysates of BmN cells transfected with *lef-9*-Re-Bacmids had obvi-

ous bands, which indicated that the *lef-9*-Re-Bacmid had been successfully constructed and that Lef-9 was correctly expressed. No obvious or very low protein expression of Lef-3, VP39, and P10 was detected in the lysates of BmN cells transfected with *lef-9*-KO-Bacmid DNA when compared with cells transfected with *lef-9*-Re-Bacmid, indicating that *lef-9* was essential for the expression of early and late viral genes (Fig. 6b).

#### Viral assembly analysis

To analyze the effect of *lef-9* deficiency on virion assembly, we examined transfected BmN cells under a transmission electron microscope. As shown in Fig. 7, no enveloped baculovirus was observed in the BmN cells transfected with *lef-9*-KO bacmids (Fig. 7 d-f). However, BmN cells

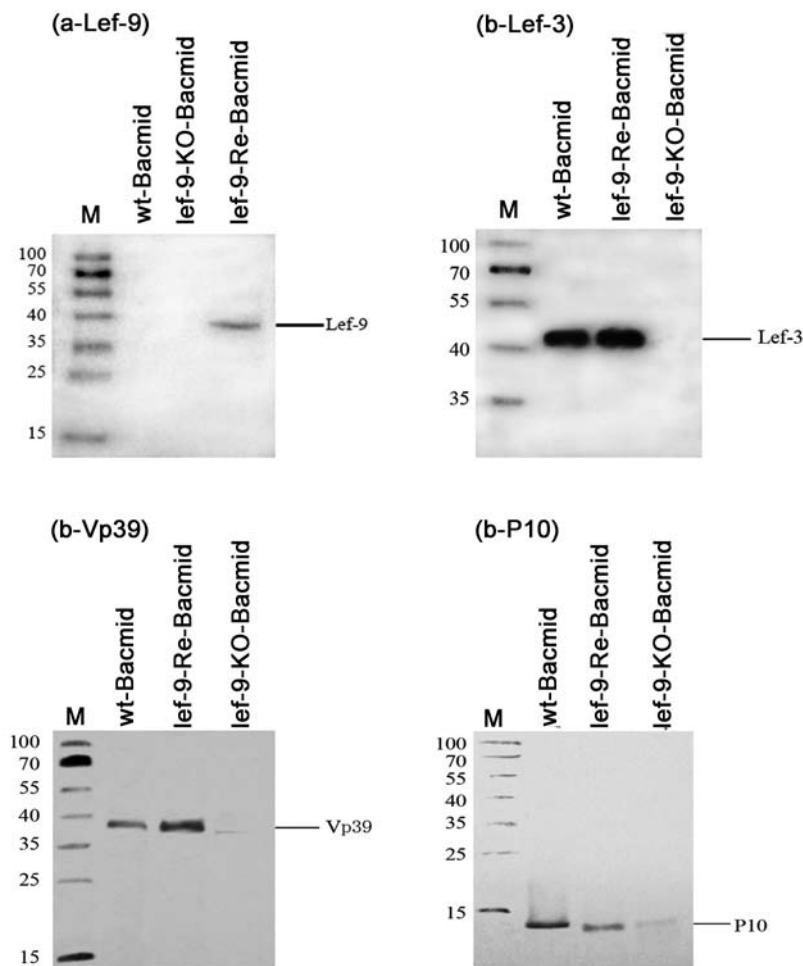


Fig. 6

#### Western blot analysis of Lef-9 (a) and Lef-3, VP39 and P10 (b)

The expression of Lef-9 was detected by 6 $\times$  His monoclonal antibody. The expressions of Lef-3, P10, and VP39 were detected by Lef-3, VP39, and P10 monoclonal antibodies, respectively. The lysates of BmN cells transfected with wt-Bacmid DNA (wt-Bacmid), *lef-9*-KO-Bacmid DNA (*lef-9*-KO-Bacmid) and *lef-9*-Re-Bacmid DNA (*lef-9*-Re-Bacmid) were used. Lane M, protein marker.

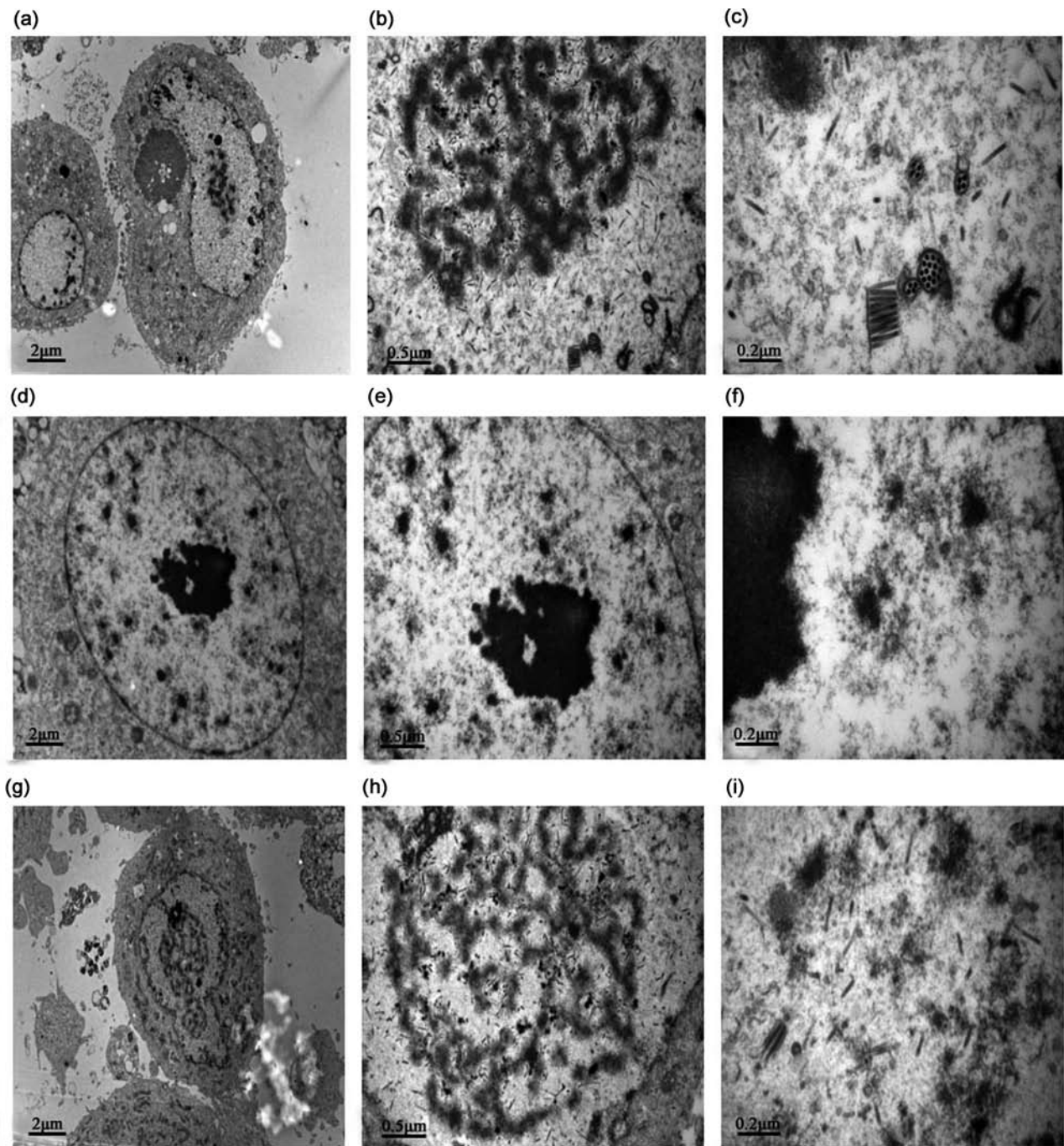


Fig. 7

**Electron microscopic analysis of BmN cells transfected with wt-Bacmid, *lef-9*-KO-Bacmid, and *lef-9*-Re-Bacmid**

A–C, 24 hr after transfection with wt-Bacmid (A, 5,000 $\times$ ; B, 20,000 $\times$ ; C, 50,000 $\times$ ); D–F, 24 hr after transfection with *lef-9*-KO-Bacmid (D, 5,000 $\times$ ; E, 20,000 $\times$ ; F, 50,000 $\times$ ); G–I, 24 hr after transfection with *lef-9*-Re-Bacmid (G, 5,000 $\times$ ; H, 20,000 $\times$ ; I, 50,000 $\times$ ).

transfected with wild-type virus (Fig. 7 a–c) or *lef-9*-repaired virus (Fig. 7 g–i) showed typical characteristics, including an electron-dense virogenic stroma enriched with rod-shaped enveloped nucleocapsids formed in the nucleus (Fig. 7c).

## Discussion

In this study, we constructed the *lef-9*-KO-Bacmid using the Red recombination technique and the Bac-to-Bac



baculovirus expression system to construct a *lef-9*-repaired virus. The deficient virus DNA was used to transfect BmN cells. We used *gp41* as a reference for determining the viral copy index. The *gp41* gene, encoding the GP41 protein, is essential for viral replication, and is required for the egress of nucleocapsids from the nucleus during BV synthesis (Zhang *et al.*, 2014). Defective viral titers in cell supernatants were 0 at all time points after transfection (Chikako *et al.*, 2012), showing that the *lef-9*-KO virus had lost its vitality and ability to proliferate; in contrast, the *lef-9*-repaired virus regained its infectivity. Studies have shown that many factors can prevent viruses from forming infectious BVs, especially, factors related to viral DNA replication (Milks *et al.*, 2003). However, our results showed that the intracellular viral genome copy number did not significantly differ between the *lef-9*-KO-Bacmid, wt-Bacmid, and *lef-9*-Re-Bacmid. Therefore, we concluded that the *lef-9* gene was not essential for BmNPV genome replication, but was vital for viral gene transcription, which explains the failure to create infectious BVs. The knockout of the late expression factors *lef-10* (Yu *et al.*, 2013) and *vlf-1* (Vanarsdall *et al.*, 2004) can reduce viral DNA copies, affect early and late gene transcription, and prevent BV production and spread. In addition, *lef-9*, a BmNPV late expression factor, has been shown to be important for viral gene transcription (Acharya and Gopinathan, 2002). Our results pertaining to the *lef-9*-KO virus were similar to these findings, for instance, the inability to produce infectious BVs (Vanarsdall *et al.*, 2004), although we found that early viral genome replication was not affected.

To further study the biological function of the *lef-9* gene, we examined the effects of a *lef-9*-KO virus on the transcription and expression of viral genes. The results showed that *lef-9* deficiency affected the transcription and expression of early, late, and very late viral genes. Lef-9 is a subunit of viral RNA polymerase (Guarino *et al.*, 1998; Vanarsdall *et al.*, 2005), so it is very reasonable that *lef-9* deficiency will affect the transcriptional levels of viral early and late genes, leading to the failure of the expression of these proteins. Western blots showed that *lef-9*-KO-Bacmid-transfected BmN cells had no obvious detectable expression of Lef-3, VP39, and P10 proteins. These results further confirmed that the deletion of the *lef-9* gene greatly reduced the transcription levels of early, late, and very late genes.

Finally, we used transmission electron microscopy to observe BmN cells transfected with wt-Bacmid, *lef-9*-KO-Bacmid, and *lef-9*-Re-Bacmid. In the *lef-9*-KO virus-infected cells, we did not observe any mature virions or the formation of a virus particle sac surrounded by a membrane. This result confirmed that the loss of *lef-9* affected virus assembly, and therefore, no virus particles with infective activity could be produced. BmN cells infected with the *lef-9*-repaired virus or wild-type virus showed typical symptoms of baculovirus infection, including a highly electron-dense virogenic stroma,

enlarged nuclei, and abundant nucleocapsid assembly in the matrix. These results were consistent with the results of virus titer measurements in cells transfected with the bacmids.

In summary, *lef-9* is necessary for the production of BVs in cells. The absence of this gene will affect viral gene transcription and expression, and prevent normal virus assembly.

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